Am J Cancer Res 2021;11(9):4485-4499
www.ajcr.us /ISSN:2156-6976/ajcr0134545

Original Article

Decreased granzyme B$^+$CD19$^+$B cells are associated with tumor progression following liver transplantation

Han Li[1*], Xian-Liang Li[2*], Shuang Cao[2], Ya-Nan Jia[2], Ruo-Lin Wang[2], Wen-Li Xu[2], Ren Lang[2], Qiang He[2], Ji-Qiao Zhu[2]

[1]Department of Head and Neck Surgery, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China; [2]Department of Hepatobiliary and Pancreaticosplenic Surgery, Medical Research Center, Beijing Organ Transplant Center, Beijing Chaoyang Hospital, Capital Medical University, No.8 Gongtinan Road, Chaoyang District, Beijing 100020, China. [*]Equal contributors.

Received April 22, 2021; Accepted July 20, 2021; Epub September 15, 2021; Published September 30, 2021

Abstract: Lymphocytes play an important role in antitumor immunity following organ transplantation. However, the function of granzyme B$^+$CD19$^+$B cells on the hepatocellular carcinoma cells from liver transplant recipients remains largely unknown; we aimed to analyze the function and elucidate the mechanisms behind it. Blood samples and clinical data from liver transplant recipients and healthy controls at Beijing Chaoyang Hospital as well as from a validation cohort were collected and analyzed. In this study, we found decreased granzyme B$^+$CD19$^+$B cells were correlated with early hepatocellular carcinoma recurrence and could further identify liver transplant recipients with poor tumor differentiation, microvascular invasion, increased total tumor diameter, and tumor beyond Milan criteria. Notably, granzyme B$^+$CD19$^+$B cells directly inhibited the proliferation, migration, and invasion of hepatocellular carcinoma cells. Upon activation regulatory B cells from liver transplant recipients with hepatocellular carcinoma recurrence displayed a CD5$^+$CD38$^+$CD27$^+$CD138$^+$CD19$^+$ granzyme B$^+$ phenotype, but the increased expression of CD5, CD38, and CD138, and the decreased protein level and transcriptional level requiring JAK/STAT signaling. In an independent validation cohort, liver transplant recipients with decreased granzyme B$^+$CD19$^+$B cells had not only early hepatocellular carcinoma cell recurrence but also shorter survival. Our study provides comprehensive data from liver transplant recipients with hepatocellular carcinoma, indicating a critical role of granzyme B$^+$CD19$^+$B cells in preventing cancer progression. Our findings warrant further investigations for the design of future immunotherapies leading to immune responses and improved patient survival.

Keywords: Granzyme B, hepatocellular carcinoma, liver transplantation, recurrence, CD19$^+$B cells

Introduction

Liver transplantation offers the highest chance of cure for hepatocellular carcinoma (HCC) among all other methods as it treats not only the tumor but also the underlying liver disease [1, 2]. However, liver transplant recipients (LTR) have been plagued by HCC recurrence following transplantation, which ranges between 8%-20% [3]. Unlike HCC recurrence after surgical resection, extrahepatic metastases are more seen following transplantation, particularly in the lungs and bones [4-6]. Studies have shown that post-transplant HCC recurrence is a result of the growth of occult metastases indicating that circulating tumor cells are the major route of HCC recurrence [7, 8]. Therefore, peripheral immune responses play a key role in HCC progression requiring further elucidation [9, 10].

B cells were once considered to act as antigen-presenting cells and provide co-stimulatory signals for T cell activation in addition to antibody production. In 2006, Jahrsdörfer B et al. first described that B-chronic lymphocytic leukemia (B-CLL) cells were characterized by inducing apoptosis of untreated B-CLL cells through granzyme B (GrB) production [11]. Subsequently, in patients with operationally tolerant kidney grafts, B cells exerted a suppressive function on CD4$^+$CD25$^-$ effector T cell by secreting

Table 1. Characteristics of liver transplant recipients and healthy controls

Parameters	Liver transplant recipients		Healthy controls (n=41)	P
	Malignant (n=57)	Benign (n=45)		
Age	51.26±7.78	53.27±7.02	50.76±7.72	0.253
Sex (male)	49	34	29	0.169
Maintenance				0.562
Tacrolimus	47	39		
Cyclosporin A	10	6		
Recurrence (cases)	22			
Tumor-free survival (months)	22.09±13.06	47.09±26.74		0.000

GrB [12]. We also confirmed GrB+CD19+B cells could maintain allospecific tolerance and enhance viral control in renal transplant recipients in a previous study [13]. Furthermore, studies reported that B cells infiltrating the tumor correlated with an increased GrB expression in addition to with reduced tumor viability suggesting B cells possessing tumor-killing potential by producing GrB [14, 15].

However, there is little data on the relation between GrB+CD19+B cells and LTR survival or HCC recurrence following liver transplantation. Here, we examined the role of GrB+CD19+B cells from LTR with and without HCC recurrence as well as from healthy controls (HC) and LTR with hepatic failure (HF) and further confirmed our findings in a validation cohort.

Materials and methods

Patients and samples

A total of 102 liver transplant recipients with HF and with pathologically confirmed HCC were enrolled in this study as well as 41 HC. Characteristics are summarized in **Table 1**. Another validation cohort was further enrolled between January 2016 and December 2020. There were neither ABO incompatible donors nor living donors. The blood samples were taken at least 6 months following liver transplantation. Basiliximab was administered for immunosuppressive induction. LTR were given a low dose of calcineurin inhibitors. Steroids were not used or gradually withdrawn within one month. Mycophenolate mofetil was adjusted according to the white blood cell count. Sirolimus was used one month after surgery.

Inclusion criteria: patients with pathologically confirmed hepatic carcinoma and hepatitis related cirrhosis; patients undergoing a first liver transplantation; no distant metastasis; patients with complete clinical and pathological data. Exclusion criteria: patients with HCC receiving anticancer therapy before sampling; patients with concurrent autoimmune disease; patients with any kind of infections, acute rejection and graft-versus-host disease; patients with diseases of hematopoietic and lymphoid systems and human immunodeficiency virus; patients undergoing combined organ transplantation.

The study was approved by the Institutional Review Board of Beijing Chaoyang Hospital (No.2016-2-19-38) in accordance with the Helsinki declaration of 1975, as revised in 1983. Written informed consent was obtained from all participants.

Flow cytometry, polymerase chain reaction, western blot and tumor cell cultivation

According to the instructions of the manufacturers, flow cytometry, polymerase chain reaction (Table S1), Western blot and tumor cell cultivation were performed, respectively (Supplementary Information).

Statistical analysis

Data analyses were carried out by using SPSS 19.0 computer software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Values were expressed as mean ± standard deviation. The Kolmogorov-Smirnov test was used for the normal distribution of continuous variables and one-way analysis of variance for the differences of multi-groups. The independent samples t-test was employed for quantitative variables. The Mann-Whitney U test was

selected due to non-normal distribution. The Chi-square or Fisher's exact test was used to compare nominal variables. Receiver operating characteristic curve (ROC) analysis and comparison of the area under the curve (AUC) was performed. The cut-off value for positive parameters was further determined by optimal sensitivity and specificity on ROC curve analysis. Multivariate Cox analysis was employed to determine the predictors. Relative risk was expressed as an odds ratio (OR) with a 95% confidence interval (CI). A *P*-value <0.05 was considered statistically significant.

Results

Decreased GrB$^+$CD19$^+$B cells are correlated with early HCC recurrence

First, we detected the GrB expression in CD19$^+$B cells from blood samples of LTR under varying conditions. Based on the previous study [13], we found CD19$^+$B cells started to produce GrB upon activation. A combination of anti-BCR and IL-21 is the most potent stimulus to induce GrB production (Figure S1A). Then, we found there was no significant difference among the groups with respect to GrA production or perforin production in resting CD19$^+$B cells (P>0.05, Figure S1B, S1C). Moreover, both GrA production and perforin production remained similar when CD19$^+$B cells were stimulated with anti-BCR and IL-21 (P>0.05, Figure S1D-F).

Subsequently, we wanted to compare the percentages of GrB$^+$CD19$^+$B cells in HC and LTR. GrB was weakly expressed in a small portion of resting B cells in both groups (P>0.05, **Figure 1A**). After stimulation, we found the frequency of GrB expression was higher in HC (P<0.05, **Figure 1B**). We further checked the percentages of GrB$^+$CD19$^+$B cells between LTR with HF and with HCC. Of note, LTR with HCC showed a lower proportion of GrB$^+$CD19$^+$B cells in activated B cells (P<0.05, **Figure 1D**) although there was no difference in resting B cells (P>0.05, **Figure 1C**). Finally, we analyzed the data from patients with HCC recurrence and without HCC recurrence. LTR with and without HCC recurrence had similar GrB production in resting B cells (P>0.05, **Figure 1E**). More importantly, upon activation LTR with HCC recurrence had a remarkably lower proportion of GrB$^+$CD19$^+$B cells (P<0.05, **Figure 1F**, **1G**).

After further analysis, a higher proportion of GrB$^+$CD19$^+$B cells were observed when patients with HCC met the Milan criteria, which is the golden criteria for liver transplantation (P<0.01, **Figure 1H**). To assess the effect of the percentages of GrB$^+$CD19$^+$B cells on tumor-free survival, we divided the patients into two groups (high frequency >25.46% and low frequency <25.46%) using the mean value of the percentages of GrB$^+$CD19$^+$B cells calculated according to the patients with and without HCC recurrence. Surprisingly, nearly 2.3 times more patients with low frequency than those with high frequency showed early recurrence (58.6% vs. 17.9%; **Figure 1I**). Collectively, these data indicate that decreased percentages of GrB$^+$CD19$^+$B cells correlate with early recurrence in LTR.

Decreased GrB$^+$CD19$^+$B cells can identify LTR with progressive HCC

Given that our data demonstrated a strong association between HCC recurrence and decreased GrB$^+$CD19$^+$B cell production, we wondered whether clinical-pathological parameters, which differentiated patients with HCC recurrence from without HCC recurrence, could have an impact on GrB expression. First, we compared the clinical parameters to check their effect. As patients with HCC had a stable preoperative liver function, the model for end-stage liver disease score was similar between LTR with and without HCC recurrence (P>0.05, Figure S2A). Although high exposure to them can increase the risk of HCC recurrence [16], calcineurin inhibitors are still our major choice due to their effective suppression. Therefore, neither the type of calcineurin inhibitors nor the tacrolimus trough level affected HCC recurrence (P>0.05, Figure S2B, S2C). HCC is commonly associated with chronic hepatitis and liver cirrhosis in China, which prevailed in both groups. Accordingly, they failed to have an impact on HCC recurrence (P>0.05, Figure S2D, S2E). Notably, preoperative transcatheter arterial chemoembolization and hepatitis recurrence which was reported to correlate with HCC recurrence [17, 18] showed a negative association as a result of a partial pathologic response and tenofovir disoproxil fumarate treatment [19], respectively (P>0.05, Figure S2F, S2G). Interestingly, levels of postoperative AFP remained similar between LTR with and without

