Increased Populations of Regulatory T Cells in Peripheral Blood of Patients with Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide with a poor prognosis and one for which immunotherapy remains a viable option. Experimental tumor models have shown that regulatory T cells, a functionally unique subset of T cells, can suppress effective antitumor immune responses. This suppression might explain the poor outcome of some of the immunotherapy protocols currently being used. A better understanding of the role of regulatory T cells in HCC is important for design of future immunotherapy-based clinical protocols. We have studied regulatory T cells from 84 patients with HCC and 74 controls, including healthy donors, patients with chronic hepatitis B virus and hepatitis C virus infection and nonviral liver cirrhosis. Regulatory T cells were identified by fluorescence-activated cell sorting using a panel of antibodies and by real-time PCR analysis for Foxp3 expression. Functional studies were done to analyze their inhibitory role. Finally, regulatory T cells were analyzed in tumors and ascites from patients with HCC. Patients with HCC have increased numbers of CD4+CD25+ regulatory T cells in their peripheral blood, which express high levels of HLA-DR, GITR, and low or no CD45RA. These cells were anergic toward T-cell receptor stimulation and, when cocultured with activated CD4+CD25+ cells, potently suppressed their proliferation and cytokine secretion. There were also high numbers of regulatory T cells in tumor-infiltrating lymphocytes of HCC patients comparable with the increase in their peripheral blood. Our data suggest that the increase in frequency of regulatory T cells might play a role in modulation of the immune response against HCC and could be important in design of immunotherapeutic approaches.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide (1) with a poor prognosis and limited survival in the majority of patients. Current treatment options include surgical resection, liver transplantation (2), and local ablative therapy, which are effective only in localized tumors (3). Therefore, identifying and establishing alternative approaches for treatment of HCC is quiet a challenge and of high interest.

One approach that has shown promising results in other tumors is immunotherapy (4). Also in HCC, several different studies suggest that immunotherapeutic approaches will be successful for the treatment of this disease. Lymphocytic infiltration of the tumors is indicative of a better survival after surgical resection of the tumors (5). Adjuvant treatment with activated lymphocytes has also been shown to increase tumor-free survival after removal of the tumor (6) and first clinical trials using dendritic cells have been initiated (7, 8).

In addition, recently, we have shown tumor-specific cellular and humoral immune responses against NY-ESO-1 in HCC patients (9). Interestingly, we have found that >50% of HCC patients develop tumor-specific immune responses. However, in most patients analyzed, tumors progressed in spite of a tumor-specific humoral and cellular immune response. Therefore, we decided to analyze possible mechanisms, which might impact the potency of these tumor-specific immune responses in patients with HCC.

CD4+CD25+ regulatory T cells have been shown to play a critical role in immunologic self-tolerance as well as immunotherapeutic responses and transplantation (10, 11). CD4+CD25+—positive regulatory T cells represent 5% to 10% of human CD4+ T cells, and are anergic, and do not proliferate on T-cell receptor stimulation in vitro (12). However, they can suppress the activation and proliferation of other CD4+ and CD8+ T cells (13, 14). Experimental tumor models have shown that removal of CD25+ T cells changes the immune response to tumors both in vitro and in vivo (15). Depletion of CD25+ T cells in mice resulted in a lower incidence or slower growth of B16 melanoma (16). Recently, the regulatory function of regulatory T cells on concomitant tumor immunity was further shown by Turk et al., who were able to show that depletion of regulatory T cells by either cyclophosphamide treatment or depletion of GITR+ cells resulted in a potent tumor-specific immune response in mice (17). In addition, it has been shown that regulatory T cells can impair induction of both antigen-specific and nonspecific T cells in autologous lymphocytes in melanoma patients (18, 19) and predict reduced survival in ovarian cancer patients (20).

