Published in final edited form as:

Cancer Cell Microenviron. 2015 ; 2(1): . doi:10.14800/ccm.677.

Immune consequences of tyrosine kinase inhibitors that synergize with cancer immunotherapy

Anna R. Kwilas, Renee N. Donahue, Kwong Y. Tsang, and James W. Hodge

Laboratory of Tumor Immunology and Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Abstract

Combination therapy for the treatment of cancer is becoming increasingly essential as we gain improved understanding of the complexity of cancer progression and the mechanisms by which cancer cells become resistant to single-agent therapy. Recent studies, both clinical and preclinical, have suggested that immunotherapy is a promising approach to the treatment of cancer; however, strategies to improve its clinical efficacy are still needed. A number of recent studies have indicated that antiangiogenic tyrosine kinase inhibitors (TKIs) target multiple components of the tumor microenvironment and are an ideal class of agents for synergizing with cancer immunotherapy. TKIs are well known to modulate tumor endothelial cells, leading to vascular normalization; however, these agents have also been recently shown to decrease tumor compactness and tight junctions, thereby reducing solid tumor pressure and allowing for improved perfusion of collapsed vessels and increased tumor oxygenation. In addition, some TKIs are capable of inducing immunogenic modulation, whereby tumor cells are sensitized to killing by T lymphocytes. Moreover, a number of TKIs have been shown to be involved in immune subset conditioning, increasing the frequency and function of effector immune elements, while decreasing the number and function of immune suppressor cells. The alteration of the immune landscape, direct modification of tumor cells, and improved vascular perfusion leads to improved antitumor efficacy when antiangiogenic TKIs are combined with immunotherapy. Collectively, the data presented in this review support the clinical combination of multi-targeted antiangiogenic TKIs, including but not limited to cabozantinib, sunitinib, and sorafenib, as well as to other antiangiogenic therapies, such as the anti-VEGF antibody bevacizumab, with cancer vaccines for improved treatment of solid tumors.

Keywords

cabozantinib; sunitinib; immunotherapy; combination therapy; immune subset conditioning; immunogenic modulation

Introduction

In recent years, the U.S. Food and Drug Administration (FDA) has approved multiple tyrosine kinase inhibitors (TKIs) for use in patients with various types of cancer; however, it is common for tumors to develop resistance to this type of therapy. Cancer vaccines, as well as other cancer immunotherapies, continue to show positive clinical trial outcomes in a variety of cancer types; however, given the immunosuppressive landscape of this disease, there is certainly room to improve the efficacy of this therapeutic modality [1-3]. Combining immunotherapy with additional agents, particularly standard-of-care therapies, that have immune modulatory capabilities in addition to their antitumor properties, could potentially enhance the clinical benefit of both anticancer therapeutics. Recently published studies have outlined the ability of antiangiogenic TKIs to modulate the immune system through direct effects on tumor vasculature as well as indirect effects on both the tumor cell and immune cells. Two mechanisms by which TKIs have been shown to mediate an antitumor immune response include immunogenic modulation and immune subset conditioning. Immunogenic modulation has been defined as an increase in tumor cell sensitivity to immune cell-mediated lysis through an alteration in tumor cell phenotype. Immune subset conditioning denotes an alteration in the frequency and/or function of immune cell subsets in the periphery or the tumor microenvironment, thus supporting more productive immune interactions. The TKIs used in these studies included sunitinib, sorafenib, and cabozantinib. In 2006, sunitinib was approved to treat advanced renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumors (GIST) [4, 5]. In 2013, sorafenib was also approved to treat advanced renal cell carcinoma, and in 2012 cabozantinib was approved for the treatment of medullary thyroid cancer. Here we review data supporting the direct and indirect immunomodulatory properties of antiangiogenic TKIs, and show how these properties allow TKIs to act synergistically when combined with immunotherapy for cancer.

Direct and indirect effects of TKIs on cancer cells

TKIs directly inhibit the growth of tumor cells by inhibiting tyrosine kinase receptor pathways. The inhibition profiles of sunitinib, sorafenib, and cabozantinib include shared as well as disparate tyrosine kinase receptors. Platelet-derived growth factor receptor signaling as well as mast/stem cell growth factor receptor (c-kit) signaling, on which many tumors depend for growth and proliferation, are inhibited by both sunitinib and sorafenib [6, 7]. Sorafenib also blocks RAF kinase which plays a role in the growth of many cancer types through activation of the MEK/ERK signaling pathway [8]. Sunitinib and cabozantinib both inhibit the Rearranged during Transfection (RET) receptor tyrosine kinase, which plays a role in the growth of thyroid cancer [9, 10]. Cabozantinib also inhibits MET signaling, which influences the survival and proliferation of tumor cells as well as the formation of distant metastases through the initiation of cell migration and invasion. MET signaling can also play a role in tumor angiogenesis through the stimulation of vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) expression, down-regulation of thrombospondin-1, and induction of tubulogenesis [11].

In addition to directly affecting tumor cell growth, cabozantinib has been shown to induce immunogenic modulation [12], improving the sensitivity of murine and human tumor cells to

T cell-mediated lysis. The sensitivity of MC38 murine colon carcinoma cells (Figure 1A) and Lewis lung carcinoma cells (Figure 1B), both engineered to express the human tumor-associated antigen carcinoembryonic antigen (CEA), to CEA-specific lysis significantly increased following 24 hours of treatment with cabozantinib. The sensitivity of SW620 human colon carcinoma cells (Figure 1C) and H441 human lung adenocarcinoma cells (Figure 1D) to mucin 1 (MUC1)-specific killing also increased following 24 hours of treatment with cabozantinib. This is in contrast to sunitinib, which did not affect the sensitivity of tumor cells to T cell-mediated lysis (data not shown). Based on these findings, one could hypothesize that combining cancer immunotherapy that stimulates anticancer immune responses with a TKI that improves the sensitivity of tumor cells to immune-mediated killing may increase the therapeutic efficacy of both treatments.

