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 […]

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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 cytoxic 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 have been shown to unleash an otherwise-ineffective immune response against melanoma (3–7), renal cell carcinoma (3), and non–small cell lung cancer (3). The antitumor responses observed in these clinical trials support the presence of naturally occurring tumor-reactive CD8+ T cells and their immunotherapeutic potential. In the particular case of TIL therapy, persistence of transferred tumor-specific T cell clones is associated with tumor regression (8). Moreover, retrospective clinical studies have shown an association of autologous tumor recognition by TILs and clinical response (9, 10), which suggests that enrichment of tumor-reactive cells could enhance clinical efficacy. However, the identification of the diverse repertoire of tumor-reactive cells limits the ability to study these cells, enhance clinical efficacy, and extend this therapy to other malignancies.

Melanoma TILs represent a heterogeneous population that can target a variety of antigens, including melanocyte differentiation antigens, cancer germline antigens, self-antigens overexpressed by the tumor, and mutated tumor neoantigens (11). The latter appear to be of critical importance for the antitumor responses observed after transfer of TILs, given the substantial regression of metastatic melanoma in up to 72% of patients in phase 2 clinical trials, in the absence of any autoimmune side effects in the great majority of patients (2). This contrasts with the modest antitumor activity but high prevalence of severe autoimmune manifestations observed after transfer of peripheral blood gene-engineered T cells expressing TCRs targeting shared melanocyte differentiation antigens MART1 and gp100 (12, 13). Furthermore, T cells targeting mutated neoepitopes are not subject to negative selection in the thymus and may constitute the predominant naturally occurring tumor-reactive population in cancer patients. In support of this notion, a recent study reported the frequent detection and dominance of T cell populations targeting mutated epitopes in melanoma-derived TILs (14). Conversely, T cells targeting shared melanocyte differentiation antigens and cancer germline antigens in bulk melanoma TILs were represented at a strikingly low frequency (15). These findings have shifted our interest from the more accessible and commonly studied T cells targeting melanocyte differentiation antigens to T cells targeting unique patient-specific mutations. However, the often rare availability of autologous tumor cell lines necessary to study these reactivities, and the hurdles associated with the identification of the unique mutations targeted, have thus far hindered immunobiological studies of these T cell populations in the tumor.

Naturally occurring tumor-reactive cells are exposed to their antigen at the tumor site. Thus, the immunobiological characterization of T cells infiltrating tumors represents a unique oppor-
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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 coactivating 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 TCR β 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...
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 (32.4% ± 5.0%, 13.0% ± 3.2%, 10.8% ± 2.4%, and 5.7% ± 2.4%, respectively). Notably, these 4 receptors displayed substantial coexpression on a subset of CD8+ TILs (32.4% ± 5.0%, 13.0% ± 3.2%, 10.8% ± 2.4%, and 5.7% ± 2.4%, respectively). Notably, these 4 receptors displayed substantial coexpression on a subset of CD8+ TILs (Figure 1B). The cumulative frequency of T cells coexpressing 2, 3, or 4 markers accounted for 16.6% ± 3.6% of TILs, com-

Figure 2

PD-1+, LAG-3+, and TIM-3+ derived CD8+ TILs, but not the negative counterparts, recognize and lyse their autologous tumor cell line. Bulk CD3+CD8+ TILs were sorted to high purity from 6 tumors based on positive or negative expression of PD-1, LAG-3, and TIM-3 and expanded in vitro. (A) Response of FrTu1913-derived TILs to TC1913. TILs were cocultured with autologous TC1913, and tumor recognition was assessed by measuring IFN-γ release (duplicates, mean ± SD), and the frequency of CD8+4-1BB+ cells. (B) Reactivity of PD-1+ and PD-1– CD8+ TILs derived from FrTu1913 against a panel of targets: TC1913 with and without HLA-I blocking antibody (W6/32), TC2448 (matched HLA-A*0201), TC2301 (allologeneic), and plate-bound anti-CD3 (OKT3). Upregulation of 4-1BB (top) and IFN-γ release (bottom, duplicates, mean ± SD) are shown. (C) Lysis of TC1913 by FrTu1913-derived TILs. Cytotoxicity of TILs against TC1913 (mean ± SD). (D) Response of PD-1+ and PD-1– derived TILs to their autologous tumor targets from all 6 fresh tumors studied. PD-1+ and PD-1– TILs were cocultured with their autologous tumor cell lines, and reactivity was assessed by measuring IFN-γ secretion (left) and 4-1BB upregulation (right). Each dot represents 1 patient’s sample. Mean ± SEM. *P ≤ 0.05, 2-tailed Wilcoxon signed-rank test.
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. 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. FrTu3289 (A), FrTu3612 (B), FrTu3713 (C), FrTu3550 (D), and FrTu2448 (E). Reactivity was assessed by measuring IFN-γ release (duplicates, mean ± SD) and frequency of 4-1BB upregulation. (F-H) Cytolytic activity of fresh tumor–derived TILs in response to their respective autologous tumor cell lines, TC3289 (F), TC3713 (G), and TC2448 (H). Percentage of specific lysis at different effector/target ratios is shown as mean ± SD.
The frequency of CDKN2A mutant-specific cells in the TIL populations was determined using a CDKN2A mutant tetramer complex after gating on CD3+CD8+ cells (gray bars). Recognition 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 is essentially undetectable in PD-1−/4-1BB− derived cells. PD-1−/4-1BB− and PD-1+/4-1BB+ expanded TILs showed comparable lysis of autologous tumor cell line TC3713 (Figure 5E). The skewed repertoire and binary distribution of CD8+ TIL TCRβ clonotypes based on PD-1 expression is indicative of local antigen-driven clonal expansion. To further explore whether the predominant CD8+PD-1− population infiltrating melanomas represented the tumor-reactive cells, we performed TCRβ deep sequencing of

