Intratumoral CD14+ Cells and Circulating CD14+ HLA-DRlo/neg Monocytes Correlate with Decreased Survival in Patients with Clear Cell Renal Cell Carcinoma


Abstract

Purpose: Immunotherapeutic strategies to treat patients with renal cell carcinoma (RCC) offer new opportunities for disease management. Further improvements to immunotherapy will require additional understanding of the host response to RCC development.

Experimental Design: Using a novel approach to understanding the immune status of cancer patients, we previously showed that patients with a certain immune profile had decreased overall survival. Here, we examine in more detail the phenotypic changes in peripheral blood and the potential consequences of these changes in RCC patients.

Results: We found that CD14+ HLA-DRlo/neg monocytes were the most predominant phenotypic change in peripheral blood of RCC patients, elevated nearly 5-fold above the average levels measured in healthy volunteers. Intratumoral and peritumoral presence of CD14 cells was an independent prognostic factor for decreased survival in a cohort of 375 RCC patients. The amount of peripheral blood CD14+ HLA-DRlo/neg monocytes was found to correlate with the intensity of CD14 staining in tumors, suggesting that the measurement of these cells in blood may be a suitable surrogate for monitoring patient prognosis. The interaction of monocytes and tumor cells triggers changes in both cell types with a loss of HLA-DR expression in monocytes, increases of monocyte survival factors such as GM-CSF in tumors, and increased production of angiogenic factors, including FGF2.

Conclusions: Our results suggest a model of mutually beneficial interactions between tumor cells and monocytes that adversely affect patient outcome.

Introduction

It is estimated that approximately 70,000 new cases and 13,000 deaths occur annually from cancer of the kidney and renal pelvis in the United States (1). Recent advances have focused on targeting receptor tyrosine kinases, angiogenesis, or mTOR pathways in the United States (1). Recent advances have focused on targeting receptor tyrosine kinases, angiogenesis, or mTOR pathways. Despite improvements in treatments, durable responses remain a challenge. Immunotherapy for renal cell carcinoma (RCC) has had mixed results. High-dose IL2 regimens have only been successful in a small percentage of patients. Other immunomodulating approaches like peptide and dendritic cell vaccines for RCC showed promising results, but additional clinical trials are needed to optimize treatment protocols to improve their efficacy (5–7). With the preliminary successes of checkpoint inhibitors to CTLA-4 and PD-1 (8, 9) in other cancers, there has been a renewed interest for adding immunotherapy to the treatment repertoire for RCC.

Tumor-mediated immune suppression remains a significant barrier to cancer treatment. RCCs can induce both local and systemic immune suppression via multiple mechanisms (reviewed by Bedke and Stenzl; ref. 10). However, it is unclear how immunosuppressive mechanisms counteract the components needed to generate an effective, multifactorial antitumor response. Thus, additional understanding of the immune status of cancer patients is critical for both improving the outcomes of a specific immunotherapy and identifying relevant biomarkers to guide the selection of patients most likely to benefit from specific immunotherapy strategies.

Recently, we used quantitative whole blood flow cytometry on 160 healthy volunteers and patients from four cancer types, including RCC, to identify immune profiles of cancer patients that were prognostic for overall survival (11). We took a comprehensive approach by measuring all of the major leukocyte subsets in both blood and tumors to identify the host immune response to RCC.
CD14⁺ Monocytes as a Prognostic Biomarker in RCC

Translational Relevance

In this study, we demonstrate that the most significant immunophenotypic change in clear cell renal cell carcinoma (RCC) patients is the induction of CD14⁺ HLA-DRLo/neg monocytes. CD14⁺ monocytes were found to accumulate in renal cell tumors, and their abundance is strongly associated with decreased patient survival. The correlation between peripheral blood CD14⁺ HLA-DRLo/neg monocytes and the accumulation of CD14⁺ monocytes in tumors suggests that these cells may be a suitable prognostic marker in RCC. CD14⁺ HLA-DRLo/neg monocytes have been shown to counteract the efficacy of immunotherapeutic approaches, and our data provide evidence that these cells may also negate the effect of angiogenesis therapies through a monocyte/tumor-coordinated induction of other angiogenic factors, such as FGF2. The presence of CD14⁺ HLA-DRLo/neg monocytes thus serves as a biomarker for patient outcome and as a therapeutic target for improving the efficacy of immunotherapies.