Currently, no information on the role of regulatory T cells and their mechanism in HCC is available. Considering the importance of regulatory T cells in inhibiting effective antitumor immune responses, we studied these T cells in HCC patients. We analyzed peripheral blood from 84 HCC patients and compared the frequency of CD4+CD25+ T cells in these samples to patients with chronic hepatitis B virus (HBV)/hepatitis C virus (HCV) infection, healthy donors, and patients with nonviral liver cirrhosis. In addition, we assessed the proliferative and suppressive ability of CD4+CD25+ T cells in HCC patients, confirming their suppressive function of proliferation and cytokine release of autologous CD4+CD25+ T cells. We have also analyzed regulatory T cells from tumor-infiltrating lymphocytes (TIL) of HCC patients as well as ascites to compare the tumor microenvironment to peripheral blood of these patients and show that, similarly, high numbers of
regulatory T cells were present in the tumor of HCC patients. The increase in number of CD4+CD25+ regulatory T cells in HCC patients might play a role in suppression of immune response to tumors in these patients.

Materials and Methods

Patients and Healthy Donors. Blood samples were collected from a total of 84 HCC patients seen at the Department of Gastroenterology, Hepatology, and Endocrinology, Hannover Medical School (Hannover, Germany). HCC was diagnosed according to the diagnostic guidelines of the European Association for the Study of the Liver (21). Written consent was obtained from all patients before blood, ascites, and tumor sampling and the Ethics Committee of Hannover Medical School approved the study protocol. Blood samples were also taken from healthy volunteers, patients with chronic viral infection (HBV/HCV), with no evidence of cirrhosis and no antiviral therapy and nonviral liver cirrhosis without a tumor. Patient characteristics and disease classification are shown in Tables 1 and 2.

Cell Isolation and Sorting. Peripheral blood mononuclear cells (PBMC) were isolated from freshly obtained blood by Ficoll density gradient (Biochrom, Berlin, Germany) as described before (22). For isolation of CD4+CD25+ and CD4+CD25- T cells, PBMCs were further separated using the regulatory T-cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) and AutoMACS separation unit (Miltenyi Biotech) according to the manufacturer's instructions. Enriched cells were >90% pure as determined by flow cytometry. For sorting of the cells, CD4+ T cells were purified from freshly isolated PBMC using the CD4+ T-cell isolation kit and CD3 and anti-CD28. Proliferation was measured after 72 hours by 3H incorporation. [3H]thymidine (Amersham, Freiburg, Germany) was added to the cultures and cell proliferation was measured by incorporation of radiolabeled thymidine for 16 to 18 hours. Incorporated radioactivity was measured using a scintillation counter (Wallac, Turku, Finland). For CD8+ T-cell proliferation, CD8+ T cells were purified by magnetic-activated cell separation affinity purification using CD8+ microbeads according to the manufacturer’s instruction. The purified cells were incubated with increasing concentrations of sorted CD4+CD25+ T cells and tested in a proliferation assay as described above.

Transwell Analysis. CD4+CD25+ and CD4+CD25- cell populations were isolated as described above and cultured in Transwell plates (Greiner bio-one, Essen, Germany). Both chambers of Transwell received soluble anti-CD3 plus soluble anti-CD28 and IL-2 as described. The cytokine release of CD4+CD25+ cells (1 x 10^6) plated in the lower chamber of each Transwell was monitored in the presence or absence of direct contact with 1 x 10^6 CD4+CD25- cells by ELISA (R&D Systems).

Table 2. HCC patient characteristics

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<th>Hepatitis</th>
<th>Cirrhosis</th>
<th>Child Pugh</th>
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<tr>
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<td>Yes: 71</td>
<td>A: 46</td>
<td>0: 15</td>
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<tr>
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<td>No: 13</td>
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<td>B + C: 1</td>
<td>C: 4</td>
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<td>Negative: 53</td>
<td>No: 13</td>
<td>3: 9</td>
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</table>

