

# The Phenotype of Hepatitis B Virus–Specific T Cells Differ in the Liver and Blood in Chronic Hepatitis B Virus Infection

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Hepatitis B virus (HBV)-specific T cells play a key role in clearance of the virus and in the pathogenesis of liver disease. Peripheral blood (n = 25) and liver biopsies (n = 19) were collected from individuals with chronic untreated HBV infection. Whole blood, cultured peripheral blood mononuclear cells (PBMCs), and cultured liver-infiltrating lymphocytes (LILs) were each stimulated with an overlapping peptide library to the whole HBV genome. The expression of T helper 1 (Th1) cytokines [interferon gamma (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), and interleukin 2 (IL-2)] and interleukin 10 (IL-10) was analyzed by intracellular cytokine staining and flow cytometry. In *ex vivo* whole blood, more lymphocytes produced Th1 cytokines than IL-10. When comparing cultured LILs with cultured PBMCs, we found a significantly higher magnitude of CD8<sup>+</sup> T cells from the liver producing IL-10 ( $P = 0.044$ ), primarily in hepatitis B e antigen positive (HBeAg<sup>+</sup>) individuals. A positive correlation resulted between the magnitude of HBV-specific TNF- $\alpha$ <sup>+</sup> CD4<sup>+</sup> T cells in the liver and the degree of liver inflammation and fibrosis ( $P = 0.002$  and  $P = 0.006$ , respectively). **Conclusion:** The differences in cytokine production from HBV-specific T cells in blood and liver may explain the capacity for HBV to persist in the absence of significant hepatic destruction and highlights the balance between cytokine-mediated viral control and liver damage. (HEPATOLOGY 2007;46:1332-1340.)

Hepatitis B virus (HBV)-specific T cells are important in the successful clearance of acute HBV infection but also mediate liver damage in both acute and chronic HBV infections. In individuals

with chronic HBV infection, circulating HBV-specific T cells are detected infrequently and are significantly reduced compared with individuals who recover from acute HBV infection.<sup>1-3</sup> We recently developed a sensitive method to assess HBV-specific T cells using an overlapping peptide library and intracellular cytokine staining (ICS).<sup>4</sup> However, despite detection of interferon-gamma (IFN- $\gamma$ ) HBV-specific T cells in blood, the magnitude of CD4<sup>+</sup> and CD8<sup>+</sup> HBV-specific T cells in chronic HBV infection was still significantly lower than that observed in other chronic infections such as human immunodeficiency virus (HIV)-1.<sup>3,4</sup>

Previous studies using tetramer staining suggested a larger population of HBV-specific T cells being sequestered within the liver than in circulation.<sup>2,5,6</sup> Although a useful tool to quantify virus-specific T cells, the use of tetramers will detect virus-specific T cells regardless of their function or ability to produce cytokine.<sup>7</sup> In addition, T cells specific for human leukocyte antigen A2–restricted HBV epitopes have been shown to be immunodominant in the peripheral blood of acutely infected individuals who clear HBV, yet immunodominance is not maintained in chronically infected individuals, and exhaustion of T cells is not uniform for all virus-specific T cells.<sup>8,9</sup>

*Abbreviations:* ALT, alanine aminotransferase; cccDNA, covalently closed circular DNA; HBeAg, precore antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; ICS, intracellular staining; IFN- $\gamma$ , interferon gamma; IL-2, interleukin-2; IL-10, interleukin-10; LIL, liver infiltrating lymphocytes; NKT, natural killer T; OR, odds ratio; PBMC, peripheral blood mononuclear cells; TNF- $\alpha$ , tumor necrosis factor alpha.

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We therefore sought to fully characterize the phenotype of circulating HBV-specific T cells in blood and liver of individuals with untreated chronic HBV infection. Using multi-parameter ICS, we also asked whether the phenotype of circulating and intrahepatic T cells differed and whether a distinct cytokine profile was associated with clinical disease.

## Patients and Methods

**Study Group.** Patients with chronic HBV infection (defined as having HBV surface antigen detected on 2 occasions more than 6 months apart) were recruited from St. Vincent's Hospital, Victoria, Australia. Participation was approved by the hospital ethics committee, and signed consent was obtained. Patients had not received any treatment for HBV and were HIV-1 and hepatitis C virus (HCV) antibody negative. Twenty-five blood samples and 19 liver biopsy specimens were collected.

**Peptides.** To examine total HBV-specific T cell responses, an overlapping peptide library was made as previously described.<sup>4</sup> The 15-residue peptides overlapping by 11 amino acids were combined to create 8 HBV peptide pools, according to the relevant protein, with each peptide represented at equal concentrations. The peptide pools therefore included peptides from precore/core [hepatitis B e antigen (HBeAg)/hepatitis B core antigen], X protein, hepatitis B surface antigen, and polymerase.

**Preparation of Intrahepatic and Circulating T Cells.** Liver biopsy was obtained for routine histology and immunological analysis using a 14-gauge or 16-gauge needle. The sizes of the liver biopsy used for immunological analysis were 1-2 × 6-15 mm. The biopsy was stored in media at room temperature and processed on the same day. Single-cell suspensions were obtained using a glass dounce tissue grinder with loose pestle (Wheaton Science Products, Millville, NJ). The mean number of liver-infiltrating lymphocytes (LILs) obtained was 0.5 to 2 × 10<sup>6</sup>. Because this was insufficient for direct ICS, the cells were expanded by culture for 2 to 3 weeks in RPMI 1640 medium, 10% fetal bovine serum, and penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA) containing 40 U/mL recombinant human interleukin-2 (Roche Applied Sciences, Indianapolis, IN), irradiated feeder cells (3,000 rads) from two normal donors, and 0.05 μg/mL anti-CD3 (Beckman Coulter, Marseille, France). Once the LILs had expanded to a minimum of 2.5 × 10<sup>7</sup>, the cells were isolated and washed and then assessed by ICS as described in the following sections. At the same time, blood was collected and peripheral blood mononuclear cells (PBMCs) isolated by Ficoll-Paque centrifugation as

previously described.<sup>10</sup> The PBMCs were then expanded using the identical method described for LILs.

