PD-1 Expression on Peripheral Blood Cells Increases with Stage in Renal Cell Carcinoma Patients and Is Rapidly Reduced after Surgical Tumor Resection

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Abstract
Programmed death-1 (PD-1) receptor is an inhibitory receptor on hematopoietic cells that can negatively regulate immune responses, particularly responses to tumors, which often upregulate PD-1 ligands. PD-1/PD-1 ligand blocking antibodies can reverse the inhibition and show significant therapeutic promise in treating renal cell carcinoma (RCC), lung cancer, and melanoma. While PD-1 expression on tumor-infiltrating lymphocytes has been associated with poor outcome in RCC, we sought to define immune cell biomarkers, including PD-1, on peripheral blood mononuclear cells (PBMC) that could predict disease progression of RCC patients before and after nephrectomy. We analyzed expression of numerous immune cell markers on fresh PBMCs from 90 RCC patients preoperatively and 25 age-matched healthy controls by 10-color flow cytometry. Postoperative blood samples were also analyzed from 23 members of the RCC patient cohort. The most striking phenotypic immune biomarker in RCC patients was a significant increase in PD-1 expression on certain PBMCs in a subset of patients. Increased PD-1 expression on CD14bright myelomonocytic cells, effector T cells, and natural killer (NK) cells correlated to disease stage, and expression was significantly reduced on all cell types soon after surgical resection of the primary tumor. The results indicate that PD-1 expression on fresh peripheral blood leukocytes may provide a useful indicator of RCC disease progression. Furthermore, measuring PD-1 levels in peripheral blood may assist in identifying patients likely to respond to PD-1 blocking antibodies, and these therapies may be most effective before and immediately after surgical resection of the primary tumor, when PD-1 expression is most prominent. Cancer Immunol Res; 2(4); 320–31. ©2013 AACR.

Introduction
Renal cell carcinoma (RCC) is among the top 10 most common malignancies, accounting for approximately 65,000 new cases and 13,500 deaths per year in the United States alone (1). More frequent use of modern imaging techniques has resulted in earlier diagnoses, largely as a result of incidental detection (2). The first line of treatment for RCC is surgical resection of the primary tumor or tumors (2, 3). Early-stage patients can be cured with this procedure, yet despite increased early detection, 30% to 40% will go on to develop metastatic disease (2, 3). Additional approved treatments for metastatic RCC include inhibitors of the VEGF pathway (sunitinib, sorafenib, pazopanib, axitinib, and bevacizumab + IFN-α), mTOR pathway (everolimus and temsirolimus), and cytokine-based immunotherapies [interleukin (IL)-2 and IFN-α, -β, -γ]2. Nonetheless, occasional long-lasting complete responses to high-dose IL-2 or IFN therapy have been reported, suggesting that the immune system can successfully respond to RCCs if appropriately stimulated (6–9). These successes fuel strong interest in immunotherapeutic approaches to treating this disease in hopes of achieving a durable complete remission. Unfortunately, severe adverse side effects of these cytokine-based therapies have significantly limited their usefulness.

PD-1 (CD279) is a member of the CD28/CTLA-4 receptor superfamily that can be induced to express on hematopoietic cells. It has both an immunoreceptor tyrosine–based inhibitory motif (ITIM) and an immunoreceptor tyrosine–based switch motif (ITSM), which can recruit anti-SRC homology phosphatase (SHP)-1 and SHP-2 protein tyrosine phosphatases to mediate inhibitory signaling (10, 11). Although much of the work describing PD-1 has been focused on T cells, it can also be

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expressed on B cells, natural killer (NK) cells, and myeloid cells (12). PD-1 is upregulated on leukocytes during the normal course of a successful immune response to suppress excessive immune cell activation, thereby limiting tissue damage and returning the immune system to its basal state. PD-1–mediated signaling can also inhibit cytolytic effector cells from attacking certain normal tissues.

