Interferon-γ Production by Peripheral Lymphocytes Predicts Survival of Tumor-Bearing Mice Receiving Dual PD-1/CTLA-4 Blockade

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Abstract

Immune checkpoint inhibitors are transforming the way cancer is treated. However, these therapies do not benefit all patients and frequently cause significant immune-related adverse events. Biomarkers that identify patients with a favorable early response to therapy are essential for guiding treatment decisions and improving patient outcomes. In this report of our study, we present evidence that shortly after administration of dual PD-1/CTLA-4 blockade, the proinflammatory capacity of peripheral lymphocytes is predictive of tumor progression and survival outcomes in multiple murine models. Specifically, we observed that the quantity of interferon-γ (IFNy) produced by peripheral lymphocytes in response to CD3/CD28 stimulation was robustly correlated with subsequent survival outcomes. In the tumor models and early time points assessed in this study, this relationship was considerably more predictive than a host of other potential biomarkers, several of which have been previously reported. Overall, these findings suggest that measuring the capacity of peripheral lymphocytes to produce IFNy may help identify which patients are benefiting from combination anti–PD-1/anti–CTLA-4 immunotherapy. Cancer Immunol Res; 4(8): 650–7. ©2016 AACR.

Introduction

Monoclonal antibody (mAb) inhibitors of the immune checkpoints, CTLA-4 (CD152) and PD-1 (CD279), have individually achieved regulatory approval in the United States. Combinations of these inhibitors may benefit some patients, and dual checkpoint blockade was recently approved for a subset of patients with melanoma. A trial in refractory melanoma revealed that this combination yielded substantial reductions in tumor burden for the majority of patients, but 98% of those patients experienced immune-related adverse events (irAE), many of which were moderate or severe (1). Head-to-head comparisons of dual PD-1/CTLA-4 blockade with monotherapy in previously untreated melanoma patients indicated that the combination significantly improved progression-free survival (2) and objective responses (3). Monotherapy also induces some irAEs with significant frequency (2–5). Overall, the inconsistent efficacy and potentially serious side effects of checkpoint blockade immunotherapy underscore the need for predictive biomarkers that identify patients with favorable early responses to treatment.

Many prognostic and predictive biomarkers for checkpoint inhibitors have been reported in clinical studies, mostly in the context of monotherapy (6). Abundant lymphocyte infiltration of the tumor (7), an immune-active microenvironment (8), and a high tumor mutational burden (9) are previously reported prognostic biomarkers for CTLA-4 blockade. Elevated PD-L1 expression in the tumor has repeatedly been associated with better responses to PD-1 blockade, although high pretreatment expression of PD-L1 may not be a prerequisite for therapeutic benefit (10, 11). With respect to predictive biomarkers, increased absolute lymphocyte counts 7 weeks into CTLA-4 blockade can be a robust biomarker for outcome (12). The same is true for increased frequencies of tumor antigen–specific T cells (9, 13); lower frequencies of peripheral myeloid-derived suppressor cells (MDSC; ref. 14); increased expression of ICOS (15), Ki-67 (16), and IFNγ by CD4 T cells (17); decreased frequencies of FoxP3+ regulatory T cells (18); and drops in serum C-reactive protein and lactate dehydrogenase. Despite the aforementioned progress, robust predictive biomarkers for therapeutic outcomes and overall survival (OS) remain elusive (19).

In this study, we investigated how the proinflammatory and cytotoxic capacity of peripheral lymphocytes was modulated 1 week after initiating dual PD-1/CTLA-4 blockade and how these changes were correlated with tumor progression and survival. Markers associated with proliferation (Ki-67) and cytotoxicity (GzmB, GzmA, and IFNγ) were elevated at this early time point, which is consistent with reports from studies involving human patients (20). We observed that the frequency of proliferative and cytotoxic markers in the peripheral CD8 T-cell population had predictive utility, but previously reported CD4 T-cell biomarkers, such as ICOS and FoxP3, did not robustly correlate with outcomes. In contrast, the most striking finding was that the quantity of IFNγ...
production by peripheral lymphocytes had the strongest correlation with tumor progression and survival.

**Materials and Methods**

**Mice**

Wild-type C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory. All mice were maintained under specific pathogen-free conditions in the Providence Portland Medical Center vivarium. Experimental procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, under an approved IACUC protocol (#39).