Table 1. Patient characteristics

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<th>n</th>
<th>Male/female</th>
<th>Age (y)</th>
<th>CD4+CD25^{hi} cells</th>
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</thead>
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<td>17</td>
<td>13/4</td>
<td>44</td>
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<tr>
<td>HCV</td>
<td>19</td>
<td>11/8</td>
<td>51</td>
<td>0.70</td>
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<td>4</td>
<td>1/3</td>
<td>56</td>
<td>0.83</td>
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<tr>
<td>Nonviral cirrhosis</td>
<td>13</td>
<td>5/8</td>
<td>47</td>
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<td>84</td>
<td>67/17</td>
<td>66</td>
<td>3.92</td>
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<tr>
<td>Healthy</td>
<td>21</td>
<td>10/11</td>
<td>37</td>
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Results

HCC Patients Have Increased Numbers of CD4+CD25+ T Cells in Their Peripheral Blood. We analyzed peripheral blood of 84 patients with HCC, 19 patients with chronic HCV infection, 17 patients with chronic HBV infection, 21 healthy donors, and 13
patients with liver cirrhosis (Table 1) for the prevalence of CD4+CD25+ T cells. The population of CD4+CD25+ T cells as a percentage of total CD4+ T cells was identified by flow cytometry after cell surface labeling for expression of CD4 and CD25 molecules. We analyzed the CD4+ cells with the highest level of CD25 expression (CD4+CD25high), which protrudes as a tail from the major population of CD4+CD25low cells (Fig. 1A). Additional surface markers, such as HLA-DR and CD45RA, were used to distinguish CD25low and CD25high cells as described previously (ref; 26; see also Fig. 2A). The CD4+CD25high population represents ~1% to 4% of the CD4+ T-cell population; however, CD4+CD25low cells compromise ~10% to 14% of CD4+ T cells. Representative dot plots of HCC patients and healthy donors are shown (Fig. 1A). Individual frequencies of CD4+CD25high cells as well as the cumulative data for all the patients and healthy donors analyzed are represented as scatter plots (Fig. 1B). As shown, the frequency of CD4+CD25high T cells in HCC patients was significantly higher in HCC patients (3.92 ± 3.3%) than in healthy donors (1.17 ± 0.87%), HBV (1.06 ± 0.68%), HCV (0.70 ± 0.45%), and liver cirrhosis (0.78 ± 0.43%) patients. The number of CD4+CD25+ T cells in HCC patients did not change depending on the presence of liver cirrhosis or hepatitis infection (data not shown). These numbers also did not correlate with stage of the disease. Thus, the prevalence of CD4+CD25+ cells in HCC patients was significantly higher (P < 0.001) than in healthy donors or patients with (P < 0.01) viral hepatitis.

Phenotypic Analysis of CD4+CD25+ T Cells. The CD4+CD25high T cells were analyzed for expression of cell surface markers to compare them with the well-documented regulatory T cells described in other studies (26). Freshly isolated PBMCs from all patient populations were labeled with CD4, CD25, and a series of cell surface markers to further characterize these cells. Representative histograms of the surface marker expression analysis of HLA-DR, CD45RO, CD45RA, CD152, and GITR on CD4+CD25high, CD4+CD25low, and CD4+CD25− cells are shown in Fig. 2A. GITR, a member of the tumor necrosis factor receptor superfamily, which is a surface marker predominantly expressed on CD4+CD25+ regulatory T cells (27), was present up to 53% in CD4+CD25high cells. CD45RA a marker for T-cell responses to recall antigens was present as high as 84% in CD4+CD25high cells and to a lesser extent in CD4+CD25− cells (50%). In contrast, the CD45RA marker (a marker for naive T cells) was expressed in only 8% of CD4+CD25high cells, whereas it was present in up to 37% of CD4+CD25− cells. CD4+CD25high cells are also distinguishable from CD4+CD25− cells by their elevated expression of CTLA-4 (CD152, 38%) which has been reported previously for human circulating regulatory T cells (28) as well as HLA class II molecule (27%). Cell surface expression of regulatory T cells for healthy donors and patients with hepatitis infection was similar to HCC patients (data not shown). Thus, the CD4+CD25+ T cells in HCC patients and healthy donors were phenotypically similar to regulatory T cells described previously.