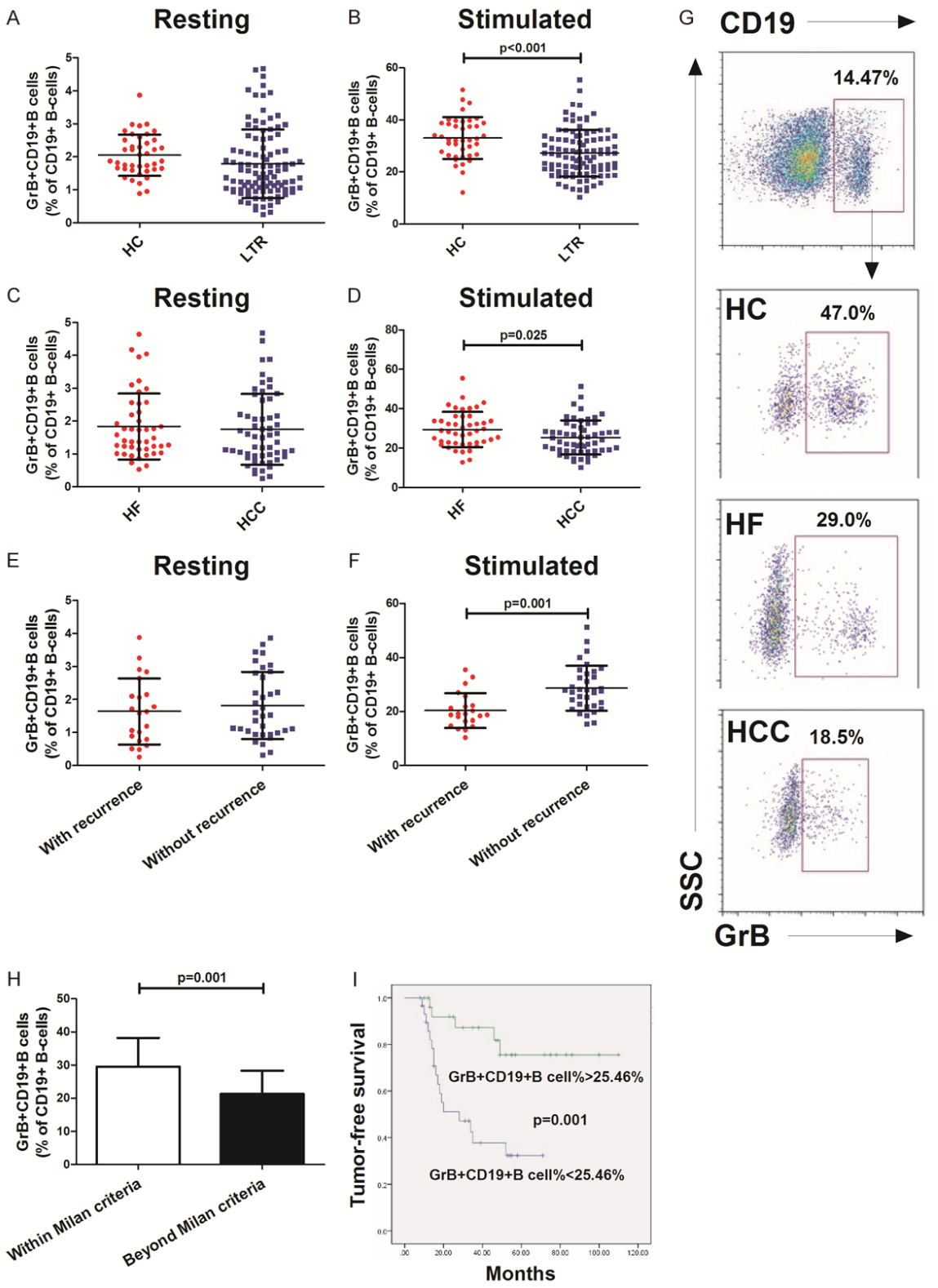


Figure 1. Decreased GrB+CD19+B cells are correlated with early HCC recurrence. Comparison of the expression of GrB in resting (P>0.05, A) and activated (P<0.01, B) CD19+B cells from HC and LTR, respectively; Comparison of the expression of GrB in resting (P>0.05, C) and activated (P<0.05, D) CD19+B cells from LTR with HF and with HCC, respectively; Comparison of the expression of GrB in resting (P>0.05, E) and activated (P<0.01, F) CD19+B cells from LTR with and without HCC recurrence, respectively; Representative flow cytometry dot plots of the expression of GrB

in activated CD19+B cells from HC and LTR with HF and with HCC, respectively (G). Comparison of the percentages of GrB+CD19+B cells between tumor within and beyond Milan criteria (P<0.01, H); Kaplan-Meier curve analysis of tumor-free survival between high and low percentages of GrB+CD19+B cells using the mean value of HCC group as the cut-off (P<0.01, I). PBMC were stimulated with IgG+IgM (5.4 μg/ml) in the presence of IL-21 (50 ng/ml) for 20 h and Brefeldin A (1 μg/ml) was added for the last 4 h of incubation. GrB expression in B cells was detected by flow cytometry. Bars represent mean and standard deviation. GrB, granzyme B; HC, healthy controls; LTR, liver transplant recipients; HF, hepatic failure; HCC, hepatocellular carcinoma; PBMC, peripheral blood mononuclear cells.

HCC recurrence (P>0.05, Figure S2H). As the patients were followed up at regular intervals, therefore, HCC recurrence was detected at an early stage.

Then, we analyzed the pathological parameters, which play a key role in HCC recurrence. We found there was no significant difference between LTR with and without HCC recurrence with respect to macrovascular invasion, capsule invasion, or lesions ≥4 (P>0.05, Figure S3A-C). However, LTR with poor tumor differentiation, microvascular invasion, HCC beyond Milan criteria, and larger total tumor diameter had a higher rate of HCC recurrence (P<0.05, Figure S3D-G). Subsequently, we checked the associations between the positive parameters and the percentages of GrB+CD19+B cells. Notably, the percentages of GrB+CD19+B cells correlated with tumor differentiation, microvascular invasion, total tumor diameter (**Figure 2A**), and tumor beyond Milan criteria (P<0.05). More importantly, using poor tumor differentiation, microvascular invasion, total tumor diameter >9 cm, and HCC beyond Milan criteria as the cut-offs to regroup the LTR, respectively, we found LTR meeting these conditions all had lower proportions of GrB+CD19+B cells (P<0.05, **Figures 2B-D**, **1H**).

To test their predictive values we performed the analysis of AUC-ROC for the positive pathological parameters and the percentages of GrB+CD19+B cells (**Figure 2E**), which revealed the latter strongly classified HCC recurrence versus non-recurrence (AUC 0.801, P<0.01) with the cut-off value of 22.45%. The corresponding sensitivity and specificity were 77.3% and 74.3%, respectively. Consequently, LTR with GrB+CD19+B cells <22.45% had shorter tumor-free survival (P<0.01; **Figure 2F**). Collectively, these data reveal that decreased GrB+CD19+B cell production is significantly associated with progressive HCC and has a better predictive value.

GrB+CD19+B cells can directly suppress HCC progression

B cells were previously regarded as antigen-presenting cells and immunoglobin-producing cells. Recently, studies have shown that B cells can exert a suppressive impact on lymphocytes through GrB secretion, which could be partially reversed by PI-9, namely the GrB inhibitor [11, 12]. Furthermore, Shi et al. reported that CD20+B cells in the tumor invasive margin possessed tumor-killing potential by producing GrB [14]. These researches and our data prompted us to find out whether GrB+CD19+B cells can directly suppress HCC progression. Therefore, we chose two HCC cell lines (Hepg2 and Huh7) for co-culture and divided them into three groups including HCC cells alone (group 1), HCC cells + GrB+CD19+B cells (group 2), and HCC cells + GrB+CD19+B cells + PI-9 (group 3). We first detected the effect of GrB+CD19+B cells on HCC cell proliferation. Using CCK8 assays, we found on day 1 the OD values were a little lower in group 1 because group 2 and group 3 had larger cell numbers. On the following days group 1 showed remarkably increased OD values compared with group 2. In contrast, the OD values rose in group 3 after adding PI-9 despite failing to reach significance. All these data meant that CD19+B cells significantly restrained HCC cell growth via GrB secretion (P<0.05, **Figure 3A**, **3B**). We also noticed a reduction in OD values of all groups on day 3, which might be a result of limited space and nutrition. Additional colony formation assay further confirmed that GrB+CD19+B cells could significantly inhibit the proliferation of HCC cells (P<0.05, **Figure 3C**, **3D**). After that, we investigated whether GrB+CD19+B cells affected HCC cell migration and invasion by taking advantage of scratch wound healing assay and transwell cell invasion assay, respectively. We found that group 1 had the higher healing rate (P<0.05, **Figure 3E**, **3F**) and the invasive cell numbers (P<0.05, **Figure 3G**, **3H**) compared

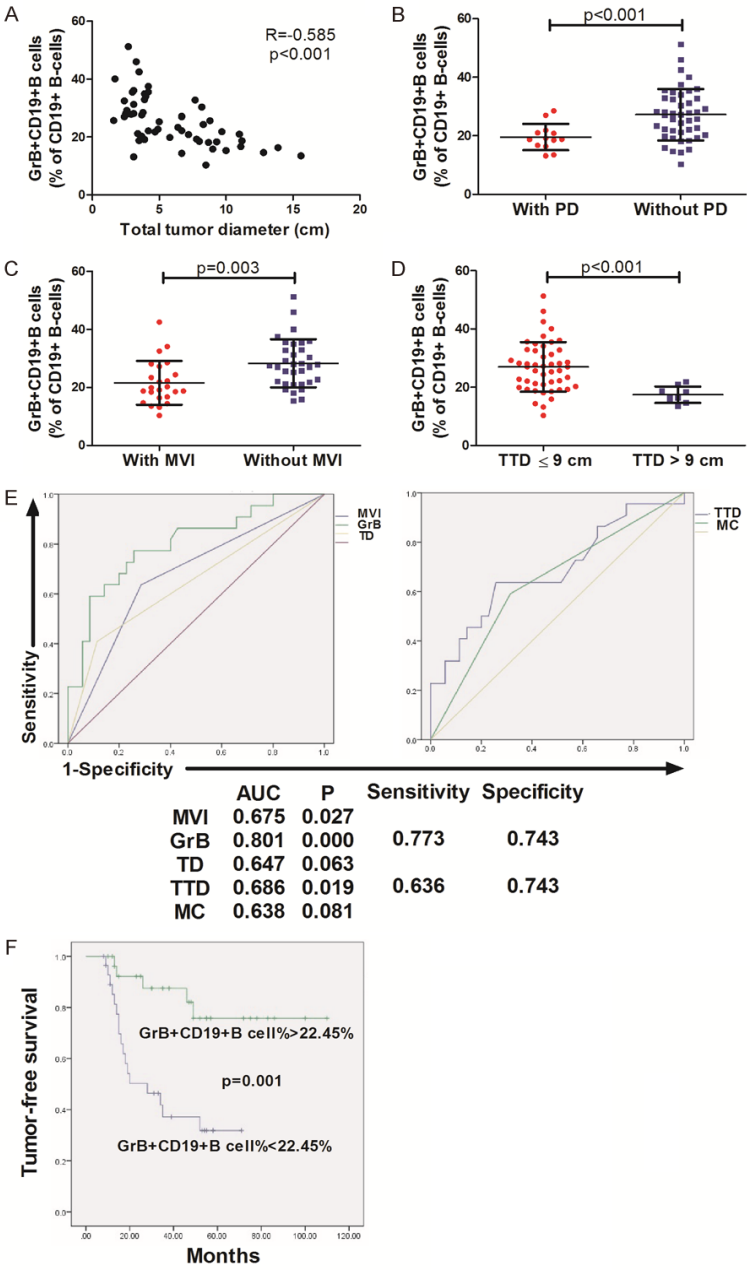
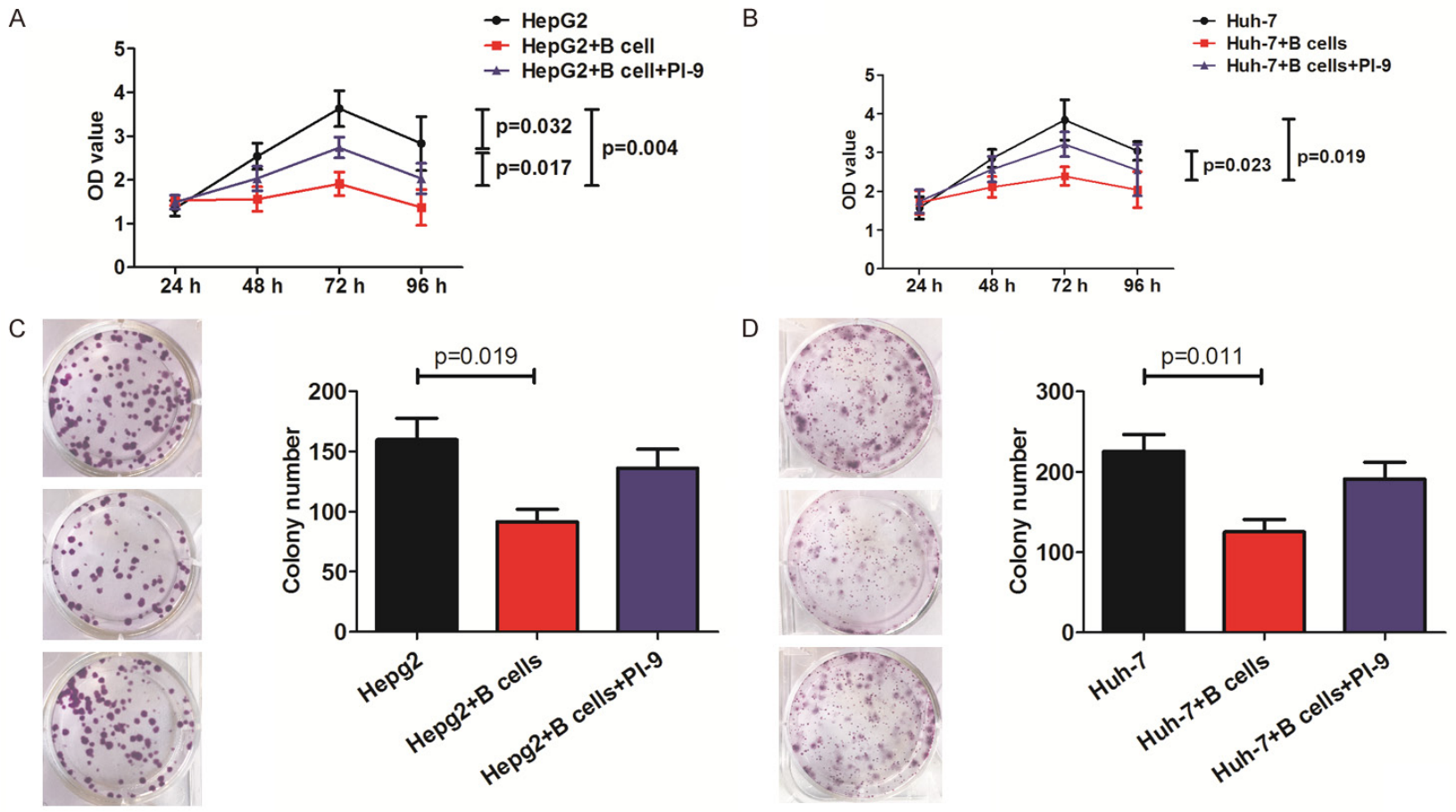