PD-1 also provides a relevant mechanism for immune escape by numerous tumor types through their expression of PD-1 ligands (PD-L1/B7-H1/CD274 and PD-L2/B7-DC/CD273; refs. 13–17). IFN-γ is a potent inducer of PD-L1 expression (17), suggesting that localized T<sub>1</sub>/NK cell–mediated immune responses or IFN-γ therapy may promote PD-L1–mediated immune evasion by tumors. PD-L1 expression has been observed on more aggressive renal tumors (18, 19), and PD-1 expression on tumor-infiltrating lymphocytes in patients with RCCs has been linked to more advanced cancer and reduced overall survival (20). Furthermore, high levels of soluble PD-L1 in the serum of RCC patients have been associated with larger tumors of advanced stages and grades and reduced survival (21).

As PD-1 ligand expression allows tumors to evade immune surveillance, preventing such evasion serves as the rationale behind therapeutic blockade of the interaction between PD-1 and its ligands. Monoclonal antibodies used for this purpose include nivolumab, lambrolizumab, MDX1105, and MPDL3280A (22, 23). Therapeutic PD-1 blockade is being investigated in clinical trials to treat several cancer types, including RCC, and has shown minimal adverse effects and durable antitumor responses, particularly in patients with tumors expressing PD-1 ligands (24–26). While these results are highly encouraging, only 27% of RCC patients responded to PD-1 blockade (24), and efficient methods are needed to identify the subset of patients that will benefit from these therapies. Improved understanding of PD-1 expression in RCC patients is necessary to identify immune cell types that will be rejuvenated by PD-1 blockade and to establish the optimal time window to administer PD-1 blocking therapies.

For this study, we prospectively analyzed cohorts of patients with renal tumors scheduled for nephrectomy and age-matched healthy donors to discover biomarkers of disease progression and components of the immune system that are therapeutically targetable. These markers were also examined after surgery in a consecutive subset of patients to determine the effect of tumor debulking. We found that increased PD-1 expression on several innate and adaptive immune cell types significantly correlates with disease stage. Furthermore, PD-1 expression is rapidly reduced to baseline levels after surgery. These results could prove useful in specifically targeting relevant cell types and determining the appropriate timing of administration of PD-1-targeted therapies.

Materials and Methods

Human subjects and blood sample preparation

Blood samples were obtained from patients with renal tumors before surgery or healthy volunteer donors and processed within 6 hours of collection. Detailed donor information is provided in Supplementary Table S1. The RCC patient cohort comprised clear cell (n = 74), papillary (n = 14), and chromophobe (n = 2) type RCC, with a mean patient age of 60.2 ± 11.6 years. The age of the healthy control donors (n = 25) was 59.2 ± 10.4 years. Postoperative sample collection was added late in the study, and these samples were collected consecutively. The postoperative sample stage distribution is representative of the total cohort, as shown in Supplementary Table S1. All donors consented in writing to have blood drawn and all consent forms and procedures were in compliance with HIPAA regulations and the policies of the Fox Chase Cancer Center (FCCC, Philadelphia, PA) Institutional Review Board. Tumor–node–metastasis (TNM) staging of patients with RCCs was based upon histopathologic analysis of surgically excised tumor and obtained from the FCCC Kidney Cancer Database. Whole blood (20 mL) was drawn into heparinized tubes and subsequently mixed in equal proportions with complete RPMI-1640 medium (supplemented with 10% FBS, 100 μg/mL penicillin/streptomycin, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, and 50 μmol/L 2-mercaptoethanol), layered over Lymphoprep (Axis-shield POC AS), and centrifuged at 900 RCF for 30 minutes. The buffy coat was removed and suspended with staining buffer (RPMI-1640 without biotin or phenol red and supplemented with 2.0 g/L NaHCO<sub>3</sub> and 2.4 g/L HEPES, pH 7.0).

