# Tumor Microenvironment Dynamics in Clear-Cell Renal Cell Carcinoma

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**ABSTRACT**

Renal cell carcinoma stands out as one of the most immune-infiltrated tumors in pan-cancer comparisons. Features of the tumor microenvironment heavily affect disease biology and may affect responses to systemic therapy. With evolving frontline options in the metastatic setting, several immune checkpoint blockade regimens have emerged as efficacious, and there is growing interest in characterizing features of tumor biology that can reproducibly prognosticate patients and/or predict the likelihood of their deriving therapeutic benefit. Herein, we review pertinent characteristics of the tumor microenvironment with dedicated attention to candidate prognostic and predictive signatures as well as possible targets for future drug development.

**Significance:** Tumor microenvironment features broadly characterizing angiogenesis and inflammatory signatures have shown striking differences in response to immune checkpoint blockade and antiangiogenic agents. Integration of stromal and immune biomarkers may hence produce predictive and prognostic signatures to guide management with existing regimens as well as future drug development.

## INTRODUCTION

Renal cell carcinoma (RCC) is the most common form of kidney cancer, claiming more than 14,000 lives per year in the United States (1). Clinically, one third of patients will present with metastatic RCC (mRCC; ref. 2). Coupled with relapsing metastases in a quarter of patients with "local" disease following curative nephrectomy, mRCC constitutes a substantial medical burden (2). Although all RCCs arise from the nephrons and receive similar clinical regimens, histologic subtypes are highly heterogeneous in their biology and therapeutic outcomes (3). The most common (70%–80%) subtype, clear-cell RCC (ccRCC), is also one of the most aggressive (3).

Generally insensitive to cytotoxic chemotherapy and uniquely dependent on tumor angiogenesis, ccRCC emerged early as the prototype solid-tumor malignancy for application of molecularly targeted agents, particularly tyrosine kinase inhibitors (TKI) targeting the VEGFR pathway (2). Additionally, ccRCC has long been categorized as "immunotherapy responsive," with cytokine-based regimens having been a standard before the advent of antiangiogenic therapy (2). More recently, immune checkpoint inhibitors (ICI) which block PD-1/PD-L1 or CTLA4 T cell–inhibitory receptors have proved highly efficacious in this disease and are now considered standards in treatment-naïve and pretreated patients (4, 5).

Recent data from large registration studies confirm that pairing VEGF-directed therapies with ICIs adds value over TKI monotherapy (6, 7), and this combination is now FDA-approved. This wealth of treatment choices will provide treating oncologists with multiple FDA-approved options in the first- and second-line setting. With no standard predictive biomarker available to help guide treatment selection, clinicians often rely on validated prognostic risk models—the International Metastatic RCC Database Consortium (IMDC) or Memorial Sloan Kettering Cancer Center (MSKCC) criteria (8, 9). These tools integrate clinical and laboratory variables and risk-stratify patients with mRCC into favorable-, intermediate-, or poor-risk disease. Poor prognostic factors include poor performance status, high serum calcium, low hemoglobin concentration, less than 1 year interval from diagnosis to treatment, high serum lactate dehydrogenase (MSKCC only), high neutrophil count (IMDC only), and high platelet count (IMDC only), with 0, >2, or >3 of these factors defining good-, intermediate-, and poor-risk groups, respectively (8, 9). As expected, recent data confirm that these categories serve as surrogates for underlying disease biology (10).

Originally developed for prognostication and stratification on clinical trials, these criteria have recently emerged as tools to help with treatment selection. Specifically,

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combination ICI therapy with ipilimumab plus nivolumab has shown superiority over TKI monotherapy with sunitinib in patients with intermediate- and poor-risk disease, while being similar in efficacy with long-term follow-up in the favorable-risk setting (11). With the approval of combination TKI plus ICI strategies for ccRCC, IMDC risk stratification is no longer sufficient for treatment selection, as benefit over TKI monotherapy was apparent across all three risk groups (6, 7). Rather, the field awaits biomarker data to recapitulate how these agents synergize to manipulate the stromal and immune tumor microenvironment (TME) to elicit an antitumor response. With that in mind, and through a series of larger-scale analyses from retrospective cohorts and clinical trials, it has become apparent that expression-based analyses are more likely to prove useful in identifying predictive signatures than tumor genomics.

In this review, we first describe the relatively unique and heterogeneous TME of ccRCC. To understand the potential mechanisms of synergy between VEGF TKIs and ICIs, we summarize how these therapies alter the immune TME and highlight specific features important for systemic therapy decision-making.

