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Immune modulation of effector CD4+ and regulatory T cell function by sorafenib in patients with hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is a difficult to treat cancer characterized by poor tumor immunity with only one approved systemic drug, sorafenib. If novel combination treatments are to be developed with immunological agents, the effects of sorafenib on tumor immunity are

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important to understand. In this study, we investigate the impact of sorafenib on the CD4+CD25⁺ effector T cells (Teff) and CD4+CD25⁺ regulatory T cells (Tregs) from patients with HCC. We isolated Teff and Treg from peripheral mononuclear cells of HCC patients to determine immune reactivity by thymidine incorporation, ELISA and flow cytometry. Teff cultured alone or with Treg were supplemented with different concentrations of sorafenib. The effects of sorafenib on Teff responses were dose-dependent. Pharmacologic doses of sorafenib decreased Teff activation by down regulating CD25 surface expression. In contrast, sub-pharmacologic concentrations of sorafenib resulted in Teff activation. These low doses of sorafenib in the Teff cultures led to a significant increase in Teff proliferation, IL2 secretion and up-regulation of CD25 expression on the cell surface. In addition, low doses of sorafenib in the suppression Teff/Treg cocultures restored Teff responses by eliminating Treg suppression. The loss of Treg suppressive function correlated with an increase in IL2 and IL6 secretion. Our findings show that sub-pharmacologic doses of sorafenib impact subsets of T cells differently, selectively increasing Teff activation while blocking Treg function. In conclusion, this study describes novel immune activating properties of low doses of sorafenib by promoting immune responsiveness in patients with HCC.

Keywords

Sorafenib; T cell; Regulatory T cells; Hepatocellular carcinoma; HCV

Introduction

Hepatocellular carcinoma (HCC) continues to have a rising global incidence and is the third leading cause of cancer-related death worldwide [1, 2]. By the time, most of the patients come to clinical attention that they have advanced cancer with limited treatment options. Previously, patients with advanced HCC did not have any viable therapeutic options, but a major milestone in the treatment for this disease has been the clinical development of sorafenib. Sorafenib is an oral multi-kinase inhibitor (MKI) that showed antitumor activity in preclinical liver cancer models and early clinical studies. The clinical success of sorafenib occurred when two pivotal studies—the SHARP and Asian Pacific trials— showed a survival benefit over best supportive care [3, 4]. This efficacy led to the approval of sorafenib by regulatory agencies as the first systemic treatment in advanced HCC and has served as a major impetus to further research in the field.

While the therapeutic effects of sorafenib in HCC are mainly explained by its pro-apoptotic and anti-angiogenic properties, additional mechanisms for its clinical benefit have been proposed. One mechanism of great interest is sorafenib's potential effect on immune responses. We have previously shown that patients with HCC have impaired immune reactivity [5, 6]. The poor immune responsiveness in HCC consists of diminished CD4 T cell function as marked by impaired cell proliferation, CD25 cell surface expression and IFN gamma secretion of peripheral blood mononuclear cells (PBMC) and CD4+CD25⁺ effector T cells (Teff). This degree of suppression correlates with tumor stage, with those patients having advanced stage showing the highest level of suppression. In addition, a subset of suppressive T cells known as CD4+CD25⁺ regulatory T cells (Tregs) contribute to the observed immune suppression in patients with HCC by inhibiting the beneficial antitumor immunity [7–9]. Clinically, a high number of Treg number is an indicator of poor prognosis, while a low number of Treg correlates with improved survival [10]. We have also shown that Tregs derived from patients with HCC are functionally more suppressive [11]. The importance of both Treg and Teff in the dynamics of tumor immunity in HCC make them critical elements to understand and potentially valuable targets as novel treatment options for HCC are needed.

The impact of sorafenib on CD4 T cell responses is presently poorly understood with only a few studies showing conflicting results [12, 13]. Characterizing the effects of sorafenib on T cell immunity will be important if we are going to improve the clinical response with single agent sorafenib through combination approaches with other agents. Future clinical trial design combining sorafenib with other therapies will need to consider the immune properties of sorafenib and the inherent immune suppression of the patients with HCC [14]. The aim of this study is to determine the effects of sorafenib on Teff and Treg function isolated from patients with HCC. We evaluate the impact of low and high doses of sorafenib on Teff responses and Treg function. We found that sorafenib has a dose-dependent effect on Teff responses. Further, sorafenib influences Teff responses in cocultures with Tregs, implicating a direct effect of Treg function. We present novel immune modulatory properties of sorafenib, with low doses leading to decreased Treg function and enhanced Teff reactivity. These findings may have major clinical implications since most of the patients with advanced HCC treated with sorafenib do not tolerate full doses [15].

