

# Kinase inhibitor Sorafenib modulates immunosuppressive cell populations in a murine liver cancer model

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Accumulating evidence suggests that regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC) are elevated in cancer patients and tumor-bearing hosts, and that depletion of Tregs and MDSC may enhance the anti-tumor immunity of the host. Sorafenib, a novel multi-kinase inhibitor, is approved for the treatment of several human cancers, including advanced hepatocellular carcinoma (HCC). Sorafenib is believed to inhibit tumor growth via anti-angiogenesis, cell cycle arrest, and inducing apoptosis. However, the impact of Sorafenib on immune cell populations in tumor-bearing hosts is unclear. In this report, we show that Tregs and MDSC are increased in the spleens and bone marrows of the BALB/c mice with liver hepatoma. The increase in Tregs and MDSC was positively correlated with tumor burden. Treatment of Sorafenib not only inhibited HCC cell growth in mice but also significantly decreased the suppressive immune cell populations: Tregs and MDSC. In conclusion, our study strongly suggests that Sorafenib can enhance anti-tumor immunity via modulating immunosuppressive cell populations in the murine liver cancer model.

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Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Most patients present in late stages of the disease when surgical resection is not feasible, which leads to dismal clinical outcomes. The annual mortality rate is almost equal to the annual incidence, which is approximately 600 000 patients.<sup>1,2</sup> Radiation therapy and chemotherapy are generally not very effective for HCC. Therefore, developing effective new strategies to target liver cancer is urgently needed. Many studies have investigated immune responses in patients with HCC,<sup>3–6</sup> and have explored approaches to harness anti-tumor immunity.<sup>3,7–10</sup> Immunotherapy for liver cancer has also been actively investigated, and some clinical trials have been reported.<sup>11</sup> Attention has mainly focused on the induction of HCC tumor-specific immunity by introducing tumor-associated antigens (TAAs).<sup>12–14</sup> Among these TAAs, alpha-fetal protein and glypican-3 have generated significant interest.<sup>15–17</sup> Both proteins are expressed during embryogenesis, but are silent

in most adult tissues. These two proteins are valuable for HCC diagnosis and they have the potential to be target antigens for immunotherapy. Although evidence of immune response to these two antigens in HCC patients has been demonstrated, eliciting anti-tumor immunity using these two antigens is still not robust.<sup>18–21</sup> One of the potential explanations is that the expression of these two proteins during embryogenesis might have caused immune tolerance in adults. However, it is still unclear how the host immunosurveillance mechanisms interact with HCC to allow tumor to grow. Recently, many studies have suggested the presence of suppressor immune cells that have an important role in immune homeostasis and cancer development/progression.<sup>22</sup>

Several studies, including ours, have shown that development of HCC is associated with a number of immunosuppressive mechanisms.<sup>5,23–25</sup> Two groups of cells are implicated as main mediators of immune suppression in

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cancer: regulatory T cells (Tregs) and regulatory myeloid cells—myeloid-derived suppressor cells (MDSC). MDSC is a heterogeneous population of cells in tumor-bearing mice and cancer patients that have been shown to inhibit T-cell responses.<sup>26–29</sup> Upregulation of Tregs and MDSC has been demonstrated in patients with HCC.<sup>25,26,30</sup> MDSC (identified as CD11b<sup>+</sup>Gr-1<sup>+</sup> cells) have also been shown to accumulate in the spleens, bone marrows, and tumor tissues in tumor-bearing mice.<sup>28</sup> It has been suggested that MDSC suppresses specific cellular response to cancer cells.<sup>26</sup> A recent report has also highlighted that MDSC can inhibit NK cells in HCC patients.<sup>30</sup> It is possible that modulation of these cells may offer a new avenue for immunotherapy against cancer. Interestingly, current chemotherapy might already take advantage of this immunoregulatory mechanism. One recent report has shown that the kinase inhibitor, sunitinib, is capable of reversing the accumulation of the suppressive cell populations: MDSC and Tregs, which are potential new mechanisms for this chemotherapy.<sup>31</sup> Sorafenib, another multiple kinase inhibitor, has recently been approved as the first agent for advanced liver cancer therapy. Besides its cell growth inhibitory activity, the precise mechanisms by which Sorafenib suppresses HCC in patients remain to be defined.

To understand the immunoregulation in HCC, to define the mechanisms of therapeutic anti-HCC agents, and to develop more effective immunotherapy, it is essential to have a robust and reliable small-animal model. Genetically engineered mouse models and xenograft liver cancer models are excellent tools to study the intracellular signaling pathways, altered in hepatocarcinogenesis, and to evaluate therapeutic efficacy for anticancer molecules. However, models that can be used for studying immunoregulation in HCC are limited. Recently, Gonzalez-Carmona *et al*<sup>32</sup> have described an immunocompetent murine model using mouse HCC cell line Hepa129 and the C3H/HeNrl mice for investigating anti-tumor dendritic cells.

In this report, we describe a robust mouse liver cancer model in a commonly used immunocompetent BALB/c mouse strain. Immune responses and suppressor cells form a complex network in this HCC cancer-bearing mouse model. Moreover, we have shown that Sorafenib is capable of suppressing HCC progression in mice and downregulating Tregs and MDSC, which suggests a novel anti-HCC mechanism in this tumor model.

## MATERIALS AND METHODS

### Tumor Cell Line and Cell Culture

A mouse liver hepatoma cell line BNL 1ME A.7R.1 (referred as 1MEA in this study), was obtained from American Type Culture Collection (ATCC), and maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS) (GIBCO) and 100 U/ml penicillin, 100 µg/ml streptomycin (Cellgro), L-glutamine (2 mM) in humidified atmosphere of

5% CO<sub>2</sub> at 37°C. This tumor cell line was developed from BALB/c mouse.<sup>33</sup>

### Mice and Tumor Injection

BALB/c mice (6–8 weeks of age) were obtained from the Jackson Laboratory. Mice were kept in pathogen-free conditions and handled in accordance with the requirements of the Institutional Guideline for Animal Experiments. The right flanks of BALB/c mice were injected subcutaneously (s.c.) with  $5 \times 10^6$  1MEA cells in 100 µl of phosphate-buffered saline (PBS). At the end point of the experiments, mice were sacrificed and spleens were collected. Tumors were separated from mice and were measured. Tumor size was indicated as weight (mg). Representative tumor tissues were carefully excised and surface blood was removed by rinsing in cold PBS, then fixed in 10% neutral formalin, and processed for histology staining, or prepared into single cells and stained with fluorescence-conjugated antibodies for flow cytometry analysis.