## THE HETEROGENEOUS TUMOR MICROENVIRONMENT OF ccRCC

The truncal event in 90% of ccRCCs is biallelic loss of the Von Hippel-Lindau (VHL) tumor suppressor, which functions as a negative regulator of the HIF1α/2α transcription factors (12). HIF accumulation drives the cellular hypoxic response, leading to increased angiogenesis, glycolysis, and aberrant fatty-acid metabolism, thus bestowing ccRCC with its characteristic glycogen- and lipid-rich cytoplasmic deposits and hypervascularity (13).

It is well appreciated that angiogenesis and immunity are closely interlinked, and targeting VEGF can promote immune surveillance by normalizing dysfunctional tumor vessels (14). In line with this, validated RNA sequencing (RNA-seq)–derived gene signatures showed that ccRCC has the highest angiogenesis score and is also highly immune-infiltrated when compared with 18 epithelial cancer types (15, 16). More specifically, ccRCC has the highest overall T-cell, $CD8^+$, T helper 1 (Th1), dendritic cell (DC), neutrophil, and cytotoxic cell and relatively low Th2 and regulatory T-cell (Treg) scores, suggesting an overall proinflammatory profile (15). However, such pooled analyses can be misleading, as accumulating data continue to highlight the heterogeneity within ccRCC, with distinct subgroups differing in expression of angiogenesis programs, immune infiltration patterns, and clinical outcomes (Fig. 1; refs. 10, 15, 17, 18).

Early efforts at molecular characterization of RCC by development of a 34-gene expression signature derived from microarray data allowed separation of patients into two broad prognostic groups, ccA and ccB (17, 19). These were characterized by high angiogenesis and improved prognosis or high lymphocytes and macrophages and worse survival, respectively (20). However, when performing simple unsupervised clustering of microarray data in 53 ccRCCs, four molecular groups could be resolved including an immune-infiltrated group and three noninfiltrated groups. The noninfiltrated groups seemed to outline a spectrum of disease progression from normal tissue-like to upregulation of hypoxia and glycolysis followed by MYC upregulation (21).

Larger analyses using single-sample gene set enrichment analysis of 415 primary tumor samples from patients with metastatic ccRCC within The Cancer Genome Atlas (TCGA) database allowed further subcategorization of these immune-infiltrated tumors (15). Again, a non–immune-infiltrated subgroup resembling normal kidney and a poorly immune-infiltrated group with high angiogenesis resembling ccA tumors were identified. Interestingly, within the T cell–enriched cluster, two distinct subclusters emerged, termed TCa and TCb. Although patients with TCa had similar survival to noninfiltrated and poorly infiltrated groups, TCb had the overall poorest survival and was significantly enriched with macrophages, stromal cells, and immunosuppressive Treg and Th2 cell signatures (15).

Similar molecular subgroups were validated at the protein level by Giraldo and colleagues when combining gene-expression data with detailed flow cytometry phenotyping of tumor-infiltrating lymphocytes (TIL). Analysis of tumors from 40 localized ccRCC tumors identified three distinct groups: immune silent, immune activated, and immune regulated (22). Whereas TILs in the immune-silent group resembled nonmalignant kidney resident TILs, the immune-activated and immune-regulated groups were infiltrated by activated $CD8^+$ T cells. However, T cells in the immune-regulated group were less clonal and poorly cytotoxic, with more Tregs and dysfunctional DCs, compared with immune-activated tumors. The immune-regulated group, analogous with TCb tumors, was enriched with high-grade tumors (74% grades 3–4) and had the highest risk of relapse. More recent studies also support that inflamed tumors have worse survival compared with noninflamed but highly angiogenic tumors, when patients were categorized using a novel deconvolution method utilizing RCC-specific immune and stromal genes (23). It would be useful to validate and refine the existence of immune-activated and immune-regulated clusters using these RCC-specific signatures.

In summary, these data suggest that highly immune-infiltrated tumors are more aggressive than angiogenic tumors, consistent with observations across IMDC risk groups (10). However, T-cell activation state within infiltrated tumors is a stronger prognostic indicator. Unlike most other cancer types, overall high $CD8^+$ T-cell infiltration in ccRCC is associated with worse prognosis, suggesting that the infiltrating $CD8^+$ T-cell pool may be dominated by suppressed and dysfunctional cells. Indeed, defective T-cell function has been reported in many ccRCC studies (24–31). Thus, simple measurement of single populations such as $CD8^+$ T cells is unlikely to provide sufficient prognostic or predictive information.

