## Cancer Therapy: Clinical

# Sunitinib Reverses Type-1 Immune Suppression and Decreases T-Regulatory Cells in Renal Cell Carcinoma Patients

James H. Finke,[1,2] Brian Rini,[2] Joanna Ireland,[1] Patricia Rayman,[1] Amy Richmond,[3] Ali Golshayan,[2] Laura Wood,[2] Paul Elson,[2] Jorge Garcia,[2] Robert Dreicer,[2] and Ronald Bukowski[2]

**Abstract**

**Purpose:** Immune dysfunction is well documented in renal cell carcinoma (RCC) patients and likely contributes to tumor evasion. This dysfunction includes a shift from a type-1 to a type-2 T-cell cytokine response and enhanced T-regulatory (Treg) cell expression. Given the antitumor activity of select tyrosine kinase inhibitors such as sunitinib in metastatic RCC (mRCC) patients, it is relevant to assess their effect on the immune system.

**Experimental Design:** Type-1 (IFN$\gamma$) and type-2 (interleukin-4) responses were assessed in T cells at baseline and day 28 of treatment with sunitinib (50 mg/d) by measuring intracellular cytokines after *in vitro* stimulation with anti-CD3/anti-CD28 antibodies.

**Results:** After one cycle of treatment, there was a significant increase in the percentage of IFN$\gamma$-producing T cells (CD3$^+$, $P < 0.001$; CD3$^+$CD4$^+$, $P = 0.001$), a reduction in interleukin-4 production (CD3$^+$ cells, $P = 0.05$), and a diminished type-2 bias ($P = 0.005$). The increase in type-1 response may be partly related to modulation of Treg cells. The increased percentage of Treg cells noted in mRCC patients over healthy donors ($P = 0.001$) was reduced after treatment, although not reaching statistical significance. There was, however, an inverse correlation between the increase in type-1 response after two cycles of treatment and a decrease in the percentage of Treg cells ($r = -0.64$, $P = 0.01$). *In vitro* studies suggest that the effects of sunitinib on Treg cells are indirect.

**Conclusions:** The demonstration that sunitinib improved type-1 T-cell cytokine response in mRCC patients while reducing Treg function provides a basis for the rational combination of sunitinib and immunotherapy in mRCC.

Immune dysfunction has been well documented in cancer patients, including renal cell carcinoma (RCC; refs. 1–4). In RCC patients, there is a shift from a type-1–mediated CD4$^+$ T-cell response producing IFN$\gamma$, which is critical for the development of effective antitumor immunity, to a type-2 cytokine response [interleukin (IL)-4, IL-5, and IL-10] that typically mediates humoral immunity (5, 6). Indeed, tumor-specific T-cell responses to the tumor-associated antigens MAGE-6 and EphA2 were characterized by a predominance of T cells that synthesize IL-5 and IL-4, with reduced levels or a complete absence of T lymphocytes expressing IFN$\gamma$ (7, 8). Patients rendered disease-free by primary tumor excision and/or immunotherapy revert to a predominance of IFN$\gamma$-producing type-1 CD4$^+$ T cells, suggesting that the tumor environment

may be skewing the response to type-2 response (7). The diminished type-1 response in RCC patients is not limited to MAGE-6–specific and EphA2-specific CD4$^+$ T cells. Following polyclonal activation, Onishi et al. (9) showed that the peripheral blood lymphocyte response changes from predominantly type 1 to type 2 with advancing stage of RCC.

There is growing evidence that CD4$^+$CD25$^{+hi}$ T-regulatory cells (Treg) may play an important role in suppressing the development of antitumor immunity in cancer patients (10). The frequency of Treg cells is elevated in tumor sites and/or the peripheral blood of patients with advanced tumors (11–15). Treg cells can impair induction of both antigen-specific and nonspecific T cells in melanoma patients (16, 17) and predict reduced survival in multiple cancer cell types (13, 18–20). Relevant to therapy, experimental models have shown that removal of Treg cells modifies the immune response to tumors. Depletion of Treg cells in mouse models with anti-CD25 antibody has been shown to enhance antitumor activity (10, 21–23). Furthermore, reducing peripheral blood Treg cell numbers in RCC patients using the recombinant IL-2 diphtheria toxin conjugate DAB$_{389}$IL-2 enhanced the immunostimulatory activity of tumor RNA-transfected dendritic cell vaccines (24).

Given the type-2 bias that develops in RCC patients and impairs antitumor immunity, it is relevant to generate strategies to promote a type-1 immune response (25, 26). To this end, we have examined the effect that treatment with the small-molecule tyrosine kinase inhibitor, sunitinib, would have on

## Translational Relevance

Tyrosine kinase inhibitors such as sunitinib represent frontline therapy for the treatment of metastatic renal cell carcinoma (mRCC) because they provide significant objective clinical responses and progression-free survival benefits to these patients. In this article, we show for the first time that sunitinib can not only induce clinical responses but also reverse some of the immune suppression that is observed in patients with mRCC. This includes a reversal in a type-1 T-cell response (IFNγ) that is critical for the generation of effective antitumor immunity along with a reduction in the development of a type-2 cytokine bias. This study also showed that the improved type-1 cytokine response following sunitinib treatment is linked to a reduction in the elevated number of T-regulatory (Treg) cells observed in RCC patients and is independent of either tumor shrinkage or objective clinical responses. The reduction in Treg cells after sunitinib treatment may be an indirect effect because sunitinib did not alter the *in vitro* expansion of either Treg cells or T-effector cells. The changes in Treg cell in mRCC patients receiving sunitinib may be related to the negative effect that sunitinib has on the expression of myeloid-derived suppressor cells, which is being reported separately.[4] Given that sunitinib is not only clinically active, likely due to its antiangiogenic effects, but can also reverse immune suppression makes this drug an ideal candidate for combining with immunotherapy for the treatment of mRCC patients.

type-1 response in RCC patients with metastatic disease. Sunitinib is a multitargeted tyrosine kinase inhibitor of multiple receptors, including vascular endothelial growth factor and platelet-derived growth factor receptors, which has produced robust objective responses in patients with metastatic RCC (mRCC) and a progression-free survival benefit over the cytokine IFNα (27). Adverse events include lymphopenia, leaving open the possibility that this drug will further impair the ability of T lymphocytes to mount an antitumor immune response. In addition, the precise mechanisms of the robust antitumor effects of sunitinib are not fully characterized. Preliminary studies reported here show that treatment with sunitinib can in fact promote a type-1 cytokine response (IFNγ) and simultaneously decrease the type-2 response (IL-4) in patients with mRCC. Additional studies suggest that Treg cells may be involved in modulating changes in the type-1 and type-2 cytokine responses. These findings could have implications for furthering the clinical benefit of sunitinib in RCC and other tumors.