Expression of Foxp3 in CD4+CD25+ Cells. Foxp3 has been described recently as an important transcription factor and the most specific molecular marker for regulatory T cells known thus far (29). We analyzed the expression of Foxp3 in CD4+CD25+ and CD4+CD25− cells from HCC patients and healthy donors using real-time PCR. RNA was isolated from either sorted or magnetically purified CD4+CD25+ and CD4+CD25− cells as described in Materials and Methods. As shown in Fig. 2B, sorted CD4+CD25+ cells from both HCC and healthy donors expressed Foxp3, whereas CD4+CD25− cells expressed no or very little Foxp3. Additionally, Western blot analysis of CD4+ cells from HCC patients and healthy donors also confirmed the expression of Foxp3 in nuclear extracts (Fig. 2C). Overall, our data show that a high frequency of circulating CD4+CD25+ cells from HCC patients express GITR, CD45RO, CTLA-4, HLA-DR, and Foxp3 mRNA. These characteristics are highly indicative of regulatory CD4+CD25+ T cells already described in both healthy donors and cancer patients (26, 30). Therefore, the CD4+CD25+ cells detected in peripheral blood of HCC patients are indeed regulatory T cells.

Cytokine Profile of CD4+CD25+ T Cells. The cytokine expression profile of CD4+CD25+ T cells from HCC patients, healthy donors, and HCV/HBV patients were analyzed and compared with each other. Freshly isolated CD4+CD25+ and CD4+CD25− cells from different patient populations were stimulated with plate-bound anti-CD3 and soluble anti-CD28 and IL-2 for 72 hours. The culture supernatants were then assayed for IFN-γ and IL-10 production by ELISA. As shown in Fig. 3, CD4+CD25− T cells from HCC patients (n = 9) predominantly secrete IFN-γ with little or no secretion of IL-10, whereas CD4+CD25+ T cells mainly produce IL-10 (which is a cytokine known to inhibit T-cell proliferation) and very low levels of IFN-γ. Similarly, both cell populations from healthy donors (n = 11) and patients with chronic HCV infection (n = 5) had the same cytokine profile (Fig. 3).
Proliferative and Suppressive Function of CD4+CD25+ T Cells.
To further characterize the function of CD4+CD25+ regulatory T cells in HCC patients, we investigated the proliferative and suppressive function of these cells from HCC patients, HCV/HBV patients, and healthy donors by coculturing sorted CD4+CD25+ cells with increasing concentrations of sorted CD4+CD25+ cells upon T-cell receptor stimulation. The cells were sorted and purified as described in Materials and Methods and the CD4+CD25+ cells were stimulated with plate-bound anti-CD3, soluble anti-CD28, and increasing concentrations of CD4+CD25+ cells for 72 hours. Cell proliferation was determined by incorporation of [3H]thymidine. The CD4+CD25+ population proliferated robustly in HCC patients (Fig. 4A) in response to anti-CD3 and anti-CD28 stimulation. The proliferative capacity of CD4+CD25+ cells was inhibited in the presence of CD4+CD25+ cells in a dose-dependent manner, where the level of suppression correlated to the ratio of the two cell populations, with more CD4+CD25+ cells resulting in more suppression of proliferation of CD4+CD25+ cells (n = 9 for HCC patients and n = 11 for healthy donors). CD4+CD25+ T cells from HCC patients show equal proliferative and suppressive effects compared with healthy donors (Fig. 4A) and HBV/HCV patients (data not shown). Similarly, the IFN-γ production by CD4+CD25+ was strongly inhibited by CD4+CD25+ cells as shown by ELISA (Fig. 4B). The supernatants from the proliferation cultures were analyzed for IFN-γ secretion by ELISA, which showed that whenever the proliferation was inhibited the secretion of IFN-γ also decreased in parallel. These data suggest that CD4+CD25+ T cells from HCC patients can also inhibit cytokine secretion and proliferation of autologous CD4+CD25+ cells on T-cell receptor stimulation.

