Intratumoral CD3 and CD8 T-cell Densities Associated with Relapse-Free Survival in HCC

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Abstract

Immune cells that infiltrate a tumor may be a prognostic factor for patients who have had surgically resected hepatocellular carcinoma (HCC). The density of intratumoral total (CD3+) and cytotoxic (CD8+) T lymphocytes was measured in the tumor interior and in the invasive margin of 65 stage I to IV HCC tissue specimens from a single cohort. Immune cell density in the interior and margin was converted to a binary score (0, low; 1, high), which was correlated with tumor recurrence and relapse-free survival (RFS). In addition, the expression of programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1) was correlated with high CD3 and CD8 density (P = 0.024 and 0.005, respectively) and predicted a lower rate of recurrence (P = 0.034), as well as prolonged RFS (P = 0.029). Immunoscore and PD-L1 expression, therefore, are useful prognostic markers in patients with HCC who have undergone primary tumor resection. Cancer Immunol Res; 4(5); 419–30. ©2016 AACR.

Introduction

Surgical resection is frequently used as a curative treatment for patients with hepatocellular carcinoma (HCC) who have well-compensated liver function and no significant portal hypertension. However, the rate of HCC recurrence within 5 years of resection has been reported as ranging from 34% to 100% in heterogeneous patient populations (1). Conventional methods of assessing clinical and pathologic risk in patients with HCC typically involve excision of the primary tumor, followed by histopathologic analysis of tissue samples collected during the resection procedure. The extent of tumor burden and aggressive phenotype of cancer cells have been integrated in the American Joint Committee on Cancer/Union of International Cancer Control—tumor-node-metastasis (AJCC/UICC-TNM) tumor staging classification method, which categorizes the extent of the tumor burden (T), the presence of tumor-derived cells in proximal lymph nodes (N), and the degree of metastasis (M; refs. 2–4). This classification has been adopted to predict relapse-free survival (RFS), disease-specific survival (DSS), and overall survival (OS) rates in patients after curative resection (5). Recently, additional tumor cell characteristics such as abnormal nuclear morphology, altered expression of oncopgenes (P53, c-myc, b-catenin, hMSH2), and increased intratumoral vascular density have been suggested as predictive of HCC recurrence, RFS, and OS (6). However, few such relationships have been independently validated.

Although the AJCC/UICC-TNM system provides information regarding patient prognosis after curative resection of HCC, large variations in RFS and OS exist among patients with the same stage (pTNM) of disease (7). Some patients with advanced-stage cancer (III/IV) can remain free of cancer progression for years, whereas patients with early-stage cancer (I/II) can experience aggressive tumor recurrence, progression, metastases, and death following apparent complete surgical resection. The AJCC/UICC-TNM system only describes tumor burden on a macro scale; it does not provide any detailed information on features of HCC biology nor the microenvironment in which HCC proliferates (8).

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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doi: 10.1158/2326-6066.CIR-15-0110

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Experimental data have already explored the concept of immunosurveillance in mouse cancer models (9, 10), but this has only recently been applied to human cancers as a prognostic tool. The presence of immune cells flanking tumor tissue reflects a distinct underlying biology of the tumor and may have a great impact on cancer cells in their response to secreted growth factors, inflammatory mediators, and immune response elements (11). In humans, the presence of tumor-infiltrating lymphocytes (TIL) has been associated with a favorable prognosis (12–19).

Programmed death ligand 1 (PD-L1) is a 40-kDa type 1 transmembrane protein that has been speculated to play a major role in suppressing the immune system during particular events such as pregnancy, tissue allografts, autoimmune disease, and other disease states, such as hepatitis and cancer. Normally, the immune system reacts to foreign antigens where there is some accumulation in the lymph nodes or spleen, which triggers a proliferation of antigen-specific CD8+ T cells. The formation of PD-1 receptor/PD-L1 or B7.1 receptor/PD-L1 ligand complexes transmits an inhibitory signal that reduces the proliferation of these CD8+ T cells in the lymph nodes (2, 20). Interaction between surface molecules and different immune cells regulates the antitumor immune response (21).

