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## 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 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 2 murine tumor models: colon carcinoma (MC38-CEA) and breast cancer (4T1). Tumor vasculature was measured by immunohistochemistry using 3 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 T 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 tumor microenvironment, 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.

### Keywords

TKI; sunitinib; sorafenib; vaccine; immunotherapy; angiogenesis; cancer

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## Introduction

Antiangiogenic tyrosine kinase inhibitors (TKI), in addition to their direct anti-vascular 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 cells (TIM), including myeloid-derived suppressor cells (MDSC) (3) and tumor-associated macrophages (TAM) (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 interferons (IFN) I and II in the tumor microenvironment (TME) (5–7). Thus, combining antiangiogenic TKIs with immunotherapy could have clinical benefit for cancer patients. 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) (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) were generated by retroviral transduction with CEA cDNA, as previously described (12). These cells are 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 other authentication assay was performed. Tumor cells were cultured as described (1). For *in vivo* studies,  $5 \times 10^5$  MC38-CEA cells were injected subcutaneously (s.c.) in the right flank of CEA-Tg mice. Tumor dimensions were measured weekly and tumor volumes were obtained using the formula  $(\text{length} \times \text{width}^2)/2$ . Because changes in tumor volume can affect vasculature and perfusion (13), tumors with similar dimensions (80–120 mm<sup>3</sup> for all treatment groups) were 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  $1 \times 10^8$  plaque-forming units/mouse (15, 16).

### Drug preparation and treatment schedule

Sunitinib malate salt > 99% diet was prepared as previously described (1). In additional experiments, sorafenib p-toluenesulfonate salt > 99% (LC Laboratories, Woburn, MA) was admixed with Open Standard Diet (Research Diets, New Brunswick, NJ), modeling the human dose of 400 mg BID (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 Supplementary Materials and Methods.

### Measurement of intratumoral pressure

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

### Flow cytometry evaluation of single-cell suspensions

CEA<sub>526-533</sub> and HIV-GAG tetramer staining were 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 (Franklin Lakes, NJ). Anti-CXCL9 Alexa Fluor 647 clone MIG-2F5.5, anti-CD105 PerCp-Cy5.5 clone MJ7/18, and anti-CD31 Pacific Blue clone 390 were purchased from BioLegend (San Diego, CA). Anti-CD45 eFluor 605NC clone 30-F11 and anti-FAS-L PerCP-eFluor 710 clone MFL3 were purchased from eBioscience (San Diego, CA). At least  $3 \times 10^5$  live cells were acquired with an LSR-II flow cytometer (BD Biosciences) and data were analyzed with FlowJo software for PC (TreeStar Inc., Ashland, OR).

### *In vivo* transfer of myeloid cells into tumor-bearing recipients

CD11b<sup>+</sup> cells were magnetically selected from the spleen or bone marrow (BM) of non-tumor-bearing C57BL/6 mice ( $n = 10$ ) (StemCell Technologies Inc., Vancouver, CA) following manufacturer's instructions. The negatively selected myeloid cells were then labeled with PKH67 or PKH26, respectively, (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<sup>+</sup> cells into the tumor.

### Statistical analysis

GraphPad Prism v. 5.04 statistical software (GraphPad Software, La Jolla, CA) was used for statistical analyses. The 2-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 Dunn's multiple comparison test was used for the other analyses. *P* < 0.05 was considered statistically significant.

## Results

### Sunitinib or sorafenib in combination with vaccine similarly enhance antitumor effect

The TKIs sunitinib and sorafenib inhibit a similar spectrum of tyrosine kinase receptors, including VEGFRs and platelet-derived growth factor receptors (PDGFR) (19). Specifically, although the two TKIs share many similar targets (VEGFR2, VEGFR3, PDGFR, 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 2 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 either 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 to those of untreated mice or mice receiving vaccine alone. In contrast, either 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, where the combination of either TKI with a vaccine targeting the transcription factor Twist resulted in smaller tumors compared to those in the control, TKI alone, or vaccine alone treatment (Suppl. Fig. 1A).

### 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 to that of control or vaccine-treated mice, while the combination of sunitinib plus vaccine had the most profound antitumor activity compared to any other group (Fig. 2A).

Sunitinib and sorafenib treatments in combination with the rMVA-CEA-TRICOM vaccine caused an increase of CD4<sup>+</sup> and CD8<sup>+</sup> TILs in 21-day old MC38-CEA tumors in CEA-Tg mice (Fig. 1B). A similar increase in CD4<sup>+</sup> and CD8<sup>+</sup> 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 (Suppl. Fig. 1B). 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 CEA<sub>524-531</sub> 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 mm<sup>3</sup> 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 to that of vaccine alone or control (Fig. 3A). To better define the effect of these therapies on mature (CD31<sup>+</sup>) or highly proliferating immature (CD105<sup>+</sup>) 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<sup>+</sup> vascular area, while the combination with vaccine resulted in a significantly smaller vascular area compared to that of either treatment alone or control. In the center of tumors, CD31<sup>+</sup> vasculature decreased with the combined regimen compared to that of either vaccine alone or control. Analysis of CD105<sup>+</sup> immature vasculature at the periphery of tumors showed that sunitinib alone or in combination with vaccine decreased the vascular area compared to that of control. In the tumor center, vaccine alone increased vascular area compared to that of any other treatment (Fig. 3B). There was no statistical difference between the mature (CD31<sup>+</sup>) and immature (CD105<sup>+</sup>) vascular area, both in the center and in the periphery of tumors. This finding suggests that newly-generated endotheliocytes, positive for CD105, and mature endotheliocytes, 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 (14, 16, 21, 22) (Suppl. Fig. 1C and D). 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 BM 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<sup>+</sup> myeloid cells were