Antibodies and cell staining

The staining panel, monoclonal antibody clones, and sources are shown in Supplementary Table S2, QDot 605 streptavidin (Life Technologies) was used at 5 nmol/L. Samples for regulatory T (Treg) cell analysis were fixed and permeabilized with the ebioscience FoxP3 fix/perm kit, and the streptavidin secondary was added with anti-FoxP3 antibody after the permeabilization step. Staining for perforin and granzyme B was done after samples were fixed with 2% paraformaldehyde and permeabilized with PBS containing 0.1% saponin, 1% bovine serum albumin (BSA), and 0.1% sodium azide. One million cells were stained in each sample on ice for 20 minutes in approximately 200 μL of staining buffer and rinsed twice. Staining tubes that were not fixed/permeabilized were subjected to 100 ng/mL propidium iodide (Invitrogen) in the second rinsing step to mark dead cells. Absolute lymphocyte counts of NK, B, CD4<sup>+</sup> T, and CD8<sup>+</sup> T cells were obtained using a BD IMK kit (Catalog # 340503).

Flow cytometry instrumentation and data analysis

Stained cells were analyzed on a Beckman Dickinson (BD) ARIA II flow cytometer with four lasers at 633-, 488-, 405-, and 365-nm wavelengths. The 365 nm laser was not used for these experiments. Absolute lymphocyte counts were analyzed on a BD FACS Calibur flow cytometer. Data were collected with BD FACS Diva software version 6 and analyzed with Flowjo v9.2 (Tree Star Inc.), Microsoft Excel (v12), GraphPad Prism v5.0d (GraphPad Software Inc.), and R (The R Foundation: www.r-project.org).

Single-cell events were first gated by a forward scatter height versus forward scatter area plot, and viable cells were then gated by lack of propidium iodide staining. Viable CD45<sup>+</sup> cells...
were split into myeloid and lymphocyte populations by applying a side scatter gate and then divided into subpopulations based on the expression of CD3, CD14, CD16, CD19, CD20, CD27, CD62L, CD45RA, and CD56 (Supplementary Figs. S1 and S2). Treg cells were quantified as a percentage of CD4+ T cells that were double positive for CD25 and FOXP3 (not shown).

Statistical methods
Flow cytometric data for distinct parameters were quantified either as mean fluorescence intensity (MFI) or percentage of cells that express a cell surface receptor. Comparisons between immune cell parameters from healthy donors and RCC patients were conducted with an unpaired Wilcoxon rank-sum test. A paired rank-sum test was used to determine whether surgical resection had a significant effect on patient immune status, with presurgical and postsurgical samples constituting a pair. P and R values for the significance of correlations between immune parameters or between immune parameters and disease stage were determined using a Spearman test.

Results
No measurable difference in most immune parameters
We conducted comprehensive immune phenotyping by multiparameter flow cytometry on fresh peripheral blood samples from 114 patients with renal tumors and 25 age-matched healthy control donors (patient and healthy donor characteristics are shown in Supplementary Table S1). Twenty-four of the 114 patients were found to have benign tumors, mostly oncocytomas, and were not included in the dataset analyzed for this study. Our final patient cohort consisted of 74 patients with clear cell RCCs, 14 with papillary RCCs, and 2 with chromophobe RCCs. The observed frequency of RCC subtypes and benign tumors is consistent with expectations, with clear cell carcinoma being the most common histologic class of RCCs and the most likely to metastasize (3, 27). The goals of our analysis were to identify immune biomarkers, including PD-1, of RCC subtype or disease progression and targets for immune therapy (antibody staining panel shown in Supplementary Table S2).

After extensive subgating analysis of the flow cytometric data (outlined in Supplementary Figs. S1 and S2), 94 immune parameters (defined in Supplementary Table S3) were compared between subgroups of samples with a Wilcoxon rank-sum test. We did not find statistically significant differences in the 94 immune parameters when comparing between the different histopathologic variants of RCCs, so all patient samples were analyzed as a single cohort. When comparing between the total patient cohort and age-matched healthy donors, no significant differences were identified in absolute cell counts and subset frequencies of blood leukocytes. Comparisons of T-cell frequencies including CD4/CD8 T-cell ratio and percentages of Treg cells or subsets of naïve, effector, or memory cells revealed no major differences between patients with RCCs and control donors. Myeloid cell subsets defined by CD14 and CD16 (CD14bright CD16– classical monocytes, CD14bright CD16– intermediate monocytes, and CD14bright CD16– nonclassical monocytes (28) were unchanged except for a slight increase in intermediate monocytes in the patient cohort (data not shown).