Materials and Methods

Patient selection, clinical and pathologic features, and patient outcome

Peripheral blood samples were collected from 25 patients with metastatic RCC before cytoreductive nephrectomy and 40 healthy volunteers. Three patients had papillary histology. The clinical characteristics of the RCC patients have been described elsewhere (11). All studies were performed under the approval of the Mayo Clinic Institutional Review Board. Informed consent was obtained from all patients. We also identified 375 patients treated with radical or partial nephrectomy for unilateral, sporadic, noncystic clear cell RCC between 2000 and 2002, from the Mayo Clinic Nephrectomy Registry. The clinical features studied included age, gender, and symptoms at presentation. Patients with a palpable flank or abdominal mass, discomfort, gross hematuria, acute onset varicocele, or the constitutional symptoms of rash, sweats, weight loss, fatigue, early satiety, or anorexia were considered symptomatic at presentation. The pathologic features studied included histologic subtype classified according to the Union Internationale Contre le Cancer, American Joint Committee on Cancer, and Heidelberg guidelines, the 2002 primary tumor classification, regional lymph node involvement, distant metastases, the 2002 TNM (tumor node metastasis) stage groupings, tumor size, nuclear grade, coagulative tumor necrosis, sarcomaoid differentiation, and SSIGN (Stage, Size, Grade, Necrosis) score (12). To obtain these features, a pathologist (J.C. Cheville) reviewed the microscopic slides from all specimens without knowledge of patient outcome. Vital status for patients in the Nephrectomy Registry is updated each year. If a patient died in the previous year, a death certificate was ordered to determine the cause of death. A visit to our institution within 6 months of the date of death for metastatic RCC was good documentation that RCC was the cause of death. If the death certificate did not support this, the medical history was reviewed to determine the cause of death. If a death certificate could not be obtained, the cause of death was verified with the patient’s family or local physician.

Peripheral blood immunophenotyping

Quantitative whole blood flow cytometry and data analysis, including hierarchical clustering, for the patient and healthy volunteer samples were previously described (11). Additional principle component analysis of flow data was performed in Partek Genomics Suite 6.6 (Partek Inc.). Color dot plots of flow data were generated using the Kaluza flow analysis software (Beckman Coulter).

Immunohistochemical staining and quantitation of CD14 expression

A formalin-fixed, paraffin-embedded block with representative tumor tissue was selected for each patient in the cohort. Staining for CD14 was conducted as follows. Tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Antigen retrieval was performed by heating tissue sections in Target Retrieval solution pH 6 (Dako #S1699) to 121°C using a Digital Decloaking Chamber (Biocare Medical). Sections were cooled to 90°C and incubated for an additional 5 minutes before the Decloaking Chamber was opened. Sections were washed in running DIH2O for 5 minutes and incubated for 5 minutes in Wash Buffer (Dako #S3006) before being placed on the Autostainer Plus (Dako) for the following protocol. Sections were blocked for endogenous peroxidase for 5 minutes using Endogenous Blocking solution (Dako #PD900M), washed twice in wash buffer, incubated 5 minutes in Background Reducer Sniper (Biocare Medical #B8966L), washed twice in wash buffer, and incubated for 60 minutes in purified rabbit anti-human CD14 antibody (Sigma PA #HPA001882) diluted 1:300 with DaVinci Green antibody diluent (Biocare Medical #PD900M). Sections were washed twice in wash buffer, incubated 15 minutes in the rabbit probe from the Mach 3 Rabbit HRP-Polymer Kit (Biocare Medical #M3R531L), washed twice in wash buffer, and incubated 15 minutes with the rabbit polymer from the Rabbit HRP-Polymer Kit. Sections were washed with wash buffer and visualized by incubating Betazoid DAB (Biocare Medical #BDB2004L) for 8 minutes. Sections were washed with DIH2O, counterstained with hematoxylin, dehydrated in ethanol, and cleared in xylene. Coverslips were mounted in permanent mounting media. All stained slides were reviewed and quantitated by a pathologist (J.C. Cheville) without knowledge of patient outcome. Intratumoral and (if possible) peritumoral CD14 expression was assessed as absent, focal, moderate, or marked.

Monocyte/cell line coculture experiments

CD14⁺ monocytes were purified from normal donor leukoreduction chambers by density gradient separation (13) and selection of CD14⁺ monocytes from peripheral blood mononuclear cells by immunomagnetic selection via the AutoMacs Cell Separator (Miltenyi Biotech). CD14⁺ cells (2 × 10⁶/mL) were plated into control medium (DMEM) alone, or incubated with 1 × 10⁶ cells/mL of RCC cell lines or normal renal cell epithelial cells. ACHN (CRL-1611, lot #57586991), 786-O (CRI-1932, lot...
#763580), and Caki-1 (HTB-46, lot #5074655) were purchased from the American Type Culture Collection (ATCC) in 2009, and primary renal epithelial cells (PCS-400-D12, lot #58488854) in 2010. RCC cell lines were cultured as per the ATCC instructions less than 6 months before the coculture experiments, and primary cells were cultured in Renal Cell Growth medium (Lonza) no more than 10 passages. ATCC uses short tandem repeats (STR) profiling to characterize most cell lines. RCC cells were cultured in Renal Cell Growth medium (Lonza) no more than 10 passages. ATCC uses short tandem repeats (SRT) profiling to characterize most cell lines. RCC cells were also cultured alone at 1 × 10^6 cells/mL in DMEM/10% FBS. Cells and conditioned supernatants were harvested after 72 hours. For cocultures, monocytes were reselected with CD14 microbeads with the negative fraction containing RCC cells. Flow cytometry was performed to assess purity of cultures.

