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Expression levels of CD28, CTLA-4, PD-1 and Tim-3 as novel indicators of T-cell immune function in patients with chronic hepatitis B virus infection

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Abstract. Chronic hepatitis B (CHB) is one of the most common types of infectious diseases worldwide. The interaction between hepatitis B virus (HBV) and the host immune response is vital for the clinical outcome of HBV infection. Costimulatory signals are key factors for the host immune response and play a critical role in innate immunity, particularly antiviral immunity. The aim of the present study was to investigate the correlation between the expression levels of costimulatory molecules and the different states of CHB infection, including the expression levels prior to and following treatment with antiviral agents. The expression levels of CD28, CTLA-4, PD-1, Tim-3 and T-cell subsets were determined by flow cytometry. The load of HBV DNA in the serum was detected by quantitative polymerase chain reaction and the serology markers, including HBeAg and alanine aminotransferase (ALT), were measured by conventional methods. Compared to the healthy control group, the expression levels of CD28 and CTLA-4 on CD4 T cells prior to and following treatment with antiviral agents (the pre- and post-treatment groups, respectively) were significantly decreased, while the expression levels of Tim-3 on CD4 and CD8 T cells were significantly increased. In addition, the expression levels of PD-1 on CD4 and CD8 T cells in the pre-treatment group were significantly increased compared to those in the post-treatment and healthy control groups. Moreover, the multivariate analysis revealed that the levels of ALT and HBV-DNA in the serum were significantly positively correlated with PD-1 expression levels. In conclusion, the expression levels of these costimulatory molecules reflect the immune dysfunction of T cells in patients with CHB and, combined with T-cell subset analysis may be used as a novel evaluation system of immune function in patients with HBV infection.

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Introduction

Chronic hepatitis B (CHB) is one of the most common types of infectious diseases worldwide. It was previously demonstrated that hepatitis B virus (HBV) is not directly cytopathic and that the interactions between HBV and the host immune response are crucial for the clinical outcome of HBV infection (1,2). The main cause of CHB is considered to be a cell immunity function disorder. Briefly, HBV evades the cellular immune response, avoids clearance and may cause persistent viral infections. During this process, T cells play a vital role in the immune function (3). During the process of immune response, the HBV antigens are processed by antigen-presenting cells (APCs) and presented to naïve T cells by major histocompatibility complex (MHC) molecules. Subsequently, these cells deliver a primary signal to initiate T-cell activation by engaging the T-cell receptor/CD3 complex with foreign antigens associated with MHC molecules (4). The activation of T cells is also regulated by the balance between positive and negative signals. This balance is maintained by the interaction of costimulatory or coinhibitory receptors on T cells and their ligands on APCs. These stimulatory and inhibitory signals, which are presented by dendritic cells, are integrated by the T cells and determine the final outcome of T-cell activation (5,6). A previous study demonstrated that there exists an altered T-cell costimulation during chronic HBV infection (7). In addition, a number of previous studies indicated that certain costimulatory molecules are highly correlated with T-cell immune function (8,9). Therefore, we hypothesized that the expression of these costimulatory molecules may be used as a novel evaluation indicator of T-cell immune function in patients with CHB.

Mature T lymphocytes expressing the αβT cell receptor are generally classified as either $CD4^+$ or $CD8^+$, based on the mutually exclusive expression of these two lymphocyte coreceptors (10). A number of diseases with immune dysfunction appear as abnormal changes of the T-cell subsets, which is also the case in patients with CHB at various stages. Therefore, it is difficult to effectively evaluate the therapeutic effects on CHB.

Table I. Clinical and virological characteristics of the subjects enrolled in the study.

Characteristics	Pre-treatment (n=30)	Post-treatment (n=32)	Healthy control (n=30)
Age, years (mean ± SEM)	41.0±3.7	38.1±2.3	44.4±2.45
Gender (F/M)	13/17	9/23	15/15
ALT (U/l) (mean ± SEM)	567±105[a]	226±78	<30
HBeAg-positive	19/11[b]	13/19	0/30
HBV DNA $\log_{10}$ copies/ml (mean ± SEM)	5.29±0.42[b]	3.56±0.27	0

The differences between two groups were assessed by the Student's t-test according to HBV DNA and ALT levels and by the Chi-square test according to HBeAg positivity. [a]P<0.05 and [b]P<0.01, compared to the post-treatment group. HBV, hepatitis B virus; ALT, alanine aminotransferase; SEM, standard error of the mean; F female; M, male.

