



Published in final edited form as:

Cancer Res. 2009 March 15; 69(6): 2514–2522. doi:10.1158/0008-5472.CAN-08-4709.

The Novel Role of Tyrosine Kinase Inhibitor in the Reversal of Immune Suppression and Modulation of Tumor Microenvironment for Immune-Based Cancer Therapies

Junko Ozao-Choy^{1,2}, Ge Ma¹, Johnny Kao³, George X. Wang¹, Marcia Meseck¹, Max Sung⁴, Myron Schwartz², Celia M. Divino², Ping-Ying Pan¹, and Shu-Hsia Chen^{1,2}

¹Department of Gene and Cell Medicine, Division of Hematology/Oncology, Mount Sinai School of Medicine, New York, New York

²Department of General Surgery, Division of Hematology/Oncology, Mount Sinai School of Medicine, New York, New York

³Department of Radiation Oncology, Division of Hematology/Oncology, Mount Sinai School of Medicine, New York, New York

⁴Department of Medicine, Division of Hematology/Oncology, Mount Sinai School of Medicine, New York, New York

Abstract

In tumor-bearing hosts, myeloid-derived suppressor cells (MDSC) and T regulatory cells (Treg) play important roles in immune suppression, the reversal of which is vitally important for the success of immune therapy. We have shown that ckit ligand is required for MDSC accumulation and Treg development. We hypothesized that sunitinib malate, a receptor tyrosine kinase inhibitor, could reverse MDSC-mediated immune suppression and modulate the tumor microenvironment, thereby improving the efficacy of immune-based therapies. Treatment with sunitinib decreased the number of MDSC and Treg in advanced tumor-bearing animals. Furthermore, it not only reduced the suppressive function of MDSCs but also prevented tumor-specific T-cell anergy and Treg development. Interestingly, sunitinib treatment resulted in reduced expression of interleukin (IL)-10, transforming growth factor- β , and Foxp3 but enhanced expression of Th1 cytokine IFN- γ and increased CTL responses in isolated tumor-infiltrating leukocytes. A significantly higher percentage and infiltration of CD8 and CD4 cells was detected in tumors of sunitinib-treated mice when compared with control-treated mice. More importantly, the expression of negative costimulatory molecules CTLA4 and PD-1 in both CD4 and CD8 T cells, and PDL-1 expression on MDSC and plasmacytoid dendritic cells, was also significantly decreased by sunitinib treatment. Finally, sunitinib in combination with our immune therapy protocol (IL-12 and 4-1BB activation) significantly improves the long-term survival rate of large tumor-bearing mice. These

© 2009 American Association for Cancer Research.

Requests for reprints: Shu-Hsia Chen, Department of Gene and Cell Medicine, Mount Sinai School of Medicine, Box 1496, One Gustave L. Levy Place, New York, NY 10029. Phone: 212-659-8256; Fax: 212-803-6740; Shu-Hsia.Chen@mssm.edu.

Note: J. Ozao-Choy and G. Ma contributed equally to this work.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

data suggest that sunitinib can be used to reverse immune suppression and as a potentially useful adjunct for enhancing the efficacy of immune-based cancer therapy for advanced malignancies.

Introduction

Immune therapies for cancer have been proposed and tested for decades. However, these efforts have not translated into improved overall long-term survival for advanced cancer patients. The myeloid-derived suppressor cell (MDSC) is found to accumulate in advanced tumor-bearing mice in organs such as the spleen, bone marrow, and tumor itself as well as in the peripheral blood of patients with various cancers, including breast, colon, pancreatic, non-small cell lung, and head and neck cancers (1–3). These cells are Gr-1⁺CD11b⁺ and are also CD115⁺. Our laboratory has previously found that these MDSCs can induce CD4⁺CD25⁺Foxp3⁺ T regulatory cell (Treg) *in vitro* and *in vivo* in murine cancer models (4, 5). MDSC and Treg are both important in the establishment and promotion of immune suppression. Several strategies have been devised to prevent their function and decrease their accumulation (6–14). Unfortunately, current strategies to effectively modulate MDSC (e.g., depletion, blockade of MDSC-secreted inhibitory factors, and promotion of differentiation) and Treg function (e.g., depletion or triggering of Toll-like receptor activation; ref. 15) are still under investigation and may not control both MDSC and Treg immune suppression function. Therefore, appropriate treatments to reverse MDSC and Treg-mediated immune suppression to enhance the efficacy of cancer immune therapies are necessary.

We have found that one of the candidate tumor-derived factors, stem cell factor (SCF), is expressed by various human and murine tumor cell lines. Mice bearing tumors with SCF small interfering RNA knockdown had significantly reduced MDSC expansion and restored proliferative responses of tumor-infiltrating T cells. In addition, blockade of the SCF receptor (ckit) by anti-ckit monoclonal antibody (mAb) prevented tumor-specific T-cell energy, Treg development, and tumor angiogenesis (5). Given this role for ckit, we hypothesized that a clinically approved small-molecule inhibitor of receptor tyrosine kinases that can interfere with ckit signaling may have a novel role in reversing immune suppression.

Sunitinib malate is an oral receptor tyrosine kinase inhibitor that inhibits some tumor growth. It is currently Food and Drug Administration (FDA) approved for the treatment of gastrointestinal stromal tumors (GIST) that have failed conventional therapy with imatinib mesylate and as the first-line treatment for metastatic renal cell carcinoma (16–18). Sunitinib has shown blocking effects on a variety of receptor tyrosine kinases, including ckit, vascular endothelial growth factor receptor 2 (VEGFR2), platelet-derived growth factor receptor (PDGFR), and Flt3 (19–21). Sunitinib is well tolerated with acceptable toxicity and good solubility, bioavailability, and protein-binding characteristics. Because of the various targets of this multikinase inhibitor, we tested whether sunitinib could decrease MDSC accumulation and prevent T-cell suppression and whether sunitinib could be used in combination with our existing Adv.mIL12 and 4-1BB ligand (4-1BBL) immune therapy.

Furthermore, given the effect of sunitinib treatment on preventing MDSC accumulation and, therefore, Treg induction, we tested whether treatment with the small molecule can alter the tolerogenic tumor microenvironment and favor tumor-specific T-cell activation.

Materials and Methods

Experimental mice and tumors

Congenic Thy-1.1⁺ BALB/c mice were a gift from Dr. Richard Dutton (Trudeau Institute, Saranac Lake, NY), and C57BL/6 and BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). Influenza hemagglutinin (HA)-specific I-E^d-restricted CD4 T-cell receptor (TCR) transgenic mice (in BALB/c background, Thy-1.2) were a gift from Dr. Constantin A. Bona (Mount Sinai School of Medicine, New York, NY). OVA TCR transgenic (OT-II) mice were purchased from The Jackson Laboratory. All animal experiments were done in accordance with the animal guidelines of the Mount Sinai School of Medicine.

Cell lines used were MCA26 (22) and LLC1 (Lewis lung carcinoma; American Type Culture Collection). To establish a model in which tumor antigen-specific T-cell responses can be tracked *in vivo*, an MCA26 line that expresses influenza HA, HA-MCA26, was used as previously reported (4).

