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Tumor-infiltrating lymphocyte activity is enhanced in tumors with low IL-10 production in HBV-induced hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common cancers and can be induced by chronic HBV infection. The role of HBV-specific immune responses in mediating tumorigenesis and HCC prognosis is debated. The effect of intratumoral microenvironment on tumor-infiltrating lymphocytes (TILs) is also unclear. Here, we examined resected tumor tissue from 36 patients with HBV-induced HCC. We categorized study cohort based on ex vivo IL-10 secretion by tumor cells into high IL-10-secreting (Hi10) and low IL-10-secreting (Lo10) groups, and found that the Lo10 group was less sensitive to TLR ligand stimulation. TILs from the Lo10 group contained higher frequencies of HBV-specific IFN-g-producing cells and total IFN-g-producing cells, and possessed higher proliferative capacity. Moreover, the proliferative capacity of TILs from the Hi10 group was negatively correlated with IL-10 secretion from tumor cells. Together, our data demonstrated that low IL-10-producing capacity in HBV-induced HCC tumors is associated with enhanced TIL activity.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers with approximately 700,000 new cases reported each year and poor prognosis in patients exhibiting intrahepatic metastasis or post-surgical recurrence [1,2]. Hepatitis B virus (HBV)-initiated tumorigenesis is the leading cause of HCC in China [3,4]. The vast majority of adults contracting HBV will spontaneously clear the virus during acute inflammation; however, in places where HBV is endemic, approximately 90% of neonates and 30% of small children (1–5 years) who contracted HBV through vertical transmission will fail to clear the virus and develop chronic HBV infection [5,6]. In these individuals, chronic HBV infection may persist for years without developing symptom, or cause cirrhosis and HCC of which the switch mechanism is not understood. The effective clearance or suppression of HBV is found to associate with many factors, most notably the age at the time of infection, and this is thought to associate with the more robust adaptive immunity in adults. People with spontaneous clearance of HBV following acute infection typically mount vigorous HBV-specific T cell responses [7,8]. In vitro depletion of $CD4^+$ and $CD8^+$ T cells also prevented HBV clearance in chimpanzee model [9]. Adoptive transfer of hepatitis B surface antigen (HBsAg)-specific $CD4^+$ and $CD8^+$ T cells into transgenic mice that have genomic expression of HBV in hepatocytes were shown to lyse HBV-expressing hepatocytes and suppress HBV replication. The downregulation of HBV was directly linked to HBsAg-specific $CD8^+$ T cell production of IFN-g [10]. The production of tissue-protective cytokine IFN-a and IFN-b also required IFN-g production [11]. On the other hand, people with chronic HBV infection were shown to have reduced frequencies of virus-specific $CD8^+$ T cells, possibly to protect the liver from immune-mediated damage [12,13].

Indeed, liver carries out complex functions, involving the uptake of nutrients, waste products and pathogens from the blood. The liver microenvironment is commonly considered tolerogenic with many mechanisms to suppress inflammation. IL-10 is abundantly produced in the liver under the stimulation of Toll-like receptor (TLR) ligands such as LPS, which signals through TLR4 to upregulate IL-10 production by Kupffer cells and tumor cells in HCC [14]. When induced in chronic HBV infections, IL-10 can act directly to suppress HBV-specific $CD8^+$ T cell response [15–17]. In addition, chronic HBV-infected subjects were found to contain lower numbers of HBV-specific T cells. Paucity of virus-specific T cells in chronic HBV infection is also attributed to their increased susceptibility to apoptosis, which may be imposed by tolerogenic intrahepatic

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priming, interaction with pro-apoptotic mediator BIM and co-inhibitory molecule CTLA4 [18–20]. The role of immune tolerance in the liver microenvironment is a heavily debated subject. Enhanced immune activation in response to HBV expression in the liver is thought to be one of the factors of tumorigenesis in chronic HBV infection, while on the other hand, some reports have shown that increased regulatory T cells and B cells is associated with poorer prognosis in HCC [21–23]. Whether the production of IL-10 in the intratumoral environment has an impact on immunity and tumorigenesis in HBV-induced HCC is currently unknown.