Previous studies demonstrated the association between the costimulatory molecules, CD28, CTLA-4, PD-1 and Tim-3 with T-cell immune function (8,9). Thus, in the present study we aimed to systematically investigate the correlation between the expression profiles of these costimulatory molecules and the different stages of CHB infection and assess the T-cell subsets in an absolute levels analysis.

Materials and methods

Subjects. A total of 62 patients with CHB, including 30 patients who had not received antiviral and immune therapy and 32 patients who had received antiviral treatment at the First Affiliated Hopital of Soochow University (Suzhou, China) were enrolled in this study. According to the total HBV-DNA load, the patients were divided into two groups: 29 patients with $\leq 10^5$ copies/ml and 33 patients with $>10^5$ copies/ml. The criteria for CHB diagnosis were previously described (11). All the patients were HBsAg-positive and hepatitis C virus-, hepatitis D virus-, human immunodeficiency virus type 1 (HIV-1)- and HIV-2-negative. Patients with other causes of chronic liver damage were excluded from the study. Table I summarizes the clinical and virological characteristics of the study population. A total of 30 healthy blood donors with normal liver function were selected as normal controls. Written informed consent was obtained from each individual according to the Declaration of Helsinki and the study protocol was approved by the Clinical Research Ethics Committee of The First Affiliated Hospital to Soochow University (Suzhou, China).

Virological assessment and liver biochemical assays. HBeAg was determined by commercial enzyme immunoassay kits (TMB HBeAg; ShanghaiBio Corporation, Pudong, China). The HBV-DNA load was determined by quantitative PCR on a Light Cycler 480 (Roche Diagnostics, Mannheim, Germany) with a detection sensitivity of 10^3 copies/ml. The serum alanine aminotransferase (ALT) levels were measured by an Autoanalyzer Au2700 (Olympus, Kobe, Japan).

Assay of CD28, CTLA-4, PD-1 and Tim-3 expression on T cells. Phycoerythrin (PE)-conjugated anti-Tim-3 was purchased from BioLegend (San Diego, CA, USA). All the other antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA). The cells were analyzed using FACS Calibur and CellQuest software (BD Biosciences). At least 500,000 events per run were acquired for pentamer and phenotypic expression analyses. The absolute value of lymphocytes was determined with the XE-2100™ Automated Hematology System (Sysmex America, Inc., Lincolnshire, IL, USA).

Statistical analysis. All the data were analyzed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). All the subjects were analyzed with one-way analysis of variance.The Student's t-test was used to compare the different groups and the Spearman's rank correlation test was used to assess the correlations between CD28, CTLA-4, PD-1 and Tim-3 with ALT and HBV-DNA levels. Count data were measured with the Chi-square test. P<0.05 was considered to indicate a statistically significant difference.

Results

T-cell subsets in absolute level analysis. The levels of CD4 and CD8 T cells were measured in the pre-treatment (n=30), post-treatment (n=32) and normal control (n=30) groups (Fig. 1). Compared to the normal control group, the levels of CD4 and CD8 T cells were significantly decreased and the CD4:CD8 ratio was significantly increased in the pre- and post-treatment groups, while there were no significant differences between the pre- and post-treatment groups.

Expression levels of costimulatory molecules. As shown in Fig. 2, compared to the normal control group, the expression levels of CD28 and CTLA-4 on CD4 T cells were significantly lower in the two CHB groups, although they did not differ significantly between the pre- and post-treatment groups. However, the expression levels of PD-1 on either CD4 or CD8 T cells in the pre-treatment group were significantly higher compared to those in the post-treatment and normal control groups. Similarly, the expression levels of Tim-3 on either CD4 or CD8 T cells were also significantly higher in the two CHB groups compared to the normal control group and the levels of Tim-3 on CD8 T cells differed significantly between the pre- and post-treatment groups (P<0.01).