### Flow Cytometric Analysis

Splenic cells were obtained by pressing splenic tissue or lymph node tissue, respectively, through a Nylon Cell Strainer of 40 µm mesh (BD Falcon). Bone marrow cells were collected by flushing femurs and tibiae with cold  $1 \times$  PBS in a syringe with a 27-gauge needle. Tumors were prepared as described above to obtain single-cell suspensions. Red blood cells were removed using red blood lysing buffer (Sigma). To decrease nonspecific binding of immunoglobulins, cells were incubated for 30 min with anti-CD16/32 (Fc block). The resulting cells were stained with fluorescence-conjugated antibodies anti-Gr-1, anti-CD11b, anti-CD3, anti-CD4, anti-CD25 (BD Pharmingen), and anti-FOXP3 (eBioscience). After washing with PBS twice, at least 200 000 events were collected and analyzed on a FACSCalibur system with CELLQuest software (Becton Dickinson) for each analysis. The data were expressed as a proportion of positive cells (compared with cells stained with an irrelevant control antibody).

### CD11b<sup>+</sup> Cell Functional Assays

CD11b<sup>+</sup> cells, with purity of more than 90%, were positively selected from splenocytes derived from tumor-free and tumor-bearing mice, by using CD11b microbeads (Miltenyi Biotec), respectively. The CD11b<sup>+</sup>-depleted splenocytes were used as responders, and placed in triplicates into a U-bottom 96-well plate ( $1 \times 10^5$ /well), and cocultured with CD11b<sup>+</sup> cells (as stimulators) at different ratios in the presence of anti-CD3 (5 µg/ml) for 48 h. At 18 h before collecting, cells were pulsed with [<sup>3</sup>H]thymidine (1 µCi/well; Perkin-Elmer). [<sup>3</sup>H]thymidine uptake was counted using a liquid scintillation counter and expressed as the mean counts per minute.

### Sorafenib Treatment *In Vivo*

Sorafenib (Nexavar, 200 mg/tablets) was dissolved in Cremophor EL/95% ethanol (termed as solvent) (50:50; Sigma), prepared stock solution at 6 mg/ml, and stored in  $-20^{\circ}\text{C}$  for *in vivo* study. After 2 weeks from tumor cells challenging, mice were divided into two groups (10 mice/group) and orally fed Sorafenib at 0.6 mg/100  $\mu\text{l}$ /mouse (30 mg/kg, once daily), or 100  $\mu\text{l}$  Cremophor EL/95% ethanol (control group), respectively, by gavage for 2 weeks. At the end point of experiments, mice were killed; tumors and spleens were dissociated and weighed, and femurs and tibias were collected. Splenocytes, bone marrow cells, and single tumor cells were prepared for further analysis.

### Immunohistochemistry Analysis of Tumor-Infiltrating Lymphocytes

Tumor tissues were immediately removed from the mice following  $\text{CO}_2$  asphyxiation and fixed in 10% formalin for 4 h. Subsequent tissue processing, including sectioning and staining, was performed using services provided by the University of Florida's Molecular Pathology and Immunology Core. Briefly, sections obtained through formalin-fixed samples were paraffin-embedded, cut, and collected 100  $\mu\text{m}$  apart, then stained with antibodies to CD4, CD8, and CD25.

To obtain tumor-infiltrating lymphocytes (TIL) from tumors, tumor tissues were cut into small pieces and placed into 40  $\mu\text{m}$  Nylon Cell Strainers in 10 ml cold PBS, and single-cell suspension was prepared by using hub of syringe.

After centrifugation, the resulting cell suspension was washed once in HBSS, resuspended in 5 ml Histopaque-1077 (Sigma), and placed in a sterile 15 ml conical tube, which was centrifuged for 30 min at 3300 r.p.m. at room temperature. The gray layer was collected. After washing with DMEM once, the cells were incubated with 1 ml red blood cell lysis buffer (Sigma) for 5 min at room temperature; subsequently, 25 ml sterile PBS was added to stop the lysis. The cells were washed, counted, and stained with antibodies to CD3, CD4, CD8, and CD25 for flow cytometry analysis.

