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Immunomodulatory effects of sorafenib on peripheral immune effector cells in metastatic renal cell carcinoma

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ABSTRACT

Background: Tyrosine kinase inhibitors (TKI) such as sorafenib have substantially improved the prognosis of metastatic renal cell carcinoma (mRCC) patients, but long-term remissions have only been reached with immunotherapy. Sequencing or combining TKI treatment with immunotherapy may represent an attractive therapeutic concept. However, *in vitro* data have shown that TKI may not only affect tumour cells, but also inhibit signalling in immune effector cells. Therefore, we asked whether sorafenib had an influence on peripheral immune effector cells in a cohort of 35 mRCC patients receiving sorafenib treatment.
Methods: Peripheral blood (pB) samples were analysed at baseline and after 8 weeks of treatment. IL-10 and TGF-ß mRNA levels were quantified by RT-PCR; regulatory T cell (Treg) counts and intracellular cytokine responses (TNF-α, IFN-γ, IL-10 and TGF-ß) of mononuclear cell subsets were determined by flow cytometry after *in vitro* stimulation with PMA/ionomycin.
Results: Sorafenib did not alter the elevated TGF-ß and IL-10 mRNA levels or elevated frequencies of IL-10 and TGF-ß producing monocytes and had no influence on type 1 cytokine responses in pB. CD4+CD25^{high} FOXP3+/CD3+ T cells, likely representing Treg cells, decreased during sorafenib therapy.
Conclusions: *In vivo*, sorafenib treatment was associated with a decrease in frequency of Treg cells without influencing the function of peripheral immune effector cells. Therefore, although sorafenib did not convert the immunosuppressive phenotype associated with mRCC, it seemed to be a possible candidate for combination with immunotherapy.

1. Introduction

For several years the reference first-line therapy for metastatic renal cell carcinoma (mRCC) has been immunotherapy with interleukin (IL)-2 and interferon (IFN)-α. However, the overall objective response rates were low (<20%).[1] The antitumour activity of cytokines might be due to pleiotropic effects, however, there exists evidence that they act – at least in

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part – via indirect immunomodulatory effects.[2–5] Their limited efficacy might be due to several immunosuppressive mechanisms.[6]

The development of targeted agents has substantially improved prognosis for patients with mRCC. Four targeted agents are approved for the treatment of mRCC by the EMEA: bevacizumab given in combination with IFN-α, sorafenib, sunitinib and temsirolimus and a number of investigational agents have shown efficacy in this setting.[7] The decision of the appropriate targeted therapy is based on the consideration of individual patient factors, such as previous treatment and prognostic risk.[8] Based on phase III trial results sorafenib (BAY43-9006, Nexavar; BayerHealthCare Pharmaceuticals Corporation, Montville, NJ; Onyx Pharmaceuticals, Emeryville, CA) is recommended as a second-line therapy following the failure of cytokine therapy.[8,9] It was originally developed as an orally bioavailable small-molecule Raf kinase inhibitor and has been approved by Food and Drug Administration for the treatment of RCC after a phase III clinical study in 2005.[10] It has an inhibitory activity for Raf-1, mutant B-RAF and p38 with additional activity against multiple tyrosine kinases, including VEGFR-2, VEGFR-3, PDGFR-a, Flt3-RTK, Flt3-ITD Flt3 and c-kit. However, although targeted therapies showed significant improvement of progression free survival, time to treatment failure remains short and long-term responses – although rare – are only reported with high dose IL-2 treatment.[11]

Combination therapy with agents with different mechanisms of action may help to enhance antitumour activity. As TKI might enhance immunogenicity of tumour cells by making them more susceptible to killing by immune cells and by reversing the immune dysbalance induced by the tumour,[12] combination with immunotherapeutic strategies tending to promote the antitumour immune response might be a feasible approach. This might include combinations with vaccination strategies based on tumour defined antigens, but also approaches that induce an antitumour immune response by unspecific immune activation like cytokine therapy.

Moreover, also combination of TKI with approaches leading to the disruption of immunosuppressive pathways might be possible.

