Analyses of Pretherapy Peripheral Immunoscore and Response to Vaccine Therapy

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Abstract

Tumor immunoscore analyses, especially for primary colorectal cancer and melanoma lesions, provide valuable prognostic information. Metastatic lesions of many carcinoma types, however, are often not easily accessible. We hypothesized that immune cells in peripheral blood may differ among individual patients with metastatic disease, which, in turn, may influence their response to immunotherapy. We thus analyzed immune cell subsets within peripheral blood mononuclear cells to determine if a "peripheral immunoscore" could have any prognostic significance for patients before receiving immunotherapy. Patients with metastatic breast cancer were randomly assigned to receive docetaxel ± PANVAC vaccine. In another trial, prostate cancer patients with metastatic bone lesions were randomly assigned to receive a bone-seeking radionuclide + PROSTVAC vaccine. Predefined analyses of "classic" immune cell types (CD4, CD8, natural killer cells, regulatory T cells, myeloid-derived suppressor cells, and ratios) revealed no differences in progression-free survival (PFS) for either arm in both trials. Predefined analyses of refined immune cell subsets for which a biologic function had been previously reported also showed no significant prognostic value in PFS for patients receiving either docetaxel or radionuclide alone; however, in patients receiving these agents in combination with vaccine, the peripheral immunoscore of refined subsets revealed statistically significant differences in PFS (P < 0.001) for breast cancer patients receiving docetaxel plus vaccine, and in prostate cancer patients receiving radionuclide plus vaccine (P = 0.004). Larger randomized studies will be required to validate these findings. These studies, however, provide the rationale for the evaluation of refined immune cell subsets to help determine which patients may benefit most from immunotherapy. Cancer Immunol Res; 4(9); 755-65. ©2016 AACR.

Introduction

Recent immune biomarker studies have indicated that in patients with some tumor types, the quantity and quality of tumor-infiltrating immune cells, analyzed prior to standard cancer therapies, can be associated with subsequent clinical outcomes. Pages and Galon reported that patients with colorectal cancer who have a high infiltration of CD8+ T lymphocytes within primary tumors survive longer (1), and that infiltration of CD8+ T cells with an effector-memory phenotype (i.e., CD45RO+ T cells) in endoscopic biopsies of colorectal cancer is a negative predictor of nodal metastasis and positive predictor of survival (2). Thus, through histopathologic analyses of human primary colorectal cancer biopsies, one can calculate a tumor immunoscore as a new component for the classification of that cancer, designated TNM-I (TNM-Immune; ref. 3). Additional studies from Fridman (4) extended these observations, suggesting that, in addition to T cells, macrophages, mast cells, granulocytes, and myeloid-derived suppressor cells (MDSC) are often found infiltrating or surrounding tumors and can also be included to help predict the likelihood of clinical response in colorectal cancer patients. However, whereas the prognostic significance of T-cell infiltration in primary tumors has been demonstrated in colorectal cancer patients, analyses of some other carcinoma types showed varying and sometimes conflicting results regarding correlations between immune infiltrate and prognosis (see ref. 5 for review).

