Resistance to Antiangiogenic Therapy Is Associated with an Immunosuppressive Tumor Microenvironment in Metastatic Renal Cell Carcinoma

Xian-De Liu, Anh Hoang, Lijun Zhou, Sarathi Kalra, Alper Yetil, Mianen Sun, Zhiyong Ding, Xuesong Zhang, Shanshan Bai, Peter German, Pheroze Tamboli, Priya Rao, Jose A. Karam, Christopher Wood, Surena Matin, Amado Zurita, Axel Bex, Arjan W. Griffoen, Jianjun Gao, Padmanee Sharma, Nizar Tannir, Kanishka Sircar, and Eric Jonasch

Abstract

Renal cell carcinoma (RCC) is an immunogenic and proangiogenic cancer, and antiangiogenic therapy is the current mainstay of treatment. Patients with RCC develop innate or adaptive resistance to antiangiogenic therapy. There is a need to identify biomarkers that predict therapeutic resistance and guide combination therapy. We assessed the interaction between antiangiogenic therapy and the tumor immune microenvironment and determined their impact on clinical outcome. We found that antiangiogenic therapy–treated RCC primary tumors showed increased infiltration of CD4+ and CD8+ T lymphocytes, which was inversely related to patient overall survival and progression-free survival. Furthermore, specimens from patients treated with antiangiogenic therapy showed higher infiltration of CD4+FOXP3+ regulatory T cells and enhanced expression of checkpoint ligand programmed death-ligand 1 (PD-L1). Both immunosuppressive features were correlated with T-lymphocyte infiltration and were negatively related to patient survival. Treatment of RCC cell lines and RCC xenografts in immunodeficient mice with sunitinib also increased tumor PD-L1 expression. Results from this study indicate that antiangiogenic treatment may both positively and negatively regulate the tumor immune microenvironment. These findings generate hypotheses on resistance mechanisms to antiangiogenic therapy and will guide the development of combination therapy with PD-1/PD-L1–blocking agents.

Introduction

Renal cell carcinoma (RCC) is the most common renal malignancy. Similar to other solid tumors, RCC is an immunogenic cancer and is frequently infiltrated with immune cells, including macrophages and T lymphocytes. Tumor-infiltrating lymphocytes (TIL) have been considered the host immune reactions to eliminate cancer cells, and TILs were generally beneficial in most cancers, including colorectal cancer, lung cancer, ovarian cancer, and melanoma (1). On the contrary, higher TILs correlated with poorer prognosis and shorter survival in RCC (1–4). The mechanism underpinning the controversial pathophysiologic significance of TILs in RCC remains unclear. It is known that immune checkpoint pathways, regulatory T cells (Treg), and myeloid-derived suppressor cells (MDSC) have a remarkable ability to suppress T-cell responses (5–7). It is thus conceivable that the activity of TILs in RCC tumors is thwarted by such immunosuppressive mechanisms.

As a proangiogenic cancer, RCC has been treated with agents, such as sunitinib and bevacizumab, that target angiogenic factors (8, 9). However, there is heterogeneity in tumor response to targeted agents, with few patients achieving a complete response, some achieving partial response or stable disease, and a significant number of patients with progressive disease (9). Furthermore, most responsive patients develop resistance over time (9). Therefore, along with the development of these new therapeutic agents, there is a need to identify mechanisms of therapeutic response and resistance that can be used to develop more effective therapy for patients with RCC. Recently, the combination of antiangiogenic therapy with immunotherapy based on the blockade of programmed death 1 (PD-1) or programmed death-ligand 1 (PD-L1) has been proposed to be a potential new therapeutic approach for RCC patients (10). However, the top priority and challenge is the selection of patients who are likely to respond to anti–PD-L1 therapy (11). Recent studies show that tumor regression after therapeutic PD-1/PD-L1 blockade requires preexisting T cells, which are negatively regulated by PD-1/PD-L1–mediated
adaptive immune resistance (12–14). Because the interaction between antiangiogenic therapy and tumor immune microenvironment and its association with patient survival in patients with RCC are unknown, such a study will not only provide a biomarker for antiangiogenic response or resistance, but also a selection marker for patients who would benefit from receiving anti–PD-L1 therapy.

