Immune Consequences of Decreasing Tumor Vasculature with Antiangiogenic Tyrosine Kinase Inhibitors in Combination with Therapeutic Vaccines

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Abstract
This study investigated the effects on the tumor microenvironment (TME) of combining antiangiogenic tyrosine kinase inhibitors (TKI) with therapeutic vaccines, and in particular, how vascular changes affect tumor-infiltrating immune cells. We conducted studies using a TKI (sunitinib or sorafenib) in combination with recombinant vaccines in two murine tumor models: colon carcinoma (MC38-CEA) and breast cancer (4T1). Tumor vasculature was measured by immunohistochemistry using three endothelial cell markers: CD31 (mature), CD105 (immature/proliferating), and CD11b (monocytic). We assessed oxygenation, tight junctions, compactness, and pressure within tumors, along with the frequency and phenotype of tumor-infiltrating lymphocytes (TIL), myeloid-derived suppressor cells (MDSC), and tumor-associated macrophages (TAM) following treatment with antiangiogenic TKIs alone, vaccine alone, or the combination of a TKI with vaccine. The combined regimen decreased tumor vasculature, compactness, tight junctions, and pressure, leading to vascular normalization and increased tumor oxygenation. This combination therapy also increased TILs, including tumor antigen–specific CD8 T cells, and elevated the expression of activation markers FAS-L, CXCL-9, CD31, and CD105 in MDSCs and TAMs, leading to reduced tumor volumes and an increase in the number of tumor-free animals. The improved antitumor activity induced by combining antiangiogenic TKIs with vaccine may be the result of activated lymphoid and myeloid cells in the TME, resulting from vascular normalization, decreased tumor-cell density, and the consequent improvement in vascular perfusion and oxygenation. Therapies that alter tumor architecture can, thus, have a dramatic impact on the effectiveness of cancer immunotherapy. Cancer Immunol Res; 2(11); 1090–102. ©2014 AACR.

Introduction
Antiangiogenic tyrosine kinase inhibitors (TKI), in addition to their direct antivascular effects, can be immunomodulatory (1, 2). This may be because the vascular endothelial growth factor receptors (VEGFR) are indispensable for the survival, migration, and suppressive function of tumor-infiltrating myeloid (TIM) cells, including myeloid-derived suppressor cells (MDSC; ref. 3) and tumor-associated macrophages (TAM; ref. 4). On the other hand, immunotherapies not only can initiate an immune attack against tumor cells, they can also reduce tumor vasculature through the release of IFN I and II in the tumor microenvironment (TME; refs. 5–7). Thus, combining antiangiogenic TKIs with immunotherapy could have clinical benefit for patients with cancer. Although sunitinib can decrease MDSCs in the peripheral blood (PB) of patients with renal cell carcinoma, this change did not correlate with clinical response (8), nor was it accompanied by a similar decrease of MDSCs in the TME (9). This dissociation between the effects of antiangiogenic TKIs on MDSCs in the PB and in the TME can raise concerns about the rationale of combining TKIs with immunotherapy to treat cancer. This study investigated the effects on the TME of combining antiangiogenic TKIs with therapeutic vaccines, and in particular how vascular changes may affect tumor-infiltrating immune cells.

Materials and Methods
Animals
Eight- to 12-week-old female carcinoembryonic antigen (CEA)–transgenic (CEA-Tg) mice that originated from a breeding pair of CEA-Tg C57BL/6 mice homozygous for the expression of CEA were provided by Dr. John Shively (Beckman Research Institute, City of Hope National Medical Center, Duarte, CA; refs. 10, 11). All animal studies were approved by the National Cancer Institute’s Intramural Animal Care and Use Committee.
Tumor-cell lines
MC38 murine colon carcinoma cells expressing human CEA (MC38-CEA, a gift from Dr. Jeffrey Schlom, LTIB, NCI, NIH, Bethesda, MD) were generated by retroviral transduction with CEA cDNA, as previously described (12). These cells were tested every month for mycoplasma and every 6 months by standard Molecular Testing of Biological Materials-Mouse/Rat (MTBM-M/R) panels and used at very low passage number. No mycoplasma and every 6 months observational assay was performed. Tumor cells were cultured as described previously (1). For in vivo studies, 5 × 10^5 MC38-CEA cells were injected s.c. in the right flank of CEA-Tg mice. Tumor dimensions were measured weekly, and tumor volumes were obtained using the formula (length × width^2)/2. Because changes in tumor volume can affect vasculature and perfusion, tumors with similar dimensions (80–120 mm^3 for all treatment groups) were further used for immunohistochemistry (IHC) studies.

Vaccination
Recombinant-modified vaccinia Ankara (rMVA) and recombinant fowlpox (rF) viruses containing transgenes for the murine costimulatory molecules B7.1, ICAM-1, and LFA-3 (designated TRICOM) in combination with the CEA transgene (rMVA/rF–CEA–TRICOM) have been described previously (14). For in vivo studies, rMVA–CEA–TRICOM was administered s.c. as a prime and rF–CEA–TRICOM as weekly boosts at 1 × 10^7 plaque-forming units per mouse (15, 16).

