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Resistance to anti-angiogenic therapy is associated with an immunosuppressive tumor microenvironment in metastatic renal cell carcinoma

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Abstract

Renal cell carcinoma (RCC) is an immunogenic and proangiogenic cancer, and anti-angiogenic therapy is the current mainstay of treatment. RCC patients develop innate or adaptive resistance to anti-angiogenic therapy. There is a need to identify biomarkers that predict therapeutic resistance and guide combination therapy. We assessed the interaction between anti-angiogenic therapy and tumor immune microenvironment, and determined their impact on clinical outcome. We found that anti-angiogenic therapy-treated RCC primary tumors demonstrated increased infiltration of CD4⁺ and CD8⁺ T lymphocytes, which was inversely related to patient overall survival (OS) and progression-free survival (PFS). Furthermore, specimens from patients treated with anti-angiogenic therapy showed higher infiltration of CD4⁺FOXP3⁺ regulatory T cells (Treg) 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 anti-angiogenic treatment may both positively and negatively regulate the tumor immune microenvironment. These findings generate hypotheses on resistance mechanisms to anti-angiogenic therapy, and will guide the development of combination therapy with PD-1/PD-L1 blocking agents.

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Keywords

Renal cell carcinoma; sunitinib; bevacizumab; T lymphocyte infiltration; regulatory T cell; PD-L1

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 pro-angiogenic 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 anti-angiogenic 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 pre-existing T cells, which are negatively regulated by PD-1/PD-L1-mediated adaptive immune resistance (12–14). Because the interaction between anti-angiogenic 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 anti-angiogenic response or resistance, but also a selection marker for patients that would benefit from receiving anti-PD-L1 therapy.

In this study, we found that anti-angiogenic therapy-treated primary tumors from patients with metastatic RCC showed increased CD4⁺ and CD8⁺ T-lymphocyte infiltration compared to that in uninvolved tissue and untreated controls, which 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. 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

anti-angiogenic 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; MS1528S0) and CD8 antibody (clone C8/144B; MS457S0) were from Fisher Scientific. CD68 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. IFN γ (285-IF-100) was from R&D systems.

Cell Culture, Establishment of Stable cell lines, Cell Lysis

RCC4, A-498, 786-O, CaKi-1, TK-10 and SN12C cell lines were obtained from ATCC. All cell lines were validated by STR DNA fingerprinting using the Promega 16 High Sensitivity STR Kit (Catalog # DC2100). The STR profiles were compared to online search databases (DSMZ/ATCC/JCRB/RIKEN) of 2455 known profiles; along with the MD Anderson Characterized Cell Line Core (CCLC) database of 2556 known profiles. The STR profiles matched known DNA fingerprints or were unique. All cell lines were grown in DMEM containing 10% fetal bovine serum. 786-O stable cell lines expressing control shRNA or *PD-L1* shRNA (Thermo Scientific, V2LHS_53668) were infected with lentiviral particles and selected in medium containing 2 μ g/ml puromycin. Cells were lysed in RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS) for immunoblot analysis.

Xenograft Tumor Models

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 are palpable (i.e., tumor volume reached 100 mm³), mice were treated with sunitinib (50mg/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 RNeasy Mini Kit (Qiagen), and converted to cDNA using cDNA Reverse Transcription kit (Applied Biosystems). *HIF2 α* , *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'-TGGCATTGCTGAACGCATTT-3' and 5'-TGCAGCCAGGTCTAATTGTTTT-3';

GAPDH, 5'-CAATGACCCCTTCATTGACC-3' and 5'-TTGATTTTGGAGGGATCTCG-3'.