In this study, we found that antiangiogenic therapy–treated primary tumors from patients with metastatic RCC (mRCC) showed increased CD4+ and CD8+ T-lymphocyte infiltration compared with that in uninvolved tissue and untreated controls, and this finding was inversely correlated with patient overall survival (OS) and progression-free survival (PFS). T-lymphocyte infiltration was correlated with infiltration of immunosuppressive Tregs and upregulation of the checkpoint ligand PD-L1. The results from this study reveal that the increase in Treg infiltration and PD-L1 expression probably can serve as biomarkers and possible drivers of resistance to antiangiogenic therapy; it may be feasible to improve therapeutic efficacy by interfering with these immunosuppressive pathways.

Materials and Methods

Antibodies and reagents
CD3 antibody (clone F7.2.38; MA5-12577) and CD45RO antibody (clone UCHL1; MA5-11532) were from Thermo Scientific. CD4 antibody (clone 4B12; MA5152850) and CD8 antibody (clone C8/1448; MA45730) were from Fisher Scientific. CD68 antibody (ab125212), CD4 antibody (ab133616), and FOXP3 antibody (clone 236A/E7; ab20034) were from Abcam. PD-L1 antibody (ab125212), CD4 antibody (ab133616), and FOXP3 antibody (clone 236A/E7; ab20034) were from Abcam. PD-L1 antibody (E1L3N; 13684) and p-STAT3 (Y701; 9167) were from Cell Signaling Technologies. GAPDH antibody (FL-335; sc25778) was from Santa Cruz Biotechnology. IFNγ (285-IF-100) was from R&D systems.

Cell culture, establishment of stable cell lines, and cell lysis
RCC4, A-498, 786-O, CaKi-1, TK-10, and SN12C cell lines were obtained from the ATCC. All cell lines were validated by short tandem repeat (STR) DNA fingerprinting using the Promega 16 High Sensitivity STR Kit (catalog # DC2100). The STR profiles were compared with online search databases (DSMZ/ATCC/JCRB/RIKEN) of 2,455 known profiles, along with the MD Anderson Characterized Cell Line Core (CCLC) database of 2,556 known profiles. The STR profiles matched known DNA fingerprints or were unique. All cell lines were grown in DMEM containing 10% FBS. 786-O stable cell lines expressing control shRNA or PD-L1 shRNA (Thermo Scientific; V2LHS_5368) were infected with lentiviral particles and selected in medium containing 2 μg/mL of puromycin. Cells were lysed in RIPA buffer (50 mmol/L Tris-Cl, pH 7.4, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Nonidet P-40, 0.1% SDS) for immunoblot analysis.

Xenograft tumor models
The RCC tumorigenesis assay was initiated by injection of 10 million 786-O RCC cells into the flank of each Ncr-nu/nu mouse. After tumors were palpable (i.e., tumor volume reached 100 mm3), mice were treated with sunitinib (50 mg/kg) by oral gavage 3 times/week for 3 weeks. Animal health was assessed daily to minimize pain and distress. Mice were monitored by veterinary staff for tumor burden, behavior, and appetite. These experiments were approved by The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee (A3343-01).

RNA isolation and real-time PCR
As previously described (15), total RNAs were isolated and purified using the RNasy Mini Kit (Qiagen) and converted to cDNA using a cDNA Reverse Transcription kit (Applied Biosystems). HIF2α, VEGFA, TGFA, and CCND1 expression was measured using a real-time PCR detection system (Applied Biosystems Viia 7) in 96-well optical plates using fast SYBR GREEN Universal PCR Master Mix (Applied Biosystems). GAPDH was used as a control. Primer sequences for RT-PCR were as follows: PD-L1 (CD274), 5'-TGGCATTGGTCGAAGCCCTTT-3' and 5'-TGGCAGCAGGTCTCTAATTGTTT-3'; GAPDH, 5'-CAATGACCCCTCATTGACC-3' and 5'-TGTATTTTGCCGGCATCTCG-3'.

Tissue microarray, image acquisition, and analysis
Human subject protocol (2007-0511) was approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center. Tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. Tissue microarrays (TMA) with tripli cate cores for each case were generated using a CHEMICON Advanced Tissue Arrayer (ATA 100). A hematoxylin- and -eosin slide of each donor tissue block was assessed to identify the desired tissue region, and donor tissue block was marked to precisely locate the same area. The donor block was punched with a 0.6-mm hollow needle, the tissue core was deposited into a prepunched recipient array block, and the identity of the transcribed core was indexed and recorded. TMA slides contained 33 unaffected kidneys, 41 untreated primary RCCs from patients with mRCC. TMAs were immunohistochemically (IHC) stained as previously described (16). Slides were scanned with the Vectra image scanning system (Caliper Life Sciences; ref. 17). The percentages of CD3+, CD45RO+, CD4+, CD8+, CD4+FOXP3+, and PD-L1+ cells in whole tissue sections were analyzed using inForm software (Caliper Life Sciences; ref. 17). An unpaired Student t test was used for statistical analysis.

