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Clinical Cancer Research

Specific Lymphocyte Subsets Predict Response to Adoptive Cell Therapy Using Expanded Autologous Tumor-Infiltrating Lymphocytes in Metastatic Melanoma Patients

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Abstract

Purpose: Adoptive cell therapy (ACT) using autologous tumor-infiltrating lymphocytes (TIL) is a promising treatment for metastatic melanoma unresponsive to conventional therapies. We report here on the results of an ongoing phase II clinical trial testing the efficacy of ACT using TIL in patients with metastatic melanoma and the association of specific patient clinical characteristics and the phenotypic attributes of the infused TIL with clinical response.

Experimental Design: Altogether, 31 transiently lymphodepleted patients were treated with their expanded TIL, followed by two cycles of high-dose interleukin (IL)-2 therapy. The effects of patient clinical features and the phenotypes of the T cells infused on the clinical response were determined.

Results: Overall, 15 of 31 (48.4%) patients had an objective clinical response using immune-related response criteria (irRC) with 2 patients (6.5%) having a complete response. Progression-free survival of more than 12 months was observed for 9 of 15 (60%) of the responding patients. Factors significantly associated with the objective tumor regression included a higher number of TIL infused, a higher proportion of $CD8^+$ T cells in the infusion product, a more differentiated effector phenotype of the $CD8^+$ population, and a higher frequency of $CD8^+$ T cells coexpressing the negative costimulation molecule "B- and T-lymphocyte attenuator" (BTLA). No significant difference in the telomere lengths of TIL between responders and nonresponders was identified.

Conclusion: These results indicate that the immunotherapy with expanded autologous TIL is capable of achieving durable clinical responses in patients with metastatic melanoma and that $CD8^+$ T cells in the infused TIL, particularly differentiated effectors cells and cells expressing BTLA, are associated with tumor regression. *Clin Cancer Res; 18(24); 6758–70. ©2012 AACR.*

Introduction

Metastatic melanoma is an aggressive form of cancer highly resistant to traditional forms of therapy, such as chemotherapy and radiotherapy (1). Response rates and survival for patients with advanced stages (IIIc and IV) in response to chemotherapy, such as dacarbazine and temozolomide, have been relatively poor (2). Drugs targeting activated oncogenes of the mitogen-activated protein kinase (MAPK) pathway (3), such as B-RAF^{V600E}, have also been actively pursued (4). Recently, a B-RAF^{V600E} inhibitor has been approved by the U.S. Food and Drug Administration (FDA) recently (5). However, although this drug was shown to induce objective tumor regression in a high percentage of patients, these responses are turning out to be of limited duration (6). Originally, due to its relative refractoriness to chemotherapy and radiotherapy, melanoma has been studied as a target for immunotherapy more than many other forms of cancer (7). Most melanoma metastases contain lymphocytic infiltrates, including T cells that recognize melanoma antigens such as Melan-A/MART-1 and NK cells

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Translational Relevance

In this study, we report on a phase II clinical trial showing that adoptive cell therapy with autologous tumor-infiltrating lymphocytes (TIL) is a powerful regimen to treat patients with metastatic melanoma after failing first- and second-line therapies. We also conducted comprehensive phenotypic analysis of T cells in the infused TIL and found a strong association between clinical response and the total number and percentage of $CD8^+$ T cells. $CD8^+$ T cells with a more differentiated effector phenotype seemed to be the most highly active component. In addition, for the first time, we screened for the expression of negative T-cell costimulatory molecules (PD-1, BTLA, and TIM-3) in TIL and how these are associated with clinical response. Unexpectedly, we found that $CD8^+$ T cells expressing BTLA (B- and T- lymphocyte attenuator) were highly associated with clinical response. This cell subset may be a new predictive biomarker in TIL therapy.

(8). A monoclonal antibody blocking CTLA-4, called ipilimumab, has resulted in durable clinical responses in a subset of patients and significantly increased median overall survival in stage IV melanoma resulting in its FDA approval recently (9). Thus, enhancing immunologic mechanisms are proving successful in the care of metastatic melanoma.

Another promising form of immunotherapy for metastatic melanoma is adoptive cell therapy (ACT) using the infusion of autologous tumor-infiltrating lymphocytes (TIL) expanded *ex vivo* combined with high-dose (HD) interleukin (IL-2) therapy (10, 11). ACT involves the isolation of viable tumor tissue and the expansion of TIL with IL-2 over 4 to 5 weeks from tumor fragments placed in culture (12). The TILs are then further expanded in a larger scale using anti-CD3 activation and exogenous IL-2 in the presence of autologous or allogeneic-irradiated feeder cells (12). This protocol has become known as the "rapid expansion protocol" (REP) and can yield as much as 100 to 150 billion cells for infusion (8, 12). Durable responses to TIL therapy have been improved by the addition of a preparative lymphodepleting regimen using a combination of cyclophosphamide and fludarabine (10, 13), which leads to an increase in the persistence of the transferred cells (13). Further improvements for this approach will be dependent on an increased understanding of the mechanism of TIL antitumor activity, such as determining which lymphocyte subtypes within the heterogeneous bulk population of cells are responsible for tumor regression. Overall, a better understanding of the nature of the T cells mediating objective antitumor responses during ACT will allow us to select the most active cell subsets to transfer into patients, or tailor TIL expansion procedures to preferentially expand these active T-cell populations for therapy to improve clinical response rates.

We have undertaken a phase II ACT clinical trial for metastatic melanoma using expanded TIL followed by HD IL-2 in patients pretreated with a cyclophosphamide and fludarabine lymphodepleting regimen (10, 13). In this article, we report results on the clinical response rates of first 31 TIL-treated patients and an analysis of possible predictive biomarkers of therapeutic effectiveness, including phenotypic markers and telomere length in the infused T cells.

Materials and Methods

Patient population, overall TIL expansion process, and therapy

Patient enrollment, TIL expansion and infusion, and HD IL-2 therapy were carried out under a protocol (2004–0069) approved by the Institutional Review Board (IRB) of the MD Anderson Cancer Center (Houston, TX) and an FDA-approved Investigational New Drug (IND) application (NCT00338377). This is an ongoing phase II study that contains a randomized component and a nonrandomized component. Data presented in this article are from the nonrandomized component whose objective is to determine clinical response rates and predictive biomarkers associated with clinical response in 50 patients. The analysis in this article is on the first 31 treated patients. Both male and female patients with stage IV melanoma, stage III in-transit disease, or recurrent regional nodal disease over the age of 18 were enrolled following informed consent. One patient under the age of 18 (a 15-year old female; patient #2247) was enrolled after a compassionate exemption was approved by the FDA. All types of prior therapy were allowed, including chemotherapy, biochemotherapy, targeted therapy with tyrosine kinase inhibitors and antiangiogenic agents, and immunotherapy. Patients with brain metastases 1 cm or less were eligible. Please refer to the clinical trial NCT00338377 in the NCI website (http://www.cancer.gov/clinicaltrials) for further details on patient inclusion and exclusion criteria. Supplementary Table S1 and Table 1 also provide further information on the accrued patient clinical and demographic characteristics for this study. All patients were HLA typed at the HLA-A locus in the MD Anderson HLA Typing Laboratory. Expanded TILs were used for functional and phenotypic analysis under an IRB-approved protocol (LAB06-0755) approved by the MD Anderson Cancer Center IRB.