The tumor immune microenvironment has been defined by Angell and Galon (22) as the type, functional orientation, density, and location of adaptive immune cells within distinct tumor regions [tumor interior (TI) and the invasive margin (IM)]. In order to quantify the immune microenvironment and apply it to a heterogeneous group of patients, the authors proposed the “Immunoscore.” Evaluation of the Immunoscore in patients with colorectal cancer has demonstrated that it is a powerful prognostic tool and may supplement the already well-established AJCC/UICC-TNM staging system (23). T-cell subpopulations (CD3+ T lymphocytes, CD8+ cytotoxic T lymphocytes, and CD45RO+ memory T cells) were studied and correlated with cancer recurrence and OS in patients with colorectal cancer (23). Because of background staining and loss of antigenicity in stored sections stained for CD45RO, the two easiest membrane stains, CD3 and CD8, were used by the worldwide Immunoscore consortium with the support of the Society for Immunotherapy of Cancer (SITC) for validation in clinical practice. The Immunoscore can be obtained by performing histopathologic analysis of tumor tissues and by applying a rubric of high/low density values to the tumor-infiltrating immune cells, with total (CD3+) and cytotoxic (CD8+) T lymphocytes being measured in the aforementioned study (23).

The prognostic value of the Immunoscore was demonstrated in patients (n = 602) with early-stage (I/II) colorectal cancer to predict OS and risk of recurrence. Five years after diagnosis, only 4.8% of patients with high Immunoscores of 3 or 4 had tumor recurrence with an OS rate of 86.2%. However, patients with Immunoscores of 0 or 1 had a tumor recurrence rate of 75% and faced an OS rate of 27.5% (24). Spranger and colleagues reported that T-cell infiltration is completely excluded from tumors with high levels of β-catenin activity in animal models, possibly due to lack of chemokine secretion and recruitment of T cells into the tumor (25).

In the current study, the clinical utility of the Immunoscore methodology was extended to assess the risk of recurrence and RFS in patients diagnosed with early- and late-stage HCC who had received curative resection. A secondary aim of this study was to identify clinicopathologic factors that were associated with immune infiltration and the corresponding Immunoscore. By identifying factors that influence tumor immune infiltration, we hope to better define risk of HCC recurrence after curative resection and, ultimately, improve the clinical management of high-risk patients.

Materials and Methods

Patients and database

The records of 65 consecutive patients with stage I to IV HCC who underwent primary tumor resection at the Medstar/Georgetown University Hospital/Lombardi Comprehensive Cancer Center (Washington, DC) between 2006 and 2015 were reviewed. Postoperative treatment was in accord with generally accepted guidelines, and the mean duration of follow-up was 39.7 months (range, 9–84 months). RFS was defined as the length of time from resection of the primary tumor to documented disease progression. Patient tumor samples were obtained from the Ruesch Center for the Cure for Gastrointestinal Cancers’ Tissue Bank at Lombardi. Patients provided consent for tissue banking prior to surgical resection of their HCC. A secure database with proper safeguards was constructed for the management of patient data. An Institutional Review Board approved the ethical, legal, and social implications of the project.

HCC and semiquantitative analysis

Pathologic slides from paraffin blocks prepared with surgical tissue specimen were stained with monoclonal antibodies to CD3 (polyclonal rabbit anti-human; Dako North America Inc.; catalog no. A0452), CD8 (monoclonal mouse anti-human; Dako North America Inc.; catalog no. M7103), PD-1 (EH12.1 from BD Biosciences/PharMingen; catalog no. 561273), PD-L1 (SP142, Spring Biosciences; catalog no. M4420; SP263, Roche/Ventana Medical Systems; catalog no. 790-4905), PD-L1, HIC-22C3 (companion diagnostics kit antibody from Dako North America, Inc.), and β-catenin (Millipore; catalog no. 05-665). All slides were then stained with hematoxylin and eosin (H&E). Slides stained for CD3+, CD8+, PD-1+, and PD-L1+ cells were first evaluated by a clinical pathologist. The TI, IM, and noncancerous liver parenchyma were marked for biomarker imaging. Expression of β-catenin expression was determined by intensity (0, +1, +2, or +3) and distribution (+1 as less than 10% of cells, +2 as 10%–50% of cells, and +3 as >50% of cells).

