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Effect of telbivudine therapy on the cellular immune response in chronic hepatitis B

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ABSTRACT

Weak T-cell reactivity to the hepatitis B virus (HBV) is believed to be the dominant cause of chronic HBV infection. Several lines of experimental evidence suggest that treatment with telbivudine increases the rate of HBV e antigen (HBeAg) loss, undetectable HBV DNA, and normalization of serum alanine aminotransferase (ALT) in chronic hepatitis B patients (CHB). However, it is still unclear how early antiviral therapy affects cellular immune responses during sustained telbivudine treatment. In order to investigate this issue, we measured detailed prospective clinical, virological, and biochemical parameters, and we examined the frequency of T cell subgroups as well as the ability of peripheral blood mononuclear cells (PBMC) to respond to stimuli at five protocol time points for 51 CHB patients who received telbivudine therapy for one year. The preliminary data from this study revealed that effective-treated patients showed an increased frequency of peripheral blood $CD4^+$T lymphocytes, an augmented proliferative response of HBV-specific T-cells to the hepatitis B core antigen (HBcAg), and the induction of cytokines, such as interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) release at the site of infection compared to non-responsive patients. Enhanced HBV-specific T-cell reactivity to telbivudine therapy, which peaked at treatment week 12, was confined to a subgroup of effective-treated patients who achieved greater viral suppression.

1. Introduction

Hepatitis B virus (HBV) infection is a complex and heterogeneous disease that can evolve in a variety of ways, such as liver failure, cirrhosis, and hepatocellular carcinoma (Lee, 1997). The pathogenesis of liver damage during an HBV infection is immune-mediated and is dependent on the balance between viral replication and the HBV-specific cytotoxic T lymphocyte (CTL) response (Guidotti and Chisari, 2006; Maini et al., 1999). Vigorous immune response against HBV has been repeatedly demonstrated in patients with acute self-limiting HBV infection, but the polyclonal CTL immune response is weak and hard to detect in peripheral circulation in chronically infected patients (Guidotti and Chisari, 2006; Sprengers et al., 2006; Sobao and Takiguchi, 2002). It has been demonstrated that the weak T cell response of chronic hepatitis B patients (CHB) is associated with persistently high viral replication (Stoop et al., 2005; Franzese et al., 2005). Such findings have led to the suggestion that early antiviral therapy may prevent down-regulation or exhaustion of HBV-specific T-cell responses.

Two major types of antiviral drugs are being used for the treatment of chronic HBV: nucleotide analogs (such as lamivudine, adefovir, entecavir, tenofovir, and telbivudine) and interferon (e.g., pegylated IFN-α2b) (Dienstag, 2008). These drugs effectively suppress HBV replication, decrease liver injury, and delay disease progression (Dienstag et al., 1999, 2003; Liaw et al., 2004; Perrillo, 2009). In a multicenter study, an efficient antiviral was found to promote T cell response in chronically infected patients. The frequency of HBV-specific CTL in the peripheral blood of responders was significantly higher than that of non-responders after lamivudine treatment (Maini et al., 2000; Boni et al., 2001; Tsai et al., 2003). It is important to know whether reduction of viral load resulting from antiviral treatment can cause a recovery of the impaired T cell response in CHB patients. To our knowledge, no data have been reported concerning the effects of telbivudine on the serial measurement of peripheral blood lymphocyte subgroups and its correlation with serum HBV DNA levels in CHB patients. In this paper, we prospectively examined T-lymphocyte immune responses in relation to viral levels and antiviral therapy in 51 patients with CHB. We tried to assess the association between T-cell responses and viremia during telbivudine therapy. Results provide insight into the effects of antiviral therapy on the cellular immune response and on the outcome of chronic hepatitis B.

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2. Materials and methods

2.1. Patients

Fifty-one patients (39 males and 12 females, aged 22–62 years) and recruited from the First Affiliated Hospital of Zhejiang Medical University, were prospectively enrolled for the study of the cell-mediated immune response before and during telbivudine therapy. All patients tested positive for the presence of hepatitis B surface antigen (HBsAg) on at least two occasions more than 6 months apart and with HBV DNA >5 $\log_{10}$ copies/mL. All presented increased serum alanine aminotransferase (ALT) levels for at least 6 months. Patients co-infected with human immunodeficiency virus (HIV), hepatitis A virus, hepatitis C virus, or hepatitis D virus and patients with resolved viral hepatitis were excluded from this study. Other possible causes of chronic liver damage, such as alcohol, drugs, autoimmune diseases, and congestive heart failure were also excluded. The baseline clinical data are shown in Table 1. All patients gave their informed consent to participate in the study. The study protocol, conforming to the guidelines of the Declaration of Helsinki, was approved by the Ethics Review Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University.

2.2. Study design

The study comprised 12 weeks of patient monitoring before the start of treatment, followed by 52 weeks of telbivudine monotherapy (600 mg once a day). Clinical, virological, biochemical, and immunological parameters were assessed in study patients at five protocol time points (baseline and weeks 12, 24, 36, and 52). At each assessment, patients were evaluated for HBV-DNA, HBsAg, hepatitis B e antigen (HBeAg), hepatitis B e antibody (anti-HBe), and hepatitis B c antibody (anti-HBc). An adverse event inquiry was completed, and blood samples were drawn for immunoassays and blood chemistry. Patients achieving a level of HBV DNA undetectable by polymerase chain reaction (PCR), HBeAg seroconversion, and normalization of serum ALT levels were defined as complete responders (CR). Cases with a transient normalization of serum ALT and HBV DNA levels followed by a relapse during continued antiviral therapies were defined as partial responders (PR). HBeAg positive patients who achieved levels of HBV DNA non-detectable by PCR and ALT normalization, but did not show HBeAg seroconversion were also defined as PR. Non-responders (NR) were defined as lacking either a biochemical or a virological response during therapy (Chien et al., 1998; Liaw et al., 2000).

Table 1
Baseline characteristics of patients.

Characteristics	All patients (n = 51)
Sex(male/female)	39/12
Age, years	32 (22–62)
Serum HBV DNA load	
>5–7 $\log_{10}$ copies (mL)	27 (52.9%)
>7 $\log_{10}$ copies (mL)	24 (47.1%)
HBV genotypic distribution	
Genotype B	16 (31.4%)
Genotype C	25 (49.0%)
Genotype D	3 (5.9%)
Genotype (B + C)/(C + D)	7 (13.7%)
HBeAg-positive patients	41 (80.4%)
Serum ALT (U/L)	160 (81–651)
Serum AST (U/L)	119 (56–575)
Serum total bilirubin (μmol/L)	25.1 (10.4–40.3)
HLA-A2 positive	26 (51%)

Data are expressed as median (range) or n (%), unless otherwise indicated.
Normal ALT range = 3–50 U/L.
Normal AST range = 3–40 U/L.
Normal total bilirubin range = 1.0–22.0 μmol/L.