Results

RCC is associated with immune-cell infiltration
RCC is considered to be an immunogenic cancer, and RCC is frequently infiltrated with immune cells (1). To confirm the immunogenic phenotype of RCC, we compared immune-cell infiltration in 42 cases of RCC with 33 cases of uninvolved renal tissue derived from patients treated with bevacizumab. We generated TMAs containing formalin-fixed triplicate cores for each case, and the TMAs were IHC stained with specific antibodies. To effectively and accurately study the infiltration of immune cells, TMA slides were scanned with the Vectra image scanning system, and the percentage of immune cells in whole tissue sections was analyzed using inForm software. The total cell number count was based on hematoxylin-positive cells. Figure 1A shows both the original and quantification images that contain different degrees of CD8+ infiltration. CD8+ cells are labeled in red, and CD8− cells are labeled in blue. Cell percentages listed below the images were calculated automatically by the inForm software. After developing this quantification algorithm, we first determined total
T-lymphocyte infiltration by IHC staining of CD3, a T-lymphocyte marker protein. RCC specimens harbored significantly more CD3⁺ T lymphocytes than the uninvolved renal tissue: 16.6% versus 4.9% (P < 0.001; Fig. 1B). CD45RO has been used as a marker protein of activated T lymphocytes, and we further observed that RCC tissues were infiltrated with a higher percentage of CD45RO⁺ T lymphocytes: 24.3% versus 5.9% (P < 0.001; Fig. 1C). Next, we checked the infiltration of CD4⁺ T lymphocytes (T-helper cells) and CD8⁺ T lymphocytes (cytotoxic T cells). We found that RCC tissues harbored more CD4⁺ T lymphocytes (16.3% vs. 2.9%) and CD8⁺ T lymphocytes (5.1% vs. 0.6%) than uninvolved renal tissues (P < 0.001; Fig. 1D and E). Similarly, RCC tissues were infiltrated with more macrophages, as indicated by the expression of CD68: 20.3% versus 4.7% (P < 0.001; Fig. 1F). These results confirm the immunogenic phenotype of RCC.

Antiangiogenic therapy–treated tumors show increased T-lymphocyte infiltrates, which are associated with poor survival

Both sunitinib and bevacizumab are used as treatment for mRCC. The former is a multikinase inhibitor with activity against
VEGFR and platelet-derived growth factor receptor (PDGFR), and the latter is a humanized monoclonal antibody that inhibits vascular endothelial growth factor A (VEGFA; ref. 18). To study the interaction between antiangiogenic therapy and immune-cell infiltration, we compared the percentage of immune cells in RCC tissues exposed to antiangiogenic therapy with control tumors in patients with mRCC. In one study (NCT00113217), patients with previously untreated mRCC were treated for 8 weeks with bevacizumab and underwent cytoreductive nephrectomy. In the second study (NCT00715442), patients with previously untreated mRCC were treated with sunitinib for 4 weeks, given a 2-week break, and then underwent cytoreductive nephrectomy during their second treatment cycle, with a 1-day break between last dose of sunitinib and surgery. We also had access to untreated primary tumors from patients with untreated mRCC who underwent treatment with sorafenib after undergoing either cytoreductive or upfront nephrectomy (NCT00126594). We generated TMA s with triplicate formalin-fixed tissue cores for each case from 42 bevacizumab-pretreated and 39 sunitinib-pretreated primary RCCs and 41 untreated primary RCCs from each of these studies.

We found that both sunitinib- and bevacizumab-pretreated cases showed increased CD3⁺ T-lymphocyte infiltration relative to untreated primary RCC controls: 19.3% and 16.6% versus 11.3% (P < 0.01), increased CD45RO⁺ T-lymphocyte infiltration: 27.2% and 24.3% versus 16.8% (P < 0.01), increased CD4⁺ T-lymphocyte infiltration: 15.3% and 16.3% versus 7.6% (P < 0.05), and increased CD8⁺ T-lymphocyte infiltration: 7.0% and 5.1% versus 2.3% (P < 0.05), respectively (Fig. 2A–D). The infiltration of CD68⁺ macrophages was higher in sunitinib- than in bevacizumab-pretreated RCC specimens (25.2% vs. 20.3%, P < 0.05) but not in bevacizumab-treated samples (21.3% vs. 20.3%, P > 0.05; Fig. 2E).

