Direct and Differential Suppression of Myeloid-Derived Suppressor Cell Subsets by Sunitinib Is Compartmentally Constrained

Jennifer S. Ko1,2,4,6, Patricia Rayman1, Joanna Ireland1, Shadi Swaidani1, Geqiang Li5,6, Kevin D. Bunting5,6, Brian Rini2,3,6, James H. Finke1,2,3,4,6, and Peter A. Cohen1

Abstract

The antiangiogenic drug sunitinib is a receptor tyrosine kinase inhibitor with significant, yet not curative, therapeutic effects in metastatic renal cell carcinoma (RCC). Sunitinib is also an immunomodulator, potently reversing myeloid-derived suppressor cell (MDSC) accumulation and T-cell inhibition in the blood even of nonresponder RCC patients. We observed that sunitinib similarly prevented MDSC accumulation and restored normal T-cell function to the spleens of tumor-bearing mice, independent of the capacity of sunitinib to inhibit tumor progression (RENCA>CT26>4T1). Both monocytic and neutrophilic splenic MDSC were highly repressible by sunitinib. In contrast, MDSC within the microenvironment of 4T1 tumors or human RCC tumors proved highly resistant to sunitinib and ambient T-cell function remained suppressed. Proteomic analyses comparing tumor to peripheral compartments showed that granulocyte macrophage colony-stimulating factor (GM-CSF) predicted sunitinib resistance and recombinant GM-CSF conferred sunitinib resistance to MDSC in vivo and in vitro. MDSC conditioning with GM-CSF uniquely inhibited signal transducers and activators of transcription (STAT3) and promoted STAT5 activation. STAT5ab(null/null) MDSC were rendered sensitive to sunitinib in the presence of GM-CSF in vitro. We conclude that compartment-dependent GM-CSF exposure in resistant tumors may account for the regionalized effect of sunitinib upon host MDSC modulation and hypothesize that ancillary strategies to decrease such regionalized escape will enhance the potency of sunitinib as an immunomodulator and a cancer therapy. Cancer Res; 70(9); 3526–36. ©2010 AACR.

Introduction

Angiogenesis is a crucial step in tumor progression and hence the rational development of several angiogenesis inhibitors. One such drug, sunitinib (Sutent, Pfizer), which inhibits signaling through receptor tyrosine kinases (RTK) including vascular endothelial growth factor (VEGF) receptor 1-3, platelet-derived growth factor (PDGF) receptor, ckit, flt3, and m-CSF receptors, is typically first-line therapy for metastatic renal cell carcinoma (RCC; refs. 1–3). Yet all patients eventually progress on sunitinib, which is thought to be due to tumor progression (i.e., sunitinib treatment failure; ref. 16). We recently showed that sunitinib therapy significantly reversed RCC-induced MDSC accumulation in patients’ peripheral blood, correlating with improved peripheral T-cell function (16). Sunitinib inhibited peripheral accumulation of all currently identified CD33+ human-MDSC subsets, including immature (lineage negative), neutrophilic (CD15+CD14−), and monocytic (CD14+HLADR−/dim) MDSC (16). Such pan inhibition was unlikely to be mediated solely through an indirect antitumor effect because sunitinib therapy inhibited peripheral MDSC accumulation even when RCC patients displayed tumor progression (i.e., sunitinib treatment failure; ref. 16). We therefore investigated whether the remarkable effect of sunitinib on peripheral MDSC was also evident when animal tumors were treated with sunitinib and whether sunitinib’s seemingly decisive negative modulation of MDSC extended into the tumor compartment.

We here report that sunitinib is uniformly effective for suppressing peripheral (spleenic) CD11b+Gr-1+ MDSC an adaptive tumor resistance to angiogenesis inhibition (4–6). Although the basis of such resistance is incompletely understood, accumulating evidence suggests that host myeloid-derived suppressor cells (MDSC) are recruited by tumors to mediate resistance to antiangiogenic drugs (7, 8). Indeed, MDSC themselves can promote angiogenesis (9–12), in addition to serving as pivotal agents of tumor-induced T2-type biasing and the escape from cell-mediated immunity (13–15).

