# Sorafenib, but not sunitinib, induces regulatory T cells in the peripheral blood of patients with metastatic renal cell carcinoma

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**Induction of regulatory T cells (Treg) is an important mechanism leading to tolerance against tumors. Increased levels of Treg have been described in renal cell carcinoma (RCC) patients and seem to correlate with an adverse outcome. Our study aimed to analyze the influence of sorafenib and sunitinib on the frequency of Treg in patients with metastatic RCC (mRCC). Treg were analyzed by flow cytometry in the peripheral blood (PB) of patients ($n=19$) with histologically confirmed mRCC under treatment with either sunitinib (50 mg/d, $n=11$) or sorafenib (800 mg/d, $n=8$). Blood samples were taken before treatment and during the first, second, and third months of therapy. Flow cytometric analysis of PB mononuclear cells was performed using fluorochrome-labeled antibodies against CD3, CD4, CD25, and FOXp3. During the first month of therapy, patients treated with sorafenib showed a significant increase in $FOXp3^+CD3^+CD4^+CD25^+$ Treg (13.5 vs. 36.3% of gated cells, $P=0.02$, or 0.35 vs. 0.49% of total cells) and the ratio $FOXp3^{pos}$ T cells/$FOXp3^{neg}$ T cells (0.16 vs. 0.56 of gated cells, $P=0.02$). These elevated levels persisted throughout the treatment period. There was no influence of sunitinib on the frequency of Treg in our cohort of patients. Sorafenib, but not sunitinib, leads to an early and sustained increase in Treg in PB of mRCC patients. In immunoresponsive tumors such as RCC, immunological effects of kinase inhibitors are particularly relevant for the design of combination trials with immunotherapeutic agents. Our study suggests that sorafenib should be avoided in such a therapeutic setting.** ***Anti-Cancer Drugs*** **23:298–302** 

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## Introduction

Significant immunosuppression has been described in several different cancer types including renal cell carcinoma (RCC) [1–4]. The mechanisms underlying the suppression of antitumor immunity are diverse and are believed to involve primarily cellular immune responses. Regulatory T cells (Treg) are important mediators in maintaining peripheral tolerance and preventing autoimmunity [5–7], and there is emerging evidence that $FOXp3^+CD4^+CD25^+$ Treg play an important role in facilitating tumor escape from immunological recognition [8]. TH2 polarization and an inverse correlation between the presence of Treg and a TH1-type of antitumor response have been reported in metastatic RCC (mRCC) patients [9–13]. Furthermore, increased numbers of Treg have been detected in previous studies with RCC patients and seem to correlate with a poorer outcome [14,15]. Sunitinib and sorafenib are oral multikinase inhibitors, which target vascular endothelial growth factor receptors, platelet-derived growth factor receptors, c-KIT (receptor for stem cell factor), and Flt-3. Sorafenib has additional inhibitory activity on Raf1-kinase. Both drugs belong to the standard treatment in mRCC and have been shown to improve progression-free survival [16–20].

As there is increasing evidence that growth factors such as vascular endothelial growth factor and stem cell factor, secreted by tumor cells, may also induce Treg by altering dendritic cell development [21,22], the aim of our study was to analyze the frequency of Treg under treatment with sunitinib or sorafenib in patients with mRCC.

## Patients and methods

Patients with histologically confirmed mRCC were treated with either sunitinib or sorafenib. Patients' characteristics are depicted in Table 1. Sunitinib-treated patients received 50 mg orally daily for 28 days followed by 14 days' rest. Sorafenib-treated patients received 800 mg orally daily. Clinical evaluation was performed by physical examination and computed tomography scans. After informed consent of the patients and approval of the local ethics committee, blood samples were taken before treatment and during the first, second, and third months of therapy. Blood sampling was generally