Tumor-infiltrating T lymphocytes are generally considered to be a host immune reaction engendered to eliminate cancer cells. We first investigated the correlation between CD4⁺ T-lymphocyte infiltration and patient survival. Based on the distribution of CD4⁺ T lymphocytes and patient OS or PFS, we divided each group of patients with or without antiangiogenic therapy into two subgroups. Unexpectedly, in sunitinib-treated patients, specimens derived from patients with shorter OS and PFS harbored a higher percentage of tumor-infiltrating CD4⁺ T lymphocytes than those derived from patients with longer OS and PFS: 22.3% versus 8.0% (P < 0.01) and 20.3% versus 8.4% (P < 0.01), respectively (Fig. 3A). Similar results were obtained with specimens from bevacizumab-treated patients (Fig. 3A). These results indicate that CD4⁺ T-lymphocyte infiltration was inversely correlated with patient OS and PFS in patients treated with antiangiogenic therapy. However, in patients who were not treated, no obvious correlation was observed between CD4⁺ T-lymphocyte infiltration and OS or PFS (Fig. 3A).

Next, we evaluated the association between CD8⁺ T-lymphocyte infiltration and patient outcome. In the untreated control group and in the sunitinib-treated group, the cases with shorter OS or PFS showed higher tumor-infiltrating CD8⁺ T lymphocytes, indicating that CD8⁺ T-lymphocyte infiltration was negatively related to patient OS and PFS (Fig. 3B). In the bevacizumab-treated group, such negative correlation was also observed between CD8⁺ T-lymphocyte infiltration and patient PFS (Fig. 3B). Although not statistically significant, the infiltration of CD8⁺ T lymphocytes in cases with shorter OS was also higher than that in cases with longer OS (6.28% vs. 3.92%). In contradistinction to the T-cell subsets, CD68⁺ macrophage infiltration was not associated with favorable or unfavorable OS or PFS in different patient groups (Fig. 3C).

These results collectively indicate that a preexisting immunosuppressive tumor microenvironment inhibited or subverted the antitumor function of T cells.

**Antiangiogenic therapy–treated tumors show increased Treg infiltration**

**TILs** are generally thought to aid in eliminating tumors, while the data above show that the increased T-lymphocyte infiltration following antiangiogenic therapy is inversely correlated to patient survival. Tumors can escape immune surveillance by initiating various immunosuppressive cells or pathways. We hypothesized that T-lymphocyte infiltration is associated with the generation of immunosuppressive tumor microenvironment, or arises as a result of a failed effector immune response.

Tregs are a subset of CD4⁺ T lymphocytes and are characterized by the expression of the transcription factor forkhead box P3 (FOXP3). Tregs secrete inhibitory cytokines and granzyme A to inhibit the function of effector T lymphocytes or induce effector T-lymphocyte apoptotic cell death (7). To explore the changes of Tregs following antiangiogenic therapy and their relationship with patient survival, we double stained TMA s with anti-CD4 and anti-FOXP3 antibodies. CD4 and FOXP3 single- or double-positive cells were quantified using Inform software. Representative images and corresponding quantification images are shown (Fig. 4A). CD4⁺ cells are labeled in red, FOXP3⁺ cells are labeled in green, and CD4⁺ FOXP3⁻ Tregs are labeled in yellow. Negative cells are labeled in blue. Compared with untreated RCC controls, the CD4⁺ FOXP3⁺ Tregs in both sunitinib- and bevacizumab-treated cases were higher: 1.1% and 0.8% versus 0.4% (P ≤ 0.01), respectively (Fig. 4A and B). However, the ratio of CD4⁺ FOXP3⁻ Tregs to total CD4⁺ T lymphocytes was not affected by sunitinib or bevacizumab treatment, and Tregs constituted fewer than 10% of CD4⁺ cells (Fig. 4C). In response to the effector immune response, immunosuppressive regulators might also increase, which will in turn lead to an increasingly frustrated feedback. There is increasing evidence for the importance of Treg/CD8 ratios in predicting the correlation with CD8⁺ T-cell infiltration and negative clinical outcomes (19, 20). Blockade of CTL-associated antigen 4 (CTLA-4) in melanoma patients and in a mouse model shifted the ratio of effector T cells to Tregs by around 5-fold and consequently led to tumor regression (19, 20). In contrast, we found that sunitinib only slightly changed the ratio of Treg to CD8⁺ T lymphocytes (Fig. 4D). These results indicate that antiangiogenic therapy may be insufficient to mount an effector T-cell response capable of overcoming the immunosuppressive tumor microenvironment.