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− 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− 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− 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− 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− 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− 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− 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− 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− 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− 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− 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− 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− 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− 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− 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− 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− derived cells were capable of lysing the autologous TC1913 (Figure 2C).

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+ lymphocytes upon TCR stimulation. We found that 4-1BB expression was preferentially upregulated in a fraction of PD-1− CD8+ TILs infiltrating freshly excised melanoma lesions (Figure 5A). PD-1+/4-1BB− cells represented a small proportion of the CD8+ 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+ 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+, 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 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− 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+ TILs.

The skewed repertoire and binary distribution of CD8+ TIL TCRβ clonotypes based on PD-1 expression is indicative of local antigen-driven clonal expansion. To further explore whether the predominant CD8+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 TCRβ 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 or 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 TCRβ clonotypes; defined by hypervariable complementarity determining region 3 [CDR3β] 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 Figure 5).

Figure 5
Expression of PD-1, rather than 4-1BB, more comprehensively identifies the repertoire of tumor-reactive cells in human tumors. (A) Representative dot plots showing PD-1 by 4-1BB expression of CD8+ TILs infiltrating FrTu1913 and FrTu3713. (B) Expression of PD-1 by 4-1BB on CD8+ TILs infiltrating melanoma tumors. The frequency of each combination of markers is shown as mean ± SEM. ***P < 0.001, Wilcoxon signed-rank test (n = 24). (C) Response of FrTu1913-derived clones to TC1913. CD8+ TILs were sorted from FrTu1913 into PD-1+/4-1BB–, PD-1+/4-1BB+, and PD-1–/4-1BB–, and clones were established. Clones derived from PD-1+/4-1BB– (n = 96), PD-1+/4-1BB+ (n = 57), and PD-1–/4-1BB– (n = 65) were cocultured with TC1913; 4-1BB upregulation upon coculture is plotted for each clone. The line represents the median. *P ≤ 0.05, ****P ≤ 0.0001, Dunn test for multiple comparisons. Pie charts depict the percentage of tumor-reactive and non–tumor-reactive clones in each population. Greater than 1% CD8+4-1BB+ and greater than twice the background percentage 4-1BB compared with no stimulation control was considered positive. (D) Response of bulk-expanded FrTu3713-derived TILs to TC3713. CD8+PD-1+/4-1BB–, PD-1+/4-1BB+, and PD-1–/4-1BB– TILs were sorted from FrTu3713 and expanded. Cells were either left unstimulated, cocultured with TC3713, or stimulated with plate-bound anti-CD3. 4-1BB upregulation was measured to assess tumor recognition. Representative dot plots of CD3–CD8+–gated cells are shown. (E) Lysis of TC3713 by TILs derived from FrTu3713.
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 suggested that these may be tumor-reactive. To investigate this, we established CD8⁺ T cell clones by limiting dilution cloning from the PD-1⁺ and PD-1⁻ populations infiltrating FrTu1913. The CD8⁺ T cells as well as PD-1⁺ and PD-1⁻ fractions were sorted to high purity from FrTu1913 and FrTu3713, respectively (Figure 6, C and F). The biased 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 Figure 3A and B). This is consistent with our previous results indicating that PD-1, rather than 4-1BB, more comprehensively identifies the diverse repertoire of tumor-reactive cells. Overall, the skewed TCRβ profile of the PD-1⁺ population and the binary distribution of TCRβ clonotypes based on PD-1 expression support the notion that this population underwent antigen-driven clonal expansion at the tumor site.

**Figure 6**

Skewed repertoire and binary distribution of CD8⁺ TIL TCRβ clonotypes based on expression of PD-1 in the tumor. CD8⁺ T cells as well as PD-1⁺ and PD-1⁻ fractions were sorted to high purity from FrTu1913 (A–C) and FrTu3713 (D–F). mRNA was extracted, and TCRβ deep sequencing was performed. (A and D) Diversity of the TCRβ repertoire within the bulk CD8⁺, CD8⁺PD-1⁺, and CD8⁺PD-1⁻ TIL populations. The relative frequencies of the most frequent TCRβ clonotype (unique CDR3β amino acid sequences), the second most frequent, the 3rd to 30th most frequent, and the rest of the clonotypes are shown. (B and E) The 30 most frequent CD8⁺ TIL clonotypes in the fresh tumor are plotted based on their frequency in PD-1⁺ and PD-1⁻ clonotypes. (C and F) Frequency of the 30 most frequent TCRβ clonotypes in the CD8⁺PD-1⁺ TIL population. Their frequency in the PD-1⁻ and PD-1⁺ subsets is represented. Each dot represents 1 unique TCRβ clonotype. The cumulative frequency (Σ freq.) of the clonotypes in each of the populations is shown below. ****P ≤ 0.0001, Wilcoxon signed-rank test.