The efficacy of cytokine immunotherapy combined with TKI has been investigated in several clinical studies for RCC.[13] The combination of sorafenib with IFN-α-2b has been confirmed to have a positive antitumour effect in advanced RCC patients.[14,15] However, many pathways targeted by small-molecule inhibitors like sorafenib which are critical for tumour cell proliferation, may also be critical for the function of immune cells.[16] The effects of sorafenib on different immune cells have been investigated by several recently published studies and immunosuppressive properties were observed: sorafenib was not only cytotoxic to regulatory T cells (Treg) cells, but also inhibited the proliferation of CD4+ and CD8+ T cells.[17] It was reported to inhibit T cell proliferation and T cell activation by targeting Lck phosphorylation[18] or by a MAPK-independent mechanism.[19] Effects on dendritic cells (DC) were contradictory. In a mouse model, Hipp et al. reported that sorafenib, but not sunitinib, inhibited function of differentiated DC, assessed through the expression of cytokines and CD1a. The inhibitory effects were mediated by the inhibition of phosphatidylinositide 3-kinase production and mitogen-activated protein kinases, as well as by nuclear factor-kappa B signalling.[20] However, Alfaro et al. found that sorafenib, but not sunitinib, reversed the inhibitory effects of VEGF on the differentiation of DC from myeloid precursors.[21]

However, all these analyses were performed *in vitro* or based on mouse models and little is known about the immunoregulatory effects of sorafenib *in vivo*. In this study we examined the influence of sorafenib on different immune effector cells in a cohort of mRCC patients receiving sorafenib treatment. We found that sorafenib treatment decreased Treg cell counts without negatively affecting immune effector cell function. However, sorafenib was not able to reverse the tumour induced immune dysbalance.

2. Material and methods

2.1. Patients and blood samples

This investigation was carried out on 35 patients with histologically proven metastatic or unresectable clear cell RCC with measureable disease starting an oral treatment with the multikinase inhibitor sorafenib within a European open access programme. The investigation of prognostic and immunologic factors had been approved by our Institutional Review Committee, and patients had given informed consent prior to blood draw.

Patients received single-agent sorafenib (NEXAVAR®, Bayer Healthcare) treatment at the standard dose of 2×400 mg per os daily. Treatment interruptions and dose modifications were done according to general recommendations for sorafenib. Patients with brain metastasis and/or patients receiving steroids were excluded from the study. Prior IFN-α and IL-2 based cytokine therapy was finished at least 8 weeks before the start of sorafenib treatment.

CT scans or MRI of chest, abdomen and brain were performed at baseline and every 8 weeks thereafter or as clinically indicated. Analysis of the scans was performed following the standard Response Evaluation Criteria in Solid tumours (RECIST).[22]

Blood samples were obtained before treatment and after 8 weeks. Mononuclear cells (MNCs) were fractionated by Ficoll Isopaque density gradient centrifugation (Pharmacia, Erlangen, Germany) and cryopreserved. Controls were blood samples of 26 healthy volunteers (12 male/14 female, median age 38.5 years [27–93 years]), who had no evidence of any clinically detectable disease at the time of blood withdrawal.

2.2. mRNA extraction and reverse transcription and quantitative real-time PCR

The method of analysis is described in detail elsewhere.[23] In brief, total RNA was isolated by RNeasy Mini Kit including RNase-Free DNase Set (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. For reverse transcription, the Omniscript Reverse Transcriptase Kit (Qiagen) was used. Quantitative real-time RT-PCR was done by LightCycler Technology (Roche) to detect transcripts of *TGF-ß1*, *IL-10* and of the housekeeping gene *porphobilinogen deaminase*

(*PBGD, also known as hydroxymethylbilane synthase*). Primer sequences were designed using the LightCycler Probe Design software, version 1.0.[24] Analysis of RT-PCR expression data was performed with the LightCycler software (version 3, Roche). Crossing points (beginning of the PCR exponential phase) were assessed by the second derivate maximum algorithm and plotted against the concentrations of the standards. Sample concentrations were calculated using the plasmid standard curve resulting in marker concentrations. All samples were analysed in duplicate. The average value of both duplicates was used as a quantitative value. To correct for differences of cDNA amount on a per-sample basis, results were provided as a ratio of housekeeping gene PBGD expression.