In the sunitinib-treated group, the cases with shorter OS or PFS showed higher tumor-infiltrating Tregs, indicating that Treg infiltration was negatively related to patient OS and PFS (Fig. 4E). In the bevacizumab-treated group, such negative correlation was only observed between Treg infiltration and patient OS but not PFS (Fig. 4E). It was recently reported that in the melanoma tumor microenvironment, CD8⁺ T-lymphocyte infiltration drives the infiltration of Tregs (21, 22). Here, we also observed a correlation...
Figure 2. Antiangiogenic therapy increases immune-cell infiltration. TMAs from untreated RCC controls or RCCs treated with sunitinib (SN) or bevacizumab (BV) were IHC stained with anti-CD3 antibody (A), anti-CD45RO antibody (B), anti-CD4 antibody (C), anti-CD8 antibody (D), or anti-CD68 antibody (E). Immunoreactivity was visualized with DAB (brown), and nuclear counterstain was shown by hematoxylin (blue). Representative images and the percentages of CD3⁺, CD45RO⁺, CD4⁺, CD8⁺, or CD68⁺ cells in each group were shown. Statistical analysis was performed with an unpaired Student t test. NT, no treatment.
between CD8\(^+\) T-lymphocyte infiltration and Treg infiltration in sunitinib-treated patients (Fig. 4F), indicating that CD8\(^+\) T lymphocytes might be involved in attracting Tregs.

Antiangiogenic therapy upregulates PD-L1 expression

PD-L1 is an immune-checkpoint receptor on T lymphocytes, and the upregulation of PD-L1 expression on tumor cells inhibits T-lymphocyte activity (6). We studied the effects of antiangiogenic therapy on PD-L1 expression in RCC tissues. To validate the specificity of PD-L1 antibody (Cell Signaling Technology; #13684) in IHC staining, we first stained human placental tissue, because PD-L1 is known to express on trophoblasts in the placenta to maintain fetomaternal tolerance (23). Figure 5A shows specific PD-L1 staining on trophoblasts. We also included a nonprimary antibody control that was only developed with the secondary antibody and detection system. In untreated RCC controls, 39% of tissue cores were PD-L1 negative, and most PD-L1\(^+\) samples showed a positivity of less than 5% (Fig. 5B). The overall positivity in our study is lower than that previously reported (24), which is probably due to a different patient population, different antibody affinity, and a different quantification method. Importantly, both sunitinib- and bevacizumab-treated cases showed increased expression of PD-L1 relative to that of untreated RCC controls: 8.0% and 6.3% versus 1.3% (\(P < 0.01\); Fig. 5B). In sunitinib-treated patients, the expression of PD-L1 negatively correlated with patient OS and PFS (\(P < 0.05\); Fig. 5C). In bevacizumab-treated patients, PD-L1 expression was inversely related to PFS (\(P < 0.01\)) but not to OS (\(P > 0.05\); Fig. 5C). PD-L1 expression can be induced by IFN\(\gamma\) secreted by TILs (6). Here, we also observed the correlation between CD8\(^+\) T-lymphocyte infiltration and PD-L1 expression in sunitinib-treated samples (Fig. 5D), implying that antiangiogenic therapy...
Figure 4. Antiangiogenic therapy increases regulatory T-cell infiltration. TMAs from untreated RCC controls or RCCs treated with sunitinib or bevacizumab were IHC stained with anti-CD4 antibody and anti-FOXP3 antibody at the same time. FOXP3 immunoreactivity is visualized with DAB (brown), CD4 immunoreactivity is visualized with warp red chromogen (red), and nuclear counterstain is shown by hematoxylin (blue). A, representative original images and corresponding quantification images. CD4+ cells are labeled in red, FOXP3+ cells are labeled in green, and CD4+ FOXP3+ Tregs are labeled in yellow. Negative cells are labeled in blue. B, percentages of CD4+ FOXP3+ Treg in each group are shown. The ratio of CD4+ FOXP3+ Tregs to C, CD4+ single positive cells or D, CD8+ positive cells. E, correlation between Treg infiltration and patient survival. Based on the distribution of tumor-infiltrating T lymphocytes and patient OS or PFS, we divided each group of patients into two subgroups. The percentages of CD4+ FOXP3+ Treg in each subgroup are shown. Statistical analysis was performed with an unpaired Student t test. F, correlation between CD8+ T-lymphocyte infiltration and Treg infiltration. NT, no treatment; SN, sunitinib; BV, bevacizumab.
may indirectly upregulate PD-L1 via CD8⁺ T-lymphocyte-mediated IFNγ secretion.