## DETERMINANTS OF EFFECTIVE T-CELL RESPONSES IN ccRCC

### Tumor Immunogenicity

It is generally thought that tumor-specific mutations give rise to immunogenic neoantigens that can drive immune infiltration, a prerequisite for ICI efficacy. Accordingly, tumor

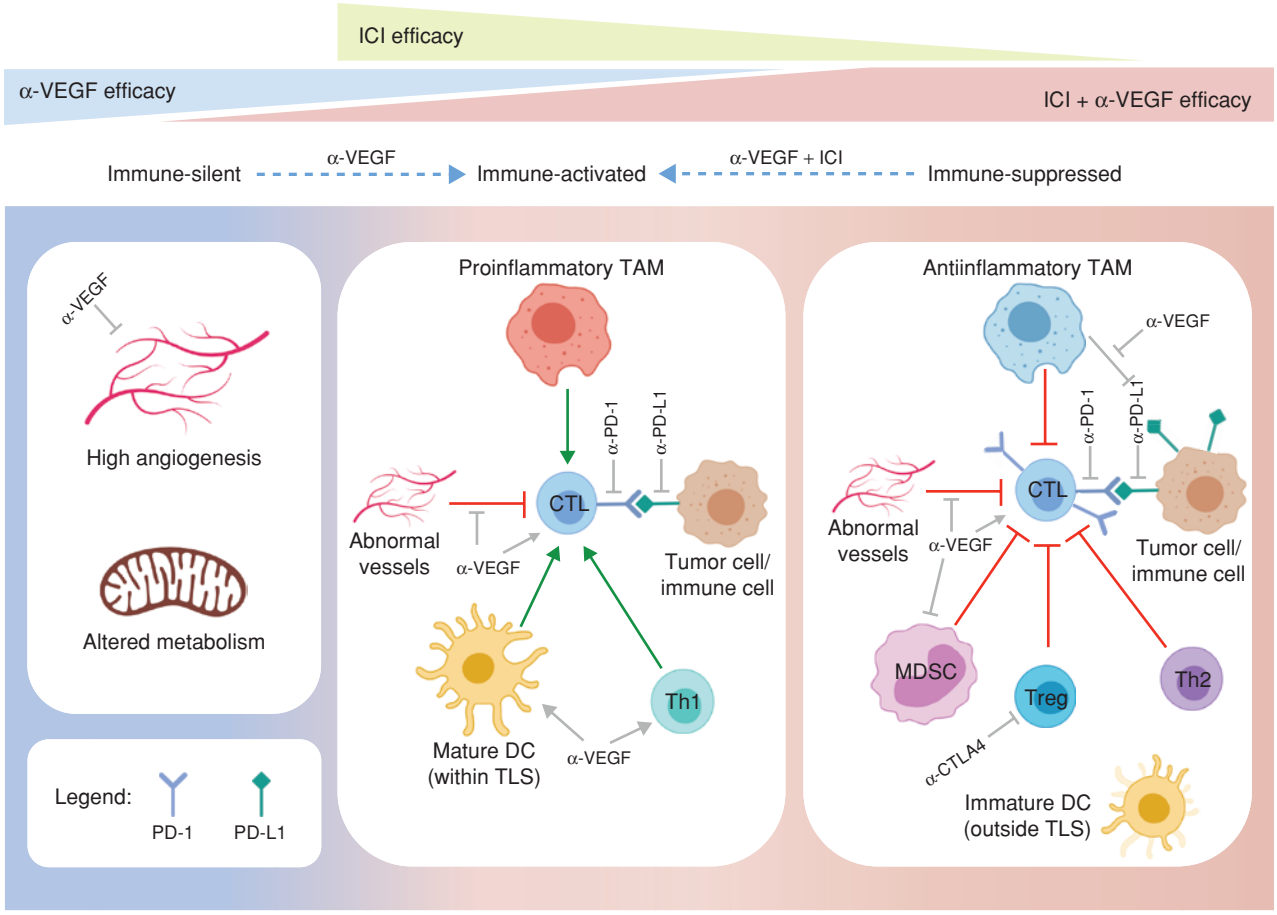


**Figure 1.** Emerging model of targeted therapy efficacy within distinct ccRCC subgroups. Transcriptomically distinct prognostic and predictive subgroups of ccRCC can be broadly defined as: (i) immune-silent, characterized by low immune infiltration, high angiogenesis, and altered metabolism; (ii) immune-activated, characterized by proinflammatory immune infiltrates; and (iii) immune-suppressed, characterized by an overall anti-inflammatory cell milieu. Anti-VEGF (α-VEGF), ICI, and combination therapy of α-VEGF and ICI are most effective in highly angiogenic immune-silent tumors, immune-activated tumors, and immune-suppressed tumors, respectively (represented by triangles at top). Anti-VEGF treatment can promote immune infiltration and activation, whereas combination treatment with α-VEGF and ICI may reinvigorate the active antitumor immune response (dashed blue arrows). White boxes below each ccRCC subgroup depict their respective TMEs and potential cellular targets of α-VEGF or ICI therapies. Generally accepted targets for ICIs include inhibiting CTL downregulatory signals derived from Tregs, tumor cells, or other immune cells. Beyond inhibiting angiogenesis, anti-VEGF therapies can also promote CTL recruitment and activation by unclear mechanisms which may include normalizing abnormal tumor vessels, inhibiting MDSCs, promoting Th1 and DC activation, and potentially by inhibiting anti-PD-L1-efficacy-limiting myeloid cells. Th1, T helper 1; Th2, T helper 2; α-PD-1, anti-programmed cell death-1; α-PD-L1, anti-programmed cell death-ligand 1. Created with Biorender.com.