Image capture and quantification

After the TI and IM were reviewed, images were captured in areas where TIL density was focally high, as well as in regions where the TIL density was representative of the TI and IM as a whole; this was done under high-power magnification (×20). Images were captured as a spectral cube with a Nikon E600 Epifluorescence microscope and Nuance FX Multiplex Biomarker Imaging. For both CD3+ and CD8+ stained slides, two images of high cell density each were taken of the TI and IM. Computer-assisted image analysis was conducted using ImageJ v1.48, a public domain, Java-based, image processing program developed at the NIH. Five built-in functions of ImageJ were used to measure the number of positively stained cells per tissue surface unit in square millimeters (see Supplementary Fig. S1 for a flowchart of the image analysis process, Supplementary Fig. S2 for an example of images obtained using computer-assisted evaluation of infiltrate densities, and Supplementary Fig. S3 for ImageJ instructions and plug-in code).
The median immune cell density was used to stratify patients into groups based on the degree of tumor infiltration. This method was independently validated by Angell and Galon in their evaluation of the Immunoscore in colorectal cancer and was thus adopted for the current study (22). The cutoff threshold for CD3⁺ and CD8⁺ cell density was determined to be 273 cells/mm² and 217.5 cells/mm², respectively (Supplementary Fig S4). Based on the established threshold, each patient was given a binary score (0 as low, 1 as high) for each immune cell type (CD3⁺ and CD8⁺) in each tumor region (TI and IM). An Immunoscore for each patient was obtained by summation of the four binary score values, the scale being from 0 to 4. Five patient groups were defined: patients with low densities of CD3⁺ and CD8⁺ T cells in both tumor regions (All-Low) were classified as Im0; patients with one high (1-High) density for one marker were classified as Im1; and patients with two (2-High), three (3-High), and four (All-High) among these two markers were classified as Im2, Im3, and Im4, respectively. Patients with a high degree of intratumoral immune cell infiltration were those with an Immunoscore of 3+, and patients with a low degree of immune cell infiltration were those with an Immunoscore of ≤2. The corresponding Immunoscore values were correlated with tumor recurrence and RFS, as well as being compared with the pathologic factors currently indicative of tumor invasiveness, such as vascular invasion, elevated α-fetoprotein (AFP), and advanced pTNM stage.

Statistical analysis

Descriptive statistics were used to summarize patients' demographic information. The χ² test or Fisher exact test was used to examine the association between categorical variables. For analysis, patients were grouped by CD3⁺ density, CD8⁺ density, and Immunoscore. The nonparametric Kaplan–Meier methodology was used to analyze the RFS time, and the log-rank test was used to compare survival curves. Multivariate logistic regression models were fit to look at the effect of CD3⁺ and CD8⁺ density on the odds of tumor recurrence, when adjusting for other significant covariates.

Results

Demographics of HCC patients treated with primary surgery

Demographic and clinical characteristics of the registered cohort treated at MedStar Georgetown University Hospital are shown in Table 1. The mean age was 61 years (range, 30–86), and the patient group was predominantly male (77%). Regarding the etiology of HCC, 16.9% of patients had viral hepatitis B (HBV), 32.3% had viral hepatitis C (HCV), 12.3% of patients had coinfection of HBV and HCV, and 38.4% of the patients had neither virus. Using the Kaplan–Meier methodology, the estimated probability of RFS following HCC was found to be 63% at 1 year and 59% at 3 years, and the estimated probability of OS was 82% at 1 year and 57% at 3 years.