We recently showed that sunitinib therapy significantly reversed RCC-induced MDSC accumulation in patients’ peripheral blood, correlating with improved peripheral T-cell function (16). Sunitinib inhibited peripheral accumulation of all currently identified CD33+ human-MDSC subsets, including immature (lineage negative), neutrophilic (CD15+CD14−), and monocytic (CD14+HLADR−/dim) MDSC (16–23). Such pan inhibition was unlikely to be mediated solely through an indirect antitumor effect because sunitinib therapy inhibited peripheral MDSC accumulation even when RCC patients displayed tumor progression (i.e., sunitinib treatment failure; ref. 16). We therefore investigated whether the remarkable effect of sunitinib on peripheral MDSC was also evident when animal tumors were treated with sunitinib and whether sunitinib’s seemingly decisive negative modulation of MDSC extended into the tumor compartment.

We here report that sunitinib is uniformly effective for suppressing peripheral (spleenic) CD11b+Gr-1+ MDSC...
accumulation in all tested tumor models, despite widely ranging antitumor effects among these models. Sunitinib inhibited the proliferation of Ly6G+ monocytic (m-) MDSC and also impaired the survival of Ly6G+ neutrophilic (n-) MDSC. We also observed, however, that sunitinib’s dramatic abrogation of splenic MDSC and normalization of bystander T-cell function did not extend to the tumor microenvironment in the most resistant 4T1 model or in human RCC. MDSC resistance to sunitinib corresponded to the compartmental availability of granulocyte macrophage colony-stimulating factor (GM-CSF) and recombinant GM-CSF itself conferred sunitinib resistance in vitro and in vivo. GM-CSF-induced sunitinib resistance in MDSC was signal transducers and activators of transcription 5 (STAT5) mediated, as it was negated in STAT5ab(null/null) MDSC, thus providing a likely rescue mechanism for MDSC in the face of STAT3 inhibition by sunitinib (24). Such regional disparities may explain how residual intratumoral MDSC contribute to antiangiogenic resistance despite pronounced drug-mediated declines in peripheral MDSC.

Materials and Methods

Mice, tumors, and treatment. Experiments performed under institutionally approved animal research committee protocols adhered to U.S. Department of Agriculture guidelines. Female BALB/c mice from National Cancer Institute were maintained pathogen free and studied at 8 to 12 weeks. 4T1-mammary, CT26-colonic, and RENCA-renal carcinomas syngeneic to BALB/c mice were maintained and injected into female BALB/c mice from National Cancer Institute were adhered to U.S. Department of Agriculture guidelines. Spleens, bone marrow, and tumors were obtained as above. Sunitinib 4, 20, or 40 mg/kg/day i.p. was initiated for 9 days after tumors reached 7 to 10 mm diameter. i.p. treatment yielded the same MDSC reductions as oral treatment. Spleens, bone marrow, and tumors were processed as previously described (25). STAT5ab(null/null) and (wild-type, WT) bone marrow was obtained as previously (26).

Reagents. Culture medium consisted of RPMI 1640 + 10% FCS and conventional additives (16, 27). Pan-T-cell isolation kit and magnetic beads conjugated to anti-CD11b, anti-FITC, or anti-APC were from Miltenyi Biotec. Functional grade anti–mouse-CD3ε and anti-CD28 were from BD Pharmingen. rmFlt3L, rmSCF, rmIL-6, rmG-CSF, and rmGM-CSF were from Peprotech. Carboxyfluorescein diacetate succinimidyl ester was from Invitrogen. Luminex Bioplex Mouse Cytokine Arrays were from Bio-Rad. Proteome Profile Arrays were from RnD Systems. Fluorochrome-coupled antibodies were purchased from ebiosciences or BD Biosciences. Fluorescence-activated cell sorting (FACS) data were collected on FACSCalibur (BD) and analyzed using the Cellquest software (BD) or FlowJo software (Tree Star).

Determination of T-cell response. IFNγ production was assayed following polyclonal stimulation as previously described (16). Proliferation was assayed with either carboxyfluorescein diacetate succinimidyl ester dilutions or tritiated thymidine incorporation as previously described (25, 28).

In vivo proliferation and viability assays. One hour following sunitinib treatment, mice were injected i.p. with 1 mg of sterile bromodeoxyuridine (BrdU) mouse. Four hours following BrdU injection, cells obtained from spleen, bone marrow, and, in some cases, tumor and blood were stained according to the BrdU-APC kit (BD). Annexin V staining was done as previously described (16).

