CD8⁺ Enriched "Young" Tumor Infiltrating Lymphocytes Can Mediate Regression of Metastatic Melanoma

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Abstract

Purpose: Tumor-infiltrating lymphocytes (TIL) and interleukin (IL)-2 administered following lymphodepletion can cause the durable complete regression of bulky metastatic melanoma in patients refractory to approved treatments. However, the generation of a unique tumor-reactive TIL culture for each patient may be prohibitively difficult. We therefore investigated the clinical and immunologic impact of unscreened, CD8⁺ enriched "young" TIL.

Experimental Design: Methods were developed for generating TIL that minimized the time in culture and eliminated the individualized tumor-reactivity screening step. Thirty-three patients were treated with these CD8⁺ enriched young TIL and IL-2 following nonmyeloablative lymphodepletion (NMA). Twenty-three additional patients were treated with CD8⁺ enriched young TIL and IL-2 after lymphodepletion with NMA and 6 Gy of total body irradiation.

Results: Young TIL cultures for therapy were successfully established from 83% of 122 consecutive melanoma patients. Nineteen of 33 patients (58%) treated with CD8⁺ enriched young TIL and NMA had an objective response (Response Evaluation Criteria in Solid Tumors) including 3 complete responders. Eleven of 23 patients (48%) treated with TIL and 6 Gy total body irradiation had an objective response including 2 complete responders. At 1 month after TIL infusion the absolute CD8⁺ cell numbers in the periphery were highly correlated with response.

Conclusions: This study shows that a rapid and simplified method can be used to reliably generate CD8⁺ enriched young TIL for administration as an individualized therapy for advanced melanoma, and may allow this potentially effective treatment to be applied at other institutions and to reach additional patients. Clin Cancer Res; 16(24): 6122–31. ©2010 AACR.

Patients with metastatic melanoma have limited treatment options, and only 2 of these treatments have received Food and Drug Administration approval. Interleukin (IL)-2 can mediate durable complete responses in 3% to 5% of treated patients, with an overall response rate of about 13% to 16% (1); decarbazine results in a 15% response rate with rare durable responses (2). Other promising experimental treatments being evaluated in randomized trials for efficacy and toxicity include inhibitors of the dominant BRAFV600E mutation (3) and an anti-CTLA-4 antibody (4, 5). Despite these recent advances, a pressing need remains for effective treatments.

Adoptive cell therapy (ACT) combines lymphodepletion with a patient’s own tumor reactive tumor-infiltrating lymphocytes (TIL) to generate an individualized therapy (6). A total of 93 refractory melanoma patients were treated at our institution with TIL selected for tumor recognition following 1 of 3 different lymphodepleting regimens (7). Forty-three patients received nonmyeloablative chemotherapy (NMA) alone, 25 patients received NMA with 2 Gy of total body irradiation (TBI), and 25 patients received NMA plus 12 Gy TBI. The objective response rates [Response Evaluation Criteria in Solid Tumors (RECIST)] for these sequential trials were 48%, 52%, and 72%, respectively, including 15 patients who had complete responses, all but 1 of which are ongoing at 31 to 89 months. Despite these promising clinical results, the extensive effort, cost, and time required to generate the individual TIL cultures limited the application of this promising therapy to only a few institutions.

Tumor-reactive TIL used in prior studies required an extended duration of multiple microcultures and an
CD8+ Enriched Young TIL

**Translational Relevance**

Cell-based immunotherapy using autologous, tumor-reactive lymphocytes can mediate the regression of bulky, established cancer in animal models and human patients. Tumor-infiltrating lymphocytes (TIL) selected for tumor recognition and highly expanded in vitro have been especially effective for treating melanoma patients, but there are logistic and technical difficulties associated with generating an individualized tumor-reactive therapy for each patient. In this report we use simple, reliable methods of generating individual, autologous TIL products and test them for the first time in a 2-arm, phase II clinical trial. Our results support a wider application of individualized cell therapies based on TIL and define correlates of clinical treatment and response for improvement of future therapies.

individualized assay to identify tumor recognition. Analysis of demographic and biological factors that correlated with TIL function indicated that the size of the lesion and the degree of lymphocyte infiltration impacted TIL generation (8). However, many patients underwent procurement of metastatic tissue for TIL generation but were not ultimately eligible for treatment because their TIL failed the test for tumor recognition, or rapid disease progression occurred during TIL establishment (8). Thus, the complex TIL production process was a substantial limitation to patient therapy. We have now simplified and standardized the TIL production process by generating “young” TIL for therapy (9). These minimally manipulated young TIL cultures consisted of bulk lymphocytes rather than microcultures, and the batch-specific assay for tumor recognition was eliminated. Young TIL have attributes associated with improved persistence and response in vivo including long telomeres and high expression of CD27 and CD28 (10–13). In a small clinical trial, young TIL were shown to be capable of mediating objective clinical responses in some patients (14, 15). We have now developed a simplified procedure to enrich for tumor-reactive CD8+ cells, eliminate nonspecific CD4+ T cells, and deplete T regulatory cells (16).