Pathology review and IHC characteristics

Slides stained with CD3 and CD8 antibodies revealed a wide spectrum of immune infiltrate densities in the TI and IM tumor regions (Fig. 1 and Fig. 2A, B, E, and G). The H&E stain of the tumor is shown in Fig. 2D, and the negative control for CD3 and CD8 staining are shown in Fig. 2F and H, respectively. The pattern of TILs in each region fell into 3 categories: (i) dense nodular clusters of lymphocytes, (ii) diffuse, evenly distributed lymphocytes, and (iii) rare or scattered lymphocytes (see Fig. 1A, B, and E, respectively). Clusters of lymphocytes are found from the IM to the TI in the first two patterns of TILs (Fig. 1C and D, migration marked by red arrows), whereas there are no obvious TILs between the IM and TI in the third pattern of TIL (Fig. 1F). Bivariate analysis showed no statistically significant association between the pattern of TIL and TNM staging (P = 0.219 for CD3, 0.377 for CD8), level of differentiation (P = 0.054 for CD3, 0.283 for CD8), or vascular invasion (P = 0.096 for CD3, 0.104 for CD8; Supplementary Fig S5). Similarly, the pattern of TILs showed no association with RFS or OS in either immune cell type (Supplementary
Tumors with different staging, differentiation, and vascular invasion were found to have either pattern of TILs. The number of PD-1–positive TILs varied from case to case, ranging from 0 to >1,000 cells per ×10 magnification field. A moderate number of PD-1–positive TILs (about 160 cells per ×20 magnification field) were both within the TI and at the IM (Fig. 2K). In the 60 HCC samples evaluated, only two samples showed positive PD-L1 stain in cancer cells; 19 samples showed PD-L1 expression within or around the tumor, but in nonmalignant (noncancer) cells (Fig. 2C, I, and J), presumably mononuclear cells such as macrophages, dendritic cells, and lymphocytes; and 39 samples showed no PD-L1 stain. The IHC analysis of PD-L1 expression using three antibodies (SP142, SP263, and PD-L1_IHC_22C3) gave identical results. PD-L1 expression showed a positive correlation with CD3+ and CD8+ cell densities. Fourteen of 31 HCC samples with high CD3+ density showed high CD8+ cell density (Fisher exact test $P = 0.0055$). IHC analysis of β-catenin expression showed a variable level of cytoplasmic, membranous, and nuclear staining (Fig. 2L). Expression of β-catenin and CD3+ or CD8+ cell density was not correlated ($P = 0.432$, $P = 0.227$, respectively, as shown in Supplementary Fig. S7).

**Association between clinical risk factors and recurrence**

Established risk factors for HCC invasiveness include tumor size, tumor stage, tumor grade, serum AFP level, cellular differentiation, HCV or HBV infection, vascular invasion, and the presence of liver cirrhosis (26–29). These elements were evaluated in our HCC patient cohort. However, bivariate analysis revealed that only tumor cell differentiation, grade, and the presence of vascular invasion were significantly correlated with HCC recurrence (Table 2). Patients with elevated serum AFP and advanced tumor stage showed an increased rate of recurrence; however, the association was not statistically significant. Tumor stage did not correlate with recurrence, but correlated with vascular invasion and cellular differentiation (as shown in Supplementary Fig. S8). Patients with well-differentiated tumors experienced a significantly reduced incidence of HCC recurrence: 5% of well-differentiated tumors recurred after primary resection compared with 33% and 48% for moderate and poorly differentiated tumors, respectively ($P = 0.011$). Furthermore, patients with HCC whose tumors did...
not reveal vascular invasion also faced a reduction in the rate of recurrence: those exhibiting tumor vascular invasion recurred in 48% of cases compared with only 22% in those without vascular invasion (\(P = 0.040\)).