**Tumor models and treatment regimens**

This study used five cell lines: CT26 (colon carcinoma, BALB/c, female, sq, 1 × 10⁶), 4T1i (mammary carcinoma, BALB/c, female, mammary fat pad, 4 × 10⁴), MCA-205 (fibrosarcoma, C57BL/6, female, sq, 1 × 10⁶), TUBO (mammary carcinoma, BALB/c, female, sq, 5 × 10⁴), and 3LL (lung carcinoma, C57BL/6, male, sq, 1 × 10⁶). MCA-205, 4T1i, and TUBO cell lines were kindly provided by Drs. Andy Weinberg and Emmanuel Akloriyoti (EACRI, Portland, OR) and Dr. Wei-Zen Wei (Wayne State University, Detroit, MI), respectively. MCA-205, 4T1i, and TUBO cell lines were obtained between 2009 and 2011. CT26 and 3LL cell lines were obtained from ATCC in 2010. These cell lines were not independently validated by our team. The 4T1i cell line is an immunogenic subclone of the parental 4T1 line that remains metastatic. Treatment regimens began between 5 and 10 days following implant, depending on when the tumors within a given model were palpable. All mice in this study received the same treatment regimen, with three doses administered (i.p.) 3 days apart (Supplementary Fig. S1). Mice received 200 µg of anti–PD-1 [proprietary clone with a single amino acid substitution within its heavy chain (D263A), which reduces Fc receptor binding; ref. 21], anti–CTLA-4 (clone 9D9), or isotype control mlgG1/mlgG2b.

Rechallenge experiments mirrored the original tumor challenge, except for the rechallenge site (opposite flank or mammary fat pad). Rechallenge began 3 weeks after all mice in a cohort reached a primary endpoint. For statistical analyses, endpoints were defined as the first time point that tumor area exceeded 150 mm² or was nonpalpable. The average rate of tumor growth was calculated as the slope of the line between the tumor size on the first day of treatment and on the endpoint day. Values for the aforementioned parameters and survival charts can be found in Supplementary Table S1 and Supplementary Fig. S2, respectively. Supplementary data from untreated and monotherapy-treated mice are included in Supplementary Table S2.

**Blood collection and processing**

For the experiment reported in Fig. 1, blood samples were drawn from tumor-bearing mice (CT26) on day 0 (tumor-bearing, pretreatment), day 8 (1 day after final treatment dose) and day 17 (10 days after final treatment dose). For the experiments reported in Figs. 2 and 3, blood samples were harvested on day 8. For each sample, 100 mL of fresh heparinized blood was diluted 1:1 with complete RPMI (cRPMI). Total lymphocytes and serum were isolated by density gradient (LymphoPrep). Purified lymphocytes were resuspended in 500 mL of cRPMI, separated into five equal aliquots (100 µL each), and analyzed by flow cytometry or functional assay, as described below. For absolute lymphocyte counts, whole blood was stained and processed with Cal-lyse (Invitrogen) following manufacturer protocols prior to flow analysis.

**Flow cytometry**

For the experiments reported in Figs. 1 to 3, freshly purified lymphocytes were stained for the following surface markers: CD4, CD8 (BD Biosciences), Thy1.2, NKp46, CD19, Fixable Viability Dye, ICOS, CD44, Fasl (eBioscience). Next, cells were fixed and permeabilized (Foxp3 Transcription Factor Staining Buffer Set; eBioscience) and stained for the following intracellular targets: Granzyme A, Granzyme B, FoxP3, and Ki-67 (Life Technologies). Flow cytometry data were acquired on an LSR-II flow cytometer running FACSDiva software (BD Biosciences), and data were processed and analyzed with FlowJo (TreeStar).

**Functional activation assays and ELISA**

Cytokine release assays were performed under three conditions: control (media only), anti–CD3/anti–CD28 stimulation (96-well round bottom plate coated with anti–CD3 (clone 145-2C11; 5 µg/mL) and anti–CD28 (clone 37.5.1; 2 µg/mL) mAb, and tumor-specific "on cell" stimulation (96-well flat bottom plate coated with a monolayer of tumor cells at 50%–60% confluency). Lymphocyte suspension (100 µL) was added to each well with an additional 150 µL of cRPMI. Plates were incubated (37°C, 5% CO₂, 95% humidity), and 18 hours later, supernatants were harvested and IFNγ and TNFα production was determined by ELISA (eBioscience). IFNγ and IL6 levels in serum samples were determined by ELISA (eBioscience).