Direct effects of TKIs on tumor vasculature, intratumoral pressure, and oxygenation

In addition to inhibiting pathways needed for tumor cell growth and proliferation, sunitinib, sorafenib, and cabozantinib all inhibit the VEGF signaling pathway, which is vital to tumor angiogenesis. VEGFRs are widely expressed in tumor vasculature, allowing these TKIs to act directly in the tumor microenvironment [6, 13]. Both cabozantinib (Figure 2A) and sunitinib (Figure 2B) alone significantly reduced the total vascularity of MC38-CEA tumors, as indicated by immunohistochemical staining that identifies CD31 (mature) and CD105 (immature) endocytic vessels. This reduction was maintained when the TKIs were combined with a poxviral-based cancer vaccine (MVA/rF-CEA/TRICOM). Further examination indicated that while sunitinib alone and the combination of sunitinib plus vaccine similarly decreased immature CD105⁺ vasculature, the combination led to a greater reduction in mature CD31⁺ vasculature [14, 15]. In addition, the periphery of tumors from sunitinib-treated mice showed decreases in both CD31⁺ and CD105⁺ vasculature, while in the center of tumors, vasculature decreased only when mice were treated with the combination of sunitinib plus vaccine [15]. Studies have shown that cells of monocytic origin may also participate in tumor vascular formation [16, 17]. Sunitinib alone decreased monocytic vasculature (50%); however, combining sunitinib with vaccine resulted in a greater reduction (91%) in CD11b⁺ monocytic vasculature. Conversely, the number of scattered intratumoral monocytes increased with sunitinib treatment. The highest increase was seen in the combination group, suggesting that this therapeutic regimen drives monocytes toward a more canonical maturation [15].

Further alterations were noted in the tumor microenvironment following TKI treatment. Tumor compactness decreased with sunitinib, sorafenib, and vaccine alone, as well as the combination of sunitinib and vaccine, most likely due to therapy-driven tumor-cell cytotoxicity [14]. JAM-A, a marker of cell-to-cell contact, became internalized following treatment with sunitinib or sorafenib alone. In contrast, vaccination with MVA/rF-CEA/TRICOM decreased JAM-A expression but did not alter its membrane localization [14]. This observation is not unexpected, as studies have shown that proinflammatory cytokines such as interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) can affect the expression pattern of tight-junction markers such as JAM-A [18]. The combination of either sunitinib or

sorafenib with vaccine, however, led to both decreased expression of JAM-A and internalization of the remaining JAM-A [15].

In contrast to direct inhibition of tumor angiogenesis, TKI-induced decreases in tumor compactness and alterations in tight-junctions may reduce the pressure that tumor cells exert against vessels within the tumor, thereby allowing them to become better perfused. Micropuncture experiments were adapted to measure the effect of TKIs and cancer vaccine-based immunotherapy on intratumoral pressure [19]. Sunitinib or sorafenib alone reduced tumor volume, but only slightly decreased intratumoral pressure. The opposite was observed with MVA/rF-CEA/TRICOM alone, which did not alter tumor dimensions but significantly decreased tumor pressure. Combining either sunitinib or sorafenib with vaccine led to both reduced tumor volume and decreased tumor pressure, combining the effects exerted by either therapy alone. Reducing intratumoral pressure and thus increasing tumor perfusion may result in improved tumor oxygenation. As indicated by pimonidazole immunoenzymatic immunohistochemistry (IHC), sunitinib alone increased zones of oxygenation by 20%; however, when combined with vaccine, areas of oxygenation increased by 40%, supporting the hypothesis that combining sunitinib with a cancer vaccine could improve tumor vascular perfusion (Figure 2C). Although the tumor vascular bed was reduced following combination immunotherapy, tumor oxygenation significantly improved, suggesting that the remaining vessels had improved functionality. Taken together, these data suggest that the combination of antiangiogenic TKIs and cancer immunotherapy reduces tumor-cell density and modulates tight-junctions between tumor cells, reducing solid-tumor stress and thereby allowing collapsed tumor vessels to reopen and better perfuse the tumor.

Indirect effects of TKIs on the immune system and immune infiltration of tumor tissue

Immune subset conditioning can apply to both the peripheral immune system and the tumor microenvironment. Studies evaluating the effects of TKIs on immune cells were initially conducted to assess their effects on the peripheral immune system. Cabozantinib-treated mice exhibited no change in total number of splenocytes, but had a trend toward increased splenic CD4⁺ T cells (Table 1). Combining cabozantinib with the cancer vaccine MVA/rF-CEA/TRICOM, however, led to a significant increase in CD4⁺ T cells. Both cabozantinib alone and its combination with vaccine significantly increased the percentage of CD8⁺ T cells (Table 1). Treatment with cabozantinib also reduced the percentage of splenic T regulatory cells (Tregs) and myeloid-derived suppressor cells (MDSCs), effects that were maintained when cabozantinib was combined with vaccine (Table 1). Clinically, a reduction in these immunosuppressive immune cell subsets has also been observed with cabozantinib [20]. As a result of the significant reduction in Treg and MDSC frequency, CD4: Treg/MDSC ratios improved even without a significant change in CD4⁺ T cell frequency. The additional increase in CD8⁺ T cell frequency led to even further improved CD8: Treg/MDSC ratios in the presence of these reduced Treg, and MDSC levels. When analyzed for functionality, significantly more IFN- γ was secreted in response to CEA from splenocytes obtained from CEA-transgenic mice receiving both cabozantinib and MVA/rF-CEA/TRICOM compared to splenocytes from control mice or mice receiving either therapy

alone (Figure 3A). These data support the hypothesis that changing the ratio between immune effector and immune regulatory cells, such that effector cells are favored, leads to the establishment of an immune stimulatory environment, in which it is easier to break tolerance and mount a successful immune response against a tumor-associated, self antigen [21-23].