Although tumor immunoscore analyses have been conducted principally on biopsies of primary tumors, metastatic tumor sites of many carcinoma types are often not easily accessible. We thus posed the question of the potential value of analyzing immune cell subsets in peripheral blood mononuclear cells (PBMC) to determine if the calculation of a "peripheral immunoscore" would be of prognostic value in determining which patients with metastatic carcinoma would most likely benefit from immunotherapy such as vaccine-mediated therapy. The recent advances in multiparametric flow cytometry, which can identify up to 18 cellular markers (6), along with the improved understanding of the significance of specific markers reflecting a given immune function (7, 8), might provide a platform for the...
analysis of immune signatures in cancer patients before therapy. Some studies suggest correlations between the frequency, prior to therapy, of various peripheral blood cell subsets and clinical outcome, such as circulating CD8⁺ CCR7⁻ T lymphocytes in patients with head and neck cancer (9), or circulating MDSCs in patients with advanced melanoma (10). In one study, low total lymphocyte count was associated with poor survival in pancreatic cancer patients receiving a granulocyte–macrophage colony-stimulating factor (GM-CSF)–secreting tumor cell vaccine (11). A comprehensive review of immunologic biomarkers, moreover, stated that "most likely the clinical effectiveness of immune-based treatments will be predicted by panels of markers rather than single assays of a specific immune effector cell" (12). Advances in immune cell biology have delineated the complexity of the human immune system in terms of the vast array of refined immune cell subsets and the immune enhancing or suppressing function of many of these individual refined subsets. Here, we sought to analyze patients' peripheral immune signatures prior to therapy to define if any correlations exist with the efficacy of immune and nonimmune treatments. We analyzed PBMCs from two randomized phase II clinical trials. In the first trial, patients with metastatic breast cancer were randomized to receive docetaxel alone or docetaxel plus PANVAC [recombinant Vaccinia (rV), recombinant fowlpox (rF)-carcinoembryonic antigen (CEA)-mucin-1 (MUC1)-tripod of human costimulatory molecules B7-1, ICAM-1, and LFA-3 (TRICOM)] vaccine (13). In the second trial, patients with metastatic castrate-resistant prostate cancer (mCRPC) with metastases to the bone received Quadramet, the bone-seeking samarium-153 (153Sm) radionuclide, with or without PROSTVAC [rV, rF-prostate-specific antigen (PSA)-TRICOM] vaccine (14, 15). A phase III study of PROSTVAC has been fully accrued in patients (n = 1,200) with metastatic prostate cancer. The rationale for these combination therapy studies was based on preclinical models using human tumor cells in vitro and murine tumors in syngeneic mice. Both docetaxel (16) and radiation (17) were shown to upregulate tumor cell surface markers, such as major histocompatibility complex (MHC)–peptide complexes, and death receptors, such as Fas, to render the tumor cells more susceptible to T-cell–mediated lysis, a phenomenon termed "immunogenic modulation" (16). Both clinical trials were designed to inform larger studies. Predetermined analyses of so-called "classic" immune cell types (CD4, CD8, natural killer (NK) cells, regulatory T cells (Treg), MDSCs, and ratios) revealed no differences in progression-free survival (PFS) for each arm in both trials. Well over 100 refined immune cell subsets of the classic immune cell types have now been delineated (18), and for some a biologic function has been identified. Analyses of refined immune cell subsets selected prospectively, for which a biologic function had been previously reported, also revealed no significant prognostic value in PFS in patients receiving either docetaxel alone or the radionuclide alone. However, in patients receiving these agents in combination with vaccine, the peripheral immunoscore based on refined subsets with a known biologic function revealed a statistically significant difference in PFS for breast cancer patients receiving docetaxel plus vaccine, and a statistical difference in prostate cancer patients receiving radionuclide plus vaccine. The studies reported here suggest that the peripheral immunoscore, based on the frequency of refined PBMC subsets with a phenotype indicating immune function, analyzed prior to therapy may help to identify those cancer patients undergoing immunotherapy who may have a more favorable clinical outcome.

Materials and Methods

Cancer patients and treatments

PBMCs were harvested at baseline (i.e., prior to treatment) from cancer patients enrolled in two phase II randomized multicenter clinical trials. The first trial consisted of docetaxel alone or in combination with PANVAC vaccine to treat breast cancer (NCT00179309; ref. 13). PANVAC vaccine consists of rV–prime and multiple rF-boosters containing transgenes for TRICOM plus the CEA and MUC1 transgenes (rV/rF-CEA-MUC1-TRICOM). In this trial, 48 patients with metastatic breast cancer were randomized to receive an initial priming vaccination with 2 × 10⁶ plaque-forming units (pfu) of rV-PANVAC followed by monthly boosting vaccinations with 1 × 10⁹ pfu of rF-PANVAC in conjunction with docetaxel therapy administered weekly for 3 weeks in each 4-week cycle (arm I) or weekly docetaxel alone for 3 weeks in each 4-week cycle (arm II; ref. 13). PBMCs from 43 of the 48 patients were assessed in the current study based on availability. The second trial consisted of 153Sm with a tetraphosphonate chelator, ethylene-diaminetetramethylenephosphonic acid (EDTMP) with or without PROSTVAC vaccination to treat men with androgen-insensitive prostate cancer (NCT00450619; refs. 14, 15). PROSTVAC vaccine consists of rV–prime and multiple rF-boosters containing transgenes for TRICOM plus the PSA transgene (rV/rF-PSA-TRICOM). In this trial, 44 men with androgen-insensitive prostate cancer and metastatic bone lesions were randomly assigned to receive Quadramet, the bone-seeking radiopharmaceutical agent 153Sm-EDTMP, alone (arm A), or Quadramet with PROSTVAC (arm B; refs. 14, 15). Quadramet was given at 1 mCi/kg i.v. on day 8 and then every 12 weeks. PROSTVAC was given on days 1, 15, 29, then every 4 weeks. PBMCs from 24 of the 44 patients were assessed in the current study based on availability.