2.3. Serological liver function tests and virological assessments

Routine liver function tests included serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBL). These assays were performed with routine automated techniques (upper limit of normal: 50 U/L, 40 U/L, and 22.0 μmol/L, respectively) (HITACHI 7600, Japan). The quantitative determinations of HBV markers (HBsAg, HBeAg, anti-HBe, and anti-HBc) were examined using a commercial Chemiluminescent Microparticle Immunoassay (CMIA) kit with the Architect-i2000 system (Abbott Laboratories).

2.4. Serum HBV DNA detection and HBV genotype analysis

Serum HBV DNA load in individuals was quantified at all time points using the Cobas HBV Amplicor Monitor assay (Roche Diagnostics, Branchburg, NJ). The experimental methods were in accordance with the instructions of the Manufacturer in the reagent kit (Roche Diagnostics, Branchburg, NJ), and the detection limit of the assay was 300 viral genomes copies/mL.

The HBV genotypes were determined using sequence detection via PCR. All products were directly sequenced with a HBV Genotype Real Time PCR Kit (ZJ bio-Tech, China) and run on MegaBACE™ 500 according to Manufacturer's instructions. HBV genome sequences analysis software was used to analyze the results.

2.5. Peripheral blood lymphocyte subgroups measurement

The fresh whole blood from each patient was stained with anti-CD3-PCy5, CD4-FITC, and CD8-PE mouse anti-human fluorescence monoclonal antibody (all from BD Biosciences) for 30 min at room temperature. Red blood cells were lysed and cells were fixed using the Coulter QPREP specimen processing instrument (Beckman Coulter). The percentages of $CD4^+$ and $CD8^+$ T cells in the total lymphocyte population were analyzed on a Becton Dickinson FACS using CELLQuest software (BD Bioscience).

2.6. In vitro HBV specific CTL cultures and proliferation assays

Peripheral blood mononuclear cells (PBMC) were obtained by separating the blood samples using Ficoll Hypaque centrifugation (Amersham Pharmacia, Uppsala, Sweden). Then, the sample was washed twice with phosphate-buffered saline (PBS) and separated into two parts, some with culture and the rest with cryopreserving in 90% newborn calf serum (NCS) −10% dimethyl sulfoxide (DMSO) at −80 °C for future use. T-cell proliferative response to hepatitis B core antigen (HBcAg) was analyzed in PBMC prepared according to the previous protocol (Li et al., 2010; Rico et al., 2001). The frequency of HBV core-specific CTL significantly increased after day 11 of the culture, when IL-2, HBcAg, and HBV core18–27 peptides were used as stimuli. Fresh PBMC and post-stimulatory PBMC were obtained and phenotypic analysis of CTL was performed using pentamer staining.

The HBV core-specific CTLs were measured using flow cytometric analysis of a human leucocyte antigen (HLA)-restricted peptide pentamer complex (Proimmune, Oxford, UK) in 26 of 51 patients who were HLA-A2 positive. The sequences of HBV peptides were FLPSDFFPSV (core 18–27). The frequency of the HBV core-specific CTL was gated on $CD3^+$, $CD8^+$, and $pentamer^+$ cells. More than 10^5 events were acquired for each sample.

2.7. HBcAg-induced cytokine secretion (ELISpot assay)

For the cultured Enzyme-Linked Immunospot (ELISpot) Assay, 1×10^6 cryopreserved PBMC were thawed and interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-10 (IL-10) ELISpot assays were performed to analyze T cell responses to HBcAg following the *in vitro* expansion of PBMC, as previously described (Rico et al., 2001; Evans et al., 2008).

PBMCs were seeded at 2.5×10^5 per well for 48 h in 96-well tissue culture plates (Mabtech, Nacka, Sweden) in the presence of commercially available recombinant HBV nucleocapsid protein (HBcAg; >95% purity; final concentration 2 μg/mL) in triplicate wells at 37 °C with 5% CO_2. PBMCs were also cultured with phytohemagglutinin (Sigma, Dorset, United Kingdom) as a positive control. Antigen-specific spot-forming cells (SFCs in the presence of antigen minus SFCs in buffer controls) were counted with an ELISpot reader (CTL Immunospot). The number of spots in wells with media alone was subtracted from each of the peptide specific wells and results are stated as number of HBV-specific SFCs per 10^6 originally cultured PBMCs. The same individuals were tested for all

Table 2
Laboratory test at baseline and at week 52 of follow-up in 51 patients with CHB.

	Baseline				Treatment over 52 weeks			
	CR (n = 13)	PR (n = 23)	NR (n = 15)	P values	CR (n = 13)	PR (n = 23)	NR (n = 15)	P values
HBV-DNA, $\log_{10}$ copies/mL	6.56 ± 1.66	7.53 ± 0.89	7.72 ± 0.53	0.0002	1.57 ± 0.59	1.96 ± 0.62	4.10 ± 0.99	<0.0001
HBV-DNA PCR undetectable (<300 copies/mL)	0 (0)	0 (0)	0 (0)	1	13 (100)	20 (87)	0 (0)	0.000
HBeAg-positive	5 (38.5)	23 (100)	13 (86.7)	0.011	0 (0)	13 (56.5)	13 (86.7)	0.000
HBeAg-negative	8 (61.5)	0 (0)	2 (13.3)	0.000	13 (100)	10 (43.5)	2 (13.3)	0.000
Serum ALT (U/L)	146 (88–417)	178 (81–290)	156 (84–651)	0.9473	19 (12–44)	26 (11–87)	24 (13–74)	0.6398
ALT normalization	0 (0)	0 (0)	0 (0)	1	13 (100)	20 (86.9)	11 (73.3)	0.000
$CD4^+$ T-lymphocyte (%)	35.6 ± 7.8	29.8 ± 9.9	32.9 ± 8.1	0.1961	40.0 ± 7.1	33.1 ± 9.6	33.9 ± 4.2	0.0266
$CD8^+$ T-lymphocyte (%)	25.8 ± 8.0	31.7 ± 8.7	30.2 ± 9.9	0.2932	25.8 ± 8.9	28.7 ± 8.6	31.3 ± 8.8	0.3228
$CD4^+/CD8^+$ T ratio	1.2 ± 0.3	1.0 ± 0.5	1.3 ± 0.6	0.237	1.8 ± 0.7	1.3 ± 0.6	1.2 ± 0.5	0.039

Data shown are mean ± standard deviation, n (%) or median (range), unless otherwise indicated. Comparisons are made among CR, PR, and NR at baseline and at week 52. One-way ANOVA and χ^2 analysis were used as appropriate.