Peptide, antibodies, and immunostaining of tumor tissue

CD4 HA peptide (¹¹⁰SFERFEIFPKE¹²⁰) was purchased from Washington Biotechnology, Inc. OVA peptide (ISQAVHAAHAEINEAGR) was purchased from AnaSpec, Inc. Mouse anti-Thy-1.2-biotin, mouse anti-Gr-1-allophycocyanin (APC) or FITC, mouse anti-CD4 FITC, mouse anti-CD115-phycoerythrin (PE) or APC, mouse anti-F4/80-FITC, mouse anti-CD11b-APC or FITC, mouse anti-CD25-APC, mouse anti-Foxp3-PE, mouse anti-PD-1 APC, mouse anti-PDL-1 PE, mouse anti-CTLA4 APC, mouse anti-CD19 PE, mouse anti-PDCA-1 FITC, mouse anti-CD11c PE-Cy7, and isotype-matched mAbs were purchased from eBioscience. Anti-ckit hybridoma (ACK2) was kindly provided by Dr. Nakashima (Chubu University, Kasugai, Japan; ref. 23). The full-length mouse 4-1BBL cDNA was obtained from pLXSHDm4-1BBL (a generous gift from Dr. Lieping Chen, Mayo Clinic, Rochester, MN; ref. 24).

Recombinant adenoviral vectors, mouse model with liver metastases, and therapeutic protocol

The construction of Adv.mIL-12 has been described previously (9). Metastatic colon cancer was induced as described previously (25). At day 9 after tumor implantation, mice with liver tumors that measured 8 × 8 to 12 × 12 mm² were given 2.5 × 10¹⁰ viral particles of Adv.mIL-12 or control DL312 (E1A-deleted control adenoviral vector) intratumorally. The day following virus injection, mice were injected i.p. with two 200 µg doses of 4-1BBL, administered 3 d apart, followed by two 100 µg doses of 4-1BBL 3 d apart. Sunitinib (0.015 mg/d) diluted in 300 µL PBS or PBS alone was administered daily for a total of 4 wk.

Isolation of MDSC and tumor-infiltrating T cells

Mice with tumors 12×12 to 15×15 mm² were sacrificed and the spleens, tibias, and femurs were harvested. After lysis of RBCs, bone marrow cells and splenocytes were fractionated on a Percoll (Amersham Biosciences) density gradient as previously described (8, 26). Cells banding between 40% and 50% were labeled as fraction 1, between 50% and 60% as fraction 2, and between 60% and 70% as fraction 3.

Tumor-infiltrating leukocytes (TIL) were isolated from tumor tissues as described previously (9). The purity of the sorted cells with >97% purity was used in subsequent experiments.

Determination of total MDSC cell number

MCA26 tumor-bearing mice with large tumors (7×7 to 10×10 mm²) were selected for treatment with PBS or sunitinib (0.005, 0.010, or 0.015 mg/d) for 10 d. Splenocytes, bone marrow cells, and TILs were harvested and stained with anti-Gr-1 and anti-CD115 followed by flow cytometry. Absolute cell numbers were determined by multiplying the percentage of Gr-1⁺CD115⁺ MDSC by the total number of Percoll fraction 2 cells.

MDSC suppression assay

The suppressive activity of MDSC was assessed in peptide-mediated proliferation assays of TCR transgenic T cells as described previously (4). Briefly, splenocytes (1×10^5) from OT-II mice were cultured in the presence of OVA peptides (1 µg/mL) and serial dilutions of irradiated (850 rad) MDSCs in 96-well microplates. [³H]thymidine was added during the last 8 h of 72-h culture.

Cytokine detection by ELISA

Cytokine concentrations in culture supernatants were measured using mouse interleukin (IL)-10 ELISA kits (R&D Systems) per manufacturer's instructions.

Adoptive transfer experiments

HA-MCA26 cells (9×10^4 ; or *neo*-transfected parental MCA26 cells as a control) were inoculated into Thy-1.1⁺ BALB/c mice, and on day 8, the tumor size was confirmed to be 7×7 to 10×10 mm². Thy-1.2⁺ congenic CD4 HA-specific TCR transgenic T cells were enriched by MACS separation (Miltenyi Biotec) and adoptive transferred via tail vein injection (5×10^6 cells per mouse) 9 d after tumor implantation. Mice were sacrificed at day 10 after the adoptive transfer and Thy-1.2⁺ T cells were recovered from spleen and lymph node cell sorting.

Proliferation assay

Sorted Thy-1.2⁺ or column-enriched T cells (1×10^4) were cocultured with irradiated (2,500 rad) naive splenic cells (4×10^3 ; as antigen-presenting cells) in the presence or absence of HA peptide (5 µg/mL) in 96-well microplates. [³H]thymidine was added during the last 8 h of 72-h culture.

CTL assay

Thy-1⁺ T cells (effector cells) in tumors were purified, by MACS (Miltenyi Biotec), from MCA26 tumor-bearing mice that had been treated with PBS or sunitinib for 10 d and then incubated with MCA26 tumor cells (target cells) at 10:1, 5:1, and 2.5:1 ratios for 4 h. Supernatants were collected from each well for measurement of lactate dehydrogenase release (CytoTox 96 Non-Radioactive Cytotoxicity Assay kit, Promega). Specific killing (%) was calculated as follows: % cytotoxicity = 100 × (experimental release – effector spontaneous release – target spontaneous release)/(total target release – target spontaneous release).

Reverse transcription-PCR and quantitative real-time PCR

Target cells were homogenized in Trizol reagent (Invitrogen) and total RNA was extracted. Reverse transcription-PCR (RT-PCR) was used to determine relative quantities of mRNA (One-Step RT-PCR kit, Qiagen) using 28 PCR cycles. The intensity of each band was further analyzed by IQ Mac v1.2 software and quantitated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control.

Primers were purchased from DNA Integrated Technologies and include the following: internal control GAPDH, 5'-GTGGAGATTGTTGCCATCAACG-3' (sense) and 5'-CAGTGGATGCAGGGATGATGTTCTG-3' (anti-sense); Foxp3, 5'-CAGCTGCCTACAGTGCCCTAG-3' (sense) and 5'-CATTTGCCAGCAGTGGGTAG-3' (antisense); CTLA4, 5'-TTACTCTGCTCCCTGAGGACC-3' (sense) and 5'-TTAAAGGTACCACTGCAGAAGG-3' (antisense); B7-H1, 5'-GCTTGCCTTAGTGGTGTACTG-3' (sense) and 5'-GTCCAGATTACCTCAGCTTCTGG-3' (antisense); B7-H4, 5'-GGTCCACGAGTTCAAAGAAGGCA-3' (sense) and 5'-AACTCAAAGCTGGTGTGGAGAC-3' (antisense); PD-1, 5'-TGCAGTTGAGCTGGCAATCAGG-3' (sense) and 5'-TTTTGCCCTGGGGTGCAGGGA-3' (antisense); transforming growth factor-β (TGF-β), 5'-TGCTACTGCAAGTCAGAGACG-3' (sense) and 5'-AAGGTGTCTCAGTATAACCACCAG-3' (antisense); IL-10, 5'-CTCTTACTGACTGGCATGAG-3' (sense) and 5'-CCTTGTAGACACCTTGGTCTTGGAG-3' (antisense); and BAFF, 5'-GTTTCACAGCGATGTCCTTG-3' (sense) and 5'-CTTGTCTGTTTCCTCTGGT-3' (antisense).

