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Research Article

Cancer Immunology Research

TK Inhibitor Pazopanib Primes DCs by Downregulation of the β-Catenin Pathway

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Abstract

Tyrosine kinase inhibitors (TKIs) target angiogenesis by affecting, for example, the VEGF receptors in tumors and have improved outcomes for patients with metastatic renal cell carcinoma (mRCC). Immune checkpoint inhibitors (ICIs) have also been proposed for treatment of mRCC with encouraging results. A better understanding of the activity of immune cells in mRCC, the immunomodulatory effects of TKIs, and the characteristics defining patients most likely to benefit from various therapies will help optimize immunotherapeutic approaches. In this study, we investigated the influence of the TKI pazopanib on dendritic cell (DC) performance and immune priming. Pazopanib improved DC differentiation and performance by promoting upregulation of the maturation markers HLA-DR, CD40, and CCR7; decreasing IL10 production and endocytosis; and increasing T-cell proliferation. PD-L1 expression was also downregulated. Our results demonstrate that pazopanib inhibits the Erk/β-catenin pathway, suggesting this pathway might be involved in increased DC activation. Similar results were confirmed in DCs differentiated from mRCC patients during pazopanib treatment. In treated patients pazopanib appeared to enhance a circulating $CD4^+$ T-cell population that expresses CD137 (4-1BB). These results suggest that a potentially exploitable immunomodulatory effect induced by pazopanib could improve responses of patients with mRCC in customized protocols combining TKIs with ICI immunotherapy. *Cancer Immunol Res; 6(6); 711–22. ©2018 AACR.*

Introduction

Tumor progression requires angiogenesis. Proangiogenic factors, such as VEGF, that are induced by hypoxia or oncoproteins can alter the equilibrium between pro- and antiangiogenic factors, resulting in the generation of new blood vessels, mostly with altered endothelium structure. Proangiogenic factors are involved in the generation of immunosuppression in tumors. Tumor neoangiogenesis is associated with immature and tolerogenic dendritic cells (DCs) and increased number of myeloid-derived suppressor cells (MDSCs), the activation of regulatory T cells (Tregs), and recruitment of tumor-associated macrophages (TAMs) in the tumor bed (1).

Various antiangiogenic and multitargeted compounds, including bevacizumab, sunitinib, pazopanib, sorafenib, axitinib, lenvatinib, and cabozantinib, have entered the clinic for use against tumors that depend on angiogenesis (2–4). For metastatic renal cell carcinoma (mRCC), VEGFR-directed tyrosine kinase inhibitors (TKIs) have demonstrated clinical benefits including improvements in progression-free survival and overall survival (5). These compounds, which target VEGF and its receptors, are likely to affect the immune repertoire of cells and molecules that interact with the growing tumor. Immunosuppression appears to be downregulated in mRCC patients treated with sunitinib or axitinib, whose Treg and MDSC cell populations are affected. Sorafenib has the opposite effect by reducing antigen-specific T-cell induction *in vitro* (6–11). The different selectivities and affinities of the various drugs are thought to account for the diverse effects on myelopoiesis and immune cells (12).

As immunotherapy using immune checkpoint inhibitors is moving to clinical application for mRCC, we must understand the immune consequences of TKI therapy. In the CheckMate 025 randomized phase III trial, Escudier and colleagues observed in mRCC patients improved overall survival and favorable hazard ratio for the anti–PD-1 nivolumab group that had previously received first-line treatment with pazopanib (HR, 0.60; 95% CI, 0.42–0.84); such results suggest an immune effect on the tumor microenvironment (13).

TKIs seem to be more effective in mRCC, suggesting that the requirement for angiogenesis increases as the disease progresses. In order to achieve maximum response from anti–PD-1 immunotherapy, the patient must be prepared to receive an immunotherapeutic regime that will expand activated and specific T cells. We have addressed this issue by studying DC performance at concentration of sunitinib and pazopanib found in plasma. DCs are antigen-presenting cells that prime antigen-naive

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T cells and perform cross-priming, thus presenting antigens both in HLA class I and II to activate immune responses. Optimal DCs express costimulatory molecules such as CD40, CD83, CD86, HLA-DR, and to a lesser extent CD14 and PD-L1. In order to migrate to the lymph node for cross talk with effector cells, DCs must express the CCR7 marker. These parameters relate to T-cell priming and activation. Failure to sustain these hallmarks will lead to tolerogenic DCs that will dampen antitumor immunity (14, 15).

We report here results that identify the TKI pazopanib as an immune stimulator, which exerts its effects by influencing DC differentiation and maturation. This activation is mediated by targeting and downregulating p-Erk and β-catenin pathway. The impact of this immune activation mediated by DCs was investigated in mRCC patients undergoing TKI treatment. Our results might influence the design of first- and second-line therapies for mRCC.

Materials and Methods

Generation of DCs

Human monocyte-derived DCs were generated from peripheral blood mononuclear cells (PBMCs) of healthy donors (Ethical Committee Protocol, RIF.CE: 4212) and of mRCC patients (Ethical Committee Protocol, RIF.CE: 4181). Monocytes ($CD14^+$) were purified from PBMCs after Ficoll–Hypaque gradient (1,077 g/mL; Pharmacia LKB) by Human CD14-Positive Selection Kit (StemCell Technologies) and cultured (5×10^5 cells/mL) in RPMI 1640 (Hyclone) supplemented with 2 mmol/L L-glutamine, penicillin 100 U/mL, streptomycin 100 μg/mL (Sigma-Aldrich), with 5% heat-inactivated Fetal Calf Serum (FCS; Hyclone). Fifty ng/mL rhGM-CSF (R&D Systems) and 2,000 U/mL rhIL4 (R&D Systems) were added at day 0 and 2. Immature DCs (iDCs) were collected at day 5 and matured with cytokine cocktail (rhIL1β, IL6, TNFα and PGE_2; all purchased by R&D Systems) for 16 hours. Sunitinib (50 ng/mL; Sigma-Aldrich) and pazopanib (19 μg/mL; Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO; Euroclone) and added to the culture during DC differentiation.

Immune phenotype

DC phenotype was analyzed by flow cytometry using the following monoclonal antibodies (mAb): anti–HLAII-DR-FITC, anti–CD86-FITC, from BD Biosciences, anti–CD14-PE, anti–CCR7-FITC, anti–CD83-PE, anti–CD40-PE, anti–PD-L1-PE from BioLegend and anti–VEGR-1 from R&D Systems. MoAbs anti–IgG1-FITC and anti–IgG1-PE (BioLegend) were used as isotype controls.

For immune profile evaluation, PBMCs were isolated from blood samples (50 mL) from six mRCC patients by Ficoll–Hypaque at different times (Ethical Committee Protocol, RIF.CE: 4181): before treatment with sunitinib or pazopanib (T0), during treatment (T1: 1 month of treatment, T2: 2 months, T3: 3 months etc.) and during progression. Various T-cell subsets were analyzed:

- T-cell subpopulations: anti–CD3-APC-H7/CCR7-PE/CD8 PerCp-Cy5.5/CD45RA-BB15.
- Treg cells: anti–CD4-APC-H7/CD25-PE/CD45RA-BB15/FoxP3-Alexa647.
- T-cell activation/proliferation: anti–CD3-APC-H7/CD8-PerCp-Cy5.5/CD137-APC/Ki67-PeCy7.
- T-cell exhaustion: anti–CD3-APC-H7/CD8-PerCpCy5.5/PD1-PE/CTLA4-APC/Tim3-BB15.