We also considered the possibility that antiangiogenic therapy may have an effect on PD-L1 expression independent of CD8⁺ T-lymphocyte infiltration. Because functional T lymphocytes are absent in the athymic nude mice, we tested this hypothesis using a xenograft model with tumors induced by injection of 786-O RCC cells into the flanks of nude mice. Mice bearing 786-O-derived xenografts were treated with sunitinib by oral gavage 3 times/week for 3 weeks. Treatment with IFNγ induced the phosphorylation of signal transducer and activator of transcription 1 (p-STAT1, Y701) and the accumulation of PD-L1 in 786-O cells, and the stable expression of PD-L1 shRNA reduced the protein level of PD-L1 (Fig. 6A), confirming the specificity of PD-L1 antibody in immunoblot analysis. Tumors from mice treated with sunitinib showed significantly higher PD-L1 protein levels than those from PBS-treated mice (Fig. 6B). Differences in tissue location, blood vessel density, oxygen stress, and immune response engaged by natural killer (NK) cells may contribute to the wide range of PD-L1 expression in human RCC.
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expression in this experimental group. As recently reported (25), prolonged treatment with sunitinib caused a decrease in tumor volume followed by a resistance phase in the xenograft model, which was possibly linked to increased PD-L1 expression. Additional experiments will be required to investigate the effect of PD-L1 blockade on tumor growth following sunitinib treatment in an immune-competent mouse model.

We tested the direct effect of sunitinib treatment on PD-L1 expression in 786-O cell lines and found that sunitinib increased PD-L1 protein levels but not mRNA levels (Fig. 6C). A recent study reported that PD-L1 is a target of the hypoxia-inducible factor 1α (HIF1α; ref. 26). However, 786-O cells do not express HIF1α and von Hippel–Lindau (VHL), the E3 ligase for HIF1α and HIF2α (17). Furthermore, sunitinib did not affect the protein level of HIF2α (Fig. 6C). These results collectively indicate that sunitinib increased PD-L1 expression independent of HIF1α or HIF2α. Similarly, bevacizumab also increased the PD-L1 protein level (Fig. 6D). We then compared the PD-L1 protein level and its response to sunitinib treatment in different RCC cell lines. Interestingly, the basal level of PD-L1 varied considerably between different RCC cell lines (Fig. 6E). The protein level of PD-L1 is fairly high in RCC4 and A-498 cells, whereas it is almost undetectable in CaKi-1, TK-10, and SN12C cells. Sunitinib treatment increased the PD-L1 protein level in several RCC cell lines (Fig. 6E). These results indicate that antiangiogenic therapy upregulates PD-L1 in a direct manner. The variability of PD-L1 expression and response to sunitinib treatment in different RCC cell lines indicates that PD-L1 might play an important role in innate and adaptive resistance to antiangiogenic therapy.