2.3. Flow cytometric analysis of T cells

Phenotypic characterisation and cytokine response assessment of T cells were done by flow cytometry. Frozen peripheral blood mononuclear cells (PBMC) samples were thawed and cultured overnight in medium (Iscovés medium supplemented with 10% human AB serum and 2% L-glutamine) prior to analysis. 2×10^6 PBMC were incubated with/without PMA/ionomycin for a total of 18 h. After 2 h 15 μg/ml of brefeldin A (Sigma, Steinheim, Germany) was added. Extracellular staining was done with fluorescence-conjugated monoclonal antibodies (mAbs) against CD3, CD4, CD8 and CD25 (Becton Dickinson, Heidelberg, Germany) and intracellular cytokine staining with mAbs against TNF-α, IFN-γ, TGF-ß and IL-10 (Becton Dickinson) following cell permeabilisation using FACS permeabilisation solution™ (Becton Dickinson). FOXP3-staining was performed intracellularly according to the manufacturer's instructions (e-bioscience, San Diego, USA). Monocytes were gated using the FS/SS scatter pattern.

Data acquisition was performed on a FACSCanto II (Becton Dickinson, Heidelberg, Germany) and data were analysed using FlowJo software version 7.2.5 (TreeStar, Ashland, USA).

2.4. Statistical considerations

The statistical analysis was performed using SPSS software (release 15.0). All tests were two sided and $P < 0.05$ was considered as statistically significant. The analysis has to be regarded as exploratory, since no correction for multiple testing was applied. The following tests were used when appropriate: Mann–Whitney *U*-test, Wilcoxon rang sum test and paired/unpaired *t*-test.

3. Results

3.1. Patients' characteristics

Blood samples from 35 patients with mRCC receiving sorafenib treatment were obtained at baseline and after 8 weeks of continuous sorafenib administration. Patients' characteristics are listed in Table 1. Twenty two patients (63%) were male and 13 patients (37%) female, median age was 65 years (37–78 years). The overall response rate was 86% (9 partial remissions, 21 stable diseases), the median progression free survival was 12 months (95% confidence interval 8–16 months) and overall survival was 20 months (95% confidence interval 12–28.3 months) with a median follow-up of 17.7 months.

Table 1 – Patients' characteristics.

	N
Patient samples available	35
Male/female	22/13
Median age, years	65 (37–78)
Prior nephrectomy	32
Cytokine pretreatment yes/no	27/8
MSKCC[a] favourable/intermediate/poor	6/25/4
Response to sorafenib therapy	30/5
PR/SD	9/21
Median PFS (95% confidence interval), months	12 (8–16)
Median OS (95% confidence interval), months	20 (12–28.3)

PFS: progression free survival and OS: overall survival.
[a] MSKCC score for pretreated patients.[30]

3.2. Sorafenib did not alter IL-10 and TGF-ß1 mRNA expression levels

We previously showed that in mRCC patients *IL-10* and *TGF-ß1* mRNA expression levels in peripheral blood are elevated compared to healthy subjects and that in contrast to *IL-10*, *TGF-ß1* mRNA expression levels were an independent good prognostic factor.[24] To address the question whether treatment with sorafenib would alter mRNA expression levels of *IL-10* and *TGF-ß1* in PBMCs, peripheral blood samples of patients of this previously analysed patient cohort were examined before treatment and after 8 weeks of sorafenib therapy by qRT-PCR (Fig. 1). Twenty-nine samples were available. Twenty-six of them were responders to sorafenib therapy and three were non-responders. As observed in our previous study,[24] compared to healthy subjects, mRCC patients before treatment showed significant higher expression levels of *IL-10* (median level 1.9×10^{-4} [range 6.2×10^{-5}–1.5×10^{-3}] versus 5.56×10^{-5} [range 1.5×10^{-5}–3.88×10^{-4}], $P < 0.001$) and *TGF-ß1* (median level 3.3×10^{-1} [range 1.2×10^{-1}–7.5×10^{-1}] versus 7.38×10^{-2} [range 9.86×10^{-3}–2.19×10^{-1}], $P < 0.001$). After 8 weeks of treatment with sorafenib there were no significant differences in mRNA levels of *IL-10* (median level 1.99×10^{-4} versus 1.36×10^{-4}, $P = 0.223$) and *TGF-ß1* (median level 3.3×10^{-1} versus 3.9×10^{-1}, $P = 0.055$; Fig. 1). There was also no difference, when analysing responders alone ($P = 0.412$ and $P = 0.071$, respectively). Prior systemic immunotherapy and response to immunotherapy had no influence on basal *TGF-ß1* and *IL-10* mRNA expression as well as on their course following sorafenib treatment (data not shown).