Unlike the activity of effector cells, the ability of Tregs to inhibit CD4⁺ T cell proliferation was significantly reduced by treatment with either cabozantinib or vaccine alone (Figure 3B). CD4⁺ T cells incubated with Tregs from mice treated with the combination of cabozantinib and MVA/rF-CEA/TRICOM, however, displayed proliferative capacity that was not significantly different from that observed when no Tregs were present, indicating a complete elimination of their regulatory function (Figure 3B). Thus, in addition to reducing the frequency of immunosuppressive immune cell subsets, cabozantinib combined with a cancer vaccine significantly reduced Treg function, leading to increased tumor-specific immune responses. When cabozantinib was examined for effects on human effector cells *in vitro*, clinical concentrations (2.5 µg/L) were shown to have no effect on cytotoxic T cells cocultured up to 144 hours. In addition, human natural killer cells treated with cabozantinib showed no change in phenotype or functional capacity. Additionally, human dendritic cells cultured with cabozantinib displayed no changes in expression of CD80, CD83, MHC-II, or CD54. These data imply that cabozantinib has no detrimental effects on these human immune cells suggesting it may be safely combined with immunotherapy.

Sunitinib increased the frequency of CD8⁺ T cells and decreased the frequency of circulating Tregs and MDSCs (Table 1), again improving the ratio between CD8 effector lymphocytes and suppressor elements. Sunitinib was administered for up to 6 months, during which time this altered immune balance was preserved and no bone marrow toxicity was noted [14]. Sunitinib alone did not alter CD4⁺ T cell proliferation; however, administration of vaccine following the initiation of sunitinib treatment led to significantly increased CD4⁺ T cell proliferation compared to that of CD4⁺ T cells from control, sunitinib alone, and vaccine alone treated mice (Figure 3C). Aside from the reduction in Tregs, a reduction in Treg function may explain this increase in CD4⁺ T cell proliferation. Treg functional analysis indicated that sunitinib alone, vaccine alone, and the combination of both agents decreased the function of circulating Tregs (Figure 3D). The inhibitory effect of sunitinib was also observed on MDSCs [15]. These results support previous findings on the effects of sunitinib on the number and function of Tregs in mouse models and in patients with renal cell carcinoma, presumably through a VEGFA-VEGFR pathway blockade [14, 24-26]. Similarly, sorafenib has been shown to inhibit the proliferation and suppressive function of Tregs in patients with kidney cancer and hepatocellular carcinoma [27, 28]. Importantly, similarly to cabozantinib, sunitinib hindered the ability of murine Tregs and MDSCs to suppress T cell proliferation, in addition to reducing the frequency of both of these inhibitory cell populations. The effect of sunitinib on MDSCs is not surprising, given that it inhibits c-KIT and VEGFR-1, two tyrosine kinase receptors expressed by this cell type [29, 30]. VEGF receptors are critical to the survival, migration, and suppressive function of tumor-infiltrating myeloid cells, including MDSCs and tumor-associated macrophages (TAMs) [31, 32]. Sunitinib has been shown to exert

immunomodulatory effects in clinical studies, similar to that described in preclinical models, including observable reductions in the numbers of circulating immunosuppressive cells in patients after 4 weeks of treatment, thus making this TKI an attractive candidate for combination with immunotherapies [33-35]. These data support the concept that the immunomodulatory effects of TKIs are further enhanced when TKIs are combined with immunotherapy.