mutation burden (TMB) and genomic instability serve as robust predictors of ICI response in many malignancies (32). ccRCC is a historically immunogenic cancer, but the precise nature of the T cell–activating neoantigens still eludes us. Interestingly, among other highly infiltrated cancer types such as lung adenocarcinoma and melanoma, ccRCC stands out as having only a modest mutation burden (15). TMB is similar between MSKCC prognostic risk groups (33), and immune infiltration is independent of predicted neoantigen affinity (15). Ultimately, TMB alone does not predict RCC response to ICI (18, 34–36).

Although nonsynonymous single-nucleotide variants (nsSNV) are the most established readout of TMB, frameshifts caused by small nucleotide insertions or deletions (indels) are predicted to generate more immunogenic neoantigens than nsSNVs. In pan-cancer comparisons, indels are strikingly abundant in ccRCC, perhaps explaining the disparity between low SNVs and high immune infiltration (34). However, in the IMmotion-150 phase II trial, indels and frameshift burdens were not significantly associated with response to atezolizumab (anti–PD-L1) alone or combined with bevacizumab (anti-VEGF; ref. 18).

Reactivated expression of the germline-encoded gene *CSAG2* (a cancer testis antigen) and expression of 7 different endogenous retroviruses (ERV) in ccRCC are positively associated with cytolytic activity score [a *GZMB*- and *PRF1*-based gene-expression metric strongly associated with cytotoxic T lymphocyte abundance (CTL); ref. 37]. Whether *CSAG2*- and ERV-derived neoantigens can elicit T-cell responses and influence ICI efficacy is unknown, but these alterations occur relatively infrequently. Ongoing efforts to identify immunogenic RCC antigens are critical for future development of novel vaccination or adoptive cell transfer approaches.

Tumor immunogenicity is also dependent on successful presentation of tumor neoantigens by antigen-presenting cells. High levels of somatic copy-number alterations (SCNA) are independently associated with immune evasion across cancer types, including ccRCC, and predict worst response to ICI in melanoma (35). Interestingly, reduction of cytotoxic CD8$^+$ and natural killer (NK) cells was predicted only by chromosomal and arm-level but not focal SCNA events, despite a significant overlap in genes. The authors speculate that the protein imbalance may impair key signals, such as neoantigen peptide presentation, which are required for immunogenicity.

Moreover, high SCNA ccRCC tumors were associated with significantly decreased ratios of pro- and anti-inflammatory cells and cytokines, and lower ratios of proinflammatory M1/anti-inflammatory M2 macrophages compared with low SCNA tumors, together suggesting that high tumor SCNA promotes both immune exclusion and an immunosuppressive microenvironment (35). Given these significant effects on the inflammatory milieu and the recurrent patterns of SCNA occurring in ccRCC (38), interrogation of the predictive power of SCNA in ICI therapy is warranted.

### T-cell Suppression

In highly infiltrated ccRCC tumors, T-cell activation state is a pivotal determinant of ccRCC prognosis and likely of immunotherapy response (discussed below). To determine which cell types may mediate T-cell suppression, coinfiltration analyses were performed on over 7,000 tumors from the TCGA database spanning 23 different cancer types (39). Consistent with the TCa and TCb groups observed in ccRCC (15), in 5 other cancer types (lung adenocarcinoma, glioma, pancreatic, ovarian, and bladder), tumors with high CD8$^+$ T-cell infiltrates could be subdivided into prognostic groups in which low macrophage levels dictated longer survival times (39). This is also true of ovarian and lung cancers with low CD8$^+$ infiltrates, highlighting the T cell–dependent and –independent tumor-promoting effects of macrophages, which are well reviewed elsewhere (40).

Recently, mass cytometry was used to comprehensively phenotype tumor resident T cells and tumor-associated macrophages (TAM) from 73 patients with ccRCC spanning stage I to IV and metastatic disease, including adjacent normal kidney (29). T cells were the most prevalent immune subset (50%) followed by TAMs (25%), NK cells (9%), B cells (4%), and other low-frequency cells including plasma cells, DCs, and neutrophils. The authors teased out 20 T-cell and 17 macrophage clusters, highlighting the sheer heterogeneity of cell subtypes and substates.

