

## Sunitinib Mediates Reversal of Myeloid-Derived Suppressor Cell Accumulation in Renal Cell Carcinoma Patients

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**Abstract Purpose:** Immune dysfunction reported in renal cell carcinoma (RCC) patients may contribute to tumor progression. Myeloid-derived suppressor cells (MDSC) represent one mechanism by which tumors induce T-cell suppression. Several factors pivotal to the accumulation of MDSC are targeted by the tyrosine kinase inhibitor, sunitinib. The effect of sunitinib on MDSC-mediated immunosuppression in RCC patients has been investigated.

**Experimental Design:** Patient peripheral blood levels of MDSC and regulatory T-cell (Treg) and T-cell production of IFN- $\gamma$  were evaluated before and after sunitinib treatment. Correlations between MDSC and Treg normalization as well as T-cell production of IFN- $\gamma$  were examined. The *in vitro* effect of sunitinib on patient MDSC was evaluated.

**Results:** Metastatic RCC patients had elevated levels of CD33<sup>+</sup>HLA-DR<sup>+</sup> and CD15<sup>+</sup>CD14<sup>+</sup> MDSC, and these were partially overlapping populations. Treatment with sunitinib resulted in significant reduction in MDSC measured by several criteria. Sunitinib-mediated reduction in MDSC was correlated with reversal of type 1 T-cell suppression, an effect that could be reproduced by the depletion of MDSC *in vitro*. MDSC reduction in response to sunitinib correlated with a reversal of CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> Treg cell elevation. No correlation existed between a change in tumor burden and a change in MDSC, Treg, or T-cell production of IFN- $\gamma$ . *In vitro* addition of sunitinib reduced MDSC viability and suppressive effect when used at  $\geq 1.0$   $\mu$ g/mL. Sunitinib did not induce MDSC maturation *in vitro*.

**Conclusions:** Sunitinib-based therapy has the potential to modulate antitumor immunity by reversing MDSC-mediated tumor-induced immunosuppression.

This year ~36,000 Americans will be newly diagnosed with kidney cancer, resulting in 12,890 deaths (1). Poor outcome in renal cell carcinoma (RCC) is related to its late disease presentation, propensity for recurrence, and refractoriness to traditional chemotherapy or radiotherapy. Additionally, treatment with cytokine therapy has resulted in limited success despite the known immunogenicity of RCC (2–6). The

receptor tyrosine kinase inhibitor sunitinib is an oral medication being used with significant clinical effect in metastatic clear cell RCC (7–10). Sunitinib inhibits signaling through the vascular endothelial growth factor receptors (VEGFR), as well as platelet-derived growth factor receptor, stem cell factor receptor (c-Kit), Flt3, and colony-stimulating factor (CSF)-1 receptor (11).

VEGF signaling plays a prominent role in the pathogenesis of clear cell RCC, particularly due to the common occurrence of von Hippel-Lindau gene inactivation in these tumors (10, 12). VEGF overproduction promotes tumor-associated angiogenesis required for tumor growth and metastasis (10, 12). VEGF may also support tumor growth via negative effects on host antitumor adaptive immunity, as increased VEGF levels have been associated with alterations in myeloid cell differentiation, which impair competent dendritic cell formation and encourage suppressive myeloid cell formation in cancer patients (13–18).

Myeloid-derived suppressor cells (MDSC) impair T-cell effector function and represent a heterogeneous population of cells that accumulate in tumor-bearing hosts as a result of tumor-induced alterations in myelopoiesis (19). MDSC accumulating in the tumors and lymphoid organs of tumor-bearing mice are CD11b<sup>+</sup>Gr1<sup>+</sup> and mediate T-cell impairment that is reversed with tumor removal or CD11b<sup>+</sup> or Gr1<sup>+</sup> cell depletion (18–21). MDSC detected in the peripheral blood of patients bearing several tumor types (16, 22–27) express the common

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## Translational Relevance

Tumor-induced immunosuppression in metastatic renal cell carcinoma patients, mediated by myeloid-derived suppressor cells, can be reversed by a sunitinib-induced reduction in myeloid-derived suppressor cell (MDSC) numbers. Because MDSC are known to inhibit T-cell sensitization to tumor antigens, their depletion may be clinically desirable before the initiation of immunotherapy modalities such as adoptive T-cell transfer, dendritic cell-based vaccines, or cytokine therapy. Other modalities such as all-*trans*-retinoic acid or certain chemotherapy regimens can also deplete MDSC. However, the well-known antiangiogenic effects of sunitinib, combined with its known tolerability and objective benefit in the clinical setting, may render it a superior adjunct agent for immunotherapy trials. The observed effect of sunitinib on host immune cells is likely to be independent of its antitumor effect, so its potential benefit in immunotherapy may not be specific to tumor type. These data should thus be applicable to the design of future clinical trials.

myeloid marker CD33 but lack markers of mature myeloid cells such as the MHC class II molecule HLA-DR. Expression of the granulocytic marker CD15 divides patient MDSC into at least two subsets. The CD15<sup>+</sup> subset has been shown to suppress T-cell function in patients with kidney cancer, among others, through an arginase and/or reactive oxygen species-dependent mechanism (23, 24). A CD15<sup>+</sup> subset of MDSC was also shown to suppress T-cell function in patients with squamous cell carcinoma of the head/neck/lung or adenocarcinoma of the breast/lung through an unclear mechanism (22). These subsets likely parallel those recently identified in the mouse model, where the CD15<sup>+</sup> (human) and the Gr1<sup>hi</sup> (mouse) MDSC are granulocytic and the CD15<sup>+</sup> (human) and the Gr1<sup>lo</sup> (mouse) MDSC are monocytic (28). New evidence in hepatocellular carcinoma and malignant melanoma patients suggests that a third, CD14<sup>+</sup>HLA-DR<sup>dim</sup> subset of MDSC also exists (26, 27).

Because VEGF has been implicated in the generation of MDSC (29, 30), we have evaluated the effect of sunitinib, which blocks signaling through multiple receptors, including VEGFRs (11), on MDSC in metastatic RCC (mRCC) patients. MDSC are reported to inhibit T-cell function directly (19) as well as indirectly via the induction of regulatory T-cell (Treg) formation (27, 31). We have therefore examined patient T-cell production of IFN- $\gamma$ , as well as patient Treg levels, before and after treatment with sunitinib. We observed an elevation in CD15<sup>+</sup> and CD15<sup>+</sup> MDSC in RCC patients compared with age-matched normal (AMN) donors. We show that treatment with sunitinib in mRCC patients reduces the elevated levels of both MDSC subsets to near normal levels. A reduction in MDSC measured by two criteria (CD15<sup>+</sup>CD14<sup>+</sup> and CD33<sup>+</sup>HLA-DR<sup>+</sup>) was correlated with a subsequent recovery in patient T-cell effector function but not with tumor regression. A reduction in CD15<sup>+</sup>CD14<sup>+</sup> MDSC was associated with a reduction in patient Treg levels. mRCC patient MDSC-mediated T-cell suppression *in vitro* was reversible with MDSC depletion and modestly with the addition of excess L-arginine. Sunitinib, when used *in vitro*,

partially blocked the suppressive function of MDSC possibly due to its effects on MDSC viability when added at  $\geq 1.0$   $\mu$ g/mL rather than its effects on MDSC maturation. Sunitinib-based combination immunotherapy may be a promising option for the future treatment of mRCC.