Materials and methods

Patients

Blood samples were collected from patients with hepatitis C virus (HCV)-related HCC and Child–Pugh A cirrhosis ($n = 20$), normal healthy controls (NHC, $n = 5$) and patients with only HCV-related Child–Pugh A cirrhosis (disease controls, DC; $n = 6$). The response to low (subpharmacologic) doses of sorafenib was evaluated using PBMC from all three study groups. The study was approved by the institutional IRB, and all patients provided informed consent.

Cell cultures and reagents

PBMC from HCC, NHC and DC patients were isolated from blood samples by the Ficoll method as previously described [6]. PBMC were immediately used as a source of Teff and Treg. Briefly, CD4+ T cells were isolated by negative selection using magnetic beads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer protocol to a final purity >85 % (flow cytometry). HCC or controls cells were cultured in the following conditions: with serum-free medium (SFM; CTL media, Shaker Heights, OH, USA), autologous serum, or allogenic serum with no mitogen (negative control), with 5 µg/ml of lectin from *Phaseolus vulgaris* PHA B (Sigma, St. Louis, MO, USA). The choice of PHA over PMA as stimulant was made to promote complete T cell activation as recommended by Houben et al. [12]. Cells were seeded in 96-well plates to a final volume of 200 µl, adding sorafenib to reach desired concentrations and culture for 72 h at 37 °C and 5 % CO₂. Sorafenib concentrations in patients normally range between 6 and 12 µM (pharmacologic concentration) [16]. We used concentrations of sorafenib that ranged between 0.1 and 3 µM to represent sub-pharmacologic concentrations (low dose of sorafenib) in our cultures.

Cell proliferation assay

The [3H]-thymidine incorporation assay was performed per standard technique. Briefly, PBMC, CD4+ T cells (1×10^5 /well) were seeded into 96-well plate in duplicate, total volume 200 µl of CTL media supplemented with autologous serum and stimulated with PHA (5 µg/ml) and cultured at 37 °C and 5 % CO₂. After 72 h, each well is pulsed with 1 µCi [3H]-thymidine in 20 µl of serum-free media for 18 h. Cells were harvested, and [3H]-incorporation measured using a β-scintillation counter. Results were expressed as the mean cell proliferation in counts per minute (cpm) ± standard error (SE).

Suppression assay

Suppression assays were performed by seeding an equal number of CD4+CD25[−] Teff and Treg (1×10^5) into 96-well plates in triplicate, total volume 200 μ l and cultured with or without PHA for 72 h in 5 % CO₂ and 37° C. After 72 h, [3H]-thymidine (Amersham Biosciences) was added (1 μ Ci/well) for 18 h, and cells were maintained at 5 % CO₂ and 37 °C. Next day, [3H]-thymidine incorporation was measured on a β -scintillation counter.

Results were expressed as mean cpm $\pm$ SE.

Flow cytometry

T cells were stained using the following antibodies: CD4 antihuman PE, CD25 antihuman PE-Cy5-A, PE mouse antihuman CD127, FITC mouse antihuman CD4, PE-Cy 7 mouse antihuman CD25, matching isotype controls (BD Biosciences Pharmingen, San Jose, CA, USA) and the Vybrant violet Stain (Life technologies, Eugene OR, USA). Cells were detected using flow cytometry performed on a flow cytometer LSR-II (Becton–Dickinson BD, Franklin Lakes, NJ, USA). One hundred thousand-gated events were acquired for each condition, and data were analyzed using FACSDiva software (BD).

ELISA

Cells were incubated in a 96-well plate alone or with different concentrations of sorafenib. Cell culture supernatant was collected and frozen at -80° C for later use. Cytokine measurements were performed using the human multicytokine detection system (Millipore, Lake Placid, NY, USA) for the following molecules: IL-1 β , -2 , -4 , -6 , -8 , -10 , -12 (p70), TNF α , IFN γ , and GM-CSF. The assay was performed according to the manufacturer's instructions.