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**Table 1 Patients' characteristics**

| Patient | Age | Sex | MSKCC risk score | Histology | Metastasis location | Medication | Clinical response after 3 months of therapy | Overall | Ratio FoxP3 before therapy | Ratio FoxP3 after therapy (third month) |
|---|---|---|---|---|---|---|---|---|---|---|
| #1 | 80 | M | 1 | Clear cell | Left kidney, lung | Sunitinib | SD | SD<br>CR | 0.26 | 0.25 |
| #2 | 55 | F | 1 | Clear cell | Bone, left adrenal gland | Sunitinib | SD | CR | 0.03 | 0.02 |
| #3 | 62 | M | 1 | Clear cell | Lung | Sunitinib | SD | SD | 0.03 | 0.02 |
| #4 | 47 | M | 2 | Clear cell | Bone, skin, lung | Sunitinib | SD | SD | 0.03 | 0.03 |
| #5 | 65 | F | 1 | Clear cell | Liver, pancreas | Sunitinib | SD | SD | 0.03 | 0.74 |
| #6 | 73 | F | 1 | Clear cell | Bone, gluteal | Sunitinib | SD | SD | 0.28 | 1.25 |
| #7 | 66 | F | 1 | Clear cell | Lung, lymph nodes | Sunitinib | SD | PR | 0.37 | 0.52 |
| #8 | 70 | M | 2 | Clear cell | Lung, liver, thyroid, adrenal gland | Sunitinib | SD | PR | 0.83 | 0.56 |
| #9 | 69 | F | 1 | Clear cell | Lung, pancreas | Sunitinib | SD | PR | 0.21 | 0.16 |
| #10 | 66 | M | 2 | Clear cell | Bone, lung | Sunitinib | SD | SD | 0.58 | – |
| #11 | 62 | F | 3 | Clear cell | Lung, liver | Sunitinib | PD | PD | 0.32 | – |
| #12 | 66 | M | 3 | Clear cell,/ chromophobe | Lung, liver, bone | Sorafenib | PD | PD | 0.03 | 0.02 |
| #13 | 61 | M | 1 | Clear cell | Lung | Sorafenib | PD | PD | 0.02 | 0.14 |
| #14 | 49 | F | 1 | Clear cell | Lung, cerebral | Sorafenib | SD | SD | 0.14 | 0.65 |
| #15 | 64 | M | 2 | Clear cell | Lung, gluteal | Sorafenib | SD | SD | 0.06 | 1.06 |
| #16 | 59 | F | 2 | Papillary | Lung, liver | Sorafenib | SD | SD | 0.74 | 0.37 |
| #17 | 70 | M | 1 | Clear cell | Lung, bone, left adrenal gland | Sorafenib | SD | SD | 1.17 | 0.53 |
| #18 | 42 | M | 1 | Clear cell | Lung | Sorafenib | SD | SD | 0.28 | 1.05 |
| #19 | 64 | F | 2 | Clear cell | Liver, pancreas, lung | Sorafenib | SD | SD | 0.17 | 0.19 |

Included patients, $n=19$.
Ratio FoxP3 = $FoxP3^{pos}/FoxP3^{neg}$ cells in $CD3^+CD4^+CD25^+$ T-cell gate.
F, female; M, male; PD, progressive disease; PR, partial remission; SD, stable disease.

performed during therapy. Only in patients 3 (sunitinib) and 14 (sorafenib), blood samples were taken during medication breaks after the third month of therapy (in patient 14 due to toxicity).

Fluorescence-activated cell sorting analysis was performed in peripheral blood (PB) mononuclear cells that had been generated by density-gradient centrifugation using Lymphoprep solution (Fresenius/Axis-Shield PoC AS, Oslo, Norway). For flow cytometric analysis, fluorochrome-labeled antibodies against CD3, CD4, CD25, and isotype control (all Becton & Dickinson, Heidelberg, Germany) were used according to the manufacturer's instructions. Intracellular FOXp3 staining was also performed according to the manufacturer's instructions using eBioscience Fixation/Permeabilisation solution (Bioscience, Hatfield, UK). A total of 60 000 lymphocytes were analyzed on a fluorescence-activated cell sorting Calibur using BD Cell Quest Pro software, version 5.2.1 (Becton & Dickinson).

The number of Treg cells was expressed as the percentage of $FOXp3^+$ cells within the $CD3^+CD4^+CD25^+$ T-cell gate (Fig. 1). The absolute number of $CD4^+$ T cells was calculated from the whole blood cell count and flow cytometric analysis.