Tissue Microarray, Image Acquisition and Analysis

Human subject protocol (2007-0511) was approved by the Institutional Review Board at UT MD Anderson Cancer Center. Tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. Tissue microarrays (TMA) with triplicate cores for each case were generated using a CHEMICON Advanced Tissue Arrayer (ATA 100). An H and E slide of each donor tissue block was assessed to find the desired tissue region, and the donor tissue block was marked to precisely locate the same area. The donor block was punched with a 0.6mm hollow needle and the tissue core was deposited into a prepunched recipient array block, and the identity of the transcribed core was indexed and recorded. TMA slides contain 33 unaffected kidneys, 41 untreated primary RCC, and 42 bevacizumab-pretreated and 39 sunitinib-pretreated primary RCC from patients with metastatic RCC. TMAs were immunohistochemically (IHC) stained as previously described (16). Slides were scanned with the Vectra image scanning system (Caliper Life Sciences, Waltham, MA) (17). The percentage of CD3⁺, CD45RO⁺, CD4⁺, CD8⁺, CD4⁺FOXP3⁺ and PD-L1⁺ cells in whole tissue sections were analyzed using inForm software (Caliper Life Sciences) (17). Unpaired Student's t-test was used for statistical analysis.

Results

RCC is associated with immune-cell infiltration

RCC is considered as 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 RCC to 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 immunohistochemically 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 were analyzed using inForm software. Total cell number count was based on hematoxylin-positive cells. Fig.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 immunohistochemical staining of CD3, a T lymphocyte marker protein. RCC specimens harbored significantly more CD3⁺ T lymphocytes than the uninvolved renal tissue: 16.6% vs 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% vs 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;1E). Similarly, RCC tissues were

infiltrated with more macrophages, as indicated by the expression of CD68: 20.3% vs 4.7% ($P<0.001$) (Fig.1F). These results confirm the immunogenic phenotype of RCC.

Anti-angiogenic therapy-treated tumors demonstrate increased T-lymphocyte infiltrates which are associated with poor survival

Both sunitinib and bevacizumab are used as treatment for metastatic RCC (mRCC). The former is a multikinase inhibitor with activity against vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR), and the latter is a humanized monoclonal antibody that inhibits vascular endothelial growth factor A (VEGFA) (18). To study the interaction between anti-angiogenic therapy and immune-cell infiltration, we compared the percentage of immune cells in RCC tissues exposed to anti-angiogenic therapy with control tumors in patients with mRCC. In one study (NCT00113217), patients with previously untreated mRCC were treated for eight weeks with bevacizumab and underwent cytoreductive nephrectomy. In the second study (NCT00715442) patients with previously untreated mRCC were treated with sunitinib for four weeks, given a two-week break, and then underwent cytoreductive nephrectomy during their second treatment cycle, with a one 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 tissue microarrays with triplicate formalin-fixed tissue cores for each case from 42 bevacizumab-pretreated and 39 sunitinib-pretreated primary RCC and 41 untreated primary RCC from each of these studies. We found that both sunitinib- and bevacizumab-treated cases showed increased CD3⁺ T-lymphocyte infiltration relative to untreated primary RCC controls: 19.3% and 16.6% vs 11.3% ($P<0.01$), increased CD45RO⁺ T-lymphocyte infiltration: 27.2% and 24.3% vs 16.8% ($P<0.01$), increased CD4⁺ T-lymphocyte infiltration: 15.3% and 16.3% vs 7.6% ($P<0.01$), and increased CD8⁺ T-lymphocyte infiltration: 7.0% and 5.1% vs 2.3% ($P<0.05$), respectively (Fig.2A–D). The infiltration of CD68⁺ macrophages was higher in sunitinib-treated versus control 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 anti-angiogenic 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% vs 8.0% ($P<0.01$) and 20.3% vs 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 anti-angiogenic therapy. However, in patients without treatment, 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 infiltrates 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 there was a preexisting immunosuppressive tumor microenvironment that inhibited or subverted the antitumor function of T cells.

Anti-angiogenic therapy-treated tumors demonstrate increased Treg infiltration

TILs are generally thought to aid in eliminating tumors, while the data above show that the increased T-lymphocyte infiltration following anti-angiogenic 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 anti-angiogenic therapy and their relationship with patient survival, we double stained TMAs 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% vs 0.4% ($P = 0.01$), respectively (Fig. 4A; 4B). 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 anti-angiogenic 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 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.