Multicolor flow cytometry

We applied a multiparametric flow cytometry platform to measure the frequency of PBMC immune subsets in patients prior to therapy. Patients' PBMCs were harvested before therapy by density gradient separation, and then 1 ml of PBMCs was cryopreserved in liquid nitrogen at a concentration of 10⁶ cells/mL per vial. On the day of staining, one vial of cryopreserved PBMCs per patient was defrosted, cells were counted, and then stained with the appropriate monoclonal antibodies (antibody panels listed in Supplementary Table S1) and analyzed using the gating strategies outlined in Supplementary Figs. S1 and S2 to identify the classic immune cell types as well as those refined subsets with a phenotype reflecting immune function (listed in Tables 1 and 2). Viability of all samples following thawing after cryopreservation was ~80% to 95%. At least 3 × 10⁶ events in the live gate were acquired with a BD LSR-II flow cytometer equipped with a UV, violet, blue, and red laser. FCS files were imported and analyzed with Flowjo V.9.7 for Macintosh (TreeStar). The frequency of individual subsets was calculated as a percentage of total PBMCs to help reduce the bias that could occur in the smaller subpopulations with fluctuations in parental leukocyte populations.

Predetermined selection of PBMC subsets for the calculation of peripheral immunoscores

The aim of this study was to test whether the clinical outcome in cancer patients treated with immune therapy in randomized trials can be predicted by measuring the frequency of specific PBMC subsets at baseline. The selection of predetermined PBMC subsets
Clinical data and patient samples; the prostate trial was initiated a later date than in the breast cancer trial, due to the availability of immunoscore. Immune cells were used for the calculation of the peripheral function and six ratios between effector and suppressor activation, or suppression (refs. 19 that have shown to be involved in immune maturation, as described in prior publications (see Tables 1 and 2).

In both trials, criterion 1, or classic cell types, consisted of the frequencies prior to therapy of CD4+ and CD8+ T lymphocytes, Tregs, NK cells, MDSCs, and the ratios between CD4+ or CD8+ T cells and Tregs or MDSCs (Tables 1 and 2). For the calculation of the peripheral immunoscore based on refined subsets with a phenotype reflecting immune function, or criterion 2, the markers were selected following an extensive investigation of the literature, which sought for markers that have shown to be involved in immune maturation, activation, or suppression (refs. 19–27; Table 1). In the breast cancer trial, seven refined PBMC subsets reflecting immune function and six ratios between effector and suppressor immune cells were used for the calculation of the peripheral immunoscore.

In the prostate cancer trial, immune analyses were performed at a later date than in the breast cancer trial, due to the availability of clinical data and patient samples; the prostate trial was initiated ~1.5 years after the breast study and was also delayed by poor patient accrual. Thus, in the prostate cancer trial, the refined PBMC subsets reflecting function were based on a subsequent updated literature search at the time of the analysis, which included the use of the marker ICOS for the identification of highly suppressive Tregs (28), Tim-3 for the identification of antitumor NK cells (29), and programmed cell death protein-1 ligand (PD-L1) in circulating conventional dendritic cells (dDCs; ref. 30) and MDSCs (refs. 31, 32; Table 2).

Calculation of tertile bins

The distribution of each predetermined PBMC immune cell type at baseline, indicated as a percentage of total PBMCs, was divided in mathematical tertile bins: low-frequency, middle-frequency, and high-frequency bins (Supplementary Fig. S3). The use of tertiles ensured that patients closer to median frequency levels were not assigned the same points as patients closer to extreme values (as would be the case if applying the median as a cutoff). The bin cutoffs for a given subset assessed in the breast cancer trial were not the same, as the tertile cutoffs were determined by the spread of a given subset within each of the patient populations examined.

### Table 1. Predetermined PBMC phenotypes for calculation of the peripheral immunoscore in patients with metastatic breast cancer treated with docetaxel alone versus docetaxel + PANVAC vaccine

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<tr>
<th>Description</th>
<th>Expected effect on PFS</th>
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<tbody>
<tr>
<td>% CD4</td>
<td>Positive</td>
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<tr>
<td>% CD8</td>
<td>Positive</td>
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<tr>
<td>% Treg(a)</td>
<td>Negative</td>
<td>% Treg</td>
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<tr>
<td>% MDSC(b)</td>
<td>Negative</td>
<td>% MDSC</td>
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<tr>
<td>% NK(c)</td>
<td>Positive</td>
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<td>Ratio CD4:Treg</td>
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**NOTE:** Frequencies of PBMC subsets represented as a percentage of total PBMCs. *(a), CD45RA+/CCR7; *(b), CTLA-4, PD-1, Tim-3, 2B4; *(c), CD4+CD25+FoxP3+CD127; *(d), CD56+CD19+CD20+CD56+CD33+HLA-DR+/low; *(e), CD14+CD15+; *(f), CD3+CD56+.