Statistical analysis

Statistical comparisons between groups were made after presenting the data as means with SD and then applying the Student's *t* test. Survival data were analyzed using the log-rank test. For samples with equal variance, the paired Student's *t* test for equal variance was used. For samples with unequal variance, the Wilcoxon signed rank test was used for statistical analysis. *P* < 0.05 was considered to be statistically significant.

Results

Dose-dependent decrease in the numbers of MDSC and Treg in sunitinib-treated tumor-bearing mice

We tested whether sunitinib, a clinically approved drug, could decrease MDSC accumulation in MCA26 colon tumor-bearing BALB/c mice. On day 10 after implantation, mice bearing hepatic metastatic colon tumors (7×7 to 10×10 mm²) were treated with sunitinib daily for 7 days at varying dosages. The maximum dose of 0.015 mg was calculated to be the mouse equivalent of the 50 mg daily human dose. Subsequently, the numbers of MDSC in various tissues were assessed by flow cytometry. We found that the absolute numbers of MDSC decreased in a dose-dependent fashion in the spleen, bone marrow, and tumor (Fig. 1A). Because MDSC can induce Treg development, we investigated whether sunitinib treatment also affected the percentage of Treg in treated tumor-bearing mice. The results indicate that although sunitinib treatment did not change the overall percentage of CD4⁺CD25⁺Foxp3⁺ Treg in the spleens of naive mice, it was able to significantly decrease the percentage of CD4⁺CD25⁺Foxp3⁺ Treg in tumor-bearing mice, from 11% to 5.07% ($P < 0.014$, paired t test; Fig. 1B). These results indicate that sunitinib can decrease the number of Gr-1⁺CD115⁺ MDSC and the percentage of Tregs in tumor-bearing mice, suggesting that sunitinib may be able to modulate the accumulation of MDSC and Treg.

Sunitinib can reduce the immune-suppressive activity of MDSC from treated tumor-bearing mice

We further investigated whether sunitinib could functionally reverse MDSC-mediated immune suppression. MDSCs were isolated from the spleens of tumor-bearing mice with and without sunitinib treatment (0.015 mg/d for 1 week). Percoll fraction 2 MDSCs were then cocultured at varying ratios with OT-II (CD4 OVA TCR transgenic) T cells in the presence of OVA. The MDSC from sunitinib-treated mice exhibited significantly reduced suppressive activity compared with MDSC from PBS-treated control tumor-bearing mice (Fig. 1C).

The effect of sunitinib on leukocyte infiltration and expression of cytokines and costimulatory molecules in TILs

To further understand the mechanisms underlying the reversion of immune suppression mediated by sunitinib, we investigated whether sunitinib treatment modulated the tumor microenvironment in treated mice. We isolated TILs from large tumor-bearing mice (7×7 to 10×10 mm² before the start of sunitinib treatment) and screened for the expression of various cytokines and costimulatory molecules, which have been implicated in tumor-mediated immune suppression, by RT-PCR. The results showed that sunitinib treatment reduced the expression of IL-10, Foxp3, PD-1, CTLA4, and BAFF (B lymphocyte-activating factor belonging to tumor necrosis factor superfamily) but increased Th1 cytokine (e.g., IFN- γ) expression in isolated TILs (Fig. 2A, left). We then investigated the effect of sunitinib treatment on CTL responses against parental MCA26 tumors. T cells isolated from treated mice were used in a direct *ex vivo* CTL assay. The T cells from sunitinib-treated mice exhibited stronger cytotoxic activity against MCA26 tumor cells when compared with

PBS-treated mice ($P = 0.009$ at E:T = 2.5:1, $22.90 \pm 0.19\%$ versus $11.20 \pm 2.40\%$; $P = 0.014$ at E:T = 5:1, $23.40 \pm 3.18\%$ versus $14.85 \pm 0.70\%$; $P = 0.034$ at E:T = 10:1, $24.75 \pm 1.41\%$ versus $5.90 \pm 3.67\%$; Fig. 2A, *top right*). IL-10 secretion by splenic T cells isolated from sunitinib-treated mice was also significantly reduced to basal levels similar to those of normal mice on stimulation by anti-CD3 and anti-CD28 (Fig. 2A, *bottom right*). Real-time PCR was done to further quantitate gene expression. This confirmed the previous results but also revealed that sunitinib treatment led to a significant reduction in TGF- β expression and an increase in IFN- γ expression (Fig. 2B), suggesting that sunitinib may modify the tumor microenvironment, resulting in a shift of cytokine and costimulatory molecule expression profiles that may favor T-cell activation and Th1 responses.

Sunitinib can modulate the composition of immune cells and the expression of costimulatory molecules in TIL

We further identified and characterized the specific subsets of leukocytes and costimulatory molecules expressed in the tumors of sunitinib- or PBS-treated mice by flow cytometry. The lymphocyte population in the tumor was gated and increased from 9.64% in PBS-treated mice to 21.3% in sunitinib-treated mice (Fig. 3A, *top left*). Among TILs, a reproducible increase in CD4⁺ and CD8⁺ T cells (from 11.7% to 13.2% and 6.78% to 15.6%, respectively; Fig. 3A, *bottom left*) in the gated lymphocyte population was observed. Furthermore, the total numbers of CD4⁺ and CD8⁺ T cells in the tumor were also significantly increased (in average, 1.10×10^5 in PBS-treated group to 1.59×10^5 in sunitinib-treated group for CD4, $P = 0.003$, and 0.6×10^5 to 2.4×10^5 for CD8, $P = 0.003$; Fig. 3B). A decrease in the percentage of pDC and MDSC (from 34.8% to 22.5% and 53.9% to 39%, respectively) was also observed in sunitinib-treated mice (Fig. 3A, *top and bottom right*). The average total numbers of MDSC and pDC in sunitinib-treated group were reduced (from 1.01×10^6 to 0.74×10^6 , $P = 0.013$, for MDSC and from 0.28×10^6 to 0.21×10^6 , $P = 0.017$, for pDC; Fig. 3B). Interestingly, the B-cell population was also decreased in sunitinib-treated mice from 0.22×10^6 to 0.19×10^6 ($P = 0.059$; data not shown).

Importantly, sunitinib treatment also resulted in phenotypical changes in the TILs. The expression of the negative costimulatory molecules PD-1 and CTLA4 was significantly reduced after sunitinib treatment (Fig. 3C). The mean fluorescence intensity (MFI) of PD-1 was decreased from a mean of 1,176 to 704 ($P = 0.02$) in Treg and from 267 to 170 ($P = 0.027$) in B cells. The CTLA4 was also reduced from 334 to 238 ($P = 0.015$) in CD8 T cells and from 875 to 561.5 ($P = 0.02$) in Treg (Fig. 3C). Furthermore, PDL-1 expression was also significantly decreased by sunitinib treatment (MFI of MDSC from 713 to 420, $P = 0.025$, and pDC from 1,191 to 577, $P = 0.02$; Fig. 3B). In addition, whereas there was a significant decrease in MFI of PD-1 on Treg, that of CD8 and CD4 cells was not affected significantly. This indicates that sunitinib treatment can modulate the composition of the immune cell subsets and results in the down-regulation of negative costimulatory molecules at the tumor site.