The hemodynamic events attributed to the TKIs in these studies, while not primarily immune-related, can have considerable immune consequences. Improved vascular perfusion can allow immune cells better access to the tumor microenvironment [36]. On the other hand, the increase in tumor oxygenation can affect the phenotype and, potentially, the function of negative regulatory immune cells, specifically MDSCs and TAMs. MC38-CEA tumors from mice receiving cabozantinib had a significant increase in immune infiltrate (Table 1), including CD4⁺ and CD8⁺ T cells as well as Tregs and MDSCs. The proportion of tumor composed of CD4⁺ T cells, CD8⁺ T cells and Tregs increased further when cabozantinib was combined with vaccine. Though the frequency of Tregs present in the tumor microenvironment increased with either therapy alone as well as combination therapy, functional data (Figure 3B) suggest that their regulatory capacity may be impaired. Increased MDSCs were also observed with this combination compared to control tumors, but MDSC infiltration was reduced with the combination compared to vaccine alone. Sunitinib administered alone and in combination with the MVA/rF-CEA/TRICOM vaccine increased tumor infiltration by CD8⁺ T cells and reduced infiltration by Tregs (Table 1). A significant reduction in MDSC infiltration was only observed when vaccine was administered following sunitinib treatment. CEA-specific tumor-infiltrating CD8⁺ T cells, measured by tetramer staining, also increased with the sequential combination of sunitinib and vaccine [14]. Sorafenib combined with MVA/rF-CEA/TRICOM also increased CD4⁺ and CD8⁺ T cell infiltration in MC38-CEA tumors [14]. However, in a follow-up study, the frequency of tumor-infiltrating MDSCs increased significantly when tumor-bearing mice were treated with either sunitinib or sorafenib, in the presence or absence of vaccine, compared to control or vaccine alone [15]. These MDSCs (and TAMs) showed a striking increase in the surface expression of the activation markers FAS-L, CXCL-9, CD31, and CD105 [15]. Sorafenib alone increased the percentage of all 4 activation markers examined, sunitinib alone increased FAS-L, while both combination therapies increased 3 of 4 activation markers examined. Up-regulation of these markers has been reported during myeloid-cell activation [37, 38], maturation [39], and a switch from type 2 to type 1 [40]. It has been suggested that, in the presence of normal tumor oxygenation, these myeloid cells could become activated and, perhaps, more tumor-lytic and less immunosuppressive. Previous studies in the B16.OVA melanoma model have shown that sunitinib alone or in combination with a dendritic-cell vaccine was associated with decreased MDSCs within the tumor microenvironment. However, this was model-dependent; in the 4T1 model, MDSCs decreased in spleens but not in the tumor microenvironment after sunitinib treatment [24]. Vaccine followed by sunitinib or concurrent administration of the treatments showed no change in intratumoral immune infiltration over sunitinib alone [14].

Combining antiangiogenic TKIs with cancer immunotherapy improves antitumor efficacy

The antitumor efficacy of the above-described TKIs was determined using MC38-CEA tumor cells in CEA-transgenic mice to mimic the targeting of a self-antigen. The growth of MC38-CEA tumors was significantly reduced by cabozantinib treatment alone. Cabozantinib treatment resulted in a single tumor regression; however, this tumor recurred prior to day 33, indicating that it was not a complete regression (Figure 4A). Concurrently administering cabozantinib with MVA/rF-CEA/TRICOM resulted in a significant reduction of MC38-CEA tumor growth rate compared to control and to the administration of MVA/rF-CEA/TRICOM alone. In addition, 5/10 mice treated with the combination of cabozantinib and MVA/rF-CEA/TRICOM underwent complete tumor regression, remaining tumor-free through day 35 (Figure 4A). Sequential administration of the combination, cabozantinib followed by MVA/rF-CEA/TRICOM, did not result in improved antitumor efficacy compared to that observed with concurrent administration (Figure 4A, insert). Thus, cabozantinib displayed increased antitumor efficacy when administered prior to or concurrent with vaccine, despite a significant increase in intratumoral Tregs (Table 1). This was possibly due to the fact that Treg functionality was significantly reduced in combination-treated mice. Cabozantinib treatment alone reduced the growth rate of tumor cells but was unable to completely eliminate the tumor, presumably as a result of decreased vascular density and a parallel increase in lymphocytic infiltration. However, in order to achieve tumor eradication the addition of an immunotherapeutic agent was required to eliminate the function of regulatory immune cells and improve the functional specificity of effector cells.

As with cabozantinib, the growth of MC38-CEA tumors was reduced by sunitinib treatment alone (Figure 4B). Also as with cabozantinib, tumor cures were only obtained when sunitinib was combined with MVA/rF-CEA/TRICOM. However, with sunitinib, the timing of vaccination was extremely important. Administration of sunitinib prior to vaccine resulted in improved antitumor efficacy compared to control, sunitinib alone, and vaccine alone (Figure 4B). Sequential treatment also improved the median survival of tumor-bearing mice [14]. In contrast, concurrent administration of sunitinib and vaccine (Figure 4B, insert), or administration of vaccine prior to sunitinib [14], reduced tumor growth similar to sunitinib alone. Moreover, like sunitinib alone, concurrent administration did not result in tumor-free mice or improved survival. Recently, sunitinib was evaluated in combination with an MVA-based cancer vaccine encoding the tumor-associated antigen 5T4 (MVA-5T4; TroVax) in a randomized phase III clinical trial. The results from this study indicated no survival benefit was obtained by combining sunitinib with this cancer vaccine compared to sunitinib alone [41]. In this trial, renal cell carcinoma patients were vaccinated prior to receiving sunitinib, which, according to preclinical observations, may explain the lack of improved clinical efficacy. Based on the observations that (a) CD8⁺ T lymphocyte levels decreased shortly after the start of sunitinib treatment and then recovered during the remaining treatment time, and (b) it took 5 to 7 days of sunitinib treatment to reduce immunosuppressive elements (Tregs and MDSCs), it is logical that antitumor efficacy is only improved when sunitinib is given prior to vaccine, when the vaccine-induced immune

response can be amplified due to the presence of an altered immune balance generated by the TKI [14]. Based on the transient reduction in CD8⁺ T cells observed following the initiation of sunitinib treatment, vaccinating concurrently with the initiation of sunitinib could result in a reduction in initial vaccine-mediated effector responses, potentially decreasing the overall magnitude of the antitumor response.