Correlation between CD28, CTLA-4, PD-1 and Tim-3 expression with HBV-DNA and HBeAg. As shown in Fig. 3, compared to the group of patients with an HBV-DNA load of $\leq 10^5$ copies/ml, the expression levels of PD-1 were significantly increased in patients with an HBV-DNA load of

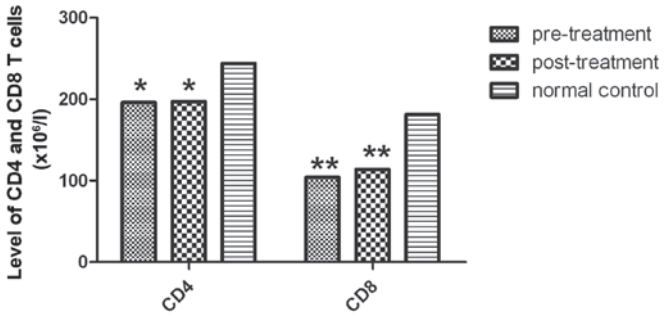

Figure 1. Histogram of CD4 and CD8 T-cell levels in normal control subjects and in chronic hepatitis B patients pre- and post-treatment with antiviral agents. The differences between patients and control subjects were determined by the least significant difference t-test. *P<0.05 and **P<0.01, compared to normal subjects.

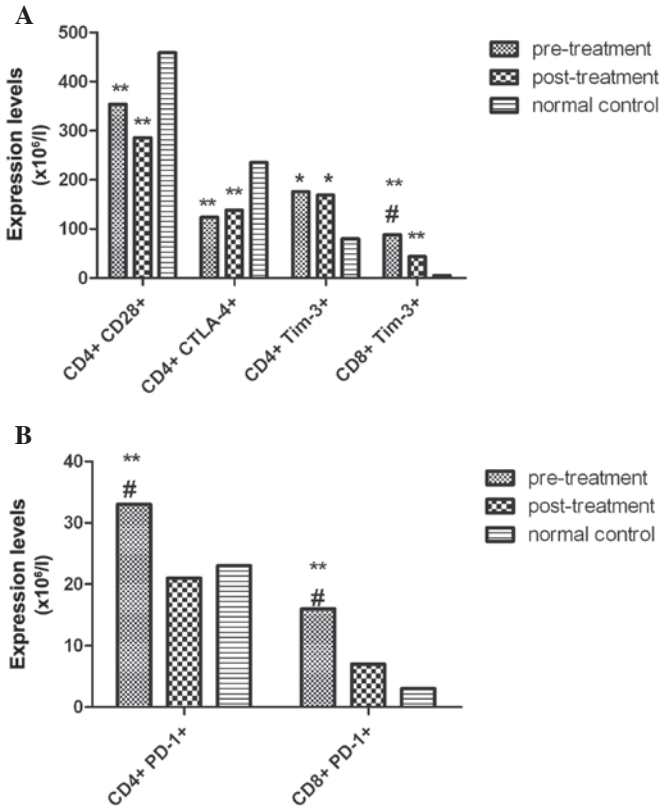

Figure 2. Histograms of the levels of (A) CD28, CTLA-4, Tim-3 and (B) PD-1 in normal control subjects and in chronic hepatitis B patients pre- and post-treatment with antiviral agents. The differences between patients and control subjects were determined by the least significant difference t-test. *P<0.05 and **P<0.01, compared to normal control subjects and #P<0.01 compared to the post-treatment group.

$>10^5$ copies/ml. There were no significant differences in the CD28, CTLA-4 and Tim-3 levels between these two groups (all P>0.05). We also observed no significant differences in the expression levels of the four costimulatory molecules between HBeAg-positive and -negative patients (all P>0.05).

We performed a correlation analysis between ALT and PD-1 levels in the post-treatment group. Our results demonstrated that there were significant and positive correlations between the levels of ALT and PD-1 on CD4 (P=0.021; Fig. 4B) and CD8 T cells (P<0.001; Fig. 4C).

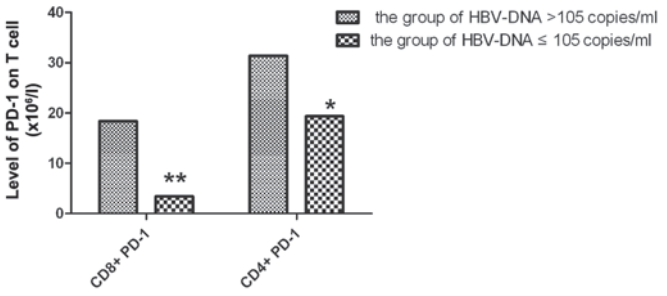

Figure 3. Correlation of the expression levels of PD-1 with the load of HBV DNA in patients with chronic hepatitis B. The differences between the two groups were determined by the Student's t-test. *P<0.05 and **P<0.01 compared to the HBV DNA of $>10^5$ copies/ml group. HBV, hepatitis B virus.