Decreased Treg percentage and prevention of anergy of tumor (HA)-specific T cells by blocking tyrosine kinase function

We then tested whether sunitinib could prevent tumor-specific T-cell anergy in a HA-MCA26 congenic tumor-bearing model. The HA TCR transgenic T cells (Thy-1.2) were adoptively transferred into HA-MCA26 tumor-bearing mice (Thy-1.1) followed by treatment with sunitinib, anti-ckit, or PBS for 10 days. The average tumor weight from sunitinib-treated and anti-ckit-treated mice was significantly decreased compared with PBS controls (Fig. 4A). We further assessed the immune response of those adoptively transferred T cells (Thy-1.2⁺) isolated from the spleens of recipient Thy-1.1⁺ mouse. T cells from the PBS-treated group had minimal proliferative response, whereas T cells from non-tumor-bearing naive HA TCR transgenic mice had a significantly higher proliferative response. Thy-1.2⁺ T cells from both the sunitinib and the anti-ckit-treated mice had significantly higher proliferative responses compared with those from control PBS-treated tumor-bearing mice (Fig. 4B). The percentage of transferred Thy-1.2, CD4⁺CD25⁺Foxp3⁺ Tregs in the spleen was determined by flow cytometry. Sunitinib treatment significantly decreased the percentage of Tregs from 20.5% to 10.9% in the PBS controls (combined reproducible experiments, $P = 0.02$), whereas a similar decrease in Treg to 8.05% was observed in the anti-ckit antibody-treated group (Fig. 4C). This suggests that sunitinib can prevent tumor-specific T cells from becoming Treg in tumor-bearing hosts.

Because sunitinib malate can block not only the ckit receptor but also VEGFR2, PDGFR, and Flt3, we further investigated whether decreased MDSC in sunitinib-treated tumor-bearing mice (7×7 to 10×10 mm²) was mediated by inhibition of ckit-dependent signaling using a ckit mutant mouse strain (Wv/Wv). After treatment of both wild-type and ckit mutant tumor-bearing mice with sunitinib, we observed a significant decreases in the populations of MDSC in the wild-type mice but not in the ckit mutants (Fig. 5). The PBS-treated ckit mutants had lower numbers of MDSCs, suggesting that ckit plays a role in the accumulation of MDSC. In our prior publication, we have shown that monoclonal anti-ckit blocking antibody can prevent MDSC accumulation (5). Taken together, these results provide evidence that ckit is required for MDSC accumulation and suggest that ckit may be the major target of sunitinib.

Blocking tyrosine kinases with sunitinib can enhance immunomodulatory therapies

To facilitate clinical translation of this novel adjunct to immune-based therapies, we next determined whether sunitinib could further enhance the therapeutic efficacy of our Adv.mIL-12 and 4-1BB immune activation therapy. Mice with tumors ranging from 8×8 to 12×12 mm² were randomly divided into various treatment groups. One day after initiation of the immune modulatory therapy (intratumoral IL-12 gene delivery by adenoviral vector + 4-1BB activation), mice began treatment with 0.010 mg/d sunitinib for the first 7 days followed by 0.015 mg/d for 21 days or PBS every day for 28 days as a negative vehicle control. Long-term survival was followed. All mice treated with control vector DL312 + control Ig were dead within 30 days after tumor implantation. As shown in Fig. 6, the long-term survival of mice treated with Adv.mIL-12 + 4-1BB activation + sunitinib was significantly higher than Adv.mIL-12 + 4-1BB activation + PBS ($P = 0.0177$) or Adv.mIL12 + sunitinib ($P < 0.0001$). Adv.mIL-12 + 4-1BBL + PBS and 4-1BBL + sunitinib also

improved the long-term survival of treated mice when compared with the control DL312 vector and PBS alone group. The results indicate that sunitinib can be used to improve the therapeutic efficacy of Adv.mIL12 + 4-1BB activation in an advanced tumor setting in which immune-based therapy alone has previously been shown to be significantly less efficacious. These *in vivo* results suggest that sunitinib, an orally ingested FDA-approved receptor tyrosine kinase inhibitor for renal cell cancer and GIST treatment, may also be used in a novel way in the immune therapy realm as an adjunct to immune therapies to reverse the immune suppression associated with advanced malignancies.

Discussion

Our proof-of-principle studies suggest a potential clinical application for SCF/ckit blockade in the prevention of immune suppression as a complement to existing immune-based cancer therapies (5). Therefore, we sought to investigate the use of the FDA-approved tyrosine kinase inhibitor, sunitinib, for translational studies to determine its efficacy in our immune therapy protocol as well as to investigate the role of tyrosine kinases in modulation of tumor-associated immune suppression.

To our knowledge, this is the first study to show a significant decrease in immune cells associated with suppression as well as an increase in other immune cells, such as CD8⁺, CD4⁺ T cells in tumor-bearing animals, providing evidence that sunitinib may be efficacious when used in combination with various immune therapeutic approaches. Our results suggest that sunitinib may modulate the tumor microenvironment not only by decreasing Treg and MDSC numbers in the tumor but also by down-regulating cytokines, such as IL-10 and TGF- β , and important immune suppressive costimulatory receptors, such as PD-1 and CTLA4 (Fig. 3C). In addition, sunitinib treatment can increase the percentage of CD8⁺ T cells in our animal model and in the peripheral blood of human patients while significantly decreasing the percentage of MDSC, Treg, and pDC (data not shown). Although the selectivity of sunitinib on MDSC could not be directly addressed in our studies, we provided strong evidence that sunitinib did alter the number of MDSC, suppressive activity of MDSC at a per-cell level, immune profile, and tumor microenvironment. Our preliminary studies⁵ with a limited number of patient blood samples showed similar results, indicating that 1 week of sunitinib treatment in metastatic cancer patients may substantially affect the composition of immune cells in the peripheral blood.

Although sunitinib is an inhibitor for multiple receptor kinases, we have provided strong evidence that ckit may be directly involved in the sunitinib-induced reduction of MDSC in the murine model (Fig. 5). Our ongoing study using Flk2-deficient mice (27) indicates that the FLK2 receptor does not play a critical role in the effect of sunitinib in our tumor models.⁵ It is possible, however, that the VEGFR2 inhibitory function of sunitinib can be synergistic due to decreased blood vessel formation because MDSCs have been implicated as having a direct role in tumor angiogenesis (28, 29) and the regulation of VEGF bioavailability in tumors by the expression of matrix metalloproteinase-9 (MMP-9; ref. 29).

⁵Unpublished results.

The inhibitory effect of sunitinib on both VEGFR2 signaling and MDSC accumulation may provide a possible double hit on tumor angiogenesis.