## Materials and Methods

*Sunitinib treatment.* Patients included in this study received sunitinib monotherapy for mRCC at a dose of 50 mg p.o. daily for 28 d followed by 14 d of rest, comprising one 6-wk cycle of therapy. Patients were excluded if they received any anticancer therapy concomitant with sunitinib, if they had a diagnosis other than clear

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[4] J. Ko, submitted for publication.

cell RCC, or if they did not receive at least 28 d of sunitinib. Patients underwent disease assessment (computed tomography scans and bone scan) at baseline and after every two cycles (approximately every 12 wk). Objective response according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (28) and tumor burden shrinkage was determined by investigator assessment of radiographs. Patients were treated until RECIST-defined disease progression or unacceptable toxicity. Dose interruption and modification was done according to treating physician discretion. All patients signed an Institutional Review Board–approved, written informed consent for collection of blood samples.

*Reagents.* RPMI 1640, HBSS without calcium or magnesium, ammonium chloride, human IgG for blocking nonspecific antibody binding, and DMSO were obtained from Sigma-Aldrich. Ficoll-Hypaque was from Amersham Pharmacia Biotech AB. Fetal bovine serum was purchased from Hyclone. T-cell stimulation beads (Dynabeads) coated with anti-CD3 and anti-CD28 were purchased from Invitrogen. Anti-CD3 (OKT3) and anti-CD28 antibodies were obtained from OrthoBiotech and BD Biosciences, respectively. Recombinant IL-2 was obtained from Chiron. GolgiPlug, Fix Perm, and Perm Wash were part of an Intracellular Cytokine Staining kit from BD Biosciences. Unconjugated anti-human IFNγ, unconjugated anti-human IL-4, anti-human IFNγ-FITC or IFNγ-APC, and anti-human IL-4-PE or IL-4-FITC were all from BD Biosciences. Mouse IgMκ-APC, mouse IgG1κ-PECy5, mouse IgG2aκ-APC, mouse IgG2a-PerCP, and mouse IgMκ-PE were from BD Biosciences. Mouse IgMκ-FITC and mouse IgG1κ-PE were from eBioscience. Anti-human CD3 and CD4 were from BD Biosciences. Anti-CD25-PE antibody was purchased from Stemcell Technologies. Anti-human FoxP3 antibody and FoxP3 buffer system was obtained from eBioscience. Secretion of IFNγ was detected using the ELISA kit from R&D Systems. Anti-rat IgG2a-FITC isotype was also obtained from eBioscience. [³H]Thymidine was purchased from Perkin-Elmer.

*Isolation and storage of peripheral blood mononuclear cells.* Peripheral blood (60 mL) was drawn at baseline (before sunitinib treatment) and on day 28 of treatment from mRCC patients and from age-matched normal donors. In a subset of patients, peripheral blood was also obtained from mRCC patients receiving a second cycle of sunitinib. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque gradient as previously described (29) and then frozen at -80°C and transferred to liquid nitrogen. Samples were thawed for analysis after all time points of a given patient were obtained. For analysis, PBMCs were removed from liquid nitrogen and thawed in a 37°C water bath. Thereafter, cells were washed with 20 mL of complete medium (RPMI 1640 plus 10% fetal bovine serum), centrifuged at 1,500 rpm for 10 min, and suspended in complete medium. The cells were plated in six-well tissue culture plates (BD Falcon) and incubated at 37°C, 5% CO₂ overnight. For the analysis of type-1 and type-2 response, patient samples were always run with PBMC from age-matched normal donors as a positive control for T-cell activation and cytokine production. Tumor-infiltrating T cells (TIL) were also examined for type-1 response ($n = 8$) and for expression of Treg cells ($n = 15$). Consented tumor tissue was obtained from surgical pathology and digested to achieve a single-cell suspension as previously described (3) and then subjected to adherence for 2 h. Thereafter, an aliquot of nonadherent cells was stained for Treg phenotype, whereas another aliquot was stimulated with anti-CD3/anti-CD28 antibody to assess intracellular levels of IFNγ.

*Type-1 and type-2 cytokine response assessment.* PBMCs (or TILs) were incubated in complete medium at a concentration of $1 \times 10^6$ to $1.5 \times 10^6$ per mL in 24-well tissue culture plates (BD Falcon) for 72 h at 37°C, 5% CO₂. PBMCs were stimulated or not with either plates precoated with anti-CD3 and anti-CD28 antibodies ($n = 20$ patients) or with 25 μL of anti-CD3/anti-CD28–coated Dynabeads ($n = 18$ patients) per $1 \times 10^6$ PBMCs along with 200 IU/mL recombinant human IL-2. There was no difference in the stimulatory capacity of either plate-bound or bead-coated anti-CD3 and anti-CD28

antibodies.[5] At the end of 72 h, GolgiPlug (BD Biosciences) was added to cells for 6 h to inhibit cytokine secretion. To detect intracellular levels of IFNγ and IL-4, the unstimulated and stimulated PBMCs were harvested and the wells were rinsed with 1× PBS. Thereafter, cells were centrifuged at 1,500 rpm for 10 min, resuspended in fluorescence-activated cell sorting (FACS) buffer (2% heat-inactivated fetal bovine serum + 1× PBS + 0.02% sodium azide), stained with CD3-APC (BD Biosciences) and CD4-PerCP (BD Biosciences) for 30 min at 4°C, and then washed with FACS buffer. The cells were permeabilized with BD Cytofix/Cytoperm Solution (BD Biosciences) for 30 min at 4°C. Next, cells were washed with BD Perm/Wash Solution (BD Biosciences), resuspended in the same solution, and stained for intracellular IFNγ using IFNγ-FITC monoclonal antibody (BD Biosciences) and intracellular IL-4 using an IL-4-PE monoclonal antibody (BD Biosciences) for 30 min at 4°C. The cells were fixed in 1% paraformaldehyde in 1× PBS and the data were acquired on the BD FACSCalibur machine. Data analysis was done using the FlowJo software (Tree Star, Inc.). Nonstimulated cells from each donor served as a negative control. Additionally, specificity of cytokine staining was confirmed in each sample via subtraction of any nonspecific staining occurring in samples pretreated with unlabeled anti-cytokine antibodies.