Statistical analysis was performed descriptively. The differences in parameters between two timepoints were calculated, and the Mann–Whitney *U*-Test was applied in order to determine statistical significance. The Wilcoxon rank sum test was used to compare variable changes on an intragroup basis in the two treatment groups. All statistical analyses were two-sided and a $P$ value of less than 0.05 was considered to be statistically significant. Analyses were performed by means of SPSS software (version 16.0, SPSS 16.0, IBM, Ehningen, Germany).

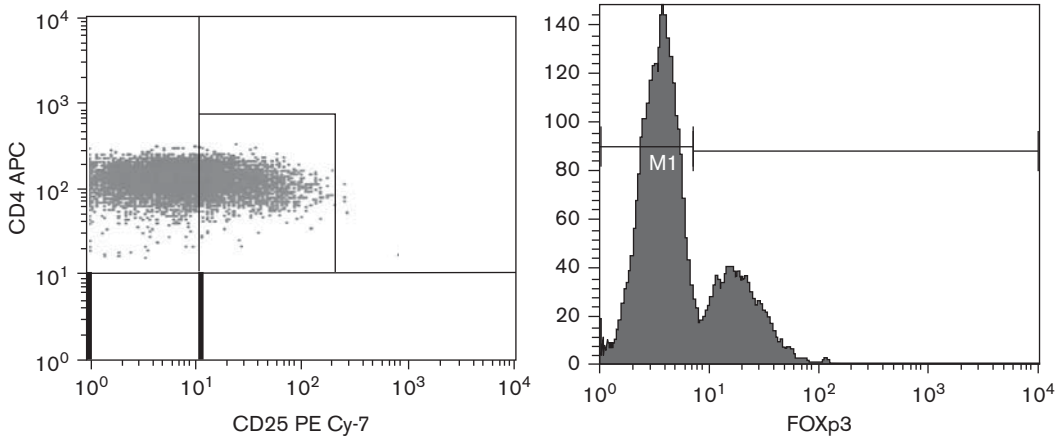
## Results

Nineteen mRCC patients treated with either sunitinib or sorafenib between May 2007 and October 2008 were evaluable. Eleven patients had been treated with sunitinib, and eight patients with sorafenib; 53% were male, and the median age was 64 years. Sixteen patients showed a Karnofsky performance status of 80% or more, and three patients had a performance status between 60 and 80% ($n=2$ in the sunitinib group, $n=1$ in the sorafenib group). All patients had undergone prior nephrectomy, and 12 had received prior systemic therapy for mRCC.

A total of 18 patients had clear cell carcinoma, and one patient had a papillary carcinoma. Applying the Memorial Sloan-Kettering Cancer Center prognostic criteria, 11 patients were considered favorable risk ($n=7$ in the sunitinib group, $n=4$ in the sorafenib group), six intermediate risk ($n=3$ in each group), and two poor risk ($n=1$ in each group).

Analysis of the baseline level of Treg in our cohort of patients did not significantly differ from healthy controls. However, during the first month of therapy, there was a significant increase in $FOXp3^+CD3^+CD4^+CD25^+$ Treg (13.5 vs. 36.3% in a $CD3^+CD4^+CD25^+$ T-cell

**Fig. 1**




Flow cytometric analysis of FOXp3+ Treg. The number of Treg is expressed as the percentage of FOXp3+ cells in a $CD3^+CD4^+CD25^+$ T-cell gate. APC, Allophycocyanine; PE, R-phycoerythrine.

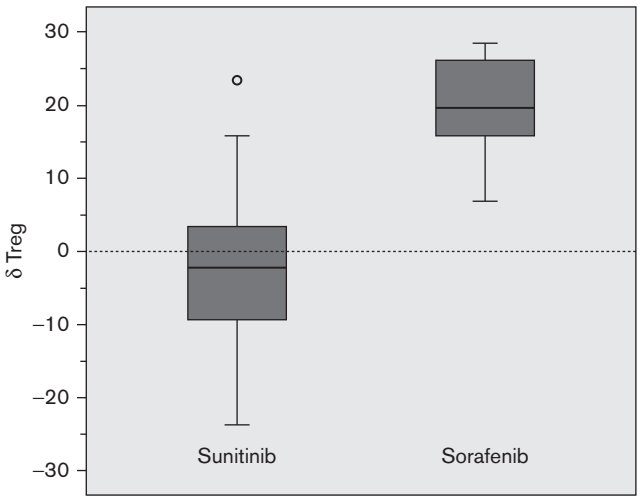
gate; or 0.35 vs. 0.49% of total lymphocytes, $P = 0.02$) in the sorafenib group (Fig. 2). This effect persisted during the second and third months of therapy (data not shown).