Figure 2. Decreased GrB+CD19+B cells can identify LTR with progressive HCC. Correlations between the percentage of GrB+CD19+B cells and total tumor diameter (P<0.01, A); The percentages of GrB+CD19+B cells between LTR with poor and non-poor tumor differentiation (P<0.01, B), with and without microvascular invasion (P<0.01, C), with total tumor diameter >9 cm and ≤9 cm (P<0.01, D), respectively; ROC curve analysis of microvascular invasion, the percentage of GrB+CD19+B cells, tumor differentiation, total tumor diameter and Milan criteria (E); Kaplan-Meier curve analysis of tumor-free survival between LTR with the percentage of GrB+CD19+B cells >22.45% and <22.45% (P<0.01, F). PBMC were stimulated with IgG+IgM (5.4 μg/ml) in the presence of IL-21 (50 ng/ml) for 20 h and Brefeldin A (1 μg/ml) was added for the last 4 h of incubation. GrB expression in B cells was detected by flow cytometry. Bars represent mean and standard deviation. HCC, hepatocellular carcinoma; GrB, granzyme B; LTR, liver transplant recipients; ROC, receiver operating characteristic; PBMC, peripheral blood mononuclear cells; MVI, microvascular invasion; TD, tumor differentiation; TTD, total tumor diameter; MC, Milan criteria.

with group 2. The results indicated invasion and migration abilities of HCC cells were greatly decreased after co-culture with GrB+CD19+B cells. Furthermore, the suppressive function of GrB+CD19+B cells can be affected in part by PI-9 in accordance with the literature. Collectively, these data indicate that GrB+CD19+B cells directly inhibits the proliferation, migration, and invasion of HCC cells.

Phenotypic characteristics of GrB+CD19+B cells from LTR with HCC

In order to investigate the phenotypic features of this cell subset isolated from LTR with HCC, we assessed the surface molecules on the CD19+B cell population identified on the basis of the GrB production. CD19+B cells were stimulated with IgG+IgM in the presence of IL-21 as either GrB (Figure S4A, S4B) or surface molecules (Figure S4C) could be hardly detected without stimulation. After analysis, we found GrB+B cells displayed a CD5+CD38+CD27+CD138+CD19+ phenotype upon stimulation (**Figure 4A**), which were different from that of typical IL-10-producing regulatory B cells [20-22]. Subsequently, we found GrB+CD19+B cells and IL-10+CD19+B cells were distinct B cell subsets (**Figure 4B**), which may explain the different phenotypes of the cell subsets. Then, we compared the frequency of each surface molecule between LTR with and without recurrence. Notably, the proportions of CD5+GrB+CD19+B cells from LTR with HCC recurrence were higher than those from LTR without HCC recurrence in activated and resting GrB+CD19+B cells,


A
HepG2
HepG2+B cell
HepG2+B cell+PI-9
OD value
24 h
48 h
72 h
96 h
p=0.032
p=0.017
p=0.004
B
Huh-7
Huh-7+B cells
Huh-7+B cells+PI-9
OD value
p=0.023
p=0.019
C
p=0.019
Colony number
Hepg2
Hepg2+B cells
Hepg2+B cells+PI-9
D
p=0.011
Colony number
Huh-7
Huh-7+B cells
Huh-7+B cells+PI-9

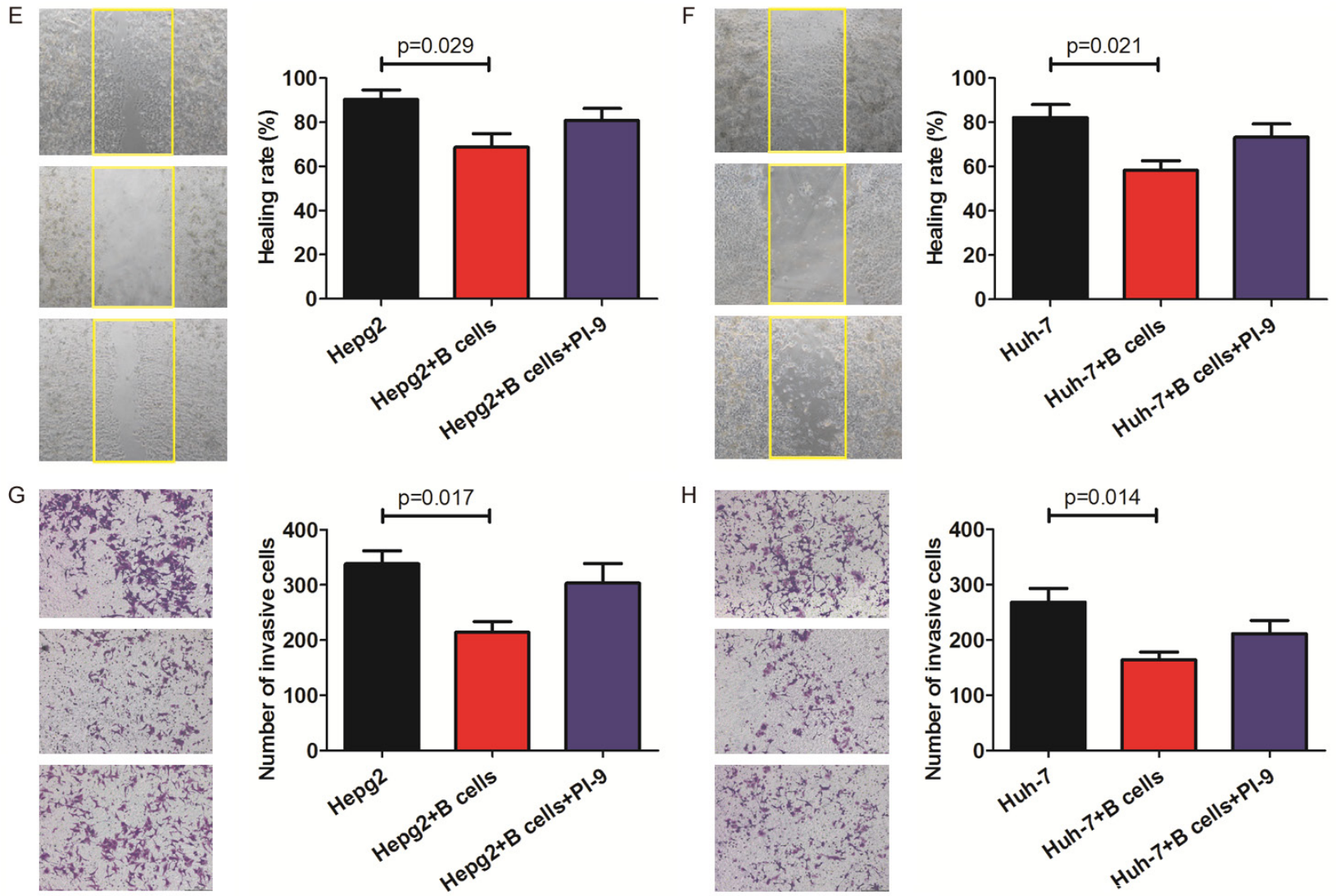


Figure 3. GrB$^+$CD19$^+$B cells can directly suppress HCC progression. The effects of GrB$^+$CD19$^+$B cells on the proliferation (A-D), migration (E, F), and invasion (G, H) in HepG2 and Huh-7 cells as analyzed by CCK-8, colony formation, scratch wound healing, and transwell cell invasion assays, respectively. Bars represent mean and standard deviation. Original magnification ×5 for colony formation assay; original magnification ×40 for scratch wound healing assay; original magnification ×100 for transwell cell invasion assay. Purified CD19$^+$B cells (10^6/ml) were stimulated with IgG+IgM (5.4 μg/ml), CpG OND 2006 (9.6 μg/ml) and IL-21 (50 ng/ml) in the presence or absence of PI-9 (5 μg/ml) for 4 d before coculture. GrB, granzyme B; CCK-8, cell counting kit-8; HCC, hepatocellular carcinoma; OD, optical density; PI-9, proteinase inhibitor-9.

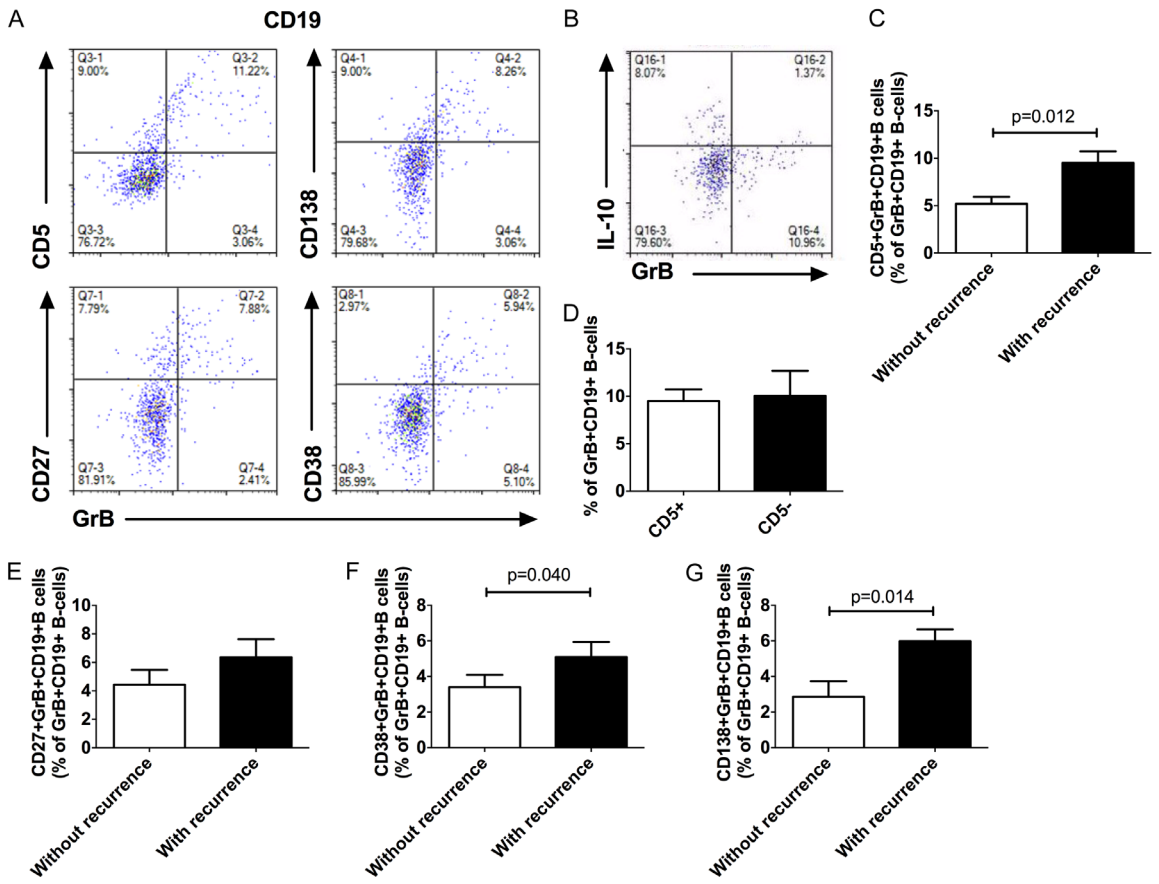


Figure 4. Phenotypic characteristics of GrB⁺CD19⁺B cells from LTR with HCC. Representative flow cytometry dot plots of the expression of surface molecules on GrB⁺CD19⁺B cells from LTR with HCC recurrence upon stimulation (A); A representative flow cytometry dot plot of GrB⁺CD10⁺CD19⁺B cells from LTR with HCC recurrence upon stimulation (B); Comparison of the percentages of CD5⁺GrB⁺CD19⁺B cells between LTR with and without HCC recurrence upon stimulation (P<0.05, C); Comparison of the percentages of CD5⁺GrB⁺CD19⁺B cells and CD5⁻GrB⁺CD19⁺B cells between LTR with and without HCC recurrence upon stimulation (P>0.05, D); Comparison of the percentages of CD27⁺GrB⁺CD19⁺B cells (P>0.05, E), CD38⁺GrB⁺CD19⁺B cells (P<0.05, F) and CD138⁺GrB⁺CD19⁺B cells (P<0.05, G) between LTR with and without HCC recurrence upon stimulation. PBMC were stimulated with IgG+IgM (5.4 μg/ml) in the presence of IL-21 (50 ng/ml) for 20 h and Brefeldin A (1 μg/ml) was added for the last 4 h of incubation. Surface markers expression on B cells was detected by flow cytometry. Bars represent mean and standard deviation. GrB, granzyme B; LTR, liver transplant recipients; HCC, hepatocellular carcinoma; PBMC, peripheral blood mononuclear cells.