Increased PD-1 expression on leukocytes from RCC patients
The most notable parameter that differed in the immune cells of patients with RCC when compared with healthy controls was increased PD-1 expression on certain subsets of lymphoid and myeloid cells (Fig. 1). We analyzed fresh blood samples within 6 hours of isolation, thereby avoiding cryopreservation that can reduce the expression of several markers on leukocytes, including the loss of PD-1 detection on CD8+ T cells and CD14+ monocytes (29). PD-1 expression was elevated on numerous myeloid subsets, with the most significant differences observed on CD14bright cells (Fig. 1A: CM, classical monocytes; IM, intermediate monocytes; DB, CD14bright CD16bright double bright myeloid cells; refs. 28, 30). Increased PD-1 expression was also observed on NK cells from patients with RCCs, but the expression was mainly restricted to the CD56dim subset, which is more mature and cytolytic than the CD56bright subset (ref. 31; Fig. 1B). Furthermore, our analysis shows increased PD-1 expression only on T cells with an effector or effector memory phenotype (Fig. 1C and D). Effector T cells are characterized by the loss of CD27 (CD27–) through ADAM17 metalloprotease-mediated cleavage immediately following activation through the TCR, presumably to facilitate escape from secondary lymphoid tissues where antigen presentation takes place (32). The selective expression of PD-1 on cytolytic NK cells and effector T-cell subsets suggests that these cells have responded to tumor-derived antigens and may be selectively suppressed through PD-1.

Correlation of PD-1 expression with disease stage
We tested for correlations between PD-1 expression and TNM staging. Statistically significant increases in PD-1 expression compared with healthy controls (P < 0.05 by a Wilcoxon rank-sum test) were observed in stages 1 and 4 on classical and intermediate monocytes, neutrophils, CD56dim NK cells, CD14bright CD16bright myeloid cells, and CD62L– (effector) subsets of CD4+ and CD8+ T cells (Fig. 2). Classical and intermediate monocytes also exhibited increased PD-1 expression at stage 2. The lack of statistically significant increases in PD-1 expression for most cell populations at stages 2 and 3 may be due to the limited number of patients within these groups. Overall correlations of PD-1 expression levels on each cell type by stage were also computed using a Spearman test (Fig. 2). Statistically significant correlations to disease stage were evident for all CD14bright myeloid subsets, CD4+ effector T cells, and CD8+ effector memory T cells (Fig. 2A–C, F, and H). Of the immune cell populations analyzed, the most significant increases in PD-1 expression by disease stage were noted on CD14bright myeloid cells and CD4+ effector T cells.

Strong correlation of PD-1 expression on different cell types
As PD-1 expression was highly elevated on CD14bright myeloid cells, we compared PD-1 expression on CD14bright (classical) monocytes with that on other leukocytes in the RCC
patient cohort, as shown in Fig. 3. Vertical and horizontal lines in these figures mark the median values on each axis. In summary, the strength of the correlation is the extent to which donors with below median PD-1 expression on classical monocytes also have below median PD-1 expression on the other cell type, whereas donors with above median PD-1 expression on classical monocytes also have above median PD-1 expression on the other cell type. Statistical correlations were analyzed by a Spearman test, and in all cases, correlation $P$ values were equal to or less than 0.001, providing credible evidence that the upregulation of PD-1 is systemic in nature. This analysis revealed that PD-1 expression on CD14$^{bright}$ classical monocytes correlates strongly with the expression on other CD14$^{bright}$ myeloid cells, neutrophils, effector T cells, and NK cells in patients with RCCs (Fig. 3).