Drug preparation and treatment schedule
Sunitinib malate salt >99% diet was prepared as previously described (1). In additional experiments, sorafenib p-toluene-sulfonate salt >99% (LC Laboratories) was admixed with Open Standard Diet (Research Diets), modeling the human dose of 400 mg twice a day (17). MC38-CEA tumor model mice were treated as follows: Control, control diet starting 7 days after tumor transplant; sunitinib alone (sun), sunitinib starting 7 days after tumor transplant; sorafenib alone (sor), sorafenib starting 7 days after tumor transplant; vaccine (vac), control diet starting 7 days after tumor transplant, vaccine prime on day 14 followed by weekly boosts; sunitinib plus vaccine (sun + vac), sunitinib starting 7 days after tumor transplant, vaccine prime on day 14 followed by weekly boosts; sorafenib plus vaccine (sor + vac), sorafenib starting 7 days after tumor transplant, vaccine prime on day 14 followed by weekly boosts.

Histologic analyses
Immunofluorescent and immunoenzymatic histochemistry as well as histopathologic analyses were conducted as described in the Supplementary Materials and Methods.

Measurement of intratumoral pressure
Intratumoral pressure was measured using a modified micropuncture technique (18) described in the Supplementary Materials and Methods.

Flow-cytometric evaluation of single-cell suspensions
CEA_20_233 and HIV-GAG tetramer staining was performed as previously described (1). To analyze TIMs, 21-day-old MC38-CEA tumors were harvested and enzymatically digested to obtain a single-cell suspension (1). Anti-CD11b Alexa Fluor 700 clone M1/70 and anti-Gr1 APC-Cy7 clone RB6-8C5 were purchased from BD Biosciences. Anti-CXCL9 Alexa Fluor 647 clone M1G-2F-5, anti-CD105 PE-Cy7 clone M72/18, and anti-CD31 Pacific Blue clone 390 were purchased from BioLegend. Anti-CD45 eFlour 605NC clone 30-F11 and anti–FAS-L PerCP-eFlour 710 clone MFL3 were purchased from eBioscience. At least 3 × 10^5 live cells were acquired with an LSR-II flow cytometer (BD Biosciences), and data were analyzed with FlowJo software for PC (TreeStar Inc.).

In vivo transfer of myeloid cells into tumor-bearing recipients
CD11b^+ cells were magnetically selected from the spleen or bone marrow of non–tumor-bearing C57BL/6 mice (n = 10; StemCell Technologies, Inc.) following the manufacturer’s instructions. The negatively selected myeloid cells were then labeled with PKH67 or PKH26 (Sigma Aldrich), and injected i.v. into syngeneic mice (n = 3) bearing established MC38-CEA tumors. Tumors were harvested 3 days after injection and analyzed by flow cytometry or IHC to assess the migration of CD11b^+ cells into the tumor.

Statistical analysis
GraphPad Prism v. 5.04 statistical software (GraphPad Software) was used for statistical analyses. The two-tailed unpaired t test was used to measure differences in junctional adhesion molecule-A (JAM-A) expression between each treatment and control; the ANOVA test with the Dunn multiple comparison test was used for the other analyses. A P value of <0.05 was considered statistically significant.

Results
Sunitinib or sorafenib in combination with vaccine similarly enhanced antitumor effect
The TKIs sunitinib and sorafenib inhibit a similar spectrum of tyrosine kinase receptors, including VEGFRs and platelet-derived growth factor receptors (PDGFR; ref. 19). Specifically, although the two TKIs share many similar targets (VEGFR2, VEGFR3, PDGFR, and c-Kit), sunitinib is also active on VEGFR1, and on the RET proto-oncogene receptor, which is a potential target for thyroid cancer. Sorafenib blocks the enzyme RAF kinase, a critical component of the RAF/MEK/ERK signaling pathway that controls cell division and proliferation of many cancer types. To evaluate whether the two TKIs had similar antitumor effects in vivo, CEA-Tg mice bearing MC38-CEA tumors were treated with either sunitinib or sorafenib, rMVA–CEA–TRICOM vaccine alone, or with each TKI plus vaccine. Over the course of 35 days, we observed similar antitumor activity with vaccine combined with either sunitinib or sorafenib (Fig. 1A). Administered alone, both sunitinib and sorafenib decreased tumor volumes compared with those of untreated mice or mice receiving vaccine alone. In contrast, each TKI combined with vaccine decreased tumor volume to a greater extent than either TKI alone or vaccine alone, indicating that sunitinib and sorafenib have similar antitumor effects when combined with vaccine. Similar
antitumor effects were observed in a second tumor model using 4T1 tumors in BALB/c mice, in which the combination of either TKI with a vaccine targeting the transcription factor Twist resulted in smaller tumors compared with those in the control, TKI alone, or vaccine alone treatment (Supplementary Fig. S1A).

