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Sorafenib combined with HER-2 targeted vaccination can promote effective T cell immunity *in vivo*

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Abstract

The tumor microenvironment (TME) is established and maintained through complex interactions between tumor cells and host stromal elements. Therefore, therapies that target multiple cellular components of the tumor may be most effective. Sorafenib, a multi-kinase inhibitor, alters signaling pathways in both tumor cells and host stromal cells. Thus, we explored the potential immune-modulating effects of sorafenib in a murine HER-2-(neu) overexpressing breast tumor model alone and in combination with a HER-2 targeted granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting vaccine (3T3neuGM). *In vitro*, sorafenib inhibited the growth of HER-2 overexpressing NT2.5 tumor cells, inducing apoptosis. Sorafenib also interfered with ERK MAPK, p38 MAPK, and STAT3 signaling, as well as cyclin D expression, but did not affect HER-2 or AKT signaling. *In vivo*, single agent sorafenib disrupted the tumor-associated vasculature and induced tumor cell apoptosis, effectively inducing the regression of established NT2.5 tumors in immune competent FVB/N mice. Immune depletion studies demonstrated that both CD4⁺ and CD8⁺ T cells were required for tumor regression. Sorafenib treatment did not impact the rate of tumor clearance induced by vaccination with 3T3neuGM in tumor-bearing FVB/N mice relative to either sorafenib treatment or vaccination alone. *In vivo* studies further demonstrated that sorafenib enhanced the accumulation of both CD4⁺ and CD8⁺ T cells into the

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Conflict of interest

Under a licensing agreement between Aduro Biotech and the Johns Hopkins University, the University and Dr. Emens are entitled to milestone payments and royalty on sales of the GM-CSF-secreting breast cancer vaccine. The terms of these arrangements are being managed by the Johns Hopkins University in accordance with its conflict of interest policies.

TME of vaccinated mice. Together, these findings suggest that GM-CSF-secreting cellular immunotherapy may be integrated with sorafenib without impairing vaccine-based immune responses.

Keywords

Breast cancer; Sorafenib; Immunotherapy; Vaccine; Immunity

1. Introduction

Signaling pathways that drive oncogenesis and promote tumor progression are key targets for cancer therapy. The biologic instability of tumors can lead to therapeutic escape through the activation of compensatory signaling pathways that promote secondary drug resistance. Increasing data support a role for oncogenic signaling pathways in both tumor-associated immune responses, suppression of tumor recognition and clearance through reinforcement of tumor tolerance [1–4]. At the same time, the high mutation rates of tumors produce tumor neo-antigens that may represent the most effective targets of the T cell-mediated immune response. Therefore, understanding the immune modulating activities of drugs that abrogate essential oncogenic signaling is critical for maximizing their therapeutic efficacy, particularly when combined with immunotherapy.

Our group has explored the impact of standard and novel cancer drugs on the immune system [5–8]. In particular, we previously reported that the vascular endothelial growth factor receptor-2 (VEGFR-2)-specific monoclonal antibody DC101 not only disrupts the tumor-associated vasculature, but also promotes T cell-dependent, immune-mediated tumor regression [9]. We showed that DC101 could also be combined with a granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting tumor cell vaccine (3T3neuGM). Our previous data support the use of highly specific therapies targeting multiple cellular components of the tumor in combination with immunotherapy. Further supporting this, several groups have shown that the tyrosine kinase angiogenesis inhibitor, sunitinib, promotes antitumor immunity [10,11]. Less is known about the effects of sorafenib on the immune system. Here we explore the immune-based activity of sorafenib, a promiscuous small molecule kinase inhibitor that blocks signaling in both tumor cells and host endothelial cells [12].

Sorafenib is a small molecule inhibitor of angiogenesis designed to inhibit RAF/MEK/ERK signaling. It has a number of off-target effects including the inhibition of wildtype and mutant BRAF, STAT3, and the receptor tyrosine kinases VEGFR2, VEGFR3, and PDGFR- β [12]. Sorafenib is FDA-approved for the treatment of hepatocellular carcinoma (HCC), renal cell carcinoma (RCC), and differentiated thyroid cancer (DTC) [12–15], and is also under investigation in other tumor types. Sorafenib has been reported to support anti-tumor immunity by decreasing the frequency of CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) without impacting the function of peripheral effector T cells in patients with RCC [16]. Conversely, sorafenib has been shown to inhibit dendritic cell (DC) function by reducing DC maturation, migration, and T cell priming [17]. The variable positive and negative effects of

sorafenib on tumor-specific immunity suggest that its immune-modulating activity could be context-and concentration-dependent [16–19].

Given the established clinical indications for sorafenib, the increasing use of immunotherapy in the clinic, and the complex immune effects of sorafenib, we investigated the immune-modulating effects of sorafenib alone and combined with the DC-based, HER-2-targeted, GM-CSF-secreting vaccine (3T3neuGM) in tumor-bearing immune-competent FVB/N mice. Here, in contrast to prior reports that sorafenib can inhibit DC-based vaccination, we show that sorafenib does not inhibit HER-2-specific T cell responses induced by 3T3neuGM [17–19]. Instead, it supports vaccine-induced tumor clearance by increasing tumor cell death, disrupting the tumor-associated vasculature, and increasing the accumulation of both CD4⁺ and CD8⁺ T cells at the tumor site.

2. Materials and methods

2.1. Materials

Sorafenib was purchased from LC Laboratories (Woburn, MA). For *in vitro* studies, sorafenib was dissolved in dimethyl sulfoxide (DMSO) and further diluted in culture medium to the required concentration with the final concentration of DMSO concentration <0.2%. The p38 pathway inhibitor SB203580 was purchased from Sigma-Aldrich (St. Louis, MO). The ERK pathway inhibitor U0126 was purchased from Invitrogen (Carlsbad, CA). Antibodies for p-STAT3 (Tyr705), STAT3, p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-p38 (Thr180/Tyr182), p38, p-AKT (Ser473), AKT, p-HER2 (Tyr877), HER2, Cyclin D1, Cyclin D2, Cyclin D3, BCLXL, BCL2, and activated caspase 3 were all purchased from Cell Signaling Technologies (Beverly, MA). The actin antibody was purchased from Calbiochem (San Diego, CA). Rabbit anti-mouse PECAM/CD31 antibody was purchased from Abcam (Cambridge, MA). Clodronate liposomes were provided by Dr. Nico van Rooijen (Vrije Universiteit, VUMC, The Netherlands). The α -asialo GM1 antibody was purchased from Wako Chemical (Richmond, VA). Anti-Ly6G FITC, Anti-PD1 PE, Anti-PDL1 PE, Anti-Tbet PE, Anti-MHCII PE, Anti-OX40 PE, Anti-Foxp3 PE, Anti-F480 PercPCy5.5, Anti-Thy1.2 APC, Anti-TIM-3 Alexa 647, Anti-CD4 APCCy7, Anti-41BB PECy7, Anti-Ki67 PECy7, Anti-IFN γ PECy7, Anti-Gata-3 PECy7, Anti-CD11b Alexa 700, Anti-FoxP3 eFluor450, Anti-Ly6C eFluor450, Anti-CD8 Pacific Blue, Anti-PD-1 BV605, antibodies were obtained from eBioscience (San Diego, CA), BioLegend (San Diego, CA) and BD Biosciences (San Jose, CA).