Anti-angiogenic therapy upregulates PD-L1 expression

PD-1 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 anti-angiogenic 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 test stained human placental tissue, since PD-L1 is known to express on trophoblasts in the placenta to maintain fetomaternal tolerance (23). Fig. 5A shows specific PD-L1 staining on trophoblasts. We also included a non-primary 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% vs 1.3% ($P<0.01$), respectively (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 γ 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 anti-angiogenic therapy may indirectly upregulate PD-L1 via CD8⁺ T lymphocyte-mediated IFN γ secretion.

We also considered the possibility that anti-angiogenic therapy may have an effect on PD-L1 expression independent of CD8⁺ T-lymphocyte infiltration. Since functional T lymphocytes are absent in the athymic nude mice, we tested this hypothesis using 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 IFN γ 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 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 786-0 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 1 α (HIF1 α) (26). However, 786-0 cells do not express HIF1 α and von Hippel-Lindau (VHL), the E3 ligase for HIF1 α and HIF2 α (17). Furthermore, sunitinib did not affect the protein level of HIF2 α (Fig. 6C). These results collectively indicate that sunitinib increased PD-L1 expression independent of HIF1 α or HIF2 α . 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, while it is almost undetectable in CaKi-1, TK-10 and SN12C cells. Sunitinib treatment increased PD-L1 protein level in several RCC cell lines (Fig. 6E). These results indicate that anti-angiogenic 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 anti-angiogenic 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, anti-angiogenic 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 to 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 anti-angiogenic 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) (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 anti-angiogenic therapy (Fig. 6). Although the directionality of sunitinib and bevacizumab effect on immune-cell infiltration was the same, the effects of sunitinib are more obvious than that 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 VEGF-A, bevacizumab disrupts the ability of VEGF-A 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). Nuclear factor kappa-B (NFκB) is a key transcription factor involved in the expression of molecules that mediated endothelial-leukocyte interaction, including ICAM1, VCAM1 and chemokines (37). NFκB activity is negatively regulated by both VEGFR2 (37) and VEGFR3 (38). It is conceivable that sunitinib more effectively inhibits NFκB 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 progression-free survival mainly through its anti-angiogenic 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 demonstrate significant heterogeneity in response to anti-angiogenic 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 anti-angiogenic therapy, CD8⁺ T-lymphocyte infiltration was associated with shorter survival. We assume that there is a pre-existing 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 anti-angiogenic therapy treatment. 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 anti-angiogenic 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 T-regulatory cell population due to the difficulties in achieving satisfactory triple antibody colorimetric staining with IHC. It is possible that further subclassification of our CD4 subpopulations will reveal additional subgroups of T cells that confer prognostic significance. As a further example, in

human ovarian cancer, CD25⁺ identifies a subset of CD4⁺FOXP3⁻ TILs that expressed the highest level of PD-1 and failed to produce common T-helper cytokines (44).

Recently, high-dose ionizing irradiation (IR) was reported to increase the expression of PD-L1 on tumor cells, and administration of anti-PD-L1 enhanced the efficacy of IR through a cytotoxic T lymphocyte-dependent mechanism (45, 46). Our experiments reveal that anti-angiogenic therapy upregulates PD-L1 in tumor tissues and in RCC cell lines. These findings collectively indicate that, as an immune checkpoint ligand, PD-L1 upregulation not only contributes to tumor escape from immune surveillance during tumor development, but also plays an important role in cancer therapy resistance. PD-L1 expression can be either an adaptive immune response driven by tumor-infiltrating CD8⁺ T lymphocytes, or an innate immune response driven by oncogenic pathways (6). IFN γ secreted by tumor-infiltrating CD8⁺ T lymphocytes has been shown to increase PD-L1 transcription. Here we also observed a correlation between CD8⁺ T-lymphocyte infiltration and PD-L1 expression in human tissues, indicating a possible negative feedback loop between T-lymphocyte infiltration and expression of PD-L1. We observed that sunitinib treatment also increased PD-L1 expression in T lymphocyte-deficient nude mice xenografts. However, since nude mice may develop NK cells that are capable of producing IFN γ , it is possible that NK cell-secreted IFN γ may be involved in sunitinib-induced PD-L1 expression. To determine whether this effect was immune cell-independent, our results show that sunitinib increased PD-L1 expression in several cell lines at a post-transcriptional level, suggesting the existence of a direct, tumor-cell autonomous mechanism of PD-L1 upregulation. The precise molecular mechanism of cell-specific PD-L1 is currently under investigation. The relative contribution of cell autonomous versus T cell-mediated PD-L1 regulation can be studied in animal models using IFN γ knockout mice or tumor cells with knockdown of the IFN γ receptor pathway.

