Clinical Studies

Higher Frequencies of GARP⁺ CTLA-4⁺ Foxp3⁺ T Regulatory Cells and Myeloid-Derived Suppressor Cells in Hepatocellular Carcinoma Patients Are Associated with Impaired T-Cell Functionality

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Abstract

The extent to which T-cell–mediated immune surveillance is impaired in human cancer remains a question of major importance, given its potential impact on the development of generalized treatments of advanced disease where the highest degree of heterogeneity exists. Here, we report the first global analysis of immune dysfunction in patients with advanced hepatocellular carcinoma (HCC). Using multi-parameter fluorescence-activated cell sorting analysis, we quantified the cumulative frequency of regulatory T cells (Treg), exhausted CD4⁺ helper T cells, and myeloid-derived suppressor cells (MDSC) to gain concurrent views on the overall level of immune dysfunction in these inoperable patients. We documented augmented numbers of Tregs, MDSC, PD-1⁺–exhausted T cells, and increased levels of immunosuppressive cytokines in patients with HCC, compared with normal controls, revealing a network of potential mechanisms of immune dysregulation in patients with HCC. In dampening T-cell–mediated antitumor immunity, we hypothesized that these processes may facilitate HCC progression and thwart the efficacy of immunotherapeutic interventions. In testing this hypothesis, we showed that combined regimens to deplete Tregs, MDSC, and PD-1⁺ T cells in patients with advanced HCC restored production of granzyme B by CD8⁺ T cells, reaching levels observed in normal controls and also modestly increased the number of IFN-γ producing CD4⁺ T cells. These clinical findings encourage efforts to restore T-cell function in patients with advanced stage disease by highlighting combined approaches to deplete endogenous suppressor cell populations that can also expand effector T-cell populations. Cancer Res; 73(8); 2435–44. ©2013 AACR.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third highest cause of cancer-related mortality globally (1). HCC develops in patients with chronic hepatitis, either due to chronic hepatitis B or C viral infection or due to inflammation following aflatoxin ingestion, or excessive alcohol consumption. Unfortunately, most patients with HCC are first diagnosed with the disease at an advanced stage or present with poor liver function, thereby preventing the use of potentially curative therapies. Thus, treatment options for patients with advanced stage disease are limited to either chemoembolization or systemic therapies, which include sorafenib an oral antiangiogenic agent that is the current backbone of HCC therapy. Although these approaches have led to improved clinical outcomes, patients remain at high risk of disease recurrence after potentially curative surgery and ablation, and survival remains less than 1 year for patients with advanced stage disease. As toxic chemotherapies are often not well tolerated by these patients due to liver dysfunction, novel immune-based therapies such as antitumor vaccination and adoptive transfer of tumor-specific CTLs hold promise; however, their impact on tumor regression remains limited (2). The lack of efficacy of such therapies implies that HCC, like many other cancers, has developed multiple strategies of escaping tumor-specific immunity (3). To develop efficacious immunotherapies for HCC, clinicians are faced with various challenges about the mechanism by which chronic viral infections and inflammation due to hepatitis and cirrhosis impacts on both tumor progression and immune cell networks. Thus, correcting the deficiencies in the basic biology of these interactions, will likely lead to a greater clinical understanding of the disease and better treatment regimens.

Immune dysfunction in a variety of patients with cancer has been studied and found to include suppression of tumor-
associated antigen-reactive lymphocytes by regulatory T cell (Treg; refs. 4–7), accumulation of myeloid-derived suppressor cells (MDSC; refs. 8–10), and dysfunctional dendritic cells (DC; refs. 11–13). Increased numbers of CD4+CD25+ Tregs have been reported in the peripheral blood mononuclear cell (PBMC) of patients with HCC (14, 15). However, as these studies characterized Tregs merely by the surface expression of CD25, there is the possibility of inadequate discrimination of Tregs from activated CD25+ effector T cells. Depletion of Tregs or blockade of CTL-associated antigen 4 (CTLA-4) in vivo in experimental tumor models (16) and patients with cancer (17, 18) has resulted in enhanced antitumor immune responses and more efficacious immunotherapy regimens.