isolated from the spleen or BM of non-tumor-bearing C57BL/6 mice, labeled with PKH67 or PKH26, and injected intravenously (i.v.) into syngeneic mice bearing MC38-CEA tumors (Fig. 4A). Cells isolated from the BM migrated into tumors in greater numbers than cells originating from the spleens (Fig. 4B). Fluorescent IHC showed the presence of CD31<sup>+</sup> and CD105<sup>+</sup> tumor vessels formed by transferred CD11b<sup>+</sup> 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 80–120 mm<sup>3</sup> MC38-CEA tumors treated as previously described. Fluorescent IHC analyses showed that, compared to 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<sup>+</sup> 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, decrease monocytic tumor vasculature and increase 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 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 Suppl. Fig. 2). Measurements showed that unpacked tumor area increased by treatment with sunitinib alone (4-fold), sorafenib alone (6-fold), vaccine alone (6-fold), sunitinib plus vaccine (8-fold), and sorafenib plus vaccine (6-fold) compared to 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 (Suppl. Fig. 3). We then studied the effects of the combined regimen on tight-junctions by staining tumor sections with junctional adhesion molecule-A (JAM-A)/DAB (Fig. 5B). In untreated tumors, JAM-A was either localized 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 to 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.

## 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 compared to 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 80–120 mm<sup>3</sup> on day 21 were evaluated. Untreated tumors showed the highest pressure at each time point compared to the tumor pressure of all other treatments (Fig. 5F); however, the pressure decreased in untreated tumors as they grew over time. Compared to 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 to a nonantigen-specific MVA vector-induced effect. Mice treated with the wild-type (WT)-MVA vaccine showed no difference in tumor pressure compared to that of unvaccinated control mice, while tumors from mice treated with rMVA-CEA-TRICOM had decreased tumor pressure compared to that of both the unvaccinated and WT-MVA groups (Fig. 5F). Altogether, these data suggest that vaccine decreases intratumoral pressure in a tumor-associated antigen-dependent fashion.

## 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/hypoxyprom probe immunoenzymatic IHC (Fig. 6). Tumors from untreated mice were normally oxygenated at the periphery and hypoxic in the center, with ~ 50% of the total tumor area positive for hypoxia marker. Compared to 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 agreed with the hypothesis that combining sunitinib with vaccine can improve tumor vascular perfusion.

## 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 were harvested and single-cell suspensions were analyzed by multi-color 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 (Suppl. Fig. 4A). In addition, the combination of sunitinib plus vaccine significantly decreased splenic MDSCs (Suppl. Fig. 4B). However, in the tumor, treatment with either sunitinib or sorafenib, with or without vaccine, significantly increased the frequency of tumor-infiltrating MDSCs compared to those of control or vaccine alone treatment (Table 1). These increases in MDSC frequency in the tumors coincided with elevated expression of the activation markers CXCL-9 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 4 activation markers examined, sunitinib alone increased FAS-L<sup>+</sup> MDSCs, while both combination therapies increased 3 of 4 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<sup>+</sup> MDSCs, while sunitinib in combination with a vaccine targeting the transcription factor Twist caused an increase of CXCL9<sup>+</sup> and CD105<sup>+</sup> MDSCs along with FAS-L<sup>+</sup>, CXCL9<sup>+</sup>, and CD105<sup>+</sup> TAMs (Suppl. Table 1). Together, these results suggest that combining antiangiogenic TKIs with vaccine can increase the number of activated MDSCs and TAMs in the TME.

## 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 3 elements of the TME: (a) targeting tumor endothelial cells can lead to vascular normalization (Figs. 3 and 4; Suppl. Fig. 1); (b) targeting tumor cells can reduce tumor compactness and allow collapsed vessels to reopen (Figs. 5 and 6; Suppl. Fig. 3); (c) 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. Tregs, MDSCs, and suppressive TAMs (Fig. 2; Table 1; Suppl. Table 1). 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). Others have reported that sunitinib in combination with an ovalbumin (OVA) peptide-pulsed dendritic cell vaccine increases OVA-specific CD8<sup>+</sup> 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, 22) decreased 4T1 breast tumors (Suppl. Fig. 1A). In both tumor models, the decrease in tumor dimensions coincided with an increase in CD4 and CD8 TILs (Fig 1B–C and Suppl. Fig. 1B–C).

It is possible that antiangiogenic TKIs used with vaccine can concur to normalize tumor vasculature. In fact, sunitinib plus vaccine increased CD3<sup>+</sup> TILs and tumor antigen-specific CD8<sup>+</sup> T cells (Fig. 2). Tumor-infiltrating CD4 (5), CD8 (7), NK, and NKT cells (28) can exert further antiangiogenic effects in an IFN $\gamma$ -dependent fashion. This hypothesis is in line with studies by Jain and Huang 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 (29).

Because of the complexity of tumor vasculature, we analyzed the effect of each TKI alone and with vaccine using 2 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 2 patterns of vasculature: highly vascularized tumor peripheries and less vascularized tumor centers (Fig. 3). While sunitinib alone and sunitinib plus vaccine similarly decreased CD105<sup>+</sup> vasculature, the combination therapy led to a greater reduction in CD31<sup>+</sup> vasculature compared to 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 (Suppl. Fig. 1C and D).

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<sup>+</sup> and maintain monocytic markers during the first 3–5 days of vascular transformation (23). We found that CD11b<sup>+</sup> monocytic cells can migrate from the spleen and, to a greater extent, from the BM into MC38-CEA (Fig. 4A–C) and 4T1 (data not shown) tumors to participate in vessel formation. For this reason, we investigated changes in monocytic CD11b<sup>+</sup> vessels. In MC38-CEA tumors, treatment with sunitinib alone decreased the monocytic vasculature compared to that of control and of vaccine alone, while the combination of sunitinib with vaccine resulted in the greatest reduction in CD11b<sup>+</sup> 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 monocytic vascular transformation and thus drive monocytes toward a more canonical maturation. These observations are the fundament for planned studies in which BM-derived myeloid cells from tumor-bearing animals will be transferred into tumor-bearing syngeneic recipients to assess whether BM-derived myeloid cells can participate in the formation of the tumor vascular tree in a model that more closely mimics the situation in cancer patients.