**Quantification of HBV-Specific T Cell Responses by ICS.** ICS was performed on whole blood as previously described,<sup>4,11,12</sup> with some minor modifications to include anti-IFN-γ-fluorescein isothiocyanate (clone B27) and anti-tumor necrosis factor-α-Allophycocyanin (MAb11) or anti-interleukin-2 (IL-2)-fluorescein isothiocyanate (clone MQ1-17H12) and anti-interleukin-10 (IL-10)- Allophycocyanin (clone JES3-19F1; all from BD Biosciences). The same ICS method was also used with expanded PBMCs and LILs resuspended at 1 × 10<sup>6</sup> cells/well in 200 μl RPMI 1640, 10% human AB serum, and penicillin/streptomycin/glutamine.

**Flow Cytometry Analysis.** All data were acquired on the FACSCalibur and were analyzed using CellQuest (BD Biosciences). Cells were gated initially on lymphocytes as determined by forward and side scatter. In most samples 10,000 to 20,000 CD4<sup>+</sup> and CD8<sup>+</sup> T cells were collected for analysis. HBV-specific cells were expressed as the percentage of cytokine producing CD8<sup>+</sup> or CD4<sup>+</sup> T cells (referred to here as the "magnitude of the HBV-specific T cell response"). A positive response was considered to be 0.05% cytokine<sup>+</sup> CD4<sup>+</sup>/CD8<sup>+</sup> T cells above background responses (responses to dimethylsulfoxide and co-stimulatory molecule stimulation) and also at least 2-fold above background (Fig. 1). The cutoff of 0.05% was determined using the mean plus 2 standard deviations of responses detected in 8 healthy controls as previously described.<sup>4</sup> The total magnitude of the HBV-specific T cell response to all 8 HBV peptide pools was calculated by adding the magnitude of cytokine-producing cells for each pool (above the 0.05% cutoff). The responses were also examined qualitatively by defining a "responder" as an individual who produced a particular cytokine to at least 1 peptide pool.

**Quantification of HBV Viral Load and Covalently Closed Circular Deoxyribonucleic Acid.** HBV viral load was determined using Bayer Versant bDNA 3.0 assay (Bayer Diagnostics, Tarrytown, NY), and HBV covalently closed circular deoxyribonucleic acid (cccDNA) levels were quantified by real-time polymerase chain reaction using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) as previously described.<sup>13</sup>

**Sequencing HBeAg/Hepatitis B Core Antigen Promoter Mutations.** HBV DNA was extracted and the catalytic domain of the reverse transcriptase/polymerase and the basal core promoter/precore regions were sequenced as previously described.<sup>14,15</sup>