MDSC subset isolation and staining. Splenic cell suspensions from 4T1+ mice were depleted of T-cells, B cells, macrophages, and dendritic cells using allophycocyanin (APC)-labeled antibodies to CD3, CD4, CD8, and MHCII and anti-APC magnetic beads. Remaining cells contained mostly MDSC and n-MDSC were isolated using anti-Ly6G-FITC and anti-FITC beads. m-MDSC were isolated from the remaining cells using anti-CD11b magnetic beads. LS magnetic columns were from Miltenyi. Cells were then stained for FACS analysis or were adhered to glass slides by cyto spun. Adherent cells were air dried then stained using Fisher Diagnostics Hema 3 Manual Staining kit, which is comparable with the Wright-Giemsa method.

Cytokine analysis using proteome profile array. Equal volumes of blood obtained through cardiac puncture were added to EDTA to prevent clotting. Cell-free plasma obtained following centrifugation at 4°C at 13,000 for 10 minutes was stored at −80°C. Five hundred microliters of pooled plasma was added to each membrane in the array. SuperSignal West Femto Maximum Sensitivity Substrate from Thermo Scientific was used for development. Additionally, lysates from frozen tumor tissue were obtained using the provided lysis buffer recipe with protease (Thermo Scientific) and phosphatase inhibitors (Pierce) added. Protein was assayed using the Bio-Rad DC Protein Assay kit as per the manual. One hundred fifty micrograms of protein were added to each membrane.

Cytokine analysis using Bioplex array. Pooled plasma obtained as above. Spleen and tumor tissue was immediately frozen in Eppendorf tubes on dry ice. Lysates were obtained from thawed, homogenized/sonicated tissue in the same lysis buffer as above and protein quantified as above. Plasma was assayed according to the Bio-Plex Pro custom assay (Bio-Rad). Tissue lysates were brought to 1 mg/mL using 1 × PBS with 0.5% bovine serum albumin and 50 μL were added to each well. A separate standard curve was made using lysis buffer as diluent for the analysis of cell lysates. Data were acquired and analyzed on a Luminex device using the Bio-Rad Bio-Plex System and Bio-Plex Manager software.

pSTAT signaling analysis in MDSC. Bone marrow–derived MDSC were cultured in stem cell factor (SCF) + Flt3L +/– either interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF), or GM-CSF. pStat staining was done according to the BD Phosflow Fix Buffer I and Perm Buffer III products. Briefly, culture plates were spun down and supernatants were removed. Fix buffer I was added to wells for 10 minutes at 37°C. Washed cells were surface stained (CD11b and Gr1), fixed/permeabilized with ice-cold Perm Buffer III for 30 minutes on ice, then blocked with 10% heat-inactivated rat serum, rat IgG, and anti-CD16/32 antibodies before staining. Anti-pStat antibodies and isotypes were Alexa Fluor 647 conjugated.

Analysis of human RCC tumor tissue. Freshly explanted clear cell RCC tumors were digested. Cells were stained for MDSC or cultured at 1 million lymphocytes/mL in complete
Sunitinib reverses systemic MDSC-mediated immune suppression in mice bearing renal and nonrenal tumors.

We first studied the ability of sunitinib to act as a broad immunopotentiator in several mouse tumor models. Treatment of either Renca–kidney, CT26–colon, or 4T1–breast tumor-bearing mice with sunitinib (40 mg/kg) daily for 9 days significantly reduced both the percentage and total numbers of CD11b+Gr1+ MDSC detected in the spleen. Significant MDSC reductions also occurred in Renca- or CT26+ with 20 mg/kg sunitinib (Fig. 1A). MDSC reductions were associated with significant disinhibition of T cells that were otherwise suppressed in the tumor-bearing state. T cells from tumor-bearing mice were less able to produce IFNγ in response to polyclonal stimulation with anti-CD3/28 when compared with naive, nontumor-bearing mice. Such T-cell suppression was reversible with either in vivo MDSC depletion using sunitinib, or in vitro MDSC depletion using anti–Gr-1 magnetic beads; finally, bead-isolated MDSC could be introduced to suppress T cells from naive mice as well (Fig. 1B). Deficits in T-cell proliferation were also attributable to the presence of MDSC (Fig. 1C), and CD4+ and CD8+ T cells regained function when MDSC were depleted with sunitinib or mechanically (Fig. 1D). These findings suggest that MDSC are major mediators of T-cell suppression in tumor-bearing hosts and that suppressed T cells can be rendered functional if activated in the presence of MDSC (Fig. 1C), and CD4+ and CD8+ T cells regained function when MDSC were depleted with sunitinib or mechanically (Fig. 1D). These findings suggest that MDSC are major mediators of T-cell suppression in tumor-bearing hosts and that suppressed T cells can be rendered functional if activated in the absence of MDSC. In addition, higher doses of sunitinib were required in 4T1+ mice to fully reverse immune suppression.