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

Suppl. Fig. 2). While 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 Jain investigated the physical forces imposed on tumor vessels, known as solid tumor stress (25), caused by overpacking of tumor cells outside of vessels. Solid tumor stress, which collapses small vessels and results in inefficient vascular perfusion, is distinct from interstitial pressure, which is isotropic stress exerted by vascular fluid leakage in perivascular areas (33). We employed a modified technique to measure tissue pressure (Fig. 5E) (18). This technique does not distinguish between solid tumor stress and interstitial pressure, but can indicate overall pressure changes 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, while 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 pressure, suggesting that the vaccine-mediated reduction in tumor pressure could be mediated by tumor antigen-specific immune cells.

Proinflammatory cytokines such as  $\text{IFN}\gamma$  and  $\text{TNF-}\alpha$  can affect the tight-junction marker JAM-A (34). By triggering a Th1-type immune response, sunitinib increases  $\text{IFN}\gamma$  production from T lymphocytes within renal cell carcinoma tumors (20). We have previously shown that vaccination with rV/F-CEA-TRICOM leads to enhanced production of  $\text{TNF-}\alpha$  and  $\text{IFN}\gamma$  by T cells in response to CEA-specific epitopes (35). While JAM-A was internalized from the cell surface into the cytosol following sunitinib treatment, vaccine alone decreased JAM-A expression without altering its localization, and combination therapy led to both internalization and decreased expression of JAM-A. Similar results were observed using sorafenib (Fig. 5B). These data suggest that reducing tumor compactness while modulating tight-junctions between tumor cells may work in concert to reduce solid tumor stress, allowing collapsed tumor vessels 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.

The hemodynamic events described above, while not primarily immune-related, can have considerable immune consequences. On 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 HIF-1 $\alpha$  mechanism. Although a decrease in the frequency of tumor-infiltrating MDSCs and TAMs may appear to be the logical consequence of using antiangiogenic TKIs, results from a number of studies belie this concept. Bose and Finke have shown that sunitinib, alone or in combination with a dendritic cell vaccine, was associated with a decrease of  $\text{CD11b}^+\text{GR1}^+$  MDSCs within the TME of B16.OVA melanoma (2); however, they reported in the 4T1 tumor model that  $\text{CD11b}^+\text{GR1}^+$  MDSCs

decreased in the spleens but not in the TME after sunitinib treatment (9). We have shown previously that sunitinib with rMVA-CEA-TRICOM vaccine decreased highly suppressive intratumoral CD11b<sup>+</sup>GR1<sup>dim</sup>IL4Rα<sup>+</sup> MDSCs in MC38-CEA tumor-bearing CEA-Tg mice (1). We document here that intratumoral CD11b<sup>+</sup>GR1<sup>+</sup> 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 1, Suppl. Table 1). 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 Gabrilovich (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 4 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 2 to type 1 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 MDSCs and TAMs 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 anti-angiogenic 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 cancer cell vaccine, enhanced anticancer efficacy in a CD8<sup>+</sup> 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).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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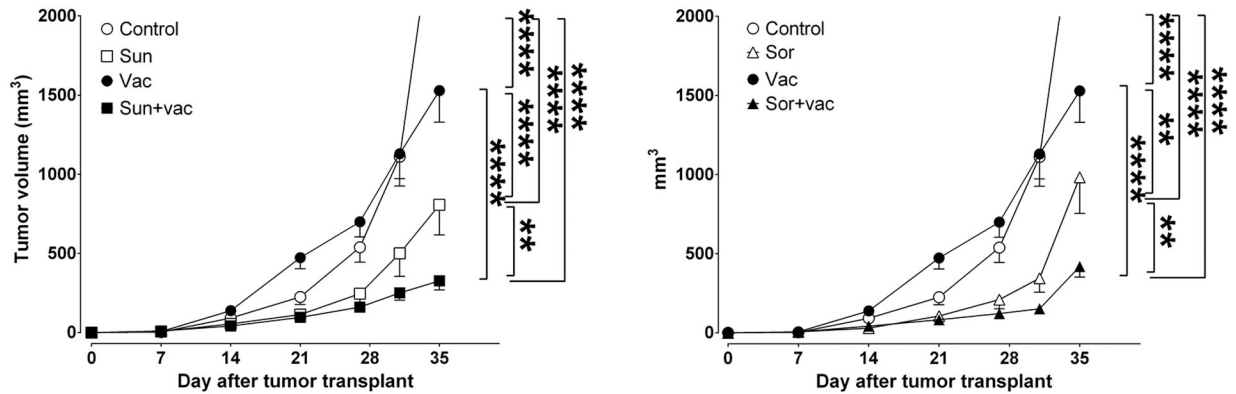
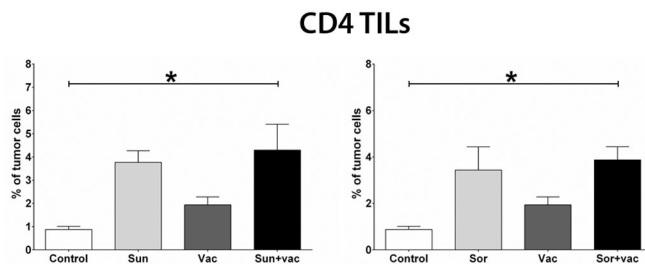
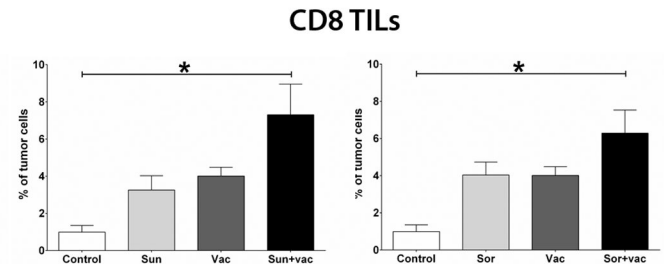
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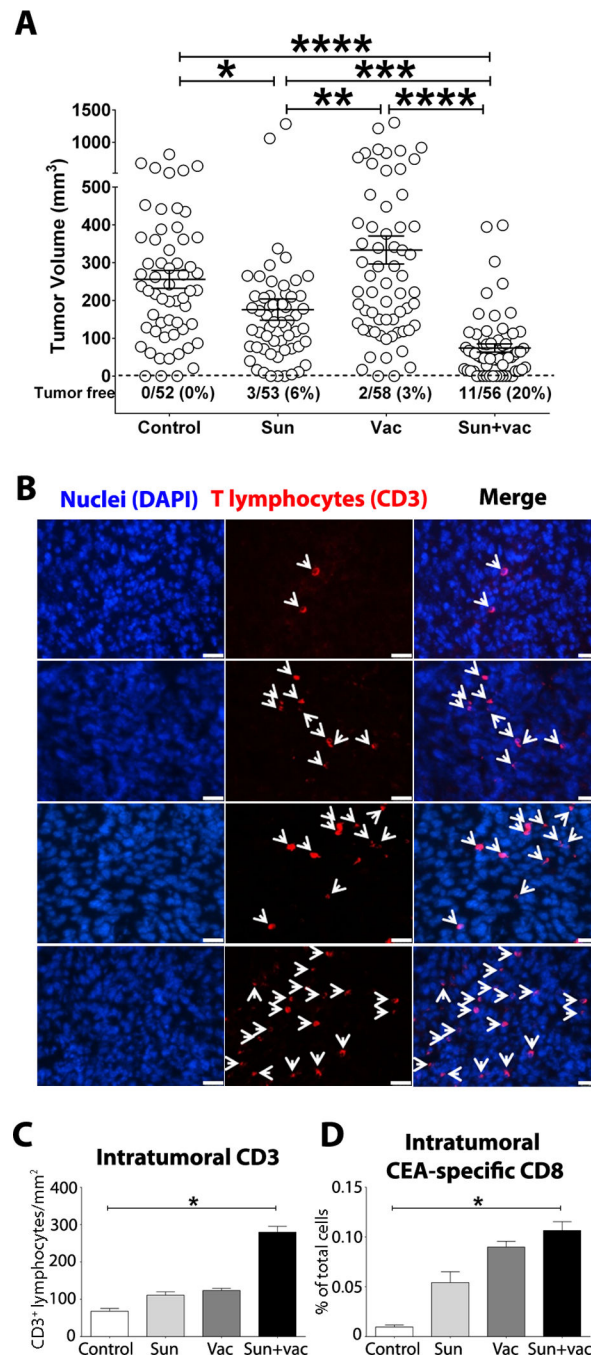
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**A****B****C****Figure 1.**