**Liver Histology.** Liver biopsy was also collected for formalin fixation and embedded in paraffin and processed

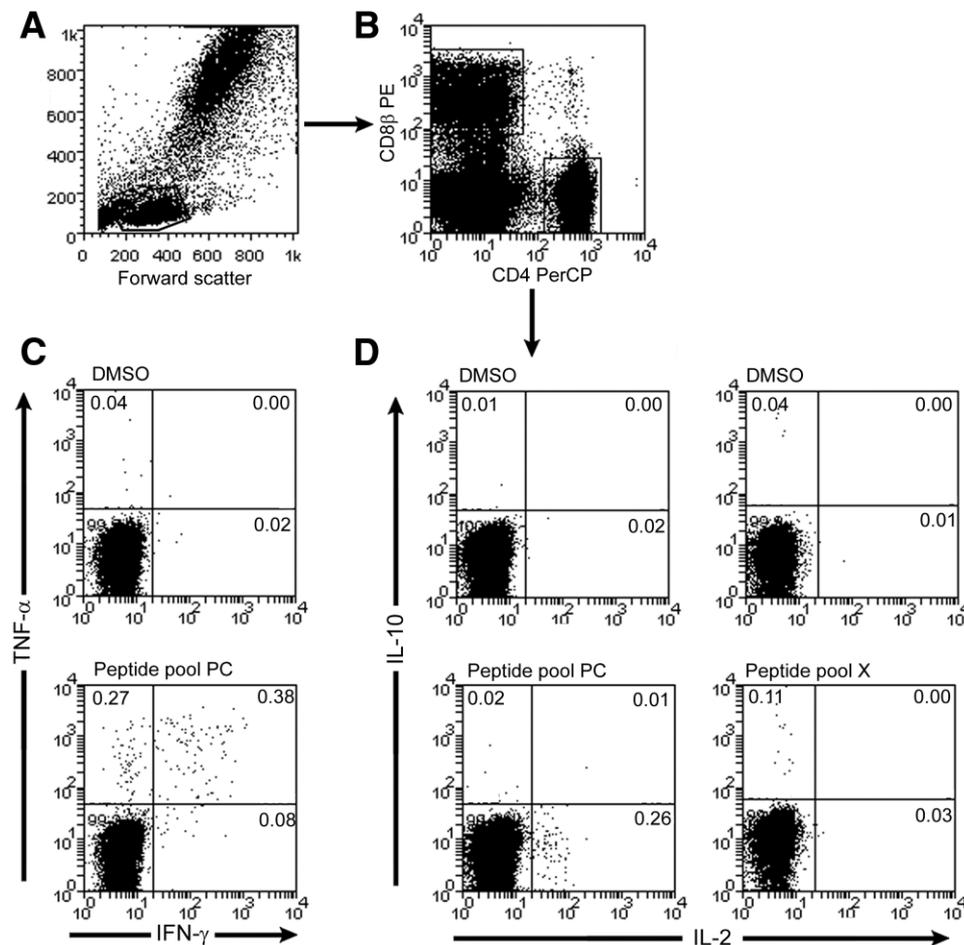


Fig. 1. Intracellular cytokine stain (ICS) for HBV-specific T cells. (A) Lymphocytes were selected by forward and side scatter and data from at least 100,000 small lymphocytes were generally collected. (B) In most samples data from at least 10,000 to 20,000 CD4+ and CD8+ T cells were collected for analysis. The percentage of cytokine producing CD8+ or CD4+ T cells were defined as the percentage of HBV-specific T cells. A positive response was considered to be >0.05% cytokine+ CD4+/CD8+ T cells above background (the response to dimethylsulfoxide and costimulatory molecule stimulation) and also at least 2-fold above background. Representative positive responses are shown for CD4+ T cell production of (C) TNF- $\alpha$  and IFN- $\gamma$ ; (D) IL-10 and IL-2.

for routine histology and hematoxylin-eosin staining. The necroinflammatory activity (A score) and fibrosis (F score) was determined by the Scheuer scoring method.<sup>16</sup>

**Statistical Analysis.** Statistical analysis was performed using SPSS version 13.0 for Windows student version (SPSS Inc., Chicago, IL) and SAS version 8.2 (SAS Institute Inc., Cary, NC). Comparisons of clinical differences between patient groups were analyzed using the Mann-Whitney *U*-test. Differences in number of responders in each group were compared using chi-squared test or Fisher exact test for small cohorts. Correlations were examined using Spearman's *rho* test for nonparametric values. For comparisons between 2 groups such as HBeAg<sup>+</sup> and HBeAg<sup>-</sup> samples and for comparisons of patient clinical data, a *P* < 0.05 was considered to be significant. Differences in magnitude of responses between 2 cell types from the same individual were examined using the paired *t* test for parametric data or Wilcoxon signed rank test and sign test for nonparametric data. Nonparametric data were considered to be significant only if they were significant for both Wilcoxon signed rank test and sign test, although only the *P*-value for the Wilcoxon signed rank test is shown. To account

for multiple comparisons between cell origins, type of cytokine produced and for individual peptide pool analysis, a *P* < 0.01 was considered statistically significant.

## Results

**Patient Cohort Demography.** Individuals with chronic HBV infection (*n* = 25) were recruited [including both HBeAg<sup>-</sup> (*n* = 16) and HBeAg<sup>+</sup> (*n* = 9) individuals; clinical details are summarized in Table 1). The patients were predominantly of Asian ethnicity (*n* = 23). All of the patients were infected with HBV in childhood, most likely by perinatal or early horizontal transmission. Individuals who were HBeAg<sup>+</sup> were significantly younger, had a significantly higher HBV viral load, cccDNA viral load and alanine aminotransferase (ALT) levels (*P* = 0.005, *P* < 0.001, *P* = 0.040 and *P* = 0.037, respectively). There was no significant association between genotype and HBeAg status. HBV ICS was performed on whole blood of all 25 patients and on the cultured PBMCs and cultured LILs of 19 of the patients (8 HBeAg<sup>+</sup> and 11 HBeAg<sup>-</sup>).

**Table 1. Demographic Details of Study Group**

|   | Total cohort   | HBeAg <sup>+</sup> group   | HBeAg <sup>-</sup> group   | P value |
|---|--|--|--|---------|
| Number of patients  | 25   | 9  | 16   |         |
| Gender; M/F   | 19/6   | 5/4  | 14/2   | 0.142*  |
| Age, median (range) in years  | 39 (22-70)   | 30 (22-39)   | 46.5 (26-70)   | 0.005†  |
| Ethnicity, %Asian   | 92.0   | 77.8   | 100.0  | 0.120*  |
| HBeAg <sup>+</sup> ; number of positives/total (%)                    | 9/25 (36)  |  |  |         |
| HBeAb <sup>+</sup> ; number of positives/total (%)                    | 15/25 (60)   |  |  |         |
| ALT; median (range), U/L  | 61 (28-2579)   | 73 (30-2579)   | 57 (28-484)  | 0.037†  |
| Precore mutants; number of positives/<br>number tested (%)            |  |  |  |         |
| Wild type   | 11/23 (47.8)   | 8/9 (88.9)   | 3/14 (21.4)  | 0.003*  |
| G1896A mutation   | 10/23 (43.5)   | 0/8 (0.0)  | 10/14 (71.4)   | 0.002*  |
| G1896A/G mutation   | 2/23 (8.7)   | 1/8 (11.1)   | 1/14 (7.1)   | 1.000*  |
| Basal core promoter mutants; number<br>of positives/number tested (%) |  |  |  |         |
| Wild type   | 15/23 (65.2)   | 8/9 (88.9)   | 7/14 (50.0)  | 0.086*  |
| A1762T/G1764A/T1753A mutation(s)                                      | 8/23 (34.8)  | 1/8 (11.1)   | 7/14 (50.0)  | *       |
| HBV viral load; Median (range), IU/mL                                 | 2.81 × 10 <sup>5</sup><br>(2.81 × 10 <sup>3</sup> – 8.72 × 10 <sup>9</sup> ) | 1.24 × 10 <sup>8</sup><br>(8.45 × 10 <sup>4</sup> – 8.72 × 10 <sup>9</sup> ) | 9.88 × 10 <sup>4</sup><br>(2.81 × 10 <sup>3</sup> – 7.64 × 10 <sup>6</sup> ) | <0.001† |
| HBV genotype, number of positives/number tested (%)                   |  |  |  |         |
| Genotype A  | 1/23 (4.3)   | 1/9 (11.1)   | 0/14 (0.0)   | 0.391*  |
| Genotype B  | 14/23 (60.9)   | 3/9 (33.3)   | 11/14 (78.6)   | 0.077*  |
| Genotype C  | 6/23 (26.1)  | 4/9 (44.5)   | 2/14 (14.3)  | 0.162*  |
| Genotype D  | 2/23 (8.7)   | 1/9 (11.1)   | 1/14 (7.1)   | 1.000*  |
| Liver biopsy  |  |  |  |         |
| A score; median (range)   | 1 (0-3)  | 1 (1-3)  | 1.5 (0-3)  | 0.718†  |
| F score; median (range)   | 1 (0-4)  | 1 (1-3.5)  | 2 (0-4)  | 0.329†  |
| cccDNA; median (range), copies/Geq                                    | 1.088 (0.159-27.545)   | 3.653 (0.211-27.545)   | 0.378 (0.159-2.825)  | 0.040†  |