Discussion

In mouse models, sunitinib treatment alone or in combination with vaccines (or adoptive transferred T cells) was observed to improve T-cell numbers in spleens or tumors (27–29). However, such a study has not been performed using human tumor tissue samples. Here, we find that, in patients with mRCC, antiangiogenic therapy–treated tumors are associated with an increased infiltration of both innate immune cells, such as macrophages, and adaptive immune cells, such as CD4+ and CD8+ T lymphocytes, compared with that in untreated control specimens. T-lymphocyte infiltration is induced by chemokines and depends on adhesion molecules (30). In addition, autophagy-dependent ATP release from dying tumor cells attracts T lymphocytes into the tumor bed (31). It was reported that antiangiogenic treatment induced the expression of intercellular adhesion molecule 1 (ICAM1) in endothelial cells (32), and enhanced the expression of vascular cell adhesion molecule-1 (VCAM1; ref. 33) and CXCR3 ligand chemokines in mice (34). Sunitinib or bevacizumab treatment also can induce autophagy (35, 36). Taken together, these data suggest that the induction of autophagy and the increased expression of adhesion molecules and chemokines may promote immune-cell infiltration in patients treated with antiangiogenic therapy (Fig. 6). Although the directional sensitivity of sunitinib and bevacizumab effect on immune-cell infiltration was the same, the effects of sunitinib are more obvious than the effects of bevacizumab on the infiltration of CD8+ T cell or CD68+ macrophages, and on the ratio of Tregs/CD8+ cells. Such differences may be due to a broader targeting of kinases by sunitinib (18). As a humanized monoclonal antibody that binds VEGFA, bevacizumab disrupts the ability of VEGFA to activate its receptors, VEGFR1 and VEGFR2 (18). In contrast, sunitinib is a direct inhibitor of the tyrosine kinase activities of VEGFR1, VEGFR2, and VEGFR3, as well as PDGFRs and other related kinases (18). NF-κB is a key transcription factor involved in the expression of molecules that mediated endothelial–leukocyte interaction, including ICAM1, VCAM1, and chemokines (37). NF-κB activity is negatively regulated by both VEGFR2 (37) and VEGFR3 (38). It is conceivable that sunitinib more effectively inhibits NF-κB signaling and consequently immune-cell infiltration by targeting both VEGFR2 and VEGFR3, whereas bevacizumab only inhibits VEGFR2 activity.

Multiple studies have demonstrated that sunitinib improved outcome in RCC patients (9, 39) and in various mouse models (28, 40, 41). Sunitinib can improve PFS mainly through its antiangiogenic activity (18). In mouse models, it has been reported that sunitinib reduced MDSCs, Tregs, and phosphorylated STAT3, which improved antitumor T-cell response and survival (28, 40, 41). However, patients showed significant heterogeneity in response to antiangiogenic treatment, and the underlying mechanism driving that heterogeneity remains unclear. Focusing on the role of T-cell infiltration in treatment resistance, we studied the association between T-cell infiltration and therapy outcome of sunitinib- or bevacizumab-treated patients. We found that patients with higher infiltrating T lymphocytes after sunitinib or bevacizumab treatment have a shorter OS and/or PFS. As previously reported (2, 3), even in RCC patients without antiangiogenic therapy, CD8+ T-lymphocyte infiltration was associated with shorter survival. We assume that there is a preexisting immunosuppressive environment in RCC tumors, which inhibits the activity of T lymphocytes. Our analyses revealed that this T-cell infiltration was associated with a commensurately increased Treg infiltration and with PD-L1 upregulation, and both immunosuppressive features were negatively related to patient survival. These findings suggest that there may be further induction of an immunosuppressive environment after antiangiogenic therapy. A recent study also showed that tumor-associated M2 macrophages, another type of immunosuppressive cells found in the tumor microenvironment, induced the skewing of blood-derived CD4+ T lymphocytes toward a more immunosuppressive phenotype (42). This observation raised the possibility that T lymphocytes recruited following antiangiogenic therapy initiation are largely reprogrammed to possess immunosuppressive characteristics, such as a TH17 phenotype (43). Limitations in antibody and IHC staining capabilities make the evaluation of additional T-lymphocyte subpopulations and MDSCs difficult on paraffin-embedded specimens. Future work will include a more elaborate evaluation of these cell subtypes and MDSCs in treated tissue specimens. In addition to Tregs and PD-L1, MDSCs, M2 macrophages, and T-helper 17 cells may also inhibit or subvert the antitumor function of T cells.