To improve our understanding of the immune regulation in HBV-induced HCC and the impact of tumor microenvironment on lymphocyte activities, we examined the surgically removed tumor tissues from 36 HBV-induced HCC patients with different tumor microenvironment condition based on the IL-10 secretion ex vivo. The corresponding HBV-specific responses from tumor-infiltrating lymphocyte (TIL) were then analyzed.

2. Materials and methods

2.1. Study subjects

A total of 36 patients with HBV-induced HCC were recruited at Affiliated Hospital of Xuzhou Medical College between 2013 and 2014. All patients gave written informed consent. All patients underwent initial curative resection for HCC, defined as the complete macroscopic removal of the tumor. All patients were positive for HBsAg for more than 6 months, showing histological evidence for chronic hepatitis, and without any prior anticancer treatment. The clinical typing of tumors was determined according to the TNM classification system (2009 Edition 7). Demographic and clinical characteristics are summarized in Table 1. This study was approved by Affiliated Hospital of Xuzhou Medical College research ethics committee.

2.2. Cell isolation and culture conditions

Fresh tumor tissues from HCC patients were obtained by surgical resection. The surrounding nontumor tissues were carefully removed. The tissues were minced and digested with 50 mL HBSS (Life Technologies) containing 40 mg collagenase, 4 mg DNase I, and 100 units of hyaluronidase (Sigma) for 2 h at room temperature. The resulting single cell suspension were then separated into two portions for TIL and non-TIL isolation by standard Ficoll gradient centrifugation. All cells were cultured in RPMI medium supplemented with L-glutamine, penicillin/streptomycin, and 10% Fetal Bovine Serum (FBS) unless otherwise noted.

2.3. ELISA

200,000 tumor cells per well were cultured in medium without stimulation, with 1 μg/mL LPS (Sigma) stimulation, or with 2.5 μg/mL CpG-B (ODN 2006, Invivogen) stimulation in 96-well plate for 24 h in 37 °C, 5% CO_2. Supernatant IL-10 concentrations from these cultures were measured using Human IL-10 ELISA kit (eBioscience) according to the manufacturer's protocol.

2.4. ELISPOT

Nitrocellulose plate (Millipore) was coated with anti-human IFN-g antibody (Mabtech) overnight, after which the plates were washed twice and blocked with culture medium for 2 h. Then TILs were plated alone, with HBV 15-mer peptide pool (Mimotopes), or with anti-CD3/CD28 monoclonal antibodies for 12 h at 37 °C 5% CO_2. Color was developed by sequential addition and incubation of biotinylated detection anti–IFN–g antibodies (Mabtech), streptavidin-alkaline phosphatase (Invitrogen), and BCIP/NBT chromogen (Invitrogen) with rigorous washing between each step. Spots were enumerated by ImmunoSpot Analyzer (CTL).

Table 1
Demographic and clinical characteristics of study subjects. Mann–Whitney test. $P < 0.05$ is considered significant.

	Hi10	Lo10	P
Number of patients	22	14	
Age (y), median (range)	49 (35–71)	52 (37–69)	>0.05
Sex (F/M)	4/18	2/12	>0.05
AFP (ng/mL), (≤25/>25)	7/15	6/8	>0.05
Liver Cirrhosis (Y/N)	14/8	10/4	
ALT (U/L), median (range)	39 (15–157)	32 (17–97)	>0.05
Tumor size (cm), (≤5/>5)	10/12	11/3	=0.017
Tumor multiplicity (single/multiple)	20/2	13/1	
Vascular invasion (absent/present)	21/1	13/1	
TNM stage (I + II/III + IV)	19/3	12/2	
Tumor differentiation (I + II/III + IV)	19/3	13/1	

2.5. Thymidine incorporation

TILs (100,000 per well) were plated in a 96-well plate in RPMI 1640 supplemented with L-glutamine, penicillin/streptomycin, and 10% FBS serum alone, with HBV-peptide pool, or with anti-CD3/CD28 monoclonal antibodies. After 5 days, wells were pulsed with 1 uCi of ^{3}H thymidine for 24 h, after which counts per minute (cpm) was measured in a β scintillation counter.