Impact of the immune response on recurrence and RFS

The densities of total (CD3\(^+\)) and cytotoxic T (CD8\(^+\)) TILs were examined in the TI and IM regions. Statistically significant associations were observed between the individual densities of CD3\(^+\) and CD8\(^+\) cells in the distinct tumor regions and frequency of HCC recurrence (Table 2). Patients with a high density of CD3\(^+\) immune infiltrates in the TI and IM experienced recurrence of their HCC in only 15% of cases compared with 44% in those with a low CD3\(^+\) cell density (\(P = 0.027\)). Similarly, 15% of patients with a high density of CD8\(^+\) immune infiltrates in the TI and IM experienced recurrence of their HCC compared with 45% in those with a low CD8\(^+\) cell density (\(P = 0.014\)). According to the Immunoscore grouping method outlined above, 27%, 14%, 16%, 16%, and 27% of the patients were classified as Im0, Im1, Im2, Im3, and Im4, respectively. The frequency of HCC recurrence in each Immunoscore subgroup was as follows: 65% for Im0, 22% for Im1, 10% for Im2, 10% for Im3, and 11% for Im4. This combined analysis revealed that patients with a high density of CD3\(^+\) and CD8\(^+\) cells in one or both tumor regions (TI or IM) experienced a significant reduction in the rate of HCC recurrence (\(P = 0.007\)). In keeping with the positive correlation between PD-L1 expression and CD3\(^+\) and CD8\(^+\) TIL density, the lack of PD-L1 expression also predicts higher risk of HCC recurrence. Fifteen of 39 HCC samples lacking PD-L1 expression recurred, while only 2 of 19 HCC samples with PD-L1 expression recurred (\(P = 0.034\)).

The densities of CD3\(^+\) and CD8\(^+\) immune cell populations were also examined in relation to RFS times, our primary endpoint. Different permutations of CD3\(^+\) and CD8\(^+\) cell populations were used in the analysis: CD3\(^+\) density in each tumor region alone, CD8\(^+\) in each tumor region alone, total CD3\(^+\) density in the TI + IM, total CD8\(^+\) density in the TI + IM, and the combined Immunoscore measuring both cell types in both tumor regions (Fig. 3). Kaplan–Meier survival curves for average CD3\(^+\) (Fig. 3A and B) and CD8\(^+\) (Fig. 3C and D) cell density in each tumor region alone were first assessed. When each cell population (CD3TI, CD3IM, CD8TI, CD8IM) was analyzed independently of one another, RFS time between patients with high...
Figure 3.
Kaplan–Meier curves comparing RFS in patients with high and low intratumoral CD3 cell density in the TI (A) and IM (B), as well as high and low intratumoral CD8 cell density in the TI (C) and IM (D). Patients were stratified in high and low groups using median immune cell density as the threshold. Kaplan–Meier curves comparing RFS in patients with high and low total intratumoral CD3 density (CD3TI + CD3IM) and total CD8 density (CD8TI + CD8IM), using median cutoff points as the threshold (E and F, respectively). Kaplan–Meier curves comparing RFS in patients with different Immunoscores (G). Kaplan–Meier curves comparing RFS in patients with and without PD-L1 expression (H).
Intratumoral CD3/CD8 Density Predicts Relapse in HCC

Figure 4.
Kaplan–Meier curves comparing OS in patients with high and low intratumoral CD3 cell density in the TI (A) and IM (B), as well as high and low intratumoral CD8 cell density in the TI (C) and IM (D). Patients were stratified in high and low groups using median immune cell density as the threshold. Kaplan–Meier curves comparing OS in patients with high and low total intratumoral CD3 density (CD3TI + CD3IM) and total CD8 density (CD8TI + CD8IM), using median cutoff points as the threshold (E and F, respectively). Kaplan–Meier curves comparing OS in patients with different Immunoscores (G). Kaplan–Meier curves comparing OS in patients with and without PD-L1 expression (H).
and low immune cell density in either region were statistically significantly different (CD3TI P = 0.044, CD3IM P < 0.001, CD8TI P = 0.002, CD8IM P = 0.01). RFS was then correlated with the total CD3+ cell density, total CD8+ cell density and the corresponding Immunoscore value. The Kaplan–Meier curves (Fig. 3E) revealed a strong positive association between high total CD3+ cell density TI+IM and prolonged RFS (P = 0.006). The Kaplan–Meier curves (Fig. 3F) for total CD8+ cell density TI+IM yielded a similar but less significant association with RFS (P = 0.01). Lastly, Kaplan–Meier curves for the Immunoscore were constructed to provide a more comprehensive picture of the two immune cell populations as they relate to survival. The curves (Fig. 3G) show three distinct patient groups with statistically significant differences in RFS times (P = 0.002). Patients with an Immunoscore of 0 experienced the worst postoperative outcome, with all patients having disease recurrence within 2 years of follow-up. The Immunoscore remained significantly associated with recurrence and RFS, whereas several clinicopathologic factors, including TNM staging, did not reach significance. PD-L1 expression was also examined in relation to RFS times. The RFS time between patients with and without PD-L1 expression was significantly different (Fig. 3H; P = 0.029).