### Table 2. Predetermined PBMC phenotypes for calculation of the peripheral immunoscore in patients with metastatic prostate cancer treated with Quadramet alone versus Quadramet + PROSTVAC vaccine

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cancer trial, the cutoff between two bins was moved by one position if the delta between the two bins with the new cutoff was >50% compared with the delta from the original mathematical tertile bin cutoff. In the prostate cancer trial, tertile bin adjustments were calculated by custom software as described below. The cutoffs used to define tertile bins were adjusted to ensure that two patients with the same, or almost the same frequency of a given subset, were assigned to the same bin, and thus assigned the same point value.

Point assignment method
Points were assigned to each PBMC subset based on the tertile distribution. For criteria 1 and 2, if a subset was expected to positively correlate with PFS, zero (0) points were assigned to the low bin, one (1) point for the middle bin, and two (2) points if in the high bin (see example of CD4 in Supplementary Fig. S3A and S3C). If a subset was expected to negatively correlate with PFS, zero (0) points were assigned to the high bin and two (2) points if in the low bin (see example of Tregs in Supplementary Fig. S3B and S3D). The peripheral immunoscore for a given patient was the sum of points assigned to the individual PBMC subsets that were included in a specific criterion used to build the immunoscore (see example in Supplementary Table S3). Equal weight was placed on each of the predetermined subsets included within the score, in an effort to be able to apply the score to multiple clinical trials of patients with different indications.

Hazard ratio and Kaplan–Meier diagrams
Given that the immunoscore is built upon pretherapy frequencies of PBMC subsets, and that the clinical outcome of patients differed depending upon the therapy received in each arm of the trials, in this study we compared the high versus low immunoscore with clinical outcome within each arm of the trials separately. To keep the peripheral immunoscore method as straightforward as possible, we used a traditional hazard analysis, with groups defined by the median when comparing the immunoscore with clinical outcome. To generate hazard ratio (HR) graphs and Kaplan–Meier curves, each PBMC subset selected for the calculation of peripheral immunoscores was individually tested with the Mantel–Haenszel method of meta-analysis. The median frequency of a specific PBMC subset was considered as the cutoff between positive and negative predictors of survival. HR and Kaplan–Meier diagrams of peripheral immunoscores were calculated as above, where the median of the immunoscores was used as the cutoff. P values < 0.05 were considered statistically significant. No adjustments were made for multiple comparisons, given the small number of P values being used as a measurement of statistical significance. The P values in the meta-style analyses graphs for the individual PBMC cell types can be considered an information-only indication of the strength of these individual cell type associations with clinical benefit, with the P value for the overall immunoscore a measure of the statistical significance of the immunoscore. Thus, there were only a handful of P values being used in the current study as a measurement of the statistical significance.

Custom software for the calculation of the peripheral immunoscore
Description of Vasco software. Vasco is a Web application that supports the exploration and analysis of immune cell subsets in the context of clinical studies through the application of exhaustive expansion (33). A detailed description is given in Supplementary Data.

Generation of immunoscores, hazard ratios, and Kaplan–Meier curves. For a given set of predetermined sets of specific phenotypes and ratios at baseline (hereafter, criterion), we compute immunoscores as follows. Immunoscore points based on bins are assigned as described above. Across both arms, we assign patients to ‘low’ or ‘high’ immunoscore groups, where patients with immunoscores less than the median are categorized as low. We perform a similar assignment for each analyte included in the criterion. Next, within each arm, we derive Mantel–Haenszel HR, P values, and 95% confidence intervals (CI), comparing the low and high groups based on immunoscore, and the low and high groups for each analyte while accounting for whether the analyte is positively or negatively associated with survival. We automatically generate corresponding HR and Kaplan–Meier diagrams for each criterion.