TIL expansion for therapy and measurement of antitumor cell reactivity

Supplementary Fig. S1A shows the overall scheme for TIL expansion first from surgical tumor harvest followed by the REP and TIL infusion together with HD IL-2 therapy. TILs were obtained from resected tumors and expanded under current Good Manufacturing Practices (cGMP) conditions in the GMP Cell Processing Facility at the MD Anderson Cancer Center. In most cases, one solitary tumor nodule was selected for TIL expansion, whereas in some cases, 2 to 3 smaller nodules were used. In previous studies, we and others have found no association between the success of TIL expansion and the site of tumor resection (14). TIL were

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Table 1. Patient number, disease stage, clinical outcome, number of TIL infused, and sites of disease

Patient number	Stage	Clinical response[a]		PFS[b] (months)	OS[c] (months)	Infused cells ($\times 10^9$)	Sites of disease						
		irRC	RECIST				SC	LN	Lung	Liver	Other visceral	Bone	Brain
2054/2256	IV M1c	PR	PR	22+	27+	80.6	X	X					
2124	IV M1a	PR	PR	31+	35+	104.9	X	X					
2131	IV M1b	PR	PR	22	37+	89		X	X				
2150/2153	IV M1c	PR	PR	22+	29+	89		X	X	X	X		
2173	IV M1c	PR	PR	8	15	115	X				X	X	
2180	IIIc	PR	PR	9	27+	58		X					
2215	IV M1c	PR	PR	28+	32+	130		X		X	X	X	
2258	IV M1c	PR	PR	25+	27+	74		X	X	X			
2261	IIIc	CR	CR	18+	21+	150	X	X					
2262	IV M1c	PR	PD[d]	22+	27+	99		X			X		X
2267	IV M1b	PR	PD[d]	2+	22+	57	X	X	X				
2340	IV M1c	PR	PR	11	19+	46	X	X	X		X		X
2350	IV M1c	PR	PR	10	17+	105	X		X		X		X
2357	IV M1c	PR	PR	13+	18+	109	X		X	X		X	X
2379	IV M1b	CR	CR	11+	14+	100	X		X				
2044	IV M1c	SD	SD	3	4	19.9		X	X	X			
2104	IV M1c	SD	PD	2	5	85		X				X	
2114	IV M1b	SD	SD	4	25	8	X	X	X				
2125	IV M1c	SD	SD	4	6	68	X	X		X	X		
2132	IV M1c	SD	SD	4	5	35	X			X	X		
2136	IV M1c	SD	PD	2	7	79.8		X	X		X		X
2144	IV M1b	PD	PD	3	18	54.6	X	X	X				
2146	IV M1c	SD	PD	6	8	8	X		X		X	X	X
2175	IV M1b	SD	SD	29+	33+	55.4		X	X				
2245	IV M1c	PD	PD	2	4	38		X				X	
2247	IV M1c	SD	PD	1	5	60	X	X	X	X		X	X
2281	IV M1c	PD	PD	2	6	62.5		X		X	X		
2284	IV M1b	PD	PD	3	19+	86		X	X				
2299	IV M1b	SD	SD	6	9+	53.8		X	X				
2338	IV M1c	SD	PD	6	14+	35		X	X				X
2373	IV M1b	SD	PD	4	5	59.2	X	X	X				

Abbreviations: LN, lymph node; PD, progressive disease; SD, stabilization of disease; SC, subcutaneous.

[a]Best overall response measured by irRC criteria or RECIST.

[b]On the basis of irRC (as of October 17, 2011).

[c]As of October 17, 2011.

[d]These patients developed small new brain metastases after receiving TIL (hence the designation as PD according to RECIST) that were effectively treated with stereotactic radiosurgery (#2262) or whole brain radiation (#2267).

expanded from 3 to 5 mm^3 cut tumor fragments according to previously published methods (11, 13) after removing extraneous connective, necrotic, and nontumor tissue using manual dissection. The TILs expanded from the tumor fragments (pre-REP TIL) to a minimum of total 48×10^6 cells were cryopreserved and kept for further expansion (Supplementary Fig. S1A) for therapy according to the criteria in the clinical protocol. TIL that did not expand to these minimal numbers were not further expanded and these patients did not go on to receive treatment. Cryopreserved pre-REP TIL from patients to be treated were thawed and further expanded using the REP to generate the final TIL infusion product (post-REP TIL; ref. 12). A small sample of the pre-REP TIL before cryopreservation was analyzed for antitumor reactivity as a possible predictive biomarker using an autologous melanoma cell line generated from the patient (when possible), or a semiallogeneic melanoma cell line that had at least one matched HLA-A allele, as targets. Details on the methodology used to expand TIL for therapy and the determination of pre-REP TIL antitumor reactivity can be found in Supplementary Methods online.

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TIL therapy and blood sampling

Each patient received a course of lymphodepleting chemotherapy before TIL infusion (day 0) with cyclophosphamide (60 mg/kg) on day −7 and −6 and fludarabine (25 mg/m^2) given from day −5 to day −1 (Supplementary Fig. S1B). The harvested autologous TIL (in ∼500 mL cell suspension in saline) were intravenously infused into each patient over a 20-minute period by gravity using a regular infusion line with an in-line 100 μm mesh to remove any large aggregates or debris. The following morning, the patients received bolus HD IL-2 (720,000 IU/kg) every 8 hours to tolerance (15). A second course of HD IL-2 therapy was given approximately 21 days after TIL infusion in a similar manner (Supplementary Fig. S1B). Hematologic and biochemical parameters were monitored daily during IL-2 administration. Intravenous blood samples (10 mL) were collected from patients before and after lymphodepletion on day 7 and day 0 before chemotherapy and before TIL infusion, respectively. Subsequent blood samples (50 mL) were collected on days 7, 14, 21, 35, and 70 after TIL infusion. The samples were analyzed for total white blood cell (WBC) count, absolute lymphocyte count (ALC), and absolute neutrophil count (ANC) in the Division of Pathology and Laboratory Medicine at MD Anderson Cancer Center.

Measurement of clinical responses by irRC and RECIST

Tumor response to therapy was done using the immune-related response criteria (irRC), a modified version of the WHO criteria (16). Response evaluation criteria in solid tumors (RECIST 1.1) was also used to assess the clinical response (17) to compare with the irRC response rates found. Throughout the study, irRC was used to determine official response rates and progression-free survival (PFS) times. For the purposes of this article, we define responders as complete and partial responders and nonresponders as patients with progressive or stable disease by determining the best overall response (BOR) using irRC (irBOR). Details on how and when tumor burden measurements were taken and irRC applied to determining the type of clinical response and whether any progression had occurred can be found in Supplementary Methods and Supplementary Table S2.

Flow cytometry analysis of infused TIL

Antibodies to human CD4, CD8, CD27, CD28, CD62L, CD45RA, and CD272 (BTLA, clone J168-540.90.22) were from BD Biosciences. Antibodies to human CD279 (PD-1, clone EH12.2H7) and Perforin (clone dG9) were obtained from BioLegend. Anti-human TIM3 (clone F38-2E2) was from eBiosciences, anti-human LAG-3 (clone 17B4) was obtained from Enzo Life Sciences, and anti-human CD270 (HVEM, clone ANC3B7) was purchased from Ancell. Staining of all TIL infusion products was conducted on samples cryopreserved immediately following the harvesting of the clinical TIL products and washing and concentrating using the Cobe 2991 machines. Details on the flow cytometry antibody staining techniques are available in Supplementary Methods online. Acquisition was done the next day after staining and fixing the samples. The samples were read on a FACSCanto II instrument (BD Biosciences) and data analysis was conducted using FlowJo software (Tree Star, Inc.). Live cells were first gated using forward scatter (FSC) and side scatter (SSC) parameters; dead cell exclusion was then conducted by gating out the Aqua-positive cells before gating on any antibody-positive populations. For $CD8^+$ differentiation status determination, the Aqua-negative cells were gated on $CD8^+CD4^-$ cells. This $CD8^+$ population was then further selected for $CD45RA^-$ (which constitute the overwhelming majority of TIL), and the resulting population was analyzed on a plot of CD62L versus CD27, where $CD27^+CD62L^+$ cells were designated TCM (central memory T cell), $CD27^+CD62L^-$ TEM (effector-memory), and $CD27^-CD62L^-$ TEFF (effector; refs. 18–20).

Tracking of TCR Vβ clonotypes after infusion by gene cloning and sequencing

Total RNA was isolated from TIL or patients' PBMC after adoptive transfer using Qiagen RNeasy Kit (74104). RNA quality was monitored by agarose gel electrophoresis to assess RNA degradation. TCR Vβ–specific cDNA was synthesized using Clonetech 5′ RACE Smarter kit. The primer in cDNA synthesis specifically binds to the Vβ constant region and can recognize both C1 and C2 (21). During cDNA synthesis, a 5′ end adapter was added to each cDNA. Nested primers for adapter and Vβ constant regions were used for PCR amplification. The resulting Vβ-specific PCR products were purified from an agarose gel and ligated into a TA cloning vector (Invitrogen). For TCR Vβ gene analysis, the CDR3 region of 96 TCR Vβ–positive DNA samples were prepared and confirmed and then sequenced. To characterize each individual TCR Vβ clonotype, sequence data from each sample was further analyzed using the international ImMunoGeneTics information system (IMGT) program. The frequency of each dominant Vβ clonotype was calculated by determining the percentage of each specific CDR3 sequence found within the 96 clones picked and sequenced.