**Immunoscore predicts risk of recurrence**

Analysis of possible links between immune infiltration and clinical risk factors is shown in Table 3. Viral hepatitis B and C were the predominant etiologies for HCC in our study population; however, the densities of CD3+ and CD8+ TILs among patients with or without HBV and HCV infection were not significantly different. Similarly, vascular invasion, cellular differentiation, and cancer stage showed no significant association with CD3+ and CD8+ cell densities in the TI and IM (Table 3). The expression of PD-L1 showed no significant association with vascular invasion, cellular differentiation, or tumor stage (Table 3). Because vascular invasion and differentiation were the only clinical risk factors that strongly correlated with HCC recurrence in our bivariate analysis, we focused on these factors in the multivariate analysis.

Table 2. Association between HCC recurrence and patient demographics and clinicopathologic factors

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In order to assess the impact of CD3⁺ and CD8⁺ cell densities on the tumor recurrence, when adjusting for the significant covariates in the bivariate analysis (specifically, vascular invasion and differentiation), multivariate logistic regression models were fit. The adjusted odds ratio (OR) of HCC recurrence was 5.8 [95% confidence interval (CI), 1.6–21.8] for CD3⁺ cell density. Similarly significant results were obtained for CD8⁺ density (the adjusted OR was 3.9; 95% CI, 1.1–14.1). The results showed that after adjusting for other covariates, CD3⁺ and CD8⁺ cell densities remained as significant risk factors for predicting tumor recurrence. Thus, the Immunoscore could predict risk of recurrence independently of the only risk factors that demonstrated predictive value (vascular invasion and cellular differentiation) in our data set. As a result, the Immunoscore seems to be a highly significant prognostic factor in the cohort of patients with HCC treated with primary surgery.

Given the great impact of CD3⁺ and CD8⁺ cell densities, and PD-L1 expression on tumor recurrence and RFS, we examined the relation between CD3⁺ and CD8⁺ cell density and OS. We found that TIL or PD-L1 expression density and OS in patients after curative resection were not significantly correlated (Fig. 4).

### Discussion

HCC is one of the most common forms of cancer and remains the second leading cause of cancer-related death worldwide (26). In areas with a high prevalence of HCC and a limited organ supply, surgical resection of HCC in patients with well-compensated liver function remains the primary curative treatment option. Unfortunately, the rate of HCC recurrence after surgical intervention is high, and no effective treatment is available to lower the risk of recurrence. Identification of biomarkers that predict recurrence is urgently needed. With a better understanding of the mechanism by which cancer recurs, new therapeutic targets that prevent recurrence may be revealed. As previously reported, the clinicopathologic factors, namely, serum AFP (27), vascular invasion (28, 29), and tumor stage (30), can predict recurrence and poor outcome. However, these factors focus specifically on the differences among HCC tumors and do not account for the interaction between the tumor and the host immune response. Improved prognostic and predictive markers for HCC recurrence after primary resection that incorporate this interaction are needed.

Our study addresses this need. We showed that the intratumoral immune cell densities of CD3⁺ and CD8⁺ cells with their associated Immunoscore were significantly associated with recurrence and RFS independently of other predictive clinicopathologic factors, such as vascular invasion and cellular differentiation. The effect of CD3⁺ and CD8⁺ density in predicting recurrence was statistically significant when adjusting for other covariates in the multivariate logistic model, even with the small sample size of the study.