In this study, we examined Tregs and soluble immunosuppressive factors as they have been implicated to be independent prognostic factors regardless of the etiology of HCC (5). Although molecules such as CD25, CTLA-4, CD62L, and CD127 are differentially expressed on Tregs, they are also expressed during chronic T-cell activation or differentiation (19), and therefore these markers may not adequately discriminate Tregs with high suppressive potential from recently activated T cells. The molecule leucine-rich repeat containing 32 (LRRC32), also known as glycoprotein A repetitions predominant (GARP), has recently been reported to be a highly specific molecule for activated Foxp3+ Tregs with high suppressive function (20). As cancer patients with advanced stage disease are likely to have greater numbers of suppressive T-cell subsets, we elected to evaluate GARP expression to distinguish highly suppressive Foxp3+ Tregs from CD4+ T cells that do not exert suppressive function.

Immunologic dysfunction observed in patients with cancer can also be attributed to MDSC, which are known to be elevated in chronic inflammation and malignancies (8, 9). Via diverse mechanisms, MDSC cause profound suppression of both innate and acquired antitumor immunity, including T cell–based immunotherapy. Considering the potential immunosuppressive role of MDSC in patients with cancer, we elected to study the prevalence of this cell subset in patients with HCC.

Interaction between programmed death-1 (PD-1) receptor and its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) also influences the immune response (21). Generation of T-cell responses is determined by the balance of activating signals, mediated by CD28:B7-1/B7-2, or inhibitory signals produced by PD-1:B7-H1 (22). Because of its importance in suppressing T-cell responses, we also compared T-cell PD-1 expression levels in patients with HCC and healthy controls to elucidate the potential role of T-cell exhaustion in the immune profile of advanced stage HCC.

Dendritic cells are essential for the induction of tumor-specific T-cell responses. Dendritic cell differentiation, maturation, and functionality are severely impaired in several human malignancies and this represents another mechanism by which potentially efficacious endogenous antitumor immune responses in patients with HCC may be dysregulated (23). Tumor-derived factors such as TGF-β and VEGF have been shown to inhibit the differentiation of monocytes into mature dendritic cell and concomitantly skew their development toward highly suppressive MDSC (24).

The rationale of the present study was to conduct a stringent multi-parameter evaluation of candidate immunosuppressive networks in conjunction with T-cell subset functionality in patients with advanced stage HCC in comparison with healthy controls and to evaluate the impact of chronic viral infection and prior treatment on the immune profiles of the patients. Additional relevance was provided by ascertaining the changes in T-cell function that could be revealed by depletion of the suppressive cells. This represents the first study that has determined whether targeted depletion of immunosuppressive cells in advanced HCC has the potential to restore endogenous antitumor T-cell function.

Materials and Methods

Blood samples

Heparinized peripheral blood samples were obtained from patients with HCC through Data Bank and Biorepository at Roswell Park Cancer Institute (Buffalo, NY) and from healthy donors after obtaining informed consent. Clinical therapy and baseline demographic data were recorded. Clinical characteristics were collected by chart review (Table 1) and merged with immune results that were analyzed in blinded fashion. PBMC were isolated by Ficoll-Paque PLUS density gradient centrifugation of blood samples (GE Healthcare), as described elsewhere (25).

Tregs

Fluorescence-activated cell sorting (FACS) analysis was conducted to measure peripheral blood Treg frequency using APC-H7 anti-CD3, V450 anti-CD4, phycoerythrin (PE) anti-CD127, PE-Cy5 anti-CTLA-4 (BD Biosciences), PE-Cy7 anti-PD-1, and Alexa488 anti-Foxp3 (Biolegend). Detection of surface GARP levels was achieved by using mouse anti-human GARP (Enzo Life Sciences) followed by staining with PE F(ab’)2 goat anti-mouse immunoglobulin G (IgG; Jackson ImmunoResearch Laboratories). Cells were incubated with normal mouse IgG for 10 minutes before surface and intracellular staining. Intracellular analysis for Foxp3 and CTLA-4 was conducted after fixation and permeabilization of cells using intracellular staining kit (eBioscience) according to manufacturer’s instructions. All samples were acquired on LSRII flow cytometer (BD Biosciences) and analyzed using Flowjo Model Fit (Tree Star).

MDSC

MDSC in the peripheral blood were detected using fluorescein isothiocyanate anti-CD11b (eBioscience), PE-Cy5 anti-CD33, APC anti-CD14, and V450 anti-HLA-DR (BD Biosciences).

Cytokine ELISA

Plasma isolated during PBMC separation was assayed to quantify the level of IFN-γ, interleukin (IL)-10, and TGF-β1 using specific ELISA kits according to the manufacturer’s instructions (eBioscience).