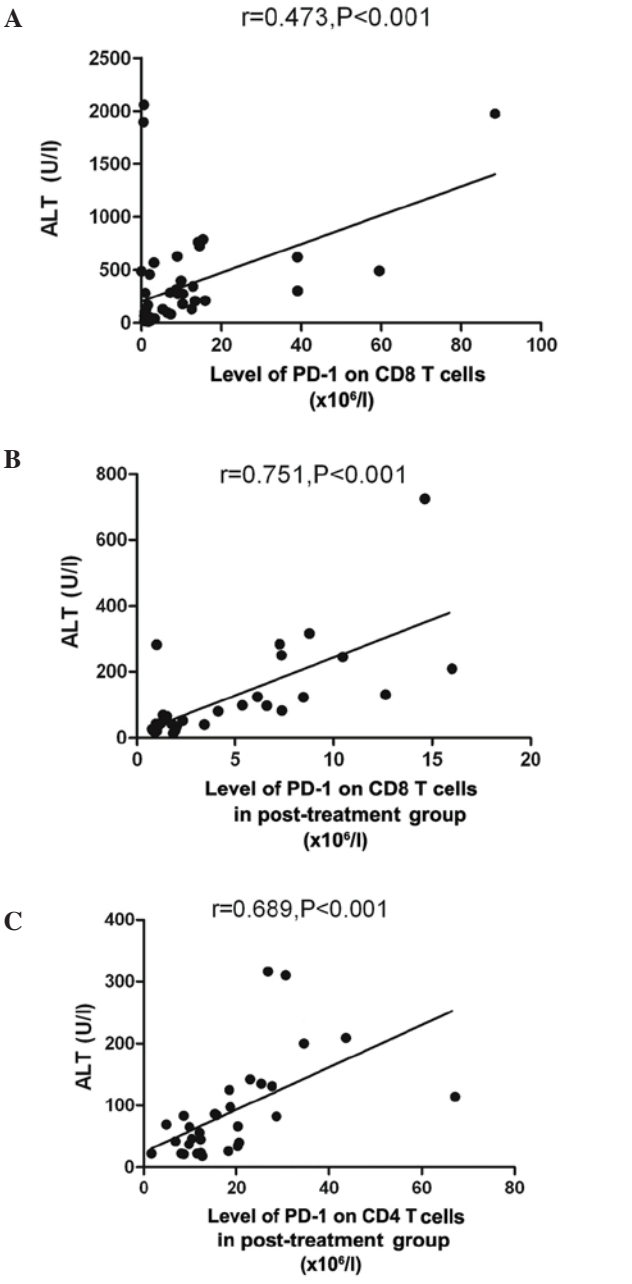

Figure 4. Correlation of the levels of alanine aminotransferase and PD-1 with chronic hepatitis B. The Spearman's rank correlation coefficient was used to assess the correlations.

Discussion

In the present study, we demonstrated that the levels of some costimulatory molecules were altered during different stages of CHB. Our results confirmed the previous hypothesis that, combined with T-cell subsets, certain costimulatory molecules may reflect T-cell immune dysfunction in CHB patients.

In our multivariate analysis, demographic factors (male gender and older age), biochemical parameters (ALT levels) and virological factors (HBeAg positivity and HBV DNA load) were found to be associated with the expression of the costimulatory molecules in the pre- and post-treatment groups to varying degrees.

From the analysis of the absolute levels of T-cell subsets in the pre- and post-treatment groups, the CD8 levels were shown to be significantly lower compared to those in the normal control group. This may be explained by the fact that excess HBV may invade and multiply rapidly in liver cells, overloading the host cell immunity and resulting in extensive immune cell death and subsequent depletion of the host's immune function (12). The CD4:CD8 ratio is an important measure of immune cell status and may reflect a certain degree of immune cell function. Once imbalanced, it may result in immune system disorders and a series of immune diseases (13). Our results demonstrated that the CD4:CD8 ratio in the pre- and post-treatment groups was significantly higher compared to that in the normal group. This may be direct evidence that patients with CHB exhibit an immune cell function disorder.