Statistical analysis

Data analysis was performed with the program SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using the Mann–Whitney test and paired *t* test. All results are expressed as mean $\pm$ SE. All *p* values are two-tailed and statistically significant when *p* < 0.05 (*) and *p* < 0.01 (**).

Results

Sorafenib improves Teff immune responses

In clinical practice, most of the patients with advanced HCC treated with sorafenib require dose reductions in sorafenib due to adverse reactions [17, 18]. Since the majority of treated patients with HCC receive sub-pharmacologic levels of sorafenib, we evaluated the effect of low doses of sorafenib on Teff responses. Initially, the effect of low doses was evaluated in patients with HCV-related HCC, HCV-related cirrhosis and NHC. This evaluation showed that low-dose sorafenib only enhanced Teff responses in HCC patients. We evaluated the effect of low doses of sorafenib (<3 μ M) on PBMC from patients with HCC (*n* = 20) using the thymidine incorporation assay to determine its effect on proliferation. Low doses of sorafenib led to a significant improvement in Teff proliferation in the study group when compared to Teff responses in the absence of sorafenib (Fig. 1a). A subset of these patients had a high degree of improvement in their Teff responses with low doses of sorafenib (Fig. 1b). In order to characterize the effect of sorafenib on Teff from patients with HCC, CD4 lymphocytes were cultured with the polyclonal stimulant PHA alone and with increasing doses of sorafenib. The sub-pharmacologic concentrations—those < μ M—had the most pronounced effect on Teff responses as measured by proliferation. Low doses of sorafenib significantly increased Teff proliferation at all tested concentrations in this range (Fig. 1c).

This pattern was observed with the 0.1, 1 and 3 μM concentrations of sorafenib when compared to the baseline, proliferation responses of Teff cultured in the absence of sorafenib. An extended dose–response curve of sorafenib shows that high concentrations of sorafenib lead to a stepwise decline in Teff responses.

Cytokine profile of Teff treated with sorafenib

We analyzed the supernatants from the proliferation assays by ELISA to characterize the cytokine profile of Teff after sorafenib treatment. We evaluated the supernatants for IL-2, -4, -6, -8, -10, TNF α , IFN γ , and GM-CSF secretion, which are known to play a role in cancer suppression or T cell activation. This analysis showed a substantial release in only IL-2 (Fig. 2a). Sub-pharmacologic doses of sorafenib generated similar levels of IL-2 release across the dose range of 0.1–3 μM . The levels of IL-2 secreted by Teff appear decline, once a threshold in the concentration of sorafenib is reached (Fig. 2b).

Sorafenib effect on cell surface CD25 expression

The effects of IL-2 on Teff (CD4+CD127+) proliferation appear to depend largely on the cell surface expression of CD25. For this reason, the impact of sorafenib on cell surface expression of CD25 was examined by flow cytometry. Subpharmacologic concentrations of sorafenib up-regulated cell surface expression of CD25 on HCC Teff. In contrast, pharmacologic concentrations of sorafenib (6 and 12 μM) suppressed cell surface expression of CD25 (Fig. 3).

Sorafenib decreases Treg-mediated suppression on NHC Teff and HCC Treg

With the purpose of determining the effect of sub-pharmacologic concentrations of sorafenib on HCC Treg function, we performed a series of 1:1 Teff/Treg suppression assays. Sub-pharmacologic concentrations of sorafenib abrogated Treg suppressive function in the suppression cocultures (Fig. 4). This loss of suppression by Treg occurred incrementally with increasing doses of sorafenib at the studied sub-pharmacologic range (Fig. 4b). This effect on Treg-mediated suppression by low doses of sorafenib may reflect a loss of their suppressive function since we did not observe changes in Treg frequency (Fig. 4c). Analysis of the supernatants from these cocultures showed IL2 and IL6 levels to be elevated. Subpharmacologic doses of sorafenib promoted significant IL-2 secretion in the cocultures (Fig. 5a, b). We also found a sizable change in IL-6 concentrations in the Teff/Treg cocultures supplemented with sorafenib 1 μM , but not in the cultures with Teff alone supplemented with a similar concentration of sorafenib (Fig. 6).