In agreement with other data, tumor-infiltrating T cells (abundant in tumors but not normal or early-stage disease) were predominantly CD8$^+$ cells expressing high levels of coinhibitory receptors including PD-1 and low levels of the proliferation marker Ki-67, indicative of a near-exhausted phenotype. Tregs also comprised a significant proportion, together depicting a largely exhausted and immunosuppressive T-cell landscape in advanced ccRCC tumors.

In contrast to T cells, human macrophage subsets are far less characterized and exhibited more heterogeneous marker expression across clusters. Interestingly, three prevalent mature TAM populations distinct from normal kidney resident macrophages were identified, termed M5, M11, and M13, and tended not to co-occur. Instead M5 TAMs, characterized by high CD38 expression, were positively associated with the number of Tregs or exhausted T cells. CD38 is upregulated in human monocytes and macrophages in response to classic proinflammatory stimulus (lipopolysaccharide) but not the alternative macrophage stimulus IL4 (41) and has been demonstrated to be critical for macrophage FcγR-mediated phagocytosis, albeit only in mice (42). This suggests that the CD38$^+$ M5 macrophage subset may be proinflammatory in nature, promoting initial T-cell activation and eventual exhaustion in ccRCC, but further functional characterization is required.

In contrast, the M11 and M13 TAMs were mutually exclusive with activated and exhausted T cells. In fact, M11 seems to be mutually exclusive with all T-cell clusters. It is tempting to speculate that these M11 TAMs may be involved in T-cell exclusion, a phenomenon recently reported in human lung tumors and mouse breast cancer models (43). Fitting with this hypothesis, low M5, high M11, and high M13 macrophages corresponded to poor survival in ccRCC, but validation is required in larger cohorts (29). Of note, three sizeable macrophage clusters expressed high levels of the commonly used protumor M2 macrophage marker CD206. These include M11 TAMs but also two potentially nonpathogenic tissue-resident macrophage populations. In sum, this crucial study highlights the complexity and need to cocharacterize TAM subsets and T-cell activation states in predictive and functional studies.

DCs can also modulate TIL function in ccRCC. Tertiary lymphoid structures (TLS) have been shown to exist in various human tumors including RCC and are sites of T-cell activation with immune organization analogous to lymph nodes (44). Localization of mature DCs within TLS was associated with better survival in patients with ccRCC with high levels of activated CD8$^+$ T cells (22, 28). In contrast, more DCs located outside of TLS were associated with coinfiltration of dysfunctional CD8$^+$ T cells and poor survival, together suggesting that local T-cell priming by DCs within TLS is a key component of effective tumor immune surveillance.

## RCC TME AND SYSTEMIC THERAPIES

### VEGF-Directed Effects on TME

Current FDA-approved antiangiogenesis agents have known immunomodulatory effects through both VEGF and their effects on other tyrosine kinase receptors. For instance, presurgical bevacizumab reduces total CD68$^+$ macrophages (45). Bevacizumab treatment has also been shown to increase gene signatures related to Th1 responses, and high CD8$^+$ T-cell infiltration has been shown with combination bevacizumab and atezolizumab (46). Presurgical pazopanib is associated with a reduction in CD8$^+$ T-cell infiltration, increased PD-L1 expression, and enhanced DC activation proposed through downregulation of the pERK/β-catenin pathway (47, 48). Sunitinib is associated with a reduction in Tregs and myeloid-derived suppressor cells (MDSC) with an improved Th1 response (49, 50). This effect has therapeutic implications, as reductions in Tregs during sunitinib treatment are associated with prolonged survival (51). Sunitinib has also been shown to enhance overall T-cell infiltration, including

Tregs, $CD8^+$ cells, and $CD4^+$ cells (52). This is accompanied by increased tumor cell PD-L1 expression and negative associations for survival, suggesting that sunitinib may enhance initial immune responses but cannot prevent subsequent immunosuppression. As each agent may demonstrate distinct immune compartmental changes and downstream impacts on survival (10), continued large-scale efforts which characterize these changes will be crucial in designing novel combination strategies.