Combined analysis revealed that patients with a high density of CD3⁺ and CD8⁺ cells in one or both tumor regions (TI or IM) experienced a significant reduction in the rate of HCC recurrence (P = 0.002). The Kaplan–Meier curves for CD3⁺ and CD8⁺ cell populations combined as an Immunoscore define three groups with distinct RFS times (based on Immunoscores 0, 1, and 2, and 3 and 4). Whereas 65% of patients with an Immunoscore of 0 experienced recurrence, 22% of patients with an Immunoscore of 1 or 2, and only 10% of patients with high Immunoscores of 3, 4, or 5 experienced recurrences. This suggests that both CD3⁺ and CD8⁺ cell densities and RFS.
and CD8+ cell populations contribute to the antitumor immune response, and the high density of both CD3+ and CD8+ T cells infiltrating the tumor generates a better antitumor response in the prevention of cancer recurrence compared with high densities of CD3+ or CD8+ T cells alone. Future studies with a larger sample size and an independent cohort of patients are warranted to confirm these findings.

Previous studies have demonstrated an association between the presence of ectopic lymph node-like structures residing within the parenchyma of solid tumors and improved survival times in patients with colorectal cancer (31, 32). In our current study focusing on HCC, we observed high densities of TILs in dense nodular clusters as well as diffuse, evenly distributed populations. Both arrangements of TILs seem to be protective in HCC. In the small dataset, the strongest predictor of no cancer recurrence was a high density of CD3+ and/or CD8+ cells within the parenchymal and invasive margin of the tumor. A possible difference in the amount of protection may exist between nodular clusters and the diffuse type of TILs, but this was not evident in our dataset. It is also possible that the mixed population of immune cells flanking the tumor was sufficient to generate an immune response against cancer, and that the presence of ectopic lymph node-like structures in the form of dense nodular clusters was not required for an antitumor immune response in HCC.

It was surprising that only 2 of 60 HCC cases showed positive PD-L1 staining in cancer cells from biopsy samples. Nineteen samples showed PD-L1 expression in or around the tumor but only in nonmalignant (noncancer) cells, presumably mononuclear cells such as macrophages, dendritic cells, and lymphocytes. PD-L1 expression was positively correlated with CD3+ and CD8+ TIL density. Tumor escape from immune-mediated destruction has been associated with immunosuppressive mechanisms that inhibit T-cell activation. It has been reported that a subset of T cell–inflamed tumors showed high expression of three defined immunosuppressive mechanisms: indoleamine-2,3-dioxygenase (IDO), PD-L1/B7-H1, and Foxp3+ regulatory T cells (Treg). This suggests that these inhibitory pathways might serve as negative feedback mechanisms that follow, rather than precede, CD8+ T-cell infiltration (33). Mechanistic studies in mice revealed that upregulated expression of IDO and PD-L1, as well as recruitment of Tregs in the tumor microenvironment depended on the presence of CD8+ T cells. The former was driven by IFNγ, and the latter by a production of CCR4-binding chemokines along with a component of induced proliferation. Cancer immunotherapy approaches that target negative regulatory immune checkpoints, such as PD-L1, have been shown to preferentially benefit patients with a preexisting T cell–inflamed tumor microenvironment (21). Furthermore, high PD-L1 expression has been correlated with improved response to immune checkpoint inhibitors, compared with low PD-L1 expression (34). High PD-L1 expression in nonmalignant cells in or around the tumor, with no expression in malignant HCC cells, may be correlated with improved response to immune checkpoint inhibitors. Nivolumab, a fully human IgG4 monoclonal antibody PD-1 inhibitor, is currently being evaluated in patients with advanced HCC (NCT01658878), and was reported to have promising clinical activity (35). Biomarker analysis for the HCC samples in this study may be available in a few years.