Discussion

Tumor immunity is a promising target for treatment in immunogenic tumors such as HCC. The MKI sorafenib is the only drug approved for treatment for advanced HCC, and its effect on immunity is poorly understood. In the present study, we report on the immunomodulatory properties of sorafenib on CD4 Teff responses isolated from patients with HCC. First, we demonstrate that the effect of sorafenib on CD4 Teff response is related to the dose of sorafenib, with sub-pharmacologic concentrations having a stimulatory effect on CD4 Teff proliferation. These stimulatory effects on CD4 Teff occur in parallel with an increase in both IL2 secretion and extracellular surface expression of CD25. Second, we show that sub-pharmacologic concentrations of sorafenib eliminate Treg suppression of CD4 Teff in one to one suppression cocultures resulting in enhanced CD4 Teff response. Third, we also show that the secretion of IL2 and IL6 is involved in the loss of Treg suppressive function with low doses of sorafenib. Our findings that sub-pharmacologic concentrations of sorafenib stimulate CD4 Teff help us understand novel mechanisms of how this drug may work in

patients with HCC. In addition to sorafenib's direct action on tumor cells causing apoptosis and anti-angiogenesis effects, the drug may also enhance tumor immunity.

Studies measuring the concentrations of sorafenib in patients with HCC after taking the approved pharmacologic dose of sorafenib (400 mg twice daily) show mean plasma concentrations that range between 3 and 6 mg/l [19, 20]. We observed a beneficial immunomodulation with sorafenib when its level is <3 mg/l. The levels of sorafenib have also been studied in the context of hepatic impairment comparing HCC patients with normal liver function (Child A cirrhosis) to those with mild hepatic function impairment (Child B cirrhosis). While this comparison showed patients with liver function impairment having slightly greater concentrations of sorafenib, the differences were not significant. MKI compounds like sorafenib display therapeutic response at doses below their optimal biological dose [21], a feature observed with other anticancer agents like cyclophosphamide. At low concentration, cyclophosphamide is well known to impair Treg suppression in addition to its direct antitumor effects at pharmacologic concentrations [22]. In this report, we show for the first time that sub-pharmacologic concentrations of sorafenib exert a positive effect on CD4 Teff responsiveness in patients with HCC. Previously, we have demonstrated that sorafenib has a positive immunomodulatory effect in an animal model and on PBMC from patients with HCC [23]. We find that high doses of sorafenib have a negative impact on CD4 Teff activation, while low doses have a positive impact on CD4 Teff activation. The stimulatory effect of low doses of sorafenib on CD4 Teff appears to involve the IL-2 signaling pathway. The IL-2 pathway is critical for naïve CD4 Teff activation and requires an increase in cell surface expression of the CD25 marker for optimal IL-2 to IL-2 receptor binding and signaling. The enhancement of CD4 Teff proliferation along with the observed increase in IL-2 secretion and expression of CD25 on the cell surface suggests that low doses of sorafenib activates and expands CD4 Teff via the IL2 pathway.

The data on the effects of sorafenib on immune cells are limited and conflicting. While some studies report that sorafenib decreases PBMC and Teff proliferation [13, 24, 25], others report little effect on PBMC responses [26]. These conflicting results about the effects of sorafenib on immune cells can be explained by the dynamic changes of CD25 expression on the cell surface with varying concentrations of sorafenib. For example, in our cell cultures, high doses of sorafenib decreased expression of CD25 on the cell surface. The loss of CD25 expression on the cell surface of T cells directly impact the ability to optimally bind IL2 despite abundance of this cytokine in the culture media. In contrast, the cultures with low doses of sorafenib show both an increase in IL2 secretion and CD25 expression on the CD4 Teff favoring IL2 signaling. Our data suggest that CD25 cell surface expression is critical for IL2 signaling and CD4 Teff responsiveness in the presence of sub-pharmacologic concentrations of sorafenib. Further, this observation is supported by our previous work showing the central role of CD25 cell surface expression on CD4 Teff in immune responsiveness and IL2 secretion [27].