*Phenotypic analysis of Treg cells in PBMC.* For the analysis of Treg cells in patient PBMCs, samples were thawed, washed, and resuspended in complete RPMI 1640 and incubated overnight (30, 31). Surface marker staining of CD3, CD4, and CD25 was done in normal FACS buffer for 30 min. Cells were then permeabilized for 1 h with eBioscience Fixation/Permeabilization solution and then stained for FoxP3 in permeabilization buffer for 30 min. All staining and permeabilization steps were done at 4°C. Cells were then resuspended in 1% paraformaldehyde and run for FACS. Data were acquired using CellQuest on a BD FACSCalibur and analyzed using FlowJo software. At least 300,000 live cell events were collected for each tube used in analysis. The gate for the CD25+ isotype was set at 0% to ensure that the analysis was done on CD25+ cells and to ensure that additional analysis included Treg cells as opposed to activated CD4+ T cells. Results are expressed as percentage of CD25+hi/FoxP3+ cells out of total CD3+/CD4+ viable cells. Nonstained cells from each donor served as a negative control. Similar analysis was also done with TIL to assess the percentage of Treg in the CD4+ T-cell population infiltrating RCC.

*Analysis of Treg cell function.* The following assay was used to examine the suppressive activity of Treg cells isolated from RCC patients to that of Treg cells from normal donors. We also compared the suppressive activity of Treg cells from patients before and after one or two cycles of sunitinib treatment. This assay consisted of testing the ability of the Treg population (CD3+CD4+CD25hi) to inhibit the proliferation of the T-effector (Teff) population (CD3+CD4+CD25- cells). This was accomplished by thawing patient PBMC and isolating the CD3+ population by negative selection using a magnetic cell sorting kit (Miltenyi Biotech). The CD3+ cell population was stained with the following antibodies: CD4-PerCP, CD25-PE, and CD3-APC. Stained cells were then subjected to high-speed cell sorting (BD FACSAria) to obtain CD4+CD25hi and CD4+CD25- populations. T-cell proliferation was assessed by stimulating the CD4+CD25hi and CD4+CD25- populations alone and in combination with cross-linked anti-CD3 antibody and soluble anti-CD28 antibody in the presence of irradiated autologous PBMC feeder cells (5 × 10^4, 3,000 Rads) for 72 h using U-bottomed 96-well plates. For the last 18 h of culture, cells were pulsed with [³H]thymidine (1 μCi/well) before harvesting and counting in β scintillation counter (Perkin-Elmer). In some experiments where we compared the suppressive activity of Treg cells from RCC patients to Treg cells isolated from normal donors, three different Teff to Treg ratios (1:1, 5:1, and 10:1) were used and the Teff cells were stimulated with irradiated (3,000 Rads) allogeneic adherent PBMC

instead of anti-CD3/anti-CD28 antibodies. The remainder of the assay was the same as described above.

To determine whether sunitinib alters the growth of either Treg or Teff cells *in vitro*, Treg and Teff cells were isolated by cell sorting. Thereafter, the cells were incubated with Dynal Treg beads (anti-CD3/anti-CD28 antibodies) and IL-2 (500 units/mL) in 96-well plates for 14 d according to the kit instructions. The beads were removed and the T cells were transferred to 24-well plates and cultured with IL-2 (100 units/mL) either alone or in the presence of sunitinib (0.2 μmol/L; Pfizer) for another 14 d to assess the fold increase of cells.

*Statistical analysis.* Data were summarized descriptively as frequency counts, means, SEs, medians, and ranges. The Wilcoxon rank sum test was used to compare mRCC patients and healthy control subjects and different patient groups with respect to the distributions of IFNγ, IL-4, type-2 bias, and Treg cells at baseline and following treatment with sunitinib. The Wilcoxon signed rank test was used to compare changes in these variables following treatment of mRCC patients. Spearman rank correlations were used to assess associations between immune variables and also their effect on tumor shrinkage. Progression-free survival was measured from the date treatment started to the date of documented progression or death. Patients who were alive and not known to have progressed were censored. Overall survival was measured from the date treatment started to the date of death or last follow-up. The method of Kaplan and Meier was used to summarize both variables. All statistical tests were two sided and $P < 0.05$ was considered statistically significant. All analyses were conducted using Statistical Analysis System (version 8; SAS Institute, Inc.).

## Results

*Patient characteristics and clinical response to sunitinib.* Data from 42 mRCC patients treated with sunitinib monotherapy between August 2005 and August 2007 and 22 healthy control subjects were available for analysis. Seventy-six percent of patients were male, median age was 55 years, and most patients had good performance status (95% Eastern Cooperative Oncology Group 0 or 1). All but two patients had prior nephrectomy, and 57% had received prior systemic therapy. As would be expected, the most common site of metastatic disease was the lung (86%). Thirty-eight percent (16 of 42) of patients were considered favorable risk using the Memorial Sloan-Kettering Cancer Center criteria for previously untreated patients (32), and 57% (27 of 42) were categorized as intermediate risk. Only two patients had an unfavorable risk profile.

Overall, 36% of patients achieved a partial response by RECIST criteria and 2 patients (5%) progressed throughout treatment. Fifty-seven percent (24 of 42) of patients have progressed; median progression-free survival is currently estimated to be 15.5 months. Median follow-up for the 18 patients still progression-free is 10.6 months (range, 3.0-26.9). Twenty-nine percent (12 of 42) of patients have died; median survival cannot be estimated as yet.

*The type-2 bias that exists in T cells from mRCC patients is reversible following treatment with sunitinib.* We previously showed that tumor-specific CD4+ T cells isolated from mRCC patients displayed a T-helper type-2 bias (7, 8). Additional studies showed that a type-2 bias was present in T cells from mRCC patients after polyclonal stimulation of PBMC with anti-CD3/anti-CD28 antibodies (9, 33).[4] When compared with healthy donors ($n = 17$), mRCC patients ($n = 38$) showed significant reduction in the percentage of CD4+ T cells expressing intracellular IFNγ (17.4 ± 1.5 versus 6.1 ± 1.0,

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[5] J.H. Finke, unpublished data.

$P < 0.001$), whereas the percentage of IL-4$^+$ T cells was similar in patients ($n = 20$) and healthy controls ($n = 6$; $15.3 \pm 3.7$ versus $22.3 \pm 6.2$, $P = 0.17$). ELISA analysis confirmed that PBMCs from mRCC patients were deficient in stimulus-dependent secretion of IFN$\gamma$ compared with PBMCs from healthy donors (normals, $n = 11$, $36.4 \pm 5.8$ ng/mL versus patients, $n = 12$, $12.3 \pm 3.0$ ng/mL, $P = 0.004$), which is consistent with data from others (33). TIL isolated from RCC tissue also showed a diminished type-1 (IFN$\gamma$) response ($n = 8$, $9.5 \pm 2.0\%$ IFN$\gamma^+$ T cells) when compared with the percentage of IFN$\gamma^+$ T cells in the peripheral blood of normal donors ($n = 19$, $18.5 \pm 1.5\%$, $P = 0.006$).