**Angiogenesis arrays and ELISAs**

The Proteome Profiler Human Angiogenesis Array kits (R&D Systems; catalog # ARY007) were used to analyze supernatants and cell lysates according to the manufacturer’s instructions. Equal volumes were used for supernatants and for lysates, and volumes were normalized to equal cell numbers. FG2 ELISAs (R&D Systems) were performed via manufacturer’s instructions.

**RNA isolation and qPCR analysis**

RNA was isolated from cells via the Qiagen RNeasy Plus Kit (Qiagen) and made into cDNA with the Transcriptor First Strand cDNA Kit (Roche Diagnostics GmbH). Primers for FG2, IL1b, GM-CSF, PTX3, and ARG-1 were designed with the Roche Universal Probe Library (UPL) system. PCR reactions were performed with the LightCycler Taqman Master Mix, ran and analyzed on the Roche Light Cycler System. Relative transcript levels were determined by assessing the crossing points (Cp) for each gene for each experiment. The relative transcript levels were first normalized to the reference gene GAPDH and calculated by the log2 difference of the Cp (ΔCp) of the reference gene and the gene of interest. Gene expression changes between samples were calculated as the log2 fold change by subtracting the average of the ΔCp from the control sample from the average ΔCp of the test sample (ΔΔCp) and their fold difference 2^-ΔΔCp.

**Statistical analyses**

Values between groups of data were tested for statistical significance using the Mann–Whitney nonparametric test for unpaired samples. Correlation analysis was performed using Pearson correlation using Prism, version 5.0 software (GraphPad Software). For the qPCR analysis, values were measure for statistical significance by using a t-test against a normalized value (1.0; representing 100%). Associations of CD14 expression with clinical and pathologic features were evaluated using χ² tests. Associations of CD14 expression with cancer-specific survival were illustrated using Kaplan–Meier curves. The magnitudes of the associations of CD14 expression with death from RCC were evaluated using Cox proportional hazards regression models and summarized with HRs and 95% confidence intervals (CI).

The duration of follow-up was calculated from the date of nephrectomy to the date of death or last follow-up. Patients who died from causes other than RCC were censored at the date of death, whereas patients who were alive at the time of analysis were censored at the date of last follow-up. Statistical analyses were performed using the SAS software package (SAS Institute). All tests were two-sided, and P values <0.05 were considered statistically significant. The 3 × 3 Fisher exact test was used for the grouping of intratumoral CD14 staining with peripheral blood phenotypes. The significance level was set at probability of significance set at less than 0.05 with specific calculated P values provided when applicable.

**Results**

Loss of HLA-DR on CD14⁺ monocytes is the dominant phenotypic change in peripheral blood of RCC patients

By combining whole blood quantitative flow cytometry and bioinformatics analyses, we generated immune profiles from 160 healthy volunteers, cancer patients, and patients with or at risk of sepsis (11). The overall survival of patients with an abnormal immune profile was significantly shorter than those patients with an immune profile similar to healthy volunteers. Twenty-five patients with RCC were included in this analysis, 9 of which were clustered into abnormal immune profiles. Here, we further investigated the immunophenotypic changes in peripheral blood leukocytes of these patients. Because the immune profiles were generated from 10 immune phenotypes [granulocytes, monocytes, lymphocytes, T cells, B cells, natural killer (NK) cells, CD4⁺ T cells, CD4⁺CD25⁺CD127lo regulatory T cells (Tregs), CD14⁺ HLA-DRlo/neg monocytes, and CD86⁺ monocytes], we wanted to identify the immune phenotypes in RCC patients that were most responsible for the variation and to determine potential relationships between seemingly unrelated cells. We performed a principal component analysis to identify the immune phenotypes with the largest variation in this dataset (Supplementary Fig. S1). Lymphocytes, CD14⁺ HLA-DRlo/neg monocytes, Tregs, and B cells were the four immune phenotypes that appeared to be responsible for most of the variability. In univariate analyses, 4 of the 10 immune phenotypes were different in RCC patients compared with healthy volunteers. B cells were lower (healthy volunteer: mean = 247 ± 131.4 (SD) cells/µL vs. RCC: 204 ± 209.6), whereas granulocytes (healthy volunteer: 4,784 ± 1,620 cells/µL vs. RCC: 7,383 ± 5,025), monocytes (healthy volunteer: 508.9 ± 145.9 cells/µL vs. RCC: 718.2 ± 229), and CD14⁺ HLA-DRlo/neg monocytes (healthy volunteer: 57.7 ± 3.6 cells/µL vs. RCC: 249.4 ± 178.6) were elevated in RCC patients (Fig. 1A). CD14⁺ HLA-DRlo/neg monocytes showed the greatest increase of any immune phenotype in RCC patients at nearly 5-fold above the mean of healthy volunteers. Correlative analyses identified expected and novel relationships (Fig. 1B, data not shown). For example, the levels of B cells correlated positively with CD4⁺ cells and granulocytes with CD14⁺ HLA-DRlo/neg monocytes in both healthy volunteers and RCC patients; however, Tregs were correlated to CD4⁺ T cells only in RCC patients. The magnitude of change in the levels of CD14⁺ HLA-DRlo/neg monocytes in RCC patients suggests that this immune phenotype is important in the pathology of RCC.