**Tables 1 and 2.** The 30 most highly expanded PD-1⁺ clonotypes were far less frequent in the PD-1⁻ populations, and they only accounted for 1.2% and 3% of the PD-1⁺ population in FrTu1913 and FrTu3713, respectively (Figure 6, C and F). The biased 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 Figure 3A and B). This is consistent with our previous results indicating that PD-1, rather than 4-1BB, more comprehensively identifies the diverse repertoire of tumor-reactive cells. Overall, the skewed TCRβ profile of the PD-1⁺ population and the binary distribution of TCRβ clonotypes based on PD-1 expression support the notion that this population underwent antigen-driven clonal expansion at the tumor site.

The frequency of specific clonotypes in the CD8⁺ and PD-1⁺ populations was 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-A11mut) and CDKN2Amut, 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-A11mut-specific clonotypes from FrTu1913 ranked among the 23 most frequent clonotypes in the PD-1⁺ population.
none of these clonotypes accounted for the most frequent CD8+ responses was first delineated in PD-1 knockout mice (38, 39). Its negative effect on T cell function and recognition of CDKN2A expressed in FrTu193. Assessment of autologous tumor recognition and HLA-A11 mut revealed that the most frequent clonotype in FrTu193 was tumor reactive (Figure 7D and Table 1). Specific target recognized by clonotypes (blank, not determined). Classification of CD3+ amino acid sequences according to their frequency in each population, 1 being the most frequent. Number of times 1 particular CD3+ sequence was found in each population. Not detected.

### 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).
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 expression of these receptors such as CTLA-4, TIM-3, BTLA, CD160, LAG-3, and 2B4 has become a hallmark of chronically stimulated T cells during chronic infections correlates with disease progression (22, 41). Expression of PD-1 in patients with chronic viral infections like HIV and HCV also 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 occurring T cell responses to cancer and providing a rationale for the treatment of cancer with immune checkpoint inhibitors. The present study, we found that expression of PD-1 on CD8+ melanoma TILs captured the diverse repertoire of clonally occurring T cell responses to cancer and providing a rationale for the treatment of cancer with immune checkpoint inhibitors.

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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, 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](image-url)

**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.
TIM-3 in IFN-γ through differentiation (17), or preferential expression of these receptors between PD-1+, LAG-3+, TIM-3+, and 4-1BB+ selected TILs, 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 cells in presence of cytokines may only 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 TCRβ 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 Ficoll-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-glucolamine {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 V500; NK-1, 3:1,000; CD57 FITC; J168-540, 1:2,100; BTLA PE), eBioscience (MIH-4, 1:6,100; PD-1 APC), Biolegend (261-9C3, 1:100; CD45RA PE-Cy7, 1:100; 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, B7-H4, 1:100, B7-H3, 1:100, 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), 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 until further analysis.

For phenotypic characterization of CD8+ T cells and cell sorting using flow cytometry, PBMCs and tumor samples were thawed in the presence of RPMI-1640 with 1-glucolamine (Lonza), 1 mg/ml collagenase IV, 30 U/ml DNase, 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.

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 RNALater (Invitrogen). We extracted mRNA and amplified TRB 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 S′ 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)-TRβ 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 × 10^6 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 × 10^6 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 cytotoxic 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 × 10^4) 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 as 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) 51Cr (PerkinElmer) and washed twice. We plated 1 × 10^6 labeled target cells in 24-well plates with effector cell at the indicated effector/target ratios in a 96-well U-bottomed plate for 4 hours at 37°C. The amount of 51Cr 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. 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...
MBMs were used as controls in coculture assays. PBMCs transduced with a MART-1 26-35 A*0201 (F5 Td) or tyrosinase 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 (COA2) 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 CRKRS mut were purchased from Peptide 2.0.

Melanoma cell lines were established from tumoral fragments or 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. T2 cells and the human melanoma tumor cell lines 624, 624CIITA, TC526, 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 transfected with HLA molecules were maintained in DMEM containing 10% FBS (Sigma-Aldrich) supplemented with 100 U/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 identified (36).

For some patients, CD4⁺CD25⁻ cells were isolated from apheresis pretreatment sample and used as normal autologous targets in coculture assays. PBMCs transduced with a frameshift mutation in CDKN2A locus that encodes for tumor suppressor proteins P14ARF and P16INK4a (CDKN2A mut) (3 different clonotypes), and a HLA-A11 mut class I gene product (2 clonotypes) expressed by TC1913 were identified (36).

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