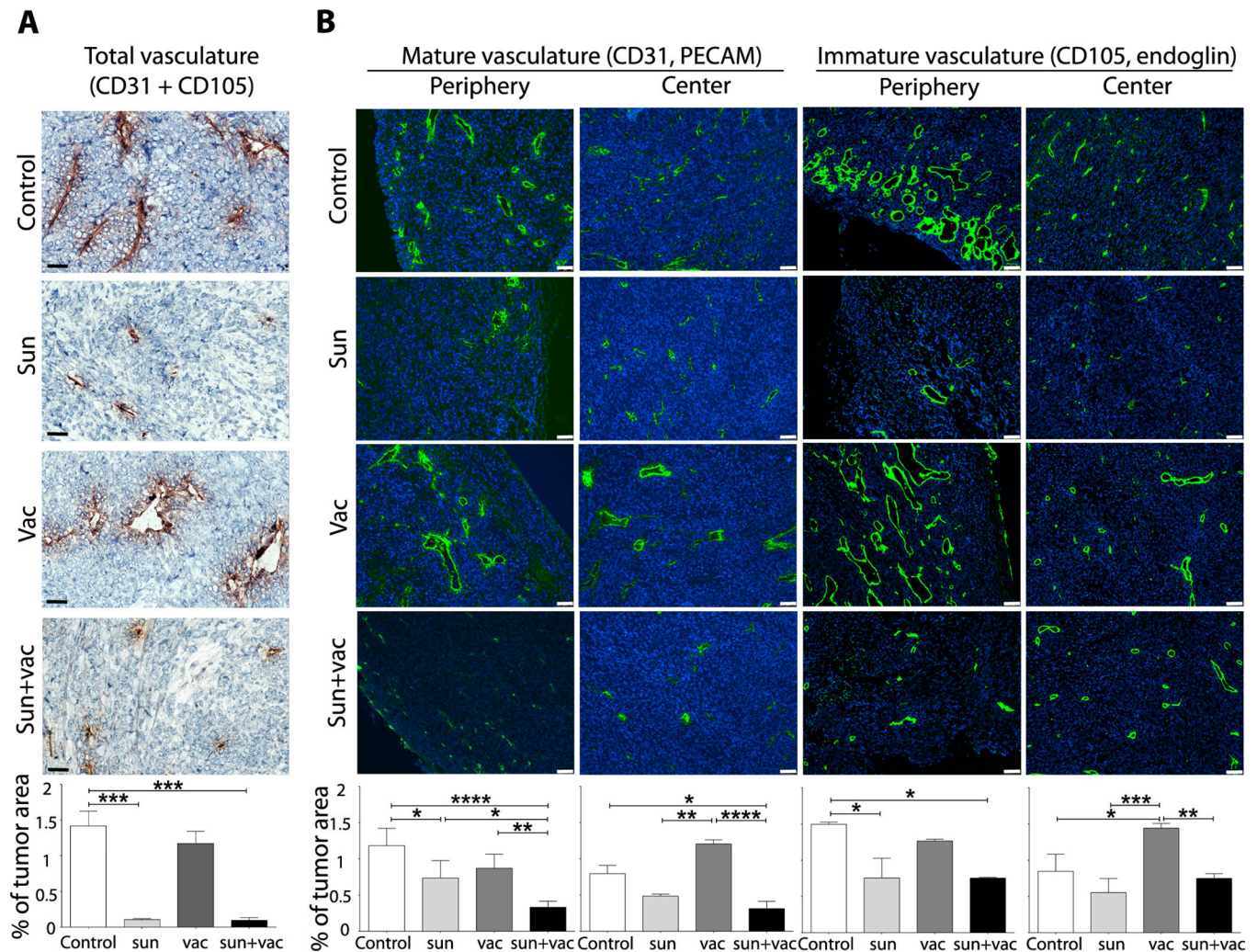
Sunitinib and sorafenib have similar antitumor effects when administered alone or in combination with recombinant vaccine. C57BL/6 mice ( $n=12-24$  from 2 independent experiments) bearing MC38-CEA tumors were treated as follows. Control: control diet starting 7 days after tumor transplant. Sun: sunitinib diet starting 7 days after tumor transplant. Sor: sorafenib diet starting 7 days after tumor transplant. Vac: control diet starting 7 days after tumor transplant, rMVA-CEA-TRICOM vaccine on day 14. Sun+vac: sunitinib diet starting 7 days after tumor transplant, rMVA-CEA-TRICOM vaccine on day 14 followed by weekly boosts with rF-CEA-TRICOM. Sor+vac: sorafenib diet starting 7 days after tumor transplant, rMVA-CEA-TRICOM vaccine on day 14 followed by weekly boosts with rF-CEA-TRICOM. A, Volumes were calculated as  $(\text{length} \times \text{width}^2)/2$ . B-C, CD4 (B) and CD8 (C) TILs measured by flow-cytometry of single-cell suspensions of enzymatically digested MC38-CEA tumors. Values: means  $\pm$  SEM. Statistically significant differences at day 35 after tumor transplant, based on ANOVA: \* $P < 0.05$ , \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ .



**Figure 2.** Sunitinib plus rMVA-CEA-TRICOM vaccine decreased tumor burden and increased intratumoral infiltration of T lymphocytes in the MC38-CEA colon carcinoma model. A, volumes of 21-day-old MC38-CEA tumors from 52–58 CEA-Tg mice/group from 6 independent experiments. Circles: individual measurements; bars: mean $\pm$ SEM. B, IHC of tumor sections harvested 21 days after tumor transplant from 3 mice/group analyzed for the T-cell marker CD3 (red-AF594) and the nuclear stain DAPI (blue). White arrows: CD3<sup>+</sup> T lymphocytes. Scale bars: 25  $\mu\text{m}$ . C, number of intratumoral CD3 T lymphocytes/ $\text{mm}^2$ . D,

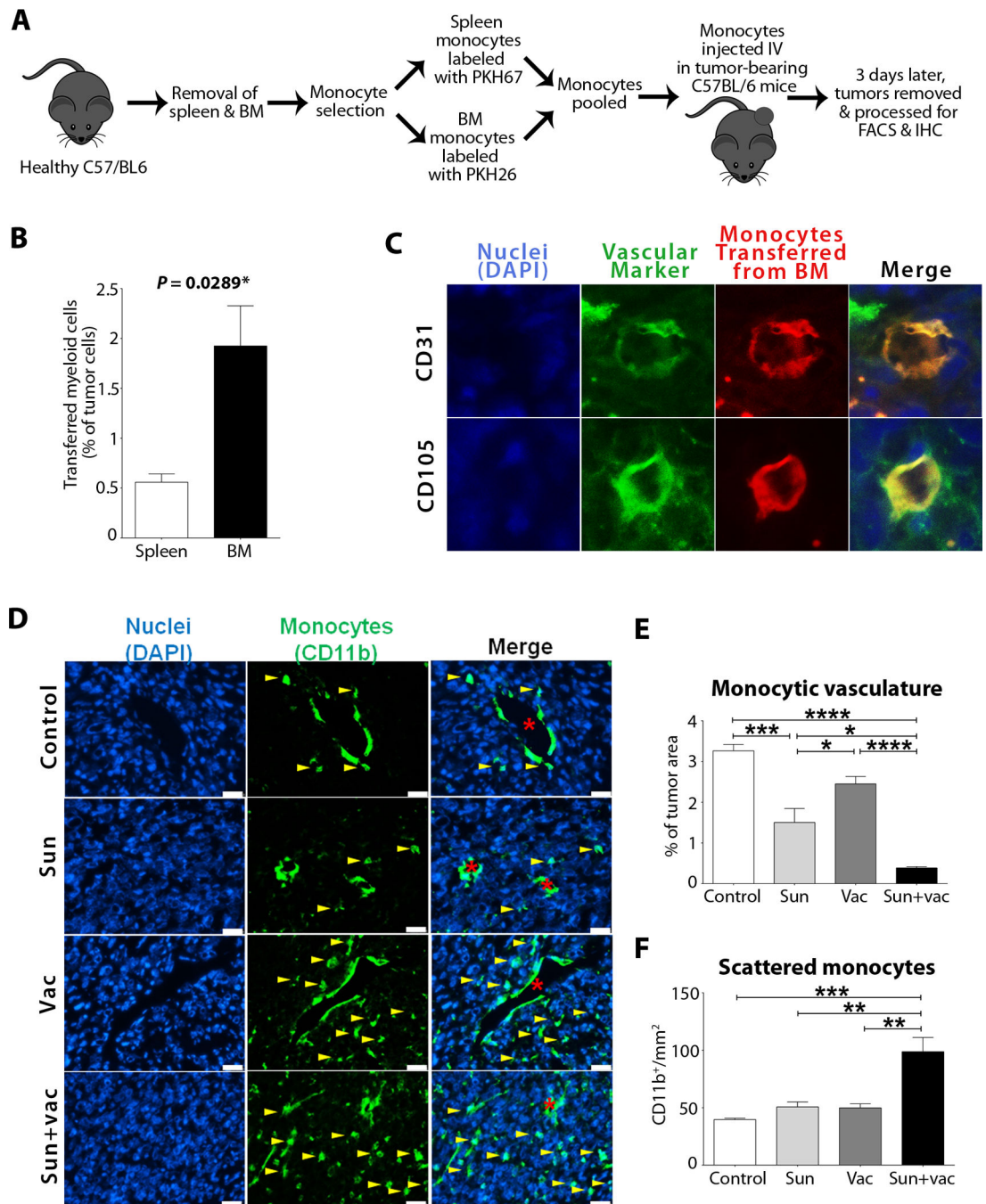


percentage of CapM8 CEA-specific tumor-infiltrating CD8 T lymphocytes, calculated by flow-cytometry. The number of HIV-GAG<sup>+</sup> lymphocytes was subtracted from the total of CapM8 CEA-tetramer<sup>+</sup> events. Bars: mean  $\pm$  SEM. Statistically significant difference based on ANOVA: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\*  $P < 0.001$ ; \*\*\*\* $P < 0.0001$ .