Depletion of suppressor cells from PBMC

HCC patient PBMC depleted of GARP+, CTLA-4+, and PD-1+ T cells (gated on the CD3+CD4+ T cells) were obtained by
Intracellular cytokine-staining assay

Four hours before harvesting PBMCs treated with PHA or anti-CD3/anti-CD28, phorbol 12-myristate 13-acetate (PMA; 20 ng/mL), 1 μL of 1 mmol/L ionomycin/mL (Sigma), and 1 μg/mL of monensin (BD Biosciences) were added to the culture. Cells were washed and stained with APC-H7 anti-CD3 for 30 minutes at 4°C. After fixation and permeabilization, intracellular staining was conducted using V450 anti-CD4, V500 anti-CD8, PE anti-IFN-γ, PE anti-granzyme B, PE isotype control, and Alexa700 anti-Foxp3 (eBioscience).

Statistical analysis

Our primary objective was to compare immunophenotypes in patients with HCC (n = 23) and healthy controls (n = 20). For each of 29 possible outcomes, the null hypothesis of no difference in the outcome distribution between the 2 groups was assessed using an Exact Wilcoxon rank sum (Mann-Whitney U) test. Per-comparison two-sided P values less than 0.05 were considered statistically significant. With 23 patients in each group, similarly conducted experiments have 80% power to detect a minimum difference in mean expression of 0.9 SDs. Tests for functional responses were done in patients with HCC (n = 8) and healthy controls (n = 8). Patients were selected on the basis of either elevated levels of Tregs/MDSC or low levels of the same. Tests of this nature have 80% power to detect a minimum difference of 1.5 SDs.

Postdepleted HCC and healthy control samples were also compared using the Exact Wilcoxon rank sum (Mann-Whitney U) test. Matched predepleted and postdepleted samples within the patients with HCC were compared using the Wilcoxon signed rank test.

Given the number of comparisons conducted, we also considered a correction for multiple testing. Methods developed by Hochberg (26) were used to identify outcome differences in the HCC and control subjects that maintained a 0.05 family-wise type I error rate. On the basis of the 29 tests considered, this method identified 15 comparisons with per-comparison P values less than 0.0027 as being interesting.

Results

Patients

Clinical characteristics of patients with HCC are summarized in Table 1. At the time of this report, 17 of 23 patients with HCC have died and median survival for these patients is 7 months (range, 1–21 months). For the surviving 6 patients, median follow-up from time of PBMC collection is 14 months (range, 12–25 months). All 23 patients with HCC analyzed in this study had locally advanced or metastatic disease and none had early-stage surgically resectable or transplantable disease.

 Cirrhosis

By radiographic criteria, 11 of 23 patients showed no signs of cirrhosis and 5 of them also had prior liver resection that confirmed this. Ten of 11 patients also had Child Pugh liver cirrhosis score class A, whereas 1 patient was of class B, confirming the limited sensitivity of radiographic methods to assess degree and/or presence of cirrhosis. Of the remaining, 12 of 23 patients had some evidence radiographically (computed
tomography, MRI, ultrasonogram, or more than one) of cirrhosis and none were deemed as surgical candidates, even though 5 of them had Child Pugh class A or good liver function.

**Tregs in advanced HCC exhibit a highly immunosuppressive phenotype**

Using the baseline parameters for Tregs that other investigators have established, we also begin our study by measuring the frequency of CD3⁺CD4⁺Foxp3⁺ Tregs. These cells were additionally identified by exclusion of CD127 expression. Representative staining and gating of CD4⁺Foxp3⁺ Tregs from 1 patient with HCC (Fig. 1A) and 1 normal donor (Fig. 1B) are shown; the Tregs were present at higher frequencies in patients with HCC compared with normal controls (HCC: 9.4% ± 5.4% vs. normal: 5.0% ± 0.78%; P = 0.001; Fig. 1C). In addition, the absolute number of Foxp3⁺ T cells in the peripheral blood was also greater in the patients with HCC compared with controls (HCC: 17,440 cells/mL ± 11,343 vs. normal: 11,282 ± 3,486 cells/mL; P = 0.015; Fig. 1D). The ratio of Foxp3 mRNA copy number was significantly increased in patients with HCC as compared with controls (Supplementary Fig. S1A). A significant correlation was seen between Foxp3 gene expression and percentage of CD4⁺Foxp3⁺ T cells (Supplementary Fig. S1B), confirming that the 2 assays measure the same cell population. Comparison of Treg frequencies or absolute number after stratification of patients based on HCV status (Supplementary Fig. S2A and S2C) or having received prior treatment (Supplementary Fig. S2B and S2D) did not influence the level of Treg accumulation in patients with HCC.