The benefit of combining antiangiogenic TKIs with therapeutic vaccines is not limited to a specific TKI, vaccine, or tumor model. In addition to sunitinib and cabozantinib, sorafenib also decreased tumor volumes in the MC38-CEA model compared to untreated mice, and showed improved antitumor efficacy when combined with vaccine [14]. Additionally, either sunitinib or sorafenib in combination with a second recombinant poxviral vaccine decreased the growth of 4T1 breast tumors [14]. As seen in the MC38-CEA model, the decrease in tumor dimensions coincided with an increase in CD4⁺ and CD8⁺ tumor-infiltrating lymphocytes [14]. Moreover, a similar alteration in the intratumoral immune landscape resulted in improved survival in the MCA26 colon tumor model upon administration of sunitinib in combination with IL-12 and 4-1BB stimulation [42]. Another recent study reported improved efficacy in the B16.OVA melanoma model by combining a peptide-pulsed dendritic-cell vaccine with a short course of sunitinib [43]. Investigators found that improved antitumor response depended on the observed increase in antigen-specific CD8⁺ T cell infiltration into the tumor microenvironment.

Conclusions

The observations reviewed here further demonstrate that TKIs induce direct effects on tumor cells leading to their destruction via apoptotic and immune-mediated mechanisms. These observations also support the conclusions that TKIs, particularly antiangiogenic TKIs, modify the tumor microenvironment in multiple ways, including the alteration of immune cell infiltration (immune subset conditioning) leading to tumor eradication particularly when these therapies are combined with cancer immunotherapy. Unfortunately, it is common for cancer cells to become resistant to targeted therapy, particularly to small molecule inhibitors such as TKIs. It is possible that drug resistance could be avoided or overcome by combining these agents with additional therapies with which synergistic antitumor effects can be observed. In addition, the effectiveness of cancer immunotherapy can be compromised when immune cells cannot penetrate the tumor due to abnormal vasculature [44]. Data obtained using the MC38-CEA tumor model indicate that TKIs synergize with the MVA/rF-CEA/TRICOM cancer vaccine to induce changes in the tumor microenvironment resulting in improved immune cell infiltration and sustained tumor regression. The antiangiogenic TKIs described here synergize with cancer immunotherapy by targeting 3 elements of the tumor microenvironment: (a) tumor endothelial cells, leading to vascular normalization; (b) tumor cells; direct growth inhibition, immunogenic modulation, and reduction of tumor compactness allows collapsed vessels to reopen; and (c) tumor-infiltrating immune cells, increasing the frequency and function of effector immune elements while decreasing the number and function of immune suppressor cells. Collectively, these data support the clinical combination of multitargeted antiangiogenic TKIs, including but not limited to cabozantinib, sunitinib, sorafenib, pazopanib, axitinib, lapatinib, and imatinib, as well as other antiangiogenic therapies such as the anti-VEGF antibody bevacizumab, with cancer

vaccines for improved treatment of solid tumors. Additional preclinical studies are warranted to determine the most appropriate schedules for combination therapies that include TKIs.

Acknowledgements

This research was supported by the Intramural Research Program of the NIH and National Cancer Institute. The authors thank Dr. Jeffrey Schlom for helpful suggestions and Bonnie L. Casey for editorial assistance in the preparation of this manuscript.

References

1. Hodge JW, Ardiani A, Farsaci B, Kwilas AR, Gameiro SR. The tipping point for combination therapy: cancer vaccines with radiation, chemotherapy, or targeted small molecule inhibitors. *Semin Oncol.* 2012; 39(3):323–39. [PubMed: 22595055]
2. Kim R, Emi M, Tanabe K, Arihiro K. Tumor-driven evolution of immunosuppressive networks during malignant progression. *Cancer Res.* 2006; 66(11):5527–36. [PubMed: 16740684]
3. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol.* 2007; 25:267–96. [PubMed: 17134371]
4. Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Oudard S, et al. Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. *J Clin Oncol.* 2009; 27(22):3584–90. [PubMed: 19487381]
5. Demetri GD, van Oosterom AT, Garrett CR, Blackstein ME, Shah MH, Verweij J, et al. Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. *Lancet.* 2006; 368(9544):1329–38. [PubMed: 17046465]
6. Mendel DB, Laird AD, Xin X, Louie SG, Christensen JG, Li G, et al. In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. *Clin Cancer Res.* 2003; 9(1):327–37. [PubMed: 12538485]
7. Fletcher JA, Rubin BP. KIT mutations in GIST. *Curr Opin Genet Dev.* 2007; 17(1):3–7. [PubMed: 17208434]
8. Gotink KJ, Verheul HM. Anti-angiogenic tyrosine kinase inhibitors: what is their mechanism of action? *Angiogenesis.* 2010; 13(1):1–14. [PubMed: 20012482]
9. Kurzrock R, Sherman SI, Ball DW, Forastiere AA, Cohen RB, Mehra R, et al. Activity of XL184 (Cabozantinib), an oral tyrosine kinase inhibitor, in patients with medullary thyroid cancer. *J Clin Oncol.* 2011; 29(19):2660–6. [PubMed: 21606412]
10. Viola D, Cappagli V, Elisei R. Cabozantinib (XL184) for the treatment of locally advanced or metastatic progressive medullary thyroid cancer. *Future Oncol.* 2013; 9(8):1083–92. [PubMed: 23902240]
11. Boccaccio C, Comoglio PM. Invasive growth: a MET-driven genetic programme for cancer and stem cells. *Nat Rev Cancer.* 2006; 6(8):637–45. [PubMed: 16862193]
12. Kwilas AR, Ardiani A, Donahue RN, Aftab DT, Hodge JW. Dual effects of a targeted small-molecule inhibitor (cabozantinib) on immune-mediated killing of tumor cells and immune tumor microenvironment permissiveness when combined with a cancer vaccine. *J Transl Med.* 2014; 12(1):294. [PubMed: 25388653]
13. Osusky KL, Hallahan DE, Fu A, Ye F, Shyr Y, Geng L. The receptor tyrosine kinase inhibitor SU11248 impedes endothelial cell migration, tubule formation, and blood vessel formation in vivo, but has little effect on existing tumor vessels. *Angiogenesis.* 2004; 7(3):225–33. [PubMed: 15609077]
14. Farsaci B, Higgins JP, Hodge JW. Consequence of dose scheduling of sunitinib on host immune response elements and vaccine combination therapy. *Int J Cancer.* 2012; 130(8):1948–59. [PubMed: 21633954]
15. Farsaci B, Donahue RN, Coplin MA, Grenga I, Lepone LM, Molinolo AA, et al. Immune consequences of decreasing tumor vasculature with antiangiogenic tyrosine kinase inhibitors in