Various strategies to modulate the suppressive functions of MDSC *in vivo* have been investigated, including depletion of Gr-1⁺ cells with anti-Gr-1 antibody (7, 13). Treatment with monoclonal anti-Gr-1 antibody led to decreased tumor growth in mice. However, translation of this approach in humans may be difficult. Chemotherapeutic agents have also been considered, including all-*trans*-retinoic acid (ATRA), pyrimidine antimetabolite, and gemcitabine (13, 14). Administration of a clinical dose to tumor-bearing mice caused a selective elimination or reduction of MDSC in the tumor (10, 14, 30, 31). However, ATRA has also been shown to favor Treg development (32, 33). Other groups have attempted to use a small-molecule approach, for example, the phosphodiesterase inhibitor sildenafil (34) or inhibitors for cyclooxygenase-2 or MMP-9, to reduce the suppressive machinery or tumor-promoting activity of the CD11b⁺Gr-1⁺ MDSCs (35–37). However, the additive effects of those reagents in combination with an immune therapeutic protocol and their effects on other critical immune cell types need to be further evaluated.

In this report, we found that sunitinib decreased the number and percentage of MDSC, Treg, and pDC (Figs. 1 and 2), thereby abrogating tumor-associated suppression and working in a synergistic manner with our immune therapy protocol in large tumor-bearing mice. More importantly, when analyzing the peripheral blood mononuclear cells from metastatic cancer patients, we found significant differences in immune cell profiles between samples collected before and after treatment with sunitinib, suggesting an immune modulatory effect of sunitinib in cancer patients.⁵ Recently, it has been shown that sunitinib treatment promotes CD1c dendritic cell differentiation. However, the effect of sunitinib on CD14⁺ myeloid suppressor cells in renal cancer patients was not statistically significant after 4 weeks of treatment (38). Finke and colleagues observed a significant increase in Th1 response and diminished Th2 cytokine expression after 4 weeks of sunitinib treatment. A decrease in the Treg population was observed but not statistically significant in treated renal cancer patients (39). In a recent report comparing two tyrosine kinase inhibitors sorafenib and sunitinib, it was found that sorafenib, but not sunitinib, exerted a negative effect on the ability of dendritic cell to migrate and stimulate a T-cell response and significantly reduced the induction of antigen-specific T cells (40). Interestingly, the number of Treg was also reduced in the peripheral blood of mice treated with sunitinib, consistent with our results.

Our studies provide evidence in an *in vivo* animal model that targeted tyrosine kinase inhibitors can be used in a novel synergistic way to enhance the therapeutic efficacy of existing immune-based therapies for metastatic cancer patients. Because this patient group has few options that lead to prolonged survival, our novel strategy to combat the immune suppression associated with advanced metastatic tumors through the use of sunitinib provides a new modality of more efficacious immune therapy protocols.

Acknowledgments

Grant support: National Cancer Institute, Sharp Foundation, and Black Family Stem Cell Foundation (S-H. Chen); Susan G. Komen Breast Cancer Foundation (P-Y. Pan); and NIH training grant fellowship T32CA078207-06 (J. Ozao-Choy).

References

1. Almand B, Clark JI, Nikitina E, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol.* 2001; 166:678–89. [PubMed: 11123353]
2. Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin Cancer Biol.* 2006; 16:53–65. [PubMed: 16168663]
3. Young MR, Lathers DM. Myeloid progenitor cells mediate immune suppression in patients with head and neck cancers. *Int J Immunopharmacol.* 1999; 21:241–52. [PubMed: 10408632]
4. Huang B, Pan PY, Li Q, et al. Gr-1⁺CD115⁺ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res.* 2006; 66:1123–31. [PubMed: 16424049]
5. Pan PY, Wang GX, Yin B, et al. Reversion of immune tolerance in advanced malignancy: modulation of myeloid-derived suppressor cell development by blockade of stem-cell factor function. *Blood.* 2008; 111:219–28. [PubMed: 17885078]
6. Young MR, Young ME, Wright MA. Myelopoiesis-associated suppressor-cell activity in mice with Lewis lung carcinoma tumors: interferon- γ plus tumor necrosis factor- α synergistically reduce suppressor cell activity. *Int J Cancer.* 1990; 46:245–50. [PubMed: 2143498]
7. Seung LP, Weichselbaum RR, Toledano A, Schreiber K, Schreiber H. Radiation can inhibit tumor growth indirectly while depleting circulating leukocytes. *Radiat Res.* 1996; 146:612–8. [PubMed: 8955710]
8. Kusmartsev SA, Li Y, Chen SH. Gr-1⁺ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 costimulation. *J Immunol.* 2000; 165:779–85. [PubMed: 10878351]
9. Pan PY, Zang Y, Weber K, Meseck ML, Chen SH. OX40 ligation enhances primary and memory cytotoxic T lymphocyte responses in an immunotherapy for hepatic colon metastases. *Mol Ther.* 2002; 6:528–36. [PubMed: 12377195]
10. Kusmartsev S, Cheng F, Yu B, et al. All-*trans*-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. *Cancer Res.* 2003; 63:4441–9. [PubMed: 12907617]
11. Imai H, Saio M, Nonaka K, et al. Depletion of CD4⁺CD25⁺ regulatory T cells enhances interleukin-2-induced antitumor immunity in a mouse model of colon adenocarcinoma. *Cancer Sci.* 2007; 98:416–23. [PubMed: 17270031]
12. Bubenik J. Depletion of Treg cells augments the therapeutic effect of cancer vaccines. *Folia Biol (Praha).* 2006; 52:202–4. [PubMed: 17184599]
13. Ko HJ, Kim YJ, Kim YS, et al. A combination of chemoimmunotherapies can efficiently break self-tolerance and induce antitumor immunity in a tolerogenic murine tumor model. *Cancer Res.* 2007; 67:7477–86. [PubMed: 17671218]
14. Suzuki E, Sun J, Kapoor V, Jassar AS, Albelda SM. Gemcitabine has significant immunomodulatory activity in murine tumor models independent of its cytotoxic effects. *Cancer Biol Ther.* 2007; 6:880–5. [PubMed: 17582217]
15. Peng G, Guo Z, Kiniwa Y, et al. Toll-like receptor 8-mediated reversal of CD4⁺ regulatory T cell function. *Science.* 2005; 309:1380–4. [PubMed: 16123302]
16. Hopkins TG, Marples M, Stark D. Sunitinib in the management of gastrointestinal stromal tumours (GISTs). *Eur J Surg Oncol.* 2008; 34:844–50. [PubMed: 18082353]
17. Motzer RJ, Hutson TE, Tomczak P, et al. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. *N Engl J Med.* 2007; 356:115–24. [PubMed: 17215529]
18. Motzer RJ, Rini BI, Bukowski RM, et al. Sunitinib in patients with metastatic renal cell carcinoma. *JAMA.* 2006; 295:2516–24. [PubMed: 16757724]
19. George S. Sunitinib, a multitargeted tyrosine kinase inhibitor, in the management of gastrointestinal stromal tumor. *Curr Oncol Rep.* 2007; 9:323–7. [PubMed: 17588358]