We expanded our analysis of Foxp3⁺ Tregs to include measurement of GARP (Fig. 1E and F) and CTLA-4 (Fig. 1I and J) to determine whether these cells in patients with HCC exhibited a highly immunosuppressive phenotype. Evaluation of GARP and CTLA-4 expression on Tregs in advanced HCC has not been previously reported, and therefore the analysis of these 2 markers of highly immunosuppressive Tregs is critical.

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**Figure 1.** Increased numbers of GARP and CTLA-4 expressing Tregs in patients with HCC. Flow-cytometric analysis was conducted on PBMCs from patients with HCC (n = 23) and healthy controls (n = 20). A and B, representative staining from an individual patient with HCC and normal healthy donor for the frequency of CD3⁺CD4⁺Foxp3⁺ T cells. C and D, frequency (C) and absolute number (D) of cells/mL of CD4⁺Foxp3⁺ Tregs in peripheral blood of patients with HCC and normal healthy subjects. E and F, representative staining of GARP on CD3⁺CD4⁺Foxp3⁺ T cells. G and H, frequency of GARP⁺ Tregs (G) and GARP expression levels (H) measured by MFI on Tregs. I and J, representative staining of CTLA-4 on CD3⁺CD4⁺Foxp3⁺ T cells. K and L, frequency of CTLA-4⁺ Tregs (K) and CTLA-4 expression levels (L). Each symbol represents an individual patient with HCC (●) or normal healthy subjects (□); lines represent median values for the group. *, P < 0.05; ***, P < 0.01; †, P < 0.001; Mann-Whitney U test; ††, P < 0.05 Hochberg adjustment for multiple comparison.
in understanding the nature of Treg-mediated immune suppression in patients with HCC. The frequency of GARP$^+$ Foxp3$^+$ Tregs was significantly higher in patients with HCC than in controls (HCC: 37.1% $\pm$ 27.1% vs. normal: 12.5% $\pm$ 15.89%; $P = 0.01$; Fig. 1G). The level of GARP expression was also elevated on Foxp3$^+$ Tregs of patients with HCC as compared with controls [HCC: 341 $\pm$ 368 mean fluorescent intensity (MFI) vs. normal: 116 $\pm$ 181 MFI; $P = 0.01$; Fig. 1H].

Neither chronic viral infection nor prior treatment of patients with HCC had any impact on the profiles of GARP expression in patients (Supplementary Fig. S3A–S3D). The frequency of Foxp3$^+$ Tregs that expressed intracellular CTLA-4 was also significantly greater in patients with HCC compared with controls (HCC: 6.4% $\pm$ 5.0% vs. normal: 4.2% $\pm$ 1.4%; $P = 0.001$; Fig. 1K). In addition, CTLA-4 expression levels were also significantly higher on Tregs from patients with HCC as compared with controls (HCC: 1.137 $\pm$ 440 MFI vs. normal: 218 $\pm$ 94.4 MFI; $P = 0.001$; Fig. 1L). Neither the frequency of CTLA-4$^+$ T cells nor the CTLA-4 expression levels were influenced by chronic viral infection or prior treatment (Supplementary Fig. S3E–S3H). The presence of GARP$^+$ CTLA-4$^+$ Tregs represents the first identification of a highly immunosuppressive Treg population in patients with advanced HCC and that these cells may pose a significant impediment to the efficacy of antitumor responses elicited by immunotherapeutic or cancer vaccine approaches.

**Elevated numbers of MDSC in HCC patients**

Just as GARP and CTLA-4 have been understudied in advanced HCC, neither have MDSC been evaluated in this patient population despite their importance as an immunosuppressive cell in many cancers. Because of the interconnectedness of MDSC and Treg generation during malignant progression, we measured the frequency of CD14$^+$ HLA-DR$^+$CD11b$^+$CD33$^+$ MDSC in each patient with HCC for which we measured Treg frequency. Representative MDSC staining patterns from 1 patient with HCC and 1 normal control are provided in Fig. 2A and B. In conjunction with the elevated Tregs levels, the frequency (Fig. 2C) and absolute number of circulating MDSC (Fig. 2D) was significantly elevated in patients with HCC. In addition, the percentage of MDSC showed excellent correlation with percentage of circulating Tregs (Fig. 2E). HCV infection or prior treatment did not impact MDSC frequency in patients with HCC (Supplementary Fig. S4A–S4D). These data formally show the use of measuring Tregs and MDSC in the same patient, as 14 of 23 patients with HCC exhibited elevated levels of both immunosuppressive cell types.