Another major finding of our study was the negative effects of sorafenib on Treg function from patients with HCC. We found that sub-pharmacologic concentrations of sorafenib led to a loss of Treg immunosuppressive properties. Naturally occurring Tregs can suppress target CD4 Teff by several mechanisms [28, 29]. Two of these mechanisms include influencing IL2 signaling by down regulation of CD25 expression and with immunosuppressive soluble factors. Our data also support an enhanced Treg suppression in HCC. The IL-2 signaling mechanism appears to be enhanced by low doses of sorafenib and may largely explain the loss of Treg-mediated inhibition of CD4 Teff in the suppression cultures. In addition, the observed increase in both IL2 and IL6 secretion in the Treg: Teff suppression cultures can also account for the loss of Treg suppressive activity on CD4 Teff,

by making Teff refractory to Treg inhibition [30]. From these observations, one can see the importance of the state of T cell activation as a key process in Treg suppression. The state of T cell activation as defined by the interaction Teff, degree of CD25 expression and IL2 availability can predict whether immune reactivity will be enhanced or suppressed [31]. In this recent study, Tregs can efficiently suppress weakly activated Teff even in the presence of large numbers, but cannot suppress strongly activated Teff even at low cell densities.

Sorafenib appears to impact the function of CD4 Teff and Treg differently depending on the dose. Low doses stimulate CD4 Teff and cause a loss of Treg suppressive activity. However, the loss of Treg suppressive function with low doses of sorafenib is not related to a decrease in Treg frequency. A plausible explanation for this observation is the differences in the activation of the IL-2 receptor in CD4 Teff versus Treg [32]. In Teff after IL2R engagement by IL2, a sequential activation of the JAK/STAT, PI3K and Ras mitogen-activated protein kinase (MAPK) signaling pathways results in cell proliferation. In contrast, IL2 signaling circuit operating in the Tregs cells prevents undesirable and unrestrained cell expansion and suppression. In addition, a secondary safety mechanism on Treg operates as a signaling blockade at the PI3 K pathway level. The permanent expression of tensin homolog (PTEN) [33] blocks IL2-dependent direct proliferation of Tregs in culture. Thus, sub-pharmacologic concentrations of sorafenib enhance IL2 signaling in Teff but not in Treg due to different intermediary controls of the IL2 pathway in the two different T cell subsets.

In conclusion, we show that the immune effects of sorafenib in patients with HCC vary from immune-activation to immunosuppression, depending on concentration of sorafenib. While high doses of sorafenib compatible with pharmacologic levels suppress CD4 Teff responses, low doses improve T cell responses. The observed improvement in T cell responses occur by enhancing Teff IL-2 signaling via increased cell surface expression of CD25 and IL2 secretion and inhibiting Treg-mediated suppression. These findings are particularly relevant in clinical practice since most patients receiving sorafenib require dose reductions due to adverse effects and suggest an immune benefit may exist with low doses of sorafenib. Understanding the immune effects of sorafenib is essential in order to improve on the modest clinical benefit observed with single agent sorafenib and deserves further study as a potential novel treatment approach with combination strategies. The current liver directed therapies for HCC all impact immunity. By clarifying the dose and immune effects of sorafenib, future treatment strategies can incorporate the optimal dose of sorafenib that favors immune reactivity against the tumor. Combination treatments that include the optimal immune promoting dose of sorafenib along with other liver targeted therapies such as chemoembolization and radiofrequency ablation may lead to improved tumor immune responses at the tumor site and better outcomes in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Sorafenib increases T cell responses in HCC patients. **a** Low doses of sorafenib significantly improved T cell proliferation in the PBMC isolated from the patients with HCC ($n = 20$) from $25,823 \pm 3,820$ cpm (no sorafenib) to $31,278 \pm 3,820$ cpm ($p = 0.025$). Results are shown as mean cell proliferation $\pm$ SE (cpm). **b** A subset of patients ($n = 6$) exhibited a high degree of improvement in their T cell responses. In this group, low doses of sorafenib led to a significant increase in T cell proliferation from a baseline $28,780 \pm 2,108$ cpm to $41,374 \pm 6,278$ cpm ($p = 0.028$). **c** CD4+CD25⁻ T_{eff} from patients with advanced HCC ($n = 6$) were cultured without ($38,520.5 \pm 4,838$ cpm) and with three concentrations of sorafenib—0.1, 1