All mAbs were purchased by BD Biosciences and BioLegend. Flow cytometric analysis was performed using FACSCanto flow cytometer running FACS Diva data acquisition and analysis software (BD Biosciences). Catalog numbers and clones for every antibody used are listed in Supplementary Table S1.

Microvesicle isolation

Microvesicles were isolated from supernatants of DCs differentiated with and without suninitib and pazopanib. Supernatants were centrifuged at 13,000 × *g* for 30 minutes at 4°C. Microvesicles were then stained with anti–PD-L1 (BioLegend) and acquired by FACSCanto flow cytometer and analyzed by FACS Diva software. Anti–IgG1-PE (BD Biosciences) was used as isotype control. Fluorescent Nile Red Particles (0.1–0.3 μm, Spherotech Inc.) were used as size control.

Western blot analysis

Immature DCs and mature DCs (iDCs and mDCs, respectively) with and without sunitinib and pazopanib were lysed using the NP-40 solution (Biocompare) in the presence of phenylmethylsulfonyl fluoride (1 mmol/L, PMES) and protease inhibitors (1X; Sigma). Proteins obtained were quantified by Bradford assay, were resolved using 4% to 12% SDS-PAGE gel and transferred to nitrocellulose. After blocking, membranes were incubated with rabbit anti–β-catenin (Bethyl Laboratories Inc.; 1:1,000), rabbit anti-pErk42/44 (Erk1/2; Cell Signaling Technologies; 1:1,000), mouse anti–β-actin (Cell Signaling Technologies; 1:1,000) and mouse anti–NF-κB (p105/p50; Cell Signaling Technologies; 1:1,000), followed by peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (H+L; Jackson Immuno Research Laboratories; 1:20,000). Protein bands were detected with Immobilon Western (Millipore Corporation) following the manufacturer's instructions. The density of protein bands was analyzed by Image J software and was normalized in terms of average intensity of bands of each protein per average intensity of bands of β-actin.

Endocytosis assay

FITC-dextran (1 mg/mL; Molecular Probes) was added to untreated iDCs and mDCs and differentiated with sunitinib and pazopanib for 2 hours at 37°C. After washing, cells were acquired by FACSCantoII flow cytometer and analyzed by FACSDiva software. DCs incubated with FITC-dextran for 2 hours at 4°C were used as negative controls.

Cytokine production

Culture supernatants from iDCs and mDCs untreated or differentiated with sunitinib and pazopanib and sera from mRCC patients were collected and analyzed using the ProcartaPlex Human Inflammation Panel (20 Plex; eBioscence). Samples were measured by BioPlex Magpix Multiplex Reader (Bio-Rad) and data analysis was performed using Bioplex Manager MP software (Bio-Rad).

T-cell proliferation

T lymphocytes were purified from Ficoll–Hypaque gradient (1,077 g/mL; Pharmacia LKB) followed by CD3 immunomagnetic isolation (StemCell). T cells were then cocultured in a 96-well round-bottom microplate (Costar) with allogeneic iDC and mDCs differentiated with and without sunitinib and pazopanib (DCs: T cells, 1:5) in the presence of PHA (5 μg/mL) for 4 days at 37°C. T cells were pretreated with CarboxyFluorescein

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Succinimidyl Ester (1 μmol/L, CFSE; Life Technologies) and cell proliferation was monitored through progressive halving of fluorescence using FACSCantoII flow cytometer and analyzed by FACSDiva software (BD Biosciences). Results were reported as the percentage of proliferation increase of T cells cultured with DCs treated with sunitinib and pazopanib compared with T cells cultured with DCs alone.

T cells were also cultured in 6-well round-bottom microplates (Costar) in the presence of sunitinib (50 ng/mL) and pazopanib (19 μg/mL) up to 24 hours. Proliferation was evaluated by FACSCantoII flow cytometer and analyzed by FACSDiva software.

Statistical analysis

Descriptive statistics (average and standard deviation) were used to describe the various data. ANOVA test was used to analyze statistical differences between three groups. Student paired *t* test was used to compare two groups. Significance is indicated when the *P* value was less than 0.05.

Results

Pazopanib improves DC activation and increases expression of DC-maturation markers

To investigate the capacity of TKIs to influence DC differentiation and maturation *in vitro*, the expression of CD14, HLA-DR, CD86, CD83, CCR7 and CD40 was evaluated by flow cytometry on monocyte-derived DCs of healthy donors. Untreated DCs were used as control. The exposure to concentrations of sunitinib and pazopanib found in plasma affected the phenotype of immature and mature DCs differently (Fig. 1A and B). Sunitinib did not affect DC differentiation and maturation, but DCs cultured in the presence of pazopanib were more activated. Pazopanib modified iDC phenotype, significantly increasing the expression of HLA-DR and CD40 molecules, compared with that of iDCs alone (HLA-DR $P < 0.01$) and iDC treated with sunitinib (HLA-DR $P < 0.05$; CD40 $P < 0.05$; Fig. 1A). The average values of mean fluorescence intensity (MFI) indicated upregulation of CD83 in DCs differentiated with pazopanib. On the other hand, CD14 expression was downregulated by pazopanib during differentiation. Similar results were obtained after DC maturation (Fig. 1B). mDCs cultured in presence of pazopanib significantly upregulated the expression of CCR7 and CD40 molecules compared with untreated mDCs (CCR7 $P < 0.05$; CD40 $P < 0.05$) and sunitinib-treated mDCs (CCR7 $P < 0.05$), suggesting that pazopanib enhances activation status of both immature and mature DCs.

VEGF-R1 expression, which is the target of both TKIs, was unaltered by treatment of DCs with pazopanib or sunitinib (Supplementary Fig. S1A)

PD-L1 is downregulated in pazopanib-generated DCs

The capacity of DCs to stimulate T cells depends on the balance between costimulatory and coinhibitory signals. Increased expression of costimulatory marker such as CD40 or CD83 can facilitate T-cell activation, whereas increased expression of inhibitory markers such as PD-L1 contributes to T cell-negative regulation (16). To evaluate the expression of coinhibitory signals in DCs in response to TKI treatment, we analyzed the expression of the PD-L1 both on DCs and on shed microvesicles (Fig. 2). During iDC differentiation, only pazopanib began to decrease PD-L1 expression, compared with untreated DC and DCs differentiated with sunitinib (Fig. 2A). The decrease in expression became significant ($P < 0.01$) in DCs after maturation (Fig. 2B).

Microvesicles released by the DCs also showed a decrease in PD-L1 expression: PD-L1 expression on microvesicles released by DCs treated with pazopanib was lower than that on microvesicles obtained by untreated DCs and DCs treated with sunitinib (Fig. 2C). The difference in expression of PD-L1 between microvesicles of mDCs and mDCs treated with pazopanib was statistically significant ($P < 0.05$).

Pazopanib treatment reduces immunosuppression downregulating IL10 production by DCs

The production of cytokines such as IL10 and IL12 during DC maturation can influence the capacity of DCs to alter Th1 or Th2 immune responses (17). Several chemokines released by DCs, such as CXCL-10, promote tumor-reactive effector T-cell recruitment (18). Thus, we evaluated cytokines and chemokines released by untreated DCs and DCs treated with TKIs (Fig. 3A). We observed a significant reduction ($P < 0.05$) of IL10 in iDCs and mDCs treated with pazopanib compared with untreated DCs (both iDC and mDCs) and DCs treated with sunitinib (both iDC and mDCs). The balance between IL12/IL10 and CXCL-10/IL10 favored immune activation when DCs were generated with pazopanib (Supplementary Fig. S1B).