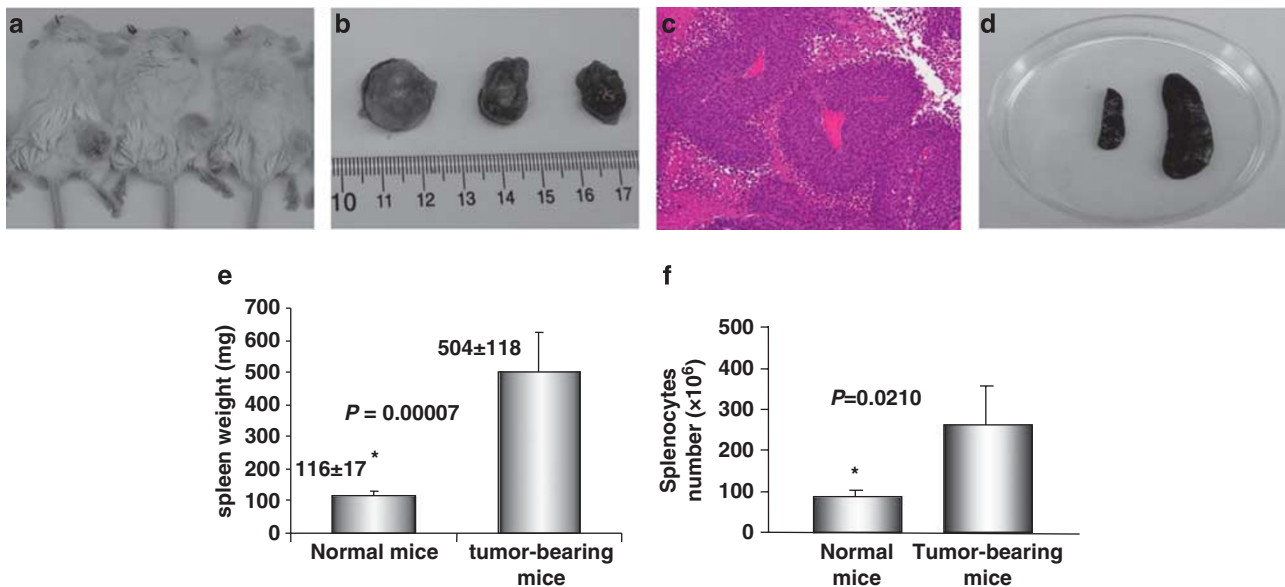
### Statistical Analysis

The statistical significance between values was determined by Student's *t*-test. All data were expressed as the mean  $\pm$  s.d. Probability values of  $P \leq 0.05$  were considered significant.

## RESULTS

### Murine Tumor Cell Line Grows and Initiates Immune Response in Immunocompetent BALB/c Mice

The murine cell line 1MEA was a chemically transformed cell line from a BALB/c mouse. To demonstrate the tumorigenic potential, we injected 5 million 1MEA cells per mouse *s.c.* into the right-hind flank of the mouse. Tumors could be palpated around 10 days in all the mice injected (total number 25). As demonstrated in Figure 1, the average tumor size was around 1.8 cm, in greatest dimension, in about 2 weeks (Figures 1a and b). Histologically, the tumor showed features of poorly differentiated HCC (Figure 1c). Marked

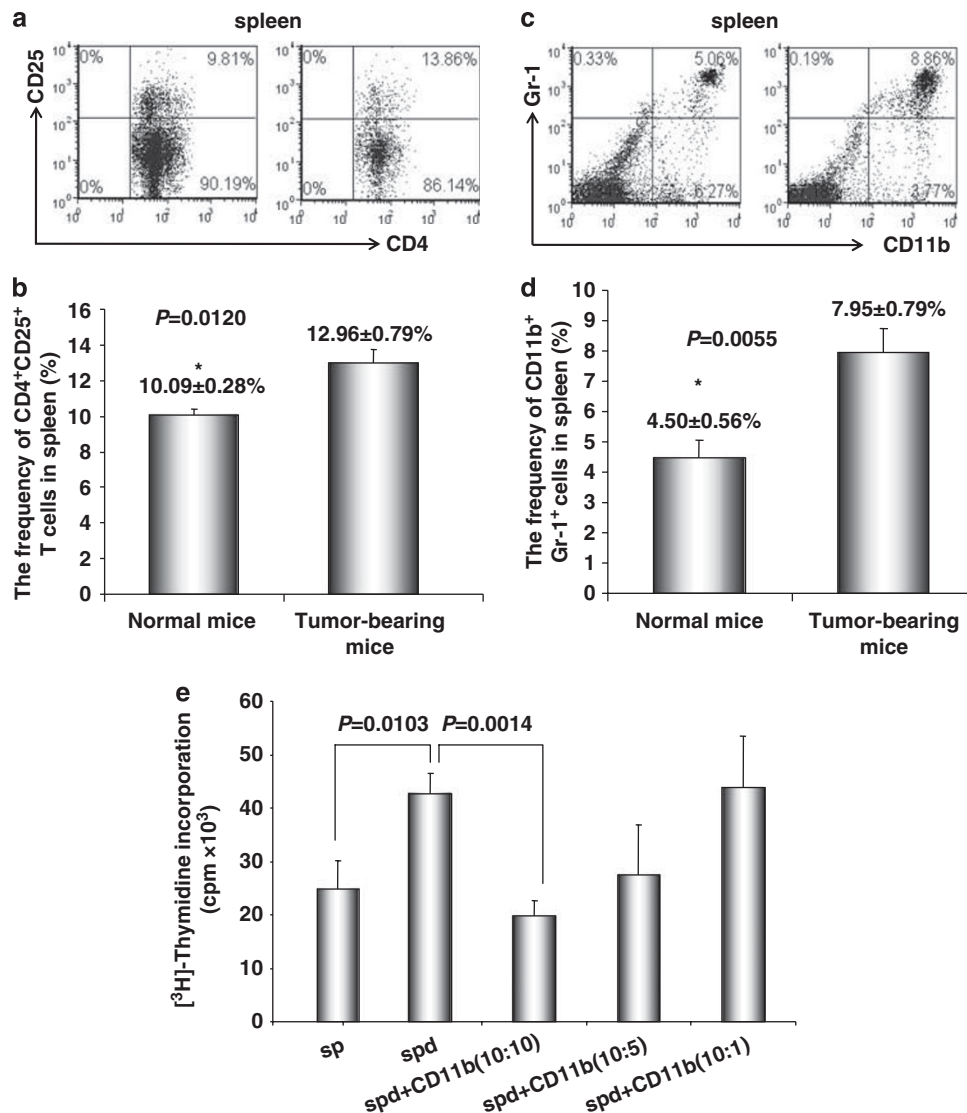


**Figure 1** Tumor growth and enlarged spleens in immunocompetent BALB/c mice. BALB/c mice (6- to 8-week old) were injected (*s.c.*) 1MEA cells ( $5 \times 10^6$ /per mouse). After 2 weeks, the tumors were measurable. As mentioned in Materials and Methods, at the end point of experiments (the end of the fourth week), spleens and tumor tissues were collected and weighed. Splenocytes were prepared and counted after red cells lysis. (a) Tumor-bearing BALB/c mice; (b) Tumor tissue separated from the mice. (c) Histological features of the liver cancer tissue (H&E stain) showing poorly differentiated hepatocellular carcinoma; (d) the size of spleens from BALB/c mice with tumor (right) and normal mice (left); (e) the weight of spleens from normal mice and tumor-bearing mice ( $n = 9$ ); (f) the splenocytes from normal mice ( $87 \pm 14 \times 10^6$ ;  $n = 3$ ) and tumor-bearing mice ( $263 \pm 95 \times 10^6$ ;  $n = 5$ ). Data represent the mean  $\pm$  s.d. from one of two similar experiments.

splenomegaly occurred in virtually all the mice that had tumor growth (Figure 1d). On average, the size of the spleen in a tumor-bearing mouse was three times larger than that of the control mouse (Figures 1e and f). The size of the spleens was consistent with the number of splenic cells when total splenic cells were counted. Both CD4 and CD8 cells were present in the spleen, and the ratio of CD4/CD8 was not significantly altered. Histologically, there was no significant morphological difference between spleens from the control mice and from the tumor-bearing mice (data not shown).