**Figure 3.**

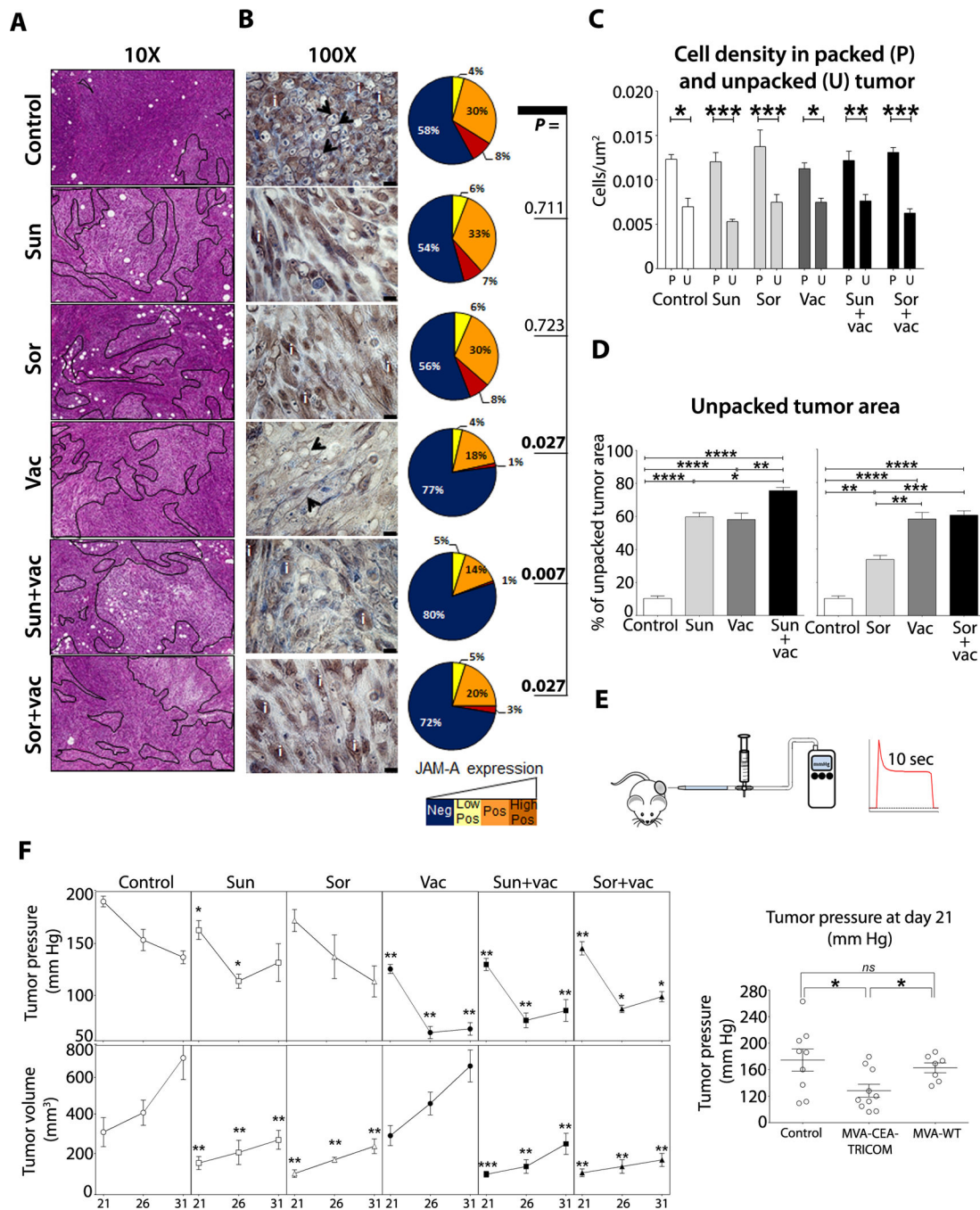
Sunitinib alone and in combination with rMVA-CEA-TRICOM vaccine reduced tumor vascular area in the MC38-CEA model. CEA-Tg mice bearing s.c. MC38-CEA tumors were treated as described in Figure 1. Slides depict tumor sections obtained 21 days after tumor transplant. A, IHC of tumor sections ( $n=3$  mice/group) analyzed for the markers CD31-PECAM1 and CD105-endoglin to assess total tumor vasculature. CD31<sup>+</sup>/CD105<sup>+</sup> tumor vessels are shown in brown. Black scale bars: 50  $\mu$ m. Columns: mean  $\pm$  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 indicate 250  $\mu$ m. Columns represent mean  $\pm$  SEM vascular area as a percentage of tumor area. Statistically significant differences based on ANOVA: \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ ; \*\*\*\* $P<0.0001$ .