2.6. Statistical analysis

Significant differences between two groups were analyzed using Mann–Whitney test for nonparametric data. Correlation analyses were done using Pearson correlation test. Differences were considered significant at $P < 0.05$. All statistical analyses were done using Prism 6 software (GraphPad).

3. Results

3.1. Resected tumor cells from a subset of ^{H}CC patients had higher ex vivo IL-10 secretion

Since IL-10 plays an important role in intrahepatic and intratumoral regulations, we first examined the IL-10 secretion from the tumor tissues ex vivo. Purified non-TIL tumor cells were cultured for 72 h, after which the supernatant was harvested and the concentration of IL-10 was measured by ELISA. We found that the IL-10 secretion pattern from the resected tumors can be categorized into two groups, the high IL-10-secreting group (Hi10) containing samples from 22 patients, which produced IL-10 of greater than 20 pg/mL to 200 pg/mL, and the low IL-10-secreting group (Lo10) containing samples from 14 patients, which produced IL-10 of less than 20 pg/mL to below detection limit (Fig. 1A). No significant differences in demographical and clinical characteristics between the two groups were found except in tumor size, in which the Lo10 group had smaller tumor sizes (Table 1).

3.2. Resected tumor cells from a subset of HCC patients exhibited reduced IL-10 production in response to LPS and CpG stimulations

Low IL-10 concentration might be due to intrinsic reduction of IL-10 secretion mechanisms or lack of external stimulation. To differentiate between the two possibilities, we examined the IL-10 production from the tumor cells of HCC patients in response to LPS and CpG stimulation. LPS is a cell wall component found in Gram-