## Materials and Methods

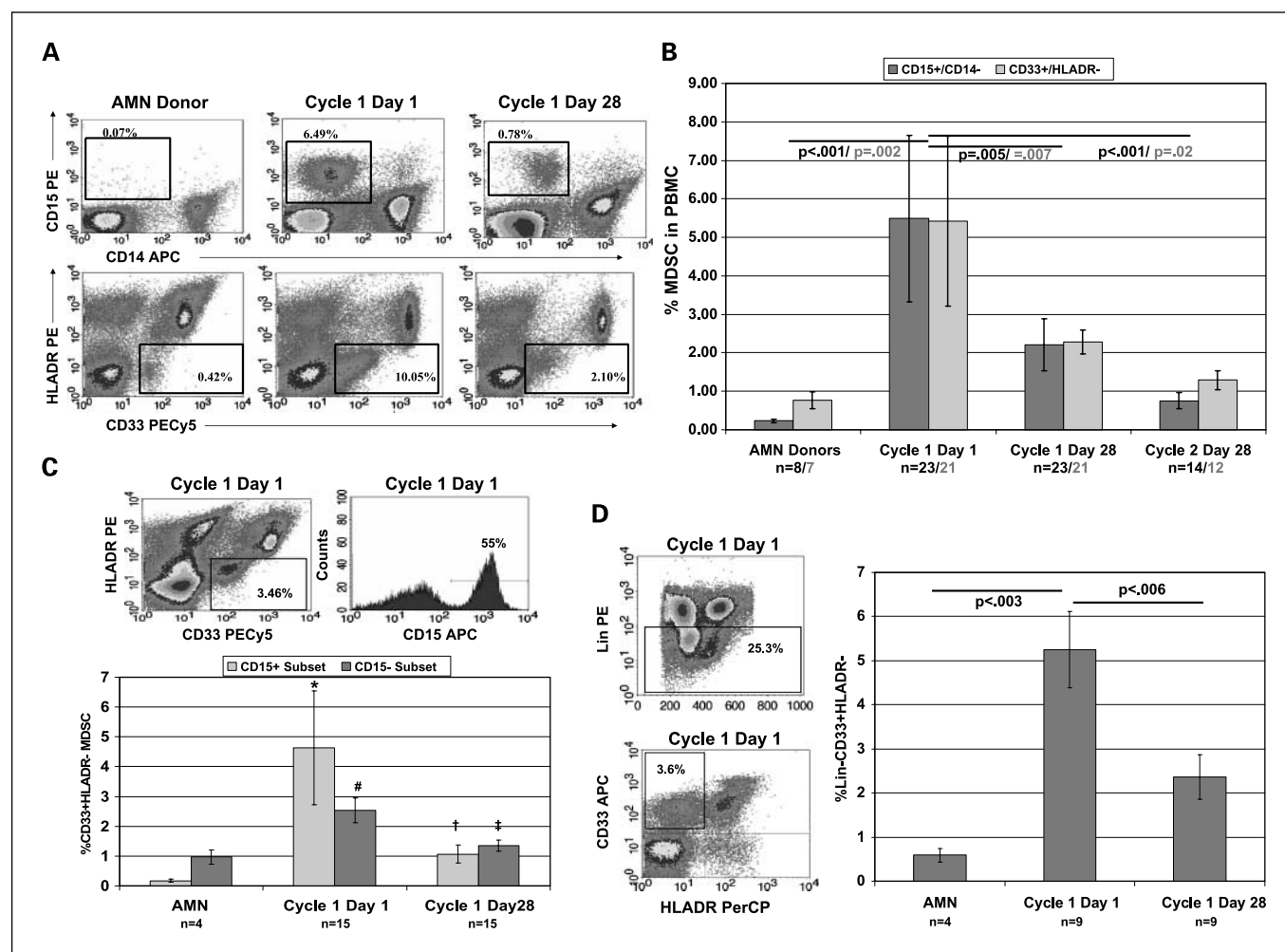
**Patient population and treatment.** Patients received sunitinib monotherapy for mRCC (50 mg by mouth daily) for 28 days followed by 14 days of rest, comprising one 6-week cycle. Patients were excluded if they received any anticancer therapy concomitant with sunitinib, if they had a diagnosis other than clear cell RCC, or if they did not receive at least 28 days of sunitinib. Patients underwent disease assessment (computed tomography and bone scans) at baseline and after every 2 cycles (every  $\sim 12$  weeks). Objective response according to the Response Evaluation Criteria in Solid Tumor (32) and tumor burden shrinkage were determined by investigator assessment of radiographs. Patients were treated until Response Evaluation Criteria in Solid Tumor-defined disease progression or unacceptable toxicity occurred. Dose interruption and modification was done according to the treating physician's discretion. All patients and healthy volunteer blood donors signed an institutional review board-approved, written informed consent for collection of blood samples. AMN donors were healthy volunteers ages  $>50$  years.

**Reagents.** Human IgG and L-arginine were from Sigma-Aldrich. Catalase was from Calbiochem. [<sup>3</sup>H]thymidine was from Amersham-Buchler. Anti-human CD3, CD4, IFN- $\gamma$ , interleukin (IL)-4, CD11c, CD13, CD14, CD15, CD33, HLA-DR, Annexin V, 7-amino-actinomycin D, and the Annexin V staining kit were from BD Biosciences. Anti-human Foxp3, CD11b, CD19, CD40, CD56, CD80, and CD86 were from eBioscience. Mouse isotype control antibodies were from BD, eBioscience, or Immunotech. Anti-human CD15 and CD33 antibody-coated magnetic microbeads and LS magnetic columns were from Miltenyi Biotec. Granulocyte macrophage-CSF (GM-CSF) and IL-4 were from R&D Systems. Sunitinib in pure powder form was from Pfizer.

**Peripheral blood mononuclear cell isolation.** Peripheral blood (60 mL) was drawn from mRCC patients before sunitinib treatment (cycle 1 day 1) and on day 28 after one cycle of treatment (cycle 1 day 28), and in a subset of patients on day 28 after two cycles of treatment (cycle 2 day 28), and from AMN donors. Peripheral blood was drawn in heparin-containing tubes. Peripheral blood mononuclear cells (PBMC) were isolated within 2 h of blood draw and either used fresh or frozen for later use according to the methods described previously (33). For phenotypic and functional studies where multiple time points were available, all time points for an individual patient were thawed together and used in the same experiment.

**Fluorescence-activated cell sorting analysis of patient PBMC.** Analysis of MDSC percentages in patient PBMC were done on thawed samples. Cells were stained in fluorescence-activated cell sorting (FACS) buffer (1 $\times$  PBS with 2% heat-inactivated fetal bovine serum and 0.02% sodium azide). Nonspecific antibody binding was blocked by pretreatment of cells with 10  $\mu$ g/mL human IgG for 20 min at room temperature. Surface stains were added to cells for 30 min at 4°C. Cells were stained with anti-human CD11b, CD11c, CD14, CD15, CD33, and HLA-DR. In a subset of patients, cells were also stained with anti-human CD3, CD19, CD56, CD80, and CD86. Cells were washed in buffer and then fixed in 1% paraformaldehyde and ran for FACS.