An important question to address was whether a single cycle of treatment with sunitinib would alter the type-2 response observed in peripheral T cells of mRCC patients. Intracellular levels of IFN$\gamma$ ($n = 38$) and IL-4 ($n = 20$) were measured in T cells from patients before and after 28 days of sunitinib treatment. Compared with pretreatment values, the percentage of IFN$\gamma$-expressing T cells increased significantly after treatment ($P < 0.001$), whereas the percentage of IL-4–expressing cells decreased ($P = 0.05$) as did the type-2 bias ($P = 0.005$; Fig. 1A). Overall, the proportion of IFN$\gamma^+$ T cells increased from day 1 to day 28 in 68% of the patients, whereas the number of IL-4–producing cells decreased in 60% of the patients. When the ratio of IL-4$^+$ to IFN$\gamma^+$ T cells was assessed, the type-2 bias that was observed in all patients at baseline ($37.0 \pm 15.0$) switched to a type-1 bias after 28 days of sunitinib treatment in 30% of the patients (6 of 20), whereas another 50% (10 of 20) showed a significant decrease in the magnitude of the type-2 bias (30-97% decrease; Fig. 1A).

Similar results were observed when analyzing the CD3$^+$CD4$^+$ subset of T cells. The percentage of IFN$\gamma^+$ CD4$^+$ T cells increased significantly after one cycle of sunitinib treatment ($P = 0.001$) and the type-2 bias decreased ($P = 0.004$). The percentage of IL-4$^+$ T cells also decreased; however, the reduction was not statistically significant ($P = 0.47$; Fig. 1B). Considering CD4$^+$ T cells, 14 of 20 patients (70%) had a type-2 bias before treatment and 6 patients (30%) had a type-1 bias. Overall, 17 of the 20 patients (85%) had an increase in IFN$\gamma$-producing cells at day 28 relative to day 1 compared with an increase in IL-4–producing cells in 9 of 20 patients (45%). Among the 14 patients with an initial type-2 bias, 6 (43%) switched to a type-1 bias following one cycle of sunitinib treatment, 7 (50%) showed a decrease in the magnitude of the type-2 bias (22-99% decrease), and one patient's type-2 bias increased (7% increase). The type-1 bias became stronger after treatment in three of six patients who showed a type-1 bias at baseline, one patient's type-1 bias decreased 19%, and one patient switched to a type-2 bias.

Given that a type-1 response was increased in sunitinib-treated patients and that a significant tumor burden reduction was also observed, it is relevant to assess whether the increase in the type-1 response was associated with (and possibly due to) tumor shrinkage. No correlation was found between the increase in IFN$\gamma^+$ T cells and the reduction in IL-4$^+$ T cells with either parameter of objective response (PR; $P = 0.95$ and 0.88, respectively) or tumor shrinkage ($P = 0.95$ and 0.97, respectively). Additionally, the reversal in immune suppression (IFN$\gamma$) did not correlate with progression-free survival ($P = 0.23$). Thus, the reduction in tumor volume did not account for the improved type-1 response.
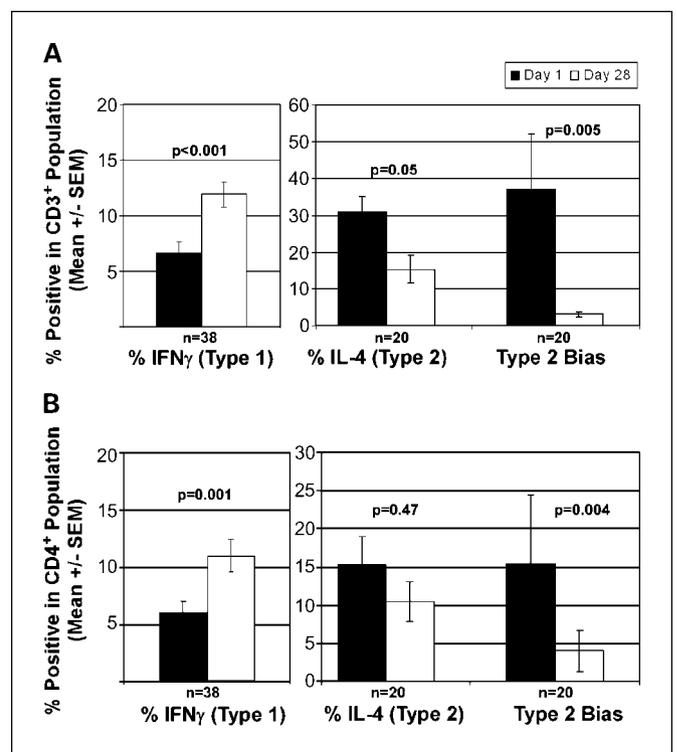


**Fig. 1.** T cells from mRCC patients display a type-2 bias that is partially reversed by sunitinib treatment. mRCC patients were treated with sunitinib (50 mg/d) for 4 wk. PBMCs obtained at baseline (pretreatment) and after 28 d of drug treatment were stimulated with anti-CD3/anti-CD28 antibodies for 72 h. Thereafter, the intracellular expression of IFN$\gamma$ and IL-4 was determined in both the total T-cell population (A) as well as the CD4$^+$ T-cell subset (B) as described in Materials and Methods. Unstimulated T cells did not express any appreciable level of either IFN$\gamma$ or IL-4 (data not shown). The mean percentage of CD3$^+$ and CD3$^+$CD4$^+$ T cells expressing either IFN$\gamma$ or IL-4 is presented. Also presented is the type-2 bias that is defined as the ratio of cells producing the type-2 cytokine (IL-4) divided by the proportion of cells producing the type-1 cytokine (IFN$\gamma$). The median plus the range for T cells expressing IFN$\gamma$ and IL-4 at pretreatment and day 28 are as follows: CD3$^+$IFN$\gamma^+$ cells: pretreatment, median, 4.2; range, 0 to 24.7; day 28, median, 10.3; range, 1.4 to 27.4; CD3$^+$IL-4$^+$ cells: pretreatment, median, 26.1; range, 6.0 to 67.8; day 28, median, 20.1; range, 0.03 to 42.6; Th2 "bias" (CD3$^+$ cells): pretreatment, median, 10.90; range, 1.16 to 234.0; day 28, median, 2.20; range, 0 to 13.01; CD4$^+$IFN$\gamma^+$ cells: pretreatment, median, 4.9; range, 0 to 29.4; day 28, median, 9.4; range, 0.2 to 43.7; CD4$^+$ IL-4$^+$ cells: pretreatment, median, 10.0; range, 0.8-60.2; day 28, median, 6.2; range, 0 to 49.3; and Th2 bias (CD4$^+$ cells): pretreatment, median, 4.69; range, 0.16 to 175.0; day 28, median, 0.83; range, 0 to 52.08.