respectively (P<0.05, **Figures 4C** and S4D). Studies have demonstrated CD5⁺B cells could play an important role in supporting tumor cell growth [23, 24], which are consistent with our data. Interestingly, in LTR with HCC recurrence the percentages of CD5⁺GrB⁺CD19⁺B cells were similar to those of CD5⁻GrB⁺CD19⁺B cells (P>0.05, **Figure 4D**). In contrast, the production of CD27⁺GrB⁺CD19⁺B cells remained similar among different populations with or without stimulation (P>0.05, **Figures 4E** and S4E). Finally, we analyzed the frequencies of CD38⁺GrB⁺CD19⁺B cells and CD138⁺GrB⁺CD19⁺B cells, which were found to be higher in LTR with HCC recurrence upon activation suggesting their potential differentiation toward plasma cells (P<0.05, **Figure 4F**, **4G**; P>0.05, Figure S4F, S4G).

GrB⁺CD19⁺B cell production decreases at both the protein level and the transcriptional level requiring JAK/STAT signaling in LTR with HCC recurrence

The expression of GrB in CD19⁺B cells isolated from LTR with HCC was confirmed by PCR and western blot analysis, respectively. Hence, GrB expression was demonstrated for both mRNA

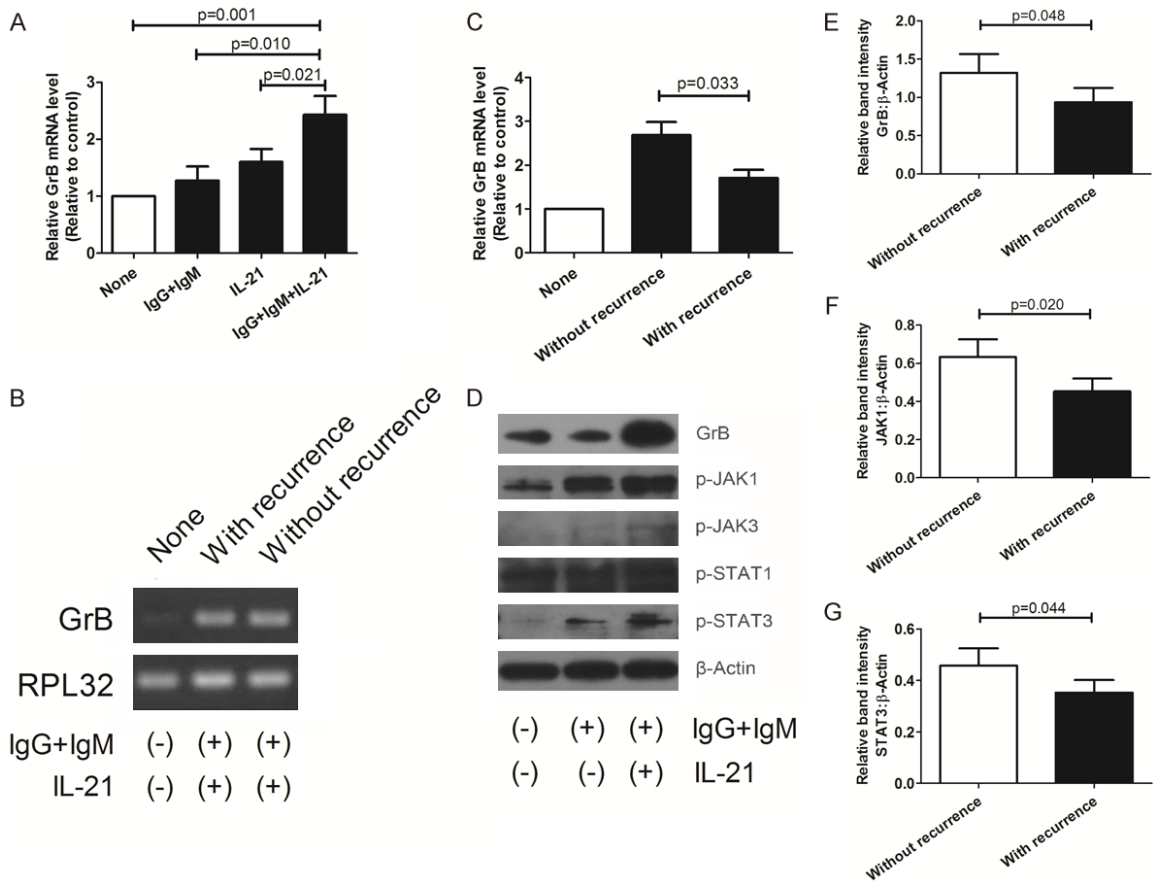


Figure 5. GrB+CD19+B cell production decreases at both the protein level and the transcriptional level requiring JAK/STAT signaling in LTR with HCC recurrence. Comparison of GrB mRNA levels upon activation under varying conditions with resting B cells as a control (A); Representative PCR for GrB in LTR with and without HCC recurrence upon activation with resting B cells as a control (B); Comparison of GrB mRNA levels in LTR with and without HCC recurrence upon activation with resting B cells as a control (P<0.05, C); Western blot analysis of GrB, pJAK1, pJAK3, pSTAT1 and pSTAT3 with β-actin as a control in CD19+B cells from LTR with HCC recurrence (D); Comparison of relative band intensity for GrB (E), pJAK1 (F) and pSTAT3 (G) in activated GrB+CD19+B cells from LTR with and without recurrence with β-actin as a control (P<0.05). CD19+B cells were stimulated in the presence or absence of IgG+IgM (5.4 μg/ml) or IL-21 (50 ng/ml) or both for 20 h and Brefeldin A (1 μg/ml) was added for the last 4 h of incubation. GrB mRNA expression was detected by PCR and GrB signal pathway by Western blot, respectively. Bars represent mean and standard deviation. GrB, granzyme B; LTR, liver transplant recipients; HCC, hepatocellular carcinoma.

and protein levels. Unlike classical cytotoxic cells such as CD3+T cells, which store preformed GrB without stimulation (Figure S5A), B cells only express GrB following activation. First, we tested the effect of varying stimuli on GrB production while the resting CD19+B cells served as a negative control confirming that GrB production was sharply enhanced upon stimulation with anti-BCR and with IL-21 (Figure S5B). Next, we found that exogenous IL-21 could increase the levels of GrB mRNA (Figure S5C) but higher levels of IL-21 could lead to an increased rate of CD19+B cell apoptosis (Figure S5D). In contrast, higher levels of anti-BCR could result in slowly elevated levels of GrB mRNA rather than an increased rate of CD19+B cell apoptosis (Figure S5E). After that, the proper levels of anti-BCR and IL-21 were reached to maximize GrB expression (**Figure 5A**). We further found LTR with HCC recurrence had lower levels of GrB mRNA compared with LTR without HCC recurrence under the same condition (P<0.05, **Figure 5B**, **5C**).

Induction of a B cell-mediated response depends on the activation of the JAK/STAT pathway and IL-21 signaling is associated with activation of a series of JAK/STAT family members [25-27]. Consequently, we stained CD19+B cells for pJAK1, pJAK3, pSTAT1, and pSTAT3

Table 2. Characteristics of liver transplant recipients with HCC from a validation cohort

Parameters	With recurrence (n=49)	Without recurrence (106)	P
Age	53.02±8.05	54.42±8.24	0.322
Sex (male)	45	99	0.988
Maintenance			0.355
Tacrolimus	38	89	
Cyclosporin A	11	17	
Tumor-free survival (months)	16.69±9.47	28.32±14.58	0.000
Status (alive)	18	102	0.000
Cum survival (months)	26.33±13.20	28.32±14.58	0.416

and included GrB and actin as controls. We found the different extent of up-regulation of GrB, pJAK1, and pSTAT3 and no up-regulation of pSTAT1 or pJAK3 (**Figure 5D**). When we compared protein expression from patients with and without HCC recurrence, the levels of GrB, pJAK1, and pSTAT3 were significantly higher in patients without HCC recurrence (**Figure 5E-G**). Collectively, these data show that CD19$^+$B cells from LTR with HCC recurrence produce less GrB at the protein level and the transcriptional level via JAK1 and STAT3 signaling.

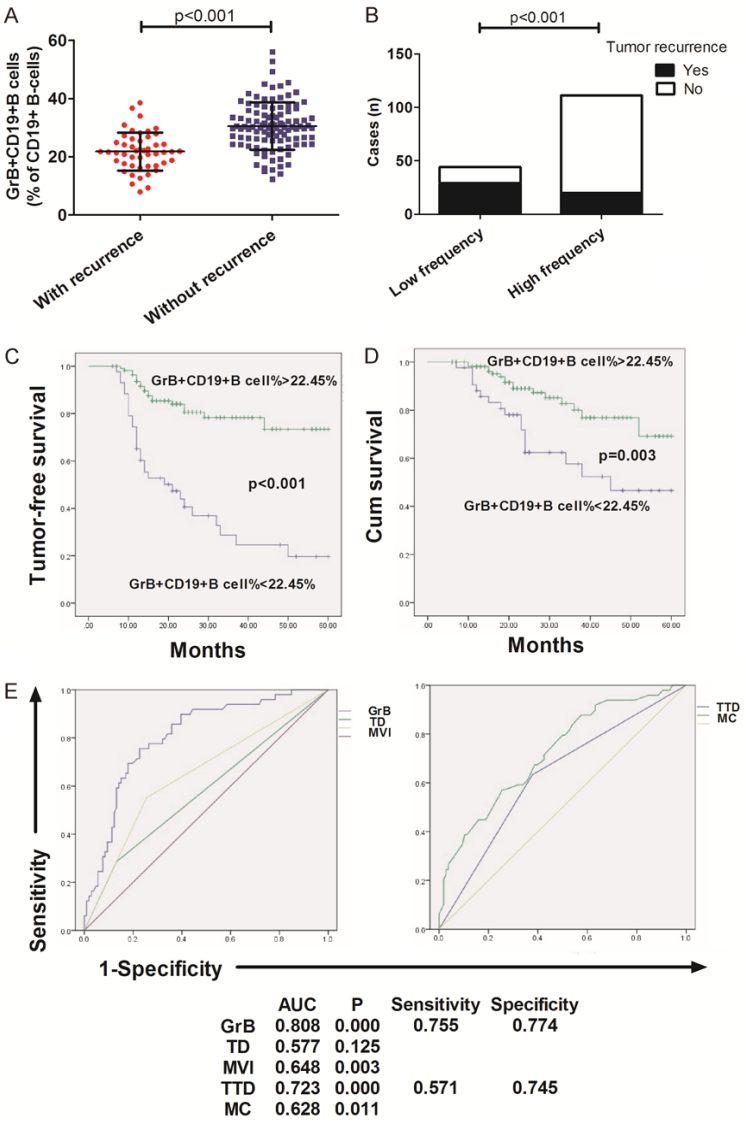
Decreased GrB$^+$CD19$^+$B cells can predict HCC recurrence in an independent validation cohort

In an attempt to validate the suppressive effect of GrB$^+$CD19$^+$B cells on tumor progression following liver transplantation, we performed an independent study. Patients lacking the follow-up data were excluded. In this validation cohort, a total of 155 LTR with HCC were enrolled, who accepted liver transplantation and were then followed up at our center. Blood samples were obtained to analyze the lymphocyte subsets. These patients were followed up at regular intervals to observe the HCC recurrence and patient's survival following liver transplantation. 49 patients had a tumor recurrence during the follow-up period and 33 patients died (**Table 2**).