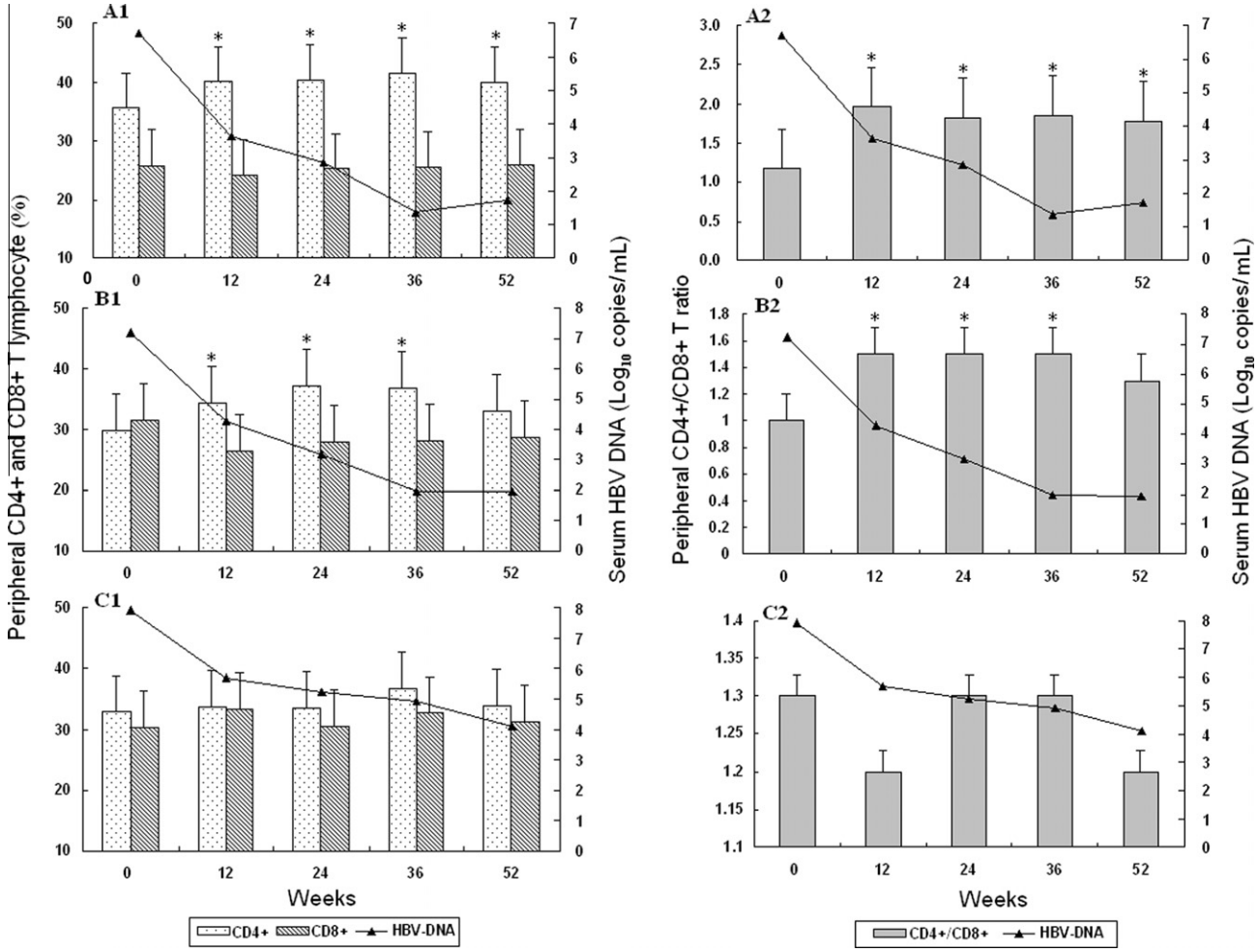

Fig. 1. Peripheral blood $CD4^+$, $CD8^+$ T-cells distribution, $CD4^+/CD8^+$ ratio, and serum HBV-DNA levels during the course of telbivudine treatment. A1 and A2, completed responders; B1 and B2, partial responders; C1 and C2, non-responders; $^*P < 0.05$, for the difference in the peripheral blood $CD4^+$ T-cells distribution and $CD4^+/CD8^+$ ratio between baseline and week 12, 24, 36,or 52.

time points; the assays were done based on availability of frozen PBMCs.

2.8. Statistics

Continuous data were presented as means ± standard deviations or median (range) and were compared using one-way ANOVA or χ^2 analysis. Correlations were examined using Spearman rank correlation analysis for nonparametric values. The differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Clinical outcome

Based on HBeAg status, serum HBV DNA levels, and normalization of the ALT at week 52, three subgroups of patients were identified (Table 2). The completed responders (CR), partial responders (PR), and non-responders (NR) represented 13 (25.5%), 23 (45.1%) and 15 (29.4%) patients in the sample of 51, respectively. Mean baseline HBV DNA levels (Log_{10} copies/mL) were 6.56 (CR), 7.53