*Treg cells are increased in mRCC and the improved type-1 response in sunitinib-treated patients correlates with reduction in Treg cells.* Treg cells are known to suppress T-cell function (10, 16, 17) and are reported to be elevated in mRCC patients (34, 35). Four-color analysis of PBMC from patients in this study ($n = 34$) confirmed that mRCC patients have elevated levels of CD3$^+$CD4$^+$CD25$^{+hi}$ ($2.0 \pm 0.11\%$ versus $3.8 \pm 0.3\%$, $P < 0.001$) and CD3$^+$CD4$^+$CD25$^{+hi}$FoxP3$^+$ cells ($1.4 \pm 0.1\%$ versus $2.7 \pm 0.3\%$, $P = 0.002$) when compared with normal healthy donors ($n = 20$; Fig. 2A). Additionally, the percentage of Treg cells within the TIL population was examined in a cohort of patients with localized RCC. Single-cell suspensions of TIL were stained for CD3, CD4, CD25, and FoxP3 and then the percentage of Treg cells within the CD4$^+$ population was determined by flow cytometry analysis. Interestingly, the percentage of Treg cells (CD3$^+$CD4$^+$CD25$^{+hi}$FoxP3$^+$) was elevated in the tumor ($10.0 \pm 2.2\%$) over that observed in

the peripheral blood of either mRCC patients or normal healthy donors ($P < 0.001$ in both cases).

Cell sorting experiments showed that the CD4$^+$CD25$^{+hi}$ cells from PBMC from RCC patients and normal donors were suppressive because they inhibited the proliferation of the Teff
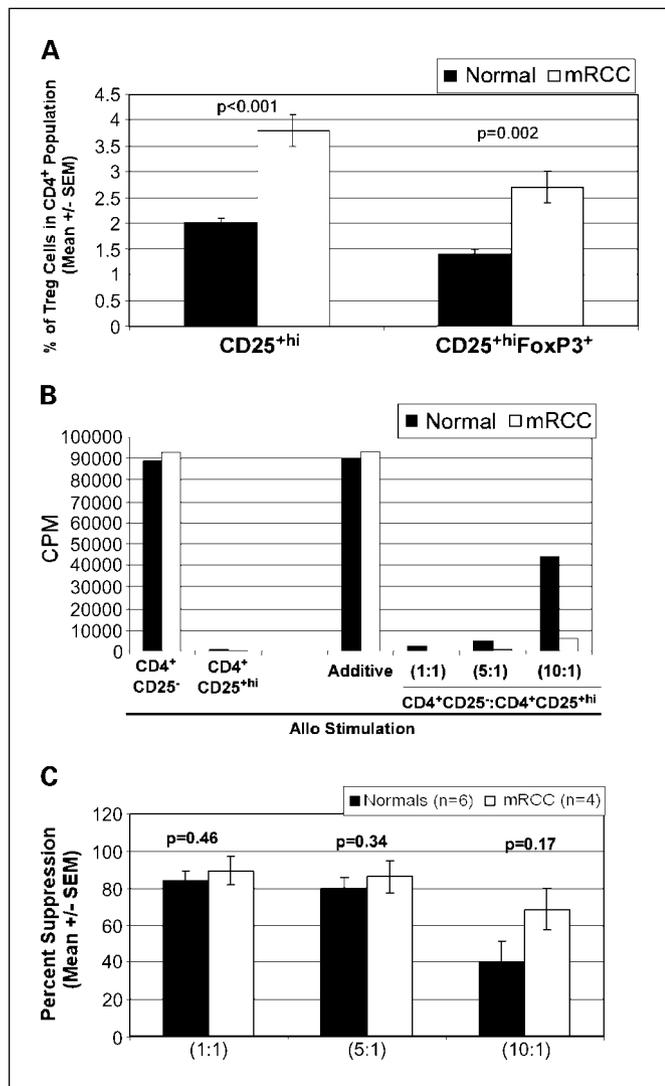


**Fig. 2.** Treg cells are increased in the peripheral blood of patients with mRCC. A, PBMCs from healthy donors and patients with mRCC were surfaced stained for CD3, CD4, and CD25 and stained intracellularly for FoxP3. Data were acquired using CellQuest and analyzed using FlowJo software. Results are expressed as mean percentage of CD25$^{+hi}$/FoxP3$^+$ cells out of total CD3$^+$/CD4$^+$ viable cells. Unstained cells from each donor served as a negative control. The median plus the range for Treg cells at pretreatment and day 28 are as follows: CD3$^+$CD4$^+$CD25$^{+hi}$: normals ($n = 20$): pretreatment, median, 2.1; range, 1.0 to 2.8; patients ($n = 34$): pretreatment, median, 3.8; range, 1.5 to 9.2; CD3$^+$CD4$^+$CD25$^{+hi}$FoxP3$^+$: normals ($n = 20$): pretreatment, median, 1.4; range, 0.4 to 2.2; patients ($n = 34$): median, 2.6; range, 0.5 to 8.2. B, representative data are presented showing the suppressive activity of Treg cells isolated from a RCC patient (white columns) and a normal healthy donor (black columns). Purified CD4$^+$CD25$^-$ Teff cells and CD4$^+$CD25$^{+hi}$ Treg cells were obtained by cell sorting (BD FACSAria) and then cultured alone or in combination with irradiated allogeneic adherent PBMC feeder cells before pulsing with [$^3$H]thymidine. The results are presented as counts per minute (cpm) and show that the CD4$^+$CD25$^+$ population displays a greater proliferative capacity compared with Treg cells (CD4$^+$CD25$^{+hi}$) cultured alone and when CD4$^+$CD25$^-$ Teff cells are cocultured with the Treg cells. The expected proliferation of Treg cells and CD4$^+$CD25$^-$ Teff when combined if there was no suppression is shown (Additive). C, summary of the suppressive activity of Treg cells derived from normal donors and mRCC patients tested at three Teff to Treg ratios ($n = 4$). The percent suppression mediated by Treg cells is calculated by the following formula: (Teff alone + Treg alone) - (combined Teff + Treg) / (Teff alone + Treg alone) × 100.
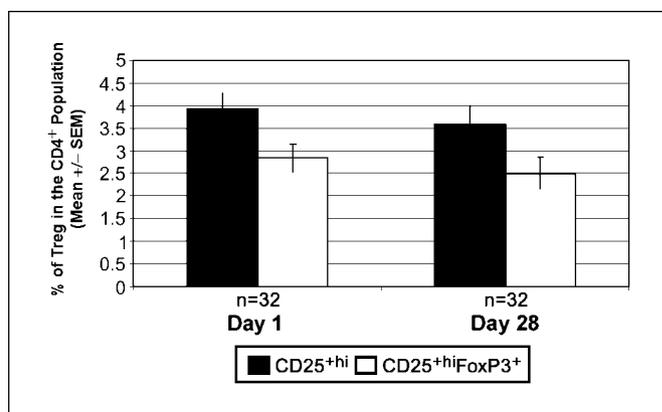