The accumulation of MDSC does not comprise the full impact of all myeloid cells in advanced HCC, as CD11c$^+$ CD123$^+$ DC (Supplementary Fig. S5A–S5D) were also reduced in patients with HCC compared with normal when measured by frequency (Supplementary Fig. S5E) or absolute number (Supplementary Fig. S5F).

**Elevated levels of immunosuppressive cytokines in HCC patients**

The phenotype of augmented immunosuppression in patients with HCC is additionally reflected by the diminished levels of plasma IFN-γ (Fig. 3A), a potent antitumor cytokine whose levels can be downregulated by the presence of Tregs. In addition to the low levels of IFN-γ, a significant increase in the levels of 2 Treg-generated immunosuppressive cytokines, IL-10 and TGF-β1, was found in patients with HCC (Fig. 3B and C). The elevated levels of Foxp3$^+$ Tregs in patients with HCC are also associated with corresponding high plasma levels of TGF-β1 (Fig. 3D). Therefore, our results show that the cytokine
Impaired T-cell proliferation in HCC patients

The presence of an extensive immunosuppressive network undermines endogenous antitumor immunity by impairment of T-cell function. Given the presence of exhausted T cells, we evaluated whether T-cell proliferation and cytokine production were also dysregulated. CD4+ and CD8+ T-cell effector function was measured with 2 polyclonal stimuli, the potent mitogen PHA and anti-CD3/anti-CD28, a surrogate for antigen-specific T-cell receptor (TCR)-mediated stimulation. Furthermore, to ascertain the contribution of Treg, MDSC, and PD-1+–exhausted T-cell accumulation on effector T-cell function, these immunosuppressive cells were depleted, and the function of the remaining effector T cells was analyzed in their absence. We have for the first time directly tested whether targeted depletion of immunosuppressive cells in advanced HCC has the potential to restore endogenous antitumor T-cell function.

Proliferation of CD4+ and CD8+ T cells assessed by CFSE dilution revealed that both subsets from patients with HCC exhibited severely impaired responses to PHA stimulation compared with T cells from normal subjects (Fig. 4C and D, open black vs. open gray and Fig. 4E and F, ● vs. □). In addition, the depletion of the 3 immunosuppressive cells resulted in only moderate improvement in PHA-mediated T-cell proliferation for both CD4+ and CD8+ T cells (Fig. 4C and D, solid gray and Fig. 4E and F, ○ vs. ●). Despite the modest improvement in T-cell proliferation, Treg, MDSC, and PD-1+–depletion did not restore HCC T-cell proliferation that was equivalent to that observed in normal T cells (Fig. 4E–H, ○ vs. □).

Selective restoration of T-cell cytokine production upon depletion of immunosuppressive cell subsets

We measured IFN-γ and granzyme B production by CD4+ and CD8+ T cells in the presence or absence of Tregs, MDSC, and PD-1+–exhausted T cells (Supplementary Fig. S6). IFN-γ–producing CD4+ or CD8+ T cells from patients with HCC were significantly lower than normal subjects following both PHA and anti-CD3/anti-CD28 stimulation (Fig. 5A–D, ● vs. □), once again showing the pervasiveness of immune function dysregulation in patients with advanced HCC. Importantly, the frequency of T cells producing this cytokine did not increase appreciably upon depletion of the 3 immunosuppressive cells (Fig. 5A–D, ○ vs. ●). Failure to restore IFN-γ production provides powerful evidence that effector T cells in advanced HCC are unlikely to be able to overcome severe immunodysregulation by targeted depletion of Tregs, PD-1+ T cells and MDSC alone.