Pazopanib-treated DCs are able to increase T-cell activity

DCs must possess specialized features to act as good antigen-presenting cells. In addition to expression of costimulatory molecules and release of cytokines, other indicators of DC function and quality include their capacity for endocytosis and T-cell activation. The exposure to maturation stimuli induces changes including downregulation of endocytosis and increase of antigen presentation to T cells. We evaluated the endocytic capacity of DCs treated with TKIs and untreated DCs by fluorescein isothiocyanate (FITC)-dextran uptake and followed by flow cytometry (Fig. 3B). We determined the ratio of the fluorescence from positive (dextran uptake obtained after 2 hours at 37°C) and negative (dextran uptake after 2 hours at 4°C) samples. The results show that pazopanib reduced endocytosis capacity by 29% for iDCs treated with pazopanib compared with untreated DCs (1.42 ratio vs. 2, respectively), and by 40% compared with iDCs treated with sunitinib (1.42 vs. 2.33). The trend persisted after maturation: the endocytic capacity of pazopanib-treated mDCs was 42% lower than that of mDCs (0.8 ratio vs. 1.37) and 57% lower than that of sunitinib-treated mDCs (0.8 vs. 1.82).

We then analyzed the capacity of DCs to stimulate the proliferation of allogeneic T cells (Fig. 3C). Lymphocytes, pretreated with CFSE, were cocultured with DCs and proliferation was evaluated after 4 days through progressive halving of fluorescence by flow cytometry. The results, plotted as percentage of fold increase (% of proliferation obtained as ratio between T cells stimulated by pazopanib-DCs/DCs or sunitinib-DCs/DCs), showed that when DCs were differentiated in the presence of pazopanib, they acquired a greater capacity to stimulate T cells than either untreated DCs (20% fold increase for iDCs, 5% for mDCs) or sunitinib-treated DCs (14% for iDC, 5,1% for mDCs). No effect was observed on T cells cultured

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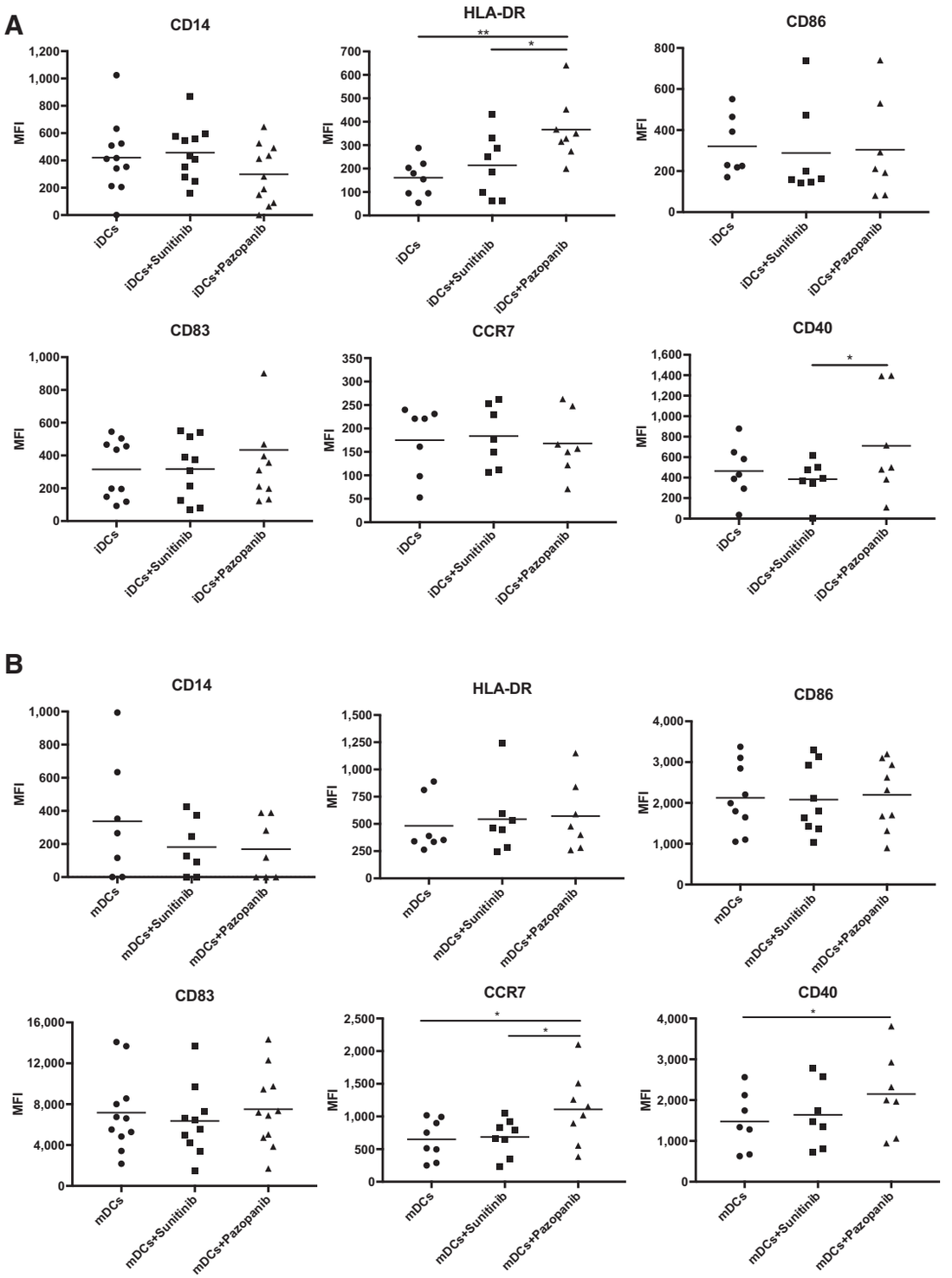

Figure 1.

Pazopanib modulates DC phenotype. The MFI values of DC phenotypic markers from healthy donors are shown. The cells were differentiated from monocytes in presence of pazopanib (20 μg/mL) and at day 5 iDCs were collected and matured with cytokine cocktail (rhIL1β, IL6, TNFα, and PGE_2). Untreated DCs and DCs differentiated with sunitinib (50 ng/mL) were used as controls. The concentration of pazopanib and sunitinib used for the culture corresponds to serum levels achieved in TKI-treated patients. **A,** The phenotype of iDCs; **B,** The phenotype of mDCs. The bars correspond to the average MFI values among donors. Statistical significance was determined by ANOVA test when comparing three groups and by Student paired *t* test for two groups. *, $P < 0.05$; **, $P < 0.01$, Student *t* test.