Several published studies have described distinct CD4+ cellular subsets that confer prognostic significance. Using immunofluorescence staining, Siddiqui and colleagues (4) reported that, in untreated patients, increased presence of CD4+ CD25+FOXP3− but not CD4+CD25−FOXP3+ cells was associated with increased probability of death from RCC. We used CD4+FoxP3+ double IHC staining to characterize our Treg population due to the difficulties in achieving satisfactory triple
A, PD-L1 antibody validation. 786-O cells stably expressing PD-L1 shRNA were treated with IFNγ (10 ng/mL) for 1 or 3 hours. Cell lysates were analyzed by immunoblot using anti–PD-L1 antibody, anti–P-STAT1 (Y701) antibody, or anti–β-actin antibody. B, sunitinib treatment increases PD-L1 protein levels in 786-O cell–induced xenograft. After the tumors are palpable (i.e., tumor volume reached 100 mm³), mice were treated with sunitinib (30 mg/kg) by oral gavage 3 times/week for 3 weeks. Each lane is a separate xenograft experiment. PD-L1 band intensity was analyzed using ImageJ software. The average level of PD-L1 in PBS-treated xenografts was normalized to 1. Statistical analysis was performed with an unpaired Student t test. C, sunitinib treatment increases PD-L1 protein level but not mRNA level in 786-O cell line. 786-O cells were incubated in the presence of sunitinib for 16 hours at a concentration of 0.5, 1, 2, 5, or 10 μmol/L. Cell lysates were analyzed by immunoblot using an anti–PD-L1 antibody, anti–HIF2α antibody, or anti–β-actin antibody. PD-L1 band intensity with or without sunitinib (5 μmol/L) treatment was analyzed using ImageJ software. The average level of PD-L1 in control cells was normalized to 1. Statistical analysis was performed with an unpaired Student t test, n = 4. Total RNAs were analyzed by real-time PCR using primers specific for PD-L1 (CD274). mRNA level in control cells was normalized to 1. GAPDH was used as endogenous control. D, bevacizumab treatment increases PD-L1. 786-O cells were treated with bevacizumab (10 or 25 μg/mL) for 16 hours. Cell lysates were analyzed by immunoblot using anti–PD-L1 antibody or anti–β-actin antibody. E, sunitinib treatment increases PD-L1 protein levels in different RCC cell lines. RCC cell lines (A-498, RCC4, 786-O, CaKi-1, TK-10, and SN12C) were treated with sunitinib (5 μmol/L) for 16 hours. Cell lysates were analyzed by immunoblot using anti–PD-L1 antibody or anti–GAPDH antibody. F, working model. Antiangiogenic therapy generates an immunosuppressive tumor microenvironment. On the one hand, antiangiogenic therapy increased T-lymphocyte infiltration to eliminate cancer cells. (Continued on the following page.)
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J.A. Karam is a consultant/advisory board member for Pfizer. A. Bex reports receiving speakers bureau honoraria from GlaxoSmithKline and Pfizer and is a consultant/advisory board member for Pfizer. P. Sharma is founder and advisor and has ownership interest (including patents) in Jouonne Therapeutics, and is a consultant/advisory board member for Amgen, Bristol-Myers Squibb, and GlaxoSmithKline. N.M. Tannir reports receiving commercial research grant from GlaxoSmithKline and Pfizer and is a consultant/advisory board member for Exelixis, GlaxoSmithKline, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: X.-D. Liu, E. Jonasch
Development of methodology: X.-D. Liu, L. Zhou, M. Sun, P. German, J. Gao, E. Jonasch
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X.-D. Liu, S. Kalra, X. Zhang, P. Tamboli, P. Rao, J.A. Karam, C. Wood, S. Matin, A. Bex, N. Tannir, K. Sircar, E. Jonasch
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X.-D. Liu, L. Zhou, S. Kalra, A. Yetil, M. Sun, X. Zhang, P. German, C. Wood, A.W. Griffioen, N. Tannir, E. Jonasch
Writing, review, and/or revision of the manuscript: X.-D. Liu, L. Zhou, S. Kalra, Z. Ding, X. Zhang, P. Tamboli, J.A. Karam, C. Wood, S. Matin, A. Zuntia, A. Bex, A.W. Griffioen, J. Gao, P. Sharma, N. Tannir, E. Jonasch
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X.-D. Liu, A. Bex, L. Zhou, A. Yetil, M. Sun, Z. Ding, S. Bai, A. Bex, A.W. Griffioen
Study supervision: X.-D. Liu, C. Wood, E. Jonasch

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(Continued) Antiangiogenic therapy might induce the expression of adhesion molecules and chemokines or the activation of autophagy, which subsequently attract and promote T-lymphocyte infiltration. On the other hand, antiangiogenic therapy promotes tumorigenesis by generating an immunosuppressive tumor microenvironment associated with PD-L1 upregulation. Antiangiogenic therapy can upregulate PD-L1 directly at the posttranscriptional level or be indirectly mediated by CD8+ T-lymphocyte infiltration and IFNγ secretion. The increased expression of PD-L1 acts as a negative feedback mechanism to inactivate tumor-infiltrating T lymphocytes or subvert T lymphocytes to play a tumor-promoting role, which leads to immune escape. Combination with anti–PD-L1 therapy will reactivate tumor-infiltrating T cells to exert anticancer cytotoxicity.
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