Several studies suggest that RNA-based molecular signatures from tumor tissue can have predictive and prognostic implications for VEGFR TKI therapy, possibly more so than traditional IMDC risk stratification. For instance, Beuselinck and colleagues performed unsupervised cluster analysis to distinguish 4 subgroups of RCC tumors: ccrcc1–4 (21). Patients with ccrcc1/4 tumors had lower response rates and shorter progression-free survival (PFS) and overall survival (OS) when compared with ccrcc2/3 tumors to both sunitinib and pazopanib therapy (21, 53). Further analyses delineated broader differences between these tumor subgroups: ccrcc2 tumors were hallmarked by angiogenesis-high gene signatures, and ccrcc3 tumors had indolent disease courses. In contrast, ccrcc1 tumors had lower rates of T-cell infiltration and were typically "immune-cold," and ccrcc4 tumors had high T-cell infiltration with high expression of inhibitory markers like PD-1, cognate ligands PD-L1/PD-L2, LAG3, and TGFβ, and high expression of activated myeloid cells with high CSF1 expression. Subsequent analyses suggest that these signatures may also inform surgical decision-making for patients considered for metastasectomy (54).

Large-scale correlative work from the COMPARZ trial, a phase III study of patients with treatment-naïve metastatic RCC treated with sunitinib or pazopanib, has uncovered several TME factors associated with outcomes on VEGFR TKI therapy (10). In this analysis, four biologically distinct groups were identified by uninformed RNA-based consensus clustering with significant differences in median PFS and OS. Among the groups, cluster 3 had the best OS and was associated with the highest angiogenesis scores. Forty-one percent of patients with ccRCC harbor *PBRM1* loss-of-function mutations (55). *PBRM1* mutations were enriched in cluster 3 and were also associated with improved response to sunitinib in a separate cohort (18), fitting with its known roles in augmenting the HIF-mediated hypoxia response signature of VHL-deficient tumors (56).

Cluster 4 had the worst OS and was enriched for *BAP1* and *TP53* mutations. In addition, cluster 4 demonstrated high enrichment for immune and particularly macrophage gene signatures with associated high $PD\text{-}L1^+$ macrophage enrichment. High macrophage infiltration was associated with poor OS (HR, 1.54; $P = 0.0018$), and angiogenesis-low/macrophage-high signatures had the worst OS with TKI therapy (HR, 3.12; $P = 0.000003$). However, when considering the two TKIs separately, angiogenesis had only marginal impact on survival within the pazopanib cohort but remained significantly predictive for improved sunitinib response. These data remain in line with results from the phase II IMmotion-150 study in which sunitinib achieved improved PFS in angiogenesis-high tumors compared with angiogenesis-low tumors [HR, 0.31; 95% confidence interval (CI), 0.18–0.55; ref. 18]. In contrast, macrophages were significantly predictive for poor response to pazopanib but not sunitinib, emphasizing that different TKIs can modulate the TME in different ways. Supporting this, hepatocellular carcinoma models implanted into immunocompetent and immunodeficient mice show that the antitumor activity of the antiangiogenesis TKI lenvatinib but not sorafenib is dependent on adaptive immunity (57). In sum, these data support the characterization of stromal and cellular immune components of the TME in an effort to develop RNA-based biomarker signatures for VEGF-directed therapies (10).

## Immune Checkpoint Blockade and TME

The differences in therapy response and survival when comparing combination ICI therapy and VEGFR TKI therapies are rooted in underlying tumor biology. In the phase III registration study of ipilimumab and nivolumab versus sunitinib, patients with intermediate- to poor-risk disease had superior survival and response compared with favorable-risk patients, setting this regimen apart from others (4). In IMmotion-151, a phase III study of atezolizumab plus bevacizumab versus sunitinib, angiogenesis-high signatures were more prevalent in MSKCC favorable-risk patients compared with intermediate- and poor-risk patients ($P = 4.28e\text{-}06$; ref. 58). TME may also significantly influence response differences seen across sarcomatoid dedifferentiated tumors. Subgroup analyses of patients with sarcomatoid treated with combination ICI or VEGF plus ICI therapy have shown impressive response rates, including a complete response in 18.3% for ipilimumab plus nivolumab (59), 11.8% for pembrolizumab plus axitinib (60), and 10% for atezolizumab plus bevacizumab (61). As these tumors have higher PD-L1 expression and T-effector gene signatures, with lower angiogenesis signatures, compared with their nonsarcomatoid counterparts (61), this may provide rationale for the robust responses seen to combination ICI therapy.