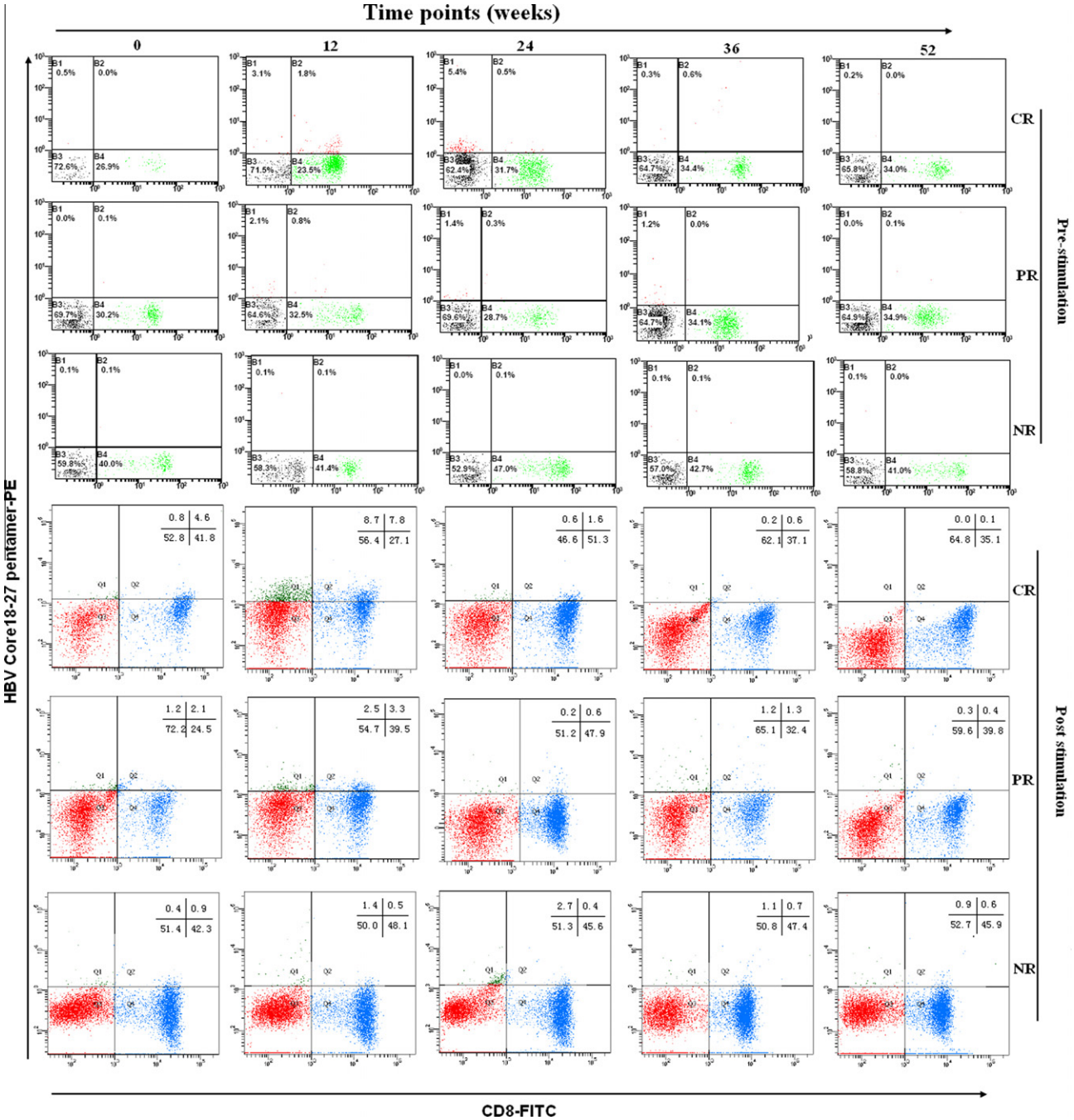

Fig. 2. The total numbers of HBV core 18–27 peptides specific T cells were assayed at both pre-stimulation and post-stimulation from one patient within each subgroup (CR group of patient 23, PR group of patient 18, and NR group of patient 5) during telbivudine treatment in HLA-A2 positive patients. Cells were stained with anti-CD3-Pcy5, anti-CD8-FITC, and pentamer-PE. Proliferation of HBV core-specific T cells were examined at day 11 following co-culture with HBcAg (1 μg/mL), HBV core 18–27 peptides (1 μg/mL), and 40 U/mL IL-2. Differential abilities of the proliferation responses of HBV core-specific T cells to telbivudine treatment are shown.

Table 3
Laboratory test at five times (weeks) of HBV-specific CTL frequency in 26 HLA-A2 positive patients.

Group	Number of HLA-A2 positive	Frequency of HBV-specific CTL (%)									
		Pre-stimulation					Post-stimulation				
		0	12	24	36	52	0	12	24	36	52
CR	6 (23.1%)	0.32 (0–1.76)	0.75 (0–2.01)	0.15 (0–0.51)	0.06 (0–0.63)	0.02 (0–0.11)	4.25 (0.43–6.17)	7.41 (3.23–9.37)	1.54 (0.32–4.05)	0.62 (0.28–1.71)	0.11 (0.33–1.12)
PR	13 (50.0%)	0.37 (0–1.03)	0.47 (0–3.51)	0.17 (0–0.85)	0.08 (0–0.31)	0.09 (0–0.26)	2.40 (0.55–4.66)	3.09 (1.46–6.87)	0.51 (0.23–2.54)	1.44 (0.29–2.72)	0.63 (0.13–1.07)
NR	7 (26.9%)	0.23 (0–1.12)	0.27 (0–0.93)	0.10 (0–0.50)	0.11 (0–0.41)	0.04 (0–0.10)	0.52 (0.21–1.65)	0.61 (0.35–1.12)	0.32 (0.13–0.98)	0.98 (0.27–1.69)	0.52 (0. 38–1.17)

Data shown are *n* (%) or median (range), post-stimulation meaning T-cell proliferative responses to HBcAg.

(PR), and 7.72 (NR). There was a significant difference in serum HBV DNA levels at baseline among these three groups of patients ($P < 0.01$) (Table 2, Fig. 1). After 52 weeks of telbivudine monotherapy, serum HBV DNA levels were markedly reduced in the CR and PR groups, being undetectable with the signal amplification assay in 33 of 51 patients, 13 in the CR and 20 in the PR. 41 HBeAg-positive patients had a loss or marked reduction of HBeAg, and five of these had the appearance of anti-HBe. Telbivudine-treated 10 HBeAg-negative patients showed higher rates of nondetectable viremia compared with 41 HBeAg-positive patients. No patient achieved HBsAg loss or seroconversion during the 52-week treatment period (data not shown).

There was a significant reduction in median ALT levels during telbivudine treatment of 51 patients with CHB (Table 2). At week 52, normal ALT were observed in 13/13 (100%), 20/23 (86.9%), and 11/15 (73.3%) of patients (CR, PR, and NR, respectively). The lower the HBV DNA levels achieved at baseline, the higher rates of HBV DNA non-detectable by PCR, ALT nomalization, and HBeAg seroconversion were achieved at week 52 (Table 2).

3.2. Peripheral blood lymphocyte subgroups response to treatment

There was a significant difference in the frequency of the response of T cell subgroups to the efficient therapy of telbivudine. The clearance of HBV DNA was relatively rapid, the percentage of peripheral blood $CD4^+$ T-cells gradually and steadily increased, and $CD8^+$ T-cells gradually and steadily decreased throughout the 52-week period (Fig. 1). It is noteworthy that only when HBV DNA levels dropped to approximately 4 $\log_{10}$ copies/mL, at week 12, could significant increases in the $CD4^+$ cells be observed ($P < 0.05$). In the CR and PR, the ratio of $CD4^+$ to $CD8^+$ T-cells ($CD4^+/CD8^+$ ratio) continued to improve and achieved a highly significant difference from the baseline ($P < 0.05$) by the 12th week. On the contrary, in the NR, the frequency of T cell subgroups exhibited no significant differences among the five time points of the study (Fig. 1). There were no statistically significant differences in $CD4^+$ and $CD8^+$ lymphocyte subset values and $CD4^+/CD8^+$ ratios between 10 HBeAg-negative and 41 HBeAg-positive patients (data not shown).

3.3. T-cell proliferation responses to HBcAg during sustained treatment

The HBV-specific CTL response was tested in 26 HLA-A2 positive patients using pentamers, and sustained lower response rates ($1.76\% \sim 0$) were observed at different time points after the start of therapy (Fig. 2, Table 3). Among these HLA-A2 positive patients, ten of them had a relatively higher frequency of CTL response before stimulation, and four in ten had a complete seroconversion at week 52.