**Fig. 3.** Changes in percentage of Treg cells after one cycle of sunitinib treatment. PBMCs were stained for Treg cells (CD3$^+$CD4$^+$CD25$^{+hi}$ and CD3$^+$CD4$^+$CD25$^+$FoxP3$^+$) before treatment and after one and two cycles of sunitinib therapy. The data are presented as the percentage of Treg cells in the CD4$^+$ population. Columns, mean; bars, SE. Below are the median and range for the CD4$^+$CD25$^{+hi}$ T cells with and without FoxP3. The median plus the range for Treg cells at pretreatment and day 28 are as follows: cycle 1: % CD3$^+$CD4$^+$CD25$^{+hi}$ ($n = 32$): pretreatment, median, 4.1; range, 1.5 to 9.2; day 28, median, 2.9; range, 1.0 to 10.0; % CD3$^+$CD4$^+$CD25$^{+hi}$FoxP3$^+$ ($n = 32$): pretreatment, median, 2.7; range, 0.5 to 8.2; day 28, median, 2.0; range, 0.4 to 9.5.

CD4$^+$CD25$^-$. In some of these experiments ($n = 4$), the suppressive activity of Treg cells isolated from mRCC patients was compared with the activity of Treg cells from healthy donors tested over a range of Teff to Treg ratios. Representative data from a single patient are presented in Fig. 2B, showing that CD4$^+$CD25$^-$ T cells proliferate to stimulus, whereas the Treg population (CD4$^+$CD25$^{+hi}$) displayed low level of [$^3$H]thymidine uptake. When incubated together, the proliferation is substantially reduced compared with the response of CD4$^+$ Teff cells alone or to the expected proliferative response when combined, showing that the CD4$^+$CD25$^{+hi}$ population is suppressive (Fig. 2B). In the same experiment, Treg cells from a normal donor were assessed for their ability to inhibit the proliferation of autologous Teff cells. As noted, the Treg cells isolated from patients tended to be more suppressive at the 10:1 ratio compared with Treg cells from normal donors, although this difference was not quite statistically significant ($n = 4$, $P = 0.17$; Fig. 2B and C).

The effect that sunitinib treatment had on the levels of Treg cells in the peripheral blood was examined in PBMC isolated from patients before treatment and on day 28 of cycles 1 and 2. Compared with pretreatment values, the percentage of Treg cells was reduced after one cycle of sunitinib treatment; however, the degree of reduction did not reach statistical significance (Fig. 3). This was true when analyzing the CD3$^+$CD4$^+$CD25$^{+hi}$ and the CD3$^+$CD4$^+$CD25$^{+hi}$FoxP3$^+$ populations after one and two cycles (data not shown).

However, there was a negative correlation between the decrease in Treg cells from pretreatment to day 28 of cycle 1 or 2 and the increase in IFNγ-producing CD4$^+$ T cells from pretreatment to cycle 2 ($n = 14$, $r = -0.64$, $P = 0.01$ in both cases; Fig. 4). A similar negative correlation was observed between the percentage of Treg cells at end of cycle 1 (and cycle 2) and the IFNγ response of the total T-cell (CD3$^+$) population at cycle 2 ($n = 14$, $r = -0.66$, $P = 0.01$ and $r = -0.70$, $P = 0.005$, respectively; data not shown). These findings are consistent with the possibility that the greater the reduction of Treg cells
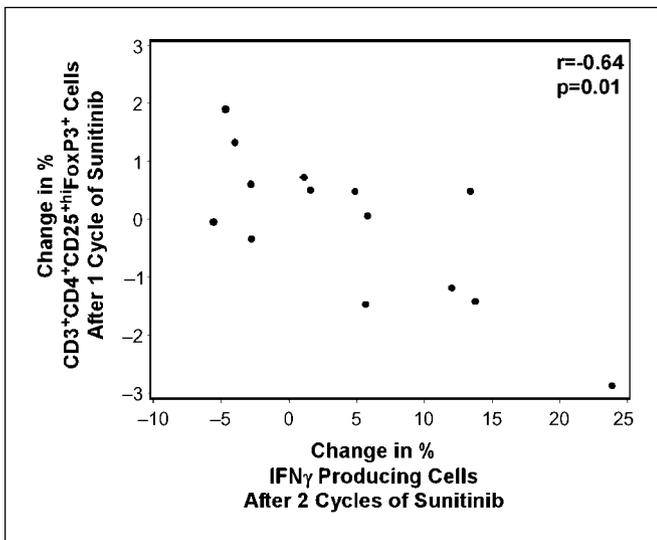
**Fig. 4.** Decrease in Treg cells correlates with an increase in IFNγ-producing T cells after two cycles of sunitinib treatment. To assess whether a correlation existed between Treg cells and type-1 T-cell response, PBMCs from mRCC patients receiving one ($n = 32$) or two ($n = 14$) cycles of sunitinib were tested for changes in the percentage of Treg cells and for changes in IFNγ as well as IL-4 production. A negative correlation was observed between the change in the % of Treg cells (CD25$^{+hi}$FoxP3$^{+}$) from pretreatment to day 28 of cycle 1 and a change in IFNγ-producing T cells (CD3$^{+}$CD4$^{+}$) from pretreatment to day 28 of cycle 2 ($n = 14$, $r = -0.64$, $P = 0.01$). Similar correlations were observed between the following: CD25$^{+hi}$FoxP3$^{+}$ T cells and all T cells (CD3$^{+}$) producing IFNγ ($n = 14$, $r = -0.66$, $P = 0.01$), CD4$^{+}$CD25$^{+hi}$ T cells and all T cells (CD3$^{+}$) producing IFNγ ($n = 14$, $r = -0.66$, $P = 0.01$), and CD4$^{+}$CD25$^{+hi}$ T cells and CD3$^{+}$CD4$^{+}$ T cells producing IFNγ ($n = 14$, $r = -0.63$, $P = 0.02$).

following one to two cycles of sunitinib treatment, the stronger the type-1 response is after two cycles of treatment.