Treg were enumerated by FACS on thawed patient PBMC rested in complete RPMI 1640 overnight (37°C, 5% CO<sub>2</sub>) and then stained with anti-CD3, CD4, CD25 (Stem Cell Technologies), and Foxp3 according to the instructions included in the BD intracellular staining kit. PBMC were incubated overnight to facilitate experiments that could examine both Treg and T-cell stimulation from the same sample. Thawed T cells stimulate better after some amount of resting



**Fig. 1.** Elevated MDSC in mRCC patients decline in response to sunitinib. **A**, PBMC from mRCC patients before sunitinib treatment (cycle 1 day 1) following one or two cycles of sunitinib (cycle 1 day 28 and cycle 2 day 28, respectively) as well as from healthy AMN donors were stored in liquid nitrogen and then thawed, washed, blocked with human IgG, and then immediately stained with monoclonal antibodies to CD14, CD15, CD33, and HLA-DR. Cells were fixed with 1% paraformaldehyde and then ran and analyzed by multicolor flow cytometry. MDSC populations in representative dot plots from an AMN donor and a mRCC patient before treatment (cycle 1 day 1) and after one cycle of sunitinib (cycle 1 day 28). Dot plots represent live gated events. MDSC populations are gated in black. **B**, columns, mean percentage of MDSC in total PBMC in patients before and after sunitinib treatment versus that in AMN healthy control donors; bars, SE (*P* values). Ranges are as follows: CD15<sup>+</sup>HLA-DR<sup>-</sup>:AMN = 0.05-0.47, cycle 1 day 1 = 0.13-37.95, cycle 1 day 28 = 0.18-15.21, and cycle 2 day 28 = 0.16-2.69; CD33<sup>+</sup>HLA-DR<sup>-</sup>:AMN = 0.1-1.91, cycle 1 day 1 = 0.31-47.1, cycle 1 day 28 = 0.33-16.42, and cycle 2 day 28 = 0.09-3.1. **C**, live gated events are displayed in dot plot (left). CD33<sup>+</sup>HLA-DR<sup>+</sup> events are gated and displayed in histogram (right) to be composed of two populations: CD15<sup>+</sup> and CD15<sup>-</sup>. Columns, mean percentage of CD33<sup>+</sup>HLA-DR<sup>+</sup> MDSC that are granulocytic (CD15<sup>+</sup>) and that are monocytic (CD15<sup>-</sup>) in total PBMC in patients before and after one cycle of sunitinib treatment versus that in AMN healthy control donors; bars, SE. \*, *P* = 0.02 versus AMN; #, *P* = 0.04 versus AMN, †, *P* = 0.02 versus cycle 1 day 1; ‡, *P* = 0.005 versus cycle 1 day 1. Ranges are as follows: CD33<sup>+</sup>HLA-DR<sup>+</sup>CD15<sup>+</sup>:AMN = 0.05-0.32, cycle 1 day 1 = 0.09-37.59, and cycle 1 day 28 = 0.09-3.26; CD33<sup>+</sup>HLA-DR<sup>+</sup>CD15<sup>-</sup>:AMN = 0.43-1.58, cycle 1 day 1 = 0.66-6.49, and cycle 1 day 28 = 0.38-3.0. **D**, PBMC were stained with PE-conjugated antibodies to CD3, CD14, CD19, and CD56 as well as CD33-APC and HLA-DR-PerCP antibodies. Live gated events are displayed in dot plot (left). Lineage-negative cells are gated and displayed in dot plot (right). Columns, mean percentage of lineage CD33<sup>+</sup>HLA-DR<sup>+</sup> MDSC in total PBMC in patients before and after one cycle of sunitinib treatment versus that in AMN healthy control donors; bars, SE (*P* values). Ranges are as follows: AMN 0.27-0.92, cycle 1 day 1 = 1.42-10.54 and cycle 1 day 28 = 0.8-5.0.

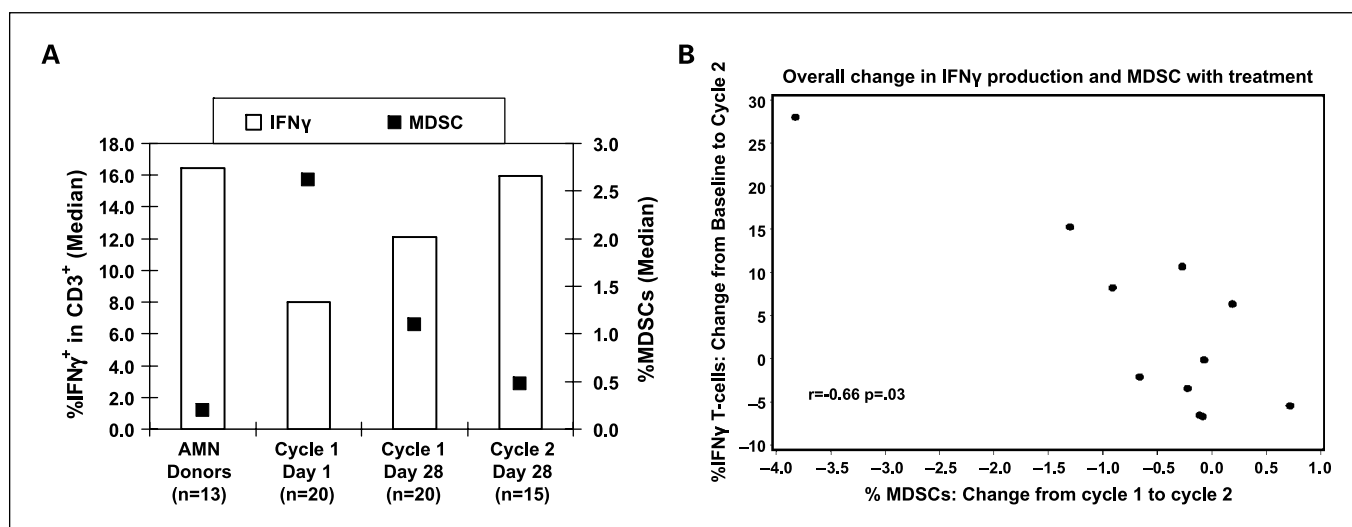
period, and multiple past experiments have shown numerically equivalent results for Treg staining done immediately after thawing samples or done after an overnight culture. All data were acquired using CellQuest on a BD FACSCalibur and analyzed using either Flow Jo (Tree Star) or CellQuest software. At least 300,000 live cell events were collected for each tube used in analysis.

**Determination of patient T-cell IFN- $\gamma$  response.** Patient PBMC samples were stimulated with anti-CD3/CD28-bound beads (Dynal) and IL-2 (Chiron) for 72 h. Golgi plug was added to cells for the last 6 h, and harvested cells were stained with anti-human CD3, CD4, IFN- $\gamma$ , and IL-4 according to the protocol provided using the BD intracellular staining kit. 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 before the addition of fluorochrome-labeled antibodies.

**MDSC depletion.** One half of each patient sample was treated with anti-CD15 antibody-coated magnetic microbeads. Cells were incubated at 4°C for 20 min and then washed and resuspended in PBE (PBS with BSA and EDTA). Cells were run over a LS magnetic column for the depletion of bead-labeled cells as per the manufacturer's instructions. FACS analysis was done on a small aliquot of cells to assure that MDSC had been effectively depleted. Cells were then resuspended in complete RPMI 1640 and then activated and stained for intracellular cytokines (IFN- $\gamma$  and IL-4) as described above.

**In vitro sunitinib culture assays.** Fresh patient PBMC were incubated with a mixture of anti-human CD33 and CD15 antibody-conjugated magnetic beads. CD33<sup>+</sup> and CD15<sup>+</sup> myeloid cells were