3.3. Sorafenib decreased peripheral Treg cell frequency

We and others showed that the frequency of peripheral CD4+CD25^{high} FOXP3+ cells likely representing Treg cells is increased in mRCC patients.[24–26] Frequencies of peripheral circulating Treg cells could be analysed in 30 patients (27 responders, 3 non-responders) by flow cytometry before treatment and after 8 weeks of therapy with sorafenib. Compared to healthy controls mRCC patients exhibited higher frequencies of CD4+CD25^{high}/CD3+ T cells (median frequency 1.3%

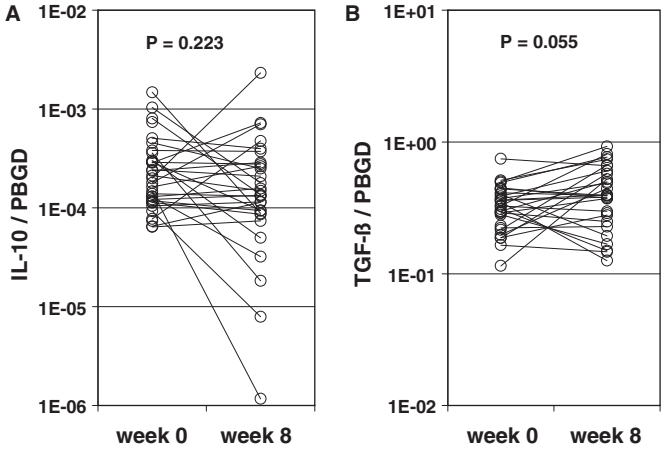

Fig. 1 – Expression level of *IL-10* and *TGF-ß1* specific mRNA in PBMCs of mRCC patients at baseline and after 8 weeks of sorafenib treatment: the relative amount was expressed as a ratio marker (pg/μl)/*PBGD* (pg/μl). The sample concentration was calculated using the plasmid standard curve. (A) *IL-10*: median level week 0: 1.99×10^{-4} versus week 8: 1.36×10^{-4} and (B) *TGF-ß1*: median level week 0: 3.3×10^{-1} versus week 8: 3.9×10^{-1}.

[range 0.2–3.7%] versus 0.72% [range 0.1–1.6%], $P = 0.01$) and CD4+CD25^{high} FOXP3+ T cells (median frequency 0.5% [range 0.1–3%] versus 0.3% [range 0–0.8%], $P = 0.02$). After 8 weeks of treatment with sorafenib there was no change in the frequency of peripheral circulating CD4+CD25^{high}/CD3+ T cells (median frequency week 0: 1.4% versus week 8: 1.2%, $P = 0.996$; Fig. 2), also if non-responders were excluded form the analysis (data not shown). However, the FOXP3+ subset (CD4+CD25^{high}FOXP3+/CD3+ T cells) decreased (median frequency week 0: 0.5% versus week 8: 0.3%, $P = 0.037$; Fig. 2).

This difference remained significant, if non-responders to sorafenib were excluded from the analysis (not shown). Prior systemic immunotherapy and response to immunotherapy had no influence on the course of CD4+CD25^{high}FOXP3+ T cells. Intracellular TGF-ß expression after polyclonal stimulation was low in the CD4+CD25^{high} T cell subset (median frequency at week 0 1.9% [range 1.4–3.4%]) and was mainly detected in the FOXP3– T cells.