**Figure 4.**

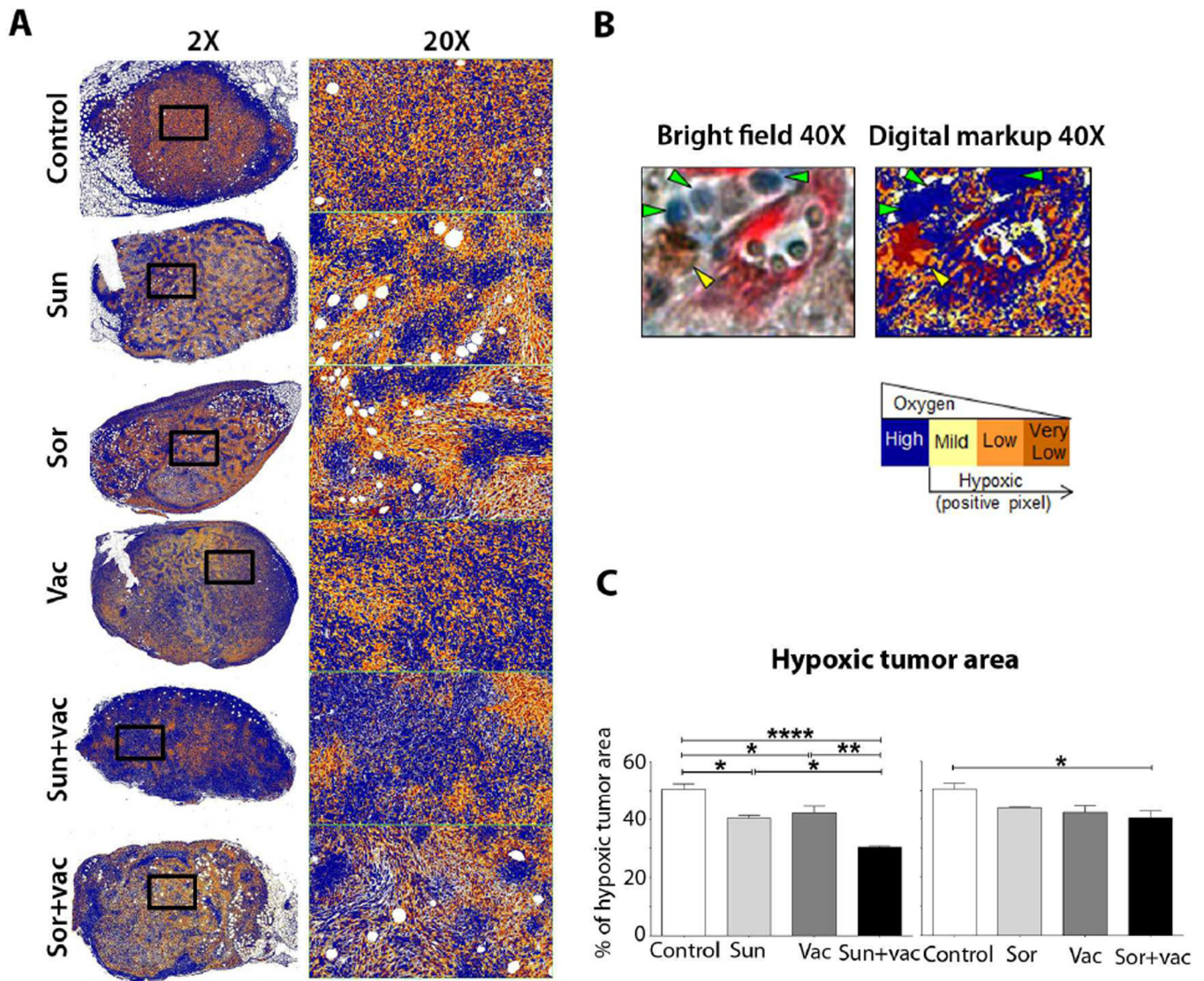
Sunitinib in combination with rMVA-CEA-TRICOM vaccine decreased monocytic vasculature and increased scattered monocytes in the MC38-CEA model. A, protocol of injection of isolated monocytic cells. B, Flow-cytometry analysis of tumor single-cell suspensions was performed 3 days after i.v. injection of CD11b<sup>+</sup> cells. Bars: mean of intratumoral CD11b<sup>+</sup> cells $\pm$ SEM that originated from the spleen (white bar) or BM (black bar). C, IHC of tumor sections prepared 3 days after injection of magnetically selected PKH26-labeled CD11b<sup>+</sup> cells from the BM of tumor-free mice. The cell tracer PKH26

spontaneously emits red light. 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 s.c. MC38-CEA tumors were treated as described in Figure 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<sup>+</sup> monocytes. Asterisks: vessels formed by monocytes. Scale bars: 25  $\mu$ m. E, tumor area occupied by monocytic vessels. F, number of scattered monocytes per mm<sup>2</sup> of tumor area. Data in E and F represent mean $\pm$ 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 s.c. MC38-CEA tumors were treated as described in Figure 1. H&E-stained tumor sections at 10X 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  $\mu$ m. Bright fields at 100X. Black arrows: intercellular