### Liver Cancer Upregulates Immunosuppressive Cell Population in Mice

As we have previously reported, HCC patients have higher percentage of Tregs ( $CD4^+ CD25^+$ ).<sup>25</sup> To investigate whether the similar phenomenon is also present in the mouse model, we firstly examined the  $CD4^+ CD25^+$  Tregs in tumor-bearing mice.  $CD3^+$  T cells were gated for the presence of  $CD4^+$  and  $CD25^+$  T cells. As shown in Figures 2a and b, tumor-bearing mice had higher numbers of  $CD4^+ CD25^+$  cells, which is consistent with findings in HCC patients. The data suggests that HCC induces immunosuppressive



**Figure 2** Increased  $CD4^+ CD25^+$  T cells and  $CD11b^+ Gr-1^+$  cells in tumor-bearing mice. (a) A representative flow cytometric data showing the  $CD4^+ CD25^+$  T-cell frequency of gated  $CD4^+$  T cells in splenocytes from normal and HCC tumor-bearing mice; (b) Mean  $CD4^+ CD25^+$  T-cells frequency in  $CD4^+$  T cells in splenocytes from normal and HCC tumor-bearing mice ( $n = 3$ ). (c, d) Representative (c) and mean (d) flow cytometry data showing increased  $CD11b^+ Gr-1^+$  cells frequency in splenocytes from normal and HCC tumor-bearing mice ( $n = 3$ ). The gated viable cells were analyzed. Numbers in the figures represent the percentage of fluorescence-positive cells in corresponding areas; (e) Functional analysis of  $CD11b^+$  cells were evaluated for their ability to suppress autologous  $CD11b^+$ -depleted splenocytes proliferation by [<sup>3</sup>H]-thymidine incorporation when stimulated with anti-CD3 and CD28 at various ratios. Values represent mean ± s.d. ( $n = 3$ ) and are representative examples of three separate experiments.

population in this mouse model that is similar to that observed in human disease.

Another population of suppressive cells, MDSC, has been recently defined as CD11b<sup>+</sup>Gr-1<sup>+</sup>. This cell population has been reported to increase in a number of cancers, including head-neck, pancreatic, HCC, renal, and breast cancers.<sup>26,34,35</sup> We next examined this population of cells in this animal model. Population of CD11b<sup>+</sup>Gr-1<sup>+</sup>MDSC was significantly expanded in tumor-bearing mice (Figures 2c and d). To confirm the immunosuppressive activity of these MDSC, we performed cell proliferation assay. As shown in Figure 2e, MDSC inhibited T-cell proliferation.

### Sorafenib Inhibits HCC Growth in the Mouse Model

Sorafenib has been approved as a systemic therapeutic agent for advanced HCC. Clinical trials indicate that treatment with Sorafenib modestly increases patient survival.<sup>36,37</sup> To validate the animal model, we tested the effect of Sorafenib on tumor growth. Mice were first injected with 1MEA cells s.c. At 2 weeks after the injection, when the tumor average size is around 0.4 cm, Sorafenib treatment was initiated with daily dose of 30 mg/kg. After 2 weeks of treatment, the mice were sacrificed and tumor sizes were measured. As shown in Figure 3, Sorafenib significantly inhibited tumor growth; the average tumor size was three times smaller than the control group.

### Sorafenib Downregulates Immunosuppressive Cell Population

To further evaluate the mechanisms of Sorafenib inhibition of HCC, we tested the hypothesis whether Sorafenib would exert anti-tumor activity through modulating suppressive populations of the immune cells.<sup>38,39</sup> We first determined the effect of Sorafenib on CD4<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>, and CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in the spleens and bone marrows of normal mice by flow cytometric analysis. As shown in Figures 4a and b, Sorafenib had no significant effects on these cells in normal mice. Then we analyzed the effects of Sorafenib on CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Tregs and CD11b<sup>+</sup>Gr-1<sup>+</sup> MDSC in tumor environments. As shown in Figures 4c and d, tumor-bearing mice had significantly elevated Tregs, whereas the treatment of Sorafenib restored the Tregs population and Foxp3 expression in CD4<sup>+</sup>CD25<sup>+</sup> T cells to the normal level. Sorafenib treatment also significantly reduced the number of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in both bone marrows and the spleens. The number of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells was even lower than the normal mice control, suggesting that Sorafenib prevented CD11b<sup>+</sup>Gr-1<sup>+</sup> cell differentiation. To evaluate overall inhibitory effect of Sorafenib on the cellular immune system, we analyzed the absolute cell numbers of CD4<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>, and CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in these mice. The data in Table 1 show that splenomegaly in tumor-bearing mice is associated with significantly increased absolute cell number of CD4<sup>+</sup>CD25<sup>+</sup> Tregs and CD11b<sup>+</sup>Gr-1<sup>+</sup> cells. Sorafenib did not alter the overall CD4<sup>+</sup> and

CD8<sup>+</sup> cells numbers and their ratio (Table 1, Figure 5). By inference, Sorafenib exhibited relative specific activity on Tregs and MDSC.

Lastly, we evaluated for the presence of tumor-infiltrating lymphocytes by using immunohistochemical staining and flow cytometry. Unexpectedly, no significant numbers of CD3, CD4, or CD8 T cells were detected in the tumor tissues (data not shown).