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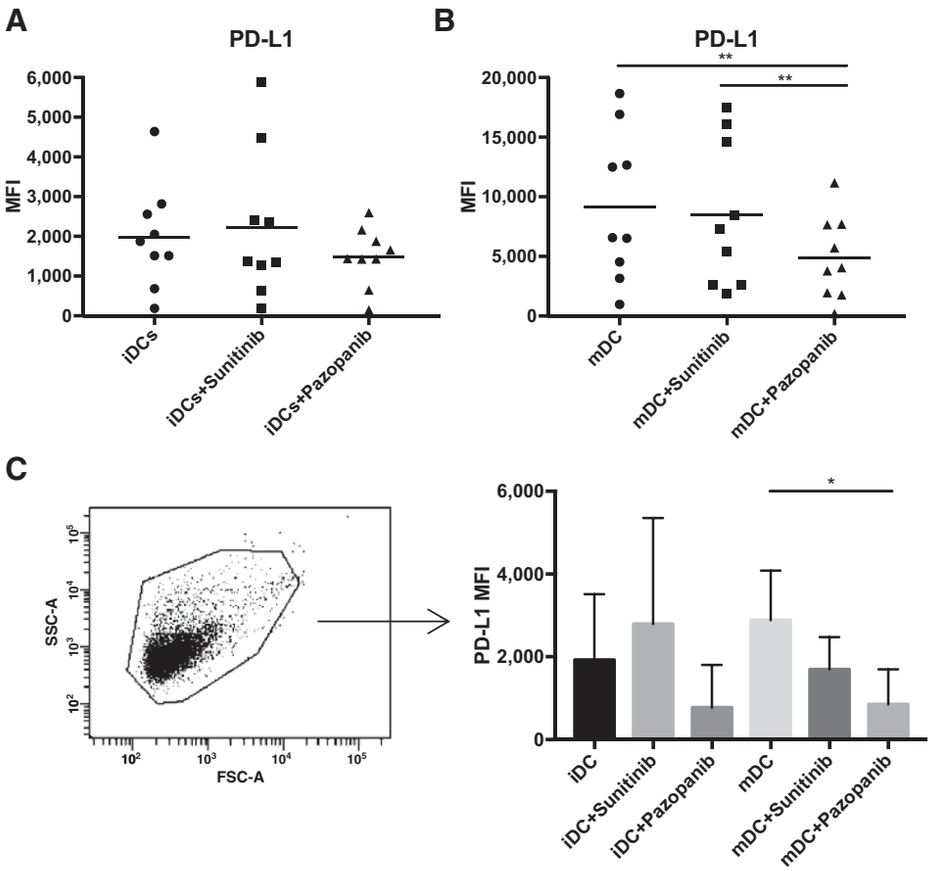

Figure 2.

Pazopanib treatment reduces PD-L1 expression. PD-L1 expression by untreated DCs and treated with sunitinib and pazopanib (iDC in **A** and mDC in **B**). Data are reported as values of MFI and the bars correspond to average of these values. **C,** Microvesicles released by DCs and analyzed by flow cytometry are represented on forward scatter/side scatter dot plot. The histogram shows the expression of PD-L1 in terms of MFI on microvesicles obtained by untreated DCs (black columns), by DCs differentiated with sunitinib (light gray column) and by DCs differentiated with pazopanib (dark gray column). ANOVA test was used to compare three groups. Student paired *t* test for two groups. *, $P < 0.05$; **, $P < 0.01$; Student *t* test.

alone for 24 hours in the presence of either TKI (Supplementary Fig. S2).

Pazopanib affects DC differentiation by inhibiting p-Erk/β-catenin signaling

The Wnt-β-catenin pathway, particularly in DCs, regulates the balance between tolerance and immune response (19). Loss of β-catenin impairs the ability of DCs to induce Tregs (20), instead the activation of β-catenin pathway increases the capacity of DC to release IL10 and promote immune tolerance, (21, 22). In DCs, β-catenin signaling synergizes with other pathways, such as the Erk pathway, to induce anti-inflammatory cytokines and proliferation of Tregs. Erk1/2 signaling retards the phenotypic and functional maturation of monocyte-derived human DCs (23).

To evaluate whether changes in DC functional activity were due to signaling differences, we examined DC intracellular pathways. Untreated DCs or DCs differentiated in the presence of sunitinib or pazopanib were lysed and probed with anti–p-Erk 1/2, anti–β-catenin and anti–NF-κB by western blot (Fig. 4). DCs treated with pazopanib expressed less p-Erk 1/2 than did DCs treated with sunitinib or untreated DCs. This downregulation associated with a significant reduction of β-catenin expression (iDCs and sunitinib-iDCs vs. pazopanib-iDCs $P < 0.05$; mDCs vs. pazopanib-mDCs $P < 0.05$). In both pazopanib-iDCs and pazopanib-mDCs, p-Erk1,2 and β-catenin antibodies detected a weaker signal than in untreated DCs and DCs treated with sunitinib, suggesting that pazopanib could act through these pathways. We analyzed the activation of NF-κB, which is essential for DC development and survival and regulates DC maturation. Results indicated that NF-κB activation was similar in all iDCs tested. Although the p50 signal was weaker in mDCs treated with pazopanib, the balance between all intracellular pathways favored the activation pathway.

Modulation of DCs generated from mRCC patients treated with pazopanib or sunitinib

To confirm the pazopanib immune-priming effect in patients, we analyzed monocyte-derived DCs differentiated from mRCC patients during TKI treatment. DCs were differentiated *in vitro* by standard methodology without the addition of TKIs. Fig. 5 shows the phenotype of DCs generated from PBMCs of RCC patients after one month of pazopanib or sunitinib treatment. CD14, a marker of DC immaturity, was less expressed in iDCs from patients treated with pazopanib ($P < 0.01$) than patients treated with sunitinib confirming data obtained *in vitro*. Moreover, DCs (both iDCs and mDCs) from pazopanib-treated patients expressed more of the activation markers HLA-DR and CCR7 and less PD-L1 (mDCs; $P < 0.01$) as compared with DCs generated from sunitinib-treated patients.

For one patient, we evaluated the modulation of some DC markers differentiated before (T0) and during pazopanib therapy (T1: after 1 month of treatment, T2: after 2 months) and results are shown in Supplementary Fig. S3A. As with DCs generated *in vitro* with pazopanib, iDCs from this mRCC patient in treatment with pazopanib showed increased

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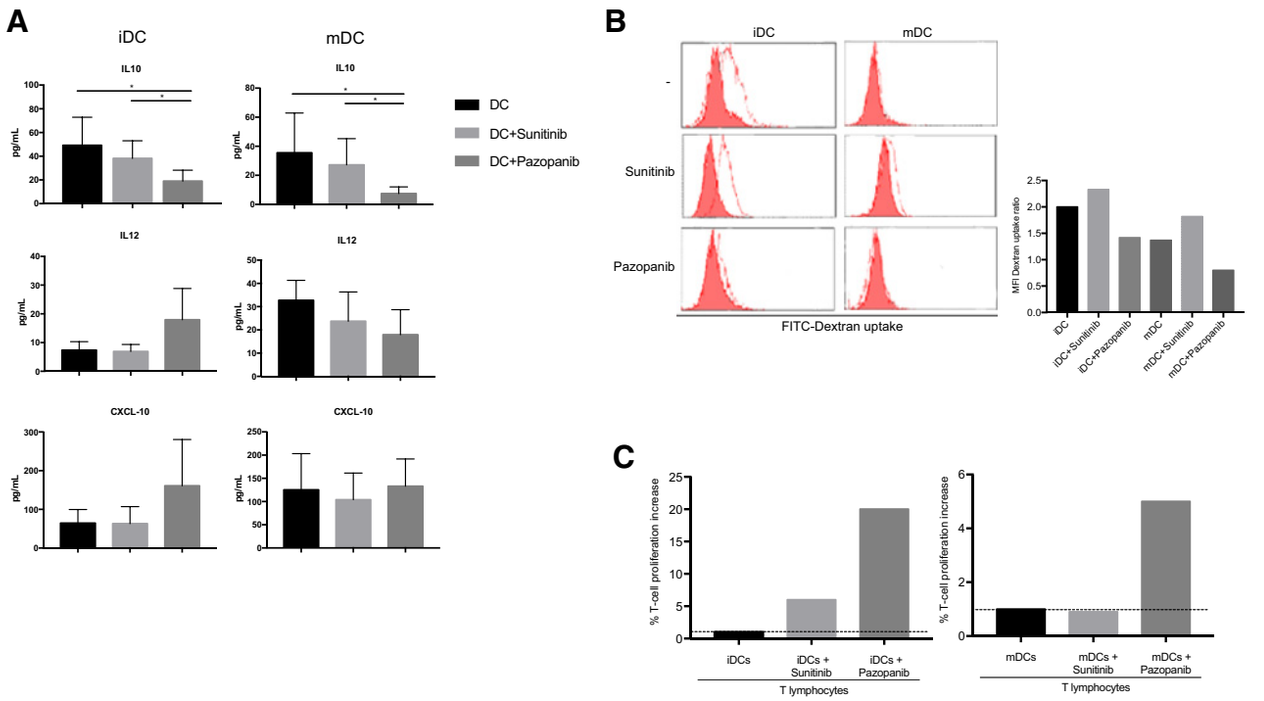