20. Dova L, Pentheroudakis G, Golfopoulos V, et al. Targeting c-KIT, PDGFR in cancer of unknown primary: a screening study for molecular markers of benefit. *J Cancer Res Clin Oncol*. 2008; 134:697–704. [PubMed: 18064489]
21. Illmer T, Ehninger G. FLT3 kinase inhibitors in the management of acute myeloid leukemia. *Clin Lymphoma Myeloma*. 2007; 8 (Suppl 1):S24–34. [PubMed: 18282363]
22. Corbett TH, Griswold DP Jr, Roberts BJ, Peckham JC, Schabel FM Jr. Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. *Cancer Res*. 1975; 35:2434–9. [PubMed: 1149045]
23. Kato M, Takeda K, Kawamoto Y, et al. c-Kit-targeting immunotherapy for hereditary melanoma in a mouse model. *Cancer Res*. 2004; 64:801–6. [PubMed: 14871802]
24. Xu DP, Sauter BV, Huang TG, Meseck M, Woo SL, Chen SH. The systemic administration of Ig-4-1BB ligand in combination with IL-12 gene transfer eradicates hepatic colon carcinoma. *Gene Ther*. 2005; 12:1526–33. [PubMed: 15973445]
25. Kwong YL, Chen SH, Woo SL. Methods for cancer gene therapy. *Curr Protoc Hum Genet*. 2001; Chapter 13(Unit 13.5)
26. Angulo I, Rullas J, Campillo JA, et al. Early myeloid cells are high producers of nitric oxide upon CD40 plus IFN- γ stimulation through a mechanism dependent on endogenous TNF- α and IL-1 α . *Eur J Immunol*. 2000; 30:1263–71. [PubMed: 10820371]
27. Mackarehtschian K, Hardin JD, Moore KA, Boast S, Goff SP, Lemischka IR. Targeted disruption of the *flk2/flt3* gene leads to deficiencies in primitive hematopoietic progenitors. *Immunity*. 1995; 3:147–61. [PubMed: 7621074]
28. Beckermann BM, Kallifatidis G, Groth A, et al. VEGF expression by mesenchymal stem cells contributes to angiogenesis in pancreatic carcinoma. *Br J Cancer*. 2008; 99:622–31. [PubMed: 18665180]
29. Yang L, DeBusk LM, Fukuda K, et al. Expansion of myeloid immune suppressor Gr⁺CD11b⁺ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell*. 2004; 6:409–21. [PubMed: 15488763]
30. Garnett CT, Schlom J, Hodge JW. Combination of docetaxel and recombinant vaccine enhances T-cell responses and antitumor activity: effects of docetaxel on immune enhancement. *Clin Cancer Res*. 2008; 14:3536–44. [PubMed: 18519787]
31. Mirza N, Fishman M, Fricke I, et al. All-*trans*-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res*. 2006; 66:9299–307. [PubMed: 16982775]
32. Elias KM, Laurence A, Davidson TS, et al. Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood*. 2008; 111:1013–20. [PubMed: 17951529]
33. Schambach F, Schupp M, Lazar MA, Reiner SL. Activation of retinoic acid receptor- α favours regulatory T cell induction at the expense of IL-17-secreting T helper cell differentiation. *Eur J Immunol*. 2007; 37:2396–9. [PubMed: 17694576]
34. Serafini P, Meckel K, Kelso M, et al. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. *J Exp Med*. 2006; 203:2691–702. [PubMed: 17101732]
35. Rodriguez PC, Hernandez CP, Quiceno D, et al. Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. *J Exp Med*. 2005; 202:931–9. [PubMed: 16186186]
36. Melani C, Sangaletti S, Barazzetta FM, Werb Z, Colombo MP. Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma. *Cancer Res*. 2007; 67:11438–46. [PubMed: 18056472]
37. Serafini P, Mgebrioff S, Noonan K, Borrello I. Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. *Cancer Res*. 2008; 68:5439–49. [PubMed: 18593947]
38. van Crujisen H, van der Veldt AA, Vroeling L, et al. Sunitinib-induced myeloid lineage redistribution in renal cell cancer patients: CD1c⁺ dendritic cell frequency predicts progression-free survival. *Clin Cancer Res*. 2008; 14:5884–92. [PubMed: 18794101]

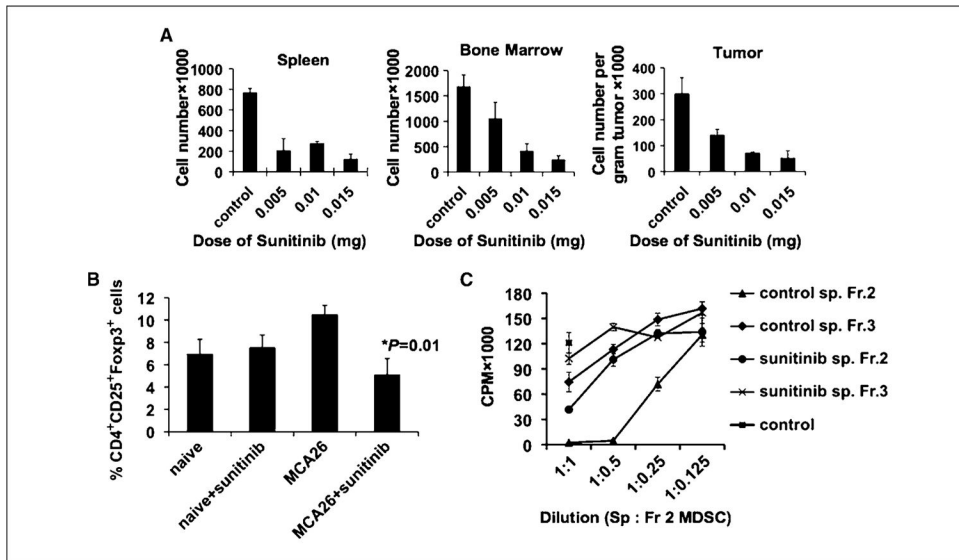
39. Finke JH, Rini B, Ireland J, et al. Sunitinib reverses type-1 immune suppression and decreases T-regulatory cells in renal cell carcinoma patients. *Clin Cancer Res.* 2008; 14:6674–82. [PubMed: 18927310]
40. Hipp MM, Hilf N, Walter S, et al. Sorafenib, but not sunitinib, affects function of dendritic cells and induction of primary immune responses. *Blood.* 2008; 111:5610–20. [PubMed: 18310500]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

**Figure 1.**

Pharmacologic disruption of ckit receptor signaling through the use of a small-molecule inhibitor can prevent the accumulation of MDSCs and reduce the suppressive activity of MDSCs isolated from tumor-bearing mice. *A, left*, total MDSC number in the spleen was assessed. A dose-dependent decrease in the number of MDSC was observed in the spleen of tumor-bearing mice treated with sunitinib. *Middle*, total MDSC number in the bone marrow was assessed. A dose-dependent decrease in the number of MDSC was observed in the bone marrow of tumor-bearing mice treated with sunitinib. *Right*, total MDSC number per gram tumor tissue was assessed. A dose-dependent decrease in the number of MDSC was observed in the tumors of tumor-bearing mice treated with sunitinib. *B*, splenocytes from MCA26 tumor-bearing mice or naive mice with and without sunitinib (0.015 mg) treatment were stained for CD4⁺CD25⁺Foxp3⁺ Treg. *C*, the suppressive activities of MDSCs were assessed using HA peptide-mediated proliferation at various ratios of CD4 HA TCR transgenic splenocytes and MDSCs. Proliferation: CPM × 1,000. Representative of three experiments. Bars, SD (using different mice).