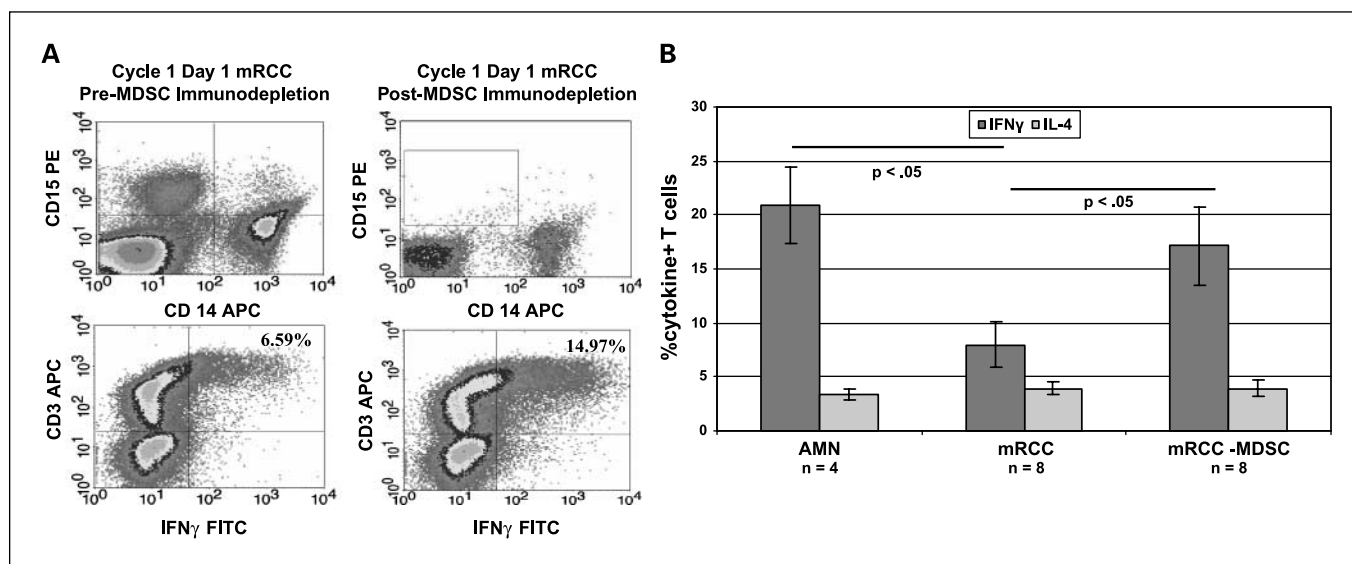


**Fig. 2.** Sunitinib-mediated normalization of MDSC is associated with sunitinib-mediated enhancement in T-cell production of IFN- $\gamma$  in mRCC patients. **A**, patient or control donor PBMC were stored in liquid nitrogen and thawed, and a portion of PBMC was analyzed immediately for MDSC as described previously. The other portion of PBMC was rested overnight in complete medium, and equal numbers of nonadherent cells were stimulated polyclonally the following day with anti-CD3/CD28 antibody-coated beads and IL-2. **Columns**, median percentage of CD3 $^{+}$  cells staining positive for IFN- $\gamma$ . **Squares**, median percentage of MDSC in PBMC. Ranges for IFN- $\gamma$  production: normal donors = 11.07-31.52, pretreatment = 2.25-38.31, cycle 1 day 28 = 2.23-21.29, and cycle 2 day 28 = 5.09-34.08. Mean  $\pm$  SE and  $P$  values are as follows: AMN donors = 18.40  $\pm$  1.78%, cycle 1 day 1 = 11.07  $\pm$  2.13% ( $P = 0.008$  for AMN versus cycle 1 day 1), cycle 1 day 28 = 11.93  $\pm$  1.19% ( $P = 0.26$  for cycle 1 day 1 versus cycle 1 day 28), and cycle 2 day 28 = 15.98  $\pm$  2.34% ( $P = 0.21$  for cycle 1 day 28 versus cycle 2 day 28). **B**, changes in patient T-cell production of IFN- $\gamma$  in response to sunitinib were compared with changes in their own levels of MDSC in response treatment with sunitinib. Each dot represents a single patient ( $r = -0.66$ ;  $P = 0.03$ ).

positively selected with a LS column. Cells that flowed through the columns were at least 80% CD3 $^{+}$  T cells, whereas positively selected myeloid cells were all CD11b $^{+}$ , and on average, 40% were negative for HLA-DR and thus represented MDSC.

For studies evaluating the *in vitro* effect of sunitinib on MDSC, a minimal concentration of 0.1  $\mu$ g/mL was used, as this is equivalent to the levels detected in human plasma (34), and sunitinib was titrated up to 10- to 50-fold from there. To measure the effect of sunitinib on MDSC viability, myeloid cells containing MDSC were incubated in

complete RPMI 1640 with 20% SK-RC26B (gift from Dr. Neil Bander, Cornell Medical Center) RCC cell line-conditioned medium (tumor conditioned medium) and 50 ng/mL GM-CSF to support cell viability over 48 h (observed and published findings; ref. 22). Sunitinib suspended in plain RPMI 1640 was added at 0.1, 1.0, and 5.0  $\mu$ g/mL to three groups of cells. After 48 h, cells were harvested, and surface stained for HLA-DR and CD33, for 15 min at 4°C in FACS buffer and then stained for Annexin V and 7-amino-actinomycin D per BD provided protocol for 15 min and then ran for FACS.



**Fig. 3.** *In vitro* depletion of MDSC restores patient T-cell production of IFN- $\gamma$ . **A**, pretreatment patient PBMC samples were thawed and immediately stained for the expression of myeloid cell surface markers with or without the removal of MDSC by anti-CD15 magnetic microbeads and magnetic column. Representative dot plots from live gated cells in patients without (mRCC) and with (mRCC-MDSC) MDSC depletion. Plots shown after MDSC depletion contain less acquired events. Pretreatment patient PBMC samples were thawed and polyclonally activated as described previously with or without the prior removal of MDSC. After 72 h, IFN- $\gamma$  and IL-4 were detected in CD3 $^{+}$  cells by FACS. Representative dot plots looking at T-cell IFN- $\gamma$  production in a patient without (mRCC) and with (mRCC-CD15) MDSC depletion. **B**, **columns**, mean percentage of CD3 $^{+}$  cells staining positive for IFN- $\gamma$  or IL-4; **bars**, SE ( $P$  values). Ranges are as follows: AMN = 13.4-35.4, mRCC = 2.4-17.6, and mRCC-MDSC = 6-39.9.



For studies examining the effect of sunitinib on MDSC differentiation, patient myeloid cells were isolated and incubated in GM-CSF and tumor conditioned medium-containing cultures as above, with the addition of 50 ng/mL IL-4, with or without sunitinib at either 0.1 or 1.0  $\mu\text{g/mL}$ . Half the medium was replaced after 3 days, and after 6 days, the cells were harvested and analyzed for the expression of HLA-DR, CD40, CD80, and CD86 by FACS. Remaining cells were irradiated at 3,000 rad and used as stimulators in mixed lymphocyte reactions with normal donor, allogeneic, fresh T cells. T-cell proliferation was determined after 6 days by the incorporation of tritiated thymidine.

Finally, for studies examining the mechanism of MDSC-mediated T-cell suppression, and the effect of sunitinib on this, patient T cells and MDSC-containing myeloid cells isolated as before were cocultured 1:1 in the presence of 200 units/mL catalase, 2 mmol/L L-arginine, or 0.1 or 1.0  $\mu\text{g/mL}$  sunitinib, and T cells were stimulated with anti-CD3/CD28-coated beads for 72 h with intracellular cytokine production being evaluated as described above.

**Statistical analysis.** Wilcoxon rank-sum test was used to compare mRCC patients and healthy donors with respect to MDSC, intracellular IFN- $\gamma$ , and Treg and to compare these parameters in patients who achieved a partial response by Response Evaluation Criteria in Solid Tumor versus patients whose best response was stable disease or progression. Spearman rank correlations were used to assess associations between immune parameters and associations with changes in tumor burden. The *t* test was used to compare results of *in vitro* experiments. All statistical tests were two-sided and all analyses were conducted using SAS version 9.1 (SAS Institute).