**Statistical analysis, data modeling, and living figure**

Biomarker expression was correlated with survival outcomes using a linear regression and classified into predictive probabilities by logarithmic regression. These probabilities were used to create receiver operating characteristic (ROC) curves to determine the predictive strength of each individual measurement as a potential threshold for survival outcome. Statistical significance was determined by an unpaired Student t test (for comparison between two groups), r² linear regression (for outcomes correlation), or Kaplan–Meier survival (for tumor survival studies) using GraphPad InStat or Prism software (GraphPad); P < 0.05 was considered statistically significant. Supplementary Table S1 contains all of the data included in the correlation analyses performed in this study. The table is formatted to be compatible with the BOSS (Biomarkers, Outcomes and Stats Software) analysis tool, which is an open-source platform for creating interactive living figures from cancer research data sets (http://www.mycancerproject.org/BOSS/). A stand-alone copy of the software that opens with the Google Chrome Web browser is included as Supplementary File S1.

**Results**

Transient increases in proinflammatory peripheral lymphocytes with cytotoxic activity

To assess expression kinetics, a selection of biomarkers (identified in the literature and in our own prior screen) were analyzed in the peripheral blood of tumor-bearing mice (CT26 tumor model) at three time points: pretreatment (day 0); early posttreatment (day 8); and late posttreatment (day 17). Dual checkpoint blockade increased expression of several markers,
particularly those associated with lymphocyte activation, cytotoxicity, and regulatory T-cell function (Fig. 1). Several of these observations were consistent with changes in human cancer patients following initiation of dual PD-1/CTLA-4 immunotherapy (20). Among peripheral CD4 T cells, the frequency of ICOS and FoxP3 cells in the dual checkpoint blockade group increased at the day 8 time point, although by day 17, these values were no longer significantly different from those in the IgG control group. Similarly, among CD8 T cells and NK cells, increased frequencies of GzmB and Ki-67 cells were observed on day 8, but these returned to near pretreatment levels by day 17 (Fig. 1B). Thus, the differential expression of the indicated activation and cytotoxicity markers was short-lived, with the greatest magnitude of change observed immediately subsequent to treatment.
Markers of cytotoxic CD8 T-cell function correlated with tumor progression and survival

We next sought to determine whether any of the aforementioned parameters were predictive for survival-associated outcomes. To account for model-specific artifacts, we used five tumor models representing different cancer types and genetic backgrounds. The complete response rate (durable cures) for dual checkpoint blockade varied greatly among models.

Predictive Biomarkers Following PD-1/CTLA-4 Blockade

Figure 2. The expression of cytotoxicity-associated markers by peripheral CD8 T cells following onset of dual PD-1/CTLA-4 blockade correlates with the rate of tumor progression. Frequencies of peripheral NK, CD4, and CD8 T cells expressing each marker are plotted against the average rate of tumor growth between the start of treatment and primary endpoint. Peripheral blood samples were acquired 1 day after final treatment dose (day 8), and each data point represents one mouse. P values reflect the probability that the slope of the line is different from the null hypothesis (slope = 0), and $r^2$ values were determined by conventional linear regression (CT26 $n = 19$, MCA-205 $n = 24$, 4T1i $n = 24$, 3LL $n = 21$, TUBO $n = 8$).
ranging from 0% to 87% (Supplementary Fig. S2). All of the mice in this study received the same three-dose regimen of anti–PD-1/anti–CTLA-4, and blood samples were analyzed on day 8 (1 day after the final treatment dose). Tumor growth was tracked until an endpoint was reached (death or nonpalpable tumor), and the relationships between biomarkers and survival outcomes were analyzed (Supplementary Table S1). Several markers of proliferation and cytotoxicity in NK and CD8 T cells, such as Ki-67 and GrzB, were correlated with a slower rate of tumor growth, consistent with a report investigating the effects of combining radiotherapy with dual checkpoint blockade (22). In contrast, the expression of ICOS and FoxP3 on CD4 T cells was poorly correlated with outcomes (Fig. 2; Supplementary Fig. S3).