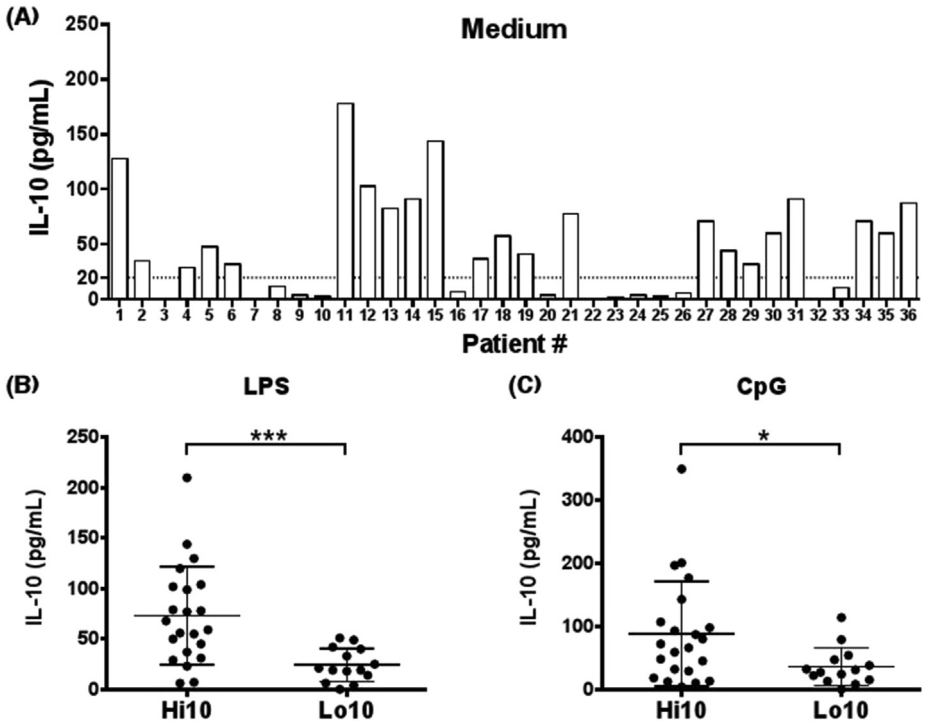

Fig. 1. Ex vivo IL-10 secretion by non-TIL tumor cells. Freshly isolated non-TIL tumor cells were incubated in (A) unstimulated medium, (B) with 1 μg/mL LPS, or (C) with 2.5 μg/mL CpG for 24 h and the concentration of IL-10 in the supernatant was measured by ELISA. Dotted line in (A) indicate the 20 μg/mL line used for the distinction between Hi10 and Lo10 subjects. Mann–Whitney test for (B) and (C). *P < 0.05. ***P < 0.001. Bar represents mean ± SD.

negative bacteria and can stimulate IL-10 secretion from TLR4 signal transduction pathway. CpG motifs can be found at elevated frequencies in bacterial DNA and can signal though the TLR9 pathway and increase IL-10 concentration. To measure the IL-10 secretion in response to TLR ligand stimulation in resected tumor cells from HBV-induced HCC patients, we repeated the above experiment with 1 μg/mL LPS or 2.5 μg/mL CpG per well added at the beginning of the incubation period. We found that the Lo10 group secreted less IL-10 in response to LPS stimulation than the Hi10 group (Fig. 1B). Similarly, 2.5 μg/mL of CpG per well stimulation resulted in lower IL-10 production in the Lo10 group than that in the Hi10 group (Fig. 1C). Together, these data suggest that the low ex vivo IL-10 production in the Lo10 group is not due to less microbial metabolite concentration in the intratumoral environment.

3.3. Increased frequencies of HBV-specific tumor-infiltrating T cells were observed in Lo10 group

Since IL-10 was shown to suppress IFN-g production from HBV-specific $CD8^+$ T cells in chronic HBV infection, we next analyzed whether IL-10 secretion in tumor affected the function of HBV-specific TILs. Isolated TILs were plated in an ELISPOT plate pre-coated with anti–IFN–g antibody alone, in the presence of HBV peptide pool, or with anti-CD3/CD28 monoclonal antibodies. The number of dark spots, each representing one IFN-g-secreting cell, was enumerated (Fig. 2A). The absolute number of HBV-specific or total IFN-g producing cells was calculated as the number of spots in the HBV-stimulated well or in the anti-CD3/anti-CD28 well minus the number of spots in the unstimulated (Medium) well, respectively, and the percentage of HBV-specific IFN-g-producing cell was calculated as the number of HBV-specific IFN-g-producing cells divided by the number of total IFN-g-producing cells. Both the absolute number of HBV-specific IFN-g-producing cells and the total IFN-g-producing cells in TILs were increased in the Lo10 group compared to the Hi10 group (Fig. 2B and C). Interestingly, when we examined the percentage of HBV-specific IFN-g-producing cells in TILs, no significant difference was found between the Hi10 group and Lo10 group (Fig. 2D), possibly reflecting the increase in the number of total IFN-g-producing cells in the Lo10 group.

3.4. TILs in the Lo10 group had increased proliferation under stimulation

Having observed the increased frequencies of IFN-g-secreting TILs in the Lo10 group compared to the Hi10 group, we examined the proliferative capacity of TILs from the two groups. TILs from each group were incubated alone, treated with HBV peptide pool or with anti-CD3/anti-CD28 monoclonal antibodies for 5d, after which the cells were pulsed with ^{3}H-Thymidine and the ^{3}H-Thymidine incorporation into the cell genome was measured as an indicator for TIL proliferation. We found that TILs from the Lo10 group had significantly higher proliferation than TILs from the Hi10 group, both under HBV-specific stimulation and under anti-CD3/anti-CD28 monoclonal antibody stimulation (Fig. 3A and B). We also examined the correlation between the amount of ex vivo IL-10 secretion in tumor cells and TIL proliferation capacity, and found that HBV-specific proliferation in neither group is correlated with tumor IL-10 secretion, while the total (anti-CD3/anti-CD28) proliferation is negatively correlated with tumor IL-10 secretion in the Hi10 group (Fig. 3C). We also examined the associations between the number of IFN-g-producing cells and proliferation. In the Hi10 group, the numbers of HBV-specific IFN-g-producing cells, as well as the numbers of total IFN-g-producing cells, were positively correlated with TIL proliferation. No such correlation was found in the Lo10 group.