\*Fisher's exact test. †Mann-Whitney U-test. Abbreviations: A score, necroinflammatory activity score; F score, fibrosis score; ALT, alanine aminotransferase; cccDNA, covalently closed circular DNA.

**HBV-Specific T Cells in Ex Vivo Whole Blood Have a Partially "Exhausted" Cytokine Production Phenotype.** Examination of HBV-specific CD4<sup>+</sup> T cells in whole blood showed significantly more responders who produced IFN- $\gamma$  than either TNF- $\alpha$  or IL-10 [ $P = 0.023$ , odds ratio (OR) = 0.259, confidence interval (CI) = 0.079-0.847 and  $P < 0.001$ , OR = 0.091, CI = 0.021-0.387, respectively) and more responders who produced IL-2 than IL-10 ( $P = 0.005$ , OR = 0.256, CI = 0.070-0.946; Fig. 2A). CD8<sup>+</sup> T cells from whole blood had significantly more responders that produced IFN- $\gamma$  and TNF- $\alpha$  than either IL-2 or IL-10 (for IL-2  $P = 0.021$ , OR = 0.248, CI = 0.074-0.833 and for IL-10  $P < 0.001$ , OR = 0.033, CI = 0.004-0.281, respectively).

In addition to differences in the proportion of subjects who were responders, the total magnitude of cytokine<sup>+</sup> HBV-specific T cells (percentage of CD4<sup>+</sup> or CD8<sup>+</sup> T cells that had a cytokine response after HBV peptide stimulation) was examined. In the peripheral blood, more CD4<sup>+</sup> and CD8<sup>+</sup> T cells produced Th1 cytokines than IL-10. Significantly more IFN- $\gamma$ <sup>+</sup> and IL-2<sup>+</sup> CD4<sup>+</sup> T cells were present in the whole blood than IL-10<sup>+</sup> CD4<sup>+</sup> T cells ( $P = 0.001$  and  $P = 0.002$  respectively; Fig. 3). The magnitude of the IFN- $\gamma$ <sup>+</sup> and TNF- $\alpha$ <sup>+</sup> CD8<sup>+</sup> T cells was also significantly higher than IL-10<sup>+</sup> CD8<sup>+</sup> T cells ( $P = 0.001$ ). The breadth

or number of HBV peptide pools that induced a response was also examined. There was a significant positive correlation between the magnitude and breadth of the HBV-specific T cell responses in whole blood ( $P < 0.001$ ,  $\rho > 0.907$ ; data not shown). This suggests that the larger magnitude reflected responses from populations of T cells that recognized a large diversity of HBV gene products rather than a single population of T cells that recognized a single peptide pool. There were no significant difference in the percentage of responders or the magnitude of responses detected in *ex vivo* whole blood and cultured PBMCs (Figs. 2B, 3). However, in cultured PBMC, there was a significantly greater percentage of responders to hepatitis B surface antigen compared with other peptide pools ( $P = 0.003$ ; data not shown). In contrast, there was no significant difference in the frequency of responses to each peptide pool in *ex vivo* whole blood.

**The Phenotype of HBV-Specific T Cells Cultured From Blood and Liver Is Different.** Characterization of LILs in humans is extremely difficult technically, given the very small number of T cells obtained by liver biopsy (maximum yield of  $2 \times 10^6$  cells). Therefore, to examine HBV-specific responses in liver to all peptide pools using the techniques described required the analysis of LILs that had been expanded in culture. PBMCs were also ex-