## Results

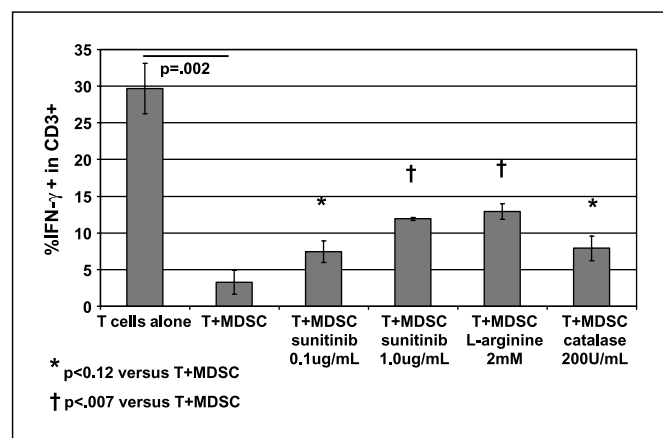
**Patient characteristics and clinical response to sunitinib.** Data from 23 mRCC patients treated with sunitinib monotherapy between August 2005 and August 2007 were available for analysis. Patient characteristics are summarized in Supplementary Table S1. Overall, 67% of patients were male, median age was 57 years (range, 41-80), and most patients (86%) had Eastern Cooperative Oncology Group performance status 0 or 1. Eighty-one percent of patients had prior nephrectomy, 43% had received prior systemic therapy (primarily sorafenib,

thalidomide, IFN- $\alpha$ , and/or IL-2), and one patient had received prior radiotherapy. Using the Memorial Sloan-Kettering Cancer Center criteria for previously untreated patients (35), 29% of patients were considered to have a favorable risk profile, 62% were considered intermediate, and 10% were considered poor risk. Forty-three percent of patients achieved a partial response by Response Evaluation Criteria in Solid Tumor; the median change in tumor burden was a 22.5% decrease (range, 60% decrease to 50% increase). Seven patients have progressed and 5 patients have died. Median follow-up for the patients still being followed is 4.4 months (range, 3.0-16.6).

**Elevated MDSC in mRCC patients decline in response to sunitinib.** mRCC patient PBMC were analyzed before the start of treatment (cycle 1 day 1) and after one or two cycles of treatment with sunitinib (cycle 1 day 28 and cycle 2 day 28, respectively) for MDSC, and their levels were compared with those in AMN donors. MDSC previously described in RCC patients, CD14<sup>+</sup>CD15<sup>+</sup> MDSC as well as CD33<sup>+</sup>HLA-DR<sup>+</sup> MDSC (24, 25), were quantified as shown (Fig. 1A). These cells were also confirmed to be positive for myeloid markers CD11b, CD11c, and CD13 in a subset of patients (data not shown). When each of the MDSC populations were calculated as a percentage of total PBMC, a highly significant increase in the number of both circulating CD14<sup>+</sup>CD15<sup>+</sup> MDSC and CD33<sup>+</sup>HLA-DR<sup>+</sup> MDSC were seen in mRCC patients (mean, 5.49% and 5.42%, respectively) when compared with healthy AMN donors (mean, 0.23% and 0.76%;  $P < 0.001$  and  $P = 0.002$ , respectively; Fig. 1B). MDSC by both criteria significantly declined after one cycle of treatment (mean, 2.21% and 2.28%;  $P = 0.005$  and  $0.007$ , respectively; Fig. 1B). In the subset of patients available who were treated with two cycles of sunitinib, MDSC continued to decline with an additional cycle of therapy (mean, 0.75% and 1.29%;  $P < 0.001$  and  $P = 0.02$ , respectively; Fig. 1B).

To confirm our suspicion that some degree of overlap existed between the two populations of MDSC as well as to assure that CD15<sup>+</sup> cells were also a target of sunitinib, a subset of 15 patients was analyzed by four-color FACS whereby anti-CD11c, CD33, HLA-DR, and CD15 were all in the same acquisition tube. A representative dot plot and histogram detail the analysis undertaken (Fig. 1C), which allowed for the quantification of both CD33<sup>+</sup>HLA-DR<sup>+</sup>CD15<sup>+</sup> immature myeloid cells, which are likely to be more monocytic in nature (22), and CD33<sup>+</sup>HLA-DR<sup>+</sup>CD15<sup>+</sup> immature myeloid cells, which are likely to be more granulocytic in nature (23, 24). Both populations of MDSC, which are likely similar to those recently characterized in the mouse tumor model (28), declined in response to treatment with sunitinib (CD15<sup>+</sup>  $P = 0.02$  and CD15<sup>+</sup>  $P = 0.005$ ; Fig. 1C). MDSC declining in response to sunitinib were confirmed to be lineage negative in a subset of patients ( $P < 0.006$ ; Fig. 1D).

**Sunitinib suppresses bone marrow production of myeloid cells but enhances lymphoid cell production.** Because sunitinib induced marked changes in MDSC, and because some of the receptors targeted by sunitinib influence hematopoiesis, we analyzed 20 patient complete blood counts with WBC differentials that have been reported at the appropriate time relative to sunitinib treatment (Supplementary Table S2). Total WBC counts declined with treatment from a median pretreatment amount of 7.7 to 4.1 K/ $\mu\text{L}$  (pretreatment to cycle 1 day 28;  $P < 0.001$ ) but stayed within the range of normal for



**Fig. 4.** Effect of sunitinib on MDSC suppressive function *in vitro*. RCC patient myeloid cells (~30-50% HLA-DR<sup>+</sup> MDSC) were positively selected with anti-CD33/CD15 magnetic beads and added or not to patient lymphoid cells (~80% T cells) at a 1:1 ratio in the presence or absence of sunitinib 0.1 or 1.0  $\mu\text{g/mL}$ , 200 units/mL catalase, or 2 mmol/L L-arginine. T cells were stimulated with anti-CD3/CD28 for 72 h and intracellular IFN- $\gamma$  production in CD3<sup>+</sup> cells was measured by FACS analysis. Pooled results are from three experiments. Mean, SE, and *P* values are shown.

most patients. The percentage of neutrophils significantly decreased but stayed within the range of normal for most patients (71% to 63%;  $P < 0.001$ ). In contrast, a decline in monocyte percentage was slight (8% to 7%), whereas the decline in absolute monocyte counts was significant (0.689-0.281;  $P < 0.001$ ). Meanwhile, the percentage of lymphocytes significantly increased (16-27%;  $P < 0.001$ ) into the normal range after treatment in most patients. Thus, sunitinib may have a myelospecific effect on bone marrow function.

**Declines in MDSC are associated with increases in IFN- $\gamma$ -producing T cells after sunitinib therapy.** In a related study, we have evaluated CD3<sup>+</sup> T-cell production of IFN- $\gamma$  in mRCC patients before and after treatment with sunitinib and found that mRCC patients have significantly reduced amounts of IFN- $\gamma$  production and that this type 1 response increases after sunitinib treatment ( $P \leq 0.001$ ;  $n = 38$ ; ref. 33). We observed similar results in this study, which included a smaller subset of patients. Pretreatment patient T-cell production of IFN- $\gamma$  was reduced (median, 7.71%) when compared with AMN donors (median, 16.43%;  $P = 0.008$ ). Treatment with sunitinib increased the amount of IFN- $\gamma$ -producing T cells in mRCC patients after one cycle (median, 13.81%) and two cycles of therapy (median, 15.93%), although this did not reach statistical significance in this subset (Fig. 2A). Type 1 T-cell IFN- $\gamma$  response normalization following sunitinib treatment was seen to coincide with normalizing numbers of MDSC, suggesting that MDSC levels may need to be reduced below a certain threshold before adaptive T-cell immunity can be recovered. Indeed, as seen in Fig. 2B, reductions in MDSC after two cycles of therapy were directly correlated with an overall increase in patient T-cell IFN- $\gamma$  production from baseline ( $r = -0.66$ ;  $P = 0.03$ ). Additionally, mRCC patients with relatively larger numbers of persisting MDSC after sunitinib treatment had relatively lower amounts of plasma IFN- $\gamma$  ( $r = -0.81$ ;  $P = 0.02$ ;  $n = 8$ ; data not shown).