Figure 3.

DCs activated by pazopanib modulate cytokine and chemokine release, reduce endocytic capacity and increase T-cell proliferation. **A,** The amount of IL10, IL12, and CXCL-10 was evaluated in differently generated DC supernatant by Luminex multiplex beads analysis. The results correspond to the mean obtained from 10 independent experiments ± SD. **B,** The endocytic capacity of iDCs and mDCs differently generated was evaluated as FITC-dextran uptake (1 mg/mL/10^6 cells). Open histograms represent the dextran uptake obtained after 2 hours at 37°C (positive), whereas filled histograms show the dextran endocytosis after 2 hours at 4°C (negative control). Results are representative of one donor out of four. The values reported on histogram indicate the ratio between MFI of positive and negative control of four independent experiments for each DC condition. **C,** The capacity of DCs to prime allogenic T-cell response *in vitro* was tested evaluating T-cell proliferation ability through progressive halving of CFSE fluorescence using FACSCanto flow cytometry. Results are reported as a percentage of increase of T-cell proliferation capacity of three independent experiments of DCs treated with pazopanib compared with sunitinib-differentiated DCs and untreated DCs. ANOVA test was used to compare three groups. Student paired *t* test for two groups. *, $P < 0.05$.

expression of HLA-DR at T2 and CD40 at T1 and T2. Expression of the coinhibitory molecule PD-L1 decreased during pazopanib treatment, both in iDCs and mDCs, confirming that pazopanib boosts DC activity.

Immune profile of mRCC patients during TKI treatment

The priming of DCs by pazopanib could impact the immune repertoire in the peripheral blood of mRCC patients. To verify this hypothesis, we followed and monitored longitudinally mRCC patients undergoing TKI therapy, either pazopanib or sunitinib. Patients had different previous therapies although all of the patients belonged to the same risk group. Six patients in treatment with TKIs for mRCC underwent peripheral blood sampling at different time points and PBMCs were isolated and analyzed by flow cytometer in order to investigate the evolution of patients' immunological profile during TKI administration. The clinical profile and the schedule of blood sampling is showed in Fig. 6A. Patients 1, 2, and 3 received pazopanib, whereas patients 4, 5, and 6 received sunitinib. The immunoassays were performed when possible at different time points, as shown in the figure. Two patients (patient 3 and 5) showed progressive disease and one of these died from the disease. The other four are still in treatment with TKIs.

Several immunological parameters were evaluated for each patient at each time point considering the limited amount of blood sampling (Fig. 6). In particular, we analyzed T-cell subpopulations, their activation status (CD137 expression), their proliferation ability as assessed by expression of Ki67, the immunosuppression as a percentage of Tregs, and T-cell exhaustion as assessed by expression of checkpoint inhibitors such as PD-1, CTLA-4, and Tim-3. Pro- and anti-inflammatory cytokines were evaluated in serum of patients. Only the assays for which we had sufficient patient samples are shown in Fig. 6.

We observed that pazopanib seemed to induce an increased number of $CD3^+CD137^+$ T cells (Fig. 6B). In patient 1, for example, activated ($CD137^+$) $CD4^+$ T cells were 30% of the activated $CD3^+$ T cells at time T2, compared with T0 (before pazopanib therapy, 0.3% of activated $CD3^+$ T cells) and T1 (4.7% of $CD3^+$ T cells). At T5, we observed a decrease of $CD4^+$ and $CD8^+$ $CD137^+$ T-cell populations. In the same patient, plasma levels of IL6 dropped from T0 to T2, both IL4 and CXCL-10 increased at T1 and T2, and ICAM decreased at T2. Ki67 expression, a proliferation signal for T cells, was higher at T2 than at other time points. Patient 2 had fewer $CD137^+$ T cells from time T0 forward. Regulatory T cells decreased during treatment and

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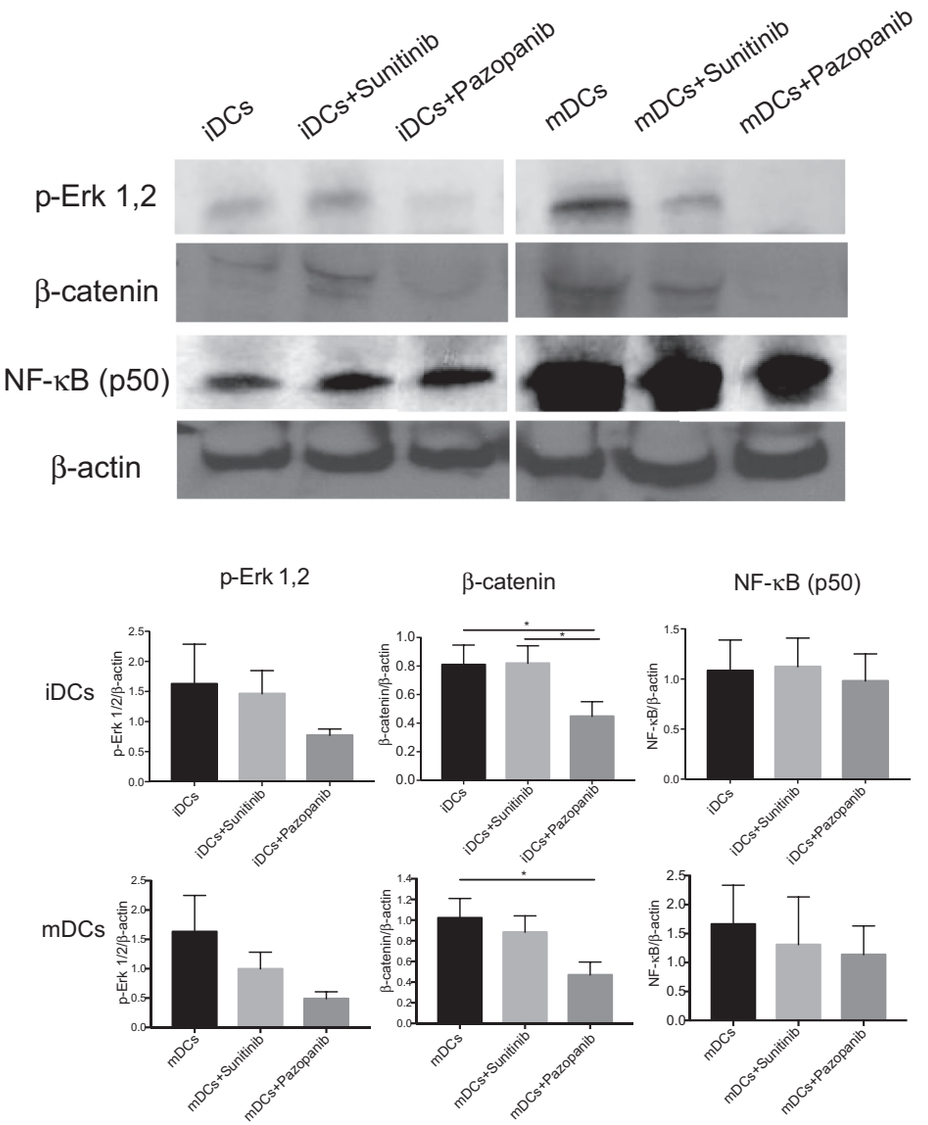