Decreased granzyme B production in CD8+ T cells was observed in patients with PHA-stimulated HCC compared with normal, but not following anti-CD3/anti-CD28 stimulation (Fig. 5E and F, ● vs. □). In contrast to IFN-γ production, the frequency of CD8+ T cells producing granzyme B following PHA stimulation was significantly augmented after targeted removal of the 3 suppressor cells and was equivalent between normal T cells and HCC T cells postdepletion (Fig. 5E, ○ vs. □). In patients with endometrial cancer, an inverse relationship has been shown between the presence of Treg and production of granzyme B expressing CD8+ T cells (27). In our studies, the depletion of highly suppressive Treg also likely accounts for the increased numbers of granzyme B+ CD8+ T cells to levels equivalent to that seen in healthy control subjects. Thus, our findings show that the restoration of T-cell

milieu in which the HCC disease progresses is skewed toward an immunosuppressive phenotype and will likely adversely impact the effector function of antitumor immune responses while simultaneously stimulating tumor-promoting immune responses.

HCC patients have increased PD-1 expression on circulating CD4+ T cells

The appearance of exhausted T cells, characterized by PD-1 expression, is a hallmark of chronic viral infections such as HCV and blockade of this molecule is currently being evaluated for cancer treatment (21). Given the widespread immunosuppressive network that we found in advanced HCC, we also examined whether PD-1+–exhausted CD4+ T cells are also present as an additional indicator of diminished effector function. Both the frequency of PD-1+CD4+ T cells (HCC: 19.0% ± 11.3% vs. normal: 8.7% ± 4.8%; P = 0.001; Fig. 4A) and PD-1 expression levels (HCC: 139 ± 72 MFI vs. normal: 46 MFI ± 31; P = 0.001; Fig. 4B) in patients with HCC was significantly higher than in healthy donors. Thus, the accumulation of exhausted CD4+ T cells in patients with HCC is another harbinger of immune dysregulation that must be overcome to elicit efficacious antitumor immune responses.

Impaired T-cell proliferation in HCC patients

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Figure 3. Elevated levels of immunosuppressive cytokines in patients with HCC. A–C, cytokine-specific sandwich ELISA of plasma from patients with HCC and healthy normal subjects were assayed to measure levels of circulating IFN-γ, IL-10, and TGF-β1. D, correlation of TGF-β1 plasma levels and frequency of CD4+Foxp3+ Tregs. Each symbol represents an individual patient with HCC (●) or normal healthy subjects (□); lines represent median values for the group. **, P < 0.01; †††, P < 0.001; Mann–Whitney U test; †, P < 0.05 Hochberg adjustment for multiple comparison.
High Frequency of GARP[^] Tregs and MDSC in HCC Patients

responses after depletion of suppressor cell subsets is restricted and does not ameliorate the entirety of immune dysregulation established in these patients.

**Discussion**

Studies have shown that HCC progresses even in the presence of tumor-specific immune responses in a majority of patients with HCC (28), indicating that HCC uses multiple mechanisms to evade host antitumor immunity. Evasion of host antitumor responses can occur by the induction of Tregs, defective antigen presentation by dendritic cells, recruitment and accumulation of MDSCs, and overproduction of inhibitory cytokines such as IL-10 and TGF-β. The effect of each of these mechanisms, and other equally important processes, has been evaluated in isolation, but no study to date has evaluated the contribution of the combined effect of immunosuppression on immune function in advanced HCC. Our study is the first of its kind to systematically measure key immunosuppressive processes, rather than individual subsets, to determine their collective effect on endogenous T-cell effector responses. As treatment options for inoperable advanced HCC are limited, physicians are seeking options involving immunotherapies. However, there are insufficient data on the immune status in these patients to assist in determining which immunotherapies can be beneficial. Our study, which involved the simultaneous measurement of multiple mediators of immune suppression, reveals a previously undescribed picture of extreme immune dysfunction in patients with advanced HCC and will

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**Figure 4.** Exhausted T cells from patients with HCC exhibit defective proliferation. A and B, frequency of PD-1[^] CD4[^] T cells (A) and PD-1[^] T cells (B) of CD4[^] T cells (C) and CD8[^] T-cell (D) proliferation measured by CFSE dilution in an HCC patient predepletion (□) and postdepletion (■) of suppressor cells and a normal healthy donor (△). C and D, representative CD4[^] T cells (C) and CD8[^] T-cell (D) proliferation index for PHA and CD4[^] T cell (G) and CD8[^] T-cell (H) proliferation index for anti-CD3/anti-CD28 stimulation. Each symbol represents an individual HCC patient predepletion (●), postdepletion (○), or normal healthy subjects (▲); lines represent median values for the group. *, P < 0.05; **, P < 0.01; ***; P < 0.001; Mann–Whitney U test; †, P < 0.05 Hochberg adjustment for multiple comparison.