**In vitro depletion of patient MDSC partially restores patient T-cell production of IFN- $\gamma$ .** Because of the negative association between mRCC patient MDSC and T-cell IFN- $\gamma$  production, we sought to determine whether removal of MDSC *in vitro* could render patient T cells capable of a type 1 response. Selected PBMC samples taken from patients with high levels of MDSC before the initiation of therapy were chosen and half of each sample was depleted of MDSC with anti-CD15 magnetic beads (Fig. 3A), and both conditions were stimulated with anti-CD3/CD28 for 72 h as described previously. CD3<sup>+</sup> cells were analyzed for IFN- $\gamma$  and IL-4 production (Fig. 3A), and mean levels of these cytokines in normal donor T cells or patient T cells with or without the removal of MDSC were calculated. MDSC depletion improved the ability of mRCC patient T cells to produce IFN- $\gamma$  ( $P < 0.05$ ; Fig. 3B). In all groups, there were low levels of IL-4 production seen at baseline that did not change with MDSC depletion.

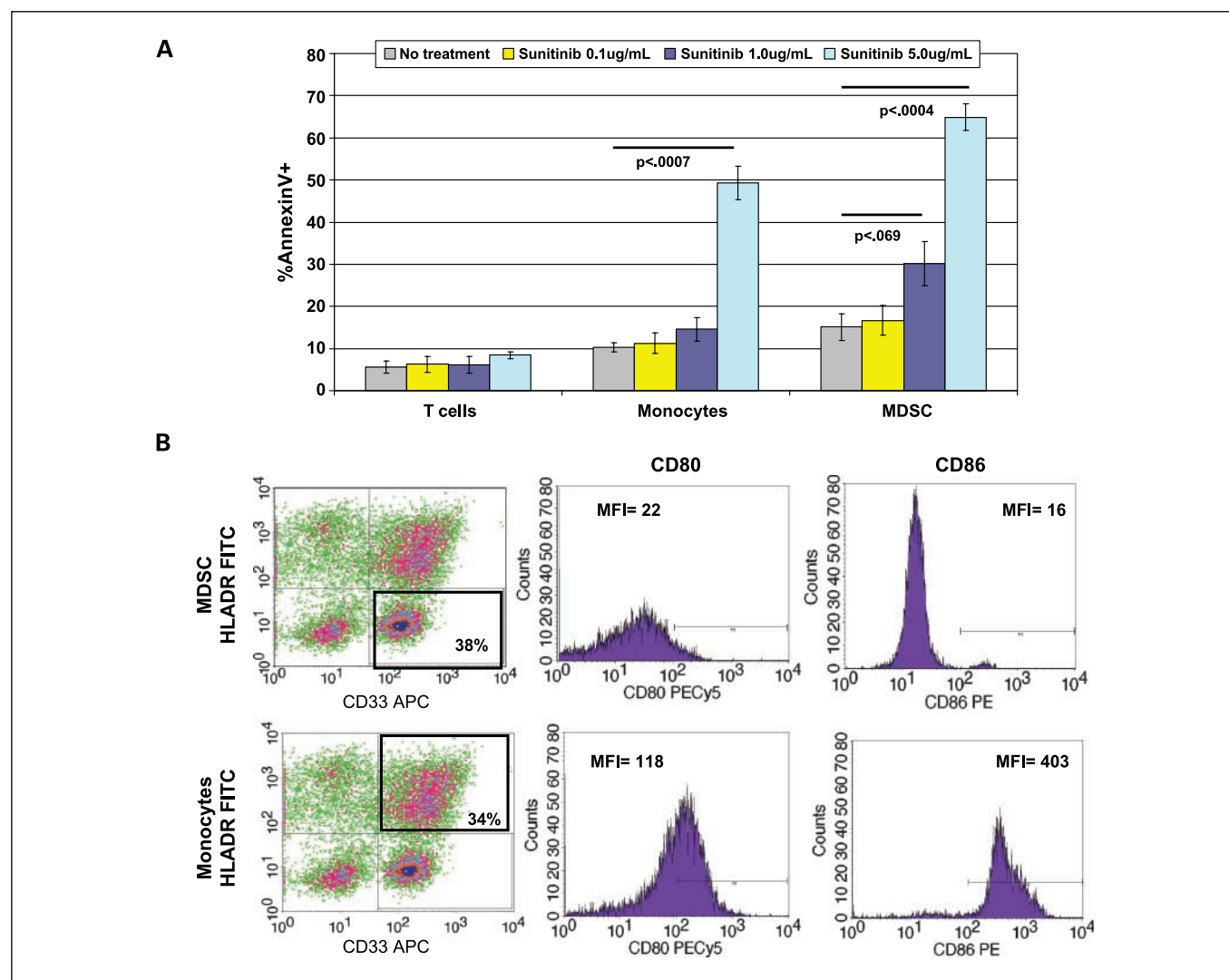
**In vitro effect of sunitinib on MDSC-mediated T-cell suppression.** MDSC characterized previously in RCC patients have been shown to inhibit T-cell function in an arginase-dependent manner (24). In addition, CD15<sup>+</sup> and CD33<sup>+</sup> MDSC were also suggested to inhibit T cells via the production of reactive oxygen species (23, 36). We therefore wanted to compare the ability of sunitinib to reverse patient MDSC-mediated T-cell suppression *in vitro* to the ability of 2 mmol/L L-arginine (24) and

200 units/mL catalase (23) to do the same. To ensure that both MDSC subtypes would be included in our experiments, we positively selected for RCC patient myeloid cells with a combination of anti-CD33 and anti-CD15 magnetic beads. Positively selected MDSC were added to patient T-cell cultures in the presence or absence of the various potential MDSC inhibitors, and T-cell production of IFN- $\gamma$  at 72 h following polyclonal stimulation was compared with cultures where no MDSC were added. MDSC isolated from patients were highly suppressive of patient T-cell function, and the addition of L-arginine to cultures resulted in significant, although modest, reversal of T-cell suppression (Fig. 4). Sunitinib used at 0.1  $\mu\text{g/mL}$ , a level equivalent to that detected in patient plasma (36), induced a trend toward normalization of T-cell function, which was equivalent to that seen with the addition of catalase, although it did not reach significance. Finally, in the presence of increased concentrations of sunitinib (1.0  $\mu\text{g/mL}$ ), there appeared to be significant, but modest, reversal of MDSC-mediated T-cell suppression (Fig. 4).

**In vitro effect of sunitinib on MDSC viability and differentiation.** We next asked whether the sunitinib-induced depression of MDSC accumulation observed in patients could be related to sunitinib-mediated MDSC apoptosis or maturation. It was necessary to add patient MDSC to cultures containing 20% SK-RC26B cell line tumor conditioned medium and/or 50 ng/mL GM-CSF to support long-term MDSC viability.<sup>7</sup> To assess the effect of sunitinib on MDSC apoptosis, patient myeloid cells were treated or not with sunitinib at 0.1, 1.0, or 5.0  $\mu\text{g/mL}$  for 48 h and then analyzed by FACS for Annexin V staining in both the CD33<sup>+</sup>HLA-DR<sup>-</sup> MDSC and the CD33<sup>+</sup>HLA-DR<sup>+</sup> monocytes/dendritic cells. Patient T cells were also separately cultured for 48 h in complete RPMI 1640 with or without the same amounts of sunitinib and assessed for viability. We found that, relative to lymphocytes, patient myeloid cells displayed an increased sensitivity to sunitinib-induced cell death (Fig. 5A). Patient MDSC seemed also to be somewhat more sensitive to sunitinib *in vitro* when compared with patient monocytes.

To assess patient MDSC differentiation in response to sunitinib, isolated myeloid cells were cultured with 50 ng/mL GM-CSF and IL-4 to stimulate dendritic cell maturation as well as 20% tumor conditioned medium to prevent indiscriminant maturation. Sunitinib was added or not to cultures at 0.1 or 1.0  $\mu\text{g/mL}$ . Figure 5B shows that patient CD33<sup>+</sup>HLA-DR<sup>-</sup> myeloid cells added to cultures were negative for the expression of CD80 and CD86 compared with CD33<sup>+</sup>HLA-DR<sup>+</sup> cells. After 6 days in culture, cells were harvested and their expression of dendritic cell maturity markers was compared by single-color FACS. Remaining cells were used in mixed lymphocyte reactions to stimulate allogeneic T-cell proliferation. Comparison of the expression of HLA-DR, CD40, CD80, and CD86, as well as the ability to stimulate T-cell proliferation in a mixed lymphocyte reaction, shows that sunitinib-treated and untreated cells became essentially equivalent dendritic cells (Fig. 5C). Sunitinib did not increase MHC class II and costimulatory marker expression on patient myeloid cells, nor did it increase the T-cell-stimulating

<sup>7</sup> Unpublished results.