Results
In the first trial analyzed, 48 patients with metastatic breast cancer were randomized to receive PANVAC vaccine plus docetaxel (arm I), or docetaxel-alone (arm II), as detailed in Materials and Methods. The study was powered to detect a trend toward improvement in PFS. The median PFS in arm I was 7.9 months compared with 3.9 months in arm II, indicating a trend toward improvement in the combination arm (P = 0.09, HR = 0.65). Clinical results of this dual center trial were detailed elsewhere (34). The purpose of the study reported here was to determine if analyses of immune cell types in PBMCs prior to therapy (i.e., baseline) of 43 of the 48 patients where blood was available would reveal any differences in clinical outcome between the two arms. It is emphasized that these markers were not selected retrospectively but were prospectively selected in the analysis of this trial. Two criteria were used. The first criterion (criterion 1) analyzed at baseline the five most studied classic immune cell types—CD4+ T cells, CD8+ T cells, Tregs, MDSCs, and NK cells—as well as the ratios between effector CD4+ or CD8+ T cells and Tregs or MDSCs (Table 1). Flow cytometry was used for analyses as described in the Materials and Methods section. These assays only required a limited amount of blood (approximately 1 mL containing 1 × 10^7 PBMCs). A peripheral immunoscore was calculated based on the frequencies of such classic immune cells before therapy per patient. As shown in Fig. 1 there was no statistically significant link between the immunoscore generated using the classic immune cell subsets and PFS in either the docetaxel-alone arm (Fig. 1E, P = 0.380, HR = 0.643) or the docetaxel plus vaccine arm (Fig. 1F, P = 0.509, HR = 0.717). The heat maps in Fig. 1A and B represent the frequency at baseline of the classic PBMC types, as well as the total immunoscore generated based on these cell types, with each row corresponding to one patient. Assessment of the impact of the individual classic immune cell types on clinical outcome showed no associations with PFS in the docetaxel-alone arm (Fig. 1C), and only a single association in the docetaxel plus vaccine arm, where a high frequency of CD8+ T cells prior to therapy was associated with clinical disadvantage (Fig. 1D). At this point, it is unknown why high numbers of CD8+ T cells were associated with clinical disadvantage. These findings indicated that the peripheral immunoscore calculated based on the frequency of classic T lymphocytes, NK cells, Tregs, MDSCs, and the
ratios of classic effectors versus suppressors could not predict the duration of PFS in this patient population if treated either with docetaxel alone or with the combination of docetaxel plus vaccine.

Numerous recent studies have revealed that each of the “classic” immune cell types, i.e., CD4, CD8, NK, Tregs, and MDSCs, contain many phenotypically different defined subset populations that can be distinguished by multicolor flow cytometry; recent studies have revealed a biologic function for some of these defined subset populations. A review of the literature at the time of the analyses described here (19–27) revealed 13 defined subset populations with either immune-enhancing or immune-suppressive properties. These are listed in Table 1 and formed the basis for criterion 2. We then calculated a peripheral immunoscore based on the frequency, prior to therapy, of defined PBMC subsets having a phenotype indicating immune function (criterion 2). As shown in Fig. 2, there was a statistically significant difference between high peripheral immunoscore at baseline and PFS in patients with breast cancer treated with docetaxel plus vaccine (Fig. 2F; \( P < 0.001, \text{HR} = 0.060 \)), but not in patients treated with docetaxel alone (Fig. 2E; \( P = 0.875, \text{HR} = 0.926 \)). The heat maps in Fig. 2A and B represent the frequency at baseline of the defined PBMC subsets indicating immune function, as well as the total immunoscore generated.
Peripheral immunoscore of PBMCs, prior to therapy, in breast cancer patients treated with docetaxel alone versus docetaxel plus vaccine. The peripheral immunoscore is based on refined PBMC subsets of cell types reflecting known function (criterion 2). A and B, heat maps representing the frequency at baseline of refined PBMC subsets with a phenotype reflecting known immune function (see references on far right of Table 1) in 43 patients with metastatic breast cancer, randomized per arm: docetaxel alone (A, \( n = 20 \)) and docetaxel + IV/F-CEA-MUC1-TRICOM vaccine (PANVAC; B, \( n = 23 \)). Each row corresponds to one patient. The total score columns show the peripheral immunoscore as a result of the sum of points assigned to each PBMC subset frequency based on the tertile distribution as described in Materials and Methods. PFS (columns at far right) is ranked from the longest (green) to the shortest (red), with censored patients indicated by a tick. The gray shadowed column shows the 95% CI for the total peripheral immunoscore compared to each individual PBMC subset.

Figure 2.

To begin to validate the process of generating a peripheral immunoscore to distinguish between clinical outcome in patients receiving immunotherapy, we analyzed a second randomized trial involving patients (\( n = 44 \)) of a different tumor type (prostatic cancer patients with bone metastases) and a different therapeutic modality (a bone-seeking radionuclide, Quadrumet, \( ^{153}\text{Sm} \) administered alone or in combination with PROSTVAC vaccine. The trial was designed to determine the proportion of patients who did not progress at 4 months (time of staging). There was both a trend in increased time to progression (TTP) and PFS,