- combination with therapeutic vaccines. *Cancer Immunol Res.* 2014; 2(11):1090–102. [PubMed: 25092771]
16. Kuwana M, Okazaki Y, Kodama H, Satoh T, Kawakami Y, Ikeda Y. Endothelial differentiation potential of human monocyte-derived multipotential cells. *Stem Cells.* 2006; 24(12):2733–43. [PubMed: 16888284]
 17. Yamaguchi Y, Kuwana M. Proangiogenic hematopoietic cells of monocytic origin: roles in vascular regeneration and pathogenic processes of systemic sclerosis. *Histol Histopathol.* 2013; 28(2):175–83. [PubMed: 23275301]
 18. Bruewer M, Utech M, Ivanov AI, Hopkins AM, Parkos CA, Nusrat A. Interferon-gamma induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. *FASEB J.* 2005; 19(8):923–33. [PubMed: 15923402]
 19. Whitesides TE Jr, Haney TC, Harada H, Holmes HE, Morimoto K. A simple method for tissue pressure determination. *Arch Surg.* 1975; 110(11):1311–3. [PubMed: 1191023]
 20. Apolo A, Tomita Y, Lee M, Lee S, Frosch A, Steinberg S, et al. Effect of cabozantinib on immunosuppressive subsets in metastatic urothelial carcinoma. *J Clin Oncol.* 2014; 32(5s) abstr 4501.
 21. Overwijk WW. Breaking tolerance in cancer immunotherapy: time to ACT. *Curr Opin Immunol.* 2005; 17(2):187–94. [PubMed: 15766680]
 22. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci U S A.* 2005; 102(51):18538–43. [PubMed: 16344461]
 23. Sinicrope FA, Rego RL, Ansell SM, Knutson KL, Foster NR, Sargent DJ. Intraepithelial effector (CD3+)/regulatory (FoxP3+) T-cell ratio predicts a clinical outcome of human colon carcinoma. *Gastroenterology.* 2009; 137(4):1270–9. [PubMed: 19577568]
 24. Bose A, Taylor JL, Alber S, Watkins SC, Garcia JA, Rini BI, et al. Sunitinib facilitates the activation and recruitment of therapeutic anti-tumor immunity in concert with specific vaccination. *Int J Cancer.* 2011; 129(9):2158–70. [PubMed: 21170961]
 25. Finke JH, Rini B, Ireland J, Rayman P, Richmond A, Golshayan A, et al. Sunitinib reverses type-1 immune suppression and decreases T-regulatory cells in renal cell carcinoma patients. *Clin Cancer Res.* 2008; 14(20):6674–82. [PubMed: 18927310]
 26. Terme M, Pernot S, Marcheteau E, Sandoval F, Benhamouda N, Colussi O, et al. VEGFA-VEGFR pathway blockade inhibits tumor-induced regulatory T-cell proliferation in colorectal cancer. *Cancer Res.* 2013; 73(2):539–49. [PubMed: 23108136]
 27. Chen ML, Yan BS, Lu WC, Chen MH, Yu SL, Yang PC, et al. Sorafenib relieves cell-intrinsic and cell-extrinsic inhibitions of effector T cells in tumor microenvironment to augment antitumor immunity. *Int J Cancer.* 2014; 134(2):319–31. [PubMed: 23818246]
 28. Cabrera R, Ararat M, Xu Y, Brusko T, Wasserfall C, Atkinson MA, et al. Immune modulation of effector CD4+ and regulatory T cell function by sorafenib in patients with hepatocellular carcinoma. *Cancer Immunol Immunother.* 2013; 62(4):737–46. [PubMed: 23223899]
 29. Kao J, Ko EC, Eisenstein S, Sikora AG, Fu S, Chen SH. Targeting immune suppressing myeloid-derived suppressor cells in oncology. *Crit Rev Oncol Hematol.* 2011; 77(1):12–9. [PubMed: 20304669]
 30. Yang Z, Zhang B, Li D, Lv M, Huang C, Shen GX, et al. Mast cells mobilize myeloid-derived suppressor cells and Treg cells in tumor microenvironment via IL-17 pathway in murine hepatocarcinoma model. *PLoS One.* 2010; 5(1):e8922. [PubMed: 20111717]
 31. Gabrilovich D, Ishida T, Oyama T, Ran S, Kravtsov V, Nadaf S, et al. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. *Blood.* 1998; 92(11):4150–66. [PubMed: 9834220]
 32. Dineen SP, Lynn KD, Holloway SE, Miller AF, Sullivan JP, Shames DS, et al. Vascular endothelial growth factor receptor 2 mediates macrophage infiltration into orthotopic pancreatic tumors in mice. *Cancer Res.* 2008; 68(11):4340–6. [PubMed: 18519694]
 33. Asemussen AM, Brossart P. Vaccination strategies in patients with renal cell carcinoma. *Cancer Immunol Immunother.* 2009; 58(7):1169–74. [PubMed: 19360405]