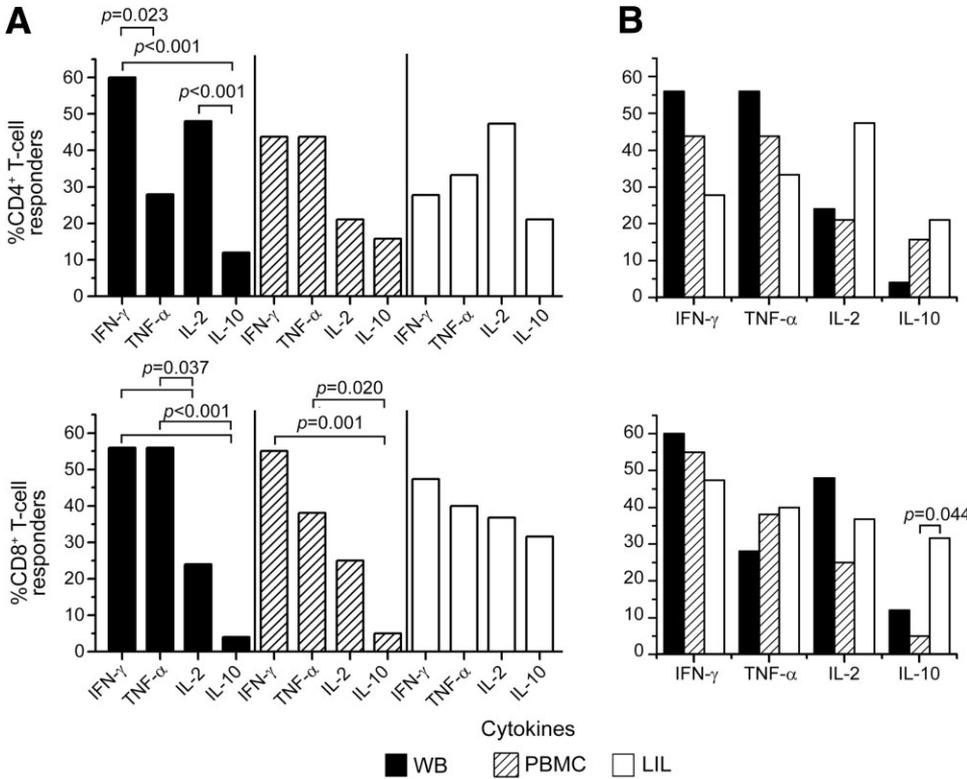


Fig. 2. Percentage of responders for each cytokine and each cell type. Comparisons between the percentage of patients who had a HBV-specific T cell response in whole blood (WB, solid bar, n = 25), cultured PBMCs (PBMC, hatched bar, n = 19), and cultured liver infiltrating lymphocytes (LIL, open bar, n = 19) in CD4+ (upper panels) and CD8+ T cells (lower panels) after stimulation with 8 HBV peptide pools. (A) Comparisons are made between the 4 cytokines measured: IFN- $\gamma$ , TNF- $\alpha$ , IL-2, and IL-10, within each group (WB, PBMC, and LIL). No significant differences were seen in the LIL group between the percentage of responders for each of the cytokines produced. (B) A significantly higher number of responders with IL-10 production from CD8+ T cells in LILs was found compared with the percentage of responders from expanded PBMCs.

panded in parallel with LILs using identical culture conditions, and comparisons were only made between cultured PBMCs and LILs.

As in whole blood, a larger number of responders produced IFN- $\gamma$  and TNF- $\alpha$  from CD8+ T cells compared

with IL-10 in cultured PBMCs ( $P = 0.001$ , OR = 0.048, CI = 0.005-0.426 and  $P = 0.020$ , OR = 0.086, CI = 0.010-0.768, respectively; Fig. 2A). Likewise, the magnitude of CD8+ T cells that produced IFN- $\gamma$  after HBV peptide stimulation was significantly greater than those

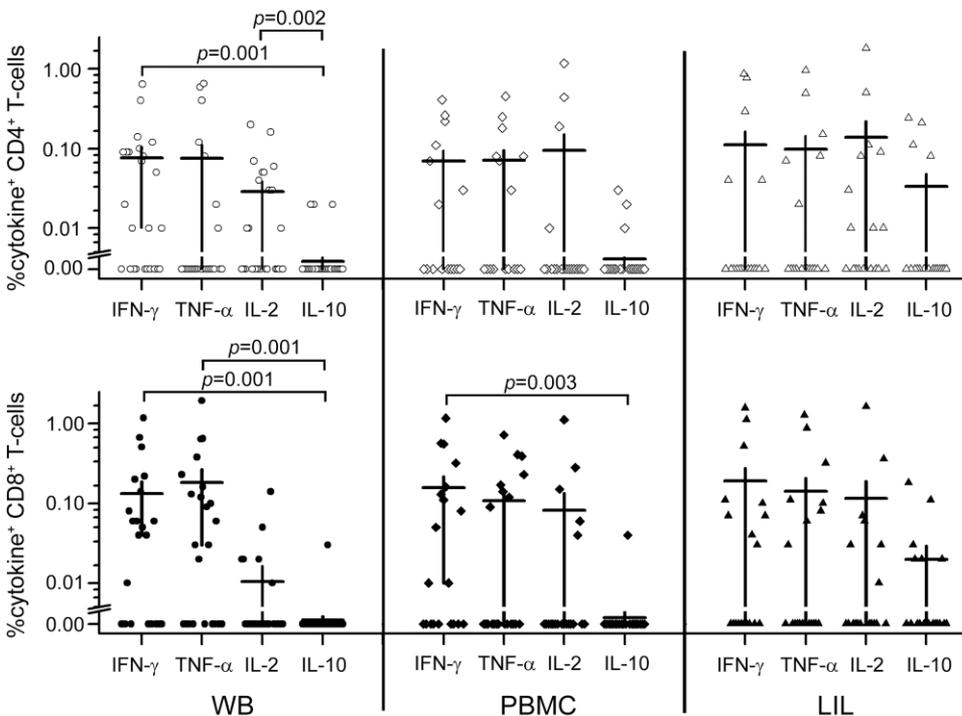


Fig. 3. Magnitude of HBV-specific T cell responses. Comparison of the total magnitude of HBV-specific T cells in whole blood (WB, circles, n = 25), cultured peripheral blood mononuclear cells (PBMC, diamonds, n = 19), and cultured liver infiltrating lymphocytes (LIL, triangles, n = 19) for multiple cytokines (IFN- $\gamma$ , TNF- $\alpha$ , IL-2, and IL-10). In whole blood, Th1 cytokine production (IFN- $\gamma$  and IL-2) from CD4+ T cells was of greater magnitude than IL-10 production, and in CD8+ T cells IFN- $\gamma$  and TNF- $\alpha$  were greater than IL-10. In cultured PBMCs a higher magnitude of IFN- $\gamma$ -producing CD8+ T cells was detected than those producing IL-10. No significant difference in the magnitude of each cytokine responses was observed in the cultured LILs. The horizontal bar represents the mean, and the vertical whiskers show the standard error.

producing IL-10 ( $P = 0.003$ ; Fig. 3). In contrast, when comparisons were made for CD4<sup>+</sup> and CD8<sup>+</sup> T cells from cultured LILs, there were no significant differences observed between the 4 cytokines in terms of both proportion of responders or the magnitude of responses (Figs. 2A, 3).