2.2. Mice

FVB/N mice were purchased from Harlan (Frederick, MD) and Jackson Labs (Bar Harbor, ME). Clone 100 T-cell receptor (TCR) transgenic mice, derived from FVB/N mice, express the high-avidity, RNEU_{420–429}-specific TCR in the majority of peripheral CD8⁺ T cells, and were generated as previously described [20]. Eight-to twelve-week old mice were used in the experiments. Animals were housed in pathogen-free conditions and were treated in accordance with institutional and AAALAC policies. All protocols were approved by the Animal Care and Use Committee of Johns Hopkins University.

2.3. Cell lines and media

The HER-2-expressing NT2.5 breast tumor cell line (derived from a spontaneous tumor explanted from a *neu*-N transgenic mouse), the GM-CSF-secreting vaccine cell lines, 3T3GM (mock) and 3T3neuGM (HER-2-specific), and the T2D^a line were derived and grown as previously described [9,21]. The cell lines used as T-cell targets, 3T3neuB7.1 and NT2.5B7.1, were produced *via* retroviral transduction as previously described [22].

2.4. Cell viability assays

NT2.5 cells were seeded in 96-well plates at 10^4 cells per well in complete growth media overnight. During drug treatments, media was replaced with RPMI + 0.5% fetal bovine serum (FBS) containing 0 μ M to 10 μ M sorafenib in 200 μ l per well. The final concentration of DMSO was normalized within each experiment. At each time point, 100 μ l of media was removed and 20 μ l of CellTiter 96 Aqueous One Solution (Promega) was added for 2 h at 37 °C. Measurements were made at 2, 24, 48, and 72 h at 490 nm with a PowerWave $\times$ 340 plate reader (Bio-tek Instruments, Inc.). Cell free wells containing media and CellTiter solution were used as blank controls.

2.5. Western blotting

2×10^6 NT2.5 cells were seeded in 6-well plates overnight in complete growth media. To analyze the effects of sorafenib on HER-2, ERK, MAPK, p38 MAPK, STAT3 and AKT signaling, media was changed to RPMI + 0.5% FBS and incubated for 2 h with 0.1 μ M to 10 μ M of sorafenib. To analyze cyclin expression, media was changed to RPMI + 0.5% FBS with 5 μ M and 10 μ M sorafenib, U0126 (MEK/ERK inhibitor) or SB203580 (p38 inhibitor) and incubated for 6–7 h. Following the incubation period, cells were lysed in ice-cold CellLytic cell lysis reagent (Sigma) supplemented with Phosphatase Inhibitor Cocktail 2 (Sigma) and EDTA-free protease inhibitor cocktail (Roche Diagnostics) for 5–10 min on ice. Cell lysates were scraped from 6-well plates, collected, and centrifuged for 10 min at 10,000 RPM. Lysates were mixed 1:1 with Laemmli sample buffer (Bio-Rad) and boiled for 8 min. Samples were subjected to SDS-PAGE on 4–15% gradient gels (Bio-Rad) and transferred to Amersham Hybond-ECL (GE Healthcare). Membranes were blocked for 1 h in 5% Milk in TBS-Tween (w/v), and then incubated overnight with primary antibodies in 5% BSA in TBS-Tween (w/v) at the dilution recommended on the product data sheet. After washing, membranes were incubated with HRP-conjugated Goat- α -Rabbit IgG (Cell Signaling Technologies) for 30 min at room temperature, washed, and developed using HyGLO Quickspray (Denville Scientific). Membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) according to the manufacturer's instructions, then blocked and reprobed.

2.6. Immunohistochemical staining

Tumors were fixed in formalin for 24 h, paraffin-embedded, and sectioned at 5 μ m by the JHMI Pathology Core. Sections were stained with H&E or retained for immunohistochemistry (IHC) at the JHMI Oncology Tissue Service and IHC Core. IHC was performed using the Power Vision+ poly-HRP IHC Kit (Novocastra). Antigen retrieval was carried out for 45 min in HTTR steam (Target Retrieval Solution; Dako) followed by

incubation of primary antibody for 45 min at room temperature. Slides were then incubated with Power Vision Poly-HRP anti-rabbit IgG secondary antibody for 30 min at room temperature. Slides were developed with 3, 3'-diaminobenzidine (Sigma Fast DAB tablets) and counterstained with Dako Mayer's hematoxylin (Sigma). Images were captured under light microscopy at 10× magnification (E600, Nikon). Three independent viewing fields were captured and staining quantified using AR-Elements Microscope Imaging Software (Nikon).

2.7. Drug treatment and vaccinations

FVB/N mice were challenged subcutaneously with 5×10^6 NT2.5 tumor cells in the right mammary fat pad, followed by treatment 10–14 days later. Sorafenib (30 mg/kg) was administered daily Monday through Friday by oral gavage with a feeding needle. Doses of 30 mg/kg sorafenib were used based on preclinical mouse models that demonstrated the efficacy of this dose for inhibiting cancer growth [12,23,24]. A viscous vehicle composed of 30% (w/v) Cremophor EL, 30% (w/v) PEG 400, and 10% ethanol, 10% glucose (Sigma-Aldrich) was used as the sorafenib diluent, and also administered as the vehicle treatment control. 3×10^6 vaccine cells per mouse were irradiated before subcutaneous injection of 1×10^6 cells in both hind limbs and the left forelimb. Doses and timing for tumor cells and vaccinations have been previously optimized [5,22]. For combination treatment studies, sorafenib treatment began on the day of vaccination. Mice were monitored for tumor growth and onset twice weekly. Tumor growth was determined by measuring tumor diameter in two perpendicular dimensions with calipers. Mean tumor size for an experimental group included only those mice with measureable tumors.