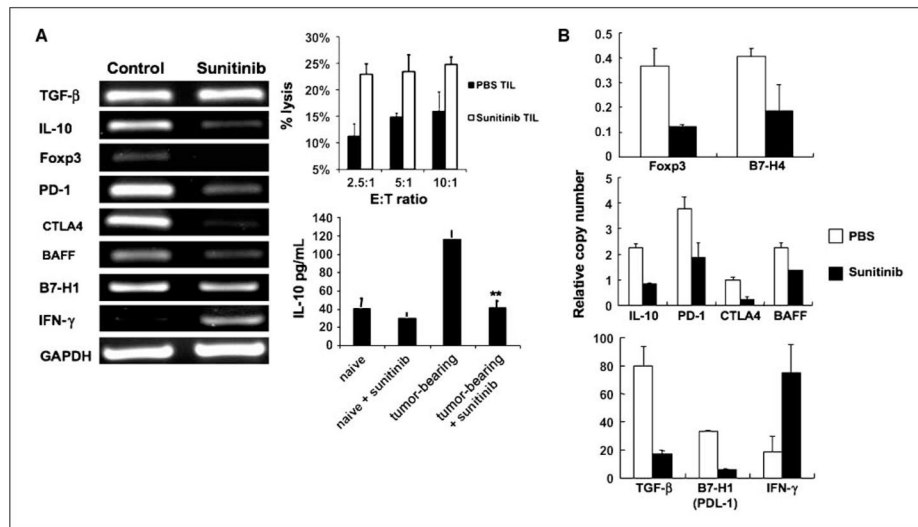
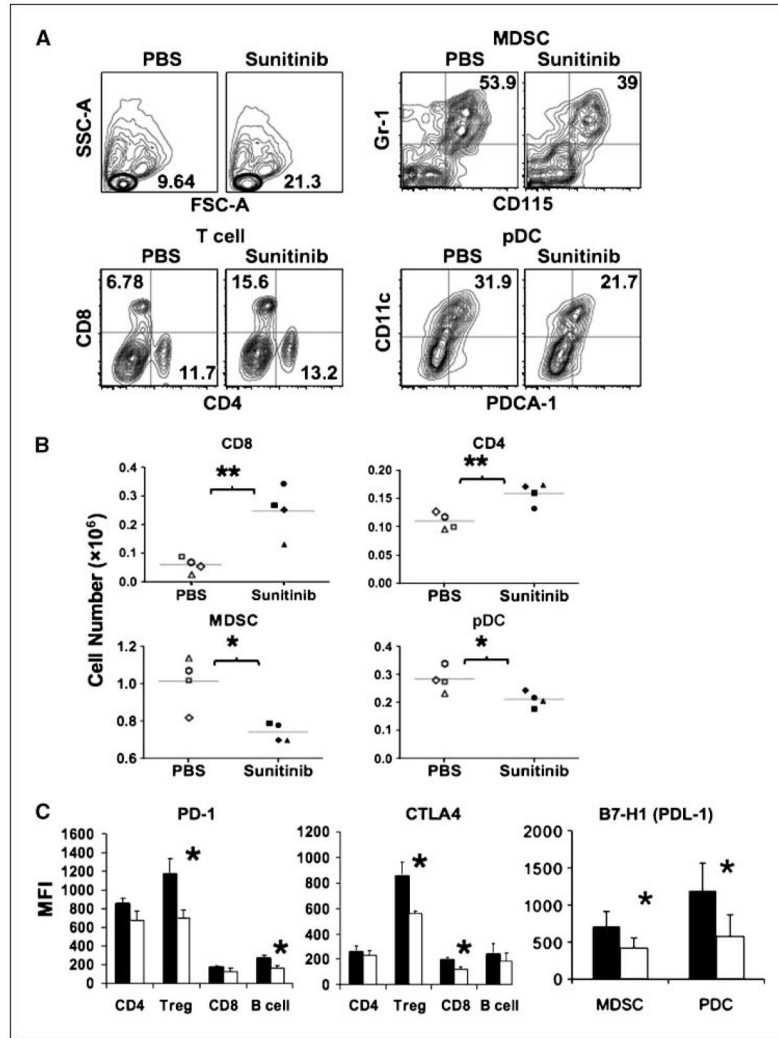


Figure 2.

The effect of sunitinib treatment on the tumor microenvironment. *A*, the expression of Foxp3, various cytokines, and costimulatory molecules by TILs. Large (7×7 to 10×10 mm²) MCA26 tumor-bearing mice were treated with PBS or 0.015 mg/d sunitinib for 7 to 10 d. *Left*, mRNA was prepared from the isolated TILs and RT-PCR analysis was done. *Top right*, T cells in the tumors of treated mice (three mice per group) were isolated and the cytotoxic activity against parental MCA26 tumor cells was assessed in an *ex vivo* CTL assay. *Columns*, mean of triplicate wells; *bars*, SD. *Bottom right*, the concentration of IL-10 in the supernatants of anti-CD3/anti-CD28-stimulated T cells isolated from the spleen of naive or tumor-bearing mice treated with PBS or sunitinib for 1 wk. Data are from the representative of three reproducible experiments. *Columns*, mean of replicate wells; *bars*, SD. *B*, quantitation of the gene expression of Foxp3, various cytokines, and costimulatory molecules in TILs as determined by real-time PCR. The RNA preparations from *A* were also used to quantitate gene expression by quantitative real-time PCR. It is of note that the RT-PCR for TGF- β expression is saturated because of the abundance of TGF- β mRNA, resulting in no significant difference. However, a significant reduction in expression after sunitinib treatment was observed using quantitative real-time PCR.

**Figure 3.**

Sunitinib treatment modulates the composition of TILs. Tumor-bearing mice were treated with sunitinib or PBS (four mice per group) for 10 d. TILs were isolated from treated tumor-bearing mice and stained with fluorochrome-conjugated antibodies followed by flow cytometric analysis. *A*, the percentages of CD8⁺ T cells, CD4⁺ T cells, MDSCs (Gr-1⁺CD115⁺), and pDC (PDCA⁺, CD111c⁺) were calculated. *B*, the cell number of various subsets was calculated. A significant increase in the percentage of CD8⁺ T cells and CD4⁺ T cells in the tumor was observed after sunitinib treatment, whereas the percentage of Treg, MDSC, and pDC was decreased. *, $P < 0.05$; **, $P < 0.01$, by unpaired Student's *t* test, equal variance. Sunitinib treatment did not significantly affect the percentage of B cells in the tumor. *C*, MFI of negative costimulatory molecules (PD-1, CTLA4, and PDL-1) on various subpopulations (Treg, CD8, B cell, MDSC, and pDC) of TILs from tumor-bearing mice treated with sunitinib (*white columns*) or PBS (*black columns*). A significant decrease in the expression of PD-1 by Treg and B cells, CTLA4 by CD8 and Treg cells, and PDL-1 by MDSC and pDC was observed after sunitinib treatment. *, $P < 0.05$, by unpaired