Detection and measurement of telomere length in TIL by Southern blotting

Telomere length analysis of a sample of the TIL infusion product was conducted using Southern blotting technique (22, 23) on isolated genomic DNA with a TeloTAGGG Telomere Length Assay kit (Roche Diagnostics). The telomere lengths were then determined using the chemoluminescent telomeric repeat probes supplied with the kit followed by exposure to BioMax Light chemoluminescent film (Sigma-Aldrich). The telomere lengths were determined using the ImageJ analysis program downloaded from the NIH (Bethesda, MD) website (rsbweb.nih.gov/ij/) using a standard curve of migration distance from the first and longest telomere standard (21.4 Kb) to the last and shortest telomere standard (1.9 Kb). Details on the methodology used with the kit to blot for telomeres and the determination of telomere lengths from the exposed blots can be found in Supplementary Methods online.

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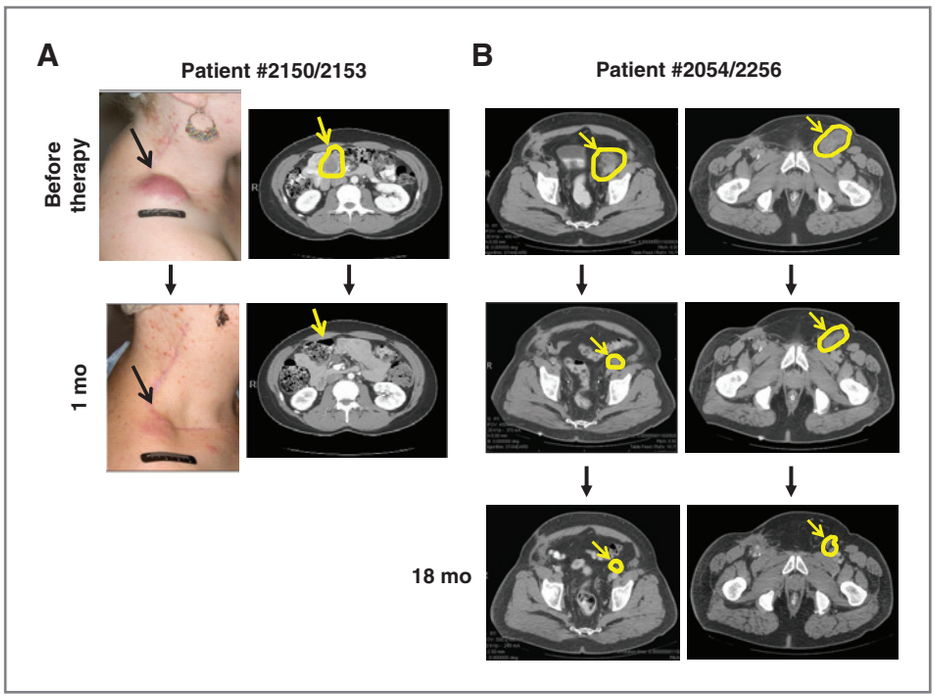

Figure 1. Objective tumor regression in patients receiving autologous TIL therapy. CT scans and photos of 2 representative patients (#2150/2153 and #2054/2256) before and after TIL therapy at different time points. Changes in a large subcutaneous mass in the right shoulder and of a large inguinal lymph node lesion after 1 month are shown for patient #2150/2153 (A). Changes are shown in abdominal tumors after 1 month and 18 months after TIL infusion in patient #2054/2256 (B).

Statistical analysis and correction for multiple variable testing

Fisher exact tests (24) were used to assess association between categorical variables and tumor response and Wilcoxon rank-sum tests (25) were used to compare continuous variables by tumor response or the type of T cell. Simple linear regression was conducted (Fig. 4D) to assess the association between a continuous predictor and percentage change in tumor burden. Box and whisker plots have been provided for several variables of interest. Because of the large number of comparisons, to account for multiple testing, we set our type 1 error rate to 0.001. All statistical analyses were conducted using SAS 9.2 for Windows (Copyright 2011 by SAS Institute Inc.) or GraphPad Prism 5 software.

Results

Patient population and TIL therapy

Patients of any HLA subtype with stage IIIc to IV (M1a-M1c) disease were recruited into the study. TILs were first expanded from tumor fragments with IL-2 for 5 weeks and cryopreserved for further expansion in the REP if a minimum of 48×10^6 cells was reached. This was required so that at least 40×10^6 cells were available for the REP after samples for quality control and antitumor analysis (8×10^6 cells) were removed. Previous data from our group have found that the success rate in meeting this threshold for pre-REP TIL expansion is about 62% regardless of the type of prior therapy before the tumor harvest (14). Patients with cryopreserved pre-REP TIL having progressive disease and who met eligibility criteria were treated after further expansion of their thawed TIL using the REP. Only patients treated with their final post-REP TIL product were included in this study. Supplementary Table S1 summarizes the demographic and clinical characteristics of the treated patients ($n = 31$). Most patients were stage IV at the time of TIL infusion and had received a variety of different prior therapies within 2 months of therapy, including biochemotherapy, radiotherapy, targeted therapy, and immunotherapy with IL-2, IFN-α, or granulocyte macrophage colony-stimulating factor (GM-CSF). Most patients received at least 2 prior therapies for metastatic disease. Only one patient was treated with a selective mutant B-RAF inhibitor (GSK-2118436; ref. 26), and none of the patients had prior ipilimumab therapy. The median age was 46 years with 65% males. Figure 1B shows the overall scheme for patient therapy, indicating the time points for the starting the lymphodepleting preparative regimen (day −7), TIL infusion (day 0), followed by the 2 cycles of HD IL-2 therapy. The total TIL infused ranged from 8 to 150×10^9 cells (Table 1).

Treatment-related toxicity and measurement of hematopoietic parameters

Patients experienced only transient, reversible adverse reactions (e.g., fever, chills, shortness of breath, increased heart rate) in the few hours following TIL transfer. No grade 3 or 4 toxicities according to the National Cancer Institute Common Toxicity Criteria (NCICTC) were noted after TIL infusion before HD IL-2 therapy (27). Hematologic toxicities due to the preparative chemotherapy were anticipated and transient. Neutropenia and lymphopenia were observed in all patients as expected. All patients were also treated with platelet transfusions and red blood cell

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transfusion as needed and their counts eventually increased back to normal levels.

Supplementary Fig. S2 shows the drop of total white blood cells (WBC), absolute neutrophil count (ANC), and absolute lymphocyte count (ALC) after the preparative chemotherapy and also shows the recovery of these populations following TIL infusion over a 70-day period in all patients, comparing the responders and nonresponders. There was no statistically significant difference in the percentage change from day −7 to day 70 between responders and nonresponders for WBC, ALC, or ANC (Supplementary Fig. S2). The usual grade 3 and 4 toxicities according to the NCICTC were found with bolus HD IL-2 infusion (pulmonary, renal, and liver dysfunction, and mental confusion). However, all of these were transient in nature and responded to standard interventions and/or resolved after IL-2 therapy. Most patients (28/31) received 2 cycles of IL-2 therapy.

Tumor response and association with patient clinical parameters

Table 1 provides a summary of major patient clinical parameters and clinical responses following TIL therapy. Clinical response rates according to the new irRC criteria established by Wolchok and colleagues (16) more closely reflects the dynamics of a response one would expect with immunotherapies as opposed to chemotherapies. We noted objective tumor regression of tumors in multiple sites in responding patients, including subcutaneous, lung, liver, lymph node, and of tumors growing attached to major organs such as the spleen and heart. Figure 1 shows examples of objective tumor regressions at visceral and nonvisceral sites following TIL infusion in 2 representative patients by CT scans and photographs. We also noted in some cases that a mass underwent a durable partial regression by CT, and the remnant lesion was no longer 2-fluorodeoxyglucose (FDG) avid by positron emission tomography (PET). An example of this is shown in patient #2262 (Supplementary Fig. S3) in which a large tumor mass attached to the aorta and the heart shrunk by more than 60% in volume at 18 months after TIL infusion and the remnant lesion persisting did not show any activity by PET scanning.

No association was found between a number of other variables, such as patient gender and age, tumor stage, number, and type of prior therapy for metastatic disease, initial tumor burden, serum LDH levels at time of TIL transfer, and number of IL-2 doses administered after TIL transfer), and clinical response (Supplementary Tables S3 and S4). Using irRC, the BOR [partial response (PR) and complete response (CR)] was 48.4% (15/31). Two patients (6.5%) experienced a CR. Many of the clinical responses have been quite extensive, with 12 of 15 responders having more than 70% reduction and 4 of 15 having a 100% reduction in their measurable tumor burden, as shown in a waterfall plot analysis (Fig. 2). Two patients had a complete regression of all measurable lesions by CT scan, but had nonmeasurable lesions in the bone that remained stable.