**Effects of surgical resection on PD-1 expression**

We examined PD-1 expression in postsurgical blood samples from 23 of the patients with clear cell RCCs in our cohort within 2 to 57 weeks after surgery. Strikingly, surgery reduced PD-1 expression to baseline levels on all immune cell types in nearly every patient with elevated pre-surgical levels at both early and late time points (Fig. 4). The reduction in PD-1 expression was statistically significant for all cell types that exhibited higher PD-1 expression before surgery when compared with healthy controls. We cannot distinguish whether the reduction in PD-1 expression is due to downregulation of the receptor from the cell surface or the loss of PD-1$^+$ expressing cells. Effector T cells are known to perish upon removal of their antigen-directed signal, and the action of PD-1 itself can have a negative impact on
Figure 2. Increased PD-1 expression on peripheral blood leukocytes as a function of TNM staging. Healthy individuals are designated as stage 0, and patients were staged (1–4) after surgery. Each hexagon represents the MFI of anti-PD-1 staining from an individual donor. Median values are marked by horizontal lines.

Values were calculated for the entire data set by a Spearman correlation (ns, not significant), and the differences between healthy donors and patients at each disease stage were calculated by an unpaired Wilcoxon rank-sum test. Individual panels show results for the following cell types: A, classical monocytes; B, intermediate monocytes; C, CD14bright CD16bright; D, neutrophils; E, CD8⁺ effector T cells; F, CD8⁺ effector memory T cells; G, CD56dim NK cells; H, CD4⁺ effector T cells; and I, CD4⁺ effector memory T cells.
Figure 3. Relationship between PD-1 expression on classical monocytes and other cell types. Correlation of PD-1 expression on classical monocytes (x-axis) to PD-1 expression on other cell types (y-axis) is shown for A, intermediate monocytes; B, CD14^{bright}CD16^{bright}; C, neutrophils; D, CD8^{+} effector T cells; E, CD8^{+} effector memory T cells; F, CD56^{dim} NK cells; G, CD4^{+} effector T cells; and H, CD4^{+} effector memory T cells. A vertical line marks the median PD-1 expression on classical monocytes, and the median PD-1 expression on other cell types is marked by a horizontal line. A least-squares linear fit is shown as a thick black line. Statistical significance was calculated by a Spearman correlation.
cellular viability (33, 34). Nonetheless, our results show that PD-1-expressing immune effector cells are far more prevalent before resection of the primary tumor. Further evidence that tumor cells are promoting the maintenance of these immune cells is shown by the observation that some of the patients that showed sustained or elevated PD-1 expression levels after surgery had stage 3 or 4 malignancy. These results suggest that remnant metastatic tumor is maintaining PD-1 expression on leukocytes in some patients, although a larger cohort and disease progression analysis are needed for definitive conclusions.