Student's *t* test, equal variance. The average results of four separate experiments are presented.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

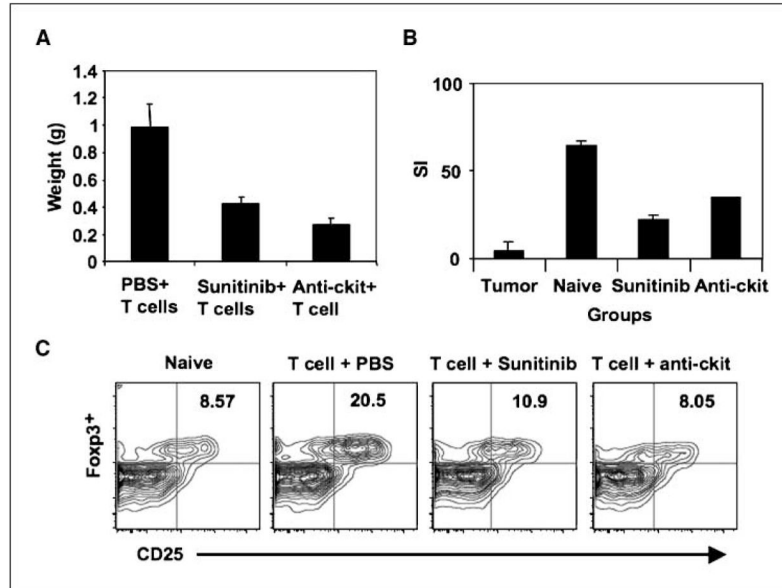


Figure 4.

Treatment with sunitinib prevents T-cell anergy in tumor-bearing mice. Thy-1.1 congenic mice were inoculated with HA-MCA26 tumor and adoptively transferred with HA-CD4 TCR T cells; mice were sacrificed and spleens were harvested after 10 d of treatment with PBS, sunitinib, or anti-ckit antibody. *A*, the tumor weight was determined in the treatment groups PBS, sunitinib, or anti-ckit antibody. *B*, stimulation index after sorting for Thy-1.2⁺ cells and HA peptide stimulation. *C*, the magnetically sorted Thy-1.2⁺ cells were also stained for CD4, CD25, and Fcγ3⁺ cells. CD4 gated cells were presented. Data from three reproducible experiments are presented. Combined statistical analysis by paired *t* test for three experiments; $P < 0.02$, for PBS versus sunitinib treatment.

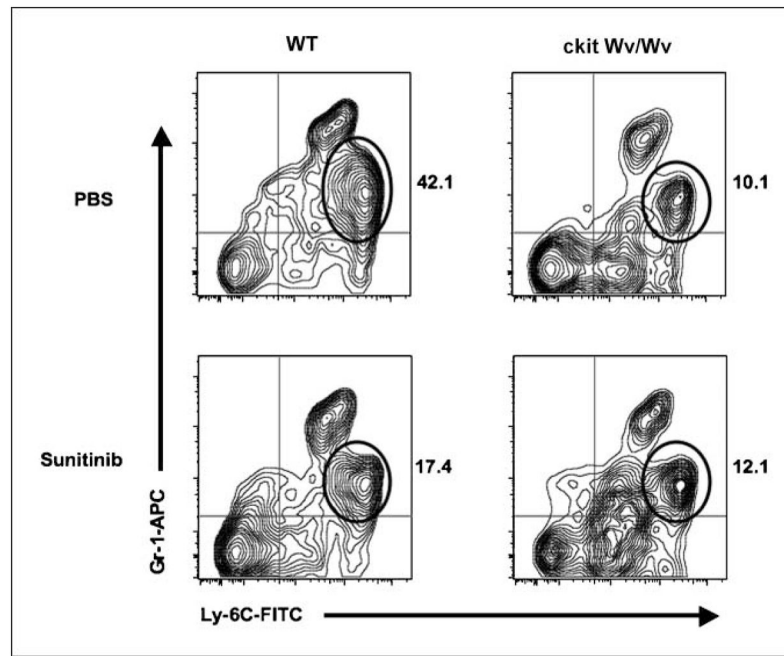


Figure 5. MDSC in the tumor of wild-type or ckit mutant (Wv/Wv) tumor-bearing mice with or without sunitinib treatment. Large tumor-bearing mice were treated with sunitinib or PBS for 10 d. The TILs were then isolated and stained for Gr-1 and Ly-6C to identify the MDSC. ckit Wv/Wv tumor-bearing mice had a lower percentage of MDSC in the tumor when compared with wild-type tumor-bearing mice. Sunitinib treatment did not further decrease MDSC in the tumor, suggesting that ckit is the direct target of sunitinib.

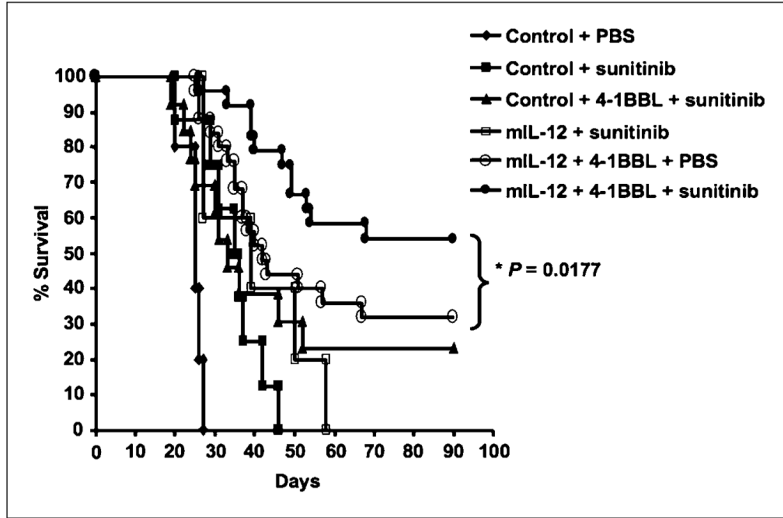


Figure 6. Sunitinib significantly improves the long-term survival rate of mice treated with Adv.mIL12 + 4-1BB activation immune modulatory therapy. Mice bearing large MCA26 tumors were divided into the following treatment groups: (a) DL312 (control vector) + PBS (*solid diamond*, $n = 5$), (b) DL312 + sunitinib (*solid square*, $n = 8$), (c) DL312 + 4-1BBL + sunitinib (*solid triangle*, $n = 10$), (d) Adv.mIL-12 + sunitinib (*open square*, $n = 5$), (e) Adv.mIL-12 + 4-1BBL + PBS (*open circle*, $n = 25$), and (f) Adv.mIL-12 + 4-1BBL + sunitinib (*solid circle*, $n = 24$). The results are combined from two reproducible experiments. The survival advantage for the Adv.mIL-12 + 4-1BBL + sunitinib-treated group was statistically significant compared with the Adv.mIL-12 + 4-1BBL + PBS group. *, $P = 0.0177$, by log rank.