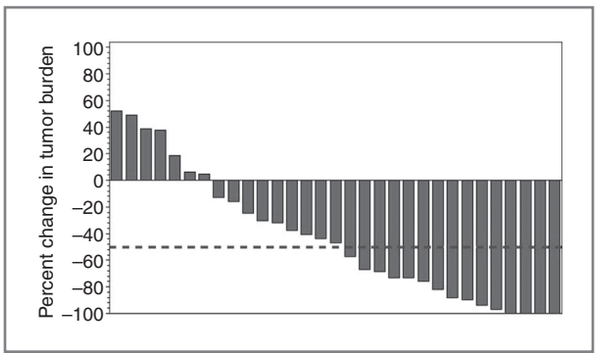

Figure. 2. Waterfall plot of percentage change in measurable tumor burden in all treated patients ($n = 31$). The patients were treated between August 23, 2007 and October 6, 2010. Clinical responses were evaluated using the irRC criteria from whole body CT scans, as described in the Materials and Methods section. The percentage change in tumor burden using the best overall irRC response after TIL infusion is shown for all patients. The dotted line indicates the −50% tumor burden reduction point needed to reach an objective clinical response according to irRC. Of the 15 patients who responded, 12 had 70% or more reduction in their tumor burden and 4 patients had a 100% reduction in measurable tumor burden. Two patients had nonmeasurable bony lesions that remained stable throughout the study period. This was not accounted for in determining the change in tumor burden shown.

We also measured responses using RECIST 1.1 (17) and found that the BOR was 41.9% (13/31). Overall, 2 patients scored as responders with irRC (16), but were nonresponders (PD) using RECIST due to the development of new lesions detected after TIL infusion. One of these patients (#2262) developed a small brain lesion 2 months after TIL ACT that was surgically resected, but was still considered as progression-free according to irRC. At follow-up, this patient was progression-free by irRC after surgery for more than 22 months and the initial sites of disease continued to respond. The other patient (#2267) had a PR by irRC but had a developing new small brain lesion by 2 months after TIL treatment. This patient was treated by whole brain radiation and was alive more than 22 months after TIL therapy.

Overall survival (OS) and PFS were estimated from the treatment start date for all 31 patients (Supplementary Fig. S4). The median OS time was not reached by the end study; the median PFS time was 7.6 months (95% CI: 4.1–22.2 months). The 6-month OS and PFS rates were 81% (95% CI: 62–91%) and 57% (95% CI: 38–73%), respectively. To compare unbiased differences in OS and PFS between responders and nonresponders, we conducted a landmark survival analysis (28) starting at the 3-month time point after TIL infusion (Supplementary Fig. S5). The landmark 6-month PFS rates for irRC responders and nonresponders were 88% (95% CI: 39–98%) and 47% (95% CI: 21–69%), respectively. The landmark 18-month OS rates for responders and nonresponders at the landmark were 87% (95% CI: 36–98%) and 48% (95% CI: 26–67%), respectively. The landmark median OS time for nonresponders was 463 days but could not be estimated for responders. The landmark median PFS times for nonresponders and

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responders were 105 days and 577 days. Change in tumor burden over time in all treated patients over the first 20 to 22 months after TIL infusion can be found in Supplementary Fig. S6. Most of the responders showed tumor regression of 50% or more by 4 months, whereas some responders showed a more protracted decrease in tumor burden. Interestingly, patient #2175 (nonresponder) exhibited a prolonged SD with more than 29 months PFS by irRC (Supplementary Fig. S6). Five responding patients, although achieving a response by irRC, were treated with additional therapies for renewed progression within the study period; these patients are listed in Supplementary Fig. S6C together with the time point and type of therapy they received after TIL.

Pre-REP antitumor reactivity and clinical response

We determined the antitumor reactivity of the pre-REP TIL as a possible predictive biomarker for irRC clinical response. Pre-REP TIL were cultured in IL-2 from tumor fragments for 5 weeks and tested with either autologous melanoma tumor cell lines (if available) or semiallogeneic cell lines matched minimally at one HLA-A allele. We have found that IL-2- expanded TIL were functional and responded to polyclonal TCR using anti-CD3 stimulation in both IFN-γ and cytotoxic T lymphocyte (CTL) assays (data not shown). The percentage of treated patients having TIL exhibiting significant antitumor–specific IFN-γ responses (≥100 pg/mL IFN-γ after subtraction of controls) either against an autologous or at least one allogeneic matched melanoma cell line was 71% (22/31). We were able to generate an autologous melanoma cell line for testing the pre-REP TIL for 17 of 31 (55%) of the patients and among these, all but 2 TILs had specific antitumor IFN-γ responses. No significant association was found between positive antitumor reactivity and clinical response ($P = 0.46$; Supplementary Table S3).

Immunophenotyping of infused TIL using flow cytometry

As shown in Table 1, the total amount of infused TIL varied across the patient population. Responding patients were infused with significantly more TIL (median 99×10^9 cells) than nonresponders (median 55×10^9; $P = 0.0003$; Supplementary Table S4, Fig 3A). We further analyzed the different subsets of T cells using multicolor flow cytometry for the content of $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells, which revealed that both the percentage and total number of $CD8^+$ T cells infused were significantly associated with clinical response ($P = 0.001$ and 0.0003, respectively; Supplementary Table S4, Fig. 3B). Nonresponders had significantly higher percentages of $CD4^+$ TIL ($P = 0.001$; Supplementary Table S4, Fig. 3C). We further analyzed the role of total $CD8^+$ TIL as a continuous predictor of percentage change in tumor burden ($P = 0.0003$, Fig. 3D).

We analyzed the state of differentiation or memory status of the $CD8^+$ subset in the infused TIL. We stained the $CD8^+$ TIL for cell surface markers associated with naïve, TEM, TCM, and more TEFF T cells, using the flow cytometry markers described in the Materials and Methods (19, 29). A viability dye was used to exclude any dead cells from the analysis. Most of the infused $CD8^+$ TIL infused were composed of cells with a TEM ($CD8^+CD45RA^-CD62L^-CD27^+$) and TEFF ($CD8^+CD45RA^-CD62L^-CD27^-$) phenotype with few TCM phenotype ($CD8^+CD45RA^-CD62L^+CD27^+$) cells (Fig. 4A). Responding patients had a significantly higher percentage of TEFF cells than nonresponders ($P = 0.0004$; Fig. 4D).

We also explored additional markers associated with $CD8^+$ cytotoxic T-cell activation and differentiation, including PD-1, BTLA, and TIM-3, all of which can function as inhibitory coreceptors on $CD8^+$ T cells (30–32). As shown in Fig. 5A, the percentage of $CD8^+$ T cells expressing PD-1, BTLA, and TIM-3 varied widely across the entire treated population. Responding patients had significantly higher percentages of $CD8^+BTLA^+$ T cells ($P = 0.0006$; Fig. 5B) and $CD8^+BTLA^+$ with a TEFF phenotype ($CD45RA^-CD62L^-CD27^-BTLA^+$; $P = 0.0002$; Fig. 5C). The percentage of $CD8^+PD\text{-}1^+$ TIL was not significantly associated with clinical response ($P = 0.07$; Fig. 5B). There was a trend towards responders receiving a higher percentage of $CD8^+TIM\text{-}3^+$ T cells in their TIL (Fig. 5B). However, analysis of BTLA coexpression with TIM-3 revealed that only $CD8^+TIM\text{-}3^+$ T cells coexpressing BTLA were associated with clinical response, whereas those lacking BTLA coexpression were not (Fig. 5D).

Analysis of TIL Vβ clonotype persistence *in vivo*

Persistence of infused TIL for at least 1 month following adoptive transfer into lymphodepleted patients with metastatic melanoma has been previously found to be associated with objective clinical response to therapy (21). We used a TCR Vβ cloning and CDR3 sequencing approach used previously to track the changes in dominant TCR Vβ clonotypes in PBMC in 5 responding patients up to 22 months after TIL infusion (patients #2131, #2150/2153, #2258, #2124, and #2180). As shown in Supplementary Table S5, some dominant Vβ clonotypes in the original TIL persisted long-term over the entire 22-month period (e.g., Vβ24-1 and Vβ12-3 in patient #2150/2153, and Vβ4-1 and Vβ29 in patient# 2131). However, a significant number of dominant TIL Vβ clonotypes also became undetectable after 1 month, while other previously undetectable Vβ clonotypes emerged and expanded over the following months (Table S5). Similar results were found in other responding patients.