High densities of immune cell infiltrates appeared to decrease the risk of cancer recurrence, yet the reason why some patients have high densities of immune cell infiltrates and others do not is not well understood. It has been speculated that both host and tumor contribute to the infiltration of immune cells. Accordingly, functional polymorphisms in Fas and Fas ligand (Fasl) of the host T lymphocytes were reported to contribute to increased apoptosis of TILs and enhanced risk of breast cancer progression (36). Polymorphisms that lead to constitutive expression of death receptor ligands, such as FasL, may cause tumor cells to adopt an activation-induced cell death mechanism (induction of apoptosis of previously activated T cells on subsequent encounters with an antigen) to delete the attacking antitumor T cells through the induction of apoptosis via death receptor and death receptor ligand interactions (37).

Analysis of human melanoma cell lines has revealed that many tumors without immune infiltration show alterations in the Wnt/β-catenin signaling pathway (25). Tumor cells with active β-catenin showed reduced expression of the chemokines CCL4 and CXCL1 and were thus associated with a diminished antitumor T-cell response (25). The effect of tumor β-catenin on the antitumor T-cell response may be tumor type–dependent because the HCC data in this study demonstrate no association between β-catenin expression and TIL density. However, we did not measure β-catenin activity, which might differ from its expression. TILs in glioblastoma were also associated with specific genomic alterations, and TILs were enriched in glioblastomas of the mesenchymal class, strongly associated with mutations in NF1 and RB1. Conversely, TILs were depleted in epidermal growth factor receptor–amplified and homozygous PTEN-deleted tumors (38). The heterogeneity of immune cell infiltration among patients may be a consequence of many such genomic alterations and may differ depending on the tumor type.

In addition, the high mutational frequency found within tumors raises the possibility that cells may preferentially invade tumors in patients whose T cells recognize mutated epitopes found within the tumor tissue. The rate of somatic mutations seems to correlate with tumor response to immune checkpoint blockers (39). Characterizing commonly mutated epitopes may provide targets for novel antitumor vaccines (40).

Identification of the defects that lead to low intratumoral immune cell density may also provide novel therapeutic targets for anticancer treatment. Type I INFs are implicated in the innate immune sensing of immunogenic tumors, leading to adaptive T-cell responses (41). A recent breakthrough in cancer immunology suggests that the stimulator of interferon genes (STING) complex is required for sensing of cancer cell–derived DNA and activation of dendritic cells, leading to CD8+ T-cell priming against tumors (42). The defective STING signaling pathway may result in low intratumoral immune cell density and a poor antitumor immune response. Multiple STING activators are being developed in promoting innate immune sensing and adaptive T-cell response against cancer (43). The combination of STING activators with inhibitors to immune checkpoints and/or antitumor vaccines may provide promising strategies for cancer treatment. The efficacy of immune checkpoint inhibition against HCC has been suggested in a phase I clinical trial that evaluated anti–PD-1 therapy (nivolumab), and a phase II clinical trial that evaluated an anti–CTLA-4 therapy (tremelimumab) in patients with HCC (35, 44). It is possible that immune infiltrates are not only prognostic, but predictive as well; identifying patients whose tumors might be sensitive to immunotherapy. Immune checkpoint inhibitors
would be of particular interest in these patients and could be evaluated in prospective clinical trials.

Disclosure of Potential Conflicts of Interest
F. Banovac reports receiving speakers bureau honoraria from Onyx Inc. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments
The authors thank Marion Hartley, PhD, for editing assistance.

Grant Support
This work was supported by American Cancer Society grant 118525-MRSG-10-068-01-TBE (A.R. He). The experiments were carried out with the help of Georgetown University Medical Center’s (GU/MC) Shared Resources (including histopathology, microscopy and imaging, genomics and epigenomics, and flow cytometry), which was supported by NIH-P30 CA51008 and NCATS 8 U11 TR000101.

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Received April 20, 2015; revised December 21, 2015; accepted January 6, 2016; published OnlineFirst March 11, 2016.

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Intratumoral CD3 and CD8 T-cell Densities Associated with Relapse-Free Survival in HCC

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doi:10.1158/2326-6066.CIR-15-0110

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