Based on these subsets, with each row corresponding to one patient. Evaluation of the impact of the individual refined subsets reflecting immune function on clinical outcome showed no associations in the docetaxel-alone arm (Fig. 2C), but several significant associations in the docetaxel plus vaccine arm (Fig. 2D). Specifically, prior to therapy, patients with high frequencies of central memory CD4\(^+\) T cells, low frequencies of lineage-negative MDSCs (CD15\(^+\)CD15\(^-\)), and immature NK cells (CD56\(^hi\)CD16\(^-\)), as well as high ratios of central memory CD4\(^+\) or CD8\(^+\) T cells/lineage-negative MDSCs, or high ratios of central memory CD4\(^+\) T cells/immature NK cells, were associated with clinical advantage when patients went on to receive docetaxel plus vaccine. These observations indicate that the measurement of refined PBMC subsets with a phenotype reflecting immune function may be helpful in distinguishing between longer versus shorter PFS in patients with metastatic breast cancer treated with the combination of docetaxel and vaccine.
and more serum PSA declines in the combination arm than in the monotherapy arm; the clinical results of this trial have been reported (14, 15, 35).

For this trial, the calculation of the peripheral immunoscores was performed in 24 of the 44 patients where blood was available by custom designed software based on the VASCO platform for the exhaustive analysis of multiple immune phenotypes (33). The use of the VASCO platform in the prostate cancer trial enabled the peripheral immunoscore to be generated in a rapid fashion, in contrast to the time-consuming manual approach that was taken with the breast cancer trial. It is again emphasized that the markers selected for analysis in this trial were not selected retrospectively but were prospectively selected. As was seen in the breast cancer trial, the peripheral immunoscore based on classic PBMC cell types (criterion 1; Table 2 and Fig. 3A–D) in this trial did not distinguish between patients with longer and shorter PFS in either the Quadramet-alone arm (Fig. 3E; $P = 0.245$, HR = 1.186) or the Quadramet plus PROSTVAC vaccine arm (Fig. 3F; $P = 0.784$, HR = 1.186). These observations again indicate that a peripheral immunoscore based on the frequency of classic PBMC cell types at baseline is unlikely to be helpful in predicting which patients will benefit from the immune-based therapy.

For the calculation of the peripheral immunoscore based on refined PBMC subsets reflecting immune function (criterion 2) in the prostate cancer trial, we applied a slightly modified list of preselected immune markers compared with the breast cancer trial, which was based on an updated knowledge of the literature (refs. 28–32; Table 2). Similar to the results observed in the breast cancer trial, patients with a high peripheral immunoscore based on refined PBMC subsets with a phenotype reflecting immune

![Image](https://example.com/image.png)

Figure 3.
Peripheral immunoscore of PBMCs, prior to therapy, in prostate cancer patients treated with the bone-seeking radionuclide $^{153}$Sm (Quadramet) with or without a vaccine. The peripheral immunoscore is based on classic cell types in PBMCs (criterion 1). A and B, heat maps representing the frequency at baseline of classic PBMC cell types in 24 patients with metastatic prostate cancer, randomized to Quadramet alone ($n = 12$) vs. Quadramet + IV/IF-PSA-TRICOM vaccine (PROSTVAC; $n = 12$). Each row corresponds to one patient. The total score columns show the peripheral immunoscore as a result of the sum of points assigned to each PBMC cell type frequency based on the tertile distribution as described in the Materials and Methods section. PFS (columns at far right) is ranked from the longest (green) to the shortest (red), with censored patients indicated by a plus.

![Image](https://example.com/image.png)

Table 2: Proportion of progression-free survival (PFS) for prostate cancer patients treated with the bone-seeking radionuclide $^{153}$Sm (Quadramet) with or without a vaccine.

<table>
<thead>
<tr>
<th>PBMC Cell Type</th>
<th>Quadramet Alone</th>
<th>Quadramet + Vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T cells</td>
<td>0.310</td>
<td>0.301</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>0.325</td>
<td>0.274</td>
</tr>
<tr>
<td>CD16+ CD56+ NK cells</td>
<td>0.255</td>
<td>0.225</td>
</tr>
<tr>
<td>CD14+ MDSC cells</td>
<td>0.245</td>
<td>0.225</td>
</tr>
</tbody>
</table>

For the calculation of the peripheral immunoscore based on refined PBMC subsets reflecting immune function (criterion 2), the peripheral immunoscore to be generated in a rapid fashion, in contrast to the time-consuming manual approach that was taken with the breast cancer trial. It is again emphasized that the markers selected for analysis in this trial were not selected retrospectively but were prospectively selected. As was seen in the breast cancer trial, the peripheral immunoscore based on classic PBMC cell types (criterion 1; Table 2 and Fig. 3A–D) in this trial did not distinguish between patients with longer and shorter PFS in either the Quadramet-alone arm (Fig. 3E; $P = 0.245$, HR = 1.186) or the Quadramet plus PROSTVAC vaccine arm (Fig. 3F; $P = 0.784$, HR = 1.186). These observations again indicate that a peripheral immunoscore based on the frequency of classic PBMC cell types at baseline is unlikely to be helpful in predicting which patients will benefit from the immune-based therapy.