Recently it was found that across multiple cancer types, tumors expressing PD-L1 and infiltrated with immune cells had particularly high response rates to anti-PD-L1 therapy (12–14). Our study shows that anti-angiogenic therapy-treated tumors are associated with an increased infiltration of T lymphocytes, although their function is probably inhibited by the simultaneous or sequential induction of PD-L1, and infiltration of Tregs. These results indicate that PD-L1 blockade might improve the antitumor activity of T lymphocytes and the efficacy of anti-angiogenic therapy in a subset of patients. It will be necessary to test this hypothesis using synergistic treatment with PD-L1 antibody and angiogenesis inhibitors in RCC animal models, and ultimately, in patients.

Not having paired intra-patient samples pre- and post-therapy, we used untreated primary tumors from patients with metastatic disease as a control group. Thus, we cannot rule out the possibility, albeit unlikely, that higher level of infiltrating T cells and PD-L1 expression in sunitinib- or bevacizumab-treated patients are related to the underlying patient characteristics, and are not induced by therapy. Even if this is the case, we may still be able to identify a group of individuals who will uniquely benefit from combination checkpoint and anti-angiogenic therapy.

In summary, our study reveals that there is a reciprocal interaction between the immune system and angiogenesis, and the immunosuppressive tumor microenvironment engendered by Treg infiltration and PD-L1 expression might inhibit the function of increased tumor-infiltrating T lymphocytes. These results support the concept of combining PD-1/PD-L1 blockade with anti-angiogenic therapy in patients whose tumors upregulate PD-L1 and immune infiltrates in an anti-angiogenic therapy-specific fashion.

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Figure 1.

RCC is associated with immune-cell infiltration. (A) Quantification of immune-cell infiltration. CD8⁺ cells were labeled in blue, and CD8⁻ cells were labeled in red. Representative original images, corresponding quantification images and the percentage of CD8⁺ cells were shown. (B) CD3⁺ T-cell infiltration. (C) CD45RO⁺ T-cell infiltration. (D) CD4⁺ T-cell infiltration. (E) CD8⁺ T-cell infiltration. (F) CD68⁺ cell infiltration. Tissue microarrays (TMA) from normal kidney or RCC were immunohistochemically stained with anti-CD3 antibody, anti-CD45RO antibody, anti-CD4 antibody, anti-CD8 antibody or anti-CD68 antibody. 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 unpaired Student's t-test.

Figure 2.

Anti-angiogenic therapy increases immune-cell infiltration. TMAs from untreated RCC controls or RCCs treated with sunitinib or bevacizumab were immunohistochemically stained with anti-CD3 antibody (A), anti-CD45RO antibody (B), anti-CD4 antibody (C), anti-CD8 antibody 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 unpaired Student's t-test.

Figure 3.

CD4⁺ and CD8⁺ T-lymphocyte infiltration inversely correlate with patient survival. Based on the distribution of tumor-infiltrating T lymphocytes and patient overall survival (OS) or progression-free survival (FPS), we divided each cohort of patients into 2 subgroups. The percentages of CD4⁺ (A), CD8⁺ (B) or CD68⁺ (C) cells in each subgroup were shown. Statistical analysis was performed with unpaired Student's t-test.

Figure 4.