TIL from responders and nonresponders did not significantly differ in telomere length

The telomere length of infused TIL products was retrospectively evaluated from cryopreserved samples. A Southern blot method using complementary telomere repeat probes on restriction enzyme-digested genomic DNA was conducted to determine not only the average telomere length found in each TIL sample, but also the range of telomere lengths that occur (23, 33). Southern blotting has

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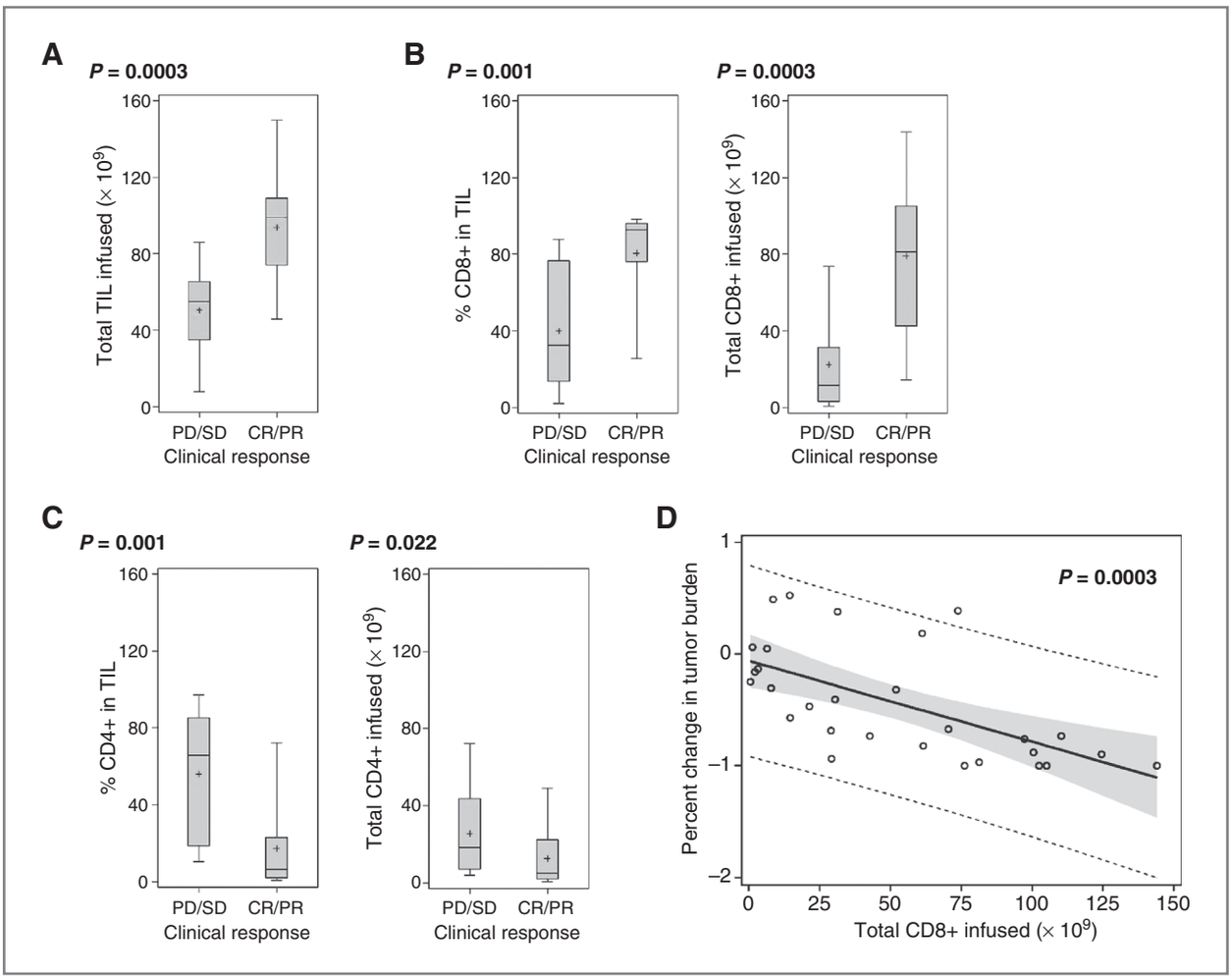

Figure. 3. Comparison of total cells infused and major T-cell subsets in the infused TIL product between responders and nonresponders. Comparison of total TIL infused (A), the percentage and total number of infused $CD8^+$ T cells (B), and the percentage and total number of $CD4^+$ T cells (C) between responders and nonresponders. Linear regression showing the relationship between total $CD8^+$ infused and percentage change in tumor burden ($P = 0.0003$), with the solid line in showing the best fit, the broken line representing the 95% prediction limits, and the gray area indicating the 95% confidence limits (D).

been the most used approach to determine both of these parameters (22, 23). This analysis revealed that each TIL sample did not have a single fixed telomere length for all the cells, but had a range of telomere lengths between 11 and 3 Kb indicating a heterogeneous population of cells of different ages that have undergone different numbers of cell divisions. We determined telomere length corresponding to the peak signal intensity in each lane (patient) representing the telomere length of highest frequency in the cell population. Using this measurement as the overall telomere length in the TIL population, there were no statistically significant differences in telomere lengths between responders and nonresponders ($P = 0.3671$; Supplementary Fig. S7) or by age ($P > 0.05$).

Discussion

In this study, we show that the adoptive transfer of highly expanded melanoma TIL into patients with metastatic melanoma who had prior transient lymphodepletion can mediate partial and even complete regression of disease at multiple organ sites. Our reported clinical response rates using irRC were 48.4% (15/31 responders) and are comparable with previously reported TIL therapy trials using RECIST (10, 11). A large proportion of our responders (12/15) had more than 70% tumor regression with 4 responders achieving a complete reduction in their measurable tumor burden. Only 2 of these patients were scored as CR, as they continued to have nonmeasurable, yet stable, lesions in the bone. Our landmark analysis revealed a significant increase in OS of TIL responders over nonresponders ($P = 0.033$). These results suggest that an initial response to TIL therapy is of significant clinical benefit in these patients.

The total number of infused TIL was a critical parameter associated with clinical response, with responders being infused on average with almost twice the number of TIL as

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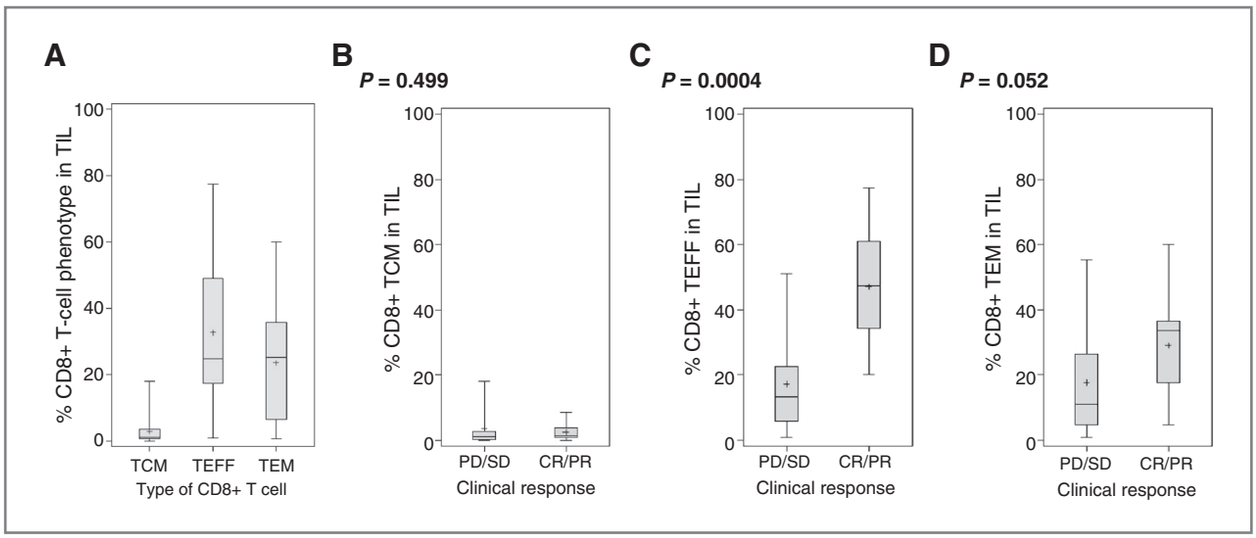

