Adoptive transfer of tumor-infiltrating lymphocytes (TILs) can mediate regression of metastatic melanoma; however, TILs are a heterogeneous population, and there are no effective markers to specifically identify and select the repertoire of tumor-reactive and mutation-specific CD8+ lymphocytes. The lack of biomarkers limits the ability to study these cells and develop strategies to enhance clinical efficacy and extend this therapy to other malignancies. Here, we evaluated unique phenotypic traits of CD8+ TILs and TCR β chain (TCRβ) clonotypic frequency in melanoma tumors to identify patient-specific repertoires of tumor-reactive CD8+ lymphocytes. In all 6 tumors studied, expression of the inhibitory receptors programmed cell death 1 (PD-1; also known as CD279), lymphocyte-activation gene 3 (LAG-3; also known as CD223), and T cell immunoglobulin and mucin domain 3 (TIM-3) on CD8+ TILs identified the autologous tumor-reactive repertoire, including mutated neoantigen-specific CD8+ lymphocytes, whereas only a fraction of the tumor-reactive population expressed the costimulatory receptor 4-1BB (also known as CD137). TCRβ deep sequencing revealed oligoclonal expansion of specific TCRβ clonotypes in CD8+PD-1+ compared with CD8+PD-1− TIL populations. Furthermore, the most highly expanded TCRβ clonotypes in the CD8+ and the CD8+PD-1+ populations recognized the autologous tumor and included clonotypes targeting mutated antigens. Thus, in addition to the well-documented negative regulatory role of PD-1 in T cells, our findings demonstrate that PD-1 expression on CD8+ TILs also […]
PD-1 identifies the patient-specific CD8+ tumor-reactive repertoire infiltrating human tumors

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Introduction
Cancer immunotherapy has experienced major progress in the last decade. Adoptive transfer of ex vivo–expanded tumor-infiltrating lymphocytes (TILs) can cause substantial regression of metastatic melanoma (1, 2). Blockade of the interaction of cytotoxic T lymphocyte antigen 4 (CTLA-4; also known as CD152) or programmed cell death 1 receptor (PD-1; also known as CD279) with their ligands using blocking antibodies alone or in combination could enhance clinical efficacy. However, the identification of tumor-reactive CD8+ lymphocytes. In all 6 tumors studied, expression of the inhibitory receptors programmed cell death 1 (PD-1; also known as CD279), lymphocyte-activation gene 3 (LAG-3; also known as CD223), and T cell immunoglobulin mucin domain 3 (TIM-3) on CD8+ TILs identified the autologous tumor-reactive repertoire, including mutated neoantigen-specific CD8+ lymphocytes, whereas only a fraction of the tumor-reactive population expressed the costimulatory receptor 4-1BB (also known as CD137). TCRβ deep sequencing revealed oligoclonal expansion of specific TCRβ clonotypes in CD8+PD-1+ compared with CD8+PD-1+ TIL populations. Furthermore, the most highly expanded TCRβ clonotypes in the CD8+ and the CD8+PD-1+ populations recognized the autologous tumor and included clonotypes targeting mutated antigens. Thus, in addition to the well-documented negative regulatory role of PD-1 in T cells, our findings demonstrate that PD-1 expression on CD8+ TILs also accurately identifies the repertoire of clonally expanded tumor-reactive cells and reveal a dual importance of PD-1 expression in the tumor microenvironment.

Adoptive transfer of tumor-infiltrating lymphocytes (TILs) can mediate regression of metastatic melanoma; however, TILs are a heterogeneous population, and there are no effective markers to specifically identify and select the repertoire of tumor-reactive and mutation-specific CD8+ lymphocytes. The lack of biomarkers limits the ability to study these cells and develop strategies to enhance clinical efficacy and extend this therapy to other malignancies. Here, we evaluated unique phenotypic traits of CD8+ TILs and TCRβ chain (TCRβ) clonotypic frequency in melanoma tumors to identify patient-specific repertoires of tumor-reactive CD8+ lymphocytes. In all 6 tumors studied, expression of the inhibitory receptors programmed cell death 1 (PD-1; also known as CD279), lymphocyte-activation gene 3 (LAG-3; also known as CD223), and T cell immunoglobulin mucin domain 3 (TIM-3) on CD8+ TILs identified the autologous tumor-reactive repertoire, including mutated neoantigen-specific CD8+ lymphocytes, whereas only a fraction of the tumor-reactive population expressed the costimulatory receptor 4-1BB (also known as CD137). TCRβ deep sequencing revealed oligoclonal expansion of specific TCRβ clonotypes in CD8+PD-1+ compared with CD8+PD-1+ TIL populations. Furthermore, the most highly expanded TCRβ clonotypes in the CD8+ and the CD8+PD-1+ populations recognized the autologous tumor and included clonotypes targeting mutated antigens. Thus, in addition to the well-documented negative regulatory role of PD-1 in T cells, our findings demonstrate that PD-1 expression on CD8+ TILs also accurately identifies the repertoire of clonally expanded tumor-reactive cells and reveal a dual importance of PD-1 expression in the tumor microenvironment.

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The opportunity to study their function and to identify the patient-specific repertoire of tumor-reactive cells. TCR stimulation triggers simultaneous upregulation of both costimulatory and coinhibitory receptors, which can either promote or inhibit T cell activation and function. Expression of the inhibitory receptors PD-1, CTLA-4, lymphocyte-activation gene 3 (LAG-3; also known as CD223), and T cell immunoglobulin and mucin domain 3 (TIM-3) is regulated in response to activation and throughout differentiation (16, 17). Chronic antigen stimulation has been shown to induce coexpression of inhibitory receptors and is associated with T cell hyporesponsiveness, termed exhaustion (18). Exhaustion in response to persistent exposure to antigen was first delineated in a murine model of chronic lymphocytic choriomeningitis virus (19), but has been observed in multiple human chronic viral infections (20–22) as well as in tumor-reactive MART1-specific TILs (23, 24), and has provided the rationale for restoring immune function using immune checkpoint blockade. Conversely, 4-1BB (also known as CD137) is a costimulatory member of the TNF receptor family that has emerged as an important mediator of survival and proliferation, particularly in CD8+ T cells (25–27). 4-1BB is transiently expressed upon TCR stimulation, and its expression has been used to enrich for antigen-specific T cells in response to acute antigen stimulation (28). However, expression of this marker has not been extensively explored in CD8+ lymphocytes infiltrating human tumors. In addition to changes in the expression of cosignaling receptors on the surface of T cells, antigen-specific stimulation typically results in clonal expansion. TCR sequence immunoprofiling can be used to monitor T cell responses to a given immune challenge even without a priori knowledge of the specific epitope targeted, through determination of the abundance of specific clonotypes (29, 30). However, there is limited knowledge regarding the TCR repertoire and the frequency of tumor-reactive clonotypes infiltrating human tumors.