**Sunitinib plus vaccine increased antigen-specific tumor-infiltrating lymphocytes**

Sunitinib treatment can condition the TME by making it more amenable to T-cell infiltration and activation (1, 2, 20). To assess the effect of combining a TKI with vaccine on T-cell infiltration, mice were treated with sunitinib alone and in combination with vaccine, as described above. On day 21 after tumor transplant, sunitinib alone markedly reduced tumor volume compared with that of control or vaccine-treated mice, whereas the combination of sunitinib plus vaccine had the most profound antitumor activity compared with any other group (Fig. 2A).

Sunitinib and sorafenib treatments in combination with the rMVA–CEA–TRICOM vaccine caused an increase of CD4+ and CD8+ tumor-infiltrating lymphocytes (TILs) in 21-day-old MC38-CEA tumors in CEA-Tg mice s.c. (Fig. 1B and C). A similar increase in CD4+ and CD8+ TILs was observed in 25-day-old 4T1 tumors in BALB/c mice after treatment with either sunitinib or sorafenib in combination with a vaccine targeting the transcription factor Twist (Supplementary Fig. S1B). In addition, we measured CD3 T-cell infiltration by fluorescent IHC and the frequency of intratumoral CEA-specific CD8 by flow cytometry of a single-cell suspension of MC38-CEA tumors. The frequency of CD3 TILs (Fig. 2B and C) as well as CD8 T cells positive for the CEA524–531 tetramer (Fig. 2D) increased with combination treatment but not with individual therapies. Taken together, these data confirm the hypothesis that combining sunitinib with vaccine significantly decrease tumor volumes while increasing tumor-associated antigen (TAA)–specific TILs.

**Both sunitinib and sorafenib, either alone or in combination with vaccine, decreased tumor vasculature**

To evaluate the effects on tumor vasculature of combining antiangiogenic TKIs with therapeutic vaccines, CEA-Tg mice bearing MC38-CEA tumors were treated as previously described. Tissue sections from tumors harvested on day 21 were double-stained for the vascular markers CD31 and CD105. Because changes in tumor volume can affect vasculature and perfusion (13), tumors with similar dimensions (80–120 mm3 for all treatment groups) were used for IHC studies. Sunitinib alone or in combination with the rMVA–CEA–TRICOM vaccine significantly reduced the total vascular area compared...
with that of vaccine alone or control (Fig. 3A). To better define the effect of these therapies on mature (CD31⁺) or highly proliferating immature (CD105⁺) vessels, we performed fluorescent IHC and assessed the vascular area in both the periphery and center of tumors. In the periphery of tumors, sunitinib alone decreased CD31⁺ vascular area, whereas the combination with vaccine resulted in a significantly smaller vascular area compared with that of either treatment alone or control. In the center of tumors, CD31⁺ vasculature decreased with the combined regimen compared with that of either vaccine alone or control. Analysis of CD105⁺ immature vasculature at the periphery of tumors showed that sunitinib alone or in combination with vaccine decreased the vascular area compared with that of control. In the tumor center, vaccine alone increased vascular area compared with that of any other treatment (Fig. 3B). No statistical difference was found between the mature (CD31⁺) and immature (CD105⁺) vascular area, both in the center and in the periphery of tumors. This finding suggests that newly generated endothelial cells, positive for CD105, and mature endothelial cells, positive for CD31, were part of the tumor vascular tree with comparable distribution.

The total vascular area was similarly decreased in a second tumor model using 4T1 tumors in BALB/c mice treated with sunitinib and a vaccine targeting the transcription factor Twist (Supplementary Fig. S1C and S1D; refs. 14, 16, 21, 22). Taken together, these data confirm the hypothesis that antiangiogenic TKIs can significantly decrease both mature and immature tumor vasculature and indicate that adding vaccine further decreases mature vessels.

**Monocytic cells from bone marrow and spleens contribute to tumor vasculature**

Proangiogenic hematopoietic cells of monocytic origin may participate in vascular formation (23, 24). We hypothesized that monocytic cells can migrate from the bone marrow into tumors, where they can either become TAMs or form tumor vessels. To investigate whether monocytic cells can form vessels in MC38-CEA tumors, CD11b⁺ myeloid cells were isolated from the spleen or bone marrow of non–tumor-bearing C57BL/6 mice, labeled with PKH67 or PKH26, and injected i.v. into syngeneic mice bearing MC38-CEA tumors (Fig. 4A). Cells isolated from the bone marrow migrated into tumors in greater numbers than cells originating from the spleens (Fig. 4B). Fluorescent IHC showed the presence of CD31⁺ and CD105⁺ tumor vessels formed by transferred CD11b⁺ myeloid cells (Fig. 4C), confirming the existence of tumor vessels of monocytic origin.