2.8. ELISPOTS

ELISPOTS were performed 14–17 days post-vaccination. Splenic CD8⁺ T cells were isolated from individual mice using the Dynabeads Untouched Mouse CD8⁺ T Cell Negative Isolation Kit (Life Technologies). RNEU_{420–429} (PDSLRLDLSVF) and NP_{118–126} (RPQASGVYM) peptides were synthesized at 95% purity by the Oncology Peptide Synthesis Facility (Johns Hopkins, Baltimore, MD). 10^5 CD8⁺ T cells were incubated in triplicate with 10^4 peptide-loaded T2D^q, NT2.5B7.1, or 3T3neuB7.1 target cells. NT2.5B7.1 and 3T3neuB7.1 cells were stimulated with IFN γ for 2 days prior to co-culture. T cell/T2D^q cells were co-cultured for 16 h and T cell/NT2.5B7.1 or T cell/3T3neuB7.1 cells were co-cultured for 24 h on pre-coated IFN- γ ELISPOT Multiscreen-HA plates (Millipore) according to the manufacturer's protocols (Ebioscience). ELISPOT plates were developed using an AEC staining kit (Sigma) according to the manufacturer's instructions. IFN γ -secreting CD8⁺ T cells were enumerated using the Immunospot counter (Cellular Technology, Ltd.). The average number of spots in control wells was subtracted from the average number of spots in each well containing both CD8⁺ T cells and targets.

2.9. ELISA

Tumors were isolated, weighed and processed using approximately 1 ml of MPER lysis buffer (Thermo Fischer Scientific) containing Halt protease and phosphatase inhibitors (Thermo Fisher Scientific) per 100 mg tumor tissue. Tumors were dissected into 2 mm pieces, placed in lysis buffer, and passed through a 70 μ m Nylon filter (Costar). The aqueous

lysate was collected and centrifuged at 20,000 rpm for 15 min to remove cellular debris and excess lipid. Multiplex cytokine and chemokine measurements were assayed using the mouse Multi-Analyte ELISArray™ Kits Th1/Th2/Th17 kit (Qiagen) and Common Chemokines kit (Qiagen) according to the manufacturers protocols. IL-1 β , TNF α , IL-6, IL-10, IL-12 were also assessed using Mouse Platinum ELISA kits (eBioscience, San Diego, CA) and all ELISAs were performed according to manufacturer's protocols. Activity was measured using the SpectraMax M3 microplate reader (Molecular Devices LLC), and calculated in pg/ml using the following equation {OD (sample) – OD (background)}/OD (Standard)} $\times$ Dilution Factor of Standard $\times$ Dilution Factor of Sample.

2.10. In vivo depletions

CD4⁺ and CD8⁺ T cells were continuously depleted using GK1.5 and 2.43 antibodies as previously described [22]. Natural killer cells were depleted by twice weekly intraperitoneal (i.p.) injections of α -asialo GM1 antibody. Macrophages were depleted by i.p. injection of clodronate liposomes weekly. Depletions were initiated one week prior to tumor challenge and maintained throughout the experiment.

2.11. Adoptive transfer experiments

Adoptive transfer of CD8⁺ T cells was carried out as described previously [20]. Briefly, FVB/N mice received subcutaneous injections of 5×10^6 NT2.5 cells into the right upper mammary fat pad. One week post-tumor challenge, mice were vaccinated with 3×10^6 irradiated 3T3neuGM cells or 3T3GM cells. Sorafenib (30 mg/kg) or vehicle was given by daily gavage starting on the day of vaccination. CD8⁺ T cells were isolated from Clone 100 TCR transgenic mice by CD8 negative selection using Dynabeads Untouched Mouse CD8⁺ T cell negative isolation kit (Life Technologies). 5×10^6 CD8⁺ T cells per mouse were adoptively transferred *via* tail vein injection one day following the initiation of treatment administration. Spleens, lymph nodes, and tumors from adoptively transferred mice were harvested five days after adoptive transfer. Spleens and lymph nodes were collected and mashed through a 70 μ M cell strainer. Tumors were minced and digested with Liberase TM (Roche) for 15 min and mashed through 70 μ M cell strainers. Isolated single cell suspensions were analyzed for Thy1.2⁺ CD8⁺ cells on a Gallios (BD Coulter) or LSRII Flow Cytometer (BD Biosciences). Data were analyzed with FlowJo software (Treestar, Inc.).

2.12. TAM isolation

Briefly, FVB/N mice received subcutaneous injections of 5×10^6 NT2.5 cells into the right upper mammary fat pad. One week post-tumor challenge, mice were vaccinated with 3×10^6 irradiated 3T3neuGM cells or 3T3GM cells. Sorafenib (30 mg/kg) or vehicle was given by daily gavage starting on the day of vaccination. Tumors were harvested Day 12 post-treatment and suspended in 5 ml RPMI 1640 containing Liberase TM (Roche). Tumors were minced and incubated at 37 °C for 30 min. Suspensions were then passed through a 100- μ m mesh nylon cell strainer (BD Falcon) to obtain single cell suspensions. Single cell suspensions were incubated with ACK lysing buffer (Sigma) for 2 min at room temperature and washed twice in FACS-staining buffer (PBS, 2% heat-inactivated FCS). Pellets were resuspended in FACS staining buffer containing Fc Block (MACs Miltenyi Biotec) and incubated on at 4 °C for 20 min. CD11b⁺ TAMs were obtained through positive

isolation (MACs Miltenyi Biotec). CD11b⁺ TAMs were incubated with FACs buffer containing biotinylated anti-F480 (eBioscience) and isolated by positive selection (MACs Miltenyi Biotec).

2.13. Statistical analysis

Statistical analysis was conducted using GraphPad Prism Software using either an unpaired, two-tailed Student's *t*-test or one-way ANOVA test, assuming equal population variances to determine the statistical significance. Student's *t*-test was used for analysis of two groups and one-way ANOVA for 3 or more groups. The significance of differences between groups of three or more was determined by pair-wise comparisons using a Bonferroni post-test after significant differences in means were determined by ANOVA. Values were considered statistically significant if $p < 0.05$.