We hypothesized that the assessment of unique phenotypic traits expressed by CD8+ TILs and TCRβ chain (TCRβ; encoded by TRB) clonotypic immunoprofiling of lymphocytes infiltrating the tumor could provide a powerful platform to study antitumor T cell responses and evaluated their usefulness in identifying the

Figure 1
CD8+ TILs exhibit unique phenotypic traits compared with PBLs. (A) Phenotypic characterization of CD8+ PBLs and TILs in melanoma patients. Percentages of PBLs and TILs expressing individual or combination of markers are shown (mean ± SEM). Each dot represents 1 sample analyzed. **P < 0.01, ***P < 0.001, Mann-Whitney test. (B) Coexpression of PD-1, LAG-3, TIM-3, and 4-1BB in CD8+ PBLs and TILs. The frequency of cells expressing 0, 1, 2, 3, or 4 markers is shown. Bars represent maximum, minimum, and mean values. **P < 0.01, ***P < 0.001, Mann-Whitney test. (C) Coexpression pattern of PD-1 and LAG-3, TIM-3, 4-1BB, CD25, and CD57 on CD8+ PBLs and TILs. Dot plots display the phenotype of CD8+ lymphocytes from matched samples from 1 representative patient. The percentage of cells expressing each combination of receptors is shown. (D) Frequency of PD-1 expression on CD8+ TIL subpopulations. TIM-3+, LAG-3+, 4-1BB+, CD25+ BTLA+, CD57+, and CD27+ CD8+ TILs were gated, and the frequency of PD-1 expression within these populations is summarized. Markers are displayed in order and color coded according to frequency of PD-1 expression. Mean ± SEM are shown. *P < 0.005 vs. BTLA+, CD57+, and CD27+, Dunn test for multiple comparisons.
diverse repertoire of tumor-reactive cells. Despite the accepted negative regulatory role of PD-1 in T cells, our findings establish that expression of PD-1 on CD8+ melanoma TILs accurately identifies the repertoire of clonally expanded tumor-reactive, mutation-specific lymphocytes and suggest that cells derived from this population play a critical role in tumor regression after TIL administration.

**Results**

CD8+ lymphocytes in the tumor display distinct phenotypic traits with increased but variable expression of PD-1, LAG-3, TIM-3, and 4-1BB. To investigate whether the state of differentiation of CD8+ TILs from freshly excised melanoma lesions or expression of markers characteristic of chronic or acute antigen-specific stimulation could guide the identification of tumor-reactive cells, we characterized the phenotype of CD8+ TILs from 24 melanomas. We measured cell surface expression of differentiation markers CD62L, CD45RO, CD27, and CD57 (31), inhibitory receptors PD-1, LAG-3, TIM-3, and BTLA, typically expressed on chronically stimulated CD8+ T cells (23, 24, 32, 33), and costimulatory receptor 4-1BB (28, 34) and IL-2 receptor alpha CD25 (35), which can be upregulated after TCR stimulation. A summary comparing the phenotypic traits of CD8+ TILs and PBLs is depicted in Figure 1A. Freshly isolated CD8+ TILs displayed a higher frequency of effector memory (EM) cells (CD62L–CD45RO+) compared with PBLs obtained from 21 of the 24 patients tested (Figure 1A). Despite this enhanced frequency of EM-like cells, CD8+ TILs did not appear to be terminally differentiated, as they maintained high expression of CD27, a marker that typically decreases throughout differentiation, and low expression of CD57, a receptor associated with terminal T cell differentiation (31). In addition, CD8+ TILs exhibited enhanced expression of PD-1, LAG-3, TIM-3, and 4-1BB, but not BTLA or CD25. PD-1 was the receptor most overexpressed in CD8+ TILs compared with PBLs, obtained from 21 of the 24 patients tested (Figure 1A). Despite this enhanced frequency of EM-like cells, CD8+ TILs did not appear to be terminally differentiated, as they maintained high expression of CD27, a marker that typically decreases throughout differentiation, and low expression of CD57, a receptor associated with terminal T cell differentiation (31).
pared with 0.3% ± 0.1% of PBLs. Figure 1C shows the coexpression pattern of some of the markers studied on CD8+ PBLs and fresh tumor CD8+ TILs for 1 representative melanoma patient. This patient’s CD8+ TILs displayed coexpression of TIM-3, LAG-3, and 4-1BB with PD-1. TIM-3, LAG-3, and 4-1BB were almost exclusively expressed on PD-1+ cells: 82.8% ± 3.1% of CD8+TIM-3+ TILs, 79.3% ± 4.9% of CD8+LAG-3+ TILs, and 78.6% ± 3.8% of CD8+4-1BB+ TILs expressed PD-1 in the fresh tumor (Figure 1D and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI73639DS1). Additionally, PD-1 and CD27 were also consistently coexpressed on a fraction of CD8+ TILs (Figure 1C). However, CD27 was not exclusively expressed on PD-1+ TILs, as there was still a substantial percentage of CD27+ TILs that were PD-1-. On average, only 37.6% of CD8+CD27+ TILs expressed PD-1 in the fresh tumor (Figure 1D). Thus, the coexpression pattern of PD-1 with LAG-3, TIM-3, and 4-1BB appeared to be different than that observed with PD-1 and the differentiation marker CD27. Overall, the unique coexpression profile of PD-1 with LAG-3, TIM-3, and 4-1BB in CD8+ TILs prompted us to study these markers and their potential significance in greater detail.