CD4+CD25+ Suppression Is Mediated through a Cell Contact Mechanism. Several studies have shown that mechanism of action of CD4+CD25+ regulatory T cells is mainly cell contact dependent (31–33). However, role of cytokines, such as IL-10 and transforming growth factor-β, in the suppressive ability of regulatory T cells has also been shown (33–35). A Transwell analysis was done to see if the suppressive effect of CD4+CD25+ T cells from HCC patients is cytokine or cell-contact mediated. Purified CD4+CD25+ and CD4+CD25+ cells were either cultured together or in Transwell-separated by a membrane. Transwell experiments from several HCC patients showed that CD4+CD25+ T cells required cell contact to suppress cytokine secretion and proliferation (data not shown) of CD4+CD25+ cells, because stimulation of CD4+CD25+ cells in the upper chamber had little effect on cytokine secretion by CD4+CD25+ cells in the lower chamber (Fig. 5). When blocking anti-IL-10 antibody was used in the proliferation experiments, the inhibition of proliferation by CD4+CD25+ cells was not significantly reversed (data not shown), suggesting that the mechanism of suppression by CD4+CD25+ regulatory T cells in HCC patients is mainly cell contact dependent.

Increased Prevalence of CD4+CD25+ T Cells in TILs. To see if regulatory T cells infiltrate the tumor microenvironment of HCC patients, we determined the prevalence of CD4+CD25+ T cells in TILs. Cancer Research

Figure 2. Phenotypic analysis of regulatory T cells and Foxp3 expression. A, freshly isolated PBMCs from HCC patients or healthy donors were labeled with anti-CD4, anti-CD25, -HLA-DR, -anti-CD45RA, -anti-CD45RO, -anti-GITR, and -anti-CTLA4. Control samples were stained with mIgG1-FITC, mIgG2a-FITC, or IgG2b-FITC. CD4+CD25+high, CD4+CD25+low, and CD4+CD25+ (see Fig. 1A) cell populations were analyzed separately. B, cDNA obtained from sorted and or magnetically purified populations of CD4+CD25+ and CD4+CD25+ cells were subjected to quantitative real-time PCR analysis using primers specific for Foxp3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). C, nuclear extracts from CD4+ T cells isolated from peripheral blood of HCC patients and healthy donors were prepared. Expression of Foxp3 was assessed in the nuclear extracts (3-6 μg of total protein) by Western blotting.
T cells in TILs of HCC patients and compared them with regulatory T cells in their peripheral blood by flow cytometry (Fig. 6A). Tumors from five HCC patients were analyzed for regulatory T cells in TILs. The frequency of CD4+CD25 high cells in TIL of HCC patients was similar to the peripheral blood with an average of 6.55 ± 3.5 of CD4+ cells for TIL and 1.84 ± 1.06 for peripheral blood (Fig. 6A). Ascites that is present within the vicinity and drainage of the tumor might very likely represent the milieu of the tumor environment (36). We determined the prevalence of CD4+CD25+ regulatory T cells also in ascites of HCC patients and compared them with regulatory T cells in their peripheral blood. There was also an increase in prevalence of regulatory T cells in ascites and this increase was similar and comparable with the increase seen in the peripheral blood of the patient. The CD4+CD25+ frequency in ascites of the HCC patients was found to be 7.2 ± 2.7% compared with the peripheral blood, which was 6.0 ± 3.7% (Fig. 6B). These data showed that regulatory T-cell frequencies were increased not only in peripheral blood but also in ascites and TILs, suggesting that these T cells might suppress immune activation at the tumor site.