3. Results

3.1. Sorafenib inhibits the growth of HER2-expressing breast tumor cells in vitro

We first examined the effect of sorafenib on the HER-2⁺ breast tumor cell line NT2.5 *in vitro*. Sorafenib treatment inhibited NT2.5 cell growth, with a decrease in cell viability observed at concentrations between 1 and 10 μ M (Fig. 1A). Flow cytometric analysis of sorafenib treated NT2.5 cells stained revealed a concentration-dependent increase in the expression of apoptotic markers Annexin V and 7-AAD (Fig. 1B), confirming that sorafenib induces NT2.5 cell death *in vitro*. We also investigated the effect of sorafenib on downstream targets of the HER-2 signaling pathway using Western blot analysis. Sorafenib interfered with phosphorylation of ERK/MAPK, p38 MAPK, and STAT3, but not HER-2 or AKT (Fig. 1C).

Sorafenib also decreased the expression of the G1/S cyclins D1, D2, and D3 in NT2.5 cells, whereas Bcl2 and BclXL expression were not affected (Fig. 1D). MAPK signaling is required for the expression of cyclin D1, whereas cyclin D3 can be controlled by additional pathways [25]. Therefore, we analyzed the effect of sorafenib on these D-type cyclins relative to specific inhibitors of the ERK/MAPK and the p38 MAPK pathways. Sorafenib inhibited cyclin D1 to a greater extent than either of the two specific MAPK pathway inhibitors, suggesting that the mechanism of NT2.5 growth inhibition by sorafenib is dependent on both arms of the MAPK signaling pathway (Fig. 1E). However, unlike the MAPK inhibitors, sorafenib also inhibited cyclin D3 (Fig. 1E). Collectively, these data demonstrate that sorafenib treatment inhibits the growth of NT2.5 cells *in vitro* by both inducing apoptosis and inhibiting cell growth through both MAPK-dependent and-independent mechanisms.

3.2. Sorafenib directly inhibits growth of HER2-expressing breast tumors in vivo

We then tested whether sorafenib could inhibit the growth of established NT2.5 tumors in immune competent FVB/N mice. Single agent sorafenib induced NT2.5 tumor regression in tumor-bearing FVB/N mice compared with vehicle-treated control mice (Fig. 2A and B). Sorafenib treatment disrupted the tumor-associated vasculature and increased tumor cell death, as shown by a reduction in endothelial cell-specific PECAM/CD31 (Fig. 2C) and an

increase in cleaved caspase-3 expression (Fig. 2D) by IHC. Collectively, these data demonstrate that sorafenib inhibits NT2.5 breast tumor cell growth *in vivo* by inhibiting angiogenesis and inducing apoptosis.

3.3. Sorafenib-mediated tumor clearance is T cell dependent

To determine whether the activity of sorafenib was dependent on the immune system, we selectively depleted distinct immune cell subsets and evaluated tumor regression in sorafenib- or vehicle-treated FVB/N mice. Selectively depleting either CD4⁺ or CD8⁺ T cells partially inhibited the efficacy of sorafenib-mediated tumor regression, whereas depleting both T cell subsets completely abrogated the anti-tumor effect of sorafenib (Fig. 3A). Depletion of NK cells or macrophages modestly affected the ability of sorafenib to inhibit tumor growth (Fig. 3B). In animals cured of their tumors by sorafenib treatment, drug was withdrawn and mice were re-challenged with 5×10^6 NT2.5 cells on the contralateral side 28 days after clearance of primary tumor. No new tumor development was observed at the second tumor challenge site, but 2 of 6 mice developed recurrence at the original tumor site, possibly through acquired drug resistance or the activation of immune evasion pathways (Fig. 3C and D). These data indicate that sorafenib-mediated tumor rejection is T cell-dependent, requiring both CD4⁺ and CD8⁺ T lymphocytes. Moreover, protection from a second tumor challenge suggests that sorafenib also leads to the induction of T cell memory.

3.4. Sorafenib can be effectively combined with GM-CSF-secreting cellular immunotherapy

We next tested sorafenib in combination with a DC-based, GM-CSF-secreting, HER-2-targeted cellular vaccine (3T3neuGM). Sorafenib treatment promoted tumor clearance when given in combination with 3T3neuGM vaccine as well as the vaccine given alone. Tumor regression was impaired when sorafenib was combined mock vaccination (3T3GM) relative to the activity of sorafenib alone, despite a significant difference in tumor growth in mice treated with 3T3GM and vehicle (Figs. 4A–B and 2A). We enumerated splenic IFN γ -secreting HER-2-specific CD8⁺ T cells using NT2.5 tumor cells or 3T3neu cells expressing B7.1 as targets by ELISPOT. Sorafenib treatment with 3T3GM did not induce a HER-2-specific CD8⁺ T cell response in mice. In contrast, vaccination with 3T3neuGM induced a robust HER-2-specific, IFN γ -secreting CD8⁺ T cell response, and adding sorafenib to 3T3neuGM increased the numbers of IFN γ -secreting CD8⁺ T cells as detected by stimulation with NT2.5 B7.1 tumor cells (32 ± 30 vs. 60 ± 36 , $p = 0.5234$) or 3T3neu B7.1 (62 ± 47 vs. 134 ± 76 , $p = 0.012$), (Fig. 4C). Further analysis of the HER-2-specific CD8⁺ T cell response revealed that 3T3neuGM-vaccinated mice receiving sorafenib developed RNEU_{420–429}-specific CD8⁺ T cell responses at levels comparable to 3T3neuGM-vaccinated mice receiving vehicle (Fig. 4D). Intracellular cytokine staining (ICS) of T cells specific for alternate HER-2 epitopes typically not found in vaccinated FVB/N mice revealed no diversification of the HER-2-specific T cell response (data not shown) [26]. Overall, these results demonstrate that sorafenib does not interfere with, and may enhance, the HER-2-specific CD8⁺ T cell response induced by 3T3neuGM vaccination.