Expression of PD-1, LAG-3, and TIM-3 in the tumor identifies tumor-reactive and mutation-specific T cells. In order to investigate whether CD8+ cells expressing PD-1, LAG-3, and TIM-3 in the tumor represented the tumor-reactive population, we isolated cells from 6 independent fresh tumors based on expression of PD-1, LAG-3, and TIM-3, expanded them in vitro for 15 days with IL-2, anti-CD3 stimulation, and irradiated feeders, and tested their ability to recognize autologous tumor cell lines. The availability of autologous tumor cell lines enabled us to assess the patient-specific repertoire of tumor-reactive cells, rather than the shared reactivities studied with HLA-I–matched tumor cell lines. We first studied fresh tumor 1913 (FrTu1913), a tumor from a patient who had objective clinical response after transfer of TIL1913. Notably, PD-1+, LAG-3+, and TIM-3+ FrTu1913-derived CD8+ TILs, but not the negative counterparts, contained all the tumor-reactive cells — as determined by IFN-γ secretion and 4-1BB upregulation — after coculture with autologous tumor cell line 1913 (TC1913) (Figure 2A). Recognition of autologous tumor appeared to be antigen specific and HLA-I restricted, as the HLA-I–blocking antibody W6/32 inhibited these responses, and TILs did not respond to HLA-I–matched
or mismatched tumor cell lines (Figure 2B). Tumor recognition by PD-1, LAG-3, and TIM-3’ derived TILs was not due to differences in the activation status of the T cells, since all the subsets were capable of responding to plate-bound anti-CD3 stimulation (Supplemental Table 1). Additionally, PD-1, LAG-3, and TIM-3’ derived cells were capable of lysing the autologous TC1913 (Figure 2C). With the exception of LAG-3’ cells isolated from FrTu2448, selection of PD-1, LAG-3, and TIM-3’ CD8 TILs reproducibly separated the tumor-reactive cells from the non-tumor-reactive cells present in all the fresh tumor tested (FrTu1913, FrTu3289, FrTu3612, FrTu3713, FrTu3550, and FrTu2448; Figure 2D and Figure 3, A–H). Recognition of the PD-1, LAG-3, and TIM-3 cells present in all the fresh tumors tested (FrTu1913, FrTu3289, and TIM-3’ FrTu1913-derived TIL populations, but not in the negative counterparts (Figure 4B). CDKN2A<sub>mut</sub>-specific cells present in these populations secreted IFN-γ in response to CDKN2A<sub>mut</sub> peptide, but not in response to an irrelevant HLA-A*1101–restricted control peptide, CRKRS<sub>mut</sub>(Figure 4B). These findings indicate that T cells targeting mutated neoepitopes express PD-1, LAG-3, and TIM-3 in the tumor and that these receptors identify and select for these T cell populations.

Expression of the inhibitory receptor PD-1, rather than 4-1BB, accurately defines the broad repertoire of tumor-reactive cells in the tumor. In contrast to expression of the inhibitory receptors PD-1, LAG-3, and TIM-3, 4-1BB is a costimulatory receptor transiently expressed on the cell surface of CD8<sup>+</sup> lymphocytes upon TCR stimulation. We found that 4-1BB expression was preferentially upregulated in a fraction of PD-1’ CD8<sup>+</sup> TILs infiltrating freshly excised melanoma lesions (Figure 5A). PD-1’/4-1BB’ cells represented a small proportion of the CD8<sup>+</sup> TILs in the 24 fresh tumors analyzed (9.8% ± 3.5%), whereas the PD-1’/4-1BB population was substantially greater (22.1% ± 2.5%) (Figure 5B). To determine whether 4-1BB or PD-1 better identified the tumor-reactive TILs, we separated CD8<sup>+</sup> TILs from 2 fresh tumors based on expression of PD-1 and 4-1BB into PD-1’/4-1BB’, PD-1’/4-1BB, and PD-1’/4-1BB’, but not PD-1’/4-1BB<sup>−</sup>, due to its low frequency (Figure 5, A and B). We either established T cell clones for the 3 subpopulations sorted from FrTu1913 or bulk-expanded them from FrTu3713, and then tested their ability to recognize autologous tumor. Both the PD-1’/4-1BB’ and PD-1’/4-1BB’ derived T cell clones from FrTu1913 and bulk-expanded cells from FrTu3713 contained tumor-reactive cells, as determined by 4-1BB<sup>+</sup> upregulation after coculture with autologous tumor cell lines TC1913 and TC3713 (Figure 5, C and D). Tumor recognition was essentially undetectable in PD-1’/4-1BB<sup>−</sup> derived cells. PD-1’/4-1BB’ and PD-1’/4-1BB’ expanded TILs showed comparable lysis of autologous tumor cell line TC3713 (Figure 5E). Given that a significant fraction of tumor-reactive T cells was found in the PD-1’/4-1BB population, our data indicate that although the costimulatory receptor 4-1BB can be used to enrich for tumor-reactive cells, it in fact misses a large proportion of tumor-reactive T cells; thus, PD-1 expression in the fresh tumor more comprehensively defines the diverse repertoire of naturally occurring tumor-reactive CD8<sup>+</sup> TILs.