34. Ko JS, Zea AH, Rini BI, Ireland JL, Elson P, Cohen P, et al. Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. *Clin Cancer Res.* 2009; 15(6):2148–57. [PubMed: 19276286]
35. Roselli M, Cereda V, di Bari MG, Formica V, Spila A, Jochems C, et al. Effects of conventional therapeutic interventions on the number and function of regulatory T cells. *Oncoimmunology.* 2013; 2(10):e27025. [PubMed: 24353914]
36. Huang Y, Yuan J, Righi E, Kamoun WS, Ancukiewicz M, Nezivar J, et al. Vascular normalizing doses of antiangiogenic treatment reprogram the immunosuppressive tumor microenvironment and enhance immunotherapy. *Proc Natl Acad Sci U S A.* 2012; 109(43):17561–6. [PubMed: 23045683]
37. Chakour R, Allenbach C, Desgranges F, Charmoy M, Mauel J, Garcia I, et al. A new function of the Fas-FasL pathway in macrophage activation. *J Leukoc Biol.* 2009; 86(1):81–90. [PubMed: 19380712]
38. McKenney JK, Weiss SW, Folpe AL. CD31 expression in intratumoral macrophages: a potential diagnostic pitfall. *Am J Surg Pathol.* 2001; 25(9):1167–73. [PubMed: 11688576]
39. Lastres P, Bellon T, Cabanas C, Sanchez-Madrid F, Acevedo A, Gougos A, et al. Regulated expression on human macrophages of endoglin, an Arg-Gly-Asp-containing surface antigen. *Eur J Immunol.* 1992; 22(2):393–7. [PubMed: 1537377]
40. Porta C, Rimoldi M, Raes G, Brys L, Ghezzi P, Di Liberto D, et al. Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB. *Proc Natl Acad Sci U S A.* 2009; 106(35):14978–83. [PubMed: 19706447]
41. Amato RJ, Hawkins RE, Kaufman HL, Thompson JA, Tomczak P, Szczyluk C, et al. Vaccination of metastatic renal cancer patients with MVA-5T4: a randomized, double-blind, placebo-controlled phase III study. *Clin Cancer Res.* 2010; 16(22):5539–47. [PubMed: 20881001]
42. Ozao-Choy J, Ma G, Kao J, Wang GX, Meseck M, Sung M, et al. The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies. *Cancer Res.* 2009; 69(6):2514–22. [PubMed: 19276342]
43. Bose A, Taylor JL, Alber S, Watkins SC, Garcia JA, Rini BI, et al. Sunitinib facilitates the activation and recruitment of therapeutic anti-tumor immunity in concert with specific vaccination. *Int J Cancer.* 2010
44. Stylianopoulos T, Martin JD, Chauhan VP, Jain SR, Diop-Frimpong B, Bardeesy N, et al. Causes, consequences, and remedies for growth-induced solid stress in murine and human tumors. *Proc Natl Acad Sci U S A.* 2012; 109(38):15101–8. [PubMed: 22932871]

Figure 1. Cabozantinib treatment improves tumor-cell sensitivity to T cell-mediated lysis
 Tumor cells were treated with cabozantinib for 24 hours, labeled with indium¹¹¹, then incubated with T cells specific for the indicated antigen for 18 hours at an effector:target ratio of 30:1. Murine tumor lines MC38-CEA (A) and LLC-CEA (B) were incubated with CEA-specific murine T cells. Human tumor lines SW620 (C) and H441 (D) were incubated with MUC1-specific human T cells. After 18 hours, supernatants were harvested and analyzed for indium¹¹¹ release. Percent lysis was calculated as follows: $[(\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm})] \times 100$. Error bars depict mean $\pm$ standard error derived from triplicate measurements. * = $P < 0.01$ as determined by Student's t test. Data adapted from [12].

Figure 2. Antiangiogenic TKIs reduce tumor vascularity, leading to increased oxygenation

(A) C57/BL6 mice were treated as indicated beginning 14 days post-implantation of MC38-CEA cells. Mice receiving vaccine received a priming vaccination of MVA-CEA/TRICOM on day 14 and a booster vaccination of rF-CEA/TRICOM on day 21. Tumors were harvested 28 days post-tumor implantation. Images show representative tumor areas at 20x magnification obtained using an Aperio ScanScope. (B) Seven days post-implantation of MC38-CEA cells, indicated mice began receiving sunitinib. MVA-CEA/TRICOM was given on day 14. Tumors were harvested 21 days post-tumor implantation. Images show representative tumor areas at 40x magnification obtained using an Aperio ScanScope. In both cases, tumors were harvested and double-stained for the vascular markers CD31 and CD105 using DAB for visualization. Representative images adapted from [12] and [15] are shown. (C) C57/BL6 mice were treated as in (B). IHC analysis of hypoxia was performed using Hypoxyprobe-DAB. Images show representative tumors at 2x magnification obtained using an Aperio ScanScope. Data adapted from [15].