3.5. Sorafenib combined with GM-CSF-secreting cellular immunotherapy primes CD4⁺ tumor-specific T cell subsets

To further elucidate the mechanisms by which sorafenib modulates T cell responses to NT2.5 breast tumors, we first examined the numbers of CD4⁺ and CD8⁺ T cells in the TME of mice treated with either sorafenib alone or sorafenib combined with 3T3neuGM. Combining sorafenib with 3T3neuGM resulted in a significant increase in CD4⁺ T cells in the TME compared to mice receiving 3T3neuGM alone (Fig. 5A). The numbers of tumor infiltrating, IFN γ -producing CD4⁺ T cells were significantly increased with the combination of sorafenib and 3T3neuGM (Fig. 5B). The combination of sorafenib and 3T3neuGM significantly increased the numbers of tumor-infiltrating CD8⁺ T cells relative to 3T3neuGM alone (4777 ± 3428 vs. 2125 ± 1788 , $p = 0.0009$), but not there was no change in CD8⁺ IFN γ -producing T cells (Fig. 5C and D). Sorafenib thus increases both CD4⁺ and CD8⁺ T cells within the tumor site *in vivo*.

We further evaluated whether sorafenib modulates the expansion and function of HER-2-specific CD8⁺ T cells by examining the fate of adoptively transferred Thy1.2⁺ RNEU_{420–429}-specific CD8⁺ T cells in tumor bearing, FVB/N recipients. Mice treated with the combination of 3T3neuGM and sorafenib displayed a significant decrease in tumor weight 5 days post-vaccination (Fig. 6A). While the combination of sorafenib and 3T3neuGM did not affect the expansion Thy1.2⁺ CD8⁺ T cells in the periphery (Fig. 6B), there was a trend toward increased recruitment of these T cells into the TME (Fig. 6C). Evaluation of Ki67 expression by CD8⁺ Thy1.2⁺ T cells revealed no significant impact of sorafenib on HER-2-specific CD8⁺ T cell proliferation in the periphery or TME (Supplemental Fig. 1A–C). In direct contrast, we found that sorafenib inhibited the proliferation of HER-2-specific CD8⁺ T cells co-cultured with CD11c⁺ DCs purified from the spleen of vaccinated, tumor-bearing FVB/N mice *in vitro* (Supplemental Fig. 1D and E). Moreover, IFN γ production from HER-2-specific CD8⁺ T cells derived from mice vaccinated with 3T3neuGM and treated with sorafenib was not diminished (Fig. 6D). Overall these findings demonstrate that combining 3T3neuGM vaccination with sorafenib *in vivo* does not inhibit RNEU_{420–429} specific CD8⁺ T cell priming, migration, or effector functions despite its inhibitory effects *in vitro*.

3.6. Sorafenib does not modulate regulatory T cells and MDSC in tumor bearing mice

To further investigate the immunomodulatory activity of sorafenib, we next evaluated suppressive regulatory T cell (Tregs) and myeloid-derived suppressor cells (MDSCs). The combination of sorafenib with 3T3neuGM did not increase intratumoral Tregs in comparison to 3T3neuGM alone (Supplemental Fig. 2A–D). Pretreatment of CD4⁺ CD25^{hi} T cells with sorafenib *in vitro* 24 h prior to co-culture with naïve CD4⁺ CD25^{lo} T cells had no effect on the ability of the Treg to suppress naïve T cell proliferation (Supplemental Fig. 3). Sorafenib alone or with 3T3neuGM did not alter CD11b⁺ CD11c[−] Ly6G^{hi} Ly6C^{int} (gMDSC) or CD11b⁺ CD11c[−] Ly6G[−] Ly6C^{hi} (mMDSC) subsets in either the periphery or TME (Supplemental Fig. 4A–D). Evaluation of PD-L1 expression on MDSC subsets revealed significant expression on both gMDSC and mMDSC in both the periphery and TME; however, sorafenib did not alter PD-L1 expression on either subset (Supplemental Fig. 4E).

Collectively, these findings suggest that sorafenib does not alter homeostasis or function of these suppressor cell populations.

3.7. Sorafenib modulates immune checkpoint receptor expression on T cells within the TME

Next we examined the effect of sorafenib on expression of the immune checkpoint receptors PD-1, TIM-3, OX40, and 41BB on both endogenous Thy1.2⁻ CD8⁺ T cells and adoptively transferred, Thy1.2⁺ HER-2-specific CD8⁺ T cells in the TME. Vaccination with 3T3neuGM was associated with decreased PD-1 expression and increased TIM-3 expression on Thy1.2⁺ HER-2-specific T cells (Supplemental Fig. 5A and B). This effect was independent of sorafenib. Sorafenib increased 41BB receptor expression on endogenous Thy1.2⁻ and adoptively transferred Thy1.2⁺ HER-2-specific CD8⁺ T cells when combined with 3T3neuGM (Supplemental Fig. 5C and D). Examination of immune checkpoint receptor expression in CD4⁺ T cell subsets revealed minimal changes in CD4⁺ Foxp3⁻ and CD4⁺ Foxp3⁺ T cell subsets (Supplemental Fig. 6A and B). Overall, these findings demonstrate that vaccination with 3T3neuGM modulates PD-1 and TIM-3 expression independently of sorafenib, and that 3T3neuGM vaccination in the presence of sorafenib increases 41BB receptor expression on Thy1.2⁺ HER-2-specific CD8⁺ T cells.

3.8. Sorafenib enhances recruitment of T_H1 CD4 T cells within the TME

Sorafenib treatment can shift the phenotype of bone marrow derived macrophages activated with LPS and PGE₂ from the pro-tumorigenic IL-10-secreting phenotype to the anti-tumor IL-12-secreting phenotype [27], suggesting that sorafenib is capable of modulating IL-12 production by TAMs *in vivo*. We therefore evaluated TAMs derived from the TME. Although there was no significant difference in TAM numbers, TAMs isolated from sorafenib-treated mice displayed a significant increase in MHC Class II expression compared to TAMs isolated from the TME of vehicle-treated animals (Fig. 7). *Ex vivo* evaluation of sorted CD11b⁺ F480⁺ TAMs revealed a significant increase in IL-12 mRNA without significant changes in IL-10 mRNA (Fig. 7).

We therefore evaluated the impact of sorafenib on chemokines and cytokines within the TME. We detected a significant increase in IL-12 and a decrease in IL-4 and IL-5 (Table 1). Although these observations suggest that sorafenib may modulate T cell polarization, evaluation of T-bet and Gata-3 expression revealed minimal changes in splenic or intratumoral CD4⁺ T cells expressing these transcription factors (Fig. 7E and F). This observation suggests that sorafenib effects likely, promotes T cell recruitment into the TME. Consistent with this, sorafenib induced expression of the T cell-recruiting chemokines CCL4 (MIP-1β), CCL5 (RANTES), and CXCL9 (MIG) and the eosinophil chemotactant CCL11 (Eotaxin), while it significantly decreased CXCL1 (KC) and CXCL10 (IP-1) (Table 1). Together, these findings suggest that sorafenib increases T cells in the TME by increasing the production of T cell-recruiting chemokines.