Proliferative responses of T cells were performed during antiviral therapy. Five in 6 CR patients showed significant proliferative

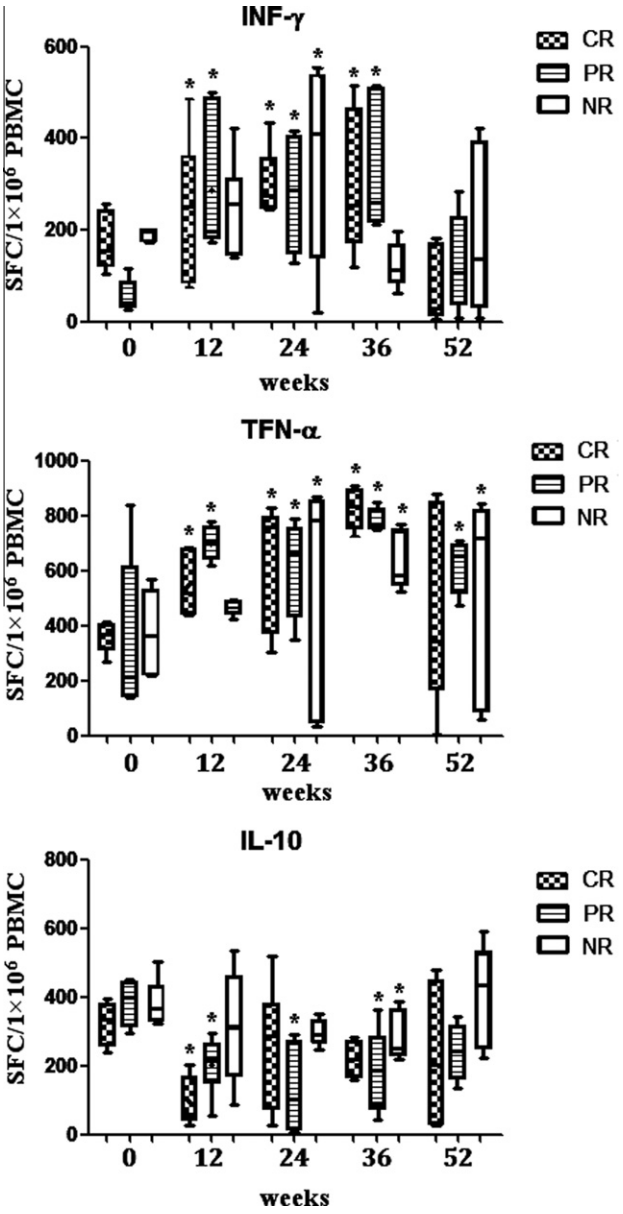

Fig. 3. Enumeration of HBcAg-specific IFN-γ, TNF-α, and IL-10 spot forming cells (SFC) per 1×10^6 peripheral blood lymphocytes during telbivudine treatment in the CR, PR, and NR groups. The results are a combination of three independent experiments. $^*P < 0.05$, for the difference in the CR, PR, and NR groups between baseline and week 12, 24, 36, or 52.

responses at week 12, and these responses were sustained until week 36. These proliferative responses were always specific to HBV core 18–27 peptides. Four in 13 PR patients also showed a

strong proliferative response at week 12. However, non-responders did not show a strong proliferative response during a sustained treatment (Fig. 2, Table 3).

3.4. Induction of HBV-peptide-specific CTL activity

We used ELISpot assays to confirm HBV-peptide-specific CTL activity. This analysis was performed retrospectively on frozen PBMC from the 51 patients initially enrolled. HBV-specific cytokine production levels of PBMC are shown in Fig. 3. Cytokine production showed secretion of IFN-γ and TNF-α and the absence of IL-10 at week 12 in CR and PR groups ($P < 0.05$). All patients exhibited remarkable increase in TNF-α production at weeks 24 and 36, as compared to baseline.

3.5. Correlation between viral suppression and peripheral blood lymphocyte phenotypes

We tried to assess the association of $CD4^+$ T cells frequency or $CD4^+/CD8^+$ ratio with viral load in the three groups during telbivudine therapy. In the CR group, there was a significant inverse correlation between the frequency of peripheral $CD4^+$ T-cells and HBV DNA levels ($r = -0.9445$, $P = 0.0156$). There was also a direct correlation between the $CD4^+/CD8^+$ ratio and HBV DNA levels ($r = -0.8305$, $P = 0.0816$). However, the relationship was not significant in PR and NR groups over the whole observation period (Fig. 4).

3.6. Correlation between HBV-DNA levels and frequency of IFN-γ-producing, TNF-α-producing, and IL-10-producing T-cells

In all patients, we assessed the frequency of HBV-specific T-cells producing IFN-γ, TNF-α, and IL-10 at the five time points specified. There was a significant increase in the frequency of IFN-γ-producing and TNF-α-producing T cells in response to HBcAg at weeks 12, 24, and 36 compared to the baseline ($P < 0.05$). In parallel, the frequency of IL-10-producing cells decreased (Fig. 3). This was inversely correlated with the increase in IFN-γ, TNF-α expression, and HBV-DNA levels in CR and PR patients, whereas there was no