**Sunitinib in combination with vaccine decreased monocytic tumor vasculature**

Because monocytes play a role in the formation of tumor vasculature, it is possible that antiangiogenic TKIs in combination with vaccine can decrease the recruitment of these cells into the tumor vasculature. We, thus, examined intratumoral vasculature of monocytic origin using the marker CD11b in CEA-Tg mice bearing 80 to 120 mm³ MC38-CEA tumors treated as previously described. Fluorescent IHC analyses showed that, compared with that of control, monocytic vascular area decreased 50% with sunitinib treatment alone, 14% with
vaccine alone, and 91% with the combination of sunitinib and vaccine (Fig. 4D and E). In contrast, the combined regimen of sunitinib plus vaccine significantly increased the number of CD11b\(^+\) scattered monocytes that were not forming vessels (Fig. 4F). These data confirm that treatment with sunitinib alone, and to a greater extent sunitinib plus vaccine, decreases monocytic tumor vasculature and increases scattered monocytes in MC38-CEA tumors.

**Sunitinib or sorafenib alone, vaccine alone, and the combination of either TKI with vaccine decreased tumor compactness and altered JAM-A expression**

Tumor vessels can collapse under the pressure exerted by tumor cells outside of the vascular wall, a phenomenon known as solid tumor stress (25). To evaluate the effect of combining antiangiogenic TKIs with therapeutic vaccines on vascular perfusion, CEA-Tg mice bearing MC38-CEA tumors were treated as previously described. Analysis of hematoxylin and eosin (H&E)–stained sections of tumors harvested on day 21 showed that cell density within the tumors was not homogeneous, as demonstrated by the presence of foci of unpacked (hypodense) tumor areas surrounded by packed (hyperdense) tumor areas (Fig. 5A and C and Supplementary Fig. S2). Measurements showed that the unpacked tumor area increased by treatment with sunitinib alone (6-fold), sorafenib alone (4-fold), vaccine alone (6-fold), sunitinib plus vaccine (8-fold), and sorafenib plus vaccine (6-fold) compared with that of control (Fig. 5D). Similar results were noted in a second tumor model using 4T1 tumors in BALB/c mice treated with sunitinib or sorafenib and a vaccine targeting the transcription factor Twist (Supplementary Fig. S3). We then studied the effects of the combined regimen on tight junctions by staining tumor sections with

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**Figure 3.** Sunitinib alone and in combination with rMVA–CEA–TRICOM vaccine reduced tumor vascular area in the MC38-CEA model. CEA-Tg mice bearing subcutaneous MC38-CEA tumors were treated as described in Fig. 1. Slides depict tumor sections obtained 21 days after tumor transplant. A, IHC of tumor sections (n = 3 mice/group) analyzed for the markers CD31-PECAM1 and CD105-endoglin to assess total tumor vasculature. CD31\(^+\)/CD105\(^+\) tumor vessels are shown in brown; black scale bars, 50 \(\mu\)m; columns, mean ± SEM total vascular area as a percentage of tumor area. B, IHC analysis of tumor sections (n = 3 mice/group) analyzed for the marker of mature endothelial cells CD31-PECAM1 (green-AF488) or for the marker of proliferating endothelial cells CD105-endoglin (green-AF488) plus the nuclear stain DAPI (blue) at the periphery and center of tumors; white scale bars, 250 \(\mu\)m; columns, mean ± SEM vascular area as a percentage of tumor area. Statistically significant differences based on ANOVA: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Sorafenib reduced intratumoral pressure

Vaccine alone and in combination with sunitinib or sunitinib reduced intratumoral pressure

We hypothesized that a decrease in tumor compactness and tight junctions could reduce the pressure that tumor cells exert against vessels within the tumor. To measure the extent of this force, we performed micropuncture experiments using an adapted protocol to measure tissue pressure (18) in MC38-CEA tumors in CEA-Tg mice on days 21, 26, and 31 following tumor transplant (Fig. 5E). On day 21, mice in the control and vaccine-alone groups had larger tumor volumes than those of the other groups (Fig. 2A); however, to exclude the effect of tumor dimension on tumor pressure, only mice with a tumor volume of 80 to 120 mm^{3} on day 21 were evaluated. Untreated tumors showed the highest pressure at each time point, compared with the tumor pressure of all other treatments (Fig. 5F); however, the pressure decreased in untreated tumors as they grew over time. Compared with that of control, treatment with sunitinib alone resulted in smaller tumors and slightly decreased pressure (except on day 31). Sorafenib alone did not change tumor pressure, but decreased tumor dimensions. Vaccine alone did not alter tumor dimensions, but significantly decreased tumor pressure. The combination of either sunitinib or sorafenib with vaccine decreased tumor pressure (except on day 31). Sorafenib alone increased the frequency of CD105^{+}, CD34^{+}, and CD105^{+},FAS-L^{+} MDSCs along with FAS-L^{+} TAMs, whereas both combination therapies increased three of four activation markers examined (Table 1). Additional studies in a second tumor model using 4T1 tumors in BALB/c mice treated with sunitinib showed similar outcomes. Sunitinib alone increased the frequency of CD105^{+} MDSCs, whereas sunitinib in combination with vaccine targeting the transcription factor Twist caused an increase of CXCL9^{+} and CD105^{+} MDSCs along with FAS-L^{+}, CXCL9^{+}, and CD105^{+} TAMs (Supplementary Table S1). Together, these results suggest that combining antiangiogenic TKIs with vaccine can increase the number of activated MDSCs and TAMs in the TME.