4. Discussion

The data presented here support several novel findings. First, the tyrosine kinase inhibitor sorafenib inhibits the growth of NT2.5 breast cancer cells *in vitro* and *in vivo*. *In vitro* studies demonstrated that cell growth inhibition occurs by both MAPK-dependent and independent mechanisms. Second, the regression of sorafenib-treated NT2.5 tumors *in vivo* is T cell-dependent, requiring both CD4⁺ and CD8⁺ T cells. Third, sorafenib treatment combined with vaccination, increased numbers of endogenous CD8⁺ T cells in the TME. Finally, sorafenib as a single agent or in combination with 3T3neuGM increases the number of IFN γ -secreting CD4⁺ T cells in the TME. Overall this study effectively demonstrates the potential for concurrent treatment with sorafenib and a tumor antigen-specific, DC-based vaccine strategy to induce effective tumor immunity.

Cancer therapies that successfully target multiple pathways in the TME will theoretically result in a greater clinical benefit in part due to their ability to target multiple oncogenic pathways essential for disease progression. Here we show that single agent sorafenib inhibits the growth of HER2⁺ breast tumors through several mechanisms, including inhibition of cell growth, induction of cell death, and inhibition of angiogenesis. *In vivo*, mice dosed with sorafenib 30 mg/kg daily completely cleared NT2.5 tumors in the majority of treated, tumor-bearing FVB/N mice and demonstrated increased tumor cell death as reflected by increased activated caspase 3 expression. These data are consistent with reported effects of sorafenib on the Ras/MEK/ERK pathway in other cancer models [12,23,28–31]. We extended these findings to show that decreased expression of the MAPK downstream target, cyclin D1, was greatest with sorafenib treatment compared to treatment with inhibitors specific for either p38 MAPK or ERK MAPK. Unlike individual p38 MAPK or ERK MAPK inhibitors, sorafenib treatment also decreased cyclin D3 expression. These findings suggest that sorafenib targets MAPK independent pathways essential for cell cycle progression and proliferation, and are consistent with previously published reports showing that cyclin D1 and cyclin D3 are differentially regulated [32]. Therapies targeting both cyclins may be most effective at inhibiting cell growth and successfully inducing tumor regression. We also showed that sorafenib substantially reduced numbers of CD31/PECAM positive microvessels within the TME, consistent with the reported anti-angiogenic effects of the drug [12].

Importantly, we demonstrate that sorafenib-mediated tumor regression *in vivo* is critically T cell-dependent. Simultaneous depletion of CD4⁺ and CD8⁺ T cells completely abrogated the efficacy of single agent sorafenib. Furthermore, sorafenib treatment protected mice from tumor growth after re-challenge, suggesting that sorafenib monotherapy can result in immunologic memory in some mice. Further investigation revealed that sorafenib increased the number of tumor-infiltrating IFN γ -secreting CD4⁺ T cells and, to a lesser extent, the accumulation of intratumoral CD8⁺ T cells. Importantly, while several studies have reported variable inhibitory and stimulatory effects of sorafenib on the immune system [16–19,33–36], this is the first study showing a T cell-dependent mechanism of action for sorafenib-mediated tumor clearance. Several *in vitro* studies have shown that sorafenib can either inhibit or enhance T cell activation, proliferation, and effector function depending on the sorafenib concentration utilized, with subpharmacologic doses (below 1 μ M) activating and

pharmacologic doses (5–10 μ M) inhibiting T cell proliferation [16,18,19]. We observed similar effects of sorafenib on naïve CD4⁺ and CD8⁺ T cell priming and proliferation *in vitro*, where doses exceeding 1 μ M tend to inhibit T cell proliferation *in vitro*. In contrast, we found that sorafenib has no detectable effect on CD4⁺ or CD8⁺ T cell proliferation in mice dosed with 30 mg/kg daily. Furthermore, pharmacologic doses of sorafenib (5–10 μ M) inhibit DC activation, proliferation, migration and cytokine secretion *in vitro* [17], and enhance IL-12 secretion while decreasing IL-10 secretion in bone marrow-derived macrophages pretreated with LPS and PGE₂ [27]. These findings indicate that pharmacologic doses of sorafenib have distinct effects on various myeloid cells, confirming that sorafenib modulates myeloid and lymphoid activation to alter T cell activity *in vitro*.

Data characterizing the immunologic effects of sorafenib *in vivo* is notably lacking to date. One *in vivo* study demonstrated that pharmacologic doses of sorafenib decreased the percentage of OVA-specific CD8⁺ T cells induced by OVA vaccination in non-tumor-bearing C57BL6 mice. A distinct study of sorafenib administered at 30 mg/kg daily in an orthotopic HCC mouse model showed increased CD8⁺ T cell activation and IFN γ secretion within TME and the regression of established tumors [17,36]. Similarly, we found that FVB/N mice bearing established NT2.5 tumors and treated with 30 mg/kg sorafenib daily developed tumor immunity capable of mediating tumor regression. Sorafenib sequenced with 3T3neuGM increased the numbers of endogenous CD4⁺ and CD8⁺ T cells in the TME. We detected a significant increase in IFN γ -producing CD8⁺ T cells as detected by *in vitro* stimulation with 3T3neu B7.1 cells, but no increase in IFN γ -producing CD8⁺ T cells as detected by cells presenting the immunodominant HER-2 epitope RNEU_{420–429}. Furthermore, sorafenib did not appear to significantly modulate the priming or accumulation of adoptively transferred RNEU_{420–429}-specific CD8⁺ T cells of 3T3neuGM-vaccinated mice, although the rapid tumor regression in these mice may have complicated detection of this effect. We did observe a significant increase in the number of IFN γ -secreting CD4⁺ T cells in the TME in response to sorafenib given with HER-2-targeted vaccination.

Interestingly, adding sorafenib to the mock vaccine (3T3GM) appeared to reduce the rate of sorafenib-mediated tumor clearance (Fig. 2 and Fig. 4). These results are consistent with the dual immune activity of GM-CSF in cancer models. Studies have shown that GM-CSF can support tumor clearance through promoting DC maturation and antigen presentation, such as in the setting of HER-2 targeted vaccination. However, GM-CSF in the absence of targeted tumor antigen, such as in the GM-CSF-secreting mock vaccine, may support tumor growth through immune suppression [37,38]. This could explain the reduced rate of tumor clearance observed with 3T3GM combined with sorafenib.