Sunitinib inhibits the intrasplenic proliferation of m-MDSC. We next asked whether sunitinib functions to inhibit MDSC expansion in vivo. The proliferative rate of MDSC was kinetically quantified by the percentage of BrdUrd" cells. Within the MDSC population, cells brightest for Gr1 staining (Gr1hi) were relatively nonproliferative in naive and tumor-bearing mice (pink squares, <10%BrdUrd"; green squares and triangles, 10–20%BrdUrd"; respectively; Fig. 2A, left (spleen) and right (bone marrow). In contrast, MDSC, which were dim for Gr1 (Gr1lo), were very proliferative in the spleens and bone marrow of tumor-bearing mice (blue squares and triangles; Fig. 2A, left and right, ∼50%BrdUrd") compared with naive mice, in which proliferation was only present in the bone marrow but not in spleen (red circles, <10%BrdUrd"; Fig. 2A, left; P < 0.0002 compared with tumor bearing), indicating the pronounced proliferative pathology of splenic Gr1lo MDSC in the tumor-bearing state. Sunitinib reversed this pathology by significantly inhibiting the expansion of splenic Gr1lo MDSC on day 6 following treatment (blue triangles; P < 0.02; Fig. 2A). The analysis undertaken is shown in Fig. 2B. Sunitinib did not have a sustained effect on bone marrow MDSC proliferation, which was reflected in sunitinib's (40 mg/kg) lack of effect on the %MDSC in naïve or tumor-positive mouse bone marrow (data not shown).

These findings suggested that MDSC could be functionally divided into two groups based on their proliferative potential in vivo. Upon isolation, as previously reported (30, 31), Ly6G" n-MDSC were indeed Gr1hi, Ly6G", and F4/80", and displayed early or completed polymorphonuclear features on cytospins (Fig. 2C), consistent with their lower rate of proliferation. Ly6G" m-MDSC were Gr1lo, Ly6G", and evenly distributed with regard to F4/80 expression and monocyte versus immature morphology in cytospins (Fig. 2C), consistent with their higher rate of proliferation. Thus, splenic m-MDSC were highly proliferative compared with n-MDSC and sunitinib inhibited this proliferation, significantly contributing to the overall reduction in splenic MDSC accumulation.

Sunitinib impairs the viability of splenic n-MDSC. We found over half the Gr1hi n-MDSC in the spleens of naïve mice were undergoing apoptosis, consistent with their being normal neutrophils with a rapid rate of turnover in naïve animals (Fig. 2D). The rate of n-MDSC apoptosis in tumor-bearing mice was significantly reduced compared with naïve mice, indicating that they have a prolonged life span in vivo. Sunitinib significantly reduced the viability of splenic n-MDSC in vivo on day 6 following treatment (P < 0.0005; Fig. 2D). In contrast, m-MDSC generally had lower rates of apoptosis that were only modestly affected by sunitinib in vivo (naïve, 25%; 4T1, 15%; 4T1+sunitinib, 32% on average; data not shown).

Compared with spleen, intratumoral MDSC are sunitinib resistant in resistant tumor models. In contrast to the global effect of sunitinib to improve splenic T-cell function through reductions in MDSC, the drug’s effect on tumor growth varied considerably. Renca kidney tumors were disproportionately sensitive to sunitinib, with tumor shrinkage occurring at both 20 and 40 mg/kg drug. In contrast, CT26 tumors continued to progress, albeit more slowly, during sunitinib treatment, and 4T1 tumor growth was not significantly affected by sunitinib treatment (Fig. 3A). Tumor cell sensitivity to drug in vitro was inversely related to in vivo sensitivity. As such, the variable effects of sunitinib on tumor growth in vivo could not be predicted by its variable toxicity to tumor cells lines in vitro (Fig. 3B).