Figure. 4. Comparison of $CD8^+$ T-cell memory phenotype in responders and nonresponders. The percentage of TCM, TEM, and TEFF in the $CD8^+$ TIL subset in all treated patients (A). Wilcoxon rank-sum tests showed significant differences at $\alpha = 0.001$ between the percentage of $CD8^+$ TIL between TCM and TEFF ($P < 0.0001$) and TCM and TEM ($P < 0.0001$). In the subsequent panels, the percentage of $CD8^+$ T cells with a TCM (B), TEM (C), and TEFF (D) in the infused TIL was compared between responders and nonresponders.

nonresponders. These results suggest that ACT using TIL should aim to infuse as many T cells as possible to ensure more consistent clinical benefit. We also tested whether analysis of TIL antitumor reactivity at the pre-REP stage can be a predictive biomarker of clinical response. This would be beneficial in selecting which TIL to further expand in large scale for therapy. However, our data were inconclusive and suggest that positive IFN-γ secretion against melanoma cells at this pre-REP stage may not be predictive and that other biomarkers in the TIL, tumor microenvironment, or differences in systemic factors may ultimately play a defining role. However, measurement of antitumor reactivity of TIL in the actual infusion products (post-REP) will be needed to make a more definitive conclusion regarding this issue.

Recently, there has been an increasing interest in identifying additional predictive biomarkers in the TIL associated with the induction objective clinical responses and durable survival. A number of markers such as telomere length of the infused T cells, the type of T cells ($CD4^+$ vs. $CD8^+$) and their differentiation status, as well as the differential expression of positive and negative T-cell costimulatory molecules, can affect both the persistence of adoptively transferred T cells and their effector function *in vivo*. In this regard, one striking observation besides the significantly higher number of TIL infused into the responding patients was that the $CD8^+$ T-cell lineage seemed to be the key active component in a large majority of patients. Both a higher percentage and higher total number of infused $CD8^+$ T cells was significantly associated with response, whereas the opposite trend was found with $CD4^+$ T cells. This suggests that $CD8^+$ T cells are the key driving force behind the antitumor activity of TIL. In certain regards, this is expected due to CTL activity attributed to $CD8^+$ T cells, but formal proof of this association in TIL therapy has been lacking until now.

Overall, our results suggest that TIL therapy protocols should aim to maximize the number of $CD8^+$ T cells generated. This can be achieved by the selective isolation of $CD8^+$ T cells at the pre-REP stage, or providing additional factors in the REP that can facilitate $CD8^+$ T-cell activation and division. In this regard, we have recently found that provision of costimulatory signals through CD137 (4–1BB) could significantly increase the survival and yield of $CD8^+$ TIL (34). Despite the positive association of $CD8^+$ T cells in TIL and clinical response, there was, however, a minority of responders (2/15 patients) that had $CD4^+$ T cells making up more than 50% of the infused TIL (#2054/2256 and #2267). Adoptively transferred $CD4^+$ T cells can in some circumstances mediate tumor regression by providing help to $CD8^+$ T cells or having a direct antitumor effector function by secreting T-helper 1 (T_H1) cytokines (e.g., IFN-γ and TNF-α) or by direct killing of HLA class II-expressing tumor targets by expressing granzymes and perforin (35). It will be important to determine what is the mechanism of action of these $CD4^+$ T cells in these selected patients and whether they act on their own or cooperate with other $CD8^+$ T cells in TIL or recruit endogenous $CD8^+$ T-cell responses to kill tumors as a results of antigen or epitope spreading.

The positive role of $CD8^+$ T cells leads to another critical question regarding the state of $CD8^+$ CTL differentiation in the infused TIL and how it is related to the response. We found that most of the $CD8^+$ T cells in the infused TIL had the phenotype of either TEM ($CD45RA^-CD62L^-CD27^+CD28^+$) or more-differentiated TEFF ($CD45RA^-CD62L^-CD27^-CD28^{+/-}$) cells, with negligible levels of naïve and few central memory cells.

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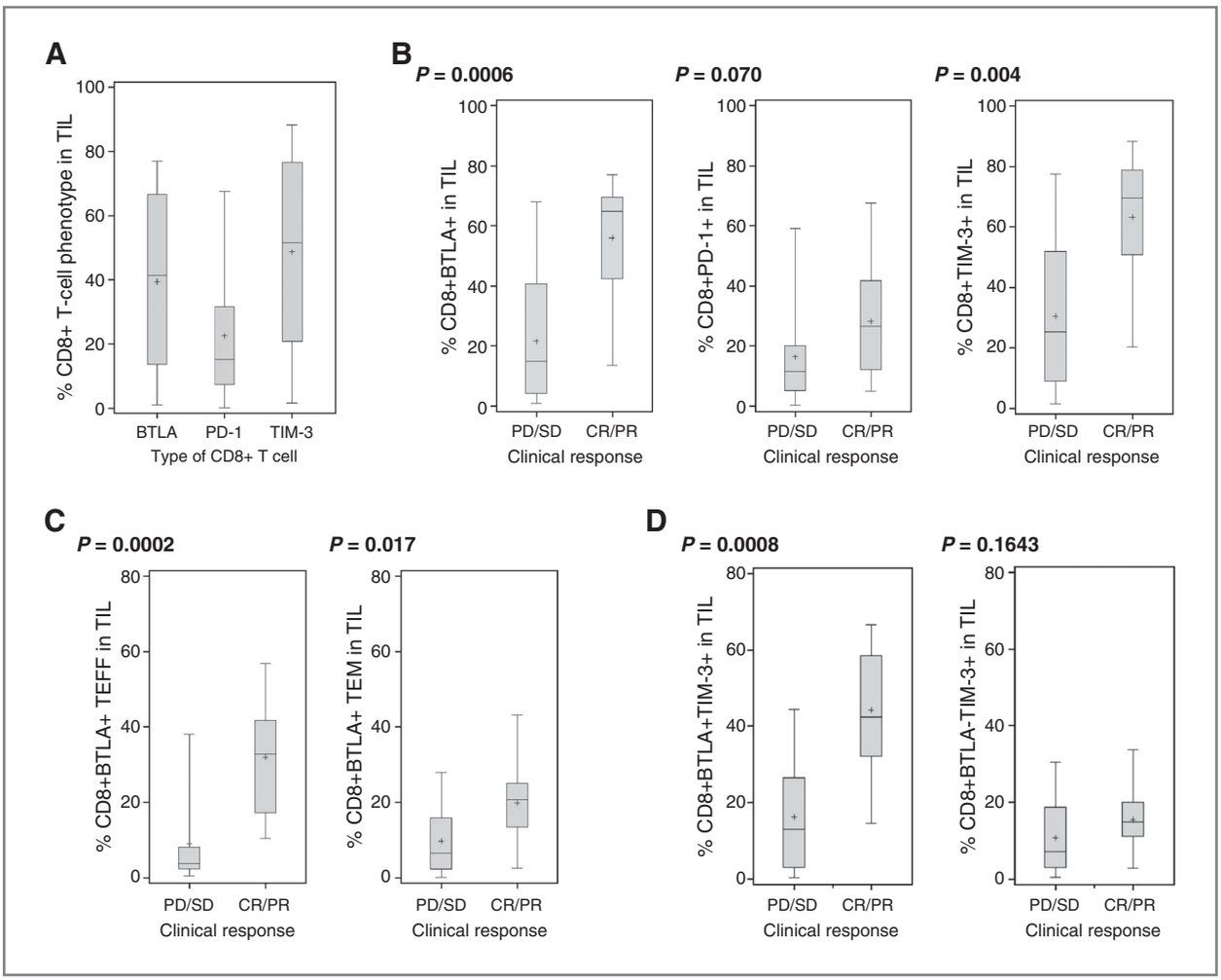

Figure. 5. Comparison of PD-1, BTLA, and TIM-3 on $CD8^+$ TIL in responders and nonresponders. The percentage of cells in the total $CD8^+$ TIL population expressing PD-1, BTLA, and TIM-3 in all treated patients (A). Wilcoxon rank-sum tests showed significant differences at $\alpha = 0.001$ between the percentage of $CD8^+$ TIL between PD-1 and TIM-3 ($P = 0.0006$). The percentage of $CD8^+PD\text{-}1^+$, $CD8^+BTLA^+$, and $CD8^+TIM\text{-}3^+$ expression in the infused TIL was compared between responders and nonresponders (B). The percentage of $CD8^+BTLA^+$ T cells in the infused TIL with a TEFF or TEM phenotype was compared between responders and nonresponders (C). The percentage of $CD8^+TIM\text{-}3^+$ T cells in the infused TIL with or without BTLA coexpression was compared between responders and nonresponders (D).