PD-1 expression on NK cells and T cells correlates with an activated phenotype

High PD-1 expression in patients with RCCs correlates strongly with numerous biomarkers indicating that these immune cell populations have been activated. For example, patients with RCCs with high levels of PD-1 expression on their CD56dim NK cells also tend to have higher than normal expression of perforin and granzyme B on those same cells (Fig. 5A and B). Perforin and granzyme B are components of cytotoxic granules, and their increased expression indicates that these PD-1+ lymphocytes are cytolytic effector cells that...
Figure 5. Correlations between PD-1 expression and other immune parameters. The relationship between intracellular perforin and granzyme B levels (y-axis) to PD-1 expression (x-axis) on CD56dim NK cells is shown in A and B, respectively. Correlation of other immune parameters to PD-1 expression on T-cell populations is shown in C–I. In each panel, individual pretreatment patient donors are represented by open hexagons. The median PD-1 expression on all T cells (CD3+CD45+ x-axis) is compared with the values for the following parameters on the same donor’s cells (y-axis): C, naive % of CD4+ T cells; D, absolute count of CD8+ T cells; E, % of T cells expressing NKG2D; F, MFI of perforin expression by CD8+ T cells; G, MFI of granzyme B expression by CD8+ T cells; H, MFI of perforin expression by CD4+ T cells; and I, MFI of granzyme B expression on CD4+ T cells. A least-squares linear fit is shown as a thick black line. Statistical significance was determined by a Spearman correlation.
have been previously activated in the immune response to malignant cells. PD-1 expression on total T cells (CD45^-CD3^+) also correlates with significant increases in perforin and granzyme B on both CD4^+ and CD8^+ subsets (Fig. 5F–I). Importantly, the T and NK cell populations expressing high levels of perforin and granzyme B also declined significantly after surgery (Supplementary Fig. S4B–S4F). The parallel post-surgical reductions in the expression of PD-1 and cytolytic granule components in these lymphocytes are striking. Other indicators that the PD-1–expressing T cells are activated include a smaller percentage of naive CD4^+ cells, greater numbers of CD8^+ cells per microliter of blood, and increased percentage of total T cells expressing NKG2D in preoperative samples (Fig. 5C–E). All of these parameters indicate previous immune activation, which is consistent with reports that PD-1 is upregulated on activated immune cells and that such upregulation leads to immune “exhaustion” (35). In our dataset, this exhaustion manifests as a negative correlation between PD-1 expression on T cells and the expression of the CD69 acute activation marker on T cells before surgery (Spearman \( P = 0.01, R = -0.27 \); Supplementary Fig. S3). In contrast, CD69 expression was significantly elevated on T cells in many postoperative samples (Supplementary Fig. S4A), indicating that the loss of PD-1 expression after resection of the primary tumor liberates T cells from suppression. Taken together, we predict that primed PD-1^+ effector cells, which are already present in the circulation of some patients with RCCs, may be able to clear tumor cells more efficiently if they are released from the inhibitory signaling of PD-1. This release of inhibition is likely the mechanism for the therapeutic efficacy of the PD-1 checkpoint inhibitors in clinical trials to date.

Discussion

Our analysis of fresh peripheral blood samples more precisely characterized the expression of PD-1 on immune cells in response to RCCs. PD-1 was significantly upregulated on effector T cells, cytolytic NK cells, and CD14^bright myeloid cells, all of which would participate in a typical inflammatory immune response. In addition, a dramatic reduction in PD-1 expression on these immune cells was observed within weeks after surgical resection of the primary tumor, which has not been reported previously. Our results could be useful for identifying patients likely to benefit from PD-1–based therapies and for establishing better timing of such treatments.

Phase I studies on the safety and efficacy of both anti-PD-1 (24) and anti-PD-L1 (25) antibodies have been conducted to treat a variety of cancers, including RCCs. Complete or partial responses were achieved for 9 of 33 patients with RCCs treated with the PD-1 inhibitor nivolumab (BMS-936558/MDX1106; ref. 24) and for 2 of 17 patients with RCCs treated with the PD-L1 inhibitor (BMS-936559/MDX1105; ref. 25). Nivolumab is being tested in a phase III randomized trial as a second-line treatment for RCCs, in hopes that the results will support the approval of the U.S. Food and Drug Administration (FDA) for metastatic RCCs. It is important to note that a kidney cancer patient with an extensive metastatic disease that was resistant to treatment with mestrinostat, sorafenib, and sunitinib achieved a durable complete remission after treatment with anti-PD-1 antibodies in the phase I studies (36), and other patients with advanced cancers have achieved remissions lasting more than a year (24, 25). A recent phase I/II trial of the PD-1 blocking antibody nivolumab in conjunction with a peptide vaccine showed similar response rates on patients with unresectable melanoma, some of whom previously had failed ipilimumab treatment (37). Interestingly, this study showed some efficacy on patients whose tumors did not express PD-L1, showing that the presence of PD-L1 alone is not an absolute predictor of clinical benefit from anti-PD-1 antibodies.