For the calculation of the peripheral immunoscore based on refined PBMC subsets reflecting immune function (criterion 2) in the prostate cancer trial, we applied a slightly modified list of preselected immune markers compared with the breast cancer trial, which was based on an updated knowledge of the literature (refs. 28–32; Table 2). Similar to the results observed in the breast cancer trial, patients with a high peripheral immunoscore based on refined PBMC subsets with a phenotype reflecting immune

![Image](https://example.com/image.png)

Figure 3.
Peripheral immunoscore of PBMCs, prior to therapy, in prostate cancer patients treated with the bone-seeking radionuclide $^{153}$Sm (Quadramet) with or without a vaccine. The peripheral immunoscore is based on classic cell types in PBMCs (criterion 1). A and B, heat maps representing the frequency at baseline of classic PBMC cell types in 24 patients with metastatic prostate cancer, randomized to Quadramet alone ($n = 12$) vs. Quadramet + IV/IF-PSA-TRICOM vaccine (PROSTVAC; $n = 12$). Each row corresponds to one patient. The total score columns show the peripheral immunoscore as a result of the sum of points assigned to each PBMC cell type frequency based on the tertile distribution as described in the Materials and Methods section. PFS (columns at far right) is ranked from the longest (green) to the shortest (red), with censored patients indicated by a plus. C and D, HR and 95% CI for each PBMC cell type are shown along with the HR and 95% CI for the total peripheral immunoscore. P values are indicated. E and F, Kaplan–Meier curves show the PFS of patients with a peripheral immunoscore higher than the median (solid line) compared with the PFS of patients with a score lower than the median (dashed line). Censored patients are indicated by a tick. P value calculated by log-rank analysis. The statistically significant difference was $P < 0.05$; HRs were calculated by the Mantel–Haenszel method. P values, HRs, and Kaplan–Meier curves were generated by custom software. The P value for correspondence of the immunoscore with PFS is $P = 0.245$ in the Quadramet-alone arm and $P = 0.784$ in the combination arm.

Published OnlineFirst August 2, 2016; DOI: 10.1158/2326-6066.CIR-16-0037

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function (Fig. 4A–D) showed a significantly longer PFS than those with a lower peripheral immunoscore in the combination arm (Fig. 4F; *P* = 0.004, HR = 0.072), but not in the arm where vaccine was not administered (Fig. 4E; *P* = 0.592, HR = 1.462). These findings further support the hypothesis that the interrogation of refined immune cell subsets indicating immune function for the calculation of a peripheral immunoscore at baseline may be helpful in predicting which patients will benefit from vaccine therapy, but may not be as informative for treatments with nonimmune therapy.

**Discussion**

The identification of a tumor immunoscore in primary tumors of colorectal cancer patients that correlates with prognosis, i.e., response to standard-of-care therapy (usually consisting of chemotherapy with or without Avastin), has been a major advance implicating the immune system in response to conventional cancer therapies (1–4). These studies analyzing primary colon tumors have been confirmed by numerous investigators (see Jochems review; ref. 5). The use of an immunoscore of primary tumors of other solid tumor types, however, has led to mixed results (5). An area of limited investigation, however, is the identification of a tumor immunoscore in patients with metastatic solid tumors, where the analysis is done on the metastatic lesion (36, 37). Unlike leukemias, lymphomas, and melanoma, metastatic sites of many carcinoma types, such as prostate cancer and breast cancer, are often difficult to biopsy. To this end, limited analyses of PBMCs of patients with metastatic carcinoma have been carried out to determine which patients may best respond to therapy (36, 37).
Immune checkpoint blockade is beneficial in the immunotherapy of several tumor types, and several therapeutic vaccines are in late-stage clinical studies (38, 39). In the studies reported here, we set out to determine whether the analysis of PBMCs of patients with metastatic carcinoma could help identify those patients more likely to respond to vaccine immunotherapy. In the first study analyzed (13), patients with metastatic breast cancer were treated with a standard-of-care agent, docetaxel, with or without PANVAC vaccine (40–42). In the second trial (14, 15), patients with prostate cancer metastatic to the bone were treated with the FDA-approved agent for palliation 131Ism (Quadramet) with or without PROSTVAC vaccine (43, 44). As these studies examined two different tumor types treated with two different standard-of-care agents, and vaccines directed against different tumor-associated antigens, the analysis of the prostate trial is not a validation cohort of the breast cancer trial, but rather a validation of the process of generating a peripheral immunoscore, based on the frequency of immune cell subsets in patients prior to therapy, to distinguish between clinical outcome in patients who receive immunotherapy.