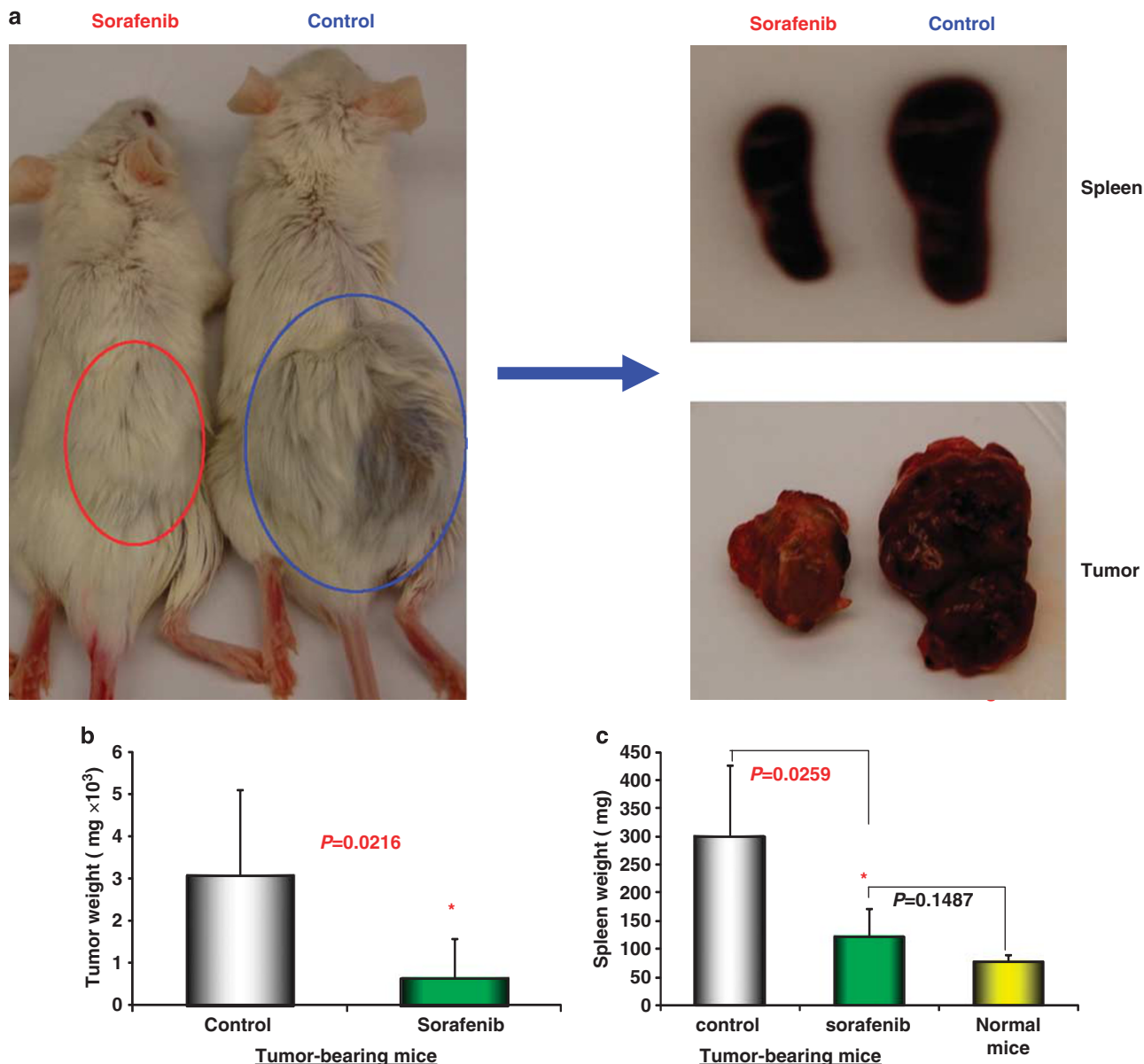
### DISCUSSION

There are a number of animal models being used for the study of liver cancer. However, most of the models rely on transgenic and knockout mice. The major disadvantage of these mouse models is the limitation of studying immune regulation because of the immune tolerance developed in these mice. Recently, an immunocompetent mouse model has been described for the study of dendritic cells.<sup>32</sup> There is emerging evidence that immune responses have a significant role in the development and progression of HCC. Thus, a robust mouse model that possesses intact immunity would be a valuable system for the study of HCC.

A murine HCC cell line, 1MEA, was first established two decades ago through chemical carcinogenesis of the immortalized cell line, BNL-derived from a BALB/c mouse.<sup>33</sup> The 1MEA cells can readily grow into tumor in BALB/c mice, indicating that there is no immune rejection to this tumor in these mice. This observation prompted us to ask the following questions: (1) Can the 1MEA tumor cells initiate immune response in the mice? (2) What is the nature of these immune responses that allow the tumor growth in an immunocompetent mouse?, and (3) Can the immune responses be modulated and tumor growth altered? Through a series of experiments, we have begun to answer these questions.

Murine HCC cell line initiates robust immune response in BALB/c tumor. Our experiments clearly demonstrated that 1MEA cell line could grow in BALB/c mice with a high rate of efficiency. With 5 million cells in the initial injection, a 1-cm tumor is visible within 2 weeks. Although we focused on tumor ectopic growth for the purpose of conveniently monitoring tumor size, we have also implanted tumor cells in the mouse livers and achieved similar tumor growth. The tumors are locally invasive to the surrounding tissue but metastasis is not observed, even in mice with primary tumor size as large as 4.5 cm.

One notable gross finding of the tumor-bearing mice is the enlargement of spleens, which has been noticed in several other cancer murine models.<sup>40,41</sup> The enlarged spleens suggest the initiation of immune responses to the tumors. The size of the spleens positively correlated with the tumor burdens, as shown in Figure 1. Extensive evaluation of splenocytes suggests that the splenic enlargement is associated with increased numbers of immune cells, mainly Tregs and MDSC cells. The overall number of CD4 and CD8 cells, and their ratios are not significantly altered. Histologically, there was no distinctive morphological difference between the spleens