Importantly, not all patients with RCCs in our study expressed high levels of PD-1 on their immune cells, suggesting that patients who do not express PD-1 may not benefit from PD-1 blockade. Analysis of tumor tissue samples of patients treated with PD-1 inhibition in clinical trials reveals that responses disproportionately clustered within the subset of patients with PD-L1 expression in their tumors (24, 25). Therefore, we hypothesize that patients with tumor expression of PD-1 ligands who did not respond to therapy may have lacked adequate PD-1 expression on their immune cells, as both are necessary to suppress immune cell function and benefit from blocking therapy. Although further verification in a clinical trial is necessary, our data suggest that quantification of PD-1 expression on fresh peripheral blood by flow cytometry may provide a valuable biomarker to select patients more likely to benefit from PD-1 blocking therapy.

One of the strongest and most consistent results was the increased PD-1 expression on classical (CD14^bright CD16^-) and intermediate (CD14^bright CD16^dim) monocytes, which correlated positively to RCC disease stage and was rapidly reduced after surgery. Despite a statistically significant increase in PD-1 expression with disease stage on CD14^bright monocytes, overlapping level of expression in some stage 4 patients with that of healthy controls shows that PD-1 will not provide a definitive clinical biomarker of disease progression. On the other hand, measuring expression would likely still be important to identify patients that are at higher risk of having advanced disease and predicting patients that will respond to PD-1–based immune therapies. Furthermore, our observation indicates that PD-1 expression on these cells has a central role in immune dysfunction in kidney cancer. It has been shown that monocyte-presented antigens can effectively prime T cells to kill renal tumor cells (38), and monocytes are capable of differentiating into antigen-presenting dendritic cells, which are important effector cells in initiating adaptive immune responses to cancer (39). In fact, vaccination with monocyte-derived dendritic cells has shown success as another promising immunotherapeutic treatment for RCCs (40). Therefore, it is possible that inhibition of monocyte functions through PD-1 expression may significantly interfere with the antigen presentation that is necessary to stimulate adaptive immune responses, and the combined use of PD-1 blocking antibodies may improve the clinical efficacy of tumor vaccine therapies. PD-1 upregulation has been reported on monocytes in response to the hepatitis C core protein, which interacts with suppressor of cytokine signaling-1 (SOCS-1) to suppress STAT-1 signaling and the
production of pro-inflammatory cytokine IL-12 (41, 42). In addition, PD-L1 engagement with PD-1 on monocytes has been shown to contribute significantly to functional suppression of T cells in patients with HIV-1 by inducing the monocytes to secrete the immunosuppressive cytokine IL-10 (43). In patients with RCCs, defects in a monocyte chemotactic protein have been shown to increase both risk and severity (44). Monocytes readily infiltrate RCCs, where the tumor microenvironment can also promote their differentiation into M2-type tumor-associated macrophages (TAM) of myeloid-derived suppressor cell (MDSC) lineage, which are associated with increased mortality in RCCs, presumably through their ability to induce CD4+ T cells to express inhibitory PD-1 and secrete the immunosuppressive cytokines, IL-10 and TGF-β (45, 46). Therefore, multiple mechanisms of PD-1-mediated suppression of antigen-presenting monocytes, macrophages, and dendritic cells could be key factors that restrain an effective immune response toward RCCs and could also severely limit the success of dendritic cell vaccination therapies in patients with RCCs.