Although previous data have suggested that the number and percentage of infused $CD8^+CD27^+$ TEM cells are critical components (36), we found that $CD8^+$ TEFF cells that are $CD27^-$ seem to be more clinically relevant than TEM cells in mediating antitumor responses. One possible caveat that has been raised previously is that the conditions during the REP of the TIL induces transient CD27 downmodulation (37), but in 2 published reports on multiple patient TIL subjected to the REP, we have found no significant downmodulation of CD27 expression on the $CD8^+$ T cells (8, 34).

The reason why $CD8^+$ TEFF phenotype cells seem to be critical in our clinical trial over TEM is unclear and seems counterintuitive. It is possible that the TEFF cells have improved migratory properties into tumors by expressing more chemokine receptors, such as CXCR3 and CX3CR1 (38, 39). However, we have not formally tested this question yet. Human $CD8^+CD27^-$ TEFF have been characterized as having a shorter lifespan than TEM (18, 40), but the situation in a prior lymphodepleted host may be different where these cells may be exposed to higher amounts of homeostatic cytokines, such as IL-15 and costimulatory signals, that may facilitate their persistence and telomerase expression. Many $CD8^+$ T-cell clones specific for chronic viruses such as cytomegalovirus (CMV) and Epstein–Barr virus (EBV) infections in adulthood are highly differentiated cells that nevertheless can persist for long periods of time and are spontaneously cytolytic, or rapidly reexpress cytolytic granule molecules upon antigen exposure and kill target cells; these cells are critical in protecting the host against viral reactivation for years (20). Another emerging issue is that adoptively transferred $CD8^+$ T cells in lymphodeleted hosts

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can exhibit plasticity, with either $CD8^+$ T cells with a TEM profile rapidly losing CD27 or with some TEFF cells gaining CD27 expression (41). Finally, our data here do not preclude a critical role for less-differentiated $CD8^+$ TEM. These cells may cooperate with TEFF cells and have a critical role in the long-term control of tumor growth not readily apparent in our study here.

One of the most unexpected findings of this study came after examining the association of the "negative" costimulatory molecules, PD-1, BTLA, and TIM-3 (30, 42, 43) on infused TIL with clinical response. Significant heterogeneity in PD-1, BTLA, and TIM-3 expression was found on $CD8^+$ T cells. Surprisingly, we found that an increased frequency of $CD8^+BTLA^+$ TIL as well as higher numbers of infused $CD8^+$ $BTLA^+$ TIL were both highly associated with a favorable clinical response. Interestingly, the extent of PD-1 expression on $CD8^+$ TIL was not observed to be related to the clinical response, and, in fact, TIL from responding patients tended to have higher frequencies of PD-1^+ T cells. Similarly, TIM-3 was also highly expressed in $CD8^+$ TIL infused into responding patients, but on its own was not observed to be predictive of response without the coexpression of BTLA. These results suggest that BTLA expression could be an important new biomarker in T-cell adoptive cell therapy that needs to be studied further in future TIL clinical trials to verify its predictive value and its possible biologic role.

BTLA is a more distant member of the immunoglobulin family binding to an unusual ligand called herpes virus entry mediator (HVEM) leading to SHP1 and SHP2 phosphatase activity dampening T-cell activation signals (44). BTLA is constitutively on naïve T cells and tends to be downmodulated in $CD8^+$ T cells as they mature into effector cells (42). Interestingly, BTLA has been found to be continually expressed (not downmodulated) during differentiation of certain melanoma antigen-specific (e.g., MART-1) $CD8^+$ T cells towards effector cells (42). The reasons for this, however, are unclear. Whether BTLA plays a functional role in $CD8^+$ TIL or whether it simply is a marker for TIL associated with improved antitumor activity *in vivo* after cell transfer needs to be determined. In addition, although BTLA is considered a negative costimulatory molecule (32), its role at different stages of $CD8^+$ T-cell differentiation has not been clearly defined, and recent data suggest that it may in fact also play a prosurvival function by binding to HVEM on activated $CD8^+$ T cells and triggering NF-κB activation (45, 46).

Another correlative parameter studied in T-cell therapy is the telomere repeat lengths of infused TIL. Telomere length has been linked to shorter persistence of transferred TIL *in vivo* (47). Using the Southern blotting approach, we found no significant difference between the mean telomere length in TIL between responders and nonresponders. Responders had a tendency to have slightly longer telomere lengths, but this was found not to be significant. It is also important to point out that the median telomere lengths found in our study for both responders and nonresponders here (median of 5.9 Kb and 5.4, respectively), as well as in previous TIL therapy studies (36), are in fact within the same range as those found in original studies on human lymphocyte senescence showing telomeric repeat lengths in the range of 4.7 to 6.3 Kb in senescing T cells by Southern blotting (23). However, our Southern blotting analysis also revealed that a considerable heterogeneity existed in the telomere lengths of infused TIL with a range of 3 and 11 Kb found in the blots of both responders and nonresponders. Thus, it is possible that smaller subpopulations of TIL still retain relatively long telomeres above this senescent range (>6.3 Kb) with a higher capacity to persist and expand *in vivo*, whereas other TIL populations with shorter telomere lengths have a reduced cell division potential and lifespan. Overall, our data underscores the difficulty in interpreting the impact of telomere length as a biomarker because there is not one single telomere length that can be assigned to the entire TIL population and considerable heterogeneity exists.

In summary, we have found that the adoptive transfer of TIL expanded *ex vivo* can induce a high rate of objective tumor regression in unresectable metastatic melanoma patients and that these responses can be long lasting in a significant fraction of treated patients. Moreover, our laboratory correlative studies have revealed potential biomarkers in adoptively transferred TIL associated with clinical response, such as the expression of BTLA. Future efforts will focus on further defining the role of BTLA in the antitumor response and enhancing predictive assays of response, developing strategies to generate more effective T cells, identifying ideal patient candidates, and exploring future TIL clinical trials in combination with new FDA-approved agents for melanoma.

Disclosure of Potential Conflicts of Interest

L.G. Radvanyi is a consultant/advisory board member in Genesis Biopharma. M.I. Ross is a consultant/advisory board member in Merck, Genomic Health, and GlaxoSmithKline. J.E. Gershenwald is a consultant/advisory board member in GlaxoSmithKline. No potential conflicts of interest were disclosed by other authors.

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Development of methodology: L.G. Radvanyi, C. Bernatchez, M. Zhang, J. Chacon, R.C. Wu, G. Lizee, J.D. McMannis, R. Ramachandran, P. Hwu

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.G. Radvanyi, C. Bernatchez, M. Zhang, P. Miller, J. Chacon, R.C. Wu, S. Mahoney, J.D. McMannis, E.J. Shpall, V.G. Prieto, K.B. Kim, J. Homsi, A.Y. Bedikian, W.-J. Hwu, S. Patel, J.E. Gershenwald, A. Lucci, R. Royal, J.N. Cormier, O.J. Fulbright, C. Toth, R. Ramachandran, S. Wardell, A. Gonzalez, P. Hwu

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.G. Radvanyi, C. Bernatchez, P. Fox, G. Alvarado, V. Johnson, J.E. Lee, R. Royal, P. Hwu

Writing, review, and/or revision of the manuscript: L.G. Radvanyi, C. Bernatchez, M. Zhang, P. Fox, G. Alvarado, V. Johnson, V.G. Prieto, N.E. Papadopoulos, K.B. Kim, A.Y. Bedikian, W.-J. Hwu, S. Patel, M.I. Ross, J.E. Lee, J.E. Gershenwald, R. Royal, J.N. Cormier, M.A. Davies, P. Hwu

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.G. Radvanyi, C. Bernatchez, M. Zhang, G. Alvarado, R. Mansaray, S. Wardell, A. Gonzalez, P. Hwu