Overall these findings demonstrate that sorafenib modulates endogenous CD4⁺ and CD8⁺ T cell responses. In contrast, sorafenib did not influence Treg or MDSC homeostasis or function in tumor bearing FVB/N mice. Furthermore, sorafenib did not modulate expression of the inhibitory receptors PD-1 or TIM-3 on CD4⁺ or CD8⁺ T cells. Our data differ from previous findings that sorafenib decreases Treg numbers and function [16,36]. We show that 3T3neuGM alone decreased PD-1 and increased TIM-3 expression, however sorafenib in combination with 3T3neuGM increased 41BB receptor expression on CD8⁺ T cells in the TME. Sorafenib combined with vaccination did not affect immune checkpoint receptor

expression on CD4⁺ T cell subsets. Collectively, our data suggest that the augmented CD4⁺ and CD8⁺ T cell responses are not due to decreasing the activity of suppressive cell populations or inhibitory checkpoint receptors such as PD-1, but may result from promoting the evolution of functional memory T cells in this model [39,40].

We explored the potential role of sorafenib in promoting T_H1 polarization. Evaluation of changes in cytokine and chemokine expression within the TME of sorafenib-treated, NT2.5 tumor-bearing FVB/N mice revealed a small but significant increase in T_H1 relative to T_H2 cytokines, where sorafenib-treated mice had a significant increase in IL-12 and a decrease in both IL-4 and IL-5. IFN γ was increased, and IL-10 and TGF β were unaffected. *Ex vivo* analysis of IL-12 expression in CD11b⁺ F480⁺ TAMs revealed an increase in IL-12 mRNA in TAMs isolated from sorafenib-treated mice, but IL-10, iNOS, or TGF β mRNA levels were unchanged. *Ex vivo* analysis of T-bet and Gata-3 expression in CD4⁺ T cells revealed a small but statistically significant increase in T-bet expression in cells derived from the spleen but not the TME. Minimal changes in Gata-3 expression were noted in each tissue, suggesting that sorafenib probably does not promote the polarization of tumor-resident CD4⁺ T cells toward the T_H1 polarized phenotype, but rather increases recruitment of T_H1-committed CD4⁺ and CD8⁺ T cells into the TME. Evaluation of the intratumoral chemokine profile revealed a significant increase in chemokines implicated in both CD8⁺ and T_H1 CD4⁺ T cell recruitment, including CCL4, CCL5, CCL11, CXCL9 [41,42]; CXCL1 and CXCL10 were significantly decreased. The differential expression of CXCL9 vs. CXCL10 is puzzling as both play important roles in recruitment of DCs, cytotoxic T cells and T_H1 polarized CD4⁺ T cells into the TME [42–44]. While the expression of CXCL9 is critically dependent on IFN γ [44], CXCL10 can be induced in human breast tumor cells in response to Raf-MAPK-kinase signaling pathways [45]. Because sorafenib clearly targets MAPK signaling in NT2.5 tumor cells, this potentially explains the differential effect of sorafenib on expression of these two chemokines.

In conclusion, we show for the first time that the activity of sorafenib is in large part dependent on T cells, and that it can be effectively combined with a DC-based, HER-2-targeted tumor cell vaccine to enhance breast tumor clearance and prolong tumor-free survival in mice. Due to its ability to target multiple aspects of the TME, including host tumor cells, endothelial cells, and immune cells, further studies utilizing sorafenib in combination with immunotherapeutic modalities in cancer are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Sorafenib inhibits growth of HER2-overexpressing cells *in vitro*. (A) NT2.5 cells were treated *in vitro* with varying concentrations of sorafenib ranging from 0 to 10 μ M and analyzed for growth by MTT assay 24, 48 or 72 hour post-treatment. (B) NT2.5 cells were treated with sorafenib for 24 h and stained for Annexin V and 7-AAD and analyzed by flow cytometry. (C) NT2.5 cells were treated with sorafenib for 2 h and then cells were harvested, quantified for protein and analyzed by Western blot for HER2 pathway targets MEK1/2, ERK1/2, p38, STAT3, and AKT. (D) NT2.5 cells were treated with 5 μ M and 10 μ M sorafenib or (E) MAPK inhibitors for 6 to 7 h and then cells were harvested for protein and

analyzed by Western blot for cyclin D1, D2, D3, BCL2 and BCLXL expression. All figures are representative of 2–3 independent experiments.

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Fig. 2.

Sorafenib inhibits growth of breast tumor cells *in vivo*. FVB/N mice ($n = 10$) were tumor challenged at Day 0 and began sorafenib or vehicle treatment on Day 10 and were followed for tumor growth (A) and overall survival (B). (C) Tumors from FVB/N mice were prepared for histological examination 3 weeks after drug treatment as described in (A). Representative samples of mice treated with vehicle (*top*) or sorafenib (*bottom*) are shown with H&E staining or immunohistochemistry to detect endothelial cells (PECAM/CD31) or apoptotic cells (activated caspase 3), at 10 $\times$ magnification. Staining was quantified using Nikon Elements software. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Fig. 3.

T cells are required for sorafenib-mediated targeting of NT2.5 cells. The experiment in Fig. 2A was repeated in the setting of T cell depletion. Additional groups were included where CD4⁺ or CD8⁺ T cells were depleted individually (Sor-CD4, Sor-CD8) or together (Sor-CD4/CD8) prior to the initiation of sorafenib therapy, and then followed for tumor growth (A). NK cells and macrophages were also depleted (B). In a separate experiment, FVB/N ($n = 6$) were tumor challenged at Day 0 and began sorafenib or vehicle treatment on Day 10. Treatment was ceased in sorafenib-treated mice that completely rejected tumors. After one week, mice were re-challenged on the contralateral side 28 days post tumor clearance and followed for tumor growth at the original site and the re-challenge site. Tumor outgrowth, (C) and survival (D) are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Fig. 4.