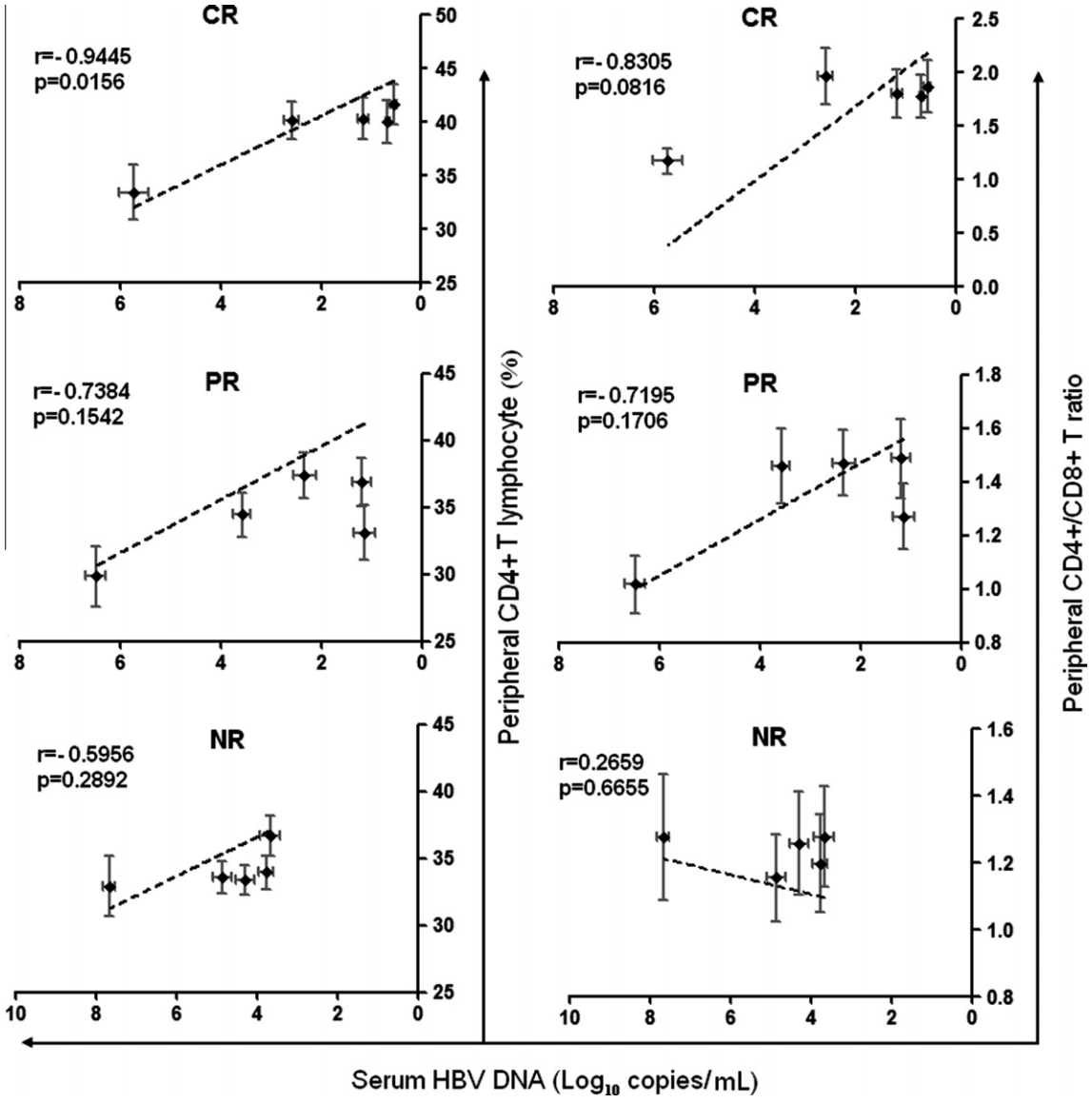

Fig. 4. Correlations between the peripheral blood $CD4^+$ T-cells, $CD4^+/CD8^+$ ratio, and HBV-DNA levels in the CR, PR, and NR groups. A increase in $CD4^+$ T-cells and $CD4^+/CD8^+$ ratio correlated with a reduction in HBV-DNA levels. The average frequency of $CD4^+$ T-cells, $CD4^+/CD8^+$ ratio and HBV-DNA levels for each time point (baseline and weeks 12, 24, 36, and 52) are represented as diamonds. Error bars illustrate standard errors.

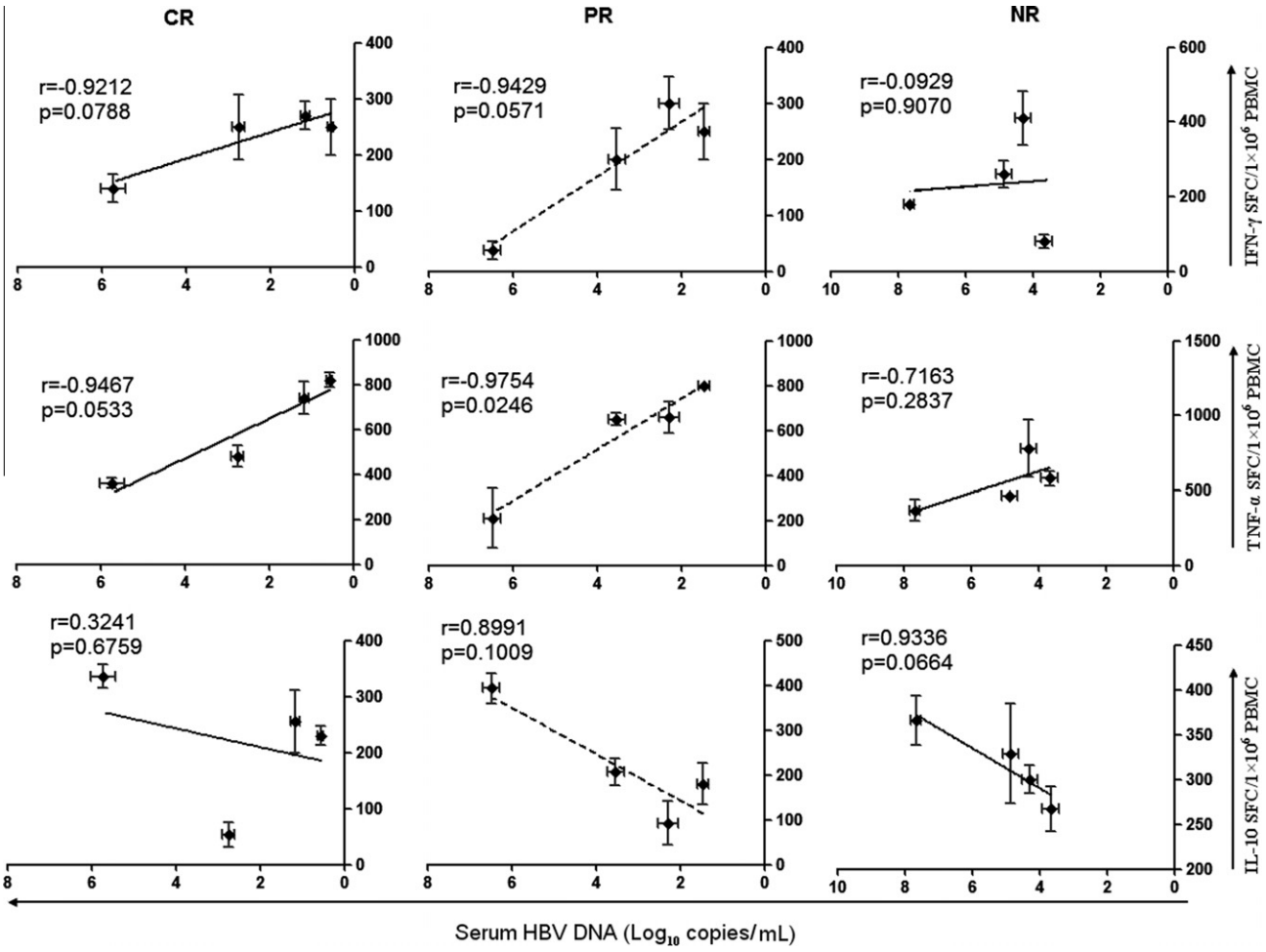

Fig. 5. Correlations between the HBcAg-specific IFN-γ, TNF-α, and IL-10 spot forming cells (SFC) and HBV-DNA levels. An increase in HBV-specific IFN-γ-producing and TNF-α-producing T cells correlates with a reduction in HBV-DNA levels. The average HBV-DNA and IFN-γ, TNF-α expression levels for each time point (baseline and weeks 12, 24, and 36) are represented as diamonds. Error bars illustrate standard errors.

correlation between IL-10-producing cells and HBV-DNA levels in the CR groups (Fig. 5).

4. Discussion

Currently, antiviral therapy has become more effective for CHB. Telbivudine is a new orally bioavailable antiviral drug with high potency and selectivity against HBV *in vitro* and in animal models (Nash, 2009; Bryant et al., 2001). The multinational GLOBE phase III study has shown that telbivudine treatment continued to be superior to lamivudine in its ability to reduce HBV load to undetectable levels, normalize serum ALT, and improve the rates of HBeAg seroconversion in CHB individuals, and there was less viral resistance in the telbivudine than in the lamivudine group (Lai et al., 2005, 2006, 2007; Liaw et al., 2009). Our results showed that telbivudine led to an early rapid viral load reduction in all patients and continued biochemical and virologic improvement through 52 weeks of treatment in patients with CHB. The improvement of virologic and biochemical parameters found in this study was in agreement with the above-mentioned studies.