We (14–16) and others (17–20) have demonstrated that CD14⁺ HLA-DRlo/neg monocytes are major mediators of tumor-induced immunosuppression. Other myeloid cells/MDSCs have been implicated in RCC-induced immunosuppression, including CD11b⁺CD14⁺ CD15⁺ cells (21), lineage-negative (LIN⁻)CD33⁻ HLA-DR⁻ CD11b⁺ (22), CD15⁻ CD11b⁺CD14⁺ CD66b⁻CD33⁺ (23), CD33⁻HLA-DR⁻, and CD15⁻CD14⁺ cells (24). Because these reports measured cell populations from density-gradient–purified mononuclear cells, we wanted to assess the levels of these cells in fresh unfractionated whole blood. Because there is considerable diversity in
the use of phenotypic markers for human MDSCs (25), we narrowed the characterization of these cells to CD14⁺ HLA-DRlo/neg monocytes, immature LIN⁺ CD33⁺ HLA-DR⁻ cells, and CD33⁺ HLA-DR⁻ cells (gating strategies outlined in Supplementary Fig. S2). Figure 2A shows the forward and side scatter properties of each of these cell types in an RCC patient to demonstrate the forward and side scatter positioning of these cells relative to each other. LIN⁺ CD33⁺ HLA-DR⁻ cells reside mainly in the lymphocyte compartment (low side scatter and forward scatter). LIN⁺ CD33⁺ HLA-DR⁻ cells and CD33⁺ HLA-DR⁻ granulocytes are distinct populations (26). CD14⁺ HLA-DRlo/neg cells have typical forward/side scatter properties of monocytes and should not be considered MDSCs as per convention, but are really monocytes characterized by the single-marker CD14 that has lost HLA-DR expression. As monocytes can be further subgrouped into classical (CD14⁺ CD16⁻), intermediate (CD14⁺ CD16⁻), and nonclassical (CD14⁻ CD16⁺; ref. 27), we measured the distribution of these subsets in RCC patients and their corresponding HLA-DR expression. The distribution of the subsets was not different in RCC patients compared with healthy volunteers, and HLA-DR was significantly lower in each of the subsets in RCC patients (Supplementary Fig. S3). Although there was some overlap with monocytes, CD33⁺ HLA-DR⁻ cells almost entirely resided in the high forward/side scatter compartment typified by granulocytes. Not unexpectedly, CD15 and CD66b also identified granulocytes. Interestingly however, as CD33⁺ HLA-DR⁻ CD15⁺ CD11b⁺ CD14⁻ CD66b⁺ granulocytes have been shown to copurify with mononuclear cells in RCC patients (21, 22, 24, 28), we rarely observed CD15⁺ CD66b⁺ cells in the mononuclear "fraction" in staining of whole blood samples. Although there was some overlap with monocytes, CD33⁺ HLA-DR⁻ CD15⁺ CD11b⁺ CD14⁻ CD66b⁺ granulocytes have been shown to copurify with mononuclear cells in RCC patients (21, 22, 24, 28), we rarely observed CD15⁺ CD66b⁺ cells in the mononuclear "fraction" in staining of whole blood samples. We did not observe an increase in the amount of LIN⁺ CD33⁺ HLA-DR⁻ cells or a difference of CD66b expression on granulocytes in RCC patients versus healthy volunteers (Fig. 2B).

The assessment of peripheral blood immune phenotypes in fresh unmanipulated cells allows us to build a complete picture of the differences between the typical peripheral blood composition (both in quantity and composition) of healthy volunteers and that of RCC patients. Figure 2C shows the distribution of immune markers from the mean of 40 healthy volunteers and 25 RCC patients. The leukocyte compartment of RCC patients is enlarged by about a third over healthy volunteers with granulocytes and CD14⁺ HLA-DRlo/neg monocytes significantly expanding in this population.