Sorafenib can be effectively combined with vaccine in FVB/N mice. FVB/N mice ($n = 10$) were tumor challenged on Day 0, vaccinated on Day 7 and then given daily ($\times 5$) sorafenib or vehicle treatment beginning on Day 7. Animals were followed for tumor growth (A) and tumor-free survival (B). FVB/N mice ($n = 10$) were tumor challenged, and on Day 7 were vaccinated and daily sorafenib or vehicle treatment was initiated. Two weeks post-vaccination, splenic CD8⁺ effector T cells were isolated and used for IFN γ ELISPOT with (C) NT2.5B7.1 or 3T3neuB7.1 or (D) p50 or NP peptide-pulsed T2d^q cells. Data is representative of 3 independent experiments of 10 mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Fig. 5.

Sorafenib treatment increases numbers of IFN γ producing CD4⁺ T and total CD8⁺ T cells in the TME. FVB/N mice ($n = 10\text{--}25$) were tumor challenged at Day 0, began sorafenib or vehicle treatment with or without 3T3GM or 3T3GMneu vaccination on Day 10 and at Day 22 (A) numbers of total CD4⁺ T cells, (B) IFN γ producing CD4⁺ T cells, (C) CD8⁺ T cells, and (D) IFN γ producing CD8⁺ T cells were evaluated within the tumor infiltrate by flow cytometry and intracellular cytokine staining. Data is cumulative from 3 experiments of 3–5 mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Fig. 6.

Sorafenib therapy does not affect HER-2 specific CD8⁺ T cell priming, migration or function. (A) FVB/N mice ($n = 13$ /group) were tumor challenged (Day 0), then vaccinated and treated with either daily doses of sorafenib or vehicle beginning on 7 days after tumor implantation. HER-2 specific CD8⁺ T cells were adoptively transferred into tumor bearing FVB/N mice 24 h after vaccination and mice were euthanized 5 days later to assess HER-2 specific CD8⁺ T cell expansion, migration and IFN γ production. (B) Tumor weights in each treatment group were assessed. (C) Numbers of adoptively transferred Thy1.2⁺ CD8⁺ T cells

in the spleen and (D) tumor were quantified. (E) IFN γ production from Thy1.2⁺ CD8⁺ T cells isolated from tumor draining lymph nodes was evaluated after a 6 hour co-culture with either p50 or NP pulsed T2D^q antigen presenting cells. Samples were analyzed by flow cytometry, with the number of positive cells normalized to cell number (spleen) or tissue weight (tumor). Data is cumulative from 3 experiments of 3–5 mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Fig. 7.

Sorafenib monotherapy activates and increases IL-12 production by tumor associated macrophage. At 7 days post-NT.25 tumor implantation, tumor-bearing FVB-N mice were treated with daily doses of either vehicle or 30 mg/kg sorafenib. Treatment was continued for 12 days. On Day 12 post-therapy tumors were harvested and TIL was evaluated for (A) Tumor associated macrophage (TAM) identified as CD11b⁺ Ly6G⁻ Ly6C⁻ F480⁺ myeloid cells. (B) Numbers of TAMs and their (C) MHCII expression were evaluated in vehicle and sorafenib treated mice. CD11b⁺ F480⁺ MHCII⁺ myeloid cells were isolated from vehicle

and sorafenib treated, tumor bearing FVB/N mice at 12 days post treatment. (D) Expression of IL-12 and IL-10 mRNA was evaluated by QPCR. (E–F) CD4⁺ T cells isolated from (E) spleens and (F) TIL were evaluated for the T_H1 polarizing transcription factor T-bet and the T_H2 polarizing transcription factor Gata-3. Data is representative of 2–3 independent experiments of 5 mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

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Table 1

Sorafenib monotherapy induces global changes in cytokine and chemokine expression in the TME. At 7 day post-NT.25 tumor implantation, tumor-bearing FVB-N mice were treated with either vehicle or 30 mg/kg daily doses of sorafenib for 12 days. Tumors were harvested and lysates were assayed for IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IL-17A, IL-23, IFN γ , TNF α , CCL2 (MCP-1), CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES), CCL11 (Eotaxin), CCL17 (TARC), CCL21 (6CKine), CCL22 (MDC), CXCL1 (KC), CXCL9 (MIG), CXCL10 (IP-10), and CXCL12 (SDF-1) using multianalyte ELISA kits from Qiagen. All values are present in pg/ml. Data is representative of 2 independent experiments with 5 mice/group.

Treatment	IL-1 β	IL-6	TNF α	IL-2	IL-12
Vehicle	250 $\pm$ 33	329 $\pm$ 13	117 $\pm$ 2	144 $\pm$ 16	116 $\pm$ 16
Sorafenib	280 $\pm$ 55	329 $\pm$ 15	163 $\pm$ 20	126 $\pm$ 2	260 $\pm$ 18 ***
IFN γ		IL-4	IL-10	IL-13	TGF β 1
Vehicle	130 $\pm$ 1.4	190 $\pm$ 4	282 $\pm$ 10	125 $\pm$ 6	763 $\pm$ 180
Sorafenib	200 $\pm$ 25	120 $\pm$ 5 *	300 $\pm$ 15	137 $\pm$ 15	970 $\pm$ 240
IL-17A		IL-23	CCL2 (MCP-1)	CCL3 (MIP-1 α)	CCL4 (MIP-1 β)
Vehicle	110 $\pm$ 6	170 $\pm$ 5	288 $\pm$ 40	114 $\pm$ 37	84 $\pm$ 7
Sorafenib	115 $\pm$ 10	167 $\pm$ 10	391 $\pm$ 67	143 $\pm$ 18	150 $\pm$ 16 *
CCL5 (RANTES)		CCL11 (Eotaxin)	CCL17 (TARC)	CCL21 (6CKine)	CCL22 (MDC)
Vehicle	362 $\pm$ 14	230 $\pm$ 20	245 $\pm$ 10	<i>nd</i>	132 $\pm$ 15
Sorafenib	516 $\pm$ 30 **	632 $\pm$ 21 ***	238 $\pm$ 8	<i>nd</i>	153 $\pm$ 10
CXCL1 (KC)		CXCL9 (MIG)	CXCL10 (IP-10)	CXCL12 (SDF-1)	
Vehicle	442 $\pm$ 17	3467 $\pm$ 173	1226 $\pm$ 105		300 $\pm$ 100
Sorafenib	293 $\pm$ 8 **	4750 $\pm$ 125 ***	731 $\pm$ 60 **		270 $\pm$ 80

* $p < 0.05$.** $p < 0.01$.*** $p < 0.001$.**** $p < 0.0001$.