Although the anti-HBV nucleoside drugs mainly target the viral polymerase activity and exhibit the early virological response, the long-term effects of most antiviral drugs is unsatisfactory (having, e.g., the emergence of HBV mutants and post-treatment relapse) (Lok and McMahon, 2004). So, more concerns have been drawn to the long-term response and especially to the continuous and stable inhibition of HBV replication. According to previous reports, in HBV infection, efficient therapy of anti-HBV drugs results in sustained inhibition of HBV replication and a significant enhancement of cellular immune responses (Boni et al., 2001; Rico et al., 2001; Tsai et al., 2003).

In this study, we found that, accompanied by a decrease in serum viral load, major changes of T lymphocyte subgroups were observed in the peripheral blood from patients with telbivudine therapy. Concomitantly with a quantitative reduction in viral replication, the frequency of $CD4^+$ T cells and the $CD4^+/CD8^+$ ratio increased during effective therapy. The results demonstrate a close positive correlation between HBV-DNA levels and frequency of $CD4^+$ T cells. The independent effect of viral load on the peripheral T-lymphocyte subgroup profile found in this study was partly in accordance with previous studies (Stoop et al., 2007; Lau et al., 2007).

Emerging evidence indicates that robust, early CD4 T-cell response is critical in the induction of effectively sustaining CD8 T-cell activity (Janssen et al., 2003; Shedlock and Shen, 2003). Studies by Boni et al. (2001) showed that lamivudine treatment for chronic hepatitis B can successfully restore CTL reactivity, creating the appropriate conditions for their therapeutic stimulation. To assess whether the antiviral effect of telbivudine also beneficially affects HBV-specific CTL function and restores efficient CTL responsiveness, we used a pentamers HLA-peptide complex technique

which could be applied to the direct measurement of each CTL population against viral peptides, including the direct measurement of the HBV core 18–27 (HBc18–27) peptide CTL population, especially the proliferation of HBV-specific $CD8^+$ T cells in response to HBcAg *in vitro* stimulation. Our data revealed that patients given the effective therapy of telbivudine not only enhanced the reconstitution of CD4 response, but also showed a significant enhancement in stimulation of HBV-specific CTL activity and reduced HBV serum titres, efficiently resulting in a significant increase in the frequency of CTL and a greater magnitude of cytokine production.

After stimulation with HBcAg, virus-specific T-cells examined directly from blood produced IFN-γ and TNF-α more frequently than IL-10. Production of proinflammatory cytokines such as IFN-γ and TNF-α may contribute to the facilitation of viral clearance. Recently, lamivudine therapy induced mainly CTL that were less frequent before the therapy. Since recovered CTL maintained the ability to produce IFN-γ in response to peptides, these CTL apparently contribute to the efficacy of lamivudine therapy in patients with hepatitis B (Kondo et al., 2004). Our results also showed that the activity of HBV-specific T lymphocyte producing IFN-γ and TNF-α showed a strong response during sustained treatment. The decrease in serum HBV DNA levels was associated with an increase in IFN-γ and TNF-α production by HBV antigen-specific T cells.

Interestingly, at the initial period of treatment ($\leqslant$12 week), the decrease of HBV DNA in all three groups was relatively rapid, but the changes in peripheral T cell subgroups, $CD4^+/CD8^+$ ratio, HBV specific T-cells proliferative response, and IFN-γ, TNF-α, and IL-10 were remarkably different. At present, virological parameters (at 12 or 24 weeks) are the most common predictors of a response to antiviral therapy in chronic HBV infection. However, the changes in virological parameters did not show significant differences during the early period ($\leqslant$12 week). Thus, these data should be combined with immunological tests, which provide information on CHB patient immune status. Early changes in immune parameters, especially, may help to predict the long-term efficacy of antiviral therapy, because these parameters were shown to be of paramount importance to evaluating the future direction of anti-viral treatment for HBV-infected patients (Tsai et al., 2003; Rico et al., 2001).

In conclusion, our data indicate that, in patients with chronic hepatitis B infection, peripheral blood $CD4^+$ T cells, the $CD4^+/CD8^+$ ratio, and the activity of HBV antigen-specific T lymphocytes correlate with continuous and stable inhibition of viral replication, which may be predictive of responsiveness during telbivudine therapy. The proper restoration of antiviral immunity was clearly associated with decreases in HBV viremia, indicating a strong correlation between viral load and T-cell function. These findings will be useful to develop appropriate therapeutic strategies for controlling viral hepatitis, as well as to improve understanding of the current knowledge regarding hepatitis prognosis.

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References

Boni, C., Penna, A., Ogg, G.S., Bertoletti, A., Pilli, M., Cavallo, C., Cavalli, A., Urbani, S., Boehme, R., Panebianco, R., Fiaccadori, F., Ferrari, C., 2001. Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B: new perspectives for immune therapy. Hepatology 4, 963–971.

Bryant, M.L., Bridges, E.G., Placidi, L., Faraj, A., Loi, A.G., Pierra, C., Dukhan, D., Gosselin, G., Imbach, J.L., Hernandez, B., Juodawlkis, A., Tennant, B., Korba, B., Cote, P., Marion, P., Cretton-Scott, E., Schinazi, R.F., Sommadossi, J.P., 2001. Antiviral L-nucleosides specific for hepatitis B virus infection. Antimicrob. Agents Chemother. 45, 229–235.

Chien, R.N., Liaw, Y.F., Chen, T.C., Yeh, C.T., Sheen, I.S., 1998. Efficacy of thymosin alpha1 in patients with chronic hepatitis B: a randomized, controlled trial. Hepatology 27, 1383–1387.

Dienstag, J.L., 2008. Hepatitis B virus infection. N. Engl. J. Med. 359, 1486–1500.

Dienstag, J.L., Goldin, R.D., Heathcote, E.J., Hann, H.W., Woessner, M., Stephenson, S.L., Gardner, S., Gray, D.F., Schiff, E.R., 2003. Histological outcome during long-term lamivudine therapy. Gastroenterology 124, 105–117.

Dienstag, J.L., Schiff, E.R., Wright, T.L., Perrillo, R.P., Hann, H.W., Goodman, Z., Crowther, L., Condreay, L.D., Woessner, M., Rubin, M., Brown, N.A., 1999. Lamivudine as initial treatment for chronic hepatitis B in the United States. N. Engl. J. Med. 341, 1256–1263.

Evans, A., Riva, A., Cooksley, H., Phillips, S., Puranik, S., Nathwani, A., Brett, S., Chokshi, S., Naoumov, N.V., 2008. Programmed death 1 expression during antiviral treatment of chronic hepatitis B: Impact of hepatitis B e-antigen seroconversion. Hepatology 48, 759–769.

Franzese, O., Kennedy, P.T., Gehring, A.J., Gotto, J., Williams, R., Maini, M.K., Bertoletti, A., 2005. Modulation of the CD8+-T-cell response by CD4+ CD25+ regulatory T cells in patients with hepatitis B virus infection. J. Virol. 79, 3322–3328.