Several studies have shown increased PD-1 expression on CD4+ and CD8+ T cells in patients with cancer (35, 47, 48); our analysis extends these observations to show that expression is only increased on the effector and effector memory T-cell subsets in patients with RCCs (Fig. 2). As there is a marked difference in PD-1 expression on T cells from patients with RCCs compared with healthy controls, and the expression significantly declines upon resection of the primary tumor, it is logical to conclude that these effector T cells have responded to tumor-specific antigens. The general T-cell phenotype of patients with high PD-1 expression is also consistent with an antigen-directed adaptive immune response, as we found that these patients have higher absolute numbers of CD8+ T cells, lower percentages of naive CD4+ T cells, and increased expression of perforin, granzyme B, and NKG2D (Fig. 5). It is certainly plausible that these T cells became activated, eliminated some tumor cells, and then were rendered inactive by natural PD-1 expression that is designed to limit aggressive immune responses and thereby prevent damage of normal tissues. Resection of primary tumor resulted in the apparent rapid loss of these cells from peripheral blood, as illustrated by coordinate reductions in the expression of PD-1, perforin, and granzyme B on T cells in most patients (Fig. 4 and Supplementary Fig. S4). Consistent with a significant role for PD-1-mediated suppression of T-cell activity by the tumor, patients with RCCs with higher levels of PD-1 expression exhibited lower expression of CD69 on T cells (Supplementary Fig. S3), whereas CD69 expression was significantly increased on T cells of many patients after surgery (Supplementary Fig. S4).

We also show that substantial levels of PD-1 are expressed on NK cells in patients with RCCs. NK cell expression of PD-1 has been reported in patients with multiple myeloma, and in vitro NK cell activity against autologous myeloma cells was enhanced by anti-PD-1 antibodies (49). We show that PD-1 upregulation is restricted to the cytolytic CD56dim NK cell subset and not in the cytokine-producing CD56bright cells. Patients with cytolytic NK cells that express high levels of PD-1 also have higher levels of perforin and granzyme B, indicative of an activated effector phenotype (Fig. 5); the expression of all 3 biomarkers declined rapidly after surgery (Fig. 4 and Supplementary Fig. S4). These results show that NK cells are also responding to tumors in patients with RCCs, but PD-1 expression is likely suppressing their responsiveness. Our result could provide a rational basis for combining other NK cell potentiating therapies (lenalidomide, IFN-α, IL-2, IL-15, etc.) with PD-1/PD-L1 blocking antibodies, as these combination therapies could synergistically increase NK responses to tumor.

Taken together, our data paint a picture of a combined innate and adaptive immune response that has been activated but subsequently was rendered incapable of completely eliminating the cancer by PD-1 expression. This provides hope that immune therapies designed to reverse the immunosuppression may allow the patient’s activated immune system to complete the tumor elimination process. Indeed recent results from clinical trials strongly suggest that blocking signaling through PD-1 could be an effective option to restore immune function in patients with RCCs. Our data indicate that PD-1 expression on CD14+CD16+ monocytes in freshly isolated peripheral blood could serve as a biomarker for identifying patients likely to benefit from PD-1 blocking therapies.

We show that surgical resection of primary tumor rapidly reverses PD-1 expression on all immune cell populations (Fig. 4), which has significant implications for the timing of PD-1–based therapies. PD-1 expression is upregulated and maintained on mouse T cells by chronic presentation of activating antigens and reverts to normal when the antigens are removed, which may be the mechanism underlying our findings (50). These results suggest that the immune system in patients with RCCs has recognized a targetable antigen but is being held back from attacking the tumor by inhibitory PD-1 signaling. This immune inhibition could occur through direct contact with PD-1 ligands on the tumor or with soluble PD-1 ligands (21). Therefore, we postulate that PD-1 blocking therapies could be more effective if started before surgery and continued immediately thereafter because this is when both the PD-1 expression on immune cells and PD-L1 expression by the tumors are the most pronounced. The existence of cytotoxic effector T cells with high levels of perforin, granzyme B, and NKG2D that also express high levels of PD-1 (Fig. 5) provides an additional rationale for starting PD-1–based therapies before surgery, as these cells are likely to die once their antigen is removed (34). The presence of PD-1 on effector memory T cells suggests that these cells could potentially mount an effective secondary immune response against a recurrence of the same tumor cells if they were not restrained by PD-1–mediated inhibitory signaling. These findings provide preclinical rationale for a clinical trial of PD-1 checkpoint inhibition in the perioperative setting for high-risk patients without radiographic evidence of metastases with the objective to prevent recurrence and increase the rate of cure over that currently obtained with surgery alone.
Disclosure of Potential Conflicts of Interest

K.S. Campbell has received research support from Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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