**Figure 5.** Diminished IFN-γ and granzyme B production by HCC patient T cells. Frequency of CD4[^] T cells (A and B) and CD8[^] T cells (C and D) producing IFN-γ upon PHA (A and C) or anti-CD3/anti-CD28 (B and D) stimulation. Frequency of CD8[^] T cells granzyme B upon PHA (E) or anti-CD3/anti-CD28 (F) stimulation. Each symbol represents an individual HCC patient predepletion (●), postdepletion (○), or normal healthy subjects (▲); lines represent median values for the group. n.s. not significant; *, P < 0.05; **, P < 0.01; Mann–Whitney U test; †, P < 0.05 Hochberg adjustment for multiple comparison.
facilitate the rationale determination of which aspects of endogenous immunity can be exploited for treatment benefit. Although tumor-mediated immune dysfunction can occur at several checkpoints, we have focused on the cell subsets, which are currently being targeted clinically in several other cancers, namely Tregs, MDSC, and PD-1 blockade. Passive or adaptive immunotherapies are likely to succeed if tumor-mediated immunosuppressive networks are mitigated (29). Because the immunosuppressive networks are interconnected, we hypothesized that future therapies are likely to succeed if the extent of immunosuppression is accurately measured. There is excellent evidence that Tregs and MDSC are detrimental for antitumor immunity and that removal of either of these cell subsets greatly improves antitumor responses; however, there is no consensus on the method to distinguish and then deplete these cells.

CD25 has been proposed to distinguish potentially suppressive T cells, but CD4+CD25+ T cells are a heterogeneous population and only a fraction of this population are immunosuppressive (20). This method of Treg identification has shown that these cells have a high prevalence in the course of HCC disease progression (14, 15, 30), but these studies could not discriminate between activated effector T cells and immunosuppressive Tregs. Because of the limitations with CD25 as a unique marker, we have used Foxp3 as a marker for circulating Tregs. The elevated frequency of CD4+Foxp3+ Tregs observed in our study, are in concordance with an earlier study describing Foxp3+ Treg accumulation in patients with HBV+ HCC of Chinese ancestry (30). However, reliance on Foxp3 as a specific marker for Tregs is not without its own limitations, as it is also expressed on nonsuppressive TGF-β–induced Tregs (20, 31). In addition, the intracellular localization of the Foxp3 transcription factor makes it difficult to target these cells in a clinical setting with anti-Foxp3 for depletion of suppressive T cells. Thus, we have extended our Treg analysis to include surface markers that identify T cells with high suppressive potential.

We have used the expression of the orphan receptor GARP to identify antigen-specific Tregs with high suppressive potential. The expression of GARP dominantly controls Foxp3 via a positive feedback loop. Thus, retroviral overexpression of GARP in T-helper (T*H) cells results in the efficient and stable reprogramming of effector T cells to become Treg cells and conversely the downregulation of GARP in human Tregs significantly impaired Foxp3 expression and suppressor function (32, 33). Ours is the first study to report that this population of highly suppressive Tregs is elevated in advanced HCC. Importantly, depletion of GARP+ cells in combination with other markers was able to restore T-cell function in a limited set of effector processes, namely CD8 T-cell granzyme B production. Studies that have observed no effect following CD25– Treg depletion may not have been effectively removing these highly suppressive Tregs. The multifactorial depletion protocol we have used provides a tantalizing possibility that certain aspects of T-cell effector function, such as granzyme B production, can be improved by removal of GARP+ Tregs, and shows that not all antitumor effector functions are rendered permanently refractory by the extensive immunosuppressive network.

There is a paucity of data on the extent of accumulation of MDSCs in patients with HCC having elevated Treg levels. We have characterized CD14+HLA-DR−CD11b+CD33+ MDSC and report for the first time that these cells are significantly elevated in patients with advanced HCC. Cells with this same phenotype, which inhibited T-cell proliferation, have been shown to be elevated in patients with lung cancer, renal cancer, and melanoma, and depletion of this cell subset restored T-cell function (8, 34–36). Although this finding is not altogether surprising as MDSC levels are often increased in patients with cancer, we have formally showed that MDSC accumulation occurs concurrently with Treg accumulation in advanced HCC. Reports in patients with pancreatic, esophageal, and gastric cancer have shown that CD11b+CD33+ MDSC are associated with elevated numbers of Tregs (37). In concordance with our results of elevated numbers of both Tregs and MDSC, depletion of only one set of suppressor cells is unlikely to exert significant benefit for antitumor immune responses in advanced HCC.