We report here the first clinical trial with CD8+ enriched young TIL administered to patients following lymphodepletion. CD8+ enriched young TIL were effective for the treatment of some patients with metastatic melanoma. The simplified methods of TIL production allowed rapid accrual to this clinical trial using methods more easily adaptable to laboratories in multiple centers. Here we analyze the clinical and immunologic features of this individualized patient therapy.

**Methods**

**Patients, clinical samples, and trial design**

Patients eligible for this study were aged 18 years or older with measurable metastatic melanoma had at least 1 lesion resectable for TIL; had good clinical performance, adequate liver and kidney function tests, and blood counts near the normal range; were free from active systemic infections, coagulation disorders, cardiovascular disease, or immunodeficiency; were negative for HIV antibody and hepatitis B and C; and had a life expectancy of more than 3 months. All patients signed an informed consent approved by the Institutional Review Board of the National Cancer Institute.

One group of 33 patients received NMA consisting of 60 mg/kg/day cyclophosphamide for 2 days followed by 5 days of 25 mg/m²/day fludarabine. A second cohort of 23 patients received 2 days of 60 mg/kg of cyclophosphamide overlapping the first 2 of 5 days of 25 mg/m²/day fludarabine. On the final day of fludarabine, patients received 2 fractions of 2-Gy TBI separated by at least 6 hours, and the following day they received 1 fraction of 2-Gy TBI. On the day following chemotherapy or radiation all patients received a bolus intravenous infusion of CD8+ enriched young TIL and started high-dose IL-2 therapy (720,000 IU/kg intravenously every 8 hours to tolerance). One day after TIL infusion, patients who received 6-Gy TBI received a minimum of 2 × 10⁹/kg autologous purified (Miltenyi) CD34+ hematopoietic stem cells from a granulocyte colony-stimulating factor ± plerixafor mobilized pheresis.

Patients received trimethoprim, sulfamethoxazole, and fungal prophylaxis following therapy; herpes virus seropositive patients also received valacyclovir. Platelets and packed red blood cells were administered as needed during hematopoietic recovery, and empiric antibiotics were initiated for neutropenic fevers (38.3°C once or for 2 temperatures of 38.0°C at least 1 hour apart and absolute neutrophil count < 500). Patient response was assessed using standard radiographic studies and physical examination at approximately 4 weeks following TIL administration and at regular intervals thereafter. The RECIST guidelines were followed and patients were categorized into complete, partial, or nonresponding categories. Complete blood counts (CBC) were obtained at least once per day while patients were in the hospital and differential counts were obtained when CBC was over 200 cells/μL.

**Generation and characterization of CD8+ enriched young TIL**

Patients with metastatic melanoma underwent biopsy and as much of the sample as possible was processed to a single cell suspension for generation of young TIL as previously described (9, 17) and detailed in Supplementary Methods. The single cell suspension was evaluated on a hemacytometer with lymphocytes and tumor cells determined on the basis of size and morphology and viability determined by Trypan blue staining. After minimum time in culture, successfully initiated “bulk” young TIL were CD8+ enriched (Miltenyi ClinMACS; ref. 16) and rapidly expanded to clinical cell numbers (18, 19). Aliquots of infused samples were evaluated by fluorescence-activated cell sorting (FACS) analysis and cytokine release assays to determine lymphocyte phenotype and antigen specificity respectively using standard techniques (18).
**Statistical analysis**

Standard statistical methods are cited in the text, and all $P$ values are 2-tailed assuming unequal variance with $P < 0.05$ considered significant.

**Results**

**CD8+ enriched young TIL mediated antitumor responses**

The safety and efficacy of therapy using CD8 + enriched young TIL following lymphodepletion was investigated in 2 cohorts of patients with metastatic melanoma. Thirty-three patients received CD8+ enriched TIL with NMA, and 23 patients received CD8+ enriched young TIL following NMA plus 6 Gy of TBI. The demographic characteristics of these patients and the treatments administered are shown in Table 1. Sixty-four percent of patients had received prior IL-2. Median follow-up as of April 1, 2010 in the NMA and 6-Gy TBI cohorts was 14 months and 7 months, respectively.