In this validation cohort, LTR with recurrence showed lower percentages of GrB$^+$CD19$^+$B cells (P<0.05, **Figure 6A**). Then, they were regrouped at the cut-off value of 22.45% calculated above, the HCC recurrence rate proved to be higher in the group with the frequency <22.45% (P<0.01, **Figure 6B**). Again, LTR with poor tumor differentiation, microvascular invasion, HCC beyond Milan criteria, and larger total tumor diameter had a higher rate of HCC recurrence (P<0.05, Figure S6A-D), which all exhibited a positive correlation with the decreased percentages of GrB$^+$CD19$^+$B cells (P<0.01, Figure S6E-H). Therefore, LTR from the group with the frequency <22.45% had the shorter tumor-free survival and cum survival (P<0.01, **Figure 6C**, **6D**). Subsequent ROC analysis further confirmed that the percentage of GrB$^+$CD19$^+$B cells was a strong predictor for HCC recurrence with the AUC of 0.808 (P< 0.01, **Figure 6E**). Finally, we asked if the percentage of GrB$^+$CD19$^+$B cells could independently predict HCC recurrence. Using multiple logistic regression analysis, the percentage of GrB$^+$CD19$^+$B cells (OR: 1.142; 95% CI: 1.064-1.225; P<0.01) was the only significant independent risk factor (**Table 3**), while tumor differentiation, microvascular invasion, tumor beyond Milan criteria or total tumor diameter did not reach statistical significance. Collectively, these data indicate that the percentage of GrB$^+$CD19$^+$B cells has a strong impact not only on the HCC recurrence but also the patient survival.

Discussion

We demonstrated that decreased GrB$^+$CD19$^+$B cells are correlated with early HCC recurrence and can identify LTR with pathological proven progressive HCC, suggesting their suppressive function on HCC. We further demonstrated that GrB$^+$CD19$^+$B cells directly inhibit the proliferation, migration, and invasion of HCC cells. GrB$^+$CD19$^+$B cells from LTR with HCC recurrence display a CD5$^+$CD38$^+$CD27$^+$CD138$^+$ phenotype, but the higher expression of CD5, CD38, and CD138, and the lower protein level and transcriptional level requiring JAK/STAT signaling. To support the role of GrB$^+$CD19$^+$B cells, we found LTR with decreased GrB$^+$CD19$^+$B cells have not only early HCC recurrence but also shorter survival in an independent validation cohort.



	AUC	P	Sensitivity	Specificity
GrB	0.808	0.000	0.755	0.774
TD	0.577	0.125		
MVI	0.648	0.003		
TTD	0.723	0.000	0.571	0.745
MC	0.628	0.011		

Figure 6. Decreased GrB+CD19+B cells can predict HCC recurrence in an independent validation cohort. Comparison of the percentages of GrB+CD19+B cells upon activation in LTR with and without HCC recurrence (P<0.05, A); Cases of tumor recurrence between LTR with the frequency of GrB+CD19+B cells >22.45% and <22.45% (P<0.05, B); Kaplan-Meier curve analysis of tumor-free survival (C) and cum survival (D) between LTR with the frequency of GrB+CD19+B cells >22.45% and <22.45% (P<0.05); ROC curve analysis of the percentage of GrB+CD19+B cells, tumor differentiation, microvascular invasion, total tumor diameter and Milan criteria (E). PBMC were stimulated with IgG+IgM (5.4 μg/ml) in the presence of IL-21 (50 ng/ml) for 20 h and Brefeldin A (1 μg/ml) was added for the last 4 h of incubation. GrB expression in B cells was detected by flow cytometry. Bars represent mean and standard deviation. GrB, granzyme B; LTR, liver transplant recipients; HCC, hepatocellular carcinoma; ROC, receiver operating characteristic; PBMC, peripheral blood mononuclear cells.

GrB is a serine protease most commonly produced by natural killer cells and cytotoxic T cells, which can also secrete the pore-forming protein, perforin, to mediate apoptosis in target cells. Recent studies have revealed that B cells can produce GrB upon stimulation [11, 28]. We demonstrated that human B cells gained regulatory potential in response to IL-21 provided additional triggering of the BCR and TLRs is present. Due to the immunosuppressive nature, regulatory B cells have been repeatedly reported to be involved in transplantation tolerance [12, 13, 29]. In contrast, its regulation of antitumor immunity is poorly understood following transplantation although the existence of tumor-infiltrating B cells has been previously described [14, 15]. In our study, we found LTR with decreased percentages of GrB+CD19+B cells showed early HCC recurrence. When we further analyzed the correlations between the frequency of GrB+CD19+B cells and pathological data, poor tumor differentiation, microvascular invasion, total tumor diameter, and HCC beyond Milan criteria, which in essence reflect the progressive nature of HCC, had a positive association with decreased frequency of GrB+CD19+B cells. Taken together, these data suggested the potential antitumor function of GrB+CD19+B cells. Subsequent co-culture experiments confirmed the suppressive role of GrB+CD19+B cells, which is consistent with the clinical observations. Interestingly, the B cells produce GrB without co-expression of perforin. Therefore, GrB uptake into target cells is independent of perforin, through either mannose-6-phosphate-receptor or fluid phase endocytosis [30, 31]. However, the specific mechanism as to how B

Table 3. Multivariate Cox analysis

Parameters	OR	95% CI	P
Poor tumor differentiation	1.508	0.542-4.195	0.431
Microvascular invasion	1.789	0.760-4.213	0.183
Total tumor diameter	0.898	0.726-1.112	0.326
Within Milan criteria	1.325	0.381-4.606	0.658
GrB+CD19+B cell%	1.142	1.064-1.225	0.000

CI, confidence interval; OR, odds ratio; GrB, granzyme B.

cells suppress HCC cells via GrB production needs to be investigated further.

In addition, we found GrB+CD19+B cells were separate from IL-10+CD19+B cells, which are the classic type of regulatory B cells [32], and only a very small portion of GrB+IL-10+B cells developed after activation. This indicates that GrB+B cells are a separate subset different from IL-10+ regulatory B cells. Until today the exact phenotype of regulatory B cells is not clearly defined. Therefore, wide discrepancies exist among varying studies [33]. In this study, we observed that GrB+CD19+B cells in LTR with HCC recurrence had elevated expression of plasma cell marker, namely CD38 and CD138, meaning the recruited GrB+CD19+B cells underwent further maturation. Of note, we also detected the elevated expression of CD5 on B cells. The role of over-expression of CD5 on B cells in cancer pathogenesis has been recognized recently [23, 24]. Thus, this might be a possible explanation at the molecular level for early tumor recurrence in LTR with decreased GrB+CD19+B cells. Induction of GrB in B cells involves both transcriptional and translational events and depends on the phosphorylation of JAK1 and STAT3. Accordingly, we noticed a reduction in GrB+CD19+B cells from LTR with HCC recurrence at both the protein level and the transcriptional level. Therefore, these data support the result that LTR with HCC recurrence exhibit decreased production of GrB+CD19+B cells.

In this study, we showed comprehensive data from LTR with HCC, indicating a critical role of GrB+CD19+B cells in preventing cancer progression. Our findings warrant further investigations for the design of future immunotherapies leading to immune responses and improved patient survival.

Acknowledgements

This work is supported by Open Project of Beijing Key Laboratory of Tolerance Induction and Organ Protection in Transplantation (2017YZNS01) and National Natural Science Foundation of China (81601392).

Disclosure of conflict of interest

None.

Address correspondence to: Qiang He and Ji-Qiao Zhu, Department of Hepatobiliary and Pancreaticosplenic Surgery, Beijing Chaoyang Hospital, Capital Medical University, No. 8 Gongtinan Road, Chaoyang District, Beijing 100020, China. Tel: +86-(0)-10-85231504; Fax: +86-(0)-10-85231503; E-mail: heqiang349@sina.com (QH); dr_zhujiqiao@163.com (JQZ)

References

[1] de Lope CR, Tremosini S, Forner A, Reig M and Bruix J. Management of HCC. J Hepatol 2012; 56 Suppl 1: S75-87.

[2] Forner A, Llovet JM and Bruix J. Hepatocellular carcinoma. Lancet 2012; 379: 1245-1255.

[3] Cescon M, Cucchetti A, Ravaioli M and Pinna AD. Hepatocellular carcinoma locoregional therapies for patients in the waiting list. Impact on transplantability and recurrence rate. J Hepatol 2013; 58: 609-618.

[4] Mehta N, Heimbach J, Harnois DM, Sapisochin G, Dodge JL, Lee D, Burns JM, Sanchez W, Greig PD, Grant DR, Roberts JP and Yao FY. Validation of a risk estimation of tumor recurrence after transplant (RETREAT) score for hepatocellular carcinoma recurrence after liver transplant. JAMA Oncol 2017; 3: 493-500.

[5] Sapisochin G, Goldaracena N, Astete S, Laurence JM, Davidson D, Rafael E, Castells L, Sandroussi C, Bilbao I, Dopazo C, Grant DR, Lazaro JL, Caralt M, Ghanekar A, McGilvray ID, Lilly L, Cattral MS, Selzner M, Charco R and Greig PD. Benefit of treating hepatocellular carcinoma recurrence after liver transplantation and analysis of prognostic factors for survival in a large Euro-American series. Ann Surg Oncol 2015; 22: 2286-2294.

[6] Roayaie S, Frischer JS, Emre SH, Fishbein TM, Sheiner PA, Sung M, Miller CM and Schwartz ME. Long-term results with multimodal adjuvant therapy and liver transplantation for the treatment of hepatocellular carcinomas larger than 5 centimeters. Ann Surg 2002; 235: 533-539.

[7] Schlitt HJ, Neipp M, Weimann A, Oldhafer KJ, Schmoll E, Boeker K, Nashan B, Kubicka S, Maschek H, Tusch G, Raab R, Ringe B, Manns MP and Pichlmayr R. Recurrence patterns of hepatocellular and fibrolamellar carcinoma after liver transplantation. J Clin Oncol 1999; 17: 324-331.

[8] Kar S and Carr BI. Detection of liver cells in peripheral blood of patients with advanced-stage hepatocellular carcinoma. Hepatology 1995; 21: 403-407.

[9] Unitt E, Marshall A, Gelson W, Rushbrook SM, Davies S, Vowler SL, Morris LS, Coleman N and Alexander GJ. Tumour lymphocytic infiltrate and recurrence of hepatocellular carcinoma following liver transplantation. J Hepatol 2006; 45: 246-253.

[10] Nguyen PHD, Ma S, Phua CZJ, Kaya NA, Lai HLH, Lim CJ, Lim JQ, Wasser M, Lai L, Tam WL, Lim TKH, Wan WK, Loh T, Leow WQ, Pang YH, Chan CY, Lee SY, Cheow PC, Toh HC, Ginhoux F, Iyer S, Kow AWC, Young Dan Y, Chung A, Bonney GK, Goh BKP, Albani S, Chow PKH, Zhai W and Chew V. Intratumoural immune heterogeneity as a hallmark of tumour evolution and progression in hepatocellular carcinoma. Nat Commun 2021; 12: 227.

[11] Jahrsdorfer B, Blackwell SE, Wooldridge JE, Huang J, Andreski MW, Jacobus LS, Taylor CM and Weiner GJ. B-chronic lymphocytic leukemia cells and other B cells can produce granzyme B and gain cytotoxic potential after interleukin-21-based activation. Blood 2006; 108: 2712-2719.

[12] Chesneau M, Michel L, Dugast E, Chenouard A, Baron D, Pallier A, Durand J, Braza F, Guerif P, Laplaud DA, Soulillou JP, Giral M, Degauque N, Chiffoleau E and Brouard S. Tolerant kidney transplant patients produce B cells with regulatory properties. J Am Soc Nephrol 2015; 26: 2588-2598.