We then examined the effect of sunitinib on cellular tumor constituents that remained following a 4 mg/kg (Renca tumors only), 20, or 40 mg/kg daily treatment schedule. Unlike Renca and CT26 tumors, 4T1 tumors retained statistically equal amounts of MDSC following treatment at 20 mg/kg, and compared with splenic MDSC in 4T1+ mice, there was a much more modest decline in tumor-associated MDSC (Fig. 3C) in response to 40 mg/kg drug.
In this relatively resistant model, tumor-infiltrating T cells from sunitinib-treated mice did not function significantly better than those from nontreated mice, in marked contrast to the improved splenic T-cell function obtainable from the same mice (Fig. 3D, left). Indeed, intratumoral MDSC from sunitinib-treated 4T1+ mice retained T cell–suppressive capabilities equivalent to those from nontreated mice (Fig. 3D, right). Our findings suggest that even when sunitinib broadly reverses peripheral MDSC accumulation (16), intratumoral MDSC can be significantly less affected.

GM-CSF is selectively expressed in resistant tumor microenvironment in vivo and uniquely protects MDSC in the presence of sunitinib in vitro. Despite their disparate susceptibilities to in vivo sunitinib exposure, purified splenic- and tumor-derived MDSC proved equally sensitive to sunitinib-mediated apoptosis in culture (data not shown), prompting us to investigate regionally produced factors that could account for in vivo disparities. To determine whether the presence of a particular cytokine or group of cytokines could predict intratumoral sunitinib resistance, we used...
Figure 2. Antiproliferative and pro-apoptotic effects of sunitinib on MDSC subsets in the spleen. A and B, after 3, 6, and 9 days ± sunitinib initiation to 4T1+ mice, mice were pulsed with BrdU (1 h following sunitinib dosing) for 4 hours. Splenic and bone marrow CD11b+Gr1+lo and CD11b+Gr1+hi cells were gated on and BrdU positivity determined by FACS. Cells from mice not given BrdU were used as staining controls. C, 4T1+ splenocytes were stained for CD11b, Gr1, Ly6G, and F4/80 before and after MDSC subset isolation with magnetic beads. Representative FACS data and cytospins shown. n = 2. D, mice were treated as above for (A) only splenocytes were stained for CD11b, Gr1, and Annexin V and immediately ran for FACS.
arrays to compare the relative levels of numerous cytokines in the plasma and tumor lysates of mice bearing Renca, CT26, or 4T1 tumors. 4T1+ mice contained an abundance of systemic and intratumoral G-CSF compared with mice bearing other tumor types (Fig. 4A). In addition, GM-CSF was selectively present in the tumor bed of 4T1+ only (Fig. 4A, representative blots below). Luminex assays of nontreated and sunitinib-treated 4T1+ mice confirmed the selective presence of GM-CSF in 4T1 tumors versus plasma and spleen (data not shown), and quantified the amount of G-CSF and GM-CSF in the plasma and tumors of 4T1+ nontreated and sunitinib-treated mice (Fig. 4B). The need to flush and disaggregate bone marrow did not permit its direct comparison with the other tissues.

Because several host- and tumor-produced factors such as SCF, IL-6, G-CSF, and GM-CSF have been implicated in MDSC accumulation (32), we cultured fresh bone marrow containing MDSC in the presence of SCF and flt3 ligand to provide basal support, with the further addition of either IL-6, G-CSF, or GM-CSF +/- sunitinib. Sunitinib significantly impaired the viability of CD11b’Gr1’ MDSC, but GM-CSF, and to a lesser extent G-CSF, could partially restore MDSC...
viability when added to cultures (Fig. 4C). Indeed, 10 ng/mL of GM-CSF protected MDSC significantly better than G-CSF added at 100 ng/mL. In addition, G-CSF and GM-CSF, when added to SCF and Flt3L, induced the strongest proliferative response in these cultures, and although sunitinib totally prevented MDSC expansion in the presence of G-CSF, it had a relatively modest effect in the presence of GM-CSF (Fig. 4D).