When cultured PBMCs and LILs were compared, the major difference was a significantly greater proportion of responders that produced IL-10 from CD8<sup>+</sup> T cells in cultured LILs compared with cultured PBMCs (31.6% vs. 5%,  $P = 0.044$ , OR = 8.769, CI = 0.942-81.671; Fig. 2B). These findings are consistent with a predominant Th1 cytokine response in HBV-specific T cells from cultured PBMCs compared with predominant IL-10 production in cultured LILs. We also examined the differences in cytokine production between cultured PBMCs and LIL after stimulation with pokeweed mitogen and staphylococcal enterotoxin B. There were significantly more IL-2-producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells and TNF- $\alpha$ -producing CD8<sup>+</sup> T cells in PBMC compared with LIL (paired Wilcoxon sign rank test;  $P = 0.015$ ,  $P = 0.007$ , and  $P = 0.003$ , respectively). However, there was no significant difference in IL-10 production that would account for the observed difference in HBV-specific T cell responses (data not shown).

**HBsAg Was Associated With Increased IL-10 Production Within the Liver.** The magnitude and breadth of the HBV-specific T cell responses was also compared between individuals who were HBeAg-positive and HBeAg-negative. HBeAg<sup>+</sup> individuals had a significantly higher serum HBV and cccDNA viral load (Fig. 4A). In HBeAg<sup>+</sup> individuals, there were significantly more intrahepatic HBV-specific IL-10<sup>+</sup> CD8<sup>+</sup> T cells than in the HBeAg<sup>-</sup> individuals ( $P = 0.041$ , Fig. 4B). Only a single HBeAg<sup>-</sup> individual (1/9) had detectable HBV-specific IL-10<sup>+</sup> CD8<sup>+</sup> T cells. The difference in the number of HBV-specific IL-10<sup>+</sup> CD8<sup>+</sup> T cells between HBeAg<sup>+</sup> and HBeAg<sup>-</sup> individuals was not observed in HBV-specific T cells in both *ex vivo* whole blood or cultured PBMCs or for any other cytokine. There was no correlation between HBV viral load and the magnitude of intrahepatic IL-10<sup>+</sup> CD8<sup>+</sup> T cells or any other HBV-specific cytokine<sup>+</sup> T cells.

**Liver Fibrosis and Activity Correlated With TNF- $\alpha$  Production by Intrahepatic CD4<sup>+</sup> T Cells.** Both A score (0-3) and F score (0-4) of the liver biopsies were strongly correlated with the magnitude of intrahepatic TNF- $\alpha$ <sup>+</sup> CD4<sup>+</sup> T cells ( $P = 0.002$ ,  $\rho = 0.672$  and  $P = 0.006$ ,  $\rho = 0.618$ , respectively; Fig. 5). This correlation was not observed in either *ex vivo* whole blood or cultured PBMC HBV-specific T cells, in relation to any other cytokines or to any clinical data (HBV viral

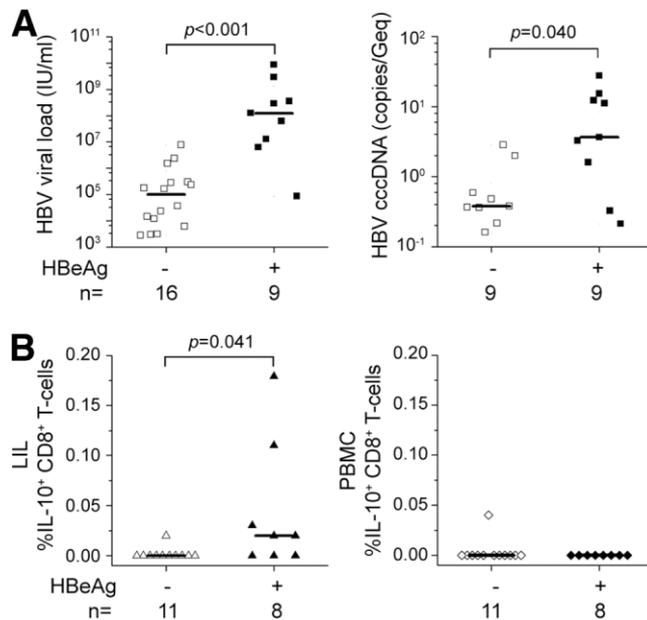


Fig. 4. Differences in immune response and viral load comparison of HBeAg-positive (closed symbols) and negative (open symbols) individuals. (A) HBV viral load and cccDNA were significantly higher in HBeAg-positive individuals. (B) The magnitude of all HBV-specific T cell responses from cultured PBMC and LILs for CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing each cytokine was compared. The only significant difference was found in the magnitude of the IL-10<sup>+</sup> CD8<sup>+</sup> T cell response in LIL (lower left panel), which was not observed in cultured PBMC (lower right panel). The horizontal bar represents the mean.

load, ALT, cccDNA levels, or HBV genotype) at the time of liver biopsy.

**cccDNA Inversely Correlated With TNF- $\alpha$  Production by Ex Vivo Whole Blood CD4<sup>+</sup> T Cells.** There was an inverse correlation between the amount of cccDNA measured and the magnitude of *ex vivo* whole blood TNF- $\alpha$ <sup>+</sup> CD4<sup>+</sup> T cells ( $n = 15$ ;  $P = 0.010$ ,  $\rho = -0.591$ , Fig. 5). Although cccDNA was positively correlated with HBV viral load ( $P < 0.001$ ,  $\rho = 0.794$ ), there was no significant linear relationship between HBV viral load and *ex vivo* whole blood HBV-specific TNF- $\alpha$ <sup>+</sup> CD4<sup>+</sup> T cells. There was also no relationship between HBV cccDNA and the magnitude of the HBV-specific T cell responses from either cultured PBMCs or LILs. No other correlation between clinical data and either the circulating or intrahepatic T cells was observed.