Removal of both Tregs and MDSC cells is also advantageous from the standpoint that these cells are known to induce the generation of the other (36, 38), thereby creating a feedback loop that undoubtedly hinders antitumor immunity. In support of this strategy, PHA-mediated proliferation and granzyme B production was improved in effector T cells stimulated in the absence of Tregs and MDSC.

Diminished granzyme B production by activated T cells of patients with HCC may render their CTLs dysfunctional. Increased prevalence of Foxp3+ Tregs in patients with HCC may compromise cytolytic effector function of CD8+ T cells at least in part by inhibiting the expression of granzyme B in CD8+ T cells, and therefore compromising CTL-mediated tumor cell killing. An inverse relationship between Tregs and granzyme B/perforin expressing CD8+ T cells in patients with endometrial cancer has been reported (27). However, in an earlier study comparing patients with HCC and normal controls of Chinese ethnicity, no difference was reported in the granzyme B/perforin expression of circulating CD8+ T cells (30). In contrast to their findings, we observed a lower frequency of granzyme B producing CD8+ T cells; this is not surprising as we have evaluated patients with advanced HCC who exhibit immune dysregulation in many compartments. In addition, we have also shown for the first time that granzyme B production by effector CD8+ T cells in patients with advanced HCC can be fully restored by targeted removal of a highly suppressive Treg population. In patients with follicular lymphoma, CD8+ T cells expressing high levels of granzyme B were correlated with prolonged progression-free survival after combination of rituximab and chemotherapy (39). It is tempting to speculate that restoration of CD8+ T cell granzyme B production may have similar benefits for patients with HCC.

Partial restoration of T-cell function in our patients indicates that antitumor immune effector cells in advanced disease patients may not be fully competent to combat tumor progression even when underlying tumor-associated immune dysfunction is mitigated. This needs to be further confirmed because of limited sample size availability for depletion and functional assays. We elected to measure T-cell responses in an antigen-independent manner using potent polyclonal T-cell...
stimuli. However, despite the strong stimulation afforded by PHA or anti-CD3/anti-CD28, only modest changes in the T-cell function either before or after depletion of suppressor cells were observed. The underwhelming response to these strong polyclonal stimuli suggests that it is quite unlikely to expect robust antigen-specific responses from T cells of patients with advanced HCC. Some of the effector responses could be attributed to the presence of chronic HBV and HCV infection; however, we did not observe any differences in the composition of the immunosuppressive network between HCV+ and HCV− patients. The failure to observe a difference in these 2 populations suggests that the malignancy itself is likely contributing to the suppression of immunity. These findings are consistent with previous studies done in patients with chronic HBV, HCV, nonviral cirrhosis, and HCC (30, 40).

In conclusion, we have shown that the augmented numbers of Foxp3+ GARP+ CTLA-4+ Tregs, MDSC, PD-1+−exhausted T cells, and increased levels of immunosuppressive cytokines represent a plethora of potential mechanisms by which HCC may foster immune dysregulation. These mediators dampen antitumor T-cell immunity and may in fact facilitate the progression of HCC. Importantly, our study represents the first demonstration that the combined depletion of Tregs, MDSC, and PD-1+ T cells from patients with advanced HCC can result in the augmentation of CD8+ T-cell granzyme B production and a modest increase in the number of CD4+ T cell IFN-γ producing cells. Our findings suggest that in the clinical setting further enhancement of endogenous antitumor responses will have to rely on the "science of combination;" thus, depletion along with concomitant expansion of effector T cells may be effective in conjunction with immunotherapeutic strategies for patients with HCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: S. Kalathil, A.A. Lugade, Y. Thanavala
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Kalathil, A.A. Lugade, R. Iyer, Y. Thanavala
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): S. Kalathil, A.A. Lugade, A. Miller, R. Iyer, Y. Thanavala
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Higher Frequencies of GARP⁺CTLA-4⁺Foxp3⁺ T Regulatory Cells and Myeloid-Derived Suppressor Cells in Hepatocellular Carcinoma Patients Are Associated with Impaired T-Cell Functionality

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