Guidotti, L.G., Chisari, F.V., 2006. Immunobiology and pathogenesis of viral hepatitis. Annu. Rev. Pathol. 1, 23–61.

Kondo, Y., Asabe, S., Kobayashi, K., Shiina, M., Niitsuma, H., Ueno, Y., Kobayashi, T., Shimosegawa, T., 2004. Recovery of functional cytotoxic T lymphocytes during lamivudine therapy by acquiring multi-specificity. J. Med. Virol. 74, 425–433.

Janssen, E.M., Lemmens, E.E., Wolfe, T., Christen, U., von Herrath, M.G., Schoenberger, S.P., 2003. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. Nature 421, 852–856.

Lai, C.L., Gane, E., Hsu, C.W., Thongsawat, S., Wang, Y., Chen, Y., Heathcote, E.J., Rasenack, J., Bzowej, N., Naoumov, N., Zeuzem, S., Bisceglie, A.D., Chao, G.C., Constance, B.F., Brown, N.A., 2006. Two-year results from the GLOBE trial in patients with hepatitis B: greater clinical and antiviral efficacy for telbivudine (LdT) vs lamivudine. Hepatology 44, 222A.

Lai, C.L., Gane, E., Liaw, Y.F., Hsu, C.W., Thongsawat, S., Wang, Y., Chen, Y., Heathcote, E.J., Rasenack, J., Bzowej, N., Naoumov, N.V., Di Bisceglie, A.M., Zeuzem, S., Moon, Y.M., Goodman, Z., Chao, G., Constance, B.F., Brown, N.A., 2007. Telbivudine versus lamivudine in patients with chronic hepatitis B. N. Engl. J. Med. 357, 2576–2588.

Lai, C.L., Leung, N., Teo, E.K., Tong, M., Wong, F., Hann, H.W., Han, S., Poynard, T., Myers, M., Chao, G., Lloyd, D., Brown, N.A., 2005. A 1-year trial of telbivudine, lamivudine, and the combination in patients with hepatitis B e antigen-positive chronic hepatitis B. Gastroenterology 129, 528–536.

Lau, G.K., Cooksley, H., Ribeiro, R.M., Powers, K.A., Shudo, E., Bowden, S., Hui, C.K., Anderson, J., Sorbel, J., Mondou, E., Rousseau, F., Lewin, S., Perelson, A.S., Locornini, S., Naoumov, N.V., 2007. Impact of early viral kinetics on T-cell reactivity during antiviral therapy in chronic hepatitis B. Antivir. Ther. 12, 705–718.

Lee, W.M., 1997. Hepatitis B virus infection. N. Engl. J. Med. 337, 1733–1745.

Liaw, Y.F., Gane, E., Leung, N., Zeuzem, S., Wang, Y., Lai, C.L., Heathcote, E.J., Manns, M., Bzowej, N., Niu, J., Han, S.H., Hwang, S.G., Cakaloglu, Y., Tong, M.J., Papatheodoridis, G., Chen, Y., Brown, N.A., Albanis, E., Galil, K., Naoumov, N.V., 2009. 2-Year GLOBE trial results: telbivudine is superior to lamivudine in patients with chronic hepatitis B. Gastroenterology 136, 486–495.

Liaw, Y.F., Leung, N.W., Chang, T.T., Guan, R., Tai, D.I., Ng, K.Y., Chien, R.N., Dent, J., Roman, L., Edmundson, S., Lai, C.L., 2000. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B, Asia Hepatitis Lamivudine Study Group. Gastroenterology 119, 172–180.

Liaw, Y.F., Sung, J.J., Chow, W.C., Farrell, G., Lee, C.Z., Yuen, H., Tanwandee, T., Tao, Q.M., Shue, K., Keene, O.N., Dixon, J.S., Gray, D.F., Sabbat, J., 2004. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N. Engl. J. Med. 351, 1521–1531.

Li, X., Chen, Y., Ma, Z., Ye, B., Wu, W., Li, L., 2010. Effect of regulatory T cells and adherent cells on the expansion of HBcAg-specific CD8+ T cells in patients with chronic hepatitis B virus infection. Cell. Immunol. 264, 42–46.

Lok, A.S., McMahon, B.J., 2004. Chronic hepatitis B: update of recommendations. Hepatology 39, 857–861.

Maini, M.K., Boni, C., Ogg, G.S., King, A.S., Reignat, S., Lee, C.K., Larrubia, J.R., Webster, G.J., McMichael, A.J., Ferrari, C., Williams, R., Vergani, D., Bertoletti, A., 1999. Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. Gastroenterology 117, 1386–1396.

Maini, M.K., Reignat, S., Boni, C., Ogg, G.S., King, A.S., Malacarne, F., Webster, G.J., Bertoletti, A., 2000. T cell receptor usage of virus-specific CD8 cells and recognition of viral mutations during acute and persistent hepatitis B virus infection. Eur. J. Immunol. 30, 3067–3078.

Nash, K., 2009. Telbivudine in the treatment of chronic hepatitis B. Adv. Ther. 26, 155–169.

Perrillo, R., 2009. Benefits and risks of interferon therapy for hepatitis B. Hepatology 49, S103–111.

Rico, M.A., Quiroga, J.A., Subirá, D., Castañón, S., Esteban, J.M., Pardo, M., Carreño, V., 2001. Hepatitis B virus-specific T-cell proliferation and cytokine secretion in chronic hepatitis B e antibody-positive patients treated with ribavirin and interferon alpha. Hepatology 33, 295–300.

Shedlock, D.J., Shen, H., 2003. Requirement for CD4 T cell help in generating functional CD8 T cell memory. Science 300, 337–339.
Sobao, Y., Takiguchi, M., 2002. The role of hepatitis B and C virus-specific CTL in the control of viral replication. Nihon Rinsho Meneki Gakkai Kaishi. 25, 79–88.
Sprengers, D., van der Molen, R.G., Kusters, J.G., De Man, R.A., Niesters, H.G., Schalm, S.W., Janssen, H.L., 2006. Analysis of intrahepatic HBV-specific cytotoxic T-cells during and after acute HBV infection in humans. J. Hepatol. 45, 182–189.
Stoop, J.N., van der Molen, R.G., Kuipers, E.J., Kusters, J.G., Janssen, H.L., 2007. Inhibition of viral replication reduces regulatory T cells and enhances the antiviral immune response in chronic hepatitis B. Virology 361, 141–148.
Stoop, J.N., van der Molen, R.G., Baan, C.C., van der Laan, L.J., Kuipers, E.J., Kusters, J.G., Janssen, H.L., 2005. Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. Hepatology 41, 771–778.
Tsai, S.L., Sheen, I.S., Chien, R.N., Chu, C.M., Huang, H.C., Chuang, Y.L., Lee, T.H., Liao, S.K., Lin, C.L., Kuo, G.C., Liaw, Y.F., 2003. Activation of Th1 immunity is a common immune mechanism for the successful treatment of hepatitis B and C: tetramer assay and therapeutic implications. J. Biomed. Sci. 10, 120–135.