Sunitinib or sorafenib alone, independent of vaccine, altered the frequency and phenotype of tumor-infiltrating MDSCs and TAMs

Increased tumor oxygenation can affect the phenotype, and potentially the function, of MDSCs (26) and TAMs (27). To test whether combining antiangiogenic TKIs with vaccine can alter the activation of myeloid cells in the TME, CEA-Tg mice bearing MC38-CEA tumors were treated as previously described. On day 21 after transplant, spleens and tumors were harvested, and single-cell suspensions were analyzed by multicolor flow cytometry to assess the frequency of MDSCs and monocytes. In the spleens, sunitinib alone or in combination with the rMVA–CEA–TRICOM vaccine significantly decreased the frequency of monocytes (Supplementary Fig. S4A). In addition, the combination of sunitinib plus vaccine significantly decreased splenic MDSCs (Supplementary Fig. S4B). However, in the tumor, treatment with either sunitinib or sorafenib, with or without vaccine, significantly increased the frequency of tumor-infiltrating MDSCs, compared with those of control or vaccine alone treatment (Table 1). These decreases in MDSC frequency in the tumors coincided with elevated expression of the activation markers CXCL9 and FAS-L. The combination of sunitinib plus vaccine also increased the expression of activation marker CD105 in MDSCs (Table 1). Evaluation of TAMs showed that sorafenib, alone or in combination with vaccine, as well as sunitinib in combination with vaccine, significantly decreased the percentage of TAMs. Furthermore, in TAMs, sorafenib alone increased the percentage of all four activation markers examined, and sunitinib alone increased FAS-L^{+} TAMs, whereas both combination therapies increased three of four activation markers examined (Table 1). Additional studies in a second tumor model using 4T1 tumors in BALB/c mice treated with sunitinib showed similar outcomes. Sunitinib alone increased the frequency of CD105^{+} MDSCs, whereas sunitinib in combination with vaccine targeting the transcription factor Twist caused an increase of CXCL9^{+} and CD105^{+} MDSCs along with FAS-L^{+}, CXCL9^{+}, and CD105^{+} TAMs (Supplementary Table S1). Together, these results suggest that combining antiangiogenic TKIs with vaccine can increase the number of activated MDSCs and TAMs in the TME.

Effect on tumor oxygenation of sunitinib, sorafenib, or vaccine alone, and in combination therapy

An indirect way to measure improvement of tumor vascular perfusion is to assess tumor oxygenation. To investigate whether the above-described changes in tumor microarchitecture resulted in changes in tumor oxygenation, we assessed hypoxia in MC38-CEA tumors from mice treated as previously described, using pimonidazole/hypoxyprobe immunoenzymatic IHC (Fig. 6). Tumors from untreated mice were normally oxygenated at the periphery and hypoxic in the center, with approximately 50% of the total tumor area positive for hypoxia marker. Compared with control, treatment with sunitinib alone or in combination with vaccine increased zones of oxygenation by 20% and 40%, respectively. Sorafenib, alone or in combination with vaccine, increased tumor oxygenation by 20%. The increased tumor oxygenation supported the hypothesis that combining sunitinib with vaccine can improve tumor vascular perfusion.

JAM-A/DAB (Fig. 5B). In untreated tumors, JAM-A was localized either in cellular membranes or in the cytosol of tumor cells. Digital analysis using the Aperio ImageScope positive pixel analysis algorithm showed that total JAM-A expression did not change compared with that of control, but became increasingly internalized into the cytosol following treatment with either sunitinib alone or sorafenib alone. In contrast, vaccination with rMVA–CEA–TRICOM decreased JAM-A expression, but did not alter the membrane localization of JAM-A. The combination of either sunitinib or sorafenib with vaccine decreased the total expression of JAM-A similar to that with vaccine alone, and led to internalization of JAM-A into the cytosol. Taken together, these data indicate that both antiangiogenic TKIs and vaccine decrease tumor-cell density and cell-to-cell contact.

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Vaccine alone and in combination with sunitinib or sorafenib reduced intratumoral pressure