Given that the improvement in type-1 response is associated with reduced Treg cell numbers after sunitinib treatment, we examined whether the functional capacity of the Treg cells remaining after sunitinib treatment was impaired. To address this issue, the suppressive activity of sorted CD4$^{+}$CD25$^{+hi}$ T cells from mRCC patients before treatment was compared with the suppressive activity of the Treg cells after one cycle of sunitinib treatment ($n = 5$) and in a subset of patients after two cycles of sunitinib treatment ($n = 3$). Following coculture of CD4$^{+}$CD25$^{+hi}$ with the CD4$^{+}$CD25$^{-}$ T-cell population for 3 days with anti-CD3/anti-CD28 antibodies, the uptake of [$^{3}$H]thymidine was determined. Before therapy, Treg cells from five of five patients showed a high level of suppression at baseline (mean 77.6 + 10.3 SE) at both 1:1 and 5:1 ratios of Teff to Treg cells. However, the suppressive activity of Treg cells was reduced >50% in three of five patients, although the peak time of suppression varied. In two patients, the reduction in Treg function was observed after the first cycle, whereas in the third patient it was observed after the second cycle of treatment. Interestingly, in one patient, the reduction in Treg suppression noted after the first cycle reversed after the second cycle. Although these preliminary findings suggest that treatment with sunitinib can lead to decreased suppressor function in Treg cells, additional samples are needed to verify these findings and to define the cycle number where sunitinib has the most effect on Treg cell function.

Whether sunitinib has a direct effect on the growth and viability of specific T-cell subsets in RCC patients has not been

defined. Although the absolute lymphocyte count decreased somewhat ($P = 0.03$), their relative proportion within the WBC increased ($P < 0.001$) in RCC patients after one and two cycles of sunitinib treatment. We did note that the number of Treg cells was decreased, although it did not reach statistical significance (Fig. 3). Consistent with our findings, Hipp et al. (36) showed that treatment of healthy mice with sunitinib reduced Treg cell numbers in the blood and this was not observed with another tyrosine kinase inhibitor, sorafenib. Given that sunitinib can have a negative effect *in vivo* on Treg cell numbers, we tested whether this drug can directly affect the expansion of Treg cells as well as the Teff population *in vitro*. This question was addressed by adding sunitinib (0.2 μmol/L) or not to cultures of Treg and Teff cells that had been previously expanded for 7 days with anti-CD3/anti-CD28 beads plus IL-2. After an additional 14 days in culture with IL-2 plus or minus sunitinib, the fold expansion of the Treg and Teff cells was determined by counting viable lymphocytes. Sunitinib (0.2 μmol/L) was chosen for T-cell treatment because this is the concentration that is achievable in the serum of patients treated with sunitinib (50 mg/d; ref. 37). As seen in Table 1, sunitinib did not seem to inhibit the expansion of either the CD4$^{+}$ Teff or Treg cells over a 14-day coincubation period ($n = 4$ experiments). Thus, it is possible that the negative effect sunitinib has on Treg cells *in vivo* is not the result of the drug directly affecting T-cell growth or viability.

## Discussion

Sunitinib has shown therapeutic activity in patients with mRCC and currently represents a frontline therapy for this disease (38). The goal of this study was to investigate the effect sunitinib therapy has on immune function in mRCC patients. Known immune dysfunction in mRCC patients includes a skewing from a T-cell type-1 cytokine response (IFNγ) to a type-2 response (IL-4), which is known to impair T-cell immunity (25, 26). The findings reported here show that mRCC patients receiving sunitinib had a significant improvement in the type-1 cytokine response with a diminished expression of a type-2

**Table 1.** Sunitinib did not block T-cell growth *in vitro*

**Teff (CD3$^{+}$CD4$^{+}$CD25$^{-}$)**

| | Purity at sort | Fold increase | |
| --- | --- | --- | --- |
| | | Medium | 0.2 μmol/L sunitinib |
| Experiment 1 | 95.2 | 45.7 | 52.2 |
| Experiment 2 | 95.8 | 268.2 | 258.5 |
| Experiment 3 | 95.4 | 12.8 | 10.1 |
| Experiment 4 | 95.2 | 78.3 | 50.5 |

**Treg (CD3$^{+}$CD4$^{+}$CD25$^{+hi}$)**

| | Purity at sort | Fold increase | |
| --- | --- | --- | --- |
| | | Medium | 0.2 μmol/L sunitinib |
| Experiment 1 | 95.9 | 36.1 | 25.4 |
| Experiment 2 | 93.7 | 3.8 | 4.0 |
| Experiment 3 | 94.1 | 4.3 | 6.1 |
| Experiment 4 | 93.7 | 10.4 | 10.4 |

response when PBMCs were stimulated *in vitro* with anti-CD3/anti-CD28 antibodies before measuring intracellular levels of IFNγ and IL-4. The increased type-1 response after therapy may be partly attributable to an effect that sunitinib treatment has on Treg cells. A negative correlation was observed between the decrease in the percentage of Treg cells from pretreatment to day 28 of cycle 1 or 2 and the increase in IFNγ-producing CD4^{+} T cells from pretreatment to cycle 2. These findings suggest that the greater the reduction of Treg cells at the end of cycle 1, the stronger the type-1 response is after two cycles of sunitinib treatment (Fig. 4). Additionally, functional studies using sorted Treg cells suggested that the suppressive activity of these cells was diminished after sunitinib treatment.

CD4^{+} T cells seem critical for the promotion of memory CD8^{+} T-cell responses (39, 40), although CD8^{+} T cells are typically effector cells associated with tumor rejection (41, 42). However, antitumor CD4^{+} Teff cells also have the capacity to mediate the regression of MHC class I–loss tumors that are resistant to CD8^{+} CTLs (43). Additionally, the quality of antitumor CD4^{+} T-cell responses seems to be a prognostic indicator of disease progression and immunotherapeutic responsiveness (44). Moreover, RCC patients whose tumor environment is biased toward a type-1 immune response have a more favorable prognosis (45). Although a type-1 immune response is the desired and appropriate antitumor response, several studies have shown that RCC patient T cells are selectively deficient in IFNγ response and biased toward a type-2 T-helper cell response (8, 9, 46). Our current study confirms that mRCCs have a type-2 bias when stimulated with anti-CD3/anti-CD28 antibodies. More importantly, the findings reported here show that partial restoration of a type-1 cytokine response is achieved in mRCC patients receiving sunitinib. The analysis of total CD3^{+} lymphocytes and the CD3^{+}CD4^{+} T-cell subset both showed increased stimulus-dependent IFNγ response after treatment compared with pretreatment values. There was also a corresponding decrease in the type-2 bias.