The skewed repertoire and binary distribution of CD8<sup>+</sup> TIL TCRβ clonotypes based on PD-1 expression is indicative of local antigen-driven clonal expansion. To further explore whether the predominant CD8<sup>+</sup>PD-1’ population infiltrating melanomas represented the tumor-reactive cells, we performed TCRβ deep sequencing of
freshly isolated CD8\(^+\)PD-1\(^+\) and CD8\(^+\)PD-1\(^{-}\) TILs. We reasoned that a less diverse TCR\(\beta\) profile in the PD-1\(^+\) population would suggest antigen-driven clonal expansion. We studied FrTu1913 (Figure 6, A–C) and FrTu3713 (Figure 6, D–F), 2 tumors from patients who had objective clinical responses after transfer of TIL1913 and TIL3713. CD8\(^+\)PD-1\(^+\) TILs infiltrating FrTu1913 (23%) and FrTu3713 (51.5%) were more oligoclonal compared with the PD-1\(^{-}\) or bulk CD8\(^+\) TILs. The 30 most frequent clonotypes (unique TCR\(\beta\) clonotypes; defined by hypervariable complementarity determining region 3 [CDR3\(\beta\)] nucleotide and amino acid sequences) in each population accounted for nearly 80% and 60% of the PD-1\(^+\) population — but only 28% and 37% of the PD-1\(^{-}\) population — in FrTu1913 and FrTu3713, respectively (Figure 6, A and D). With rare exceptions, PD-1 expression separated the most frequent CD8\(^+\) clonotypes into 2 populations, given that they were either highly expanded in the PD-1\(^+\) TILs (PD-1\(^+\)/PD-1\(^{-}\) frequency ratio, >1) or dominant in the PD-1\(^{-}\) population (PD-1\(^+\)/PD-1\(^{-}\) frequency ratio, <1) (Figure 6, B and E, and Table 3).
The frequency of specific clonotypes in the CD8⁺ and PD-1⁺ populations is associated with tumor recognition. The presence of clonotypes that were highly represented in the CD8⁺ TIL population and preferentially expanded in the CD8⁺PD-1⁺ population infiltrating FrTu1913 and FrTu3713, respectively (Figure 6, C and F). The skewed TCR repertoire displayed by CD8⁺PD-1⁺ TILs was also observed in FrTu3612, a tumor from a patient who did not respond following transfer of TIL3612 (Supplemental Figure 2). Notably, the binary distribution of TCRβ clonotypes observed based on PD-1 expression was less evident for CD8⁺ TILs separated based on 4-1BB expression, given that some of the most frequent clonotypes present in the 4-1BB⁺ population where still detected at a substantial frequency in the 4-1BB⁻ population (Supplemental Table 2). The 30 most frequent CD8⁺ TCRβ clonotypes in the fresh tumor are plotted based on their frequency in PD-1⁻ and PD-1⁺ populations. Among the tumor-reactive clones sequenced, we detected the CDR3β sequence corresponding to the most highly expressed CD8⁺ TCRβ clonotype in FrTu3713, representing 12.3% (Table 2). Moreover, this clonotype was preferentially expanded in the PD-1⁺ population, but not in the PD-1⁻ population (representing 18.6% and 0.1%, respectively). The cumulative frequency of all the tumor-reactive clones sequenced represented 29.2% of the PD-1⁺ fraction in FrTu3713 and only accounted for 0.4% of the PD-1⁻ clonotypes. These data support that the increased clonotypic frequency in the CD8⁺ and PD-1⁺ populations in FrTu3713 can be used to predict autologous tumor recognition, regardless of the knowledge of the specific antigen targeted by these cells.

CD8⁺ TCRβ clonotypes recognizing mutated tumor neoantigens are highly expanded in the CD8⁺ and PD-1⁺ populations infiltrating FrTu1913. The previously reported tumor-reactive clonotypes specific for mutated HLA-A*1101 (referred to herein as HLA-A11_mut and CDKN2A_mut derived from FrTu1913 (36), allowed us to further investigate whether clonotypic frequency in the CD8⁺ and PD-1⁺ populations in the fresh tumor could predict tumor recognition. The 3 known CD8⁺ HLA-A11_mut-specific clonotypes from FrTu1913 ranked among the 23 most frequent clonotypes in the PD-1⁺ population.
The 2 known mutation-specific clonotypes were also predominantly detected in the PD-1+ population (Table 1). However, none of these clonotypes accounted for the most frequent CD8+ TCR clonotype in FrTu1913, representing 26.9% and 0.2% of the PD-1+ and PD-1- populations, respectively.

We next sought to determine whether the most frequent clonotype in tumor FrTu1913 was capable of recognizing autologous tumor cell line TC1913 and either of the 2 known mutations expressed by this tumor. To identify this clonotype, we screened the clones established from FrTu1913 (Figure 3C) for the TRBV12-3 family region using a TCR Vβ 8-specific antibody and sequenced some of the clones. The CDR3β hypervariable region of clone 41 was identical to the most frequent clonotype expressed in FrTu1913.

Assessment of autologous tumor recognition and recognition of CDKN2A<sub>mut</sub> and HLA-A11<sub>mut</sub> revealed that the most frequent clonotype in FrTu1913 was tumor reactive (Figure 7B) and specifically recognized the mutation in HLA-A*1101 uniquely expressed by TC1913 (Figure 7C). In FrTu1913, mutation-specific clonotypes were preferentially expanded in the PD-1+ population, and the most frequent clonotype in the CD8+ population was specific (Figure 7D and Table 1).

**Discussion**

PD-1 was initially described to be expressed on a T cell hybridoma undergoing cell death (37). Its negative effect on T cell responses was first delineated in PD-1 knockout mice (38, 39).

### Table 1

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Freshly isolated CD8+ melanoma TIL were sorted to high purity from FrTu1913 (bulk, 1 x 10<sup>6</sup> cells; PD-1+, 5 x 10<sup>4</sup> cells; PD-1-, 5 x 10<sup>4</sup> cells), and TCRβ deep sequencing was performed. TRBV, TRBD, and TRBJ represent consensus nomenclature for TCR variable β chain sequences with the CDR3β amino acid sequences according to their frequency in each population, 1 being the most frequent. DNumber of times 1 particular CDR3β sequence and the added counts for all the CDR3β sequence in the different populations is shown. Totals show the number of unique CDR3β sequences and the added counts for all the CDR3β sequences in each population. AThe PD-1/PD-1- ratio was calculated; values >1 are shown in bold to denote the clonotypes that were enriched in the PD-1+ population. BSpecific target recognized by clonotypes (blank, not determined). CClassification of CDR3β according to their frequency in each population, 1 being the most frequent. DNumber of times 1 particular CDR3β sequence was found in each population. ENot detected.
Since then, PD-1 expression and coexpression of other inhibitory receptors such as CTLA-4, TIM-3, BTLA, CD160, LAG-3, and 2B4 have become a hallmark of chronically stimulated T cells during chronic infection or in the tumor microenvironment. This altered phenotype, and the interaction of these receptors with their corresponding ligands on target cells, is associated with impaired proliferation and effector function frequently referred to as exhaustion (18, 24, 40). Expression of PD-1 in patients with chronic viral infections correlates with disease progression (22, 41). Additionally, CD8+ lymphocytes targeting melanoma differentiation antigens in the tumor express PD-1, CTLA-4, TIM-3, and LAG-3 and exhibit impaired IFN-γ and IL-2 secretion (23, 24), supporting a negative regulatory role of PD-1 and inhibitory receptors in naturally occurring T cell responses to cancer and providing a rationale for the treatment of cancer with immune checkpoint inhibitors.