We hypothesized that a decrease in tumor compactness and tight junctions could reduce the pressure that tumor cells exert against vessels within the tumor. To measure the extent of this force, we performed micropuncture experiments using an adapted protocol to measure tissue pressure (18) in MC38-CEA tumors in CEA-Tg mice on days 21, 26, and 31 following tumor transplant (Fig. 5E). On day 21, mice in the control and vaccine-alone groups had larger tumor volumes than those of the other groups (Fig. 2A); however, to exclude the effect of tumor dimension on tumor pressure, only mice with a tumor volume of 80 to 120 mm^{3} on day 21 were evaluated. Untreated tumors showed the highest pressure at each time point, compared with the tumor pressure of all other treatments (Fig. 5F); however, the pressure decreased in untreated tumors as they grew over time. Compared with that of control, treatment with sunitinib alone resulted in smaller tumors and slightly decreased pressure (except on day 31). Sorafenib alone did not change tumor pressure, but decreased tumor dimensions. Vaccine alone did not alter tumor dimensions, but significantly decreased tumor pressure. The combination of either sunitinib or sorafenib with vaccine decreased tumor pressure and reduced tumor burden. Additional experiments were performed to investigate whether the observed decrease in tumor pressure measured in the vaccine-alone group was due to antigen-specific immune stimulation or due to a nonantigen-specific MVA vector–induced effect. Mice treated with the wild-type (WT)-MVA vaccine showed no difference in tumor pressure compared with that of unvaccinated control mice, whereas tumors from mice treated with rMVA–CEA–TRICOM had decreased tumor pressure compared with that of both the unvaccinated and WT-MVA groups (Fig. 5F). Altogether, these data suggest that vaccine decreases intratumoral pressure in a TAA-depencen.
Removal of spleen and BM

Monocyte selection

Spleen monocytes labeled with PKH67

BM monocytes labeled with PKH26

Monocytes injected i.v. in tumor-bearing C57BL/6 mice

3 days later, tumor removed and processed for FACS and IHC

Healthy C57/BL6

Figure 4. Sunitinib in combination with rMVA–CEA–TRICOM vaccine decreased monocytic vasculature and increased scattered monocytes in the MC38-CEA model. A, protocol of injection of isolated mononuclear cells. B, flow-cytometric analysis of tumor single-cell suspensions was performed 3 days after i.v. injection of CD11b+ cells; bars, mean of intratumoral CD11b+ cells ± SEM that originated from the spleen (white bar) or bone marrow (BM; black bar). C, IHC of tumor sections prepared 3 days after injection of magnetically selected PKH26-labeled CD11b+ cells from the bone marrow of tumor-free mice. The cell tracer PKH26 spontaneously emits red light. (Legend continued on the following page.)
Discussion

The effectiveness of cancer immunotherapy can be compromised when immune cells cannot penetrate the tumor (25). We propose here that combining an antiangiogenic TKI with a cancer vaccine can increase antitumor response by targeting three elements of the TME: (i) targeting tumor endothelial cells can lead to vascular normalization (Figs. 3 and 4; Supplementary Fig. S1); (ii) targeting tumor cells can reduce tumor compactness and allow collapsed vessels to reopen (Figs. 5 and 6; Supplementary Fig. S3); and (iii) targeting tumor-infiltrating immune cells can increase the frequency and function of effector immune elements, i.e., TILs and antitumor myeloid cells, and decrease the number and function of immune-suppressor cells, i.e., regulatory T cells (Treg), MDSCs, and suppressive TAMs (Fig. 2; Table 1; Supplementary Table S1). We have shown recently that the combination of sunitinib with the therapeutic vaccine rMVA–CEA–TRICOM increases CD4 and CD8 CEA–specific immune responses (1). Other authors have reported that sunitinib in combination with an ovalbumin (OVA) peptide-pulsed dendritic cell (DC) vaccine increases OVA-specific CD8+ splenocytes in C57Bl/6 mice (2). However, this benefit had not been compared with another antiangiogenic TKI such as sorafenib. Similar to sunitinib, sorafenib inhibits angiogenesis by blocking the phosphorylation of VEGFRs, PDGFRs, and other receptors on the cell membrane of tumor endothelial cells (19). The benefit of combining antiangiogenic TKIs with therapeutic vaccines is not limited to a specific TKI (Fig. 1), vaccine, or tumor model. In fact, similar to that shown in the MC38-CEA model, either sunitinib or sorafenib in combination with a recombinant vaccine targeting the transcription factor Twist (14, 16, 21, 23) decreased 4T1 breast tumors (Supplementary Fig. S1A). In both tumor models, the decrease in tumor dimensions coincided with an increase in CD4 and CD8 TILs (Fig. 1B and C and Supplementary Fig. S1B and S1C).

It is hypothesized that antiangiogenic TKIs used with vaccine can concur to normalize tumor vasculature. In support of this, sunitinib plus vaccine increased CD3+ TILs and tumor antigen–specific CD8+ T cells (Fig. 2). Tumor-infiltrating CD4 (5), CD8 (7), natural killer (NK), and NKT cells (28) can exert further antiangiogenic effects in an IFNγ-dependent fashion. This hypothesis is strengthened with a study by Huang and colleagues (29) showing that low-dose anti-VEGF antibody therapy, which normalizes rather than eradicates tumor vasculature, can facilitate the penetration of immune effector elements into the tumor parenchyma.