Figure 4.
Pazopanib modulates DC signaling turning off the p-Erk/β-catenin pathway. Western blotting of iDCs (left) and mDCs (right) untreated and generated in presence of sunitinib and pazopanib. Samples were analyzed for p-Erk 1/2 (42–44 kDa), β-catenin and for the presence of NF-κB. β-Actin was used as a loading control. Proteins were resolved in 4% to 12% SDS-PAGE gel. Densitometric evaluation of the signals for β-catenin, p-Erk1/2 and p50 were normalized to the levels of β-actin (under the blot). ANOVA test was used to compare three groups. Student paired *t* test for two groups. *, $P < 0.05$.

Ki67$^+$ T cells increased. Patient 3, who was monitored during pazopanib treatment and immediately after progression, showed enhancement of CD4$^+$CD137$^+$ T cells at T3 (54%) compared with T0 (24.9%) and T1 (23.2%). This population decreased during progression under nivolumab treatment (T4, T5). This patient's Treg population was unchanged. PD1$^+$ T cells increased slightly from T0 to T1, then decreased during pazopanib and nivolumab treatment.

When we analyzed mRCC patients treated with sunitinib, we observed that CD137$^+$ T cells were less evident, thus suggesting that this population could be influenced by the effect of pazopanib. In Patient 4, under sunitinib therapy, CD4$^+$CD137$^+$ T cells were barely detectable at all three time points. This patient presented with 29% of T cells being CD8$^+$CD137$^+$ T cells at T1; however, this population decreased to 14% at T2 and T3. In the same patient, the serum concentrations of IL10 were increased at T2 compared with T1, whereas serum concentrations of IL12, IL4, and CXCL-10 decreased. In accordance with the increase of IL10, we observed an increase of Treg cells during sunitinib treatment, accompanied by upregulation of CTLA-4 expression on T cells at T3. Patient 5 showed a similar decreasing trend in CD137$^+$ T-cell population. Coinhibitory markers, such as CTLA-4 and Tim-3, increased during sunitinib treatment. Ki67$^+$ T cells were decreased, and Treg cells were reduced. Patient 6 presented low and stable fractions of CD137$^+$ T cells during sunitinib treatment when there time points were analyzed, although CD3$^+$CD137$^+$ T cells doubled at T7. At the same time IL10 and IL4 decreased compared with T5, and IL12 slightly increased. Tregs decreased at T7 but increased at T9.

Discussion

RCC is the tumor that has most benefited from clinical use of TKIs. The clear cell histotype drives VEGF overproduction via inactivation of the tumor-suppressor Hippel–Lindau (VHL) gene

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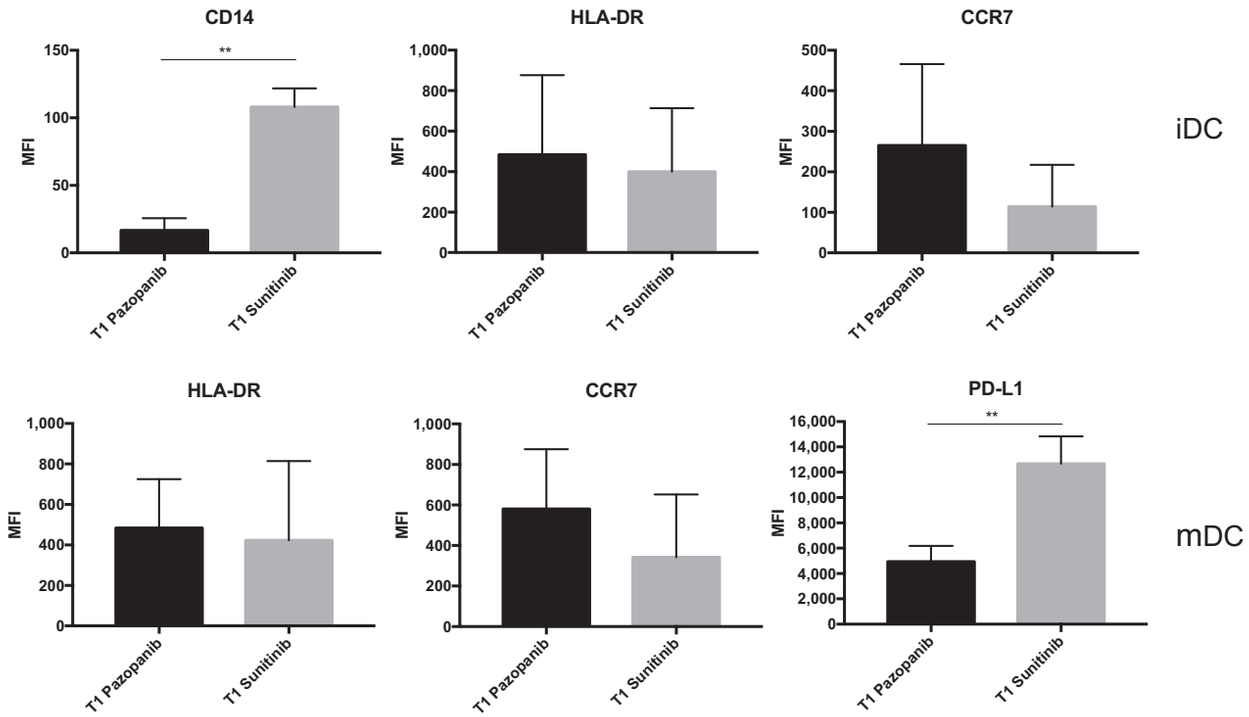

Figure 5.

DC phenotype obtained from TKI-treated patients. Comparison of iDCs and mDCs at T1 of pazopanib (black column) and sunitinib (gray column) treatment. Data are reported as values of MFI ± SD. Statistical significance was determined by Student paired *t* test. **, $P < 0.01$.

(24, 25). Immune-targeting molecules have added additional possible treatment choices for RCC. The increase in treatment options has added complexity to clinical questions on how to choose first-line treatment and treatments after recurrence (26). Although VEGF- and mTOR-targeted therapies have improved clinical outcome in metastatic RCC, durable responses remain rare despite efforts to design sequential or combined treatment modalities.