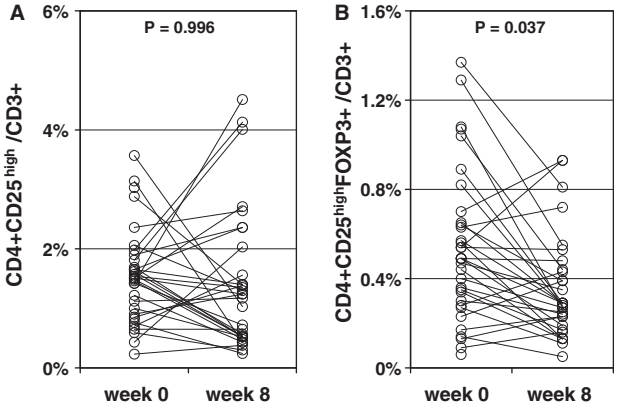

Fig. 2 – Frequency of CD4+CD25^{high} and CD4+CD25^{high}-FOXP3+ T cells per CD3+ T cells in RCC patients at baseline and after 8 weeks of sorafenib treatment. (A) Median frequency of CD4+CD25^{high}/CD3+ T cells week 0: 1.4% versus week 8: 1.2% and (B) median frequency of CD4+CD25^{high}-FOXP3+/CD3+ T cells week 0: 0.5% versus week 8: 0.3%.

3.4. *Influence of sorafenib on cytokine production of peripheral mononuclear cells*

Additionally we analysed the functional T cell responses after polyclonal stimulation in these 30 patients. As observed in the previously analysed larger cohort[24] production of both the immunosuppressive cytokines, IL-10 and TGF-ß was low (median frequency of IL-10+ CD3+ T cells 0.13% [range 0–1%] and of TGF-ß+ CD3+ T cells 0.4% [range 0–2.7%]) and remained low during sorafenib treatment (data not shown).

The type 1 cytokine response of PBMC after polyclonal stimulation in mRCC patients was not different from healthy subjects: mean IFN-γ+/CD3+ T cells: 21.9% (SD 12%) in mRCC versus 18.5% (SD 12.6%) in healthy subjects; mean TNF-α+/CD3+ T cells: 26.0% (SD 14.7%) in mRCC versus 24.6% (SD 12.2%) in healthy subjects. Neither in CD8+ T cells nor in CD4+ T cells type 1 cytokine production was influenced by 8 weeks sorafenib treatment (Fig. 3).

As we previously identified monocytes as the main producers of IL-10 and TGF-ß in peripheral blood of mRCC patients,[24] we also compared cytokine production by monocytes before treatment and after 8 weeks therapy of sorafenib (Fig. 4). As observed in the previously analysed larger cohort of mRCC patients, frequencies of monocytes producing the immunosuppressive cytokines IL-10 and TGF-ß were higher in mRCC patients compared to healthy controls: mean frequency of IL-10+ monocytes: 6.4% (SD 6.%) versus 1.9% (SD 1.9%), $P = 0.003$; mean frequency of TGF-ß monocytes: 4.5% (SD 5%) versus 0.5% (SD 0.6%), $P = 0.005$. Both the frequencies of IL-10+ and TGF-ß+ monocytes did not change during sorafenib treatment (Fig. 4).

Frequencies of TNF-α and IFN-γ producing monocytes were lower in RCC patients compared to healthy controls: mean frequency of TNF-α+ monocytes: 19.3% (SD 7.8%) versus 23.2% (SD 13.1%), $P = 0.118$; mean frequency of IFN-γ+ monocytes: 12.5% (SD 8%) versus 23% (SD 17.3%), $P > 0.001$. Sorafenib treatment had no influence on both the frequencies of TNF-α+ and IFN-γ+ monocytes (mean frequency of TNF-α monocytes week 0: 20.4% versus week 8: 19%; mean