In the present study, we found that expression of PD-1 on CD8+ melanoma TILs captured the diverse repertoire of clonally expanded tumor-reactive lymphocytes. TCRβ sequencing revealed that tumor-reactive and mutation-specific clonotypes were highly frequent among the CD8+ TIL population. The 30 most frequent TCRβ clonotypes in the CD8+ population are shown. The frequency of each TCRβ in the different populations is shown. Totals show the number of unique CDR3β sequences and the added counts for all the CDR3β sequences in each population. AThe PD-1+CD8+ ratio was calculated; values >1 are shown in bold to denote the clonotypes that were enriched in the PD-1+ population. BAbility of specific clonotype to recognize autologous tumor cell line TC3713 (Y, yes; blank, not determined). CClassification of CDR3β was performed. TRBV, TRBD, and TRBJ represent consensus nomenclature for TCR V (V, D, and J) chain sequences with the hypervariable region translated into amino acid sequences. The 30 most frequent TCRβ clonotypes in the CD8+ population are shown. The frequency of each CDR3β sequence in the different populations is shown. Totals show the number of unique CDR3β sequences and the added counts for all the CDR3β sequences in each population. ENot detected.
identifying the diverse repertoire of tumor-reactive cells infiltrating melanoma tumors, although the less frequent PD-1+/TIM-3+ and PD-1+/LAG-3+ subpopulations could also represent tumor-reactive cells (Supplemental Figure 4 and Supplemental Table 6). Additionally, previous studies from our laboratory showing coexpression of PD-1 and CTLA-4 (23), and our preliminary data supporting coexpression of PD-1 and ICOS (Supplemental Figure 5), suggest that other receptors may also be used to distinguish tumor-reactive cells. Our present results further support immunotherapeutic intervention using immune checkpoint blockade using PD-1, TIM-3, and LAG-3 blocking antibodies or 4-1BB agonistic antibody to restore the function of tumor-reactive lymphocytes, which is currently being actively pursued in the clinic (3, 4, 6, 7, 43). The potential cooperative mechanisms of inhibition of these receptors when engaged with their ligands (44, 45) suggests that the combined targeting of different inhibitory receptors can further enhance antitumor efficacy, as already shown with the combination of anti–PD-1 and anti–CTLA-4 (5). Our present results demonstrate that expression of PD-1 on CD8+ TILs can be used as a potential predictive biomarker of antitumor efficacy using immune checkpoint inhibitors.

Naturally occurring tumor-reactive cells play a pivotal role in mediating antitumor responses after TIL transfer. Currently, expansion of TILs for patient treatment involves nonspecific growth of TILs from tumor fragments in IL-2, and the diversity and frequency of antitumor T cells present in the final T cell product used for treatment remains largely uncharacterized. Prospective clinical studies have reported that in vitro recognition of autologous tumor by TILs is associated with a higher probability of clinical response (9, 10), which suggests that enrichment of tumor-reactive cells could enhance clinical efficacy. This is consistent with the idea that both tumor-reactive and non–tumor-reactive cells may compete for cytokines in vivo, especially in the absence of vaccination. However, the isolation of the patient-specific repertoire of tumor-reactive cells is not possible with current technologies (14, 28, 46–50). Our findings established that expression of PD-1, TIM-3, LAG-3, and 4-1BB in CD8+ TILs can be used to enrich for tumor-reactive cells, regardless of the specific antigen targeted. One potential concern with isolating T cells expressing inhibitory receptors for therapy is that these cells may be exhausted or functionally impaired (23, 24, 44, 51, 52). However, we found that PD-1+, TIM-3+, and LAG-3+ CD8+ cells expanded in IL-2 were capable of secreting IFN-γ and lyse tumor in vitro. This supports the notion that immune dysfunction associated with coexpression of inhibitory receptors on CD8+ TILs can be reversed (21, 41, 51, 53), and may enable the reproducible enrichment of tumor-reactive cells for patient treatment. Notably, in a preliminary experiment (n = 8 nonresponders; 14 responders), there was no association between the frequency of expression of any of the

Figure 7 Tumor-reactive and mutation-specific clonotypes are highly expanded in the CD8+ and PD-1+ populations. (A) Response of CD8+PD-1- (n = 44) and CD8+PD-1+ (n = 109) FrTu3713-derived T cell clones to TC3713. Each dot represents 1 clone; line represents median IFN-γ release for all clones tested. The percentage of tumor-reactive clones (>50 pg/ml IFN-γ) is shown above. ****P ≤ 0.0001, Mann-Whitney test. (B) Reactivity of FrTu1913-derived clones against autologous tumor TC1913 or allogeneic TC624 (matched HLA-A*0201). Clone 41 expressed the CDR3β amino acid sequence corresponding to the most frequent CD8+ clonotype in FrTu1913; clone 208 recognized CDKN2Amut HLA-A*1101 restricted epitope; clone 199 was a tumor-reactive clone of yet-unknown specificity; clone 88 did not recognize TC1913. Recognition of targets was measured by assessing 4-1BB upregulation. (C) Recognition of CDKN2Amut and HLA-A11mut by FrTu1913-derived T cell clones. COS-A2 cells were transiently transfected with plasmids encoding HLA-A11WT or HLA-A11mut, and recognition of CDKN2Amut peptide was assessed by measuring 4-1BB upregulation. (D) Frequency of the 30 most frequent TCRβ clonotypes in the CD8+PD-1+ population in FrTu1913. The cumulative frequency (Σ freq.) of the HLA-A11mut–specific clonotypes in each population is specified below.
markers studied in the CD8+ TILs in the fresh tumor and the clinical response to TILs derived from these tumor samples. However, the fresh TILs included in this study belonged to patients treated in several TIL protocols over the course of 10 years, and TILs were generated from these tumors using different methods, which makes these data difficult to interpret. In addition, the frequency of cells initially expressing PD-1 in the tumor may not reflect the frequency of the PD-1 derived cells in the infusion bag. For example, a low frequency of PD-1+ cells may be highly enriched during the process of TIL culture as a result of the presence of tumor cells. Although in vivo antitumor activity of tumor-isolated TILs based on PD-1 expression requires testing in a clinical trial, the observation that the overwhelming majority of tumor-reactive cells were derived from cells expressing PD-1 suggests that cells expressing PD-1 and inhibitory receptors in the tumor play a critical role in tumor regression after TIL administration.