One plausible explanation for these findings is that the restoration of a type-1 IFNγ response is related to a decrease in the immunosuppressive tumor burden. Although small numbers of patients may preclude detection of a meaningful association, there was no correlation between the increase in the type-1 bias and tumor shrinkage or objective response. This suggests that there are alternative or additional explanations for the improved T-cell response. There did, however, seem to be some association between baseline type-2 bias and tumor shrinkage (Spearman $r = 0.59$, $P = 0.006$). That is, the greatest percentage of tumor shrinkage tended to occur in patients with a lower type-2 bias (lower type-2 to type-1 ratio). Additionally, there is some suggestion that the achievement of a PR is related to lower baseline type-2 response: the mean ± SE type-2 bias was 8.8 ± 2.3 for patients who achieved a PR ($n = 9$) compared with 60.0 ± 25.6 for patients that did not respond ($n = 11$, $P = 0.07$). One interpretation of these findings is that the clinical response induced by sunitinib may be influenced by the degree of the type-2 bias at baseline. This hypothesis could be explained by an immune mechanism of sunitinib (which is enhanced by a reduced type-2 bias at baseline) or that parallel mechanisms of antitumor effect (sunitinib-induced antiangiogenic and an innate immune response) are relevant.

A significant increase in Treg cells has been reported in the peripheral blood of mRCC patients when compared with normal donors, and when isolated, these Treg cells inhibited the proliferation of Teff cells (CD4^{+}CD25^{-}) in coculture (34, 35). In one of these studies, the increased numbers of Treg cells (CD4^{+}CD25^{+}FoxP3^{+}) in the peripheral blood correlated with poor clinical outcome (35). However, immunostaining of RCC tissue from patients with localized disease showed that CD4^{+}CD25^{+}FoxP3^{-}IL-10^{+} T cells, perhaps representing the Tr1 variety of Treg cells, were significantly associated with poor outcome and death from RCC (30). Thus, additional studies are needed to better define the role of Treg subsets regulating T-cell immunity and clinical outcome in mRCC patients. The importance of the Treg cells is further supported by the demonstration that reduction of Treg cells in mRCC patients using a recombinant IL-2 diphtheria toxin conjugate (DAB389IL-2) combined with vaccination increased the number of tumor antigen–specific CD8^{+} and CD4^{+} T cells producing IFNγ (24). The findings reported here suggest that sunitinib treatment may have an effect on the Treg population resulting in improved type-1 cytokine response. Indeed, the decrease in the percentage of Treg cells after one or two cycles of sunitinib correlated with an increase in IFNγ^{+} T cells at cycle 2 (Spearman $r = -0.64$, $n = 0.01$ in both cases). One explanation for these findings is that Treg cells contribute to the suppression of the type-1 response and the reduction in Treg cells observed after sunitinib treatment allows for a recovery of IFNγ-producing T cells. It is possible that Treg cells reduced the type-1 response by inhibiting the proliferation of the CD4^{+}CD25^{-} T cells. Indeed, our findings did show that patient Treg cells were efficient at inhibiting the proliferation of autologous CD4^{+}CD25^{-} T responders *in vitro*. Although preliminary, it seems that sunitinib also reduced the functional activity of the Treg cells that remained after therapy. In three of five patients where Treg function was compared before treatment and after 28 days of therapy, posttreatment Treg displayed diminish ability to suppress the proliferation of CD4^{+}CD25^{-} T cells.

The mechanism by which Treg numbers increase in mRCC patients and how sunitinib treatment can reduce their numbers and possibly their function in mRCC patients is not clear. One possibility is that the interaction of CD4^{+}CD25^{-} T cells with tumor cells results in the development of Treg cells as reported in the renal cell cancer murine model Renca (47). We have found that incubation of purified CD4^{+}CD25^{-} T cells with two different RCC lines for 72 to 96 h in the presence of anti-CD3 stimulation induced ∼15% of these cells to express the phenotype of Treg cells (CD4^{+}CD25^{+}FoxP3^{+}).[6] We are currently testing whether the addition of sunitinib to the cultures will block the induction and suppressive function of these induced Treg cells. However, our *in vitro* studies with sunitinib suggest that it does not directly affect the ability of either Treg or Teff cells to proliferate. The expansion of Treg and CD4^{+} Teff cells was similar in cultures that did and did not contain sunitinib (0.2 μmol/L; Table 1). Alternatively, Treg cells may be induced from the CD4^{+}CD25^{-} T population by interaction with immature myeloid dendritic cells (48). RCCs secrete several factors that could combine to result in the accumulation of

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[6] J. Ireland, in review.

immature myeloid dendritic cells, such as vascular endothelial growth factor, IL-6, granulocyte macrophage colony-stimulating factor, macrophage colony-stimulating factor, and gangliosides (49). These myeloid-derived suppressor cells (MDSC) describe a heterogeneous group of immature cell types that induces Treg cells and/or T-cell tolerance (50, 51) and is elevated in mRCC patients (33, 52). We have recently observed that the percentage of MDSC ($CD15^+CD14^-$ and $CD33^+HLADR^-$) decreased after sunitinib treatment of mRCC patients ($n = 19$, $P = 0.04$).[4] It is possible that the MDSC may partly promote the increase in Treg cells because a positive correlation between Treg cells at cycle 2 (day 28) and $CD15^+CD14^-$ cells was observed (Spearman $r = 0.97$, $P < 0.001$). Further studies are needed to clarify the mechanism by which sunitinib can modulate Treg cells and MDSC function in RCC patient. Additional studies are also needed to determine whether the positive effect that sunitinib has on reversing immune suppression in mRCC patients is unique or is generalized to other targeted agents, such a bevacizumab or temsirolimus. However, a recent report comparing sunitinib and sorafenib in both *in vitro* studies with human dendritic cells and *in vivo* murine studies examining specific T-cell response to OVA peptides suggest that sorafenib but not sunitinib may be suppressive (36). This study also showed that in non–tumor-bearing mice sunitinib reduced the number of Treg cells, a finding that is consistent with the notion that this drug may have the potential to promote more effective T-cell immunity.

The demonstration that sunitinib treatment improved type-1 T-cell cytokine response in RCC patients while reducing Treg number and function provides a basis for the rational combination of sunitinib and immunotherapy in mRCC. To this end, we propose to combine sunitinib with a vaccine consisting of autologous dendritic cells pulsed with a mixture of peptides previously shown to be expressed on RCC (MAGE-2, EphA2, and G250; ref. 53). The proposed study will be done in collaboration with Drs. Strokus and Whiteside (University of Pittsburgh, Pittsburgh, PA). The dendritic cell–based vaccine will be administered either before or after sunitinib. The monitoring of type-1 and type-2 responses along with Treg and MDSC should provide some insight into the effect that sunitinib/vaccine combination has on T-cell responses as well as immune suppression in this patient population.

## Disclosure of Potential Conflicts of Interest

## Acknowledgments

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