CD4+CD25+ T cells from TILs and ascites were further analyzed for expression of cell surface markers, such as HLA-DR and GITR, and a similar pattern as seen in peripheral blood of these patients was found (Fig. 6C and D). GITR expression on CD4+CD25+ regulatory T cells in both tumor and peripheral blood was ~68% and 46%, respectively. HLA-DR expression was also similar in both (65% for tumors and 62% for blood). Similar pattern was observed in ascites (Fig. 6D). Expression of Foxp3 was also found in CD4+CD25+ purified from TILs (Fig. 6E), reconfirming that the regulatory T cells were present in TILs of HCC patients.

Our results suggest that in the tumor microenvironment as well as the peripheral blood of HCC patients there is a significant increase in the number of CD4+CD25+ T cells.

Discussion
This study presents evidence for increase in frequency of CD4+CD25+ regulatory T cells in peripheral blood as well as tumor microenvironment of HCC patients. Eighty-four HCC patients were compared with healthy donors and patients with other liver diseases but no liver malignancies.

We used several cell surface markers to distinguish CD4+CD25+ regulatory T cells from activated cells. Although CD25 is the typical cell surface marker used to identify regulatory T cells, its specificity is not limited to regulatory T cells but also to other types of activated T cells. Therefore, we decided to include other, more specific surface markers, such as GITR, HLA-DR, CD45RO, CD152, and CD45RA, to identify regulatory T cells by flow cytometry (26, 27). This allowed for distinguishing T cells with regulatory properties from other activated CD4+ T cells in peripheral blood.
from HCC patients and controls. The stringent conditions used in our study led to sorting out a smaller population of CD4+CD25+ (i.e., CD4+CD25^{high} cells), which represent ~1% to 2% of CD4+ T cells in healthy people. In addition, we have analyzed Foxp3 expression in CD4+CD25^{high} cells from HCC patients and healthy donors by real-time PCR, because it has been shown recently that CD4+CD25^{high} Treg specifically express this transcription factor (37, 38) and it is currently considered to be the most accurate marker to identify Tregs (29). We also found Foxp3 to be expressed almost exclusively by the CD4+CD25^{high} T-cell population. The cell surface marker analysis combined with Foxp3 expression led us to believe that the population detected in the peripheral blood of HCC patients is indeed CD4+CD25^{high} regulatory T cells.

We have shown that the prevalence of CD4+CD25^{high} cells in peripheral blood of HCC patients was significantly higher in peripheral blood from patients with HCC than in healthy controls or patients with HBV/HCV infection. Our data show no significant differences in the number of regulatory T cells between healthy controls and patients with chronic HCV/HBV infection and therefore confirms data shown previously by others.

**Figure 5.** Suppressive function of CD4+CD25^{high} cells is cell-contact dependent. Isolated CD4+CD25^{high} and CD4+CD25^{low} cell populations were either cultured together or separated by Transwell at a 1:1 ratio. IFN-γ release was measured by ELISA. Data shown are representative of three separate experiments for healthy controls and HCC patients. Results are shown as % activation with CD4+CD25^{high} cells stimulated with anti-CD3/anti-CD28 in the absence of any CD4+CD25^{low} cells [anti-CD3/anti-CD28-stimulated CD4+CD25^{high} cells released 830 pg (healthy) and 450 pg (HCC) of IFN-γ].

**Figure 6.** Regulatory T cells are present in tumor infiltrating cells and ascites of HCC patients. A, lymphocytes from tumor infiltrating cells and peripheral blood of HCC patients (n = 5) were analyzed in parallel. Similar frequencies of CD4+CD25^{high} regulatory T cells are found in peripheral blood as well as in TILs. B, ascites from HCC (n = 3) patients were also tested in parallel to peripheral blood for presence of CD4+CD25^{high} regulatory cells. C and D, regulatory T cells from peripheral blood, ascites, and tumors showed similar expression patterns for HLA-DR and GITR expression. E, real-time PCR analysis for FoxP3 expression in CD4+CD25^{high} in TILs.
(39). One study has shown an increase of CD4+CD25+ cells in patients with HCV infection; however, in this study, less stringent criteria were used to identify Tregs and activated CD4+ T cells were not excluded (40).