**CD14⁺ monocytes accumulate in human RCCs, and their presence is predictive of outcome**

Monocytes are capable of migrating from blood to tissues. Therefore, we investigated if the increase in the periphery of CD14⁺ HLA-DRlo/neg cells was associated with the presence of monocytes in the tumor. We examined the associations of tumor...
CD14 expression with clinical and pathologic features and patient outcome using a large cohort (n = 375) of RCC patients. Mean age at surgery was 63 years (median, 63; range, 26–87), mean tumor size was 6.8 cm (median, 5.8; range, 1.0–22.0), and mean SSIGN score was 3.9 (median, 3; range, 0–15). Tumors from 375 RCC patients were stained by immunohistochemistry for CD14 expression. These sections were evaluated and divided into four groups classified as high (marked CD14 expression), moderate (intermediate CD14 expression), low (focal) or none (absent CD14 expression) intratumoral, peritumoral, and maximal CD14 expressions are summarized in Supplementary Table S1. Associations of maximal CD14 expression with clinical and pathologic features are summarized in Table 2. The association of maximal intratumoral and peritumoral CD14 expression with cancer-specific survival is illustrated in Fig. 3A. The magnitudes of the associations of CD14 expression with death from RCC are summarized in Supplementary Table S2. At last follow-up 159 patients had died, including 100 who died from RCC at a mean of 2.3 years following surgery (median, 1.7; range, 0–9). Among the 216 patients who were still alive at last follow-up, the mean duration of follow-up was 7.1 years (median, 7.3; range, 0–10); only 5 (2.3%) patients had fewer than 2 years of follow-up. Estimated cancer-specific survival rates (95% CI, number still at risk) at 1, 3, 5, and 7 years following surgery were 91.0% (88.1–94.0; 328), 79.0% (74.9–83.4; 268), 75.3% (70.9–80.0; 232), and 71.9% (67.3–77.0; 136), respectively. By univariate analysis, patients whose tumors contained moderate CD14 expression were nearly 5 times more likely to die from RCC compared with patients whose tumors contained absent/focal CD14 expression (HR, 4.79; P < 0.001). Patients whose tumors contained marked CD14 expression were over 11 times more likely to die from RCC compared with patients whose tumors contained absent/focal CD14 expression. The geographic mean fluorescent intensity on granulocytes was measured from a subset of RCC patients and healthy volunteers. C. pie graphs representing the mean of the entire leukocyte compartment of RCC patients and healthy volunteers. The mean values of immune phenotypes, including T cells, B cells, NK cells, CD14+ HLA-DR+ monocytes, CD14+HLA-DR+monocytes, Lin CD33+HLA-DR+ MDSCs, and granulocytes, were quantified from RCC patients, and healthy volunteers in cells per microliter were combined to represent the total leukocyte compartment. The RCC pie graph is sized in relation to the healthy volunteer graph (140% vs. 100%).
Table 1. Clinical and pathologic features for 375 ccRCC patients

<table>
<thead>
<tr>
<th>Feature</th>
<th>N (%)</th>
</tr>
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<tbody>
<tr>
<td>Age at surgery (years)</td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>204 (54.4)</td>
</tr>
<tr>
<td>≥65</td>
<td>171 (45.6)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>113 (30.1)</td>
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<tr>
<td>Male</td>
<td>262 (69.9)</td>
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<tr>
<td>Symptoms</td>
<td></td>
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<tr>
<td>Constitutional symptoms</td>
<td>192 (51.2)</td>
</tr>
<tr>
<td>Symptoms</td>
<td>50 (13.3)</td>
</tr>
<tr>
<td>2002 Primary tumor classification</td>
<td></td>
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<tr>
<td>pTa</td>
<td>114 (30.4)</td>
</tr>
<tr>
<td>pT1b</td>
<td>94 (25.3)</td>
</tr>
<tr>
<td>pT2</td>
<td>71 (19.8)</td>
</tr>
<tr>
<td>pT3a</td>
<td>40 (10.7)</td>
</tr>
<tr>
<td>pT3b</td>
<td>50 (13.3)</td>
</tr>
<tr>
<td>pT4</td>
<td>3 (0.8)</td>
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<tr>
<td>Regional lymph node involvement</td>
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<td>pNX and pN0</td>
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<tr>
<td>pN1 and pN2</td>
<td>26 (6.9)</td>
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<tr>
<td>Distant metastases</td>
<td></td>
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<tr>
<td>M0</td>
<td>324 (86.4)</td>
</tr>
<tr>
<td>M1</td>
<td>51 (13.6)</td>
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<tr>
<td>2002 TNM stage groupings</td>
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<tr>
<td>I</td>
<td>203 (54.1)</td>
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<tr>
<td>II</td>
<td>54 (14.4)</td>
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<tr>
<td>III</td>
<td>62 (16.5)</td>
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<td>IV</td>
<td>56 (14.9)</td>
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<td>Tumor size (cm)</td>
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<td>&lt;5</td>
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<td>5 to &lt;7</td>
<td>73 (19.5)</td>
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<td>7 to &lt;10</td>
<td>75 (20.0)</td>
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<td>≥10</td>
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</tr>
<tr>
<td>High (≥7)</td>
<td>89 (23.7)</td>
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</tbody>
</table>