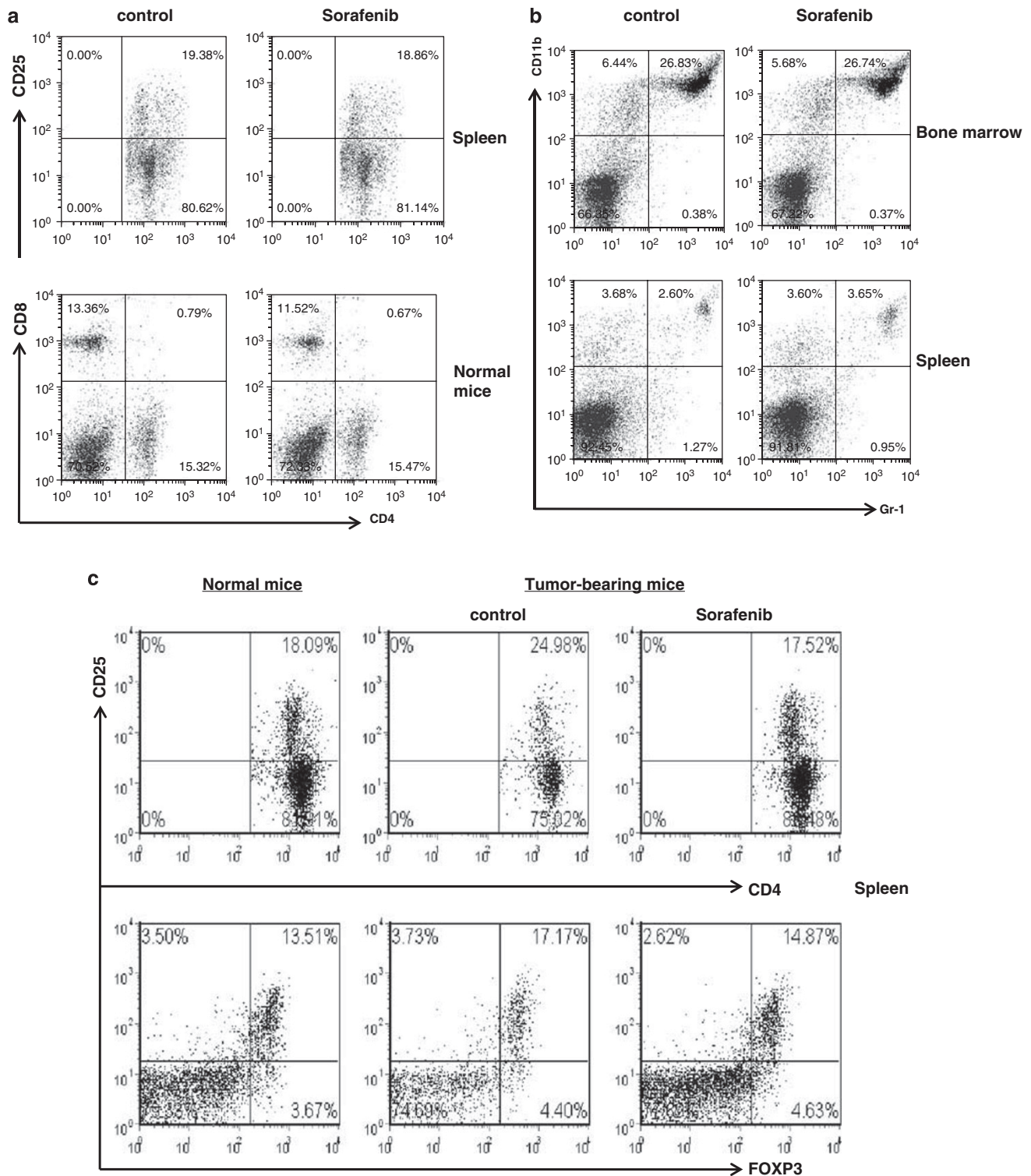


**Figure 3** Sorafenib inhibits HCC growth in BALB/c mice. BALB/c mice (6–8 weeks old) were s.c. injected with 1MEA cells (4 million/mouse) in the right flank. At 2 weeks later, the mice with measurable tumor were divided into two groups. Each mouse was gavage-fed with Sorafenib (0.6 mg/100  $\mu$ l) or solvent (100  $\mu$ l), once daily, 5 days each week, for 2 weeks. At end point of experiments, mice were sacrificed; tumors and spleens were collected, and weighed. (a) Representative of HCC tumor-bearing mice after 2 weeks treatment with Sorafenib (left) or solvent (right), spleen (right upper), and tumor (right lower) size. (b) The weight of tumor tissues from tumor-bearing mice after 2 weeks treatment with Sorafenib or solvent ( $n=5$ ). (c) The weight of spleens from normal mice or tumor-bearing mice after 2 weeks treatment with Sorafenib or solvent ( $n=5$ ). Data represent the mean  $\pm$  s.d. of five mice per group from one representative experiment of two similar experiments. \* $P \leq 0.05$ .

taken from normal and tumor-bearing mice. The observation of the splenomegaly in the mice is interesting considering that such phenomenon does not exist in human liver cancer patients.

As our experiments demonstrated, HCC could readily grow in the mice in spite of the robust immune responses. This observation would suggest that specific anti-tumor activity must have been inhibited. We have reported before that human HCC induces an immunosuppressive phenotype,

as reflected by the increased circulating Tregs.<sup>25</sup> Recently, another suppressive population of cells MDSC have been shown to be elevated in HCC patients.<sup>26</sup> Therefore, we decided to examine Tregs and MDSC present in the tumor-bearing mice. Our data show that both Tregs and MDSC were significantly upregulated in these mice, and they were positively correlated with tumor burden. Moreover, the MDSC cells also exhibited typical suppressive activity when co-cultured with effector T cells. We have previously reported



**Figure 4** Sorafenib treatment significantly decreased the percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD11b<sup>+</sup> cells in tumor-bearing mice. **(a, b)** Representative of flow cytometry data showing the frequency of CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in spleens and bone marrow from normal mice or tumor-bearing mice treated with solvent or Sorafenib; **(c, d)** Representative **(c)** and collective **(d, n = 3)** of flow cytometry data showing the expression of FOXP3 in CD4<sup>+</sup>CD25<sup>+</sup> T cells in spleens from normal mice or tumor-bearing mice treated with solvent or Sorafenib; **(e)** A representative of flow cytometry data showing the frequency of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in bone marrow (upper panel) and spleen (lower panel) from normal mice or tumor-bearing mice treated with solvent or Sorafenib; **(f)** Mean CD11b<sup>+</sup>Gr-1<sup>+</sup> cells frequency bone marrow and spleen from normal and HCC tumor-bearing mice treated with solvent or Sorafenib (n = 5). Data represent the mean ± s.d. of three or five mice per group from one representative experiment of two similar experiments.