The functional implications of selecting PD-1–, LAG-3–, TIM-3–, or 4-1BB–expressing T cells to enrich for tumor-reactive cells for patient treatment remain unclear. Although previous studies have reported differential expression of PD-1, LAG-3, and TIM-3 throughout differentiation (17), or preferential expression of TIM-3 in IFN-γ-secreting cells (54), our preliminary results have failed to show consistent phenotypic or functional differences between PD-1–, LAG-3–, TIM-3–, and 4-1BB+ selected TILs, including cytokine secretion, proliferation, and susceptibility to apoptosis (data not shown). We found that PD-1 expression was almost completely lost in the PD-1+ derived populations upon in vitro culture in IL-2. Conversely, TIM-3 and LAG-3 expression increased in the TIM-3– and LAG-3– populations after expansion. Overall, there were no differences in the expression of PD-1, TIM-3, or LAG-3 between any the populations after expansion. Thus, in agreement with previous reports (55, 56), we conclude that expansion in IL-2 alters the expression of these markers and compromises the potential use of inhibitory receptors to select for tumor-reactive cells after in vitro expansion. Recent work in animal models suggests that chronic antigen stimulation (57–59) or a tolerizing microenvironment (60) may lead to permanent epigenetic changes in T cells, raising the possibility that the restoration of function observed in previously exhausted or tolerized T cells may be transient. These results have not yet been corroborated in human tumor-specific cells. However, given that the overwhelming majority of tumor-reactive cells appear to derive from cells expressing PD-1 in the tumor, studying permanent versus transient reversion of exhaustion may have important implications for adoptive cell transfer of TILs.

Tumor-reactive cells can also be found infiltrating other tumor malignancies, such as renal cell carcinoma (61) or ovarian (62), cervical (63), or gastrointestinal tract cancers (64), albeit at lower frequencies. Our findings provide alternatives to enrich and study tumor-reactive CD8+ TILs through selection of cells expressing the cell surface receptors PD-1, LAG-3, TIM-3, and 4-1BB, a hypothesis that we are actively investigating. Additionally, our present findings showed that the frequency of a specific clonotype in the CD8+ and PD-1+ populations can be used to predict its ability to recognize tumor and isolate tumor-specific TCRs, thus providing means to overcome potential irreversible functional impairments of TILs (52).

2 reports with opposing results have generated controversy regarding which may be the optimal marker for the identification of the tumor-reactive repertoire, PD-1 or 4-1BB. In one report studying PD-1 expression in the tumor, the authors showed promising although inconsistent ability to enrich for shared melanoma-reactive cells (55). In a more recent article studying the role of 4-1BB in fresh ovarian TILs, Ye et al. concluded that expression of 4-1BB, but not PD-1, on lymphocytes defines the population of tumor-reactive cells in the tumor (65). The results of Ye et al. appear to contradict our present findings, showing that expression of PD-1 rather than 4-1BB more comprehensively identifies the repertoire of tumor-reactive cells in the tumor. However, these inconsistencies can be explained by different experimental approaches undertaken to study the immunobiology of TILs. First, Ye et al. found that expression of 4-1BB in fresh ovarian TILs and tumor-associated lymphocytes was low, and thus exposed the tumor to IL-7 and IL-15 (65). In the 1 patient sample in which the authors enriched for tumor-reactive cells from fresh ovarian TILs or tumor-associated lymphocytes exposed to IL-7 and IL-15, expression of 4-1BB was dependent on in vitro activation, but no longer represented the natural expression of 4-1BB in the fresh tumor. Second, with the exception of the 1 experiment described above, the enrichment experiments reported were carried out with melanoma or ovarian TIL lines expanded in IL-2 and cocultured with tumor cell lines in vitro. It is well known that IL-2 can change the activation status and also the expression of inhibitory receptors on T cells (data not shown and ref. 56). Thus, the experiment comparing expression of PD-1 and 4-1BB performed by Ye et al. (65) addressed the significance of these receptors after in vitro coculture of a highly activated melanoma TIL line with a tumor cell line, rather than the role of PD-1 and 4-1BB expression in CD8+ lymphocytes in the fresh tumor. Finally, both Inozume et al. and Ye et al. used matched HLA-A2 cell lines to assess tumor reactivity (55, 65). However, the use of HLA-matched tumor cell lines does not enable the assessment of reactivities against unique mutations that are present only in the autologous tumor cell line. In our current study, we used fresh melanoma tumors for all our experiments, and these were rested in the absence of cytokines to preserve the phenotype of TILs. Moreover, we used autologous tumor cell lines to assess tumor recognition. We believe that our experimental approach overcomes the limitations described above, enabling us to conclude that tumor-reactive cells can be detected in both the PD-1+/4-1BB+ and PD-1+/4-1BB– CD8+ TIL populations.

In summary, expression of PD-1 in CD8+ TILs in the fresh tumor identified and selected for the diverse patient-specific repertoire of tumor-reactive cells, including mutation-specific cells. In addition, analysis of the CD8+ TIL TCR repertoire in 2 melanomas showed that the frequency of a specific TCRβ clonotype in the CD8+ and PD-1+ populations could be used to predict its ability to recognize the autologous tumor. The use of inhibitory receptors and the frequency of individual TCRs to prospectively identify and select the diverse repertoire of tumor-reactive cells holds promise for the personalized treatment of cancer with T cell therapies, but may also facilitate the dissection and understanding of the immune response in human cancer patients.