Robust IFNγ production by peripheral lymphocytes predicted survival

The capacity of peripheral lymphocytes to produce proinflammatory cytokines (IFNγ and TNFα) in response to a generic stimulation (anti-CD3/anti-CD28) was robustly correlated with tumor progression (Fig. 3A). Dual checkpoint blockade did not significantly alter lymphocyte counts at this early time point (data not shown), suggesting that the modulation of IFNγ production derives from a phenotypic change, rather than an absolute increase in the number of proinflammatory lymphocytes. IFNγ production by unstimulated lymphocytes (Fig. 3A) and serum IFNγ (Fig. 3B) concentrations were often below the detection threshold of the assay and poorly predictive. Overall, IFNγ production in response to anti-CD3/anti-CD28 stimulation was the best predictive biomarker for tumor progression, as defined by the area under the curve (AUC) of the receiver operating characteristic (ROC; Fig. 4A and B). Based on the vertex of the ROC curve for anti-CD3/anti-CD28–induced IFNγ production, a threshold of 20.9% was selected for the false-positive rate (probability of identifying a nonresponder as a responder) as it maximized the true-positive rate (probability of correctly identifying a positive responder), while minimizing the false-positive rate. The true-positive rate at this threshold was 88.49%. In comparison, the expression frequency of GrzA among CD8 T cells was the second best predictor at the chosen false-positive rate, but yielded a significantly lower true-positive rate (69.2%; Fig. 4B). Furthermore, the average rate of tumor growth was significantly

Figure 3.
Increased capacity of peripheral lymphocytes to generate proinflammatory cytokines following onset of combination PD-1/CTLA-4 blockade is correlated with outcome. Peripheral blood samples were acquired 1 day after final treatment dose (day 8), and each data point represents one mouse. P values reflect the probability that the slope of the line is different from the null hypothesis (slope = 0), and r² values were determined by conventional linear regression. A, purified peripheral lymphocytes were cultured for 24 hours under one of three conditions: normal culture media (“No stim”/control); plate-bound αCD3/αCD28 mAb; or a viable monolayer of autologous tumor cells (“On-cell stim”). Concentrations of IFNγ and TNFα in the supernatant were quantified by ELISA. B, serum was isolated from peripheral blood samples, and IFNγ and IL6 concentrations were quantified by ELISA (CT26 n = 19, MCA-205 n = 24, 4T1i n = 24, 3LL n = 21, TUBO n = 8).
different between the groups of mice above and below the IFNγ level (1,385 pg/mL) corresponding to the selected false-positive rate threshold (Fig. 4C).

Data sharing and development of an open-source living figure

The data set from this study can be used to generate thousands of individual correlation analyses, depending on the tumor models, biomarkers, and outcomes selected for analysis. To facilitate these analyses, we used the BOSS analysis platform to create an interactive visualization of this study’s data set (e.g., “living figure”). This living figure uses a structured data set (Supplementary Table S1) and the stand-alone BOSS analysis tool (Supplementary File S1), which runs in the Google Chrome Web browser. Additional outcomes, experimental parameters, cancer types, treatment types, and biological specimens can be independently added to the data. The goal is that addition of independently
generated data will help eliminate artifacts and further clarify the predictive utility of the putative biomarkers observed in this study and others.

Discussion

Several observations from this study were consistent with peripheral immune correlates in human patients with advanced melanoma treated with combined nivolumab/ipilimumab (NCT01024231; refs. 10, 20), including that dual therapy did not significantly alter peripheral absolute lymphocyte counts, unlike anti–CTLA-4 monotherapy (12). They also found that ICOS and Ki-67 expression were increased in peripheral T-cell populations following treatment. These observations were mirrored across the tumor models we investigated, which offers hope that some of the immune correlates we identified may be conserved between mice and humans.

Tumors that are well infiltrated by lymphocytes, have a high mutational burden, or are otherwise immunogenic are more likely to respond productively to checkpoint blockade (7–9), but these metrics have not helped estimate the likelihood of a therapeutic response. We sought to identify immunologic biomarkers that can be noninvasively monitored (in peripheral blood) and are predictive for tumor progression/survival in mice receiving dual PD-1/CTLA-4 blockade. Our data indicate that the capacity of peripheral lymphocytes to produce IFNγ after CD3/CD28 stimulation was the strongest predictor of survival outcomes. We also found that frequency of CD8 T cells expressing granzyme B and Ki-67 may have predictive utility, which is consistent with previous reports (20, 22, 23).

The assay necessary to measure IFNγ production is a derivative of the well-established IFNγ-release assay, which is FDA approved for several indications, including determination of *Mycobacterium tuberculosis* exposure (QuantiFERON-TB Gold; ref. 24). Thus, the capacity of peripheral lymphocytes to produce IFNγ may be a viable strategy for assessing patients’ response to the onset of checkpoint blockade and a valuable addition to existing immune monitoring portfolios.

Disclosure of Potential Conflicts of Interest

W.L. Redmond reports receiving commercial research support from Bristol-Myers Squibb, Merck, Galectin Therapeutics, and Nektar Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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