**GM-CSF confers sunitinib-resistance to peripheral MDSC and relies on STAT5 signaling to rescue MDSC from sunitinib toxicity.** 4T1+ mice received systemic GM-CSF (i.p.) with and without sunitinib treatments to test whether systemic resistance to sunitinib would result. Indeed, daily GM-CSF significantly inhibited the remarkable reductions in splenic MDSC otherwise seen in response to sunitinib in 4T1+ mice (Fig. 5A). The persistence of 30% MDSC in GM-CSF–treated mice was sufficient to significantly inhibit the recovery in T-cell function that was otherwise induced in sunitinib-treated 4T1+ mice (Fig. 5B).

To test whether a particular signaling profile could distinguish sunitinib-sensitive from sunitinib-insensitive MDSC, we performed intracellular staining for steady-state levels of pY705STAT3 and pY694STAT5 expression in MDSC cultured in nonprotective (SCF, Flt3L, ± IL-6, or G-CSF) or protective (SCF, Flt3L, + GM-CSF) conditions. Consistent with our previous studies (24), MDSC maintained in the absence of GM-CSF displayed a STAT3-driven signaling signature (Fig. 5C); and, consistent with prior reports, these STAT3-driven MDSC showed sensitivity to sunitinib, resulting in pSTAT3 downregulation (25). In contrast, GM-CSF dominantly activated pSTAT5 (Fig. 5C) rather than pSTAT3, suggesting that STAT5 could provide an alternate survival signal for MDSC in the presence of sunitinib-mediated pSTAT3 inhibition in MDSC. This was formally tested by comparing sunitinib sensitivity in the presence of GM-CSF in WT versus STAT5ab(null/null) MDSC. We found that the absence of STAT5 rendered MDSC sensitive to sunitinib-mediated toxicity in the presence of GM-CSF, whereas having no significant effect on cultures driven by pSTAT3-promoting IL-6 and/or G-CSF (Fig. 5D).

**Tumor microenvironment limits the anti-MDSC effect of sunitinib in RCC patients.** We next quantified MDSC subsets in RCC patients’ tumor specimens (described in Supplementary Table S1) using the gating methods shown for HLADR−CD33hiCD15−CD14+ monocytic, HLADR−CD33hiCD15−CD14− lineage-negative, and HLADR−CD33hiCD15−CD14+ neutrophilic MDSC. Figure 6A shows the average percentage of each MDSC subset detected in tumors from nontreated patients, as well as the amount detected in two tumors from sunitinib-treated patients. Unlike the pronounced decline in peripheral blood MDSC...
observed in RCC patients treated with sunitinib (16), tumors obtained from sunitinib-treated patients have not shown declines in MDSC. The continued presence of MDSC in the tumors of sunitinib-treated patients was associated with continued T-cell suppression, as measured by IFNγ production, compared with normal donor T cells and T cells from nontreated tumors (Fig. 6B). In addition, several short-term tumor cell lines derived from surgical patients’ RCC tumors (one to two passages) produced abundant GM-CSF in vitro and this production was not significantly reduced in the presence of sunitinib (Fig. 6C). GM-CSF levels equivalent to those detected in RCC supernatants could also induce pSTAT5 activation in patient-derived MDSC (Fig. 6D). This suggests that compartmental constraints limit the ability of sunitinib to act as an immunopotentiator and that locally produced GM-CSF may promote local sunitinib resistance.

Discussion

Although the role of immunosurveillance to inhibit tumor progression is well documented, there are many obstacles that prevent the destruction of advanced tumors by immune cells, one of which is MDSC accumulation. Sunitinib targets several RTKs and has experienced relative clinical success, especially in the setting of mRCC (1). We recently reported the ability of sunitinib to reverse immune suppression in mRCC patients through MDSC inhibition (16), yet the broad potential of sunitinib to modulate immune function independently of its antitumor effect was unknown. We here report that sunitinib, when used at clinically relevant doses (33, 34), can inhibit MDSC accumulation, and thereby restore normal T-cell function, in the spleens of mice bearing both sunitinib-sensitive and sunitinib-insensitive tumors. This suggests that the immunomodulatory activities of sunitinib occur independently of its antitumor potency. As such, sunitinib may prove as a useful adjunct agent in immunotherapy trials (35).

We here show two mechanisms by which sunitinib inhibits MDSC accumulation. Pilot studies identified the spleen and bone marrow as sites of MDSC proliferation, with BrdUrd-positive cells appearing after only a 1-hour BrdUrd pulse. Using in vivo BrdUrd administration, Grllo m-MDSC were found to proliferate rapidly in the bone marrow of naïve and tumor-bearing mice, and in the spleens of tumor-bearing
mice, suggesting that the extramedullary proliferation is the most pathologic proliferation because it does not normally occur in naïve mice under steady-state conditions. Sunitinib had an antiproliferative effect on splenic m-MDSC in vivo and this effect could be duplicated in vitro.