Specific TME features that correlate with combination VEGF plus ICI therapy response have been gleaned from the above-mentioned IMmotion-150 study, a phase II 3-arm randomized study of atezolizumab and bevacizumab, and atezolizumab and sunitinib monotherapy. Consistent with data from the COMPARZ cohort (10), sunitinib had improved PFS and response rate in those tumors harboring angiogenesis-high versus angiogenesis-low signatures. This difference in both response and survival rates was not seen in the atezolizumab monotherapy arm, suggesting that angiogenesis is not predictive of anti–PD-L1 response. When directly comparing outcomes for atezolizumab and sunitinib monotherapy, no difference was seen in PFS overall nor when stratified by levels of angiogenesis or T-effector cells. Overall, the combination arm was only significantly superior to sunitinib in patients with >1% PD-L1–expressing immune cells, emphasizing the necessity for prior immune activation in tumors for ICI combination efficacy. Further, angiogenesis-low tumors had improved PFS with combination ICI plus VEGF therapy when compared with sunitinib monotherapy. Interestingly, when stratifying T-effector–high tumors by myeloid signature abundance, the combination arm was superior to atezolizumab monotherapy in myeloid-high but not myeloid-low groups, suggesting that myeloid immunosuppression hinders single-agent ICI therapy,

and that the immunomodulatory effects of concurrent VEGF inhibition can help overcome this (18). Validation of these molecular analyses was confirmed in the phase III IMmotion-151 study, where T-effector signatures were associated with superior PFS with combination atezolizumab plus bevacizumab when compared with sunitinib monotherapy (HR, 0.76; 95% CI, 0.59–0.99), and high T-effector gene signatures were associated with increased PD-L1 expression. Further, combination therapy is associated with superior PFS compared with sunitinib in angiogenesis-low tumors (HR, 0.68; 95% CI, 0.52–0.89; refs. 58, 62). Recently, PBRM1 was discovered to confer resistance to T cell–induced apoptosis, and deletion in a B16F10 melanoma mouse model increases susceptibility to anti–PD-1 and anti-CTLA4 (63). In small RCC cohorts, PBRM1 status was also suggested to be a positive predictor of anti–PD-1 efficacy (64), but this signal was not seen in the context of anti–PD-L1 therapy (18). Given these conflicting data, validation in larger cohorts is required, and *PBRM1* mutations should not be used as biomarkers at present.

### Novel Approaches to Manipulating the TME

With the identification of specific TME constituents and their recognized impact on treatment responses, combination strategies that target distinct cell populations may help overcome treatment resistance. As highlighted here, TAMs significantly contribute to VEGFR TKI and ICI therapy resistance, and targeting of macrophage-specific pathways may represent a novel treatment for patients with advanced RCC. Direct blockade of CSF1 and its cognate receptor CSF1R, responsible for macrophage recruitment and differentiation, and CD47/SIRPα, responsible for transmitting a protective "don't eat me" signal, may represent new therapeutic avenues for investigation for patients harboring macrophage-high tumors (65, 66). The institution of these new therapies may be facilitated by the integration of rapid biomarker assessment tools and incorporation of novel radiomic approaches. Ferumoxytol-enhanced MRI or hybrid MR/PET scans using alternative tracers can identify TAMs (67, 68), and combination imaging modalities have shown high concordance between imaging and RNA-seq data distinguishing angiogenesis-low or angiogenesis-high tumors (69). Application of these modalities may also yield similar classifying metrics; for example, hybrid PET/MRI has been shown to distinguish tumors into ccA and ccB classifications represented by either angiogenesis-high or TGFβ/epithelial-to-mesenchymal transition–high tumor differentiation (70). Integration of these tools will lead to future biomarker-driven studies which help select and stratify patients based on individual TME characteristics for response evaluation.

Agents that additionally target other immunosuppressive populations through alternative mechanisms serve as attractive strategies for paired or enhanced ICI response. Entinostat, a histone deacetylase inhibitor, has shown inhibitory effects on monocytes and neutrophils (71), and paired ICI therapies to target immunosuppressive MDSCs or TAMs using this approach are under way (NCT02526017; ref. 72). Early-phase studies of X4P-001, a CXCR4 inhibitor which downregulates HIF2α with preliminary efficacy in patients with advanced RCC, also may downregulate MDSC trafficking (73). Further, pegilodecakin, a pegylated IL10 which increases $CD8^+$ T cells and reduces TGFβ, has shown preliminary efficacy when combined with an anti–PD-1 agent, but interestingly has shown hemophagocytic lymphohistiocytosis (HLH; 7.9% any grade, $n = 38$) as a reported treatment side effect. As HLH only occurred in this small cohort of patients with RCC and preliminarily not in other solid tumors, whether this effect represents a heightened macrophage activation state due to the high infiltration of TAMs in RCC specifically is unclear. The prodrug NKTR-214, a CD-122 agonist designed to provide sustained signaling through the IL2 receptor pathway, has been shown to activate and expand NK and $CD8^+$ effector T cells over Tregs. In a phase I trial, serial TME analyses during treatment showed that NKTR-214 induces an increase in total and proliferating NK cells, $CD8^+$ cells, and $CD4^+$ cells, with correlations between peripheral immune cells and immune infiltration in the tumor (74). Preliminary results for the combination of NKTR-214 with ICI have shown clinical responses, and higher clinical benefit for those patients whose tumors were $PD\text{-}L1^+$ at baseline or those tumors which converted to $PD\text{-}L1^+$ during treatment (75).