and 3 μM . A significant increase in Teff proliferation was observed with all three low doses of sorafenib. The low doses of sorafenib resulted in a general improvement in Teff proliferation: $51,764 \pm 1,847$ cpm with 0.1 μM ($p = 0.04$); $57,953 \pm 5,225$ cpm with 1 μM ($p = 0.02$) and $59,701 \pm 5,138$ cpm with 3 μM ($p = 0.013$). $*p < 0.05$; $**p < 0.01$. **d** Extended dose response of sorafenib on CD4+CD25⁻ Teff from patients with HCC ($n = 2$). Low doses of sorafenib result in improved T cell responses: $18,565 \pm 49$ cpm with 0.1 μM ($p = 0.008$); $19,768 \pm 145$ cpm with 1 μM ($p = 0.005$); ($18,937 \pm 1,402$ cpm with 3 μM ($p = 0.13$); $15,848 \pm 652$ cpm with 6 μM ($p = 0.65$); while higher doses result in a stepwise decline in T cell responses: ($9,381 \pm 909$ cpm with 12 μM ($p = 0.02$); $3,821.5 \pm 492$ cpm with 15 μM ($p = 0.002$); 256 ± 154 cpm with 18 μM ($p = 0.004$); 41 ± 492 cpm with 21 μM ($p = 0.0003$); and 34 ± 6 cpm with 25 μM ($p = 0.0003$)).

Fig. 2.

Sorafenib stimulates HCC Teff to secrete IL-2. **a** IL-2 levels were analyzed in the supernatants from CD4+CD25 – Teff cultures treated with sorafenib ($n = 2$). IL-2 content was significantly higher in the supernatants from the Teff cultures containing 0.1 and 3 μ M sorafenib when compared to the cultures without sorafenib. The IL-2 levels in the Teff cultures without sorafenib (62 ± 12 pg/ml) significantly increased with the addition of low doses of sorafenib: 112 ± 12 pg/ml with 0.1 μ M ($p = 0.05$); 109 ± 23 pg/ml with 1 μ M ($p = 0.15$); 110 ± 10 pg/ml with 3 μ M ($p = 0.02$). **b** This figure represents the IL2 levels recovered from the sample of HCC Teff cells plotted in Fig. 1d. Baseline IL2 secretion from

—HCC Teff without sorafenib 111 ± 3 pg/ml; 0.1 μ M sorafenib 104 ± 5 pg/ml ($p = 0.4$); 2 μ M 10 ± 2 pg/ml ($p = 0.5$); 3 μ M 114 ± 5 pg/ml ($p = 0.4$), 6 μ M 87 ± 9 pg/ml ($p = 0.12$); 12 μ M 49 ± 4 pg/ml ($p = 0.007$); 15 μ M 42 ± 0.6 pg/ml ($p = 0.002$); 18 μ M 17 ± 1 pg/ml ($p = 0.001$); 21 μ M 7 ± 0.7 pg/ml ($p = 0.0008$); and 25 μ M (11 ± 0.9 pg/ml, $p = 0.00091$)

Fig. 3.

Sorafenib increases the cell surface expression of CD25 on HCC Teff. HCC PBMC cells were stimulated with PHA and cultured without sorafenib or with increasing concentrations of sorafenib ranging from 0.1 to 12 μ M. Flow cytometry was used to determine the surface expression of CD25 on the CD4+CD127+ T effector cell population in the cultures. A representative patient is shown. The addition of sorafenib significantly increased the percentage of CD25 expression on the CD4+CD127+ Teff compartment from 9.5 (no sorafenib) to 16.0 % with 1 μ M. Further supplementation of sorafenib above 1 μ M failed to substantially increase CD25 expression

Fig. 4.