CD14+ Monocytes as a Prognostic Biomarker in RCC

tumor tissue lack expression of granulocytic cell markers (CD15), indicating that these are not granulocytes with aberrant CD14 expression (Fig. 3C). Taken together, these results confirmed positive correlation between increased intratumoral CD14 monocytes and increased CD14+ HLA-DRlo/neg monocytes in systemic circulation, suggesting that CD14+ HLA-DRlo/neg monocytes can be a surrogate marker for intratumoral CD14 and a predictor of decreased survival.

The interaction of CD14+ monocytes and tumor cells leads to loss of monocyte HLA-DR expression and release of proangiogenic factors

Knowing that patients with high intratumoral CD14 have decreased survival, we developed an in vitro model to examine monocyte/tumor cell interaction. We isolated CD14+ monocytes from healthy volunteers and cocultured them for 72 hours with the renal carcinoma cell line ACHN. HLA-DR expression was reduced by more than 75% upon coculture with ACHN cells versus monocytes in media alone (Fig. 4A, left graph; experimental replicates = 7; P = 0.026). This result was specific for tumor cells as HLA-DR expression was not downregulated during coculture with normal primary renal epithelial cells. We also observed loss of HLA-DR expression when monocytes were cocultured with another RCC cell line, 786-0 (Supplementary Fig. S4). Tumor cells promoted survival of monocytes in these coculture experiments as recovery of the CD14+ monocytes was 31% compared with 3% for monocytes alone (P = 0.004; experimental replicates = 5; Fig. 4A, right graph).

Because myeloid cells have been implicated in the process of angiogenesis (reviewed by Schmid and colleagues; ref. 29), we hypothesized that the interaction between the tumor and monocytes highlighted by the loss of DR may also represent the increase of an angiogenesis program by monocytes. We used a proteomics approach to test supernatants and cell lysates from CD14+ and ACHN cells following coculture for the simultaneous expression of 55 proteins involved in angiogenesis (Fig. 4B). We detected specific increases in a number of secreted proteins in the supernatant following monocyte/tumor cell coculture including IL1β, FGF2, and CSF2/GM-CSF. We also found other angiogenic factors, PTX3 and VEGF, that were secreted by monocytes and/or tumor cells but were not changed upon coculture (Fig. 4B). By examining the cell lysates, we could further determine the cell types (CD14+ or ACHN cells) that were producing the proteins detected in the supernatant. For example, IL1β and FGF2 were specifically induced in monocytes when cocultured with tumor cells, whereas GM-CSF and PTX3 were induced in tumor cells when cocultured with monocytes. Again, there were other angiogenic factors, like VEGF, that were detected in the cell lysates but did not change upon coculture. These proteins were not induced in cocultures of monocytes with primary renal epithelial cells (Supplementary Fig. S4). To further verify these changes, we repeated these experiments with up to 6 other normal donor monocyte preparations but assessed changes by quantitative RT-PCR (Fig. 4C). The RNA transcript levels of GM-CSF and PTX3 were significantly induced in tumor cells, whereas IL1β and FGF2 were induced in monocytes. Interestingly, FGF2 levels were not detectable in monocytes cultured in control media, but were greatly induced upon coculture with tumor cells. Since FGF2 expression in tumors has been implicated as a prognostic factor in RCC (30), we measured the plasma levels of FGF2 in our cohort of RCC patients. Although we failed to see a significant difference...
between healthy volunteer and RCC patient plasma FGF2 levels, we found that FGF2 levels inversely correlated with the expression of HLA-DR on CD14⁺ monocytes (Fig. 4D). Taken together, these data demonstrate that monocytes and tumor cells are undergoing reciprocity of induction where monocytes are changing the tumor microenvironment and the tumor environment is changing the monocytes leading to monocytic survival, increased immune suppression, and increased angiogenesis.