Increasing numbers of $FOXp3^+CD3^+CD4^+CD25^+$ Treg in PB under sorafenib treatment were confirmed by a statistically significant increase in the ratio of $FOXp3^{pos}/FOXp3^{neg}$ T cells (0.16 vs. 0.56 in a $CD3^+CD4^+CD25^+$ T-cell gate, $P = 0.02$) (Fig. 3).

Patients' characteristics are shown in Table 1.

The median count of $CD4^+$ T cells did not significantly change during treatment (sutent group: $830 \pm 430/\mu l$ before therapy vs. $870 \pm 250/\mu l$ after 3 months; sorafenib group: $1080 \pm 330/\mu l$ before therapy vs. $950 \pm 230/\mu l$ after 3 months, NS). The effect of sorafenib treatment on the number of circulating Treg was confirmed by an intragroup analysis (Wilcoxon rank sum test), also showing a significant increase in Treg during therapy. In our cohort of patients, there was no influence of sunitinib on the frequency of Treg. Furthermore, we could not demonstrate a significant correlation between changes in the number of Treg in PB and the clinical response, neither for sorafenib nor for sunitinib (Table 2).

**Fig. 2**




The difference between $CD3^+CD4^+CD25^+FOXp3$ Treg (percentage of marker-positive cells) before therapy (t0) and during the first month of treatment (t1) ($=\delta$ Treg t1 – t0) in sunitinib-treated and sorafenib-treated patients ($P=0.02$).

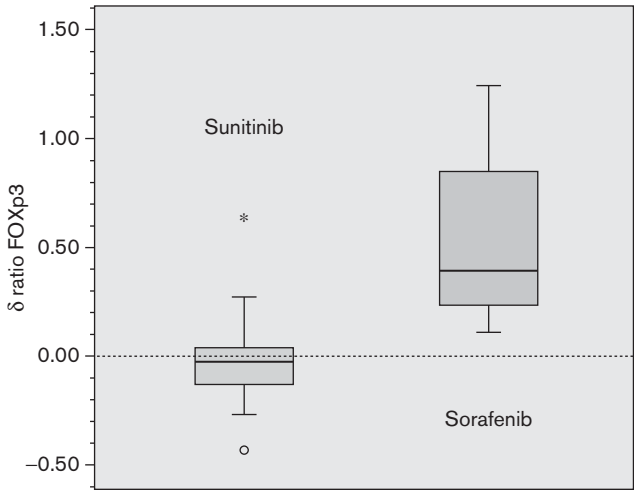
## Discussion

In our study, sunitinib and sorafenib showed substantial differences in their ability to influence the number of Treg in PB of mRCC patients. Sunitinib treatment did not affect Treg numbers, whereas sorafenib led to a significant and sustained increase in Treg. The increase in Treg under sorafenib treatment occurred early after 4 weeks of therapy and persisted over the whole treatment period. Analysis of the Treg number, the ratio of $FoxP3^{pos}$ versus $FoxP3^{neg}$ cells within the $CD3^+CD4^+CD25^+$ T-cell population, and the $CD4^+$ T-cell count showed that the effect was not simply due to changes in the total number of $CD4^+$ T cells.

As the two patient groups did not substantially differ with respect to baseline characteristics and tumor burden, it can be assumed that the observed difference is treatment related.

Both sorafenib and sunitinib belong to the group of multikinase inhibitors, although vascular endothelial

**Fig. 3**




The difference between the ratio of $FOXp3^{pos}/FOXp3^{neg}$ cells in a $CD3^+CD4^+CD25^+$ T-cell gate before therapy (t0) and during the first month of treatment (t1) (=δ ratio FOXp3 t1 – t0) in sunitinib-treated and sorafenib-treated patients ($P=0.02$).