Sorafenib decreases HCC Treg-mediated suppression. **a** Suppression assays were performed with CD4+CD25⁻ Teff and CD4+CD25⁺ Tregs from patients with HCC. Teff cultured alone and stimulated with PHA proliferate up to $47,458 \pm 2,462$ cpm. The addition of Treg decreased cell proliferation. 1Treg:1Teff; $14,668 \pm 161$ cpm ($p = 0.047$). The addition of sorafenib to the cocultures (0.1 μ M) did not decrease HCC Treg-mediated suppression $16,253 \pm 161$ cpm ($p = 0.13$). In contrast, sorafenib 1 μ M and recombinant IL2 significantly decreases HCC Treg-mediated suppression, $25,750 \pm 1,253$ cpm ($p = 0.013$) and $27,220 \pm 1,760$ cpm ($p = 0.02$) respectively. **b** CD4+CD25⁻ Teff cells from a patient with HCC were

stimulated with PHA ($27,432 \pm 1,415$ cpm) and also cocultured with CD4+CD25+ T cells at ratios of 1:10 and 1:1. The suppression cocultures showed a progressive decrease in target T cell proliferation from $19,620 \pm 2,493$ cpm with the 1:10– $1,230 \pm 121$ cpm with the 1:1 cultures. The CD4+CD25– target T cell proliferation was restored by the addition of low doses of sorafenib as follows: $8,314 \pm 1,309$ cpm with $0.1 \mu\text{M}$ ($p = 0.016$); $45,011 \pm 6,896$ cpm with $1 \mu\text{M}$ ($p = 0.023$); $34,662 \pm 6,141$ cpm with $2 \mu\text{M}$ ($p = 0.032$); and $28,648 \pm 2,155$ cpm with $3 \mu\text{M}$ ($p = 0.006$). **c** HCC PBMC cells were stimulated with PHA and cultured alone or supplemented with increasing concentrations of sorafenib ranging from 0.1 to $25 \mu\text{M}$. Flow cytometry was used to determine the Treg (CD4+CD25+CD127–) frequency in the cultures. A representative patient is shown. The percentage of CD4+CD25+CD127– T cells at baseline (PHA stimulated in the absence of sorafenib) was 5.3% . Addition of increasing doses of sorafenib did not significantly change the frequency of Tregs which was 6.5% with $0.1 \mu\text{M}$, 4.5% with $1 \mu\text{M}$, 6.2% with $3 \mu\text{M}$, 4.7% with $6 \mu\text{M}$, 5.7% with $12 \mu\text{M}$, and 6.5% with $25 \mu\text{M}$.

Fig. 5.

Sorafenib significantly increases IL-2 content in HCC Teff/ Treg cocultures. **a** IL2 content in the supernatants from the suppression assays was determined by ELISA. Teff cells alone produced at the end of the culture 32 ± 6 pg/ml of IL2. IL-2 levels in the 1:1 cocultures alone, with $0.1 \mu\text{M}$ of sorafenib and $1 \mu\text{M}$ of sorafenib were 36 ± 1 ($p = 0.6$), 51.3 ± 1 ($p = 0.07$) and 73.5 ± 3.4 ($p = 0.024$) pg/ml, respectively. The IL-2 control showed the largest content of IL2 at 582 ± 52 ($p = 0.008$), although the degree of cell proliferation was similar to the cocultures treated with $1 \mu\text{M}$ sorafenib. **b** This figure represents the IL2 levels from the supernatants of Fig. 4b. The introduction of Tregs to cocultures with target CD4+CD25-

Teff cells lead to a twofold decrease in IL2 secretion from 74 ± 13 pg/ml by CD4+CD25–Teff to 33 ± 6 pg/ml ($p = 0.11$) in the 1:10 and to even lower levels in the 1:1 (3.2 ± 1 pg/ml) cocultures. The addition of sorafenib to the 1:1 cocultures significantly elevates IL2 levels, particularly with 1 μ M (73 ± 0.5 , $p = 0.00005$), 2 μ M (103 ± 21 , $p = 0.04$), and 3 μ M (73 ± 2 , $p = 0.0008$)

Fig. 6.

Sorafenib significantly increases IL-6 content in HCC Teff/ Treg cocultures. ELISA was used to determine IL6 concentration in the supernatants from the suppression assays. Teff cells from patients with HCC alone generated at the end of the culture $1,241 \pm 108$ pg/ml of IL 6. Next, the IL-6 levels in the first coculture 1–1 without sorafenib and with 0.1 and 1 μ M sorafenib were $1,612 \pm 95$ ($p = 0.12$), $1,734 \pm 54$ ($p = 0.15$) and $1,896 \pm 78$ ($p = 0.039$) pg/ml, respectively. Finally, the IL2 control showed not significant increase in IL6 content when compared to HCC Teff cells alone, $1,633 \pm 29$ ($p = 0.86$)