RCC is been considered an immunogenic tumor and therapy with cytokines such as IL2 and IFNα has been used although the responses have been mild and associated with toxicity. Long-term responses and complete remissions have been occasionally observed in mRCC patients treated with immunotherapy (27, 28). The induction of an immune response such as the one elicited by the multi-peptide IMA901 vaccine was associated with a clinical benefit. Over the next decade, immunotherapy trials in kidney cancer will focus on learning how to combine PD-1/PD-L1 inhibitors with immune-modifying agents such as those targeting the VEGF pathway (29, 30).

In this scenario, and with the possibility to introduce the immune checkpoint inhibitors (ICI) in RCC patient treatment, we must consider the impact of TKIs on the immune system of the patient. TKIs currently approved for first-line treatment of mRCC inhibit tumor cell growth and angiogenesis but also show immune-regulatory effects. How these immune effects impact subsequent immune-targeted therapies and affect treatment outcome needs to be understood.

Several molecules and immune cell types are involved in the interaction between the growing tumor and the immune system. The balance between immunosuppression and immune activation dictates to some extent the prognosis and the response to treatment, particularly for immune-based treatments. Several biomarkers and or immune-molecular portraits have been studied in the attempt to identify responder patients, such as those who most likely will benefit from anti–PD-L1 treatment (31). One of the mechanism of tumor silencing of immunity involves DCs, the main orchestrators of the immune system (32, 33). DCs in the tumor microenvironment may be tolerized and therefore unable to present tumor antigens or unable to present antigens in the appropriate setting within a costimulatory background. Several molecules released by the tumor can induce this freezing effect (34, 35). VEGF is one of them and this mechanism has been well characterized in RCC (36). The immediate consequence of induction of tolerogenic DCs will be the lack of T-cell infiltration, particularly tumor-specific T cells that represent the target for ICI immunotherapy.

Results reported in this article show that the TKI pazopanib could potentiate and differentiate human DCs. Immature and mature DCs showed increased expression of HLA-DR, CCR7, and CD40. PD-L1 decreased in the DCs after maturation, as compared with untreated mature DCs or DCs differentiated in the presence of sunitinib. PD-L1 was expressed in microvesicles released by DCs confirming the role of cell particles in delivering remote signaling (37). The phenotypic changes observed in the pazopanib-treated DCs were accompanied with biological behavior consistent with an immune priming effect. IL10 production was reduced, whereas IL12 and CXCL10 production increased. The process of endocytosis was reduced as well, and

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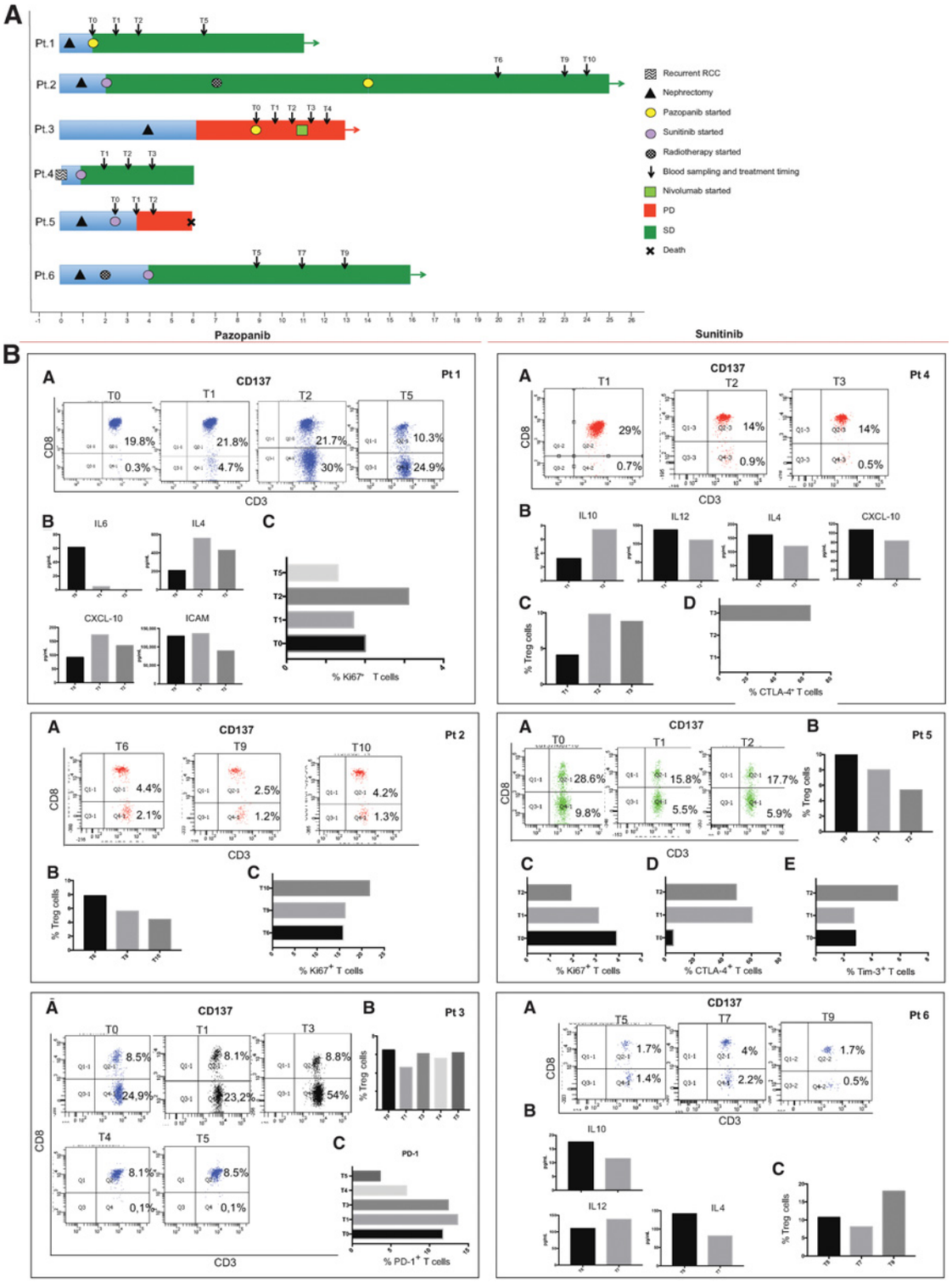

Figure 6.

Immunological profile of six mRCC patients during TKI treatment. **A,** Swimmer Plot showing the clinical history and the blood sampling of six mRCC patients: Patients 1, 2, and 3 treated with pazopanib, patients 4, 5, and 6 with sunitinib. **B,** Immunological analysis performed on PBMCs and sera collected from blood samples for each patient. Sera were used to evaluate cytokines and chemokines released during TKIs treatment and analyzed by Luminex multiplex beads. Immune cell subpopulations were monitored by flow cytometer and analyzed to FACSDiva Software. To analyze $CD137^+$ T cells, lymphocytes were first gated on FSC-A and SSC-A, then the $CD3^+$ T-cell subpopulation was selected on lymphocytes. These $CD137^+CD3^+$ T cells were then selected and analyzed for CD4 and CD8 (gating scheme in Supplementary Fig. S3B).

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amplified MLRs indicated an overall acquisition of immunogenicity by DCs.

DCs are the critical cells in immune activation as they are responsible for antigen processing/presentation, cross-priming and trafficking to the lymph nodes (38, 39). The pazopanib *in vitro* and *in vivo* "treated" DCs, both from donors and from patients, have all these hallmarks.