The hypothesis set forth in these studies is that immune cells in the periphery may differ among individual patients with metastatic disease, which, in turn, may influence their response to immunotherapy. Differences in specific immune cell types could be due to (i) the number of prior therapies; (ii) tumor type and stage; (iii) size of tumor; (iv) the microbiome; (v) stress; and (vi) genetic factors such as HLA type and cellular polymorphisms, etc. For example, it is known that certain tumors will secrete immunosuppressive factors such as TGFβ, IL10, and/or IL8, which can all potentially affect specific immune subsets in the periphery (45). Genetics can clearly play a role in immunity, one example being specific polymorphisms in NK cells that can affect antibody-dependent cell-mediated cytotoxicity in the use of monoclonal antibody therapy (46). The use of prior immunotherapy may also affect a patient's PBMC profile and will likely become more of a factor in this era in which immunotherapy is being used and/or evaluated in many tumor types. The analysis of “classic” immune cell types, i.e., CD4, CD8, MDSCs, Tregs, and NK cells, in our study was of little prognostic value. In contrast, we analyzed refined immune cell subsets of these different cell types that have previously been shown in the literature to have immune-enhancing or immune-suppressing biologic function, as well as ratios between effector and regulatory refined subsets. These markers range from indicators of T-cell maturation, such as CD45RA/RO and CCR7/CD62L, that can classify T lymphocyte maturation from a naïve to a terminally differentiated status (47) to markers of immune activation/exhaustion, such as the immune checkpoint molecule PD-1 and its ligand PD-L1 (7), CTLA-4 (8), Tim-3, LAG-3, 2B4, and many others (20, 21). These last functional markers can be ubiquitously expressed in lymphoid and myeloid cells, and can represent either a functionally active phenotype or immune exhaustion (48). It has been proposed that the coexpression of multiple immune checkpoint molecules indicates T lymphocytes that have lost the ability to lyse tumor cells as well as functional exhaustion (20). On the immune suppressive side, FoxP3+ CD4+ T lymphocytes, which are conventionally classified as Tregs, can be “contaminated” by effector CD4+ T cells that express the activation marker CD49d (22). In order to identify “true” suppressive Tregs, the markers CTLA-4 (49) or ICOS (28) can be included in the Treg phenotype. The absence of monocytic and granulocytic markers in MDSCs has been associated with a highly immature status of these immune-suppressive myeloid cells (24, 25). In addition to T lymphocytes and myeloid cells, NK cells can also be characterized by functional markers. The brightness of CD56 and the expression of the FcγR CD16 can distinguish between immature/nonlytic and mature/lytic NK cells (26, 27). The expression of Tim-3 correlates with NK production of IFNγ (29) and with clinical outcome in patients with mCRPC treated with the combination of ipilimumab plus PROSTVAC (50). The expression of PD-L1 on the surface of dendritic cells impairs antigen presentation (30), whereas PD-L1 expression in MDSCs has been correlated with malignancy and immune suppression (31, 32).

In both trials analyzed, these markers on refined immune cell subsets were of little or no value in identifying patients receiving only standard-of-care therapy, but the analyses did identify patients most likely to benefit by the use of those therapies in combination with vaccine. These markers were not selected retrospectively, but were prospectively selected in the analysis of both trials. The markers used in the prostate cancer trial varied slightly from those used in the breast cancer trial due to the different indication and stage of disease. Much larger studies, complete with validation cohorts, are needed to determine the appropriate cutoff points for high, medium, and low bins of a given PBMC subset used in the current studies to generate the peripheral immunoscore will be representative of larger populations, or of patient populations with other indications and/or stages of disease. Much larger studies, complete with validation cohorts, are needed to determine the appropriate cutoffs between bins of a given refined PBMC subset included in the generation of a peripheral immunoscore for it to be potentially used as a prognostic biomarker. Moreover, these types of results may not be obtained with all forms of immunotherapy. Larger multicenter randomized studies are needed to further validate the prognostic value of these immune cell subsets in PBMCs in terms of response to vaccine therapy or other immunotherapy regimens.

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

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Published OnlineFirst August 2, 2016; DOI: 10.1158/2326-6066.CIR-16-0037

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Acknowledgments

The authors thank Debra Weingarten for her editorial assistance in the preparation of this article.

References


Pretherapy Peripheral Immunoscore Analyses in Immunotherapy