Study supervision: L.G. Radvanyi, C. Bernatchez, M. Zhang, O.J. Fulbright, C. Toth, P. Hwu

Other: Research nurse in charge of the study, M. Glass

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References

1. Trinh VA. Current management of metastatic melanoma. Am J Health Syst Pharm 2008;65:S3–8.
2. Quirt I, Verma S, Petrella T, Bak K, Charette M. Temozolomide for the treatment of metastatic melanoma: a systematic review. Oncologist 2007;12:1114–23.
3. Fecher LA, Amaravadi RK, Flaherty KT. The MAPK pathway in melanoma. Curr Opin Oncol 2008;20:183–9.
4. Flaherty KT, McArthur G. BRAF, a target in melanoma: implications for solid tumor drug development. Cancer 2010;116:4902–13.
5. Flaherty KT, Yasothan U, Kirkpatrick P. Vemurafenib. Nat Rev Drug Discov 2010;10:811–2.
6. Flaherty KT. Targeting metastatic melanoma. Annu Rev Med 2012; 63:177–83.
7. Weber J. Immunotherapy for melanoma. Curr Opin Oncol 2011;23: 163–9.
8. Li Y, Liu S, Hernandez J, Vence L, Hwu P, Radvanyi L. MART-1-specific melanoma tumor-infiltrating lymphocytes maintaining CD28 expression have improved survival and expansion capability following antigenic restimulation *in vitro*. J Immunol 2010;184:452–65.
9. Hoos A, Ibrahim R, Korman A, Abdallah K, Berman D, Shahabi V, et al. Development of ipilimumab: contribution to a new paradigm for cancer immunotherapy. Semin Oncol 2011;37:533–46.
10. Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J Clin Oncol 2005;23:2346–57.
11. Besser MJ, Shapira-Frommer R, Treves AJ, Zippel D, Itzhaki O, Hershkovitz L, et al. Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. Clin Cancer Res 2010;16: 2646–55.
12. Dudley ME, Wunderlich JR, Shelton TE, Even J, Rosenberg SA. Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients. J Immunother 2003;26:332–42.
13. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 2002;298:850–4.
14. Joseph RW, Peddareddigari VR, Liu P, Miller PW, Overwijk WW, Bekele NB, et al. Impact of clinical and pathologic features on tumor-infiltrating lymphocyte expansion from surgically excised melanoma metastases for adoptive T-cell therapy. Clin Cancer Res 2011;17:4882–91.
15. Atkins MB, Kunkel L, Sznol M, Rosenberg SA. High-dose recombinant interleukin-2 therapy in patients with metastatic melanoma: long-term survival update. Cancer J Sci Am 2000;6 Suppl 1:S11–4.
16. Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbe C, et al. Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. Clin Cancer Res 2009; 15:7412–20.
17. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 2009;45:228–47.
18. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, et al. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med 2002;8:379–85.
19. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 2004;22:745–63.
20. Wherry EJ, Ahmed R. Memory CD8 T-cell differentiation during viral infection. J Virol 2004;78:5535–45.
21. Huang J, Khong HT, Dudley ME, El-Gamil M, Li YF, Rosenberg SA, et al. Survival, persistence, and progressive differentiation of adoptively transferred tumor-reactive T cells associated with tumor regression. J Immunother 2005;28:258–67.
22. Kimura M, Stone RC, Hunt SC, Skurnick J, Lu X, Cao X, et al. Measurement of telomere length by the Southern blot analysis of terminal restriction fragment lengths. Nat Protoc 2010;5:1596–607.
23. Vaziri H, Schachter F, Uchida I, Wei L, Zhu X, Effros R, et al. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. Am J Hum Genet 1993;52:661–7.
24. Fisher RA. On the interpretation of *X2* from contingency tables, and the calculation of P. J R Stat Soc. 1922;85:87–94.
25. Randles R, Wolfe D. Introduction to the Theory of Nonparametric Statistics. New York: John Wiley; 1979.
26. Arkenau HT, Kefford R, Long GV. Targeting BRAF for patients with melanoma. Br J Cancer 2010;104:392–8.
27. Shimizu T, Saijo N. [Common toxicity criteria: version 2.0, an improved reference for grading the adverse reaction of cancer treatment]. Nihon Rinsho 2003;61:937–42.
28. Dafni U. Landmark analysis at the 25-year landmark point. Circ Cardiovasc Qual Outcomes 2011;4:363–71.
29. Romero P, Zippelius A, Kurth I, Pittet MJ, Touvrey C, Iancu EM, et al. Four functionally distinct populations of human effector-memory CD8+ T lymphocytes. J Immunol 2007;178:4112–9.
30. Ahmadzadeh M, Johnson LA, Heemskerk B, Wunderlich JR, Dudley ME, White DE, et al. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. Blood 2009;114:1537–44.
31. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. J Exp Med 2010;207:2175–86.
32. Paulos CM, June CH. Putting the brakes on BTLA in T cell-mediated cancer immunotherapy. J Clin Invest 2010;120:76–80.
33. Effros RB, Allsopp R, Chiu CP, Hausner MA, Hirji K, Wang L, et al. Shortened telomeres in the expanded CD28-CD8+ cell subset in HIV disease implicate replicative senescence in HIV pathogenesis. AIDS 1996;10:F17–22.
34. Hernandez-Chacon JA, Li Y, Wu RC, Bernatchez C, Wang Y, Weber JS, et al. Costimulation through the CD137/4–1BB pathway protects human melanoma tumor-infiltrating lymphocytes from activation-induced cell death and enhances antitumor effector function. J Immunother 2011;34:236–50.
35. Qui HZ, Hagymasi AT, Bandyopadhyay S, St Rose MC, Ramanarasimhaiah R, Menoret A, et al. CD134 plus CD137 dual costimulation induces Eomesodermin in CD4 T cells to program cytotoxic Th1 differentiation. J Immunol 2011;187:3555–64.
36. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res 2011;17:4550–7.

Published OnlineFirst October 2, 2012; DOI: 10.1158/1078-0432.CCR-12-1177

37. Huang J, Kerstann KW, Ahmadzadeh M, Li YF, El-Gamil M, Rosenberg SA, et al. Modulation by IL-2 of CD70 and CD27 expression on CD8+ T cells: importance for the therapeutic effectiveness of cell transfer immunotherapy. J Immunol 2006;176:7726–35.
38. Dengel LT, Norrod AG, Gregory BL, Clancy-Thompson E, Burdick MD, Strieter RM, et al. Interferons induce CXCR3-cognate chemokine production by human metastatic melanoma. J Immunother 2010;33:965–74.
39. Wenzel J, Bekisch B, Uerlich M, Haller O, Bieber T, Tuting T. Type I interferon-associated recruitment of cytotoxic lymphocytes: a common mechanism in regressive melanocytic lesions. Am J Clin Pathol 2005;124:37–48.
40. Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol 2002;2:251–62.
41. Powell DJ Jr, Dudley ME, Robbins PF, Rosenberg SA. Transition of late-stage effector T cells to CD27+ CD28+ tumor-reactive effector memory T cells in humans after adoptive cell transfer therapy. Blood 2005;105:241–50.
42. Derre L, Rivals JP, Jandus C, Pastor S, Rimoldi D, Romero P, et al. BTLA mediates inhibition of human tumor-specific CD8+ T cells that can be partially reversed by vaccination. J Clin Invest 2010;120:157–67.
43. Zhou Q, Munger ME, Veenstra RG, Weigel BJ, Hirashima M, Munn DH, et al. Coexpression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. Blood 2011;117:4501–10.
44. Murphy KM, Nelson CA, Sedy JR. Balancing co-stimulation and inhibition with BTLA and HVEM. Nat Rev Immunol 2006;6:671–81.
45. Cheung TC, Steinberg MW, Oborne LM, Macauley MG, Fukuyama S, Sanjo H, et al. Unconventional ligand activation of herpesvirus entry mediator signals cell survival. Proc Natl Acad Sci U S A 2009;106:6244–9.
46. Sakoda Y, Park JJ, Zhao Y, Kuramasu A, Geng D, Liu Y, et al. Dichotomous regulation of GVHD through bidirectional functions of the BTLA-HVEM pathway. Blood 2011;117:2506–14.
47. Zhou J, Shen X, Huang J, Hodes RJ, Rosenberg SA, Robbins PF. Telomere length of transferred lymphocytes correlates with *in vivo* persistence and tumor regression in melanoma patients receiving cell transfer therapy. J Immunol 2005;175:7046–52.

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