Because of the complexity of tumor vasculature, we analyzed the effect of each TKI alone and with vaccine using two endothelial markers: CD31 (PECAM1) and CD105 (endoglin). CD31 is an endothelial marker expressed in vessels in normal tissues and tumors. CD105, a marker of activated endothelium, is expressed only in proliferating cells (30) and observed almost exclusively in tumor vessels (31). We identified two patterns of vasculature: highly vascularized tumor peripheries and less-vascularized tumor centers (Fig. 3). Although sunitinib alone and sunitinib plus vaccine similarly decreased CD105+ vasculature, the combination therapy led to a greater reduction in CD31+ vasculature compared with sunitinib alone (Fig. 3B). Sunitinib plus a vaccine targeting the transcription factor Twist in BALB/c mice bearing established 4T1 tumors similarly decreased the total vascular area (Supplementary Fig. S1C and S1D).

Bone marrow–derived monocytes can participate in vascular regeneration after injury (32). Moreover, monocyte-derived multipotent cells can differentiate along the endothelial lineage upon stimulation with angiogenic growth factors. These endothelial cells are CD31+ and maintain monotypic markers during the first 3 to 5 days of vascular transformation (23). We found that CD11b+ monocytes can migrate from the spleen and, to a greater extent, from the bone marrow into MC38-CEA (Fig. 4A–C) and 4T1 (data not shown) tumors to participate in vessel formation. For this reason, we investigated changes in monocyte CD11b+ vessels. In MC38-CEA tumors, treatment with sunitinib alone decreased the monocytic vasculature, compared with that of control and of vaccine alone, whereas the combination of sunitinib with vaccine resulted in the greatest reduction in CD11b+ vasculature. In contrast, the number of scattered monocytes was increased, with the highest increase in the combination group (Fig. 4D–F). It is possible that, in untreated tumor-bearing mice, immature monocytes are recruited into the TME to differentiate into endothelial cells. The combination of sunitinib plus vaccine could inhibit monocyctic vascular transformation and, thus, drive monocytes toward a more canonical maturation. These observations are the fundament for planned studies in which bone marrow–derived myeloid cells from tumor-bearing animals will be transferred into tumor-bearing syngeneic recipients to assess whether bone marrow–derived myeloid cells from tumor-bearing mice can participate in the formation of the tumor vascular tree in a model that more closely mimics the situation in patients with cancer.

We observed that tumor compactness was not homogeneous. Zones of low cell density (unpacked) appeared adjacent to areas of high cell density (packed; Fig. 5A and C and Supplementary Fig. S2). Although most areas of untreated MC38-CEA tumors were hyperdense, treatment with sunitinib alone, vaccine alone, and the combination of sunitinib plus vaccine significantly increased the extent of unpacked areas (Fig. 5D). This effect was probably caused by therapy-driven tumor-cell cytotoxicity. Similar results were observed using sorafenib. Stylianopoulos and colleagues (25) investigated the physical forces imposed on tumor vessels, known as solid tumor stress, caused by overpacking of tumor cells outside of vessels. Solid tumor stress, which collapses small vessels and

(Continued.) Tumor sections were stained for the nuclear marker DAPI (blue) and for the vascular markers CD31 or CD105 (green-AF488). D, CEA-Tg mice (n = 3/group) bearing subcutaneous MC38-CEA tumors were treated as described in Fig. 1. IHC analysis of MC38-CEA tumor sections evaluated for the monocytic marker CD11b (green-AF488) and the nuclear stain DAPI (blue); yellow triangles, scattered CD11b+ monocytes; asterisks, vessels formed by monocytes; scale bars, 25 μm. E, tumor area occupied by monocytic vessels, F, number of scattered monocytes per mm2 of tumor area. E and F, data, mean ± SEM. Statistically significant differences based on ANOVA: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Figure 5. Effect of antiangiogenic TKIs and rMVA–CEA–TRICOM vaccine on tumor compactness, tight junctions, and intratumoral pressure in the MC38-CEA model. A, effect of antiangiogenic TKIs, vaccine, and their combination on tumor compactness. CEA-Tg mice (n = 3/group) bearing subcutaneous MC38-CEA tumors were treated as described in Fig. 1. H&E-stained tumor sections at ×10 magnification show tumor compactness. Less-compact areas are outlined in black. B, IHC analysis of tumor sections stained for the tight-junction-associated JAM-A; scale bars, 10 µm. Bright fields at ×100; black arrows, intercellular JAM-A; i, internalized JAM-A; pie charts, digital analysis of JAM-A expression. (Legend continued on the following page.)
results in inefficient vascular perfusion, is distinct from interstitial pressure, which is isotropic stress exerted by vascular fluid leakage in perivascular areas (33). We used a modified technique to measure tissue pressure (Fig. 5E; ref. 18). This technique does not distinguish between solid tumor stress and interstitial pressure, but can indicate overall pressure changes.