**Discussion**

The relationship between the power of the immune system to eliminate tumors and the tumors’ ability to stifle that power has been a source of intense investigation. Most models of tumor/immune system interaction consist of the tumor actively and unilaterally inhibiting an antitumor response through the release of tumor-suppressing growth factors, or by specific antigen escape. The data we have presented here provide an alternative and powerful model. Our data suggest that tumors interact with monocytes resulting in altered monocytes with both immunosuppressive and immune-independent functional characteristics. This model also provides a framework to describe systemic immune suppression as the monocytes have the capacity to leave the tumor environment and move to the periphery.

FD14⁺HLA-DRlow/neg monocytes have known immunosuppressive properties via multiple mechanisms, including effector T-cell inhibition, decreased antigen presentation, and defective dendritic cell maturation (14, 15, 18, 19, 31). The degree by which these cells were increased in RCC patients suggests a dominant role in mediating tumor progression. Other myeloid cells, including CD33⁺HLA-DR/C0 CD15⁺CD66b⁺ granulocytes, were also elevated in RCC patients. This observation is generally consistent with previous findings that granulocytic MDSCs are elevated in RCC patients in the mononuclear fraction of density gradient centrifugation–purified cells (21–24). However, these results are not directly comparable because we performed our flow assays on unfractionated whole blood. In our experience, flow cytometry performed on whole blood is more amenable to standardization and direct inter-laboratory comparisons than flow cytometry performed on fractionated samples (32). The correlation between the levels of CD14⁺HLA-DRlow/neg monocytes and granulocytes is quite interesting. We do not have any data suggesting that these cells influence each other; however, it is possible that tumor-
The presence of intratumoral CD14 is prognostic for survival in RCC and correlates with the frequency of peripheral blood CD14+/HLA-DRlow/neg monocytes. A, cancer-specific survival following surgery determined by maximal intratumoral and peritumoral CD14 immunohistochemistry for 375 RCC patients based on no staining (blue), focal staining (green), moderate staining (red), and marked staining (yellow; \( P < 0.001 \)). Estimated cancer-specific survival rates at 5 years (involvement; 95% CI; number still at risk) following surgery were 100% (no staining; 100–100; \( n = 6 \)), 92.2% (focal staining; 88.2–96.4; \( n = 134 \)), 64.1% (moderate staining; 56.4–73.0; \( n = 75 \)), and 41.8% (marked staining; 29.6–58.9; \( n = 17 \)) for patients with tumors with absent, focal, moderate, and marked CD14 expression, respectively. B, seventeen patients with corresponding blood and tumor tissue were analyzed for the frequency of CD14+/HLA-DRlow/neg in peripheral blood and CD14 staining by immunohistochemistry in their tumor. Intensity of tumor tissue CD14 positivity was related to loss of HLA-DR expression on peripheral blood CD14+ monocytes (\( P < 0.01 \)). C, tumor CD14+ cells are CD15 and CD66b negative. Representative tissue costaining CD14 (brown) with CD15 or CD66b (pink) at low and high magnifications.

Derived factors that promote the survival, mobilization, and/or proliferation of monocytes also influence granulocytes. Our in vitro monocyte/coculture system supports this observation with increased GM-CSF induced during cocultures. It is likely that this observation is an indication of increased program of survival for certain classes of leukocytes.

We found considerable variability in the amount of CD14 staining in RCC samples but found a strong adverse correlation to the amount of intratumoral CD14 staining and survival. Sections of the highest grade from the tumor blocks were selected for staining and were generally consistent. Multiple blocks were not assessed; therefore, little is known regarding whole tumor infiltration of monocytes. In addition, it is not known if the tumor heterogeneity or specific oncogene expression influences the recruitment of monocytes differently at primary sites as opposed to metastatic sites.