Nineteen of the 33 patients (58%) in the NMA cohort exhibited an objective tumor regression by RECIST criteria, including 16 partial responders (48%) and 3 complete responders (9%). Eleven of 23 patients (48%) in the 6-Gy TBI cohort achieved an objective response, including 2 complete responders (9%). Illustrative examples of clinical tumor regression are shown in Figure 1. Fifteen of 24 patients with M1a or M1b melanoma and 15 of 32 patients with M1c disease responded to therapy. As reported previously (7, 18), all patients experienced transient hematologic toxicities from the lymphodepleting conditioning and received platelet and red blood cell transfusions as medically indicated. Patients were also treated for symptoms associated with high-dose IL-2 therapy. All toxicities typically returned to baseline within a few days. All nonhematologic grade 3 and 4 toxicities not attributable to IL-2 are listed in Table 2. There were no grade 3 or 4 toxicities directly attributable to the infused cells. There were 2 treatment-related mortalities, 1 in each cohort, that resulted from acute sepsis during the neutropenic period associated with lymphodepletion about 5 days after TIL infusion.

**Generation of young TIL for treatment was reliable and rapid**

During the period of this study (August 2008 through September 2009), 176 tumors from 122 patients were processed to establish young TIL cultures. TIL were successfully grown ($>50 \times 10^6$ cells within 5 weeks) from 124 of the 176 lesions (70%), comprising 101 of the 122 patients (83%). A striking correlation was observed between the success of establishing TIL and the initial proportion of lymphocytes in the single cell suspension (Fig. 2A). Tumors that successfully yielded TIL had an initial median of 52% lymphocytes whereas tumors that failed to grow TIL cultures in vitro had a median of 8% lymphocytes ($P = 5 \times 10^{-5}$). Among these 122 patients, 53 were treated (3 additional patients received cryopreserved TIL from prior resections). 21 had samples that failed to grow TIL cultures, 20 developed rapidly progressive disease that prevented treatment, 13 were resected free of evaluable disease (although 9 patients recurred and received TIL treatment subsequently), 9 received other treatments including 1 complete responder to high dose IL-2 therapy, and 6

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**Table 1. Demographics of patients and characteristics of treatments administered**

<table>
<thead>
<tr>
<th>NMA (n = 33)</th>
<th>6 Gy TBI (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
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<tr>
<td>Female</td>
<td>19</td>
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<tr>
<td><strong>Age (years)</strong></td>
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<td><strong>Stage of disease</strong>*</td>
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</tr>
<tr>
<td>M1a</td>
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<tr>
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<tr>
<td>M1c</td>
<td>20</td>
</tr>
<tr>
<td><strong>Cell number (×10^6)a</strong></td>
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<tr>
<td>IL-2 (doses)</td>
<td>47.7 (±3.3)</td>
</tr>
<tr>
<td>IL-2 (doses)</td>
<td>6.3 (±0.3)</td>
</tr>
<tr>
<td><strong>Age of cells at Infusion (days)</strong></td>
<td></td>
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<tr>
<td>CD4+ cells (%)</td>
<td>32.7 (±0.7)</td>
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<tr>
<td>Prior to CD8+ enrichment</td>
<td>21.8 (±3.2)</td>
</tr>
<tr>
<td>Infused</td>
<td>0.6 (±0.3)</td>
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<tr>
<td>CD8+ cells (%)</td>
<td>57.2 (±4.3)</td>
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<tr>
<td>Prior to CD8+ enrichment</td>
<td>96.0 (±0.6)</td>
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<tr>
<td><strong>Tissue of TIL originb</strong></td>
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<tr>
<td>Lymph node</td>
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<tr>
<td>Other visceral site</td>
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*aAverage (± SE).  
*bSome patients were treated with TIL from multiple tissues of origin.*
Fig. 1. CD8+ enriched young TIL caused regression of bulky melanoma lesions at multiple sites. A, computed tomography (CT) scans of metastatic melanoma lesions (arrows) before CD8+ enriched young TIL therapy in the sacrum and ilium (top left), lung (middle left), and spleen (lower left). All lesions showed regression at 2 months (right) with visible signs of recalcification of prior metastatic sites in the sacrum and ilium. B, subcutaneous melanoma around the ear and in the auditory canal caused complete hearing loss (left); 11 days after CD8+ enriched young TIL infusion, gross necrosis of the melanoma was visible (middle); at 76 days after treatment the patient experienced a partial response at all sites including liver and subcutaneous lesions (right). Tumor was absent from the auditory canal and the patient’s hearing returned to normal. C, CT scans show mediastinal, lung, nodal, and subcutaneous metastatic deposits before (left) and 1 month after (right) treatment with CD8+ enriched young TIL, demonstrating the rapid initial pace of tumor regression in a patient who eventually achieved a complete response.
experienced individual laboratory or clinical issues, including 1 patient whose CD8+ TIL failed to expand during the rapid expansion protocol.