Sunitinib, another TKI used in mRCC treatment, did not have the effects on DC differentiation as have been described by others (11). Sunitinib and pazopanib have a similar pattern of receptor recognition but display different affinities for VEGF-R1, which could explain the diversity of immune modulation effect (12). In particular, sunitinib affects the immunosuppressive repertoire, Treg and MSDC, in RCC patients. The two drugs appear, however, to be similar in terms of clinical outcome when used in first-line therapy in mRCC (40, 41). Immunologically this could be explained by arguing that one (pazopanib) is more active in the rescue of DC from a tolerogenic state, inducing activation signals, whereas the other (sunitinib) is more efficient in making space for the antitumor effector cells by eliminating immunosuppression in the microenvironment.

The consequences of receptor inhibition were studied in the pazopanib-treated DC, as we investigated the signal transduction involved. We found that Erk signaling was shut down, leading to downregulation of β-catenin. Wnt/β-catenin signaling plays a role in cell differentiation, proliferation, survival, and immune cell function. In tumors, the Wnt/β-catenin pathway is activated in DCs leading to immune tolerance and immune evasion. This results in suppression of effector T cells and recruitment of Treg cells. Increased Wnt ligands released in the tumor microenvironment activate β-catenin signaling in DCs, resulting in production of IL10 and induction of tumor tolerance (22). In melanoma, intrinsic activation of the Wnt/β-catenin pathway correlates with absence of T cells in the microenvironment (42). The same pathway is involved in bladder cancers, generating the non-T cell–inflamed tumors that represent most cases in which tumors are unresponsive to ICI therapy (43). A DC subtype is enriched in RCC that expresses CD14, high TNFα, and low CXCL-10, which is responsible for preventing a T-cell infiltrate from mediating antitumor functions (35). Indeed, the presence of circulating, intratumoral, and peritumoral $CD14^+$ cells was a prognostic factor for decreased survival in a cohort of 375 RCC patients (44).

Pazopanib can therefore have an antagonistic effect in the tumor, releasing DCs from the tolerogenic/immature/$CD14^+$ state and tentatively restoring antitumor immune activation. The goal of such therapy is to turn cold tumors into hot tumors, which would be more responsive to ICI therapy. To understand if pazopanib treatment could indeed exert this effect *in vivo*, we utilized two different approaches. First, we selected $CD14^+$ cells from peripheral blood of mRCC patients during treatment with pazopanib or sunitinib. The immune-priming effect of pazopanib in DCs was confirmed. DCs derived from mRCC patients in treatment with sunitinib expressed high amounts of $CD14^+$ marker and PD-L1. This may suggest no effect by sunitinib on the DCs that remain in a more immature state as seen in other cancer patients (44).

The second approach was to longitudinally follow and monitor mRCC patients undergoing TKI therapy. This was not a homogeneous patient population because patients had different previous therapies, although all of them belong to the same risk group. Nevertheless, we wanted to assess if we could detect immunological changes in the peripheral blood that could be associated with pazopanib treatment. Various conclusions are supported although results are preliminary. First, patients 1, 2, and 3 who received pazopanib showed a $CD137^+$ T-cell population. Patient 1 presented an ideal setting to study the effect of pazopanib *in vivo* because this patient had no previous possibly confounding therapy. Patient 2 had a recent radiotherapy to control bone pain, which might have influenced the immune system (45). Patient 3 is the only patient we have monitored during anti–PD-1 nivolumab treatment. This patient had a drop of $CD137^+$ $CD4^+$ T-cell population at time T4 and T5 just after treatment with ICIs for progression. PD-1 expression also decreased. This could be explained by recruitment of exhausted T cells to the tumor or by difficulty in detecting PD-1 cells due to the covering of the circulating nivolumab antibody.

CD137 (4-1BB, a member of the TNF-receptor family) is considered a biomarker of tumor-reactive cells. The signaling with its ligand or an agonistic antibody promotes expansion of T cells, sustains survival, and enhances cytolytic function. This marker has been used to select tumor-specific T cells and can be upregulated in an antigen-dependent fashion (46, 47). Agonist antibodies recognizing the CD137 receptor are part of the vast repertoire of immune-modulatory antibodies being prepared for the clinic. The Treg population was unchanged or diminished in all mRCC patients. Immune monitoring on patient 4, the only patient with recurrent mRCC we tested, showed the worst-case scenario during sunitinib treatment, characterized by no $CD137^+$ T cells, high IL10, high percentages of Treg, low IL12, and upregulation of CTLA-4 at 3 months of therapy.

The other consideration is the upregulation of checkpoint inhibition markers on T cells as a sign of T-cell exhaustion. This evaluation is relevant for clinical decisions because ICI are proposed as second-line therapies for mRCC after TKIs. We saw upregulation of CTLA-4 early in 2 of 3 sunitinib-treated patients. In patient 5, we observed upregulation of both CTLA-4 and Tim-3. This patient progressed rapidly and died from the disease.

Pazopanib can have an immunological effect as early as the second month of treatment. Our experience with ICIs showed that immune modifications can happen sooner than their impact on the tumor becomes evident (48). Our data are part of an ever-evolving clinical arena. CheckMate 214, a phase III, randomized, open-label study evaluating the combination of nivolumab (anti–PD-1) plus ipilimumab (anti–CTLA-4) versus sunitinib in patients with previously untreated advanced or mRCC, showed improved OS, ORR, and PFS in the nivolumab plus ipilimumab group in intermediate/poor risk patients, with the greatest improvements in those with PD-L1 expression on $\geq$ 1% of biopsied tumor cells. These data suggest that ICIs could be used as front-line treatment in mRCC even though only the knowledge of the biological and immunological features of the tumor and patient could indicate the best individual sequence/combination of treatment.

In this context, we are recruiting mRCC patients with no previous therapies and collecting tumor specimens to confirm the immunological observations that we observed from this preliminary study. Longitudinal study methods combined with an individualized approach based on the immunological fitness of the patient hold the greatest likelihood of clinical success. Confounding influences that might affect the immune status of the patient, such as other therapies, could limit our understanding

of the activity and efficacy of the immune-modulating agents we wish to study.

In conclusion, the immune-priming effects of pazopanib open therapeutic avenues for this TKI in the mRCC and probably for other cancers. Variations of treatment sequences, schedules, doses, and combinations with other TKIs or immunotherapy compounds or vaccines should be tested, taking into account the immunological effects of TKIs and the updated insights into oncology and the interactive tumor microenvironment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: I.G. Zizzari, A. Botticelli, S. Caponnetto, F. Calabrò, A. Gelibter, E. Rossi, G. Schinzari, P. Marchetti, M. Nuti
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Napoletano, A. Botticelli, S. Caponnetto, F. Calabrò, A. Gelibter, E. Rossi, G. Schinzari, M. Nuti
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I.G. Zizzari, C. Napoletano, A. Gelibter, I. Ruscito, H. Rahimi, M. Nuti
Writing, review, and/or revision of the manuscript: I.G. Zizzari, C. Napoletano, A. Botticelli, S. Caponnetto, F. Calabrò, A. Gelibter, A. Rughetti, I. Ruscito, E. Rossi, G. Schinzari, P. Marchetti, M. Nuti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Gelibter, P. Marchetti, M. Nuti
Study supervision: F. Calabrò, A. Gelibter, P. Marchetti, M. Nuti

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TK Inhibitor Pazopanib Primes DCs by Downregulation of the β -Catenin Pathway

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