In *ex vivo* assays, we also studied the function of regulatory T cells in HCC patients by analyzing their proliferative and suppressive abilities as well as cytokine profile. The CD4+CD25+ T cells from HCC patients had a potent suppressive activity, as they inhibited the proliferative response of CD4+CD25+ T cells significantly upon T-cell receptor stimulation. This suppressive effect was similar in both HCC patients and healthy donors. Thus, the regulatory T cells from HCC patients do not have an impaired proliferative capacity. In addition, these cells secreted inhibitory cytokines, such as IL-10. The suppressive effect seen by CD4+CD25+ regulatory T cells in HCC patients was not cytokine mediated but rather cell-contact mediated as shown by Transwell analysis. Several groups have found the suppressive effect of regulatory T cells to be cell-contact dependent and not mediated by cytokines. However, other studies have shown that IL-10 and transforming growth factor-β are responsible for the suppressive effect of regulatory T cells (41, 42). It is possible that although in our assays IL-10 does not seem to play a role in *in vitro*–mediated suppression, this cytokine might act synergistically in vivo with cell-contact mechanism to mediate suppression possibly by affecting other cell types.

To analyze the role of the regulatory T cells in tumor environment, we have also analyzed TILs and lymphocytes isolated from ascites. The CD4+CD25+ regulatory T cells isolated from HCC tumors were HLA-DR positive and GITR positive and also expressed Foxp3. Our data clearly showed that regulatory T-cell frequencies were increased not only in peripheral blood but also in ascites and in the tumor, suggesting that these T cells might suppress immune activation at the tumor site. Thus, the CD4+CD25+ T cells found in the TILs in HCC patients are clearly regulatory T cells and might play a role in hampering antitumor immune responses.

Recently, several studies have reported an increase in number of regulatory T cells in patients with different cancers (20, 23, 43, 44). Several experimental models have shown that elimination of CD4+CD25+ cells can lead to effective antitumor immune responses. In mice, treatment with anti-CD25 monoclonal antibody led to regression of leukemia and fibrosarcoma (45). In another study, depletion of regulatory T cells resulted in a slower growth of B16 melanoma (16, 17). These studies combined with our data suggest that Tregs in human cancer might suppress tumor-specific immune responses.

In addition, we have started to analyze the influence of current treatment options for HCC on Treg frequencies. Our preliminary data suggest that elevated numbers of regulatory T cells drop after removal of HCC tumors, suggesting a clear dependency of an increase in the frequency of regulatory T cells and occurrence of HCC. Due to the low frequency of tumor-specific CD4+ T cells in HCC as described by us previously (9), analysis of the antigen specificity by classic MHC peptide tetramers (46) or cytokine secretion analysis has not been feasible. However, work is in progress to address antigen and tumor specificity of regulatory T cells in HCC patients.

Our data at this point do not provide any mechanistic explanation for role of regulatory T cells in HCC patients. It remains to be shown as to how the increase in regulatory T cells contributes to immunological tolerance or inhibition of effective antitumor immune responses in HCC. It is possible that the presence of regulatory T cells at the tumor site promote the tumor growth. On the other hand, secretion of inhibitory factors by tumors might cause expansion of regulatory T cells.

In summary, our data suggest that in HCC patients there is an increase of regulatory T cells in peripheral blood and tumor microenvironment. These T cells might prevent effective antitumor immune responses, and in designing immunotherapy protocols for HCC, regulatory T cells are one more obstacle to overcome. These findings will prove to be important for design of immunotherapeutic approaches to HCC.

**Acknowledgments**

Received 9/7/2004; revised 12/3/2004; accepted 1/5/2005.

**Grant support:** Wilhelm Sander Foundation, Deutsche Forschungsgemeinschaft (KFO 119).

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We thank Matthias Ballmaier for technical assistance with the cell sorting using the FACSAria (Central Sorter Facility, Medizinische Hochschule Hannover) and Monique Hörring for collecting patient samples.

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