The CD8+ enrichment was highly effective for reducing the fraction of CD4+ cells in the infused TIL (Table 1 and Supplementary Table S1). The CD4 cell component comprised an average of 22% of the cells in bulk young TIL prior to CD8 enrichment, and natural killer cells comprised 21% (Supplementary Table S1). TIL from prior protocols administered without CD8 enrichment after NMA conditioning also contained about 21% CD4+ cells. Following CD8+ enrichment and expansion, CD4+ cells were reduced to an average of 2% of the infused young TIL.

The age of TIL was compared for patients who received CD8+ enriched TIL and patients on prior TIL protocols at our institution (Fig. 2B). Microculture generated, tumor-selected TIL administered to responding patients were significantly younger than TIL given to patients who did not respond. In the cohorts treated in this report, there was no difference between the age of CD8+ enriched young TIL cultures for responding and nonresponding patients but these cultures were significantly younger than TIL cultures administered on prior protocols ($P = 3 \times 10^{-7}$).

### CD8+ enriched young TIL frequently exhibited tumor recognition

We retrospectively evaluated the ability of the administered CD8+ enriched TIL to recognize tumor and looked for any correlation with clinical efficacy. Recognition of autologous or HLA-matched tumor was evaluated by cytokine release assay (Fig. 3). Twenty-three of the 33 NMA patients had autologous tumor available (18 with cryopreserved tumor digest and 5 with a tumor cell line). Eight of 10 nonresponding patients and 8 of 13 objective responders demonstrated autologous tumor recognition. Four additional objective responding patients recognized HLA-A–matched tumor cell lines. Nineteen of 23 patients treated with 6-Gy TBI had autologous tumor available (15 with fresh frozen tumor and 4 with a cell line). Eight of 11 nonresponding patients and 5 of 8 responding patients demonstrated specific tumor recognition. In addition, 3 patients’ TIL recognized HLA-matched tumor cell lines, including 1 nonresponding patient (patient SA, Fig. 6B), whose TIL failed to recognize autologous tumor. In total, 29 of 42 evaluable CD8+ enriched young TIL samples (69%) demonstrated specific autologous tumor recognition and 36 of 56 (64%) patients demonstrated specific recognition in all. Strikingly, 11 of 30 objective responses were mediated by CD8+ enriched young TIL with no evidence of specific tumor recognition as defined in prior TIL clinical protocols.

### CD8+ enriched young TIL influenced lymphocyte reconstitution of the host

The average absolute lymphocyte count (ALC) for all patients who received CD8+ enriched young TIL after NMA or 6-Gy TBI lymphodepletion is plotted in Figs. 4A and B, respectively. For comparison, lymphocyte reconstitution from patients who received antigen-selected TIL derived from microculture expansions after NMA or 12-Gy TBI (7) lymphodepletion are also shown. Interestingly, patients who received CD8+ enriched young TIL demonstrated higher peak ALCs suggesting that CD8+ enriched young TIL have increased capacity for in vivo expansion compared with selected TIL.

Peripheral blood lymphocytes (PBL) from 47 of the 56 patients who received CD8+ enriched young TIL were sampled at approximately 1 month after cell infusion. ALC and absolute CD3+CD8+ and CD3+CD4+ cell numbers were assessed by FACS analysis in a blinded manner. The treatment resulted in low CD4+ counts at 1 month in most patients (average: 109 CD4+ cells/µL).
with no difference between responding and nonresponding patients ($P = 0.5$; Fig. 4D). Patients in prior clinical trials who received TIL that contained CD4$^+$ lymphocytes had higher peripheral CD4$^+$ cell counts at 1 month after TIL infusion (average: 187 cells/$\mu$L). In contrast to CD4$^+$ cells, CD8$^+$ ALC for most patients treated with CD8$^+$ enriched young TIL was in the normal range at 1 month, and there was a significant increase in CD8$^+$ ALC in responding patient compared with nonresponders ($P = 0.002$; Fig. 4C). Patients who responded had CD8$^+$ ALC about 2-fold higher than nonresponders (1,504 versus 696 cells/$\mu$L). In prior clinical trials the average CD8$^+$ cell count was 652 CD8$^+$ cells/$\mu$L at 1 month after TIL infusion. Infused TIL were examined by FACS for expression of additional markers including CD27, CD28, CD62L, and CCR7, but none correlated with clinical response.