## Discussion

We report the first comprehensive comparison of the magnitude and phenotype of HBV-specific T cell responses in the blood and liver from individuals with untreated chronic HBV infection. After stimulation with HBV peptides, T cells examined directly from blood produced IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 more frequently than IL-10. Using cultured T cells from PBMCs and LILs, we

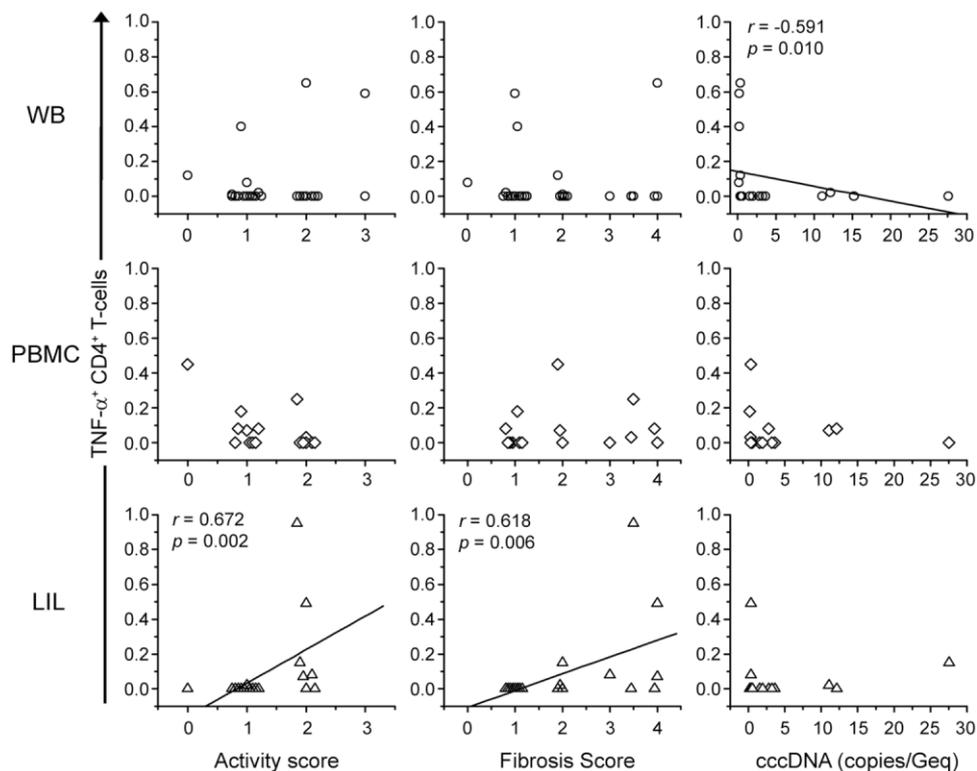


Fig. 5. Relationship of disease activity to the magnitude of cytokine-producing HBV-specific T cells. The magnitude of HBV-specific TNF- $\alpha$ + CD4+ T cells in whole blood (WB, upper panels), cultured PBMCs (middle panel), and LIL (lower panels) are shown. The magnitude of TNF- $\alpha$ + CD4+ T cells in LIL ( $n = 19$ ) was positively correlated with both activity and fibrosis scores, and this association was not observed in WB ( $n = 25$ ) or cultured PBMCs ( $n = 19$ ). On the contrary, TNF- $\alpha$ + CD4+ T cells in WB ( $n = 18$ ) were inversely correlated with the concentration of cccDNA, and this correlation was not seen in LILs ( $n = 15$ ) and cultured PBMCs ( $n = 14$ ).

found no increase in the overall magnitude of responses or the proportion of responders within the liver, although the cytokine profile of HBV-specific CD8<sup>+</sup> T cells was different to the periphery. There was an increased magnitude and proportion of responders with HBV-specific CD8<sup>+</sup> T cells that produced IL-10, especially in individuals who were HBeAg<sup>+</sup>. Despite the presence of intrahepatic HBV-specific IL-10<sup>+</sup> CD8<sup>+</sup> T cells, the proinflammatory cytokine TNF- $\alpha$  was still present, and production of this cytokine by intrahepatic CD4<sup>+</sup> T cells was significantly associated with liver damage.

Although more than half of the individuals studied had a cytokine response to at least 1 HBV peptide pool, the magnitude of cytokine<sup>+</sup> HBV-specific T cells from blood was low, with responses ranging from 0.0% to 1.93% of total CD4<sup>+</sup> or CD8<sup>+</sup> T cells. This is lower than typically observed in other chronic viral infections such as in HIV-1, where the magnitude of HIV-1-specific IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells ranges from 0.8% to 18.0%.<sup>12,17</sup> The magnitude of intrahepatic cytokine<sup>+</sup> HBV-specific T cells was similar to that seen in cultured PBMC (0.0%-1.80%). The low magnitude of HBV-specific T cells found in blood was therefore not explained by sequestration of HBV-specific T cells to the liver, which has been previously shown to be true in both HBV and HCV infections when using virus-specific tetramer<sup>+</sup> T cells.<sup>5,6,18-20</sup> It is likely that not all tetramer-positive HBV-specific T cells will produce cytokines in response to HBV peptide stim-

ulation, as has been shown in HCV and cytomegalovirus infections.<sup>3,19,21</sup> Furthermore, recently published data also show that tetramer<sup>+</sup> HBV-specific T cells can recover the capacity to produce IFN- $\gamma$  after inhibition of programmed death ligand-1.<sup>3</sup> In this study we were unable to show sequestration of functional HBV-specific T cells in the liver.