4. Discussion

Our data demonstrated a role of intratumoral IL-10 in negatively regulating TIL inflammation. In this study, we first examined the IL-

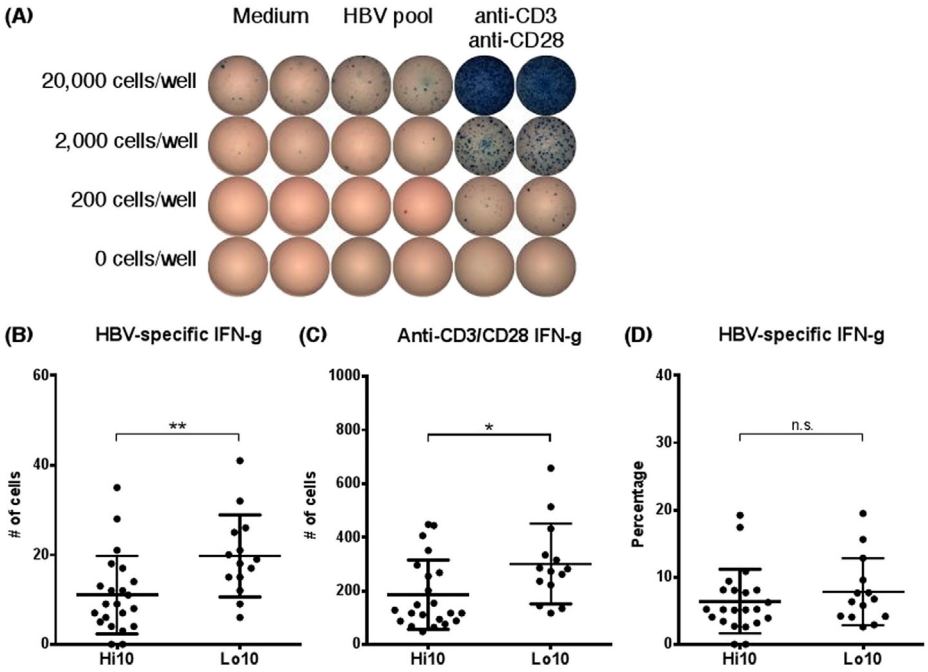

Fig. 2. Frequencies of IFN-g producing cells in TILs. Different numbers of freshly isolated TILs were plated in an anti–IFN–g antibody-pre-coated ELISPOT plate for 12 h, alone (Medium), in the presence of HBV peptide pool (HBV-specific) or in the presence of anti-CD3/anti-CD28 monoclonal antibodies (anti-CD3/anti-CD28). (A) Representative ELISPOT plate layout from a subject in the Lo10 group. (B) The number of HBV-specific IFN-g-secreting cells, as calculated by the number of spots in the HBV-specific well minus the number of spots in the medium well. (C) The number of total IFN-g-secreting cells, as calculated by the number of spots in the anti-CD3/anti-CD28 well minus the number of spots in the medium well. (D) The percentage of HBV-specific IFN-g-producing cells as calculated by the value in (B) divided by the value in (C). Mann–Whitney test. $^{*}P < 0.05$. $^{**}P < 0.01$. n.s.: not significant. Bar represents mean ± SD.