JAM-A. i: internalized JAM-A. Pie charts: digital analysis of JAM-A expression. Statistical analysis based on *t* test compared to control. Bold numbers: statistically significant difference ( $P < 0.05$ ). C, cell density in packed areas (P) was higher than in unpacked areas (U) of tumor, independent of treatment. Columns: averages of packed (P) or unpacked (U) tumor area for each treatment. Bars: SEM. Statistical analysis based on *t* test comparing packed and unpacked areas with each treatment. D, each individual treatment and the combination of TKI plus vaccine increased the extent of unpacked tumor area. Columns: averages of unpacked tumor areas. Bars: SEM. Statistically significant differences based on ANOVA. E, intratumoral pressure analysis of MC38-CEA tumors from CEA-Tg mice ( $n = 15-20$ /group from 2 independent experiments) treated as described in Figure 1. Vaccinated mice also received rF-CEA-TRICOM boosts on days 21 and 28. F, intratumoral pressure was measured on days 21, 26, and 31 after tumor transplant. Right graph: CEA-Tg B57BL/6 mice ( $n = 7-10$ /group) bearing s.c. MC38-CEA tumors were vaccinated with rMVA-CEA-TRICOM, WT-MVA, or left untreated. Intratumoral pressure was measured 21 days after tumor transplant. Statistically significant differences based on ANOVA. \* $P < 0.05$ ; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . \*\*\*\* $P < 0.0001$



**Figure 6.** Antiangiogenic TKIs and rMVA-CEA-TRICOM vaccine increased tumor oxygenation in the MC38-CEA model. CEA-Tg mice ( $n=3$ /group) bearing s.c. MC38-CEA tumors were treated as described in Figure 1. A, digital markup of hypoxia of tumor sections using the marker pimonidazole (Hypoxyprobe-DAB). Magnifications of 2X and 20X are shown; 20X magnifications correspond to squares drawn in the related 2X 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, 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: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ .



Effect of sunitinib, sorafenib, and rMVA-CEA-TRICOM vaccine on the phenotype of tumor-infiltrating MDSCs and TAMs in the MC38-CEA tumor model.

Table 1

	Control % (± SEM)	Sun % (± SEM)	Sor % (± SEM)	Vac % (± SEM)	Sun+vac % (± SEM)	Sor+vac % (± SEM)
<b>Total MDSCs<sup>a</sup></b>	6.3 (1.1)	<b>20.3 (2.5)**</b>	<b>21.2 (1.1)**</b>	8.4 (1.5)	<b>22.7 (3.0)**</b>	<b>22.1 (4.9)**</b>
<b>MDSCs</b>						
<b>FAS-L<sup>b</sup></b>	18.8 (3.2)	<b>35.2 (5.5)*</b>	<b>41.7 (3.3)**</b>	19.9 (3.6)	<b>39.6 (2.3)**</b>	<b>33.5 (3.1)*</b>
<b>CXCL-9<sup>b</sup></b>	33.1 (5.6)	<b>59.3 (9.6)*</b>	<b>65.2 (8.8)*</b>	40.0 (2.2)	<b>59.9 (2.6)*</b>	<b>57.0 (5.2)*</b>
<b>CD31<sup>b</sup></b>	1.5 (0.2)	6.8 (3.1)	12.2 (5.1)	2.3 (0.6)	8.5 (0.7)	8.1 (2.6)
<b>CD105<sup>b</sup></b>	38.3 (4.0)	60.2 (10.6)	65.9 (12.6)	40.3 (2.0)	<b>71.1 (2.4)*</b>	54.6 (3.7)
<b>Total TAMs<sup>b</sup></b>	65.0 (4.2)	54.7 (5.2)	<b>44.5 (6.6)*</b>	62.5 (1.7)	<b>42.9 (4.4)*</b>	<b>44.1 (1.1)*</b>
<b>TAMs</b>						
<b>FAS-L<sup>d</sup></b>	18.0 (2.2)	<b>45.7 (8.9)*</b>	<b>55.3 (14.1)*</b>	21.3 (3.7)	<b>58.6 (3.9)*</b>	<b>47.2 (4.1)*</b>
<b>CXCL-9<sup>d</sup></b>	0.5 (0.2)	4.3 (2.7)	<b>14.3 (2.7)***</b>	0.8 (0.3)	5.9 (0.8)	<b>11.0 (2.2)**</b>
<b>CD31<sup>d</sup></b>	2.9 (0.8)	11.1 (2.7)	<b>14.9 (3.8)*</b>	3.5 (0.8)	<b>14.5 (0.7)*</b>	<b>13.1 (3.1)*</b>
<b>CD105<sup>d</sup></b>	33.4 (3.7)	59.6 (14.8)	<b>70.0 (13.4)*</b>	34.9 (3.7)	<b>74.5 (2.0)*</b>	58.0 (5.9)

Flow cytometry analysis of single-cell suspensions of 21-day-old MC38-CEA s.c. tumors from CEA-ig C57BL/6 mice ( $n = 3/\text{group}$ ). Control: no treatment. Sor: sorafenib on day 7. Sun: sunitinib on day 7. Vac: rMVA-CEA-TRICOM vaccine on day 14. Sor+vac: sorafenib on day 7 followed by rMVA-CEA-TRICOM vaccine on day 14. Sun+vac: sunitinib on day 7 followed by rMVA-CEA-TRICOM vaccine on day 14.

<sup>a</sup> percentage of MDSCs identified as CD45<sup>+</sup> intratumoral cells CD11b<sup>+</sup>Gr1<sup>+</sup>.

<sup>b</sup> frequency of MDSCs positive for the activation marker.

<sup>c</sup> percentage of TAMs identified as CD45<sup>+</sup> intratumoral cells CD11b<sup>+</sup>Gr1<sup>+</sup>.

<sup>d</sup> frequency of TAMs positive for the activation marker. SEM: standard error of the mean. Bold values: statistically significant difference compared to control based on one-way ANOVA test vs. control group.

\*  $P < 0.05$ ;

\*\*  $P < 0.01$ ;

\*\*\*  $P < 0.001$ .