**Fig. 5.** Effect of sunitinib on MDSC viability and differentiation *in vitro*. **A**, RCC patient myeloid cells (~30-50% HLA-DR<sup>+</sup> MDSC) were positively selected with anti-CD33/CD15 magnetic beads and cultured with 50 ng/mL GM-CSF and 20% SK-RC26B tumor conditioned medium to maintain viability in the presence or absence of 0.1 or 1.0  $\mu$ g/mL sunitinib for 48 h. Patient lymphoid cells were cultured separately in complete medium with or without sunitinib. Cells were stained with anti-CD33 and HLA-DR antibodies for 15 min in FACS buffer and then with Annexin V and 7-amino-actinomycin D in Annexin V binding buffer for 15 min and ran for FACS. Columns, mean percentage of Annexin V-positive CD3<sup>+</sup> T cells, CD33<sup>+</sup>HLA-DR<sup>+</sup> monocytes, and CD33<sup>+</sup>HLA-DR<sup>+</sup> MDSC, respectively; bars, SE. Pooled results are from three experiments. **B**, isolated RCC patient MDSC. Gated HLA-DR<sup>+</sup> MDSC are negative for HLA-DR, CD80, and CD86 compared with gated patient HLA-DR<sup>+</sup> monocytes.

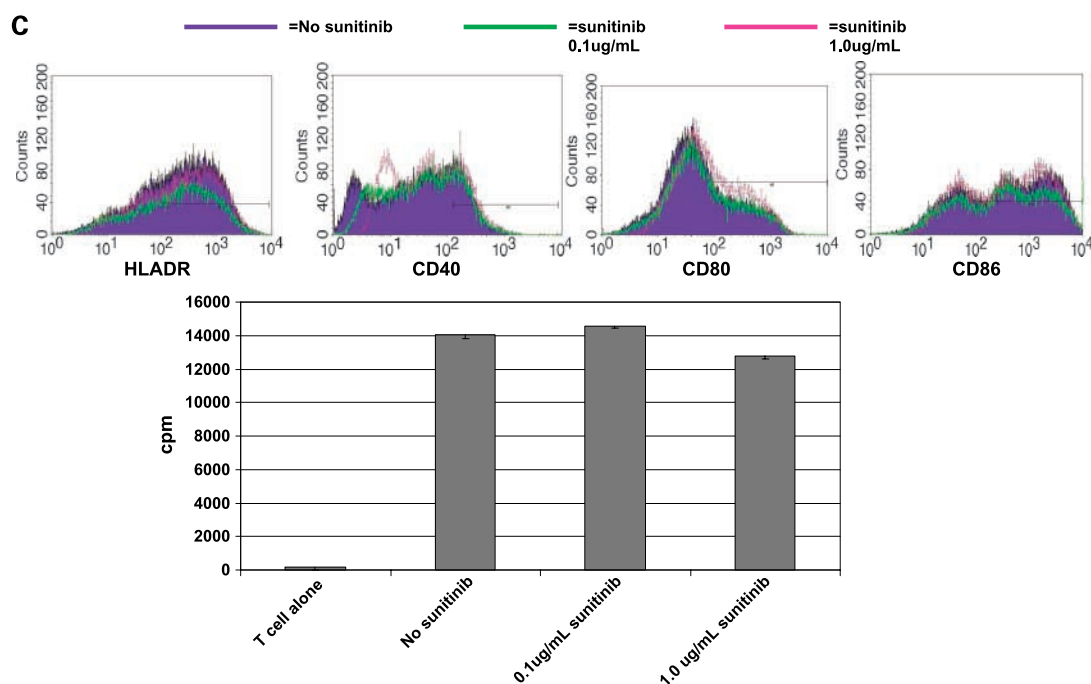
capabilities of these cells, suggesting that it is not functioning to induce MDSC differentiation.

**Changes in patient MDSC and Treg in response to sunitinib are directly associated.** Elevated Treg have been observed in the blood of cancer patients and are believed to suppress the development of antitumor immunity (37, 38). It has been shown that MDSC in tumor-bearing mice and hepatocellular carcinoma patients can induce CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> Treg formation (27, 31), and several clinical strategies aimed at Treg depletion are under investigation (39). We thus investigated modulations in mRCC patient Treg levels in response to sunitinib. Elevated CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> Treg (median, 2.59% of CD3<sup>+</sup>CD4<sup>+</sup> cells in mRCC versus 1.41% in AMN;  $P = 0.002$ ; ref. 33), quantified as shown (Fig. 6A) and confirmed to be suppressive in multiple *in vitro* experiments (33), also declined after treatment with sunitinib (although

this decline did not reach statistical significance). In the cohort of patients included in this study, we detected a positive correlation between the numbers of Treg and MDSC remaining after two cycles of therapy ( $r = 0.75$ ;  $P = 0.008$ ; data not shown). In addition, a change in MDSC levels between cycles of treatment was positively correlated with a change in Treg levels over two treatments ( $r = 0.93$ ;  $P < 0.001$ ; Fig. 6B). This warrants further investigation into the possibility that sunitinib may interfere with Treg formation possibly via MDSC or possibly via a shared sunitinib target, which affects both MDSC and Treg.

## Discussion

It is now widely accepted that there are several tumor-mediated immunosuppressive networks operational in kidney



**Fig. 5 Continued.** C, RCC patient myeloid cells were isolated and cultured as above (GM-CSF and tumor conditioned medium) with the addition of IL-4 at 50 ng/mL to aid in the potential differentiation of myeloid cells into dendritic cells in the presence or absence of sunitinib. After 6 d, cells were harvested and a portion was stained for dendritic cell maturation markers as seen in four histograms: purple, untreated cells; green, 0.1  $\mu$ g/mL sunitinib-treated cells; pink, 1.0  $\mu$ g/mL sunitinib-treated cells. Another portion of cells was used in mixed lymphocyte reaction with freshly isolated, normal donor, allogeneic T cells. T-cell proliferation was measured after 6 d in culture by tritiated thymidine incorporation. Representative experiment from a total of three experiments.

cancer that impede the success of immune-based therapies (3, 4, 40, 41). One of these networks involves the tumor-induced accumulation of MDSC (42–44). Elevated levels of peripheral blood MDSC in mRCC patients have been shown to decline after treatment with all-*trans*-retinoic acid presumably due to all-*trans*-retinoic acid-induced maturation of these cells (25). Here, we report that sunitinib monotherapy, a frontline treatment for patients with mRCC, induces a significant reduction in circulating MDSC in mRCC patients. In addition, this reduction was associated with an improvement in effector T-cell function, and direct correlations were observed between the drug-mediated reduction in MDSC numbers and an improvement in T-cell IFN- $\gamma$  production as well as a decline in Treg numbers. Interestingly, we did not see a correlation between changes in any of the immune parameters tested and changes in tumor burden, response to treatment, or survival.