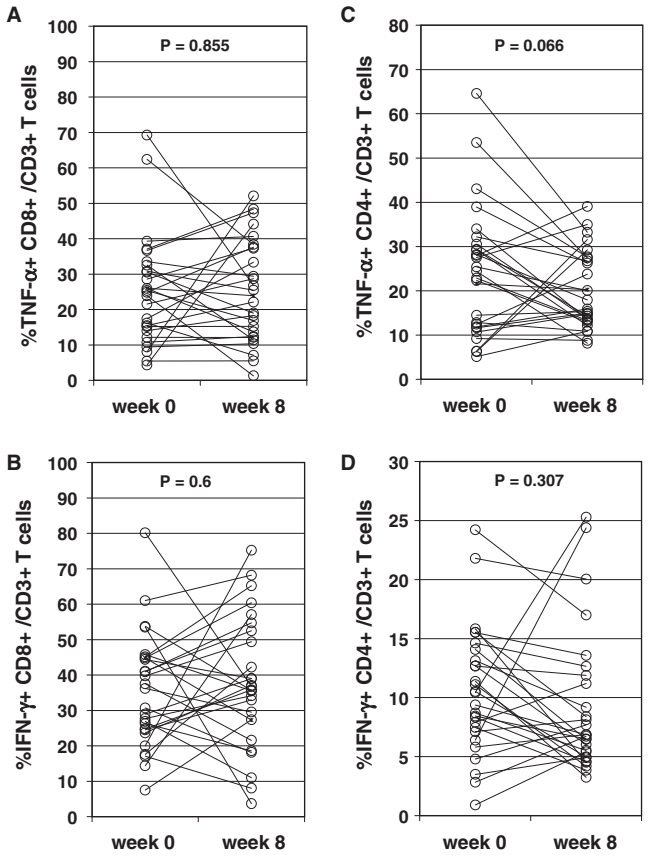

Fig. 3 – Intracellular cytokine response of CD4+CD3+ T cells and CD8+CD3+ T cells in RCC patients at baseline and after 8 weeks of sorafenib treatment. Cytokine production was determined by intracellular flow cytometry after polyclonal stimulation with PMA/ionomycin. (A) Mean frequency of TNF-α+CD8+CD3+ T cells: week 0: 27% versus week 8: 27.6%; (B) mean frequency of IFN-γ+CD8+CD3+ T cells: week 0: 38% versus week 8: 40.1%; (C) mean frequency of TNF-α+CD4+CD3+ T cells: week 0: 24.9% versus week 8: 20.1%; and (D) mean frequency of IFN-γ+CD4+CD3+ T cells: week 0: 10.9% versus week 8: 6.7%.

frequency of IFN-γ+ monocytes week 0: 11.9% versus week 8: 13.7%).

4. Discussion

Sequencing or combining targeted therapies such as TKI with immunotherapeutic strategies – including both, mechanisms of immune activation and disruption of immunosuppressive pathways – may represent an attractive therapeutic concept for treatment of mRCC to reach long-term remission. However, for combinatory immunotherapeutic approaches, the choice of TKI requires careful consideration due to potential immunosuppressive side effects of different compounds. Herein, we analysed the immunoregulatory properties of sorafenib in mRCC patients and demonstrate that *in vivo* sorafenib although it did not inhibit immune effector cell function, could not reverse the immune dysbalance observed in RCC patients.

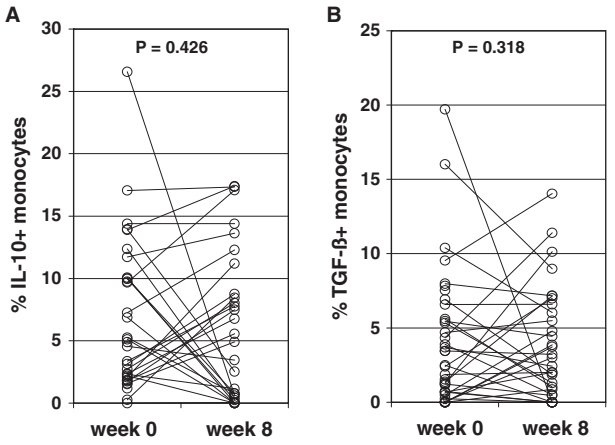

Fig. 4 – Frequency of cytokine producing monocytes in mRCC patients at baseline and after 8 weeks of sorafenib treatment. Cytokine production was determined by intracellular flow cytometry after polyclonal stimulation with PMA/ionomycin. (A) Mean frequency of IL-10+ monocytes week 0: 8% versus week 8: 6.4% and (B) mean frequency of TGF-ß+ monocytes week 0: 4.7% versus week 8: 3.6%.