**Methods**

*Patient characteristics and PBL and tumor samples.* Matched PBL (*n* = 21) and tumor specimens (*n* = 24) were obtained from patients with metastatic melanoma. Patients included in this study were not undergoing therapy when their samples were collected, and they all had metastatic melanoma. Patients had undergone a wide range of prior therapies, including
surgery, chemotherapy, radiotherapy, and immunotherapy, or none of the above. See Supplemental Table 7 for patient characteristics. A fraction of the patients had received immunotherapy (including high-dose IL-2, anti-CTLA-4, or anti-PD-1) between 8 years to 2 months prior to tumor resection, but none had received adoptive cell therapy. See Supplemental Table 8 for details of the 6 tumors studied more in depth, specific immunotherapeutic regimens to which they were exposed, and times prior to phenotypic assessment. PBLs were obtained by either leukapheresis or venipuncture, prepared over Pricoll-Hypaque gradient (LSM; ICN Biomedicals Inc.), and cryopreserved until analysis. After surgical resection, tumor specimens were processed as previously described (66). Briefly, tumor specimens were minced under sterile conditions, followed by enzymatic digestion (RPMI-1640 with 1-glutamine [Lonza], 1 mg/ml collagenase IV [Sigma-Aldrich], 30 U/ml DNAse [Genentech], and antibiotics) overnight at room temperature or for several hours at 37°C and intermittent mechanical tissue separation using gentleMACS (Miltenyi Biotech). Tumor single-cell suspensions were cryopreserved until further analysis.

Antibodies, flow cytometry, and cell sorting. We purchased fluorescently conjugated antibodies from BD Biosciences (UCHT1, 1:6.100, CD3 PE-CF594; RPTA-T4, 3.4:100, CD4 VS00; NK-1, 3:100, CD57 FITC, J168-540, 1:2.100, BTLA PE), eBioscience (MIH-4, 1:6.100, PD-1 APC), Biolegend (O323, 2:100, CD27 BV605; BD96, 1:4.100, CD25 BV650; EH12.2H7, 0.7:100, PD-1 BV421; c398.4A, 1:100, ICOS Pacific Blue), R&D (344823, 2.6:100, TIM-3 PE and APC), Enzo Life Sciences (17B4, 1:100, LAG-3 FITC), and Miltenyi (4B4-1, 2.6:100, 4-1BB PE). We carried out flow cytometry acquisition on a modified Fortessa instrument (BD Biosciences), equipped to detect 18 fluorescent parameters. We compensated and analyzed data with FlowJo software (Treestar). We sorted T cell subsets using a modified FACSAria instrument (BD Biosciences). Antibodies and tetramers were titrated for optimal staining.

For phenotypic characterization of CD8+ T cells and cell sorting using flow cytometry, PBMCs and tumor samples were thawed in the presence of 3 U/ml DNAse (Genentech Inc.), and rested 16–24 hours in a 1:1 mix of RPMI-1640 with 1-glutamine (Lonza) and AIMV (Gibco) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 12.5 mM HEPES, and 5% human serum (noncommercial, prepared from healthy donors) without cytokines (T cell media) at a concentration of 10^5 live cells (determined by trypan blue exclusion) per well in a 24-well plate at 37°C in 5% CO2. This overnight resting period was carefully assessed and necessary for the recovery of expression cell surface receptors affected by cryopreservation or subject to cleavage during the enzymatic digest. Cells were harvested, counted, and blocked with Fc block for human cells (Miltenyi). 1×10^5 live cells were stained for 30 minutes in 50 μl volume of FACS buffer (PBS containing 0.5% BSA and 2 mM EDTA) with the antibodies specified, washed, and acquired. Propidium iodide (PI) was added to exclude dead cells. Phenotypic analysis and sorting of CD3+CD8+ subsets from the tumor single-cell suspensions were performed after gating on lymphocytes (FSC by SSC, excluding aggregates and dead cells (PI)). Gates were set according to isotype, and fluorescence minus one (FMO) controls. See Supplemental Figure 6 for 2 examples of the post-sort purity of CD3+CD8+ subsets sorted from FrTu1913 for TCRβ sequencing or for in vitro expansion.

RNA preparation and TCRβ deep sequencing. Cells of interest were sorted into a 1:1 mix of PBS and FBS (Hyclone Defined, Logan), and cell pellet was resuspended in 200 μl RNA-later (Invitrogen). We extracted total RNA and amplified TCRβ gene products of sorted CD8+ T cell subsets using a template-switch anchored RT-PCR, as previously described (67), followed by Illumina sequencing. TCRβ annotation was performed by combining a custom Java program written in house and NCBI BLAST+. Briefly, BLAST+ was used to identify the V and J germline genes of a TCRβ read. The CDR3 was then determined by finding the conserved cysteine at the 5′ end of the CDR3 and the conserved phenylalanine at the 3′ end of the CDR3. Unique TCRβ species, in which species is defined as a unique TRBV-CDR3 (nucleotide)-TRBJ combination, were then collapsed to determine the count for each species.

Similar cell numbers were sorted from each subset from the fresh tumor to ensure comparable coverage (total number of TCRβ sequence counts from a population/initial cell input of population) to enable comparison of clonotypic diversity among different populations.

Expansion of CD8+ T cells and establishment of T cell clones. FACS-sorted CD8+ T cells (5×10^5–1×10^6 cells) were expanded using a rapid expansion protocol (REP) in T25 flasks in T cell media containing 30 ng/ml soluble anti-CD3 (OKT3, Miltenyi), 3,000 IU/ml rhIL-2 (Chiron), and 3×10^5 irradiated PBMCs pooled from 3 allogeneic donors. For establishment of T cell clones, cells were plated at 2 cells/well in 96-well U-bottomed plates in T cell media containing OKT3 and IL-2 (as described above) and 7.5×10^5 irradiated allogeneic PBMCs/well. After day 5, media was replaced every other day with fresh media containing IL-2. Cells were split when confluent or when they reached a concentration exceeding 3×10^6 cells/ml. At day 14–15, bulk-expanded populations were counted and frozen down. Cell expansion was variable among different populations and patients (Supplemental Table 9). Cloning efficiency (percentage of clones plated at 2 cells/well that grew) ranged 9%–26%. T cell clones were maintained in 500 CU/ml IL-2 and tested for recognition of autologous tumor 3–6 weeks after cell sorting.