The antiproliferative effect of sunitinib is unlikely to account solely for the remarkable declines seen in MDSC in response to drug. Because previous studies had shown sunitinib to have a toxic effect on RCC patient MDSC in vitro (16), its effect on Gr1hi, neutrophilic MDSC (n-MDSC) viability was also studied in vivo. Our findings show that Gr1hi n-MDSC were relatively nonproliferative and suggest that the accumulation of this MDSC subset in tumor-bearing mice is more related to an abnormally prolonged life span. Indeed, n-MDSC from tumor-bearing mice were much less likely to be undergoing apoptosis at any given time, compared with naïve mice. Sunitinib seemed to normalize this in vivo by increasing the frequency of n-MDSC apoptosis, as measured by Annexin V staining, an effect that was reproducible in vitro. The data thus show that sunitinib acts to inhibit both the abnormal expansion of m-MDSC and the abnormally extended survival of n-MDSC. An alternative interpretation is that sunitinib prevents the expansion of mononuclear MDSC, which may, in part, represent n-MDSC precursors (36). Such activity alone could lead to the perceived increase in n-MDSC apoptosis because terminal n-MDSC would eventually undergo spontaneous apoptosis and fail to be replaced. This possibility is currently being tested.

Figure 6. Tumor microenvironment limits the local anti-MDSC effect of sunitinib in RCC patients. A, MDSC subsets in tumors of nontreated patients as well as % found in two patients treated with sunitinib before surgery. B, IFNγ+ tumor-infiltrating T cells (FACS) following anti-CD3/28 stimulation from tumors of nontreated patients (compared with normal donor T cells) as well as patients treated with sunitinib before tumor removal. C, GM-CSF levels (ELISA) in short-term and long-term RCC cultures. The effect of sunitinib on SK-RC-26b tumor cell production of GM-CSF. D, pSTAT5 activation in gated CD33+HLADR-RCC patient–derived MDSC was measured with intracellular phosphoflow staining 48 hours into culture with varying concentrations of GM-CSF.
In addition to being MDSC subset specific, our data strongly suggest that sunitinib’s inhibition of MDSC is direct. Proteome profile and luminex arrays did not identify a decline in any of the factors implicated in MDSC expansion or activation. In fact, several cytokines such as G-CSF and IFNγ were present at increased amounts in sunitinib-treated mice, consistent with the parallel observations of Ebos and colleagues (37) in naive mice.

The receptors involved in the anti-MDSC activity of sunitinib are currently under investigation. Putative RTKs inhibited by sunitinib include targets of VEGF, SCF (c-kit), Flt3L (flt3), m-CSF, and PDGF (1–3). SCF has recently been implicated in MDSC accumulation (38) and sunitinib inhibits its receptor, c-kit. However, culture with RTK ligands such as SCF and/or flt3L produced only limited MDSC expansion and viability unless combined with proliferatively synergizing cytokines such as IL-6, G-CSF, or GM-CSF (25). This suggests that although the sunitinib RTK targets c-kit and/or flt3 may provide permissive signals for MDSC expansion, tumor-promoted excessive MDSC accumulation requires synergy from additional cytokines.

G-CSF and GM-CSF have both previously been implicated in MDSC expansion (21, 32, 39, 40) and vaccines that produce higher concentrations of GM-CSF have been reported to be less effective as a consequence of promoting MDSC (39). Additionally, however, our studies show the divergent abilities of G-CSF and GM-CSF to confer resistance to sunitinib in vitro and in vivo, paralleling the compartmental disparities of these cytokines in vivo. Because the Renca model is paradoxically far more vulnerable to sunitinib treatment than human RCC, we believe other models such as 4T1 are potentially more predictive of escape mechanisms relevant to human cancer. Notably, both mouse 4T1 and human RCCs displayed disparately greater GM-CSF production than sunitinib-sensitive peripheral compartments (plasma), whereas all compartments were similarly rich in G-CSF.