TKI inhibit signalling pathways that are not only necessary for the survival of tumour cells but also for the proliferation and function of immune effector cells.[16]

Both, sunitinib and sorafenib are not specific inhibitors of a single tyrosine kinase but have been shown to inhibit various kinases like VEGFR (types 1–3), platelet-derived growth-factor receptor-ß (PDGFR-ß), Flt3 and c-kit with significantly different inhibitory concentrations.[27] Moreover, sorafenib also inhibits c-Raf and b-Raf whereas sunitinib lacks anti raf activity, but inhibits PDGFR-α, macrophage colony-stimulating factor 1 (CSFR1) and fibroblast growth-factor receptor-1 (FGFR1). The difference in affinities for 'off-target kinases' may not only account for their lack of cross-resistance but let also suggest different immunoregulatory effects.[16] Indeed, sunitinib, approved for first-line treatment of mRCC seems to have favourable immunoregulatory properties. Sunitinib treatment has been shown to improve type I responses by T lymphocytes and to decrease regulatory T cell frequencies and myeloid derived suppressor cells in pB of mRCC patients.[26,28] In contrast, several *in vitro* studies showed an unfavourable effect of sorafenib on immune effector cells: sorafenib was not only reported to be cytotoxic to Treg cells, but also growth inhibitory to CD4+ and CD8+ T cells, to impair T cell and NK cell activation and to inhibit the immune stimulatory function of differentiated DC *in vitro* and *in vivo*.[17,19,20,29] However, *in vitro* studies do not necessarily represent the situation in the more complex *in vivo* system. In our patient cohort, we found sorafenib to slightly decrease the peripheral frequencies of $CD4+CD25^{high}FOXP3+$ T cells (likely representing Treg), which is in line with the previous studies. However, we found no inhibition of type I cytokine production by T cells during treatment; therefore, T cell signalling might not be inhibited *in vivo*.

How can the discrepancies between the *in vitro* data and our *in vivo* data be explained?

The immunoregulatory effects of sorafenib *in vivo* might be more complex and the inhibitory effects on T cell function

observed in previous *in vitro* studies might be only one piece of the puzzle. Sorafenib could also exhibit some immune response promoting effects that counterbalance (or even prevail) the potential immunosuppressive effects observed *in vitro*. This might be simply by reducing tumour burden and therewith reducing the tumour induced immune dysbalance or by direct effects on tumour cells increasing their immunostimulatory properties, i.e. by increasing MHC molecules due to inhibition of the RAS pathway[30] or by suppression of immunosuppressive cytokines as it has been shown for MEK inhibitors in melanoma cells.[31] Moreover, Alfaro et al found that sorafenib reversed the inhibitory effects of VEGF on the differentiation of DC from myeloid precursors.[21] Interestingly, studies in mice revealed that combining rhIL-2 with sorafenib significantly augmented antitumour efficacy in comparison with either rhIL-2 or sorafenib alone.[32,33] Moreover, the combination of sorafenib and IFN-α-2b, which exerts its antitumour effects at least in part via indirect immunomodulatory effects, has been confirmed to have a positive antitumour effect in advanced RCC patients.[14,15] However, from our *in vivo* data it needs to be considered that the decrease in peripheral CD4+CD25^{high}FOXP3+ T cell frequencies that we observed in our patient cohort was not accompanied by decrease of IL-10 and TGF-ß production by monocytes. Additionally, sorafenib therapy did not decrease *IL-10* and *TGF-ß* mRNA levels in PBMCs. Although CD4+CD25^{high}FOXP3+ T cells might most likely represent Treg cells, we cannot exclude that this population also compromises activated T cells as the expression of CD25 and FOXP3 is not confined to Treg but also found to be transiently expressed in activated T cells. Unfortunately, due to paucity of patients' material further characterisation of the suppressive activity of CD4+CD25^{high}FOXP3+ T cells by functional assays was not possible.

In conclusion, our findings of the immunomodulatory effects of sorafenib *in vivo* are of importance for the development of combinatory approaches of TKIs with immunotherapy. Herein we showed in our exploratory analysis that sorafenib treatment was associated with a decrease in frequency of peripheral CD4+CD25^{high}FOXP3+ T cells that might represent Treg cells without influencing the cytokine response of peripheral immune effector cells. Therefore – although the effect of sorafenib on peripheral immune effector cells might not necessarily reflect its effect on those effector cells infiltrating the tumour – it appears to be a possible compound for combination with immunotherapy. Our observations are in line with recent reports of an additive effect of sorafenib compared to cytokine therapy alone. Furthermore, by the improvement of progression free survival following sorafenib treatment a tumour-directed immune response might be established by combination of TKI therapy with immunotherapy. However, it needs to be considered that sorafenib does not convert the immunosuppressive phenotype associated with mRCC as reported for sunitinib.

Conflict of interest statement

All authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within that could inappropriately influence their work.

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