10-producing capacity in freshly resected tumors from patients suffering HBV-induced HCC, and found that a subset of subjects contained reduced levels of IL-10 in the tumor microenvironment ex vivo. The mechanism of reduction in ex vivo IL-10 production might be due to an observed insensitivity toward TLR ligand stimulation in these Lo10 tumors. The reduction of IL-10 may act in favor of better immunity in two ways. First, IL-10 production in chronic HBV infection were found to suppress HBV-specific T cell responses directly [16]. Second, reduced IL-10 production was associated with enhanced type I IFN activity in the liver microenvironment, which has potent antiviral effects [13,24]. Here, we found that the reduction of IL-10 in the tumor microenvironment is directly correlated with decreased tumor size and increased number of HBV-specific IFN-g-producing TILs as well as total IFN-g-producing TILs.

When examining the TILs from high IL-10 tumor compared to those from low IL-10 tumor, we found that TILs from Hi10 tumors proliferated less than TILs from lo10 tumors. The proliferative capacity in TILs from the Hi10 group but not the Lo10 group is negatively correlated with IL-10 concentration in tumor microenvironment, suggesting that the proliferation of TILs can be heavily affected by IL-10 when the IL-10 concentration is high. At low IL-10 concentration, other factors may play a more important role. Furthermore, the frequencies of IFN-g-producing HBV-specific TILs and total IFN-g-producing TILs were positively correlated with proliferative capacity in the Hi10 group but not the Lo10 group, suggesting that the low frequency of IFN-g producing cells in TILs in Hi10 group is at least in part due to the suppressed proliferative capacity. Together, our data demonstrated a role of intratumoral IL-10 in negatively regulating TIL inflammation. Previously, HBV infection was shown to upregulate IL-6 and IL-10 but not type I IFN, which was associated with protection from liver injury [15,25,26]. Our data demonstrated a more complicated role of IL-10 in HBV-induced HCC.

Our study is limited by a number of factors. First, due to ethical concerns, we limited the study samples to the resected tumor tissue. Thus, it is difficult to infer the physiological impact of more robust TIL response in chronic HBV infection, HBV-induced tumorigenesis, and HCC progression. We did found that the resected tumor sizes were smaller in the Lo10 group; however, a host of other clinical attributes, such as cancer stage, liver cirrhosis, and liver functions, were comparable between the two groups. As many other factors may contribute to the physiological disease progression, it is still unclear how intratumoral IL-10 concentration may affect HCC, and whether IL-10-depleting immunotherapy might improve disease prognosis. Although it is possible that the reduced tumor size in the Lo10 group is reflecting an earlier cancer stage; however, this is unlikely since the Hi10 group contained more Stage I + II tumors. In the future, liver biopsy samples from chronic HBV patients without cancer development as well as biopsy samples from HCC patients at earlier stages might be obtained and time course experiments need to be performed to explore the impact of liver/tumor microenvironment in intrahepatic/intratumoral immunity. Second, the dividing line between Hi10 group and Lo10 group is artificially determined, though the Hi10 group and Lo10 group did have a number of differences between the two, including insensitivity toward LPS and CpG in the Lo10 group. Since the liver is constantly filtering blood for pathogens and waste products, the liver cells might experience persistent stimulation through TLRs, and may induced a phenomenon termed LPS tolerance in a subset of people [27]. Our results suggested that LPS tolerance may also exist in tumors, but how LPS tolerance might affect tumorigenesis in chronic HBV-infection requires more study. Better understanding of molecular