In this study, we observed a significant decrease in the CD28 levels on CD4 T cells in the pre- and post-treatment groups. We also observed that the levels of CD28 in the post-treatment group were decreased more significantly, indicating that CD28 may not be an efficient indicator of therapeutic effect. It is hypothesized that CHB T cells were not effectively activated, which would result in a decrease in the specific killing with allergen antigenic target cells, allowing the virus to evade immune recognition and cause persistent chronic liver disease. Therefore, the analysis of CD28 levels via long-term monitoring may contribute to the elucidation of the immune response in CHB patients with immune escape.

Furthermore, we observed that the levels of CTLA-4 on CD4 T cells were significantly lower in the pre- and post-treatment groups compared to those in the normal control group. Previous studies demonstrated that Thl/Th2 dysfunction may contribute to chronic HBV infection (14). It was demonstrated that the predominance of Th1 cells is associated with acute self-limiting infection and HBV removal, whereas Th2-cell predominance is associated with the duration of chronic HBV infection (15,16). Furthermore, CTLA-4 has the ability to inhibit the Thl/Th2 balance from shifting towards Th2 (17,18). Thus, we hypothesized that CHB patients may be in a state of Th2 predominance. Furthermore, we observed that the expression of CTLA-4 on CD4 T cells was increased in the post-treatment group, indicating that CTLA-4 expression on CD4 T cells may gradually return to normal with treatment. Therefore, we hypothesized that the expression levels of CTLA-4 may be used to evaluate T-cell immune function in patients with CHB.

As a negative costimulatory receptor, PD-1 attenuates T-cell responses and is crucial in the regulation of T-cell tolerance. The expression levels of PD-1 were found to be high on the virus-specific T cells during persistent chronic HIV and HCV infection (19,20). In the present study, we observed a significant elevation in the expression of PD-1 on either CD4 or CD8 T cells in the pre-treatment group compared to the normal group, which was similar to the observations of recent studies on HBV (21-24). Notably, the levels of PD-1 on CD8 T cells in the post-treatment group were significantly decreased compared to those in the pre-treatment group. Therefore, we inferred that PD-1 expression may be used as an efficient indicator of CHB patients receiving antiviral treatment. Moreover, we observed that the levels of PD-1 either on CD4 or CD8 T cells were significantly positively correlated with the HBV-DNA load. It was demonstrated that the HBV-DNA load is a measure of the persistence of HBV and it was hypothesized that PD-1 is associated with HBV infection. The PD-1 levels were found to be correlated significantly and positively with serum ALT levels in patients with chronic HBV. Furthermore, we found that, following antiviral treatment, the PD-1 levels were significantly positively correlated with the serum ALT levels. As ALT is a laboratory parameter commonly used in screening for the progression of liver diseases (25), the results of this correlation further suggest that PD-1 may be a novel parameter indirectly reflecting the activity of chronic HBV infection.

The overexpression of Tim-3 on T cells and the Tim-3/galectin-9 signaling pathway have been associated with disease progression and immune suppression in CHB (26). Moreover, the overexpression of PD-1 and Tim-3 on CD8 T cells was associated with the CD8 T-cell dysfunction and co-blockade of the PD-1 and Tim-3 pathways may improve CD8 T-cell responses and viral control in chronically infected mice (27). It was demonstrated that the two negative costimulatory molecules may lead to further disruption of T-cell function (28). Our study demonstrated that the expression of Tim-3 on either CD4 or CD8 T cells was upregulated compared to that in normal subjects and the level of Tim-3 tended to normalize with treatment, reflecting the development of immune homeostasis in patients with CHB.

There were no significant differences in the levels of the costimulatory molecules between HBeAg-positive and -negative patients, indicating that HBeAg may not be a crucial factor regulating their expression. However, our study demonstrated that HBeAg positivity in the post-treatment group was significantly decreased compared to that in the pre-treatment group. Therefore, HBeAg positivity may only be used as an independent screening process laboratory parameter of liver disease.

In conclusion, to the best of our knowledge, this study was the first to evaluate the expression levels of CD28, CTLA-4, PD-1 and Tim-3 on the T cells of patients with CHB. We also combined the analysis of T-cell subsets in absolute levels in order to accurately evaluate T-cell immune function at different stages of CHB. Based on our results, we aim to investigate the association of HBV infection in cirrhosis and hepatocellular carcinoma with the quantitative detection of soluble molecules, in order to further optimize the evaluation system. Future studies addressing this issue may contribute to clinical diagnosis, treatment and prognosis of patients with CHB.

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