[13] Zhu J, Zeng Y, Dolff S, Bienholz A, Lindemann M, Brinkhoff A, Schedlowski M, Xu S, Sun M, Guberina H, Kirchhof J, Kribben A, Witzke O and Wilde B. Granzyme B producing B-cells in renal transplant patients. Clin Immunol 2017; 184: 48-53.

[14] Shi JY, Gao Q, Wang ZC, Zhou J, Wang XY, Min ZH, Shi YH, Shi GM, Ding ZB, Ke AW, Dai Z, Qiu SJ, Song K and Fan J. Margin-infiltrating CD20(+) B cells display an atypical memory phenotype and correlate with favorable prognosis in hepatocellular carcinoma. Clin Cancer Res 2013; 19: 5994-6005.

[15] Garnelo M, Tan A, Her Z, Yeong J, Lim CJ, Chen J, Lim KH, Weber A, Chow P, Chung A, Ooi LL, Toh HC, Heikenwalder M, Ng IO, Nardin A, Chen Q, Abastado JP and Chew V. Interaction between tumour-infiltrating B cells and T cells controls the progression of hepatocellular carcinoma. Gut 2017; 66: 342-351.

[16] Rodriguez-Peralvarez M, Tsochatzis E, Naveas MC, Pieri G, Garcia-Caparros C, O'Beirne J, Poyato-Gonzalez A, Ferrin-Sanchez G, Montero-Alvarez JL, Patch D, Thorburn D, Briceno J, De la Mata M and Burroughs AK. Reduced exposure to calcineurin inhibitors early after liver transplantation prevents recurrence of hepatocellular carcinoma. J Hepatol 2013; 59: 1193-1199.

[17] Allard MA, Sebagh M, Ruiz A, Guettier C, Paule B, Vibert E, Cunha AS, Cherqui D, Samuel D, Bismuth H, Castaing D and Adam R. Does pathological response after transarterial chemoembolization for hepatocellular carcinoma in cirrhotic patients with cirrhosis predict outcome after liver resection or transplantation? J Hepatol 2015; 63: 83-92.

[18] Faria LC, Gigou M, Roque-Afonso AM, Sebagh M, Roche B, Fallot G, Ferrari TC, Guettier C, Dussaix E, Castaing D, Brechot C and Samuel D. Hepatocellular carcinoma is associated with an increased risk of hepatitis B virus recurrence after liver transplantation. Gastroenterology 2008; 134: 1890-1899; quiz 2155.

[19] Choi J, Jo C and Lim YS. Tenofovir versus entecavir on recurrence of hepatitis B virus-related hepatocellular carcinoma after surgical resection. Hepatology 2021; 73: 661-673.

[20] Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, Szabolcs PM, Bernstein SH, Magro CM, Williams AD, Hall RP, St Clair EW and Tedder TF. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood 2011; 117: 530-541.

[21] Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR and Mauri C. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic lupus erythematosus patients. Immunity 2010; 32: 129-140.

[22] Liu Y, Cheng LS, Wu SD, Wang SQ, Li L, She WM, Li J, Wang JY and Jiang W. IL-10-producing regulatory B-cells suppressed effector T-cells but enhanced regulatory T-cells in chronic HBV infection. Clin Sci (Lond) 2016; 130: 907-919.

[23] Zhang C, Xin H, Zhang W, Yazaki PJ, Zhang Z, Le K, Li W, Lee H, Kwak L, Forman S, Jove R and Yu H. CD5 binds to interleukin-6 and induces a feed-forward loop with the transcription factor STAT3 in B cells to promote cancer. Immunity 2016; 44: 913-923.

[24] Pylayeva-Gupta Y, Das S, Handler JS, Hajdu CH, Coffre M, Koralov SB and Bar-Sagi D. IL35-producing B cells promote the development of

pancreatic neoplasia. Cancer Discov 2016; 6: 247-255.
[25] Lee HK, Keum S, Sheng H, Warner DS, Lo DC and Marchuk DA. Natural allelic variation of the IL-21 receptor modulates ischemic stroke infarct volume. J Clin Invest 2016; 126: 2827-2838.
[26] Davis ID, Skak K, Smyth MJ, Kristjansen PE, Miller DM and Sivakumar PV. Interleukin-21 signaling: functions in cancer and autoimmunity. Clin Cancer Res 2007; 13: 6926-6932.
[27] Rozovski U, Wu JY, Harris DM, Liu Z, Li P, Hazan-Halevy I, Ferrajoli A, Burger JA, O'Brien S, Jain N, Verstovsek S, Wierda WG, Keating MJ and Estrov Z. Stimulation of the B-cell receptor activates the JAK2/STAT3 signaling pathway in chronic lymphocytic leukemia cells. Blood 2014; 123: 3797-3802.
[28] Hagn M, Ebel V, Sontheimer K, Schwesinger E, Lunov O, Beyer T, Fabricius D, Barth TF, Viardot A, Stilgenbauer S, Hepp J, Scharffetter-Kochanek K, Simmet T and Jahrsdorfer B. CD5+ B cells from individuals with systemic lupus erythematosus express granzyme B. Eur J Immunol 2010; 40: 2060-2069.
[29] Durand J, Huchet V, Merieau E, Usal C, Chesneau M, Remy S, Heslan M, Anegon I, Cuturi MC, Brouard S and Chiffoleau E. Regulatory B cells with a partial defect in CD40 signaling and overexpressing granzyme B transfer allograft tolerance in rodents. J Immunol 2015; 195: 5035-5044.
[30] Ahmed KA and Xiang J. mTORC1 regulates mannose-6-phosphate receptor transport and T-cell vulnerability to regulatory T cells by controlling kinesin KIF13A. Cell Discov 2017; 3: 17011.
[31] Gross C, Koelch W, DeMaio A, Arispe N and Multhoff G. Cell surface-bound heat shock protein 70 (Hsp70) mediates perforin-independent apoptosis by specific binding and uptake of granzyme B. J Biol Chem 2003; 278: 41173-41181.
[32] Wilde B, Thewissen M, Damoiseaux J, Knippenberg S, Hilhorst M, van Paassen P, Witzke O and Cohen Tervaert JW. Regulatory B cells in ANCA-associated vasculitis. Ann Rheum Dis 2013; 72: 1416-1419.
[33] Rosser EC and Mauri C. Regulatory B cells: origin, phenotype, and function. Immunity 2015; 42: 607-612.

Supplementary Information

Supporting materials and methods

Cell culture

Frozen stored peripheral blood mononuclear cells (PBMC) were thawed gently in 10% fetal calf serum (FCS, Gibco) supplemented RPMI 1640 medium (Gibco) and suspended (1×10^6/ml) in RPMI-1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml Streptomycin (Gibco). For cytokines produced by CD19$^+$B cells, PBMC were stimulated with IgG+IgM (anti-BCR, 5.4 μg/ml, Jackson ImmunoResearch Laboratories), lipopolysaccharide (LPS, 10 μg/ml, Sigma-aldrich), CD40L (1 μg/ml, Enzo Lifesciences) and CpG OND 2006 (9.6 μg/ml, Sigma-aldrich) in the presence or the absence of IL-21 (50 ng/ml, Sigma-aldrich), seeded in a 24-well flat-bottom plate, and incubated for 20 h at 37°C, 5% CO_2. Brefeldin A (1 μg/ml, Sigma-aldrich) was added for the last 4 h of incubation.

Antibodies (Abs) and flow cytometric measurement

Anti-human Abs included PE-Cy7-CD3 (Biolegend), PE-Granzyme A (GrA, eBioscience), APC-perforin (Biolegend), FITC-Zombie (Biolegend), APC-fire750-CD69 (Biolegend), PerCP-Cy5.5-TGF-β (Biolegend), APC-IL-10 (Biolegend), PE-GrB (eBioscience), APC-fire750-CD138 (Biolegend), APC-CD27 (Biolegend), PerCP-Cy5.5-CD38 (Biolegend), FITC-CD5 (Biolegend), PE-Cy7-CD19 (Biolegend). After stimulation, PBMC were harvested and first stained with surface Abs followed by fixation/permeabilization (Cytofix/Cytoperm kit, BD Biosciences) and subsequent intracellular staining. Flow cytometry was performed in NovoCyte D2060R (ACEA Biosciences Inc). NovoEXpress software (San Diego, CA, USA) was used for analysis.

CD19$^+$ B cell purification

PBMC were isolated by ficoll density gradient centrifugation and resuspended in isolation Buffer I (PBS+ 0.5% BSA+ 2 mm EDTA) and Biotin Antibody cocktail (Miltenyi Biotec). The mixture was incubated for 10 minutes at 4°C followed by adding isolation buffer I and Anti-Biotin Microbeads (Miltenyi Biotec). After incubation for another 15 minutes, the cells were passed through the column placed in the magnetic field. After that, the purified cells were collected and resuspended at a concentration of 1 Mio/ml for further use.

GrB polymerase chain reaction (PCR)

B cells from patients with HCC were magnetically isolated to a purity of ≥95% and incubated for 20 h with either no treatment or in the presence of IL-21 (10 ng/ml, 50 ng/ml, and 100 ng/ml) or anti-BCR (2.7 μg/ml, 5.4 μg/ml and 10.8 μg/ml) in addition to the above stimuli. After incubation, the cells were counted and total RNA from each sample was isolated using Trizol (Beyotime). Then, cDNA was prepared using PrimeScript RT reagent Kit with gDNA Eraser (Takara). The housekeeping gene RPL32 was run simultaneously as a standard. Primers were designed as summarized in Table S1. After reverse transcription, products were run on a 2% agarose gel. All PCR assays were performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Data are representative of four independent experiments.

Western blot

B cells from patients with HCC were isolated and incubated for 15 and 20 min (for JAK and STAT detection) or overnight (for GrB detection) with anti-BCR in the presence or absence of IL-21. After washing and blocking, primary Abs were added followed by a washing step and incubation with the secondary Ab conjugated to HRP for 1 h. Primary Abs against GrB, pJAK1, pSTAT1, and pSTAT3 were from Abcam and the Ab against pJAK3 was from CST. The Ab against β-Actin was purchased from Santa Cruz. Secondary

Abs were anti-rabbit IgG from Santa Cruz and anti-mouse IgG from Solarbio. Data are representative of four independent experiments.

Cell counting kit-8 (CCK-8) assay

The hepatic cell lines HepG2 and Huh7 (Xiehe Cell Resource Center of Beijing) were cultured in 96-well plates alone or with activated B cells (1:5) from patients with HCC in the presence or absence of GrB Inhibitor IV (PI-9, 5 μg/ml, Calbiochem) and separately incubated for 0, 24, 48, 72 and 96 h. In accordance with the manufacturer's protocol, optical density values were measured using the CCK-8 solution. The absorbance values at each point were measured at 450 nm. Data are representative of three independent experiments.

Colony formation assay

HepG2 and Huh7 cells alone or with activated B cells (1:10) in the presence or absence of PI-9 were seeded in 12-well plates. Colony formation occurred within 12 days. After culturing for 12 days, we observed the cells fixed with 4% paraformaldehyde (KeyGEN BioTECH) stained with 1% crystal violet (Sigma-aldrich) at a magnification of ×5 for each group. Data are representative of three independent experiments.

Scratch wound healing assay

HepG2 and Huh7 cells were plated on a 6-well plate and scraped by a pipette tip to generate uniform wounds, respectively. After that, activated B cells with or without PI-9 were added to one well of each cell line. The initial gap length at 0 h and the residual gap length at 72 h after wounding were observed and photographed using photomicrographs at a magnification of ×40 for each group. Data are representative of three independent experiments.

Transwell cell invasion assay

Transwell chambers coated with Matrigel (Corning) were employed to assess cell invasive ability. Cells (1:5) were seeded in the upper chamber and the modified Eagle medium consisting of FCS was added to the lower chamber. After incubated for 24 h, those cells adherent to the underside of the membrane were fixed with 4% paraformaldehyde stained with 1% crystal violet. The stained cells were visualized in random fields with the help of an optical microscope at a magnification of ×100 for each group. Data are representative of three independent experiments.

Table S1. DNA primers used in this study

Gene	Sequence
GrB-F	GCAGGAAGATCGAAAGTGCG
GrB-R	TTGAGACTTTGGTGCAGGCT
RPL32-F	GCGTAACTGGCGGAAACCCA
RPL32-R	TTGTGAGCGATCTCGGCACA

GrB, granzyme B.