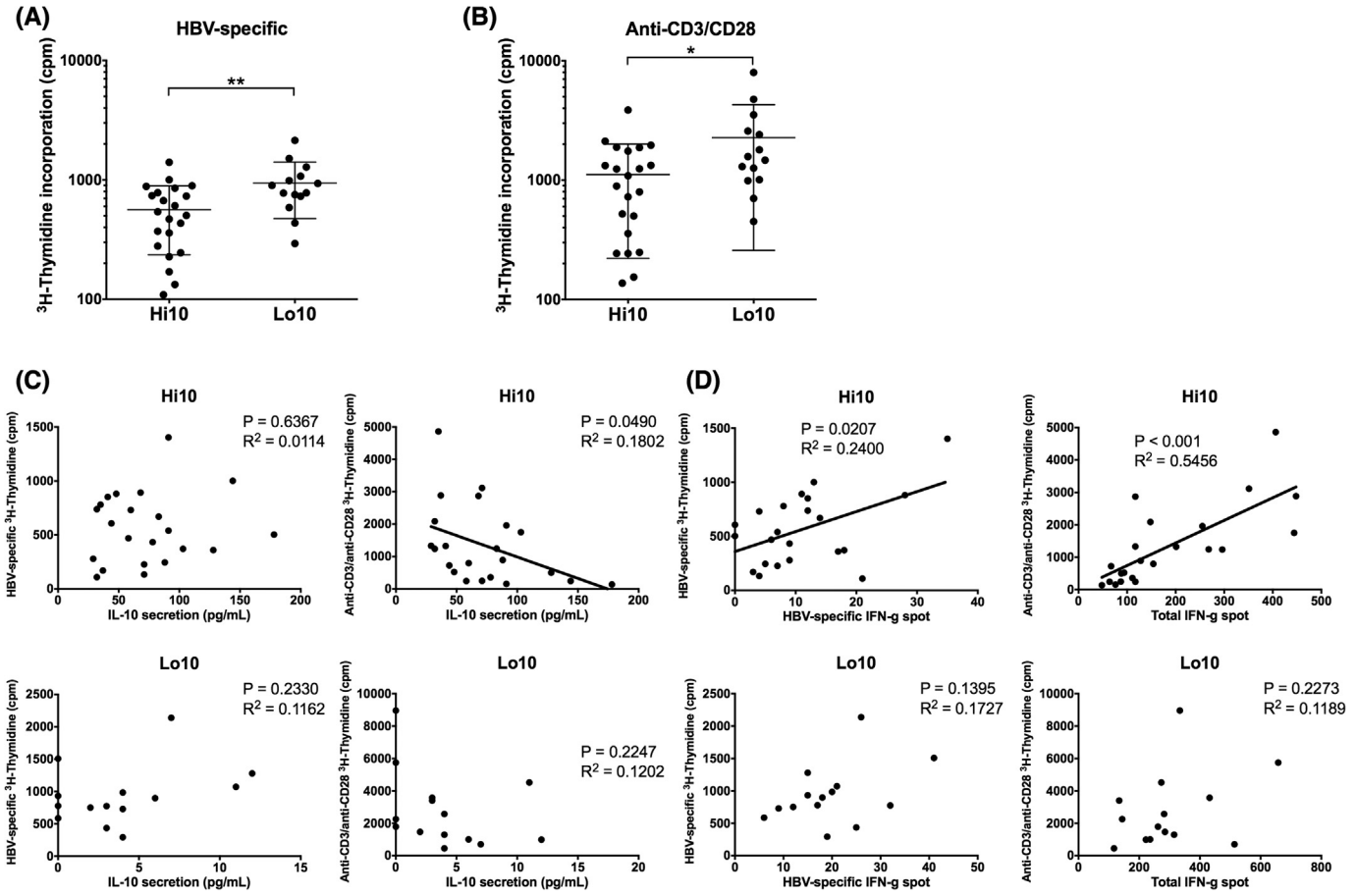

Fig. 3. TIL proliferation by TILs in the Hi10 group and the Lo10 group. The proliferation of TILs in response to (A) HBV-peptide pool or (B) anti-CD3/anti-CD28 was measured. Mann–Whitney test. *P < 0.05. **P < 0.01. Bar represents mean ± SD. The correlation between TIL proliferative capacity and ex vivo IL-10 secretion by non-TIL tumor cells was shown in (C), and the correlation between TIL proliferative capacity and the frequency of IFN-g-secreting cells was shown in (D). Pearson correlation. P < 0.05 is considered significant. Line of best fit (solid line) is drawn if P < 0.05.

mechanisms of LPS tolerance may assist with less artificial grouping of Hi10 and Lo10 groups.

Conflict of interest

None.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.177.

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