**Discussion**

Adoptive cell therapy (ACT) can mediate durable objective regression (including durable complete responses) in patients with metastatic melanoma refractory to other treatments. The complexity of preparation of cell products unique for each patient has limited the widespread use of this method. In this study we developed a simplified method for cell preparation that may allow wider application of this effective treatment.

Previously, 93 patients were treated over 84 months using highly expanded TIL selected for specific tumor recognition (7). This corresponds to about 1 treatment per month and about 27% of resected patients who finally received a TIL product (8). In the current report 56 additional patients were treated with CD8$^+$ enriched young TIL over 14 months, corresponding to about 3 to 4 patients treated per month, with 53% of eligible patients who underwent resection able to receive TIL therapy. With both methods, clinical response rates were about 55%, but strikingly 11 of 30 objective responders in this study had TIL that would have been ineligible for the prior study. Although comparisons of sequential clinical trials should be made with caution, the changes in the CD8$^+$ enriched young TIL methods were apparently effective for improving delivery of individualized TIL therapy to eligible patients without compromising therapeutic efficacy.

Prior clinical trials with TIL in our institution (7) and preclinical mouse models (20–22) suggested that increased...
lymphodepletion improves ACT. This study comparing 2 cohorts of patients treated with different conditioning regimens found similar objective response rates and toxicity profiles. This outcome could result from relatively small patient numbers, sequential cohort enrollment, and short follow-up. Alternately, 6 Gy of TBI delivered in fractionated doses may only minimally impact host mechanisms that support the transferred CD8$^+$ cells compared with 12 Gy of TBI (23).

Despite methodologic improvements, 21 of the 123 patients (17%) had TIL that failed to grow in culture. On average, these melanoma lesions started with a median of only 8% lymphocytes (Fig. 2A). In the future, changing the lymphocyte/tumor ratio in vitro could improve the generation of TIL for some tumors. Additionally, some studies have noted a correlation between good prognosis and T-cell infiltration for nonmelanoma cancers including colon cancer (24), breast cancer (25), ovarian cancer (26), and non–small cell lung cancer (27), suggesting that CD8$^+$ enriched young TIL from metastatic lesions from nonmelanoma histologies may be therapeutically active in ACT treatments.

The enrichment of CD8$^+$ cells prior to rapid expansion has practical benefits for TIL generation (16) but the enrichment process is expensive, and depletion of CD4$^+$ cells raises questions about lymphocyte persistence, tumor regression, and treatment toxicity. CD4$^+$ T helper cells are involved in generating and maintaining CD8$^+$ T memory cells in mice (28, 29) and a CD4$^+$ clone was associated with melanoma regression in a patient (30). However,
CD4+ T regulatory cells may block CD8+ cell function in tumor immunity (20, 31). The impact of CD4+ cells on the persistence and function of CD8 TIL in vivo is not known. In this study, the CD8+ enriched young TIL repopulated peripheral blood and persisted at high levels for over a month. Furthermore, the objective response rate of 54% reported here is comparable to the 56% objective response rate observed in prior trials with CD4+ replete TIL. These data suggest that elimination of the CD4+ cells was beneficial to the therapy. More study is needed to understand the role of CD4+, CD25+ and FoxP3+ cells in the regulation of TIL responses after infusion and CD4+ T helper cells in the duration of responses. To directly address the impact of CD4+ cells in young TIL therapy, we have initiated a randomized clinical trial where one arm receives unselected young TIL that contain both CD4+ and CD8+ cells (Fig. 4).
CD8+ cells, whereas a second arm receives CD8+ enriched young TIL. This study presents data from 2 cohorts of patients treated with autologous TIL following lymphodepletion for metastatic melanoma. The production process for CD8+ young TIL was relatively rapid and reliable, and the manufactured product was capable of generating tumor regression and objective responses in patients. The minimally manipulated CD8+ cell product had a high frequency of autologous tumor reactivity, and repopulated the host PBL compartment rapidly. Persistently high CD8+ cell numbers were correlated with tumor regression in treated patients. This method for the generation of CD8+ enriched young TIL represents a major simplification in the application of therapeutically effective TIL.

References
27. Kawai O, Ishii G, Kubota K, Murata Y, Naito Y, Mizuno T, et al. Predominant infiltration of macrophages and CD8+ T Cells in cancer because of the ease of cell production (one culture versus many microcultures) and the elimination of testing for antitumor specificity. These simplifications can serve to extend the ability to apply this treatment approach to additional institutions.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Received 05/13/2010; revised 07/01/2010; accepted 07/10/2010; published OnlineFirst 07/28/2010.
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