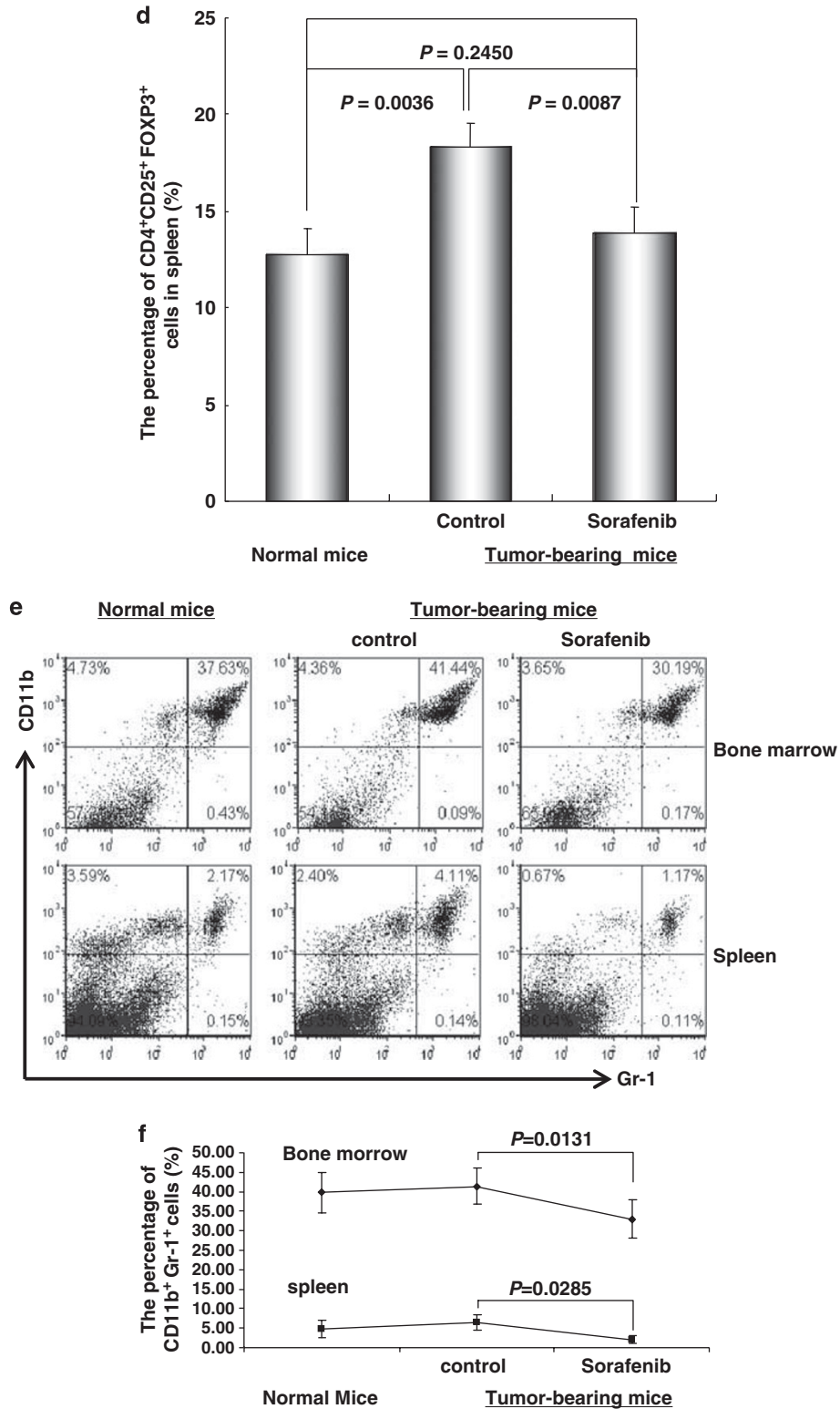


Figure 4 Continued.

that supernatants from human HCC cell lines can promote Tregs *in vitro*, indicating that tumor cells can secrete factors that modulate host immune system by promoting immunosuppressive populations.<sup>23</sup> Identification of these

factors would have a significant impact on the understanding of the tumor immunity and immune modulation for anti-tumor activity. One potential limitation of our current model is the fact that we have injected tumor in an ectopic



site rather than in the natural location of the liver. Further experiments will validate these findings using an orthotopic model.

The precise mechanism by which Sorafenib influences HCC progression and patient survival is unclear. As it is a kinase inhibitor, one possible mechanism is that it directly induces cancer cell apoptosis or inhibits cancer cell proliferation. Such activity has been reported previously.<sup>42</sup> We tested the *in vitro* activity of Sorafenib in IMEA cells and

no significant tumor cell death was seen, but there was a significant impact on IMEA cell proliferation (data not shown). We then tested the effect of Sorafenib in the mouse model. As our data shown in Figure 3, Sorafenib exhibited overt anti-tumor activity, which is unlikely because of anti-proliferation alone. We then examined the possibility of the immunomodulatory effect of Sorafenib in the HCC model. Indeed, the animals treated with the drug exhibited significantly reduced numbers of both Tregs and MDSC. This data suggests an immunomodulatory mechanism of Sorafenib on HCC *in vivo*. The marked decrease in MDSC from the bone marrow also suggests a direct effect on progenitor cell production, which requires further investigation. One note of caution is that we cannot exclude the possibility of the secondary effect on the suppressive populations, ie, the slow growth of HCC caused by Sorafenib would have less capacity to induce Tregs and MDSC. We believe that both direct and indirect effects may exist in the Sorafenib-treated mice.