The phenotype of intrahepatic HBV-specific T cells was different from cultured PBMC. The major difference was the elevated number of IL-10<sup>+</sup> CD8<sup>+</sup> T cells measured in the liver. The increased frequency of IL-10-producing T cells within the liver may be a mechanism to protect against hepatic cell destruction and limit liver damage.<sup>22,23</sup> One potential weakness of our methodology was that the effects of culturing T cells from blood and liver may not be identical. It is possible that "exhausted" HBV-specific T cells may have been primed for apoptosis after stimulation, and the extent of exhaustion may differ between intrahepatic and circulating T cells. The proliferative capabilities of PBMCs and LILs therefore may also vary given the difference in antigen exposure *in vivo*. However, given there were too few cells obtained from a liver biopsy, we had no alternative to this approach.

The presence of intrahepatic HBV-specific IL-10<sup>+</sup> CD8<sup>+</sup> T cells was increased in the setting of HBeAg<sup>+</sup> disease. The HBeAg protein has previously been shown to be a tolerogen possibly leading to an anti-inflammatory state as the body attempts to minimize liver damage.<sup>22</sup>

The presence of IL-10 therefore potentially allows for protection against immune-mediated disease but may be at the cost of persistence of viral infection.<sup>24</sup> Given that the HBV viral load was significantly higher in HBeAg<sup>+</sup> individuals, we cannot exclude the possibility that a high HBV viral load or elevated ALT may be associated with increased IL-10 production in the liver and not HBeAg itself. In a larger cohort this could have been addressed with a multivariate analysis, but this was not possible here. However, we found no correlation between the IL-10<sup>+</sup> CD8<sup>+</sup> T cells in the liver and either HBV viral load or ALT, suggesting that it is unlikely for the differences between the HBeAg<sup>+</sup> and HBeAg<sup>-</sup> groups to be attributed solely to either of these factors.

During chronic HBV infection, there is elevated IL-10 production by CD4<sup>+</sup> CD25<sup>+</sup> T-regulatory cells in both the blood and liver compared with individuals who have resolved HBV infection.<sup>25-27</sup> In contrast, we found no statistically significant increase in IL-10<sup>+</sup> CD4<sup>+</sup> T cells in the liver or blood, and the production of IL-10 was largely attributed to CD8<sup>+</sup> T cells rather than from conventional T-regulatory cells. Another possible source of IL-10 producing T cells in the liver are natural killer T (NKT) cells.<sup>28,29</sup> However, previous characterization of liver and peripheral NKT cells show that it is the CD4<sup>+</sup>CD8<sup>-</sup> NKT cells that produce IL-10.<sup>28,29</sup> Therefore it is unlikely that NKT cells are contributing to the IL-10<sup>+</sup> CD8<sup>+</sup> T cell population detected in this study.

We identified an association between the presence of intrahepatic TNF- $\alpha$ <sup>+</sup> CD4<sup>+</sup> T cells and both higher A and F scores. TNF- $\alpha$  is a well-known proinflammatory cytokine and can also induce apoptosis<sup>30,31</sup> or induce activation of hepatic stellate cells that drive fibrosis, as seen in rat models.<sup>32,33</sup> Other studies have shown an association between the infiltration of HBV-tetramer<sup>-</sup> CD8<sup>+</sup> T cells and an elevated ALT in chronic HBV infection but did not specifically look at the relationship of these non-HBV specific CD8<sup>+</sup> T cells to histological changes.<sup>2,34</sup> The frequency of intrahepatic HBV-specific TNF- $\alpha$ <sup>+</sup> CD4<sup>+</sup> T cells is likely to be much lower than non-HBV-specific CD8<sup>+</sup> T cells in chronic HBV infection, but the relative importance of these populations in the generation of liver disease will be important to dissect in future studies. Despite the correlation with liver damage, the magnitude of TNF- $\alpha$  CD4<sup>+</sup> T cells was also found in our study to be inversely correlated with HBV cccDNA. The importance of TNF- $\alpha$  in control of HBV replication was previously demonstrated in the HBV transgenic mouse model, in which blocking of both IFN- $\gamma$  or TNF- $\alpha$  inhibited CD8<sup>+</sup> T cell antiviral activity.<sup>35,36</sup>

In other chronic viral infections, it has been shown that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells have a hierarchy of "ex-

haustion." In chronic viral infections with low antigen load, the CD8<sup>+</sup> T cells are able to produce IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 in addition to having cytolytic and proliferative function. However as antigen load increases, such as in HIV-1 or HCV, the ability to produce cytokines in response to viral antigens is progressively lost starting with IL-2 then TNF- $\alpha$ , and finally IFN- $\gamma$ .<sup>37</sup> Indeed, in our cohort of individuals with chronic HBV infection, we found that both circulating and intrahepatic HBV-specific T cells had low production of IFN- $\gamma$ , TNF- $\alpha$ , and even lower IL-2 production. This phenomenon was more evident within the liver, where exposure to antigen was highest. Recent work has also shown that programmed death ligand-1 expression is upregulated on hepatocytes by both HBV itself and IFN- $\gamma$ .<sup>37</sup> Programmed death ligand-1 has also been shown to be expressed at high levels in chronic HBV infection.<sup>3</sup> Together with our findings, this suggests that HBV-specific T cells have an "exhausted" phenotype in chronic HBV infection as found in HIV and HCV infection.<sup>38,39</sup>

In conclusion, we have demonstrated a difference in the cytokine profile of cultured circulating and intrahepatic HBV-specific T cells primarily in HBeAg<sup>+</sup> disease and a significant relationship between cytokine production and liver histology. The differences seen in cultured circulating and intrahepatic HBV-specific T cells may explain the capacity for HBV to persist with limited hepatocyte destruction. Interventional strategies that modulate HBV-specific T cell responses require careful consideration of both the antiviral and inflammatory consequences.

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