Figure 6. Antiangiogenic TKIs and rMVA–CEA–TRICOM vaccine increased tumor oxygenation in the MC38-CEA model. CEA-Tg mice (n = 3/group) bearing subcutaneous MC38-CEA tumors were treated as described in Fig. 1. A, digital markup of hypoxia of tumor sections using the marker pimonidazole (Hypoxyprobe-DAB). Magnifications of ×2 and ×20 are shown; ×20 magnifications correspond to squares drawn in the related ×2 images. B, example of the computer software–performed positive pixel count. Negative pixels are blue and indicate highly oxygenated cells; low positive, positive, and high positive pixels are yellow, orange, and brown, respectively, and correspond to mild, low, and very low oxygenated cells, respectively. Sections were also stained for the endothelial cell markers CD31 and CD105 (both vector-red). Green triangles, examples of cells negative for pimonidazole that were calculated as negative pixels (blue in the digital markup); yellow triangles, examples of cells positive for pimonidazole that were calculated as positive pixels (brown in the digital markup). Endothelial cells are red in the bright field. C, the percentage of hypoxic tumor area measured as number of positive pixels divided by total number of pixels. Positive pixels are the sum of low positive, positive, and high positive pixels; bars, SEM. Statistically significant differences based on ANOVA: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
within tumors after treatment. Untreated MC38-CEA tumors had the highest tissue pressure, which decreased over time, perhaps as a result of the formation of necrotic regions in the poorly vascularized tumor centers during tumor expansion. Notably, although treatment with either TKI alone caused the greatest decrease in tumor dimensions, treatment with vaccine alone mediated the greatest reduction in tumor pressure. Treatment with the combination of either sunitinib or sorafenib with vaccine led to both a reduction in tumor volume and a decrease in tumor pressure (Fig. 5F). WT-MVA did not reduce tumor volume (Fig. 5F).

The hemodynamic events described above, although not primarily immune related, can have considerable immune consequences. On the one hand, improved vascular perfusion can allow immune cells better access to the TME. On the other hand, the increase in tumor oxygenation can affect the phenotype and, potentially, the function of MDSCs (26) and TAMs (27) via an HIF1α mechanism. Although a decrease in the frequency of tumor-infiltrating MDSCs and TAMs may seem to reopen. Although the tumor vascular bed shrank following combination immunotherapy, tumor oxygenation significantly improved, especially at the center of tumors (Fig. 6), indicating that the remaining vessels had improved functionality.

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MC38-CEA tumor-bearing CEA-Tg mice. We document here that intratumoral CD11b+GR1+ MDSCs increased in MC38-CEA after treatment with either sunitinib or sorafenib, independent of the addition of vaccine but did not change in 4T1 tumors. However, TAMs decreased following treatment with sorafenib alone, sorafenib plus vaccine, and sunitinib plus vaccine in MC38-CEA tumors, but did not change in the 4T1 tumor model (Table I and Supplementary Table S1). These inconsistencies in the effect of antiangiogenic TKIs on the intratumoral frequency of tumor-infiltrating MDSCs and TAMs could be explained by the fact that the markers used to identify them may not be associated with their function (36, 37). For example, Ortiz and colleagues (37) have shown that, in healthy and control mice, some cells with a typical MDSC phenotype are actually immature myeloid cells, which lack immunosuppressive activity and, therefore, must be distinguished from suppressive MDSCs. In support of this hypothesis, we report here that MDSCs and TAMs in both the MC38-CEA and 4T1 tumor models showed a striking increase in the surface expression of four activation markers: FAS-L, CXCL-9, CD31, and CD105. These markers have been reported to be upregulated during myeloid cell activation (38, 39), maturation (40), and a type II to type I immune activation switch (6). It is possible that, in the presence of normal tumor oxygenation, these myeloid cells could become activated and, perhaps, more tumor lytic and less immunosuppressive. Studies to evaluate the suppressive function of tumor-infiltrating MDSCs and TAMs following treatment with antiangiogenic TKIs have been planned to confirm this hypothesis. The switch of tumor-infiltrating MDSC and TAM cells from an immune-suppressive to an immune-active phenotype, driven by both sunitinib and sorafenib, coincides with a decrease in the number and function of Tregs caused by both antiangiogenic TKIs. In fact, sunitinib can decrease the number and function of circulating Tregs in mouse models (1, 2) and in patients with renal cell carcinoma (20) via a VEGFA–VEGFR pathway blockade (41). Similarly, sorafenib has been shown to inhibit the proliferation and suppressive function of Tregs in patients with kidney cancer (42) and hepatocellular carcinoma (43).

If confirmed by additional studies, these observations could be extended to other multitargeted antiangiogenic TKIs, including but not limited to cabozantinib, pazopanib, axitinib, lapatinib, or imatinib, as well as to other antiangiogenic therapies, such as the anti-VEGF antibody bevacizumab. In preclinical models, low-dose anti-VEGFR2 antibody DC101, which normalized tumor vasculature (44), in combination with a whole-cell cancer vaccine, enhanced antitumor efficacy in a CD8+ T-cell–dependent manner in both immune-tolerant and immunogenic murine breast cancer models (29). In contrast, therapy with a high dose of the anti-VEGF antibody, which ablated tumor vasculature, in combination with vaccine failed to both increase T-cell tumor infiltration and improve antitumor responses (29). The architectural changes in the TME caused by combining antiangiogenic agents with cancer immunotherapy could be an integral step of the process that mediates improved antitumor responses.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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