Figure 3. TKIs alone and with a cancer vaccine alter immune-cell function

For cabozantinib studies, C57/BL6 CEA-Tg mice received cabozantinib beginning on day 0. Mice treated with vaccine received MVA-CEA/TRICOM on day 0 and rF-CEA/TRICOM on days 7 and 14. On day 35, spleens were harvested and a single cell suspension of splenocytes was obtained. (A) CEA peptide was incubated with the splenocytes for 7 days after which cells were incubated with fresh, irradiated, naïve splenocytes and either CEA or HIV-gag peptide for 24 hours at 37°C. After 24 hours, a cytometric bead array was used to analyze the supernatants for murine IFN- γ . CEA-specific cytokine production was determined by subtracting cytokine production induced by the HIV-gag peptide from that induced by the CEA peptide. Error bars depict the mean $\pm$ standard error. * = $P < 0.05$ relative to control and single agents as determined by Student's t test. (B) Tregs (CD3⁺CD4⁺CD25⁺FoxP3⁺ cells) were isolated from spleens using negative selection. Tregs were cultured with CD4⁺ T cells from naïve mice, antigen-presenting cells (APCs, irradiated allogeneic splenocytes) and soluble anti-CD3 for 72 hours. The background level of CD4⁺ T cell proliferation was determined by incubating the naïve CD4⁺ T cells with APCs and anti-CD3 in the absence of purified Tregs. Error bars depict the mean $\pm$ standard error. * = $P <$

0.01 compared to the proliferation of CD4⁺ T cells incubated with Tregs from untreated mice; ns = no significant difference between CD4⁺ T cell proliferation in the absence of Tregs and the proliferation of CD4⁺ T cells incubated with Tregs isolated from mice treated with the indicated therapy. Significance was determined Student's t test. Data adapted from [12]. For sunitinib studies, C57/BL6 mice received sunitinib beginning on day 0. Mice treated with vaccine received MVA-CEA/TRICOM on day 7, and rF-CEA/TRICOM on day 14. On day 35, spleens were harvested and a single cell suspension of splenocytes was obtained. (C) CD4⁺ lymphocytes were isolated from spleens and cocultured with APCs and 6.25 g/mL CEA protein for 5 days at 37°C. One μ Ci [³H] thymidine was added to each well for the last 24 hours. Mean cellular proliferation was dictated by [³H] thymidine incorporation. Error bars depict the mean $\pm$ standard error. * = P < 0.01 relative to control and single agents as determined by Student's t test. (D) Treg functional assay performed as in (B) using splenic Tregs purified from mice treated with sunitinib $\pm$ vaccine. Data adapted from [14].

Figure 4. Combining TKIs with a cancer vaccine results in improved antitumor efficacy
 (A) Mice were treated as indicated ($n = 10/\text{group}$) beginning 4 days post-implantation of MC38-CEA cells. Ratios denote the number of tumor-free mice at day 35. (B) Indicated groups ($n = 12$) began receiving sunitinib 7 days post-implantation of MC38-CEA cells. Vaccine administration began 14 days post-implantation. Ratios denote the number of tumor-free mice at day 42. Tumor volumes were calculated as follows: $(\text{length} \times \text{width}^2)/2$. Data adapted from ^[12] and ^[14], respectively.

Table 1

Effects of Cabozantinib or Sunitinib on immune cell frequencies

Splenic	Control	Vaccine	Cabozantinib	Cabozantinib + Vaccine	Control	Vaccine	Sunitinib	Sunitinib > Vaccine
% CD4⁺ T cells	15.86 (0.36)	16.92 (1.20)*	17.6 (2.48)	18.26 (1.16)*	24.52 (2.38)	nd	20.32 (0.41)*	nd
% CD8⁺ T cells	9.79 (1.20)	12.11 (2.15)*	12.86 (2.04)*	13.10 (1.31)*	15.34 (2.54)	nd	18.26 (1.71)*	nd
% Treg cells	1.18 (0.12)	1.18 (0.10)	0.98 (0.18)*	1.11 (0.04)*	2.04 (0.28)	nd	1.50 (0.09)*	nd
% MDSCs	3.82 (0.18)	3.89 (0.70)	2.43 (0.28)*	2.53 (0.22)*	0.93 (0.24)	nd	0.37 (0.03)*	nd

Intratumoral	Control	Vaccine	Cabozantinib	Cabozantinib + Vaccine	Control	Vaccine	Sunitinib	Sunitinib > Vaccine
% CD4⁺ T cells	0.03	0.75*	0.46*	1.13*	3.86 (3.28)	3.41 (2.94)	2.03 (0.75)	1.90 (0.86)
% CD8⁺ T cells	0.03	1.10*	0.22*	1.49*	0.32 (0.12)	0.46 (0.22)	0.69 (0.22)*	0.66 (0.03)*
% Treg cells	0.01	0.09*	0.13*	0.24*	0.11 (0.06)	0.07 (0.02)*	0.07 (0.02)*	0.05 (0.02)*
% MDSCs	0.02	0.61*	0.21*	0.46*	1.48 (0.23)	1.35 (0.99)	1.16 (0.46)	0.48 (0.13)*

Flow cytometric analysis of splenic immune cell subsets or tumor infiltrating lymphocytes in MC38-CEA tumors. Cabozantinib and vaccine were administered 14 days post tumor implantation. Tumors were 28 days post tumor implantation. Sunitinib was administered 7 days post tumor vaccination and in this case vaccine was administered 14 days post tumor implantation. Tumors were 21 days post tumor implantation. Splenic studies, n = 5 mice/group. Intratumoral studies, cabozantinib, n = 2 mice/group; sunitinib, n = 12-17 mice/group.

* = p<0.01 as determined by Student's t test or the Kolmogorov-Smirnov test, nd = not determined