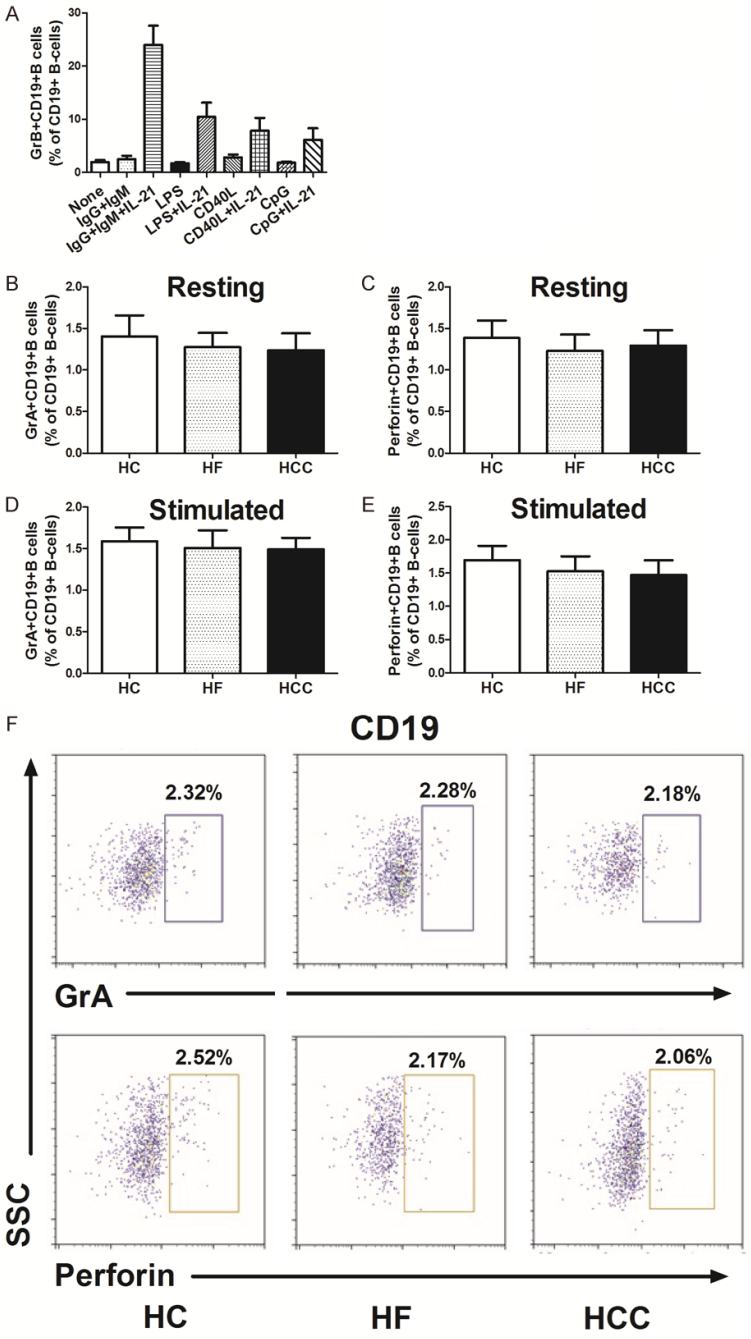


Figure S1. Decreased GrB^{+}CD19^{+}B cells are correlated with early recurrence of HCC. GrB expression in CD19^{+}B cells from LTR with HCC upon stimulation (IgG+IgM, 5.4 μg/ml; LPS, 10 μg/ml; CD40L, 1 μg/ml; CpG OND 2006, 9.6 μg/ml; IL-21, 50 ng/ml; A); Comparison of the expression of GrA (B) and perforin (C) in resting CD19^{+}B cells from HC, LTR with HF and with HCC (P>0.05); Comparison of the expression of GrA (D) and perforin (E) in activated CD19^{+}B cells from HC, LTR with HF and with HCC (IgG+IgM, 5.4 μg/ml; IL-21, 50 ng/ml; P>0.05); Representative flow cytometry dot plots of the expression of GrA and perforin in activated CD19^{+}B cells from HC, LTR with HF and LTR with HCC (F). Intracellular expression of cytokines in B cells was detected by flow cytometry. Bars represent mean and standard deviation. GrB, granzyme B; LPS, lipopolysaccharide; GrA, granzyme A; HC, healthy controls; HF, hepatic failure; HCC, hepatocellular carcinoma; LTR, liver transplant recipients.

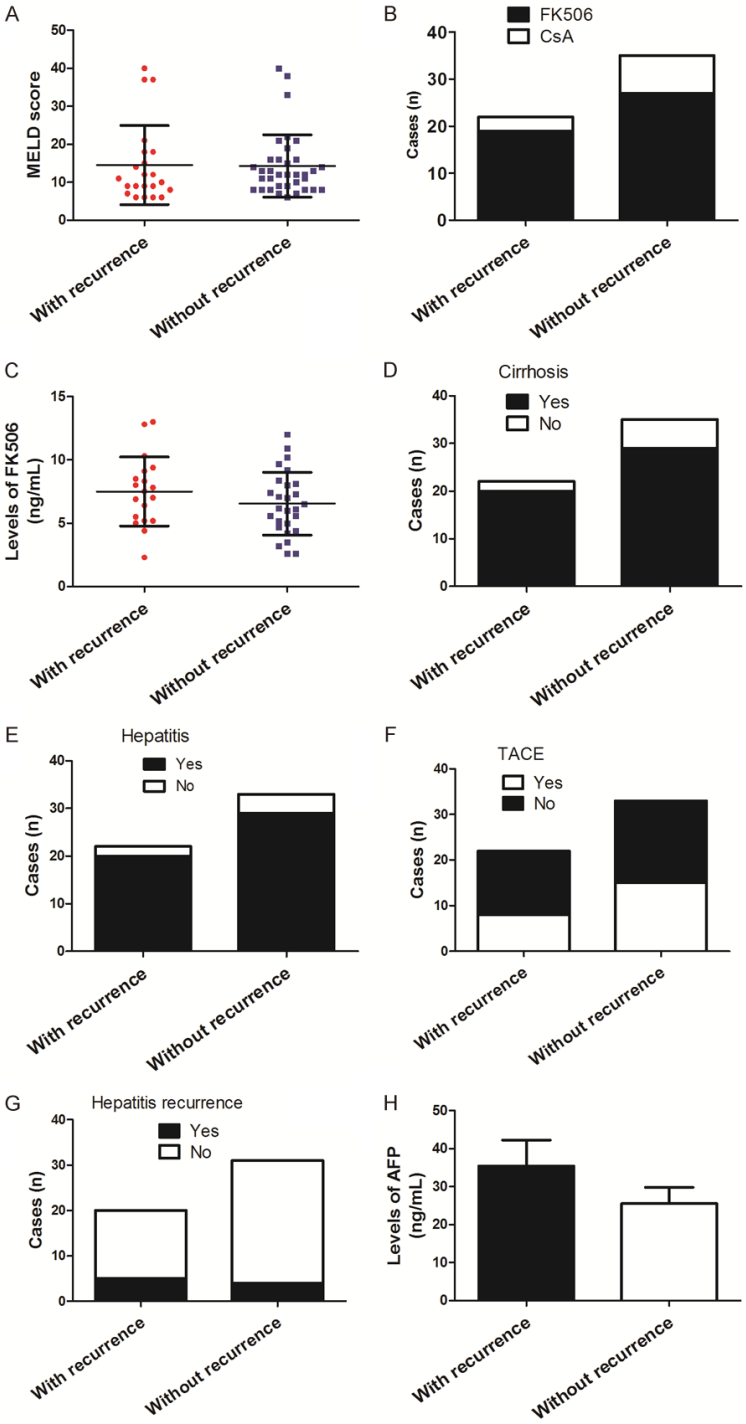


Figure S2. The correlations between clinical parameters and HCC recurrence. Comparison of MELD score (A), type of the calcineurin inhibitors (B), levels of tacrolimus (C), cases of pre-transplant cirrhosis (D), cases of pre-transplant hepatitis (E), cases of pre-transplant treatment (F), cases of post-transplant hepatitis recurrence (G) and levels of AFP (H) at the time of sampling between LTR with and without HCC recurrence, respectively. Bars represent mean and standard deviation. HCC, hepatocellular carcinoma; MELD, model for end-stage liver disease; AFP, alpha fetal protein; LTR, liver transplant recipients.

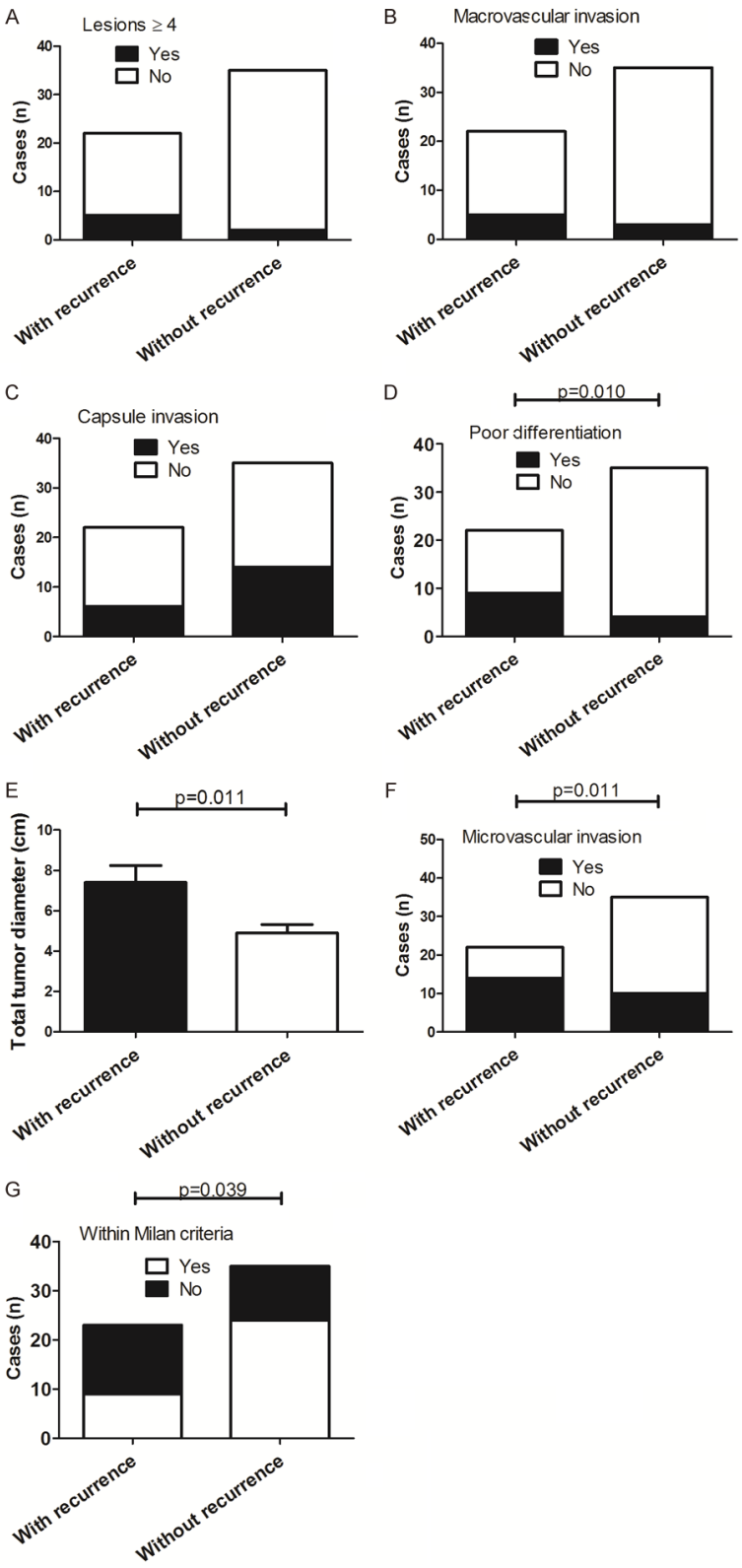


Figure S3. The correlations between pathological parameters and HCC recurrence. Comparison of cases of lesions ≥4 (A), cases of macrovascular invasion (B), cases of capsule invasion (C), cases of poor tumor differentiation (D), cases of microvascular invasion (E), total tumor diameter (F) and cases of HCC within Milan criteria (G) between LTR with and without HCC recurrence, respectively. Bars represent mean and standard deviation. HCC, hepatocellular carcinoma; LTR, liver transplant recipients.