Our in vitro coculture model of CD14+ monocytes and RCC cell lines demonstrated that the interaction between monocytes and tumor cells leads to the upregulation of factors that favor tumor progression. Monocytes induced GM-CSF production from tumor cells, a known potent survival factor for monocytes. Tumor cells caused the downregulation of HLA-DR on monocytes and induced expression of the angiogenic factors FGF2 and IL1β (RNA transcript and protein levels). IL1β is a potent inducer of angiogenesis and has been reported to contribute to tumor invasive-ness, metastasis, and immune suppression (33). In the RCC setting, Chittezhath and colleagues recently identified the IL1-IL1R pathway via transcriptomic and molecular profiling as an important mechanism for the tumor-promoting role of monocytes (34). They found that peripheral blood monocytes from RCC patients displayed elevated expression of genes involved in angiogenesis and invasion. Blockade of the IL-1/IL1R pathway in tumor/monocyte cocultures inhibited the induction of these genes. Through their analysis of tumor-gene expression data from 34 RCC patients, they found that IL1β expression correlated with myelomonocytic markers, including CD14. We identified that IL1β protein levels were induced in monocytes cocultured and assessed CD14 accumulation in tumors by immunohistochemistry. Taken together, our results combined with those of Chittezhath and colleagues independently confirm the role of tumor-promoting effects of monocytes and their impact on patient survival. FGF2 has been shown to be a prognostic factor in RCC (35). The high levels of intratumoral CD14+ cells combined with the observation that FGF2 was predominantly induced in monocytes may provide a mechanistic explanation to the failure of anti-VEGF therapies in some RCC patients. In many respects, the actions of tumor-infiltrating monocytes mimic the natural phenomena of tissue repair. Circulating monocytes are very efficient at homing to sites of injury, whereby they initiate proinflammatory...
FGF2, are additional functions that these cells provide. This functions of monocytes (36, 37). The process certainly demonstrates the remarkable plasticity of the selected proteins after coculture of CD14+ monocytes with tumor increased monocyte recovery (gray) compared with CD14+ monoculture (black; n = 6; right). Coincubation of CD14+ monocytes with tumor increased monocyte recovery (gray) compared with CD14+ monoculture (black; n = 6; right). B, angiogenesis array blots showing selected proteins after coculture of CD14+ monocytes with ACHN cells from culture medium supernatants and separated cell lysates. C, quantitative RT-PCR results from the change in gene expression in CD14+ monocytes (left) and ACHN (right) after coincubation and reseparation as in B. D, measurements of FGF2 plasma levels and correlation between plasma FGF2 and corresponding HLA-DR expression on circulating CD14+ cells in RCC patients. Dotted line represents the mean HLA-DR MFI of healthy volunteers. Figure 4. Functional and phenotypic consequences of the interaction of CD14+ monocytes and tumor cells. CD14+ monocytes from healthy volunteers were cultured with an RCC cell line (ACHN) for 72 hours. CD14+ monocytes were reisolated and analyzed for flow cytometric, protein, and gene expression analysis. A, representative histogram plot showing HLA-DR levels on CD14+ monocytes in control media (black line), mixed with ACHN cells (gray line), and primary normal renal epithelial cells (dotted line); representative results from seven similar experiments (left). Coincubation of CD14+ monocytes with tumor increased monocyte recovery (gray) compared with CD14+ monoculture (black; n = 6; right). B, angiogenesis array blots showing selected proteins after coculture of CD14+ monocytes with ACHN cells from culture medium supernatants and separated cell lysates. C, quantitative RT-PCR results from the change in gene expression in CD14+ monocytes (left) and ACHN (right) after coincubation and reseparation as in B. D, measurements of FGF2 plasma levels and correlation between plasma FGF2 and corresponding HLA-DR expression on circulating CD14+ cells in RCC patients. Dotted line represents the mean HLA-DR MFI of healthy volunteers.

responses as part of the innate immune response. The monocytes/macrophages then switch to an antiinflammatory response that is required for efficient tissue regeneration. Angiogenesis and fibroblast recruitment, mediated in part by FGF2, are additional functions that these cells provide. This process certainly demonstrates the remarkable plasticity of the functions of monocytes (36, 37).

Our data additionally suggest that CD14+ HLA-DRlow/neg monocytes are both an important therapeutic target and a strong prognostic biomarker for outcome in RCC patients. Perhaps by targeting these cells through elimination or blocking their migration to tumors, the efficacy of conventional therapies will likely improve. For example, we have recently shown that in a similar in vitro model with non-Hodgkin lymphomas, the presence of monocytes protected the tumor from direct killing with doxorubicin by altering the expression patterns of proapoptotic and antiapoptotic proteins (38). This study identifies an additional mechanism, protection from chemotherapy-induced cytotoxicity, in which the interaction of monocytes and tumor cells reduces the efficacy of anticancer treatments.

Because CD14+ HLA-DRlow/neg monocytes have multiple immunosuppressive functions, they will likely negatively impact immunotherapeutic approaches. There is emerging evidence that this phenomenon is already happening in RCC patients. These cells have been shown to adversely affect survival of RCC patients treated with a multipeptide cancer vaccine (6) and impede the differentiation into fully mature dendritic cells (31). Circulating monocytes and granulocytes have previously been independently associated as prognostic factors for poor clinical responses to IL2 immunotherapy (39, 40). Therefore, the synergistic interaction between tumor and monocytes may explain the ineffectiveness of many immune therapies by shutting down both local and systemic antitumor immunity. Taken together, our results reveal why the presence of CD14+ cells may reflect the aggressiveness of RCC tumors and the failure of these tumors to respond to current treatments.

Disclosure of Potential Conflicts of Interest

E.D. Kwon has ownership interest (including patents) in Bristol-Myers Squibb and MedImmune. No potential conflicts of interest were disclosed by the other authors.

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