Anti-angiogenic therapy increases regulatory T-cell infiltration. TMAs from untreated RCC controls or RCCs treated with sunitinib or bevacizumab were immunohistochemically stained with anti-CD4 antibody and anti-FOXP3 antibody at the same time. FOXP3 immunoreactivity was visualized with DAB (brown), CD4 immunoreactivity was visualized with warp red chromogen (red), and nuclear counterstain was shown by hematoxylin (blue). (A) Representative original images and corresponding quantification images. CD4⁺ cells were labeled in red, FOXP3⁺ cells were labeled in green, CD4⁺FOXP3⁺ Tregs were labeled

in yellow. Negative cells were labeled in blue. (B) Percentages of CD4⁺FOXP3⁺ Tregs in each group were 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 FPS, we divided each group of patients into 2 subgroups. The percentages of CD4⁺FOXP3⁺ Tregs in each subgroup were shown. Statistical analysis was performed with unpaired Student's t-test. (F) Correlation between CD8⁺ T-lymphocyte infiltration and Tregs infiltration.

Figure 5.

Anti-angiogenic therapy increases PD-L1 expression in human RCC. Human placenta tissues and TMAs from untreated RCC controls or RCCs treated with sunitinib or bevacizumab were immunohistochemically stained with anti-PD-L1 antibody. Immunoreactivity was visualized with DAB (brown), and nuclear counterstain was shown by hematoxylin (blue). (A) Human placenta tissues were immunohistochemically stained with anti-PD-L1 antibody (left), and non-primary antibody control (right) was included. (B) Representative images and the percentage of PD-L1 positive cells in whole RCC tissue were shown. (C) Correlation between PD-L1 expression and patient survival. Based on the distribution of tumor-infiltrating T lymphocytes and patient OS or FPS, we divided each group of patients into 2 subgroups. The percentages of PD-L1 positive cells in each subgroup were shown. Statistical analysis was performed with unpaired Student's t-test. (D) Correlation between CD8⁺ T lymphocyte infiltration and PD-L1 expression.

Figure 6.

(A) PD-L1 antibody validation. 786-O cells stably expressing *PD-L1* shRNA were treated IFN γ (10ng/ml) for 1 or 3 hrs. 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 cells-induced xenograft. After the tumors are palpable (i.e., tumor volume reached 100 mm³), mice were treated with sunitinib (50mg/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 unpaired

Student's 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 hrs at the concentration of 0.5, 1, 2, 5 or 10 μ M. Cell lysates were analyzed by immunoblot using an anti-PD-L1 antibody, anti-HIF2 α antibody or anti- β -actin antibody. PD-L1 band intensity with or without sunitinib (5 μ M) treatment was analyzed using ImageJ software. The average level of PD-L1 in control cells was normalized to 1. Statistical analysis was performed with unpaired Student's t-test, n=4. Total RNA were analyzed by real-time PCR using primers specific for *PD-L1* (*CD274*). mRNA level in control cells was normalized to 1. *GAPDH* were used as endogenous control. (D) Bevacizumab treatment increases PD-L1. 786-O cells were treated with bevacizumab (10 μ g/ml or 25 μ g/ml) for 16 hr. 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 μ M) for 16 hr. Cell lysates were analyzed by immunoblot using anti-PD-L1 antibody or anti-GAPDH antibody. (F) Working model. Anti-angiogenic therapy generates an immunosuppressive tumor microenvironment. On the one hand, anti-angiogenic therapy increased T-lymphocyte infiltration to eliminate cancer cells. Anti-angiogenic 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, anti-angiogenic therapy promotes tumorigenesis by generating an immunosuppressive tumor microenvironment associated with PD-L1 upregulation. Anti-angiogenic therapy can upregulate PD-L1 directly at post-transcriptional level or indirectly mediated by CD8⁺ T-lymphocyte infiltration and IFN γ secretion. The increased expression of PD-L1 act 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 re-activate tumor-infiltrating T cells to exert anticancer cytotoxicity.