Metabolic-based therapeutic strategies which alter the TME also remain under active investigation in early-phase studies. CB-839, a selective glutaminase inhibitor which blocks formation of glutamate metabolites and cell proliferation, may have additional effects as other constituents of the TME compete for nutrients (76). Combination strategies utilizing CB-839 with VEGFR TKI, mTOR inhibitors, and ICI agents are ongoing (NCT03428217, NCT03163667, NCT02771626). Metabolic changes with CB-839 and downstream oxidative stress may be captured radiographically, and new PET avid tracers of glutamine metabolism which capture glutamine and glutamate conversion may aid in monitoring treatment response with therapeutic use (77). In a small cohort of patients with advanced RCC, peripheral blood metabolic analysis highlights additional metabolic pathways including indoleamine 2,3-dioxygenase and adenosine and associations with ICI therapy response. Patients with no response to PD-1 monotherapy had significantly higher adenosine levels at baseline and while on-treatment, and high levels were associated with a significantly worse PFS ($P = 0.004$; ref. 78). The use of CPI-444, an A2A receptor antagonist, may overcome this dampening and promote immune responses when combined with ICIs (NCT02655822). On-treatment tumor biopsies also show high associations between $CD8^+$ T-cell infiltration and response with CPI-444 treatment when compared with baseline samples ($P < 0.016$), and implementation of an adenosine gene signature composed of chemokines and cytokines associated with myeloid-derived suppression was also associated with response ($P < 0.008$; ref. 79). Interestingly, this adenosine signature is similar to the myeloid-high signature previously used for characterization in the IMmotion-150 trial, supporting the role of adenosine as an additional mechanism for tumor escape (80).

## CONCLUSION AND FUTURE DIRECTIONS

Molecular classification of RCC into tumors harboring high expression of angiogenic or inflammatory signatures is associated with differential responses to VEGF-directed and ICI-based therapies. Whether these distinct ccRCC subgroups exist along a spectrum of high angiogenesis followed by immune infiltration and eventual immunosuppression is not

yet known, but given their associations with prognosis and stage, this would not be surprising.

TAMs play a significant role in both VEGFR TKI and ICI therapy resistance, and directed attention to macrophages and other immunosuppressive compartments in the RCC TME provides opportunity to salvage therapeutic response in refractory patients. Although several macrophage-depletion agents are under clinical evaluation, TAMs are highly heterogeneous and it is likely that targeting specific TAM subpopulations may lead to better outcomes than pan-macrophage ablation. Further comprehensive functional characterization of ccRCC TAMs and their interplay with T cells is greatly needed and will likely be guided by emerging high-resolution technologies such as single-cell RNA-seq (scRNA-seq).

We have discussed potentially treatment-altering insights made possible by large-scale genomic analyses of clinical trial specimens, but it is important to note the limitations of current genomic tools. Conflicting reports on the prognostic effects of macrophages in ccRCC (10, 22, 39) may be confounded by the high degree of macrophage heterogeneity and highlight the inherent challenge this poses for the generation of cell type–specific gene signatures for accurate quantification of immune populations from bulk RNA-seq data. Indeed, existing immune gene signatures were not sufficiently representative of RCC-specific transcripts and immune populations (23). Development of RCC-specific signatures could greatly improve the accuracy of ccRCC immune deconvolution, but further refinement of these signatures is needed, as a small proportion of leukocytes were still misidentified (23).

Finally, deep sequencing of multiple regions of primary and metastatic tumors from more than 100 patients with ccRCC revealed that single-region biopsies relied on to date are unlikely to capture the full spectrum of tumor subclones and that metastases are significantly more clonal than primary tumors (81, 82). This has fundamental implications for the interpretation of the majority of published studies that infer TME characteristics and therapeutic outcomes of metastatic clones from the phenotypes of fully excised primary tumors. Moreover, important spatial or coinfiltration patterns can be missed when relying fully on genomics and protein cytometry (28, 83). Going forward, multiregion assessments and integrative genomic and imaging-based models will be critical for robust clarification of the relationships between tumor genomics, the TME, and their impact on immunotherapy outcomes.

## Disclosure of Potential Conflicts of Interest

M.H. Voss reports receiving commercial research grants from BMS and Genentech and is a consultant/advisory board member for Alexion, Bayer, Calithera Biosciences, Corvus Pharmaceuticals, Exelixis, Eisai, GSK, Natera, Novartis, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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