Assessment of target cell recognition: IFN-γ release, 4-1BB upregulation, and cytolytic assay. Both IFN-γ release and 4-1BB upregulation were used to measure recognition of targets. After 15 days of in vitro expansion of clones or TILs, basal expression of 4-1BB was consistently negative. 4-1BB is upregulated transiently in response to TCR stimulation, regardless of the effector cytokines produced or the differentiation state of the cell (28), and its expression on stimulated CD8+ cells peaks at 24–36 hours. For assessment of target cell recognition by TILs after in vitro expansion, we thawed the effector cells 2 days prior to coculture and cultured them at 1×10^6 cells/ml in 3,000 IU/ml IL-2 at 37°C in 5% CO2. 2 days later, effector cells were washed and cultured, either alone or with target cells (1×10^5–1×10^6) or plate-bound anti-CD3 (OKT3, Miltenyi), in 96-well plates in T cell media without IL-2. In antibody-blocking experiments, we preincubated melanoma cells with 0.05 mg/ml HLA-I or HLA-II blocking antibodies (clones W6/32 and IVA12, respectively) for 3 hours, followed by coculture with T cells. 24 hours later, supernatants were harvested (duplicates) and analyzed by previously described (47), and cells were stained in 40 μl PBS containing 0.5% BSA and 2 mM EDTA with anti-CD3, anti-CD8, and anti-4-1BB antibodies and acquired on Canto II (BD Biosciences). Data were analyzed with FlowJo software (Treestar).

For cytolytic assays, target cell lines were pulsed with 100 μCi (1 Ci = 37 GBq) 35Cr (PerkinElmer) and washed twice. We plated 1×10^4 labeled target cells per well with effector cells at the indicated effector/target ratios in a 96-well U-bottomed plate for 4 hours at 37°C. The amount of 35Cr released was determined by γ-counting, and specific lysis was calculated from triplicate samples as [(experimental cpm – spontaneous cpm)/(maximal cpm – spontaneous cpm)] and expressed as a percentage.

T cell lines, controls, cell lines, and reagents. Infusion products TIL3309, TIL3289, and TIL1700 were expanded from fresh tumor digests as previously described (68). Briefly, single-cell suspensions of tumor digests were cultured in media containing 6,000 IU IL-2 (Chiron). TIL cultures that expanded were screened for recognition of autologous or HLA-matched tumor, and reactive TIL cultures were expanded further — using a REP with IL-2, anti-CD3 antibody, and irradiated feeder cells — to large numbers for patient infusion. A small portion of TILs underwent a second REP
and were used as controls in coculture assays. PBMCs transduced with a MART-1 26-35 A*0201 (F5 Td) or tyrösinease 450-462 HLA-DR4 specific (CD4 T4) T cell receptor were previously described (69). Evaluation of reactivity of FrTu1913-derived TILs or T cell clones was carried out by pulsing peptides (1 μM for 1 hour) onto COS-A11 cells or COS-7 cells stably transduced with A*0201 (COS-A2 cells) and transiently transfected with HLA-A11 wt or HLA-A11 mut plasmids with Lipofectamine 2000 (Invitrogen). CDKN2A mut peptide and the control HLA-A*1101-restricted peptide CRKRSmut were purchased from Peptide 2.0.

Melanoma cell lines were established from tumor fragments or were obtained from mechanically or enzymatically separated tumor cells (as described above), which were cultured in RPMI 1640 plus 10% FBS (Hyclone Defined, Logan) at 37°C in 5% CO2 and TC1913 were identified (36).

Streptomycin at 37°C in 5% CO2. Expression of HLA-II molecules on the surface of A2 cells and the human melanoma tumor cell lines 624, 624CiTA, TCS26, TC1913, TC3289, TC3612, TC3713, TC3550, and TC2448 were cultured in RPMI 1640 plus 10% FBS (Sigma-Aldrich) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO2. COS-7 cells and COS-7 cells stably transduced with HLA molecules were maintained in DMEM containing 10% FBS (Sigma-Aldrich) supplemented with 100 μg/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO2. Expression of HLA-II molecules on the surface of melanoma cell line 624 was upregulated by transduction with a retroviral construct encoding the HLA-II transactivator gene (CIITA) as previously described (70).

TC1913 and recognition by TIL1913 grown from FrTu1913 were reported previously (36). Briefly, a cDNA library was generated from autologous tumor cell line TC1913, and CD8+ T cell clones recognizing a frameshift mutation in CDKN2A locus that encodes for tumor suppressor proteins P14ARF and P16INK4a (CDKN2A mut) were purchased from Peptide 2.0.

For some patients, CD4+CD25– cells were isolated from a pheresis pretreatment sample and used as normal autologous targets in coculture, with CD25 depletion followed by CD4-positive selection using BD IMag cell separation system (BD Biosciences).

Statistics. Values are reported as mean ± SEM, mean ± SD, or median, as indicated. We used Mann-Whitney test, Dunn test for multiple comparisons, and Wilcoxon signed-rank test to determine the statistical significance of the data. To determine the statistical difference in marker expression between CD8+ T cells in PBLs and TILs, we adjusted P values for the number of comparisons (Figure 1A; n = 11 comparisons). To determine whether there were differences in the frequency of PD-1 expression between CD8+ TIL populations, 21 multiple comparisons were performed, and only the ones that were statistically significantly different are shown (Figure 1D). P values were also adjusted for the number of comparisons. A P value of 0.05 or less was considered significant. We performed statistical calculations with Prism program 6.0 (GraphPad Software Inc.).

Study approval. All patient samples were obtained in the course of a National Cancer Institute Institutional Review Board–approved clinical trial, and patients provided informed consent.

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