**Table 2 Changes in the number of Treg during therapy in patients treated with sunitinib and sorafenib**

| | Treg stable or decreasing | Treg increasing |
|---|---|---|
| Progressive disease | | |
| Sunitinib | #11 | |
| Sorafenib | #12 | #13 |
| Stable disease | | |
| Sunitinib | #1. #3. #4. #10 | #5. #6 |
| Sorafenib | #16. #17 | #14. #15. #18. #19 |
| Objective response | | |
| Sunitinib | #2. #8. #9 | #7 |
| Sorafenib | – | – |

Treg, regulatory T cells.

growth factor receptor-related pathways can be considered as a main target of these drugs [16–20]. As sorafenib, in contrast to sunitinib, inhibits Raf1-kinase [17,19,20], it is tempting to speculate that Raf inhibition might cause the observed differences. However, it was shown in mice that Raf signaling is involved in the differentiation of Treg populations [23], suggesting that the effect observed in our study is not caused by Raf inhibition. Whether differential effects of sunitinib and sorafenib on molecular targets other than Raf are responsible for these results remains an open question in the light of the current literature.

Interestingly, Hipp and colleagues have previously demonstrated an inhibitory effect of sorafenib on dendritic cell maturation, and antigen presentation by immature antigen-presenting cells may lead to induction of Treg [24–26]. Therefore, the increase in the number of Treg observed in our study is not necessarily caused by direct effects of sorafenib on T cells. Instead, our results might as well be explained by the inhibitory effects on antigen presentation in the tumor microenvironment or in lymphatic tissues.

In contrast to our results, reduction of Treg by sunitinib has been reported previously in mRCC patients by other authors [27,28]. An obvious difference, which might explain the conflicting results, is the baseline number of Treg in these studies; in the cohort of patients reported by Finke and colleagues the Treg number was elevated compared with healthy controls, whereas it was normal in our study. This might indicate more severe immunosuppression at baseline in the study by Finke and colleagues and this might have resulted in more pronounced immunological effects of sunitinib treatment on Treg numbers.

Immunosuppression in cancer is an increasingly recognized phenomenon, and recent results from animal models suggest that it is not restricted to antitumor immune responses [29]. Treg play an important role in maintaining peripheral tolerance, and particularly in mRCC, increased levels of Treg have been correlated with an adverse outcome [14,15]. Our study did not show any significant correlation between changes in the number of PB Treg and clinical response (Table 2); however, this might be due to the limited number of patients in each group. In fact, this finding is not necessarily surprising, as clinical effects of kinase inhibitors (KIs) cannot be assumed to be mainly immune-mediated. Our results are in line with previous reports on the induction of Treg by sorafenib in mRCC patients. Desar *et al.* [30] described a reduction of tumor-infiltrating Treg under sorafenib treatment, but these authors also observed increasing numbers of Treg in PB, raising the question whether sorafenib differentially affects Treg in these compartments or whether it influences the distribution of Treg by unknown mechanisms.

Recent studies on combining KIs with immunotherapeutic agents such as a-IFN did not show superior efficacy, but rather added toxicity [31,32]. Considering these negative results with combinations of KIs and a-IFN, it should be realized that a-IFN is a nonspecific immunostimulatory agent. Therefore, negative studies with KIs and a-IFN do not prove a general lack of efficacy of combinations with immunotherapeutic agents, particularly as far as antigen-specific approaches such as vaccination or adoptive T-cell transfer are concerned. Moreover, biologicals that suppress immunoregulatory networks, such as anti-CTLA-4 antibody or anti-PD-1 antibody, may be potential combination partners. Combination therapy with immunotherapeutic agents remains a promising strategy in immunoresponsive tumors such as RCC, particularly in the setting of minimal residual disease. Further clinical studies are warranted. However, our data indicate that sorafenib treatment should be avoided in such an experimental setting.

## Acknowledgements

### Conflicts of interest

Anne Flörcken has received travel allowances from Bayer Schering Pharma. All other authors declare that they do not have any affiliations that would lead to a conflict of interest with respect to this work.

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