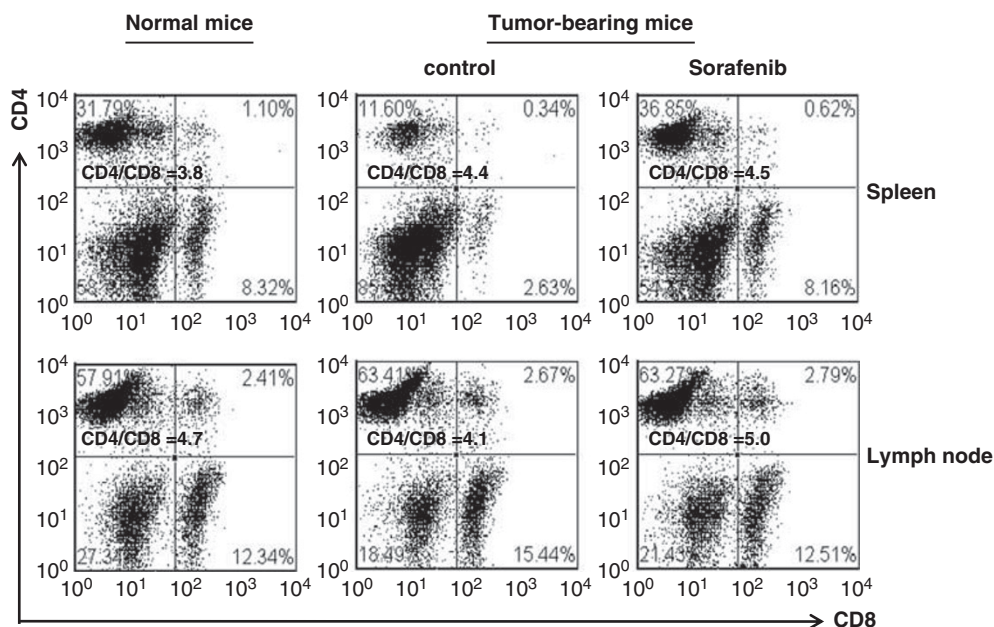
There are recent reports about the effect of sunitinib, another kinase inhibitor, on the inhibition of MDSC.<sup>31</sup> Ozao-Choy *et al*<sup>38</sup> hypothesized that malate, a receptor tyrosine kinase inhibitor, could reverse MDSC-mediated immune suppression and modulate the tumor microenvironment, thereby improving the efficacy of immune-based therapies. Treatment with sunitinib decreased the number of MDSC and Treg in advanced tumor-bearing animals. Furthermore, it not only reduced the suppressive function of MDSC but also prevented tumor-specific T-cell anergy and Treg development. Interestingly, sunitinib treatment resulted in

**Table 1 Splenomegaly and increased absolute numbers of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells and CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in tumor-bearing mice**

Cell number ( $\times 10^6$ )	Normal mice	Tumor-bearing mice (control group)	Tumor-bearing mice (Sorafenib group)
CD4	32.8 $\pm$ 4.3	32.3 $\pm$ 7.4	36.8 $\pm$ 6.2
CD8	20.9 $\pm$ 2.8	23.7 $\pm$ 5.4	21.8 $\pm$ 3.7
CD4 <sup>+</sup> CD25 <sup>+</sup>	3.6 $\pm$ 0.5 <sup>a</sup>	5.3 $\pm$ 1.2	2.8 $\pm$ 0.5 <sup>a</sup>
CD11b <sup>+</sup> Gr-1 <sup>+</sup>	7.2 $\pm$ 0.9 <sup>a</sup>	19.2 $\pm$ 4.4	5.6 $\pm$ 0.9 <sup>a</sup>

<sup>a</sup>Normal vs control group  $P < 0.05$ .

Spleens from normal or tumor-bearing mice treated with or without Sorafenib were prepared into single-cell suspension. After red cell lysing and wash, cells were suspended, counted, and stained with antibodies for CD3, CD4, CD8, CD25, CD11b, and Gr-1, then analyzed by flow cytometry. The absolute numbers of cells were figured out based on the total number of splenocytes and percentages of CD4<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup> and, CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in spleens. Values represent mean  $\pm$  s.d. from four mice per group from one of two independent similar experiments.



**Figure 5** Sorafenib treatment maintained the CD4 and CD8 cells in spleen and lymph nodes. A representative flow cytometric data showing the CD4<sup>+</sup> and CD8<sup>+</sup> T-cells frequency of gated lymphocytes in spleen and lymph nodes from normal and HCC tumor-bearing mice treated with solvent or sorafenib. Numbers in the figures represent the percentage of fluorescence-positive cells in corresponding areas, representative data of three independent experiments.

reduced expression of interleukin (IL)-10, transforming growth factor-beta, and Foxp3, but enhanced expression of Th1 cytokine IFN-gamma and increased CTL responses in isolated tumor-infiltrating leukocytes.

In a variety of human solid tumors, TIL are considered to have an important role in anticancer immune surveillance. Among the mature human T-cell population, three subtypes appear to have a major role in immunosurveillance against tumor growth: CD8<sup>+</sup>, CD4<sup>+</sup>, and Treg cells. Curiel *et al*<sup>43</sup> reported that tumor infiltration by Treg cells predicted reduced survival, but others have failed to reproduce this finding.<sup>44</sup> In many rodent tumor models, the mobilization of T-cell responses is also sufficient to eradicate tumors early during their establishment.<sup>45,46</sup> However, such mobilization is clearly less effective when it occurs late during tumor progression, in both rodent tumor models and in human cancer patients.<sup>47,48</sup> Studies suggested an inability of functionally normal T cells to infiltrate or persist in late-stage solid tumors because of physical barriers and/or emigration from the tumor site.<sup>48,49</sup> In our study, we did not detect significant infiltration of T cells in the tumor tissues. The reason may be partially because of late stage of tumor progression or the ectopic nature of the tumor.

In summary, we have established a novel, immunocompetent HCC mouse model that demonstrates the relevance to human HCC. Using this model, we have studied the immune response to HCC and examined the treatment effect of kinase inhibitor, Sorafenib. Our results show that HCC can upregulate immunosuppressive population of cells: Tregs and MDSC. We also found that Sorafenib modulated immune responses by suppressing Tregs and MDSC. We believe that this validated model can be used for understanding the immunoregulations in HCC, immunotherapy, and evaluating novel therapeutic agents.

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#### DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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