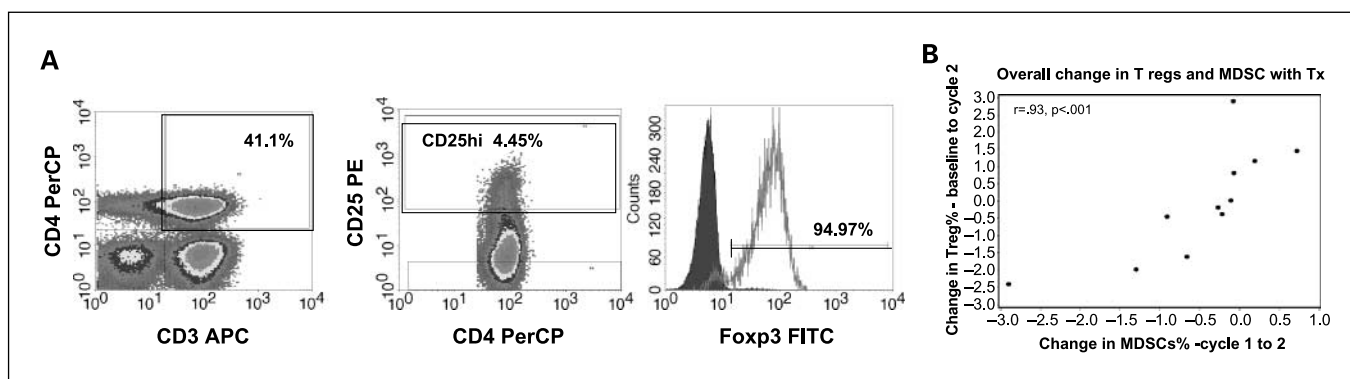
The mechanism by which sunitinib improves type 1 T-cell function in mRCC patients is currently under investigation. It is likely to be in part due to the reduction of MDSC, which inhibit effector T-cell function directly. Results from *in vitro* experiments indicate that a portion of MDSC-mediated T-cell suppression in patients was mediated by L-arginine depletion and perhaps ROS production; however, further investigation is currently under way to identify additional mechanisms that may be relatively active in all MDSC or MDSC subsets. Importantly, patient T cells can be rendered functional when MDSC are depleted. Indeed, removal of MDSC from the PBMC of selected patients before the stimulation of their T cells

resulted in a significant improvement in the ability of those T cells to produce IFN- $\gamma$ .

Our *in vitro* results support the notion that patient T cells are viable and functional even in the presence of concentrations of sunitinib that depress MDSC viability and function. Exposure to 1  $\mu$ g/mL sunitinib induced 30% of patient MDSC to undergo apoptosis, and >60% of MDSC were killed with 5.0  $\mu$ g/mL sunitinib over 48 h, although no effect on patient T-cell viability was seen. Thus, it is likely that the recovery of IFN- $\gamma$  production by patient T cells that were cocultured with their own MDSC in the presence of 1.0  $\mu$ g/mL sunitinib was a result of sunitinib-induced MDSC apoptosis. Modest recovery in T-cell IFN- $\gamma$  production in cocultures exposed to 0.1  $\mu$ g/mL sunitinib may relate to sunitinib-mediated inhibition of MDSC suppressive function. Sunitinib-mediated improvements in patient T-cell function likely occur via effects on MDSC that are exerted by multiple mechanisms including an effect on MDSC viability and function. Because it is difficult to directly recalculate the *in vitro* and *in vivo* doses of sunitinib, we have begun parallel experiments in several mouse tumor models where we have also observed a dramatic suppression of MDSC in response to sunitinib.<sup>8</sup> We plan to evaluate the effects of sunitinib on MDSC function and viability in tumor-bearing mice receiving sunitinib and also to determine the

<sup>8</sup> Cohen et al., in preparation.





**Fig. 6.** Patient MDSC correlate with Treg in response to sunitinib treatment. Patient PBMC were thawed and rested overnight. Nonadherent cells were stained for CD3, CD4, CD25, and Foxp3 and analyzed by FACS analysis. Representative dot plots and histograms show the analysis undertaken. Patient levels of MDSC at the indicated time points relative to sunitinib treatment are positively correlated with patient levels of Treg. Each dot represents a single patient ( $r = 0.93$ ;  $P < 0.001$ ).

effects of drug on MDSC formation in the bone marrow and tumor.

We have shown previously that sunitinib induces a reduction in Treg levels in mRCC patients and that Treg reductions are associated with increases in patient T-cell function as measured via IFN- $\gamma$  production (33). In the current study, an increase in T-cell function was also directly correlated with a reduction in MDSC numbers, suggesting that both MDSC and Treg are contributing to immune dysfunction in mRCC patients. Sunitinib may be unique because it not only reduces the numbers of MDSC in mRCC patients but also reduces the number of Treg. Similar to what we have reported in patients receiving sunitinib, we have seen that tumor-bearing mice receiving sunitinib experience a decline in Treg levels.<sup>7</sup> This is in agreement with what was recently published by Hipp et al., where it was observed that sunitinib, but not another tyrosine kinase inhibitor, sorafenib, reduced Treg levels (45). In the current study, a highly significant correlation between the decline in MDSC and the decline in Treg in response to sunitinib was seen. Further investigation is therefore warranted to determine whether this is due to the influence of MDSC on Treg formation or rather due to a common target of sunitinib, which is shared by MDSC and Treg.

It is still possible that the immune effects of sunitinib observed in patients could be direct, via its interactions with receptors on hematopoietic cells, or indirect, via its effects on tumor cells. No statistically significant associations between any of the immunological parameters investigated and either objective response or tumor shrinkage were found ( $P > 0.12$  in all cases; data not shown). Similarly, even those patients whose tumors progressed during the course of treatment saw a decline in MDSC with sunitinib treatment. In parallel, our ongoing mouse experiments have shown a reduction in MDSC in tumor models such as 4T1 and CT26 where virtually no direct antitumor effects were evident.<sup>8</sup> These findings are consistent with the possibility that sunitinib has a direct effect on host immune cells, that is, MDSC, which occurs independently of the antitumor effect of sunitinib. However, the possibility that sunitinib induces specific, functional changes in tumor makeup, which in turn affect MDSC without inducing an apparent change in tumor size, is currently under investigation.

It has been shown that several tumor-produced growth factors targeted by sunitinib are implicated in MDSC accumulation. Continuous VEGF infusion in naive mice induced MDSC formation via VEGFR2 signaling (28) in one report, whereas MDSC accumulation in mice bearing colon tumors has been attributed to stem cell factor (46). Indeed, experiments ongoing in our laboratory as well as those published have identified a portion of MDSC, which express VEGFR1 and VEGFR2 (47).<sup>9</sup> Sunitinib-targeted receptors include all of those implicated thus far (VEGFR and c-kit), among others such as platelet-derived growth factor, Flt3, and CSF-1 receptor whose mechanistic importance cannot be ruled out (11).

The accumulation of MDSC and Treg as well as the suppression of T-cell IFN- $\gamma$  in mRCC patients is consistent with previous reports (24, 25, 39). Here, we show that MDSC elevation as well as IFN- $\gamma$  suppression can be reversed in response to sunitinib, a drug with unparalleled activity in mRCC. The generation of an effective antitumor adaptive immune response requires the elimination of MDSC, which likely initiate several T-cell deficits (48). The inclusion of clinical treatments aimed at MDSC removal may be an important part of future immunotherapeutic protocols. These data further the rationale for sunitinib-based combination therapy with immunomodulators to enhance the antitumor effect and the effect on patient survival.

<sup>9</sup> George S, Rayman P, Biswas S, et al. Expression of Flt-3 and VEGFR1 on myeloid derived suppressor cells in renal cell carcinoma [abstr]. ASCO; Chicago, IL; 2008.

## Disclosure of Potential Conflicts of Interest

J. Garcia, J.H. Finke, B.I. Rini, commercial research grant, Pfizer; R. Bukowski, honoraria, Wyeth, Genentech, Novartis, Bayer; J. Garcia, honoraria, Pfizer; R. Dreicer, honoraria, Sanofi Aventis; other, Lilly, Astra Zeneca.

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# Clinical Cancer Research

## Sunitinib Mediates Reversal of Myeloid-Derived Suppressor Cell Accumulation in Renal Cell Carcinoma Patients

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