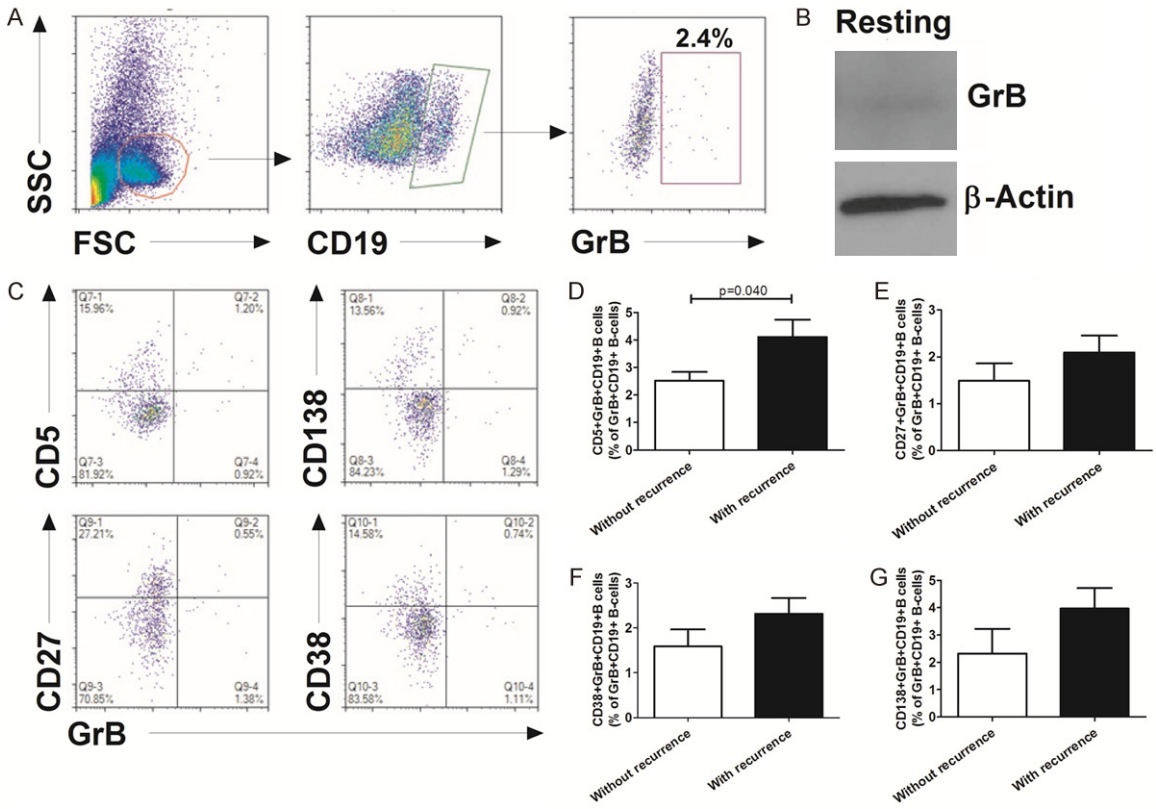


Figure S4. Phenotypic characteristics of GrB$^+$CD19$^+$B cells from LTR with HCC recurrence. Representative flow cytometry dot plots of the GrB expression in resting CD19$^+$B cells from LTR with HCC recurrence (A); A representative Western blot of GrB expression in resting CD19$^+$B cells from LTR with HCC recurrence (B); Representative flow cytometry dot plots of the expression of surface molecules on resting GrB$^+$CD19$^+$B cells from LTR with HCC (C); Comparison of the percentages of CD5$^+$GrB$^+$CD19$^+$B cells ($P<0.05$, D), CD27$^+$GrB$^+$CD19$^+$B cells ($P>0.05$, E), CD38$^+$GrB$^+$CD19$^+$B cells ($P>0.05$, F) and CD138$^+$GrB$^+$CD19$^+$B cells ($P>0.05$, G) on resting CD19$^+$B cells between LTR with and without HCC recurrence. The expression of surface markers and cytokines was detected by flow cytometry and GrB signal pathway by Western blot, respectively. Bars represent mean and standard deviation. GrB, granzyme B; LTR, liver transplant recipients; HCC, hepatocellular carcinoma.

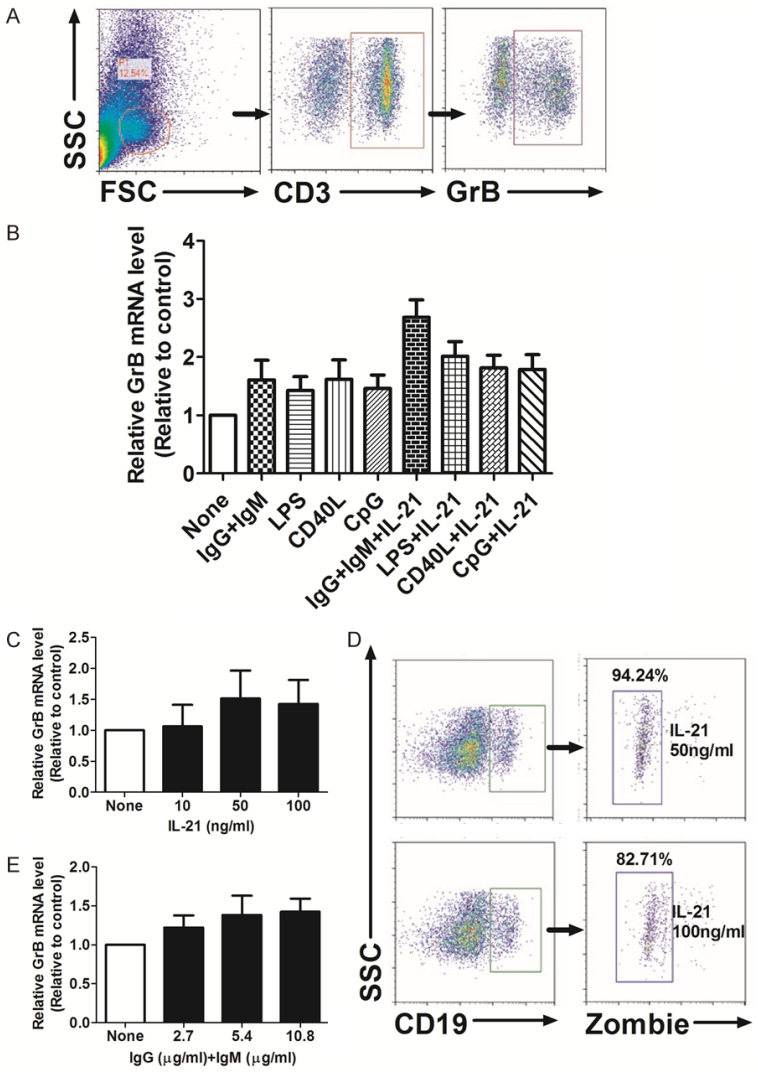


Figure S5. GrB$^+$CD19$^+$B cell production in LTR with HCC recurrence decreases at both the protein level and the transcriptional level requiring JAK/STAT signaling. Representative flow cytometry dot plots of the GrB expression in resting CD3$^+$T cells from LTR with HCC recurrence (A); Comparison of GrB mRNA levels upon activation under varying conditions with resting B cells as a control (IgG+IgM, 5.4 μg/ml; LPS, 10 μg/ml; CD40L, 1 μg/ml; CpG OND 2006, 9.6 μg/ml; IL-21, 50 ng/ml; B); Comparison of GrB mRNA levels in B cells stimulated with different levels of IL-21 (10 ng/ml, 50 ng/ml, 100 ng/ml, C) or IgG+IgM (2.7 μg/ml, 5.4 μg/ml, 10.8 μg/ml, E) with resting B cells as a control, respectively; Representative flow cytometry dot plots of CD19$^+$B cell apoptosis stimulated with different levels of IL-21 (50 ng/ml, 100 ng/ml, D). CD19$^+$B cells were stimulated under different conditions for 20 h and Brefeldin A (1 μg/ml) was added for the last 4 h of incubation. The expression of surface markers and cytokines was detected by flow cytometry and GrB mRNA expression by PCR, respectively. Bars represent mean and standard deviation. GrB, granzyme B; LTR, liver transplant recipients; HCC, hepatocellular carcinoma; LPS, lipopolysaccharide.

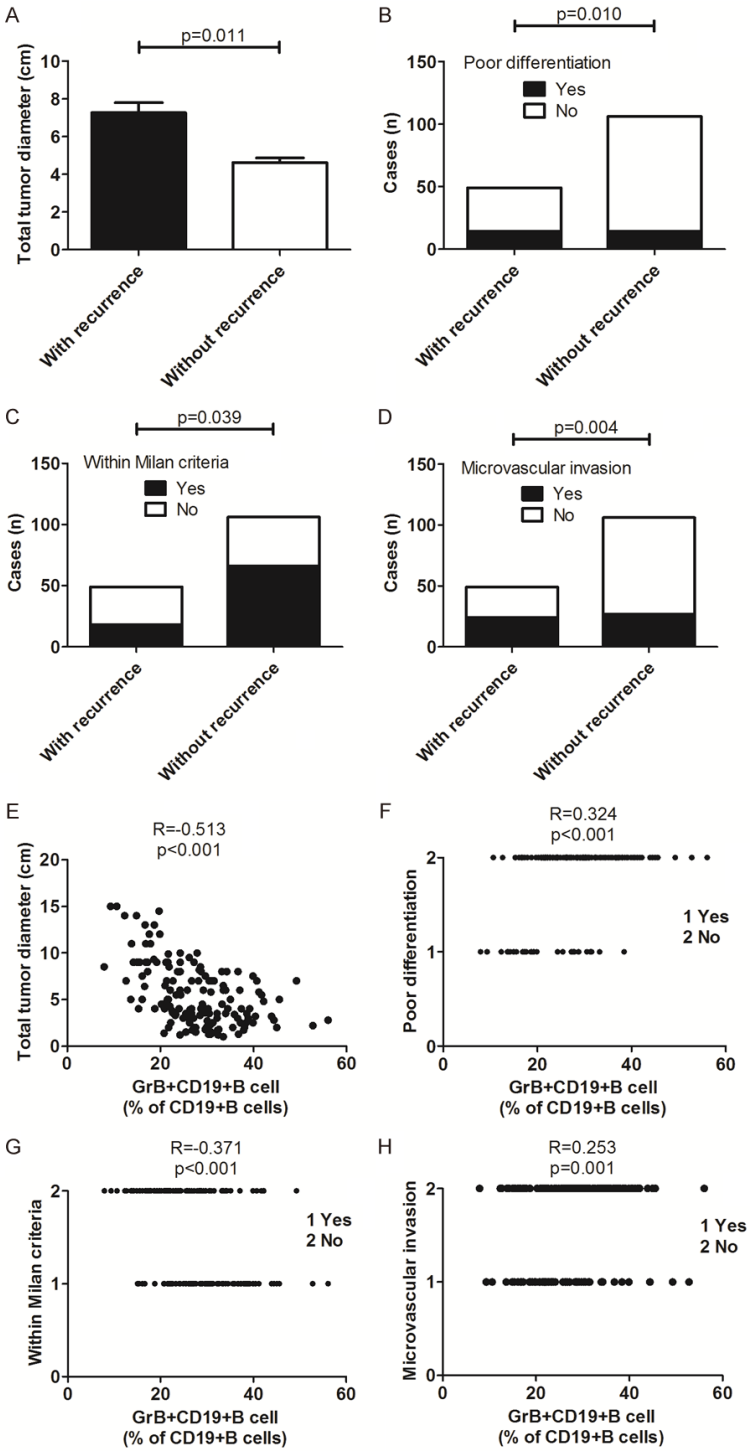


Figure S6. Decreased GrB+CD19+B cells can predict LTR with HCC recurrence in an independent validation cohort. Comparison of total tumor diameter (A), cases of poor tumor differentiation (B), cases of HCC within Milan criteria (C), and cases of microvascular invasion (D) between LTR with and without HCC recurrence, respectively; Correlation analysis between total tumor diameter (E), tumor differentiation (F), Milan criteria (G) and microvascular invasion (H), and the percentages of GrB+CD19+B cells, respectively. PBMC were stimulated with IgG+IgM (5.4 μg/ml) in the presence of IL-21 (50 ng/ml) for 20 h and Brefeldin A (1 μg/ml) was added for the last 4 h of incubation. GrB expression in B cells was detected by flow cytometry. Bars represent mean and standard deviation. GrB, granzyme B; LTR, liver transplant recipients; HCC, hepatocellular carcinoma.