We hypothesize that the striking ability of GM-CSF but not other pro-proliferative cytokines to promote sunitinib resistance is due to its preferential activation of STAT3, which cannot be appreciably repressed by sunitinib. Our previous studies showed that treatment of CD34+ hematopoietic progenitors with GM-CSF induced proliferative synergy with Flt3L/IL-6 despite GM-CSF-mediated inhibition of STAT3 activation in favor of dominant STAT5 programming (25). Despite preventing STAT3 activation, GM-CSF continued to display proliferative synergy with Flt3L and IL-6, which was undiminished even for STAT3 knockout bone marrow (25). We here show a similar pSTAT5-dominated signaling pathway for MDSC exposed to GM-CSF. Conversely, in the absence of GM-CSF, combinations of SCF, Flt3L, IL-6, and/or G-CSF all promoted STAT3-activated MDSC that were highly susceptible to inhibition by sunitinib. Although previous studies have highlighted the importance of STAT3 signaling in MDSC accumulation and function (41, 42), as well as in emergency granulopoiesis (43), the present report is the first to show the generation of MDSC in a steady state of STAT5 rather than STAT3 activation, the consequence of GM-CSF exposure. Because sunitinib was recently shown to inhibit MDSC expression of STAT3 (24), we hypothesize that GM-CSF, when available, can rescue MDSC by reprogramming them to function independently of STAT3, rendering them resistant to sunitinib. Indeed, in vitro experiments with STAT5ab(null/null) mice further confirmed an important role for STAT5 in MDSC rescue from sunitinib-toxicity.

MDSC-mediated diminutions in T cell–mediated immunoresponse may contribute to the currently limited effectiveness of immunotherapy in RCC and other tumors (16, 44–47). Our previous studies in RCC patients showed that MDSC declines and improvements in T-cell function were not contingent upon tumor shrinkage in response to sunitinib and even sunitinib-induced tumor cytoreduction is not associated with cure (16, 47). Our data thus far obtained in human RCC patients support the model established in 4T1 tumor–bearing mice, in which peripheral compartment reductions in MDSC ubiquitously occur in response to sunitinib, but in which the anti-MDSC activity of the drug is much less pronounced intratumorally as a result of a relative abundance of nonsunitinib-targeted growth factors, such as GM-CSF, which provide alternative sunitinib-resistant survival signals. We are investigating ancillary strategies to abrogate such regional resistance, to enhance the potency of sunitinib both as an immunomodulator and as a cancer therapy.

Disclosure of Potential Conflicts of Interest

B. Rini: commercial research grant, Pfizer; consultant/advisory board, Pfizer, Bayer, and Genentech. J.H. Finke: commercial research grant, Pfizer. The other authors disclosed no potential conflicts of interest.

Grant Support

Work supported by Pfizer Corporation and the Frank Rudy Fund for Cancer Research (J.H. Finke). Work supported by R01-CA129815 (P.A. Cohen).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 09/02/2009; revised 01/08/2010; accepted 01/26/2010; published OnlineFirst 04/20/2010.

Ko et al.
Correction: Direct and Differential Suppression of Myeloid-Derived Suppressor Cell Subsets by Sunitinib Is Compartmentally Constrained

In this article (Cancer Res 2010;70:3526–36), which was published in the May 1, 2010 issue of Cancer Research (1), a new reference has been added for the acquisition of STAT5ab(null/null) and WT bone marrow. The final sentence of the paragraph "Mice, tumors, and treatment" on page 3527 should read "STAT5ab(null/null) and (wild-type, WT) bone marrow was obtained as previously (26, 48)." The new reference is as follows:


Also, the authors acknowledge Dr. Lothar Hennighausen and the NIH as the originating source of STAT5-floxed mice in these studies, and also acknowledge that this article represents partial fulfillment of J.S. Ko’s Doctoral Thesis in the Department of Pathology at CWRU and the Department of Immunology, Cleveland Clinic.

Reference

Direct and Differential Suppression of Myeloid-Derived Suppressor Cell Subsets by Sunitinib Is Compartmentally Constrained

Jennifer S. Ko, Patricia Rayman, Joanna Ireland, et al.

Cancer Res 2010;70:3526-3536. Published OnlineFirst April 20, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-3278

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/04/19/0008-5472.CAN-09-3278.DC1

Cited articles
This article cites 47 articles, 30 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/9/3526.full#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/70/9/3526.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.