Effects of Co-infection With Clonorchiasis Sinensis on T Cell Exhaustion Levels in Patients With Chronic Hepatitis B

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Research

Keywords: Clonorchiasis Sinensis, Chronic hepatitis B, T cell exhaustion, Inhibitory receptors, Suppressive cytokines

Posted Date: August 18th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-789633/v1>

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Abstract

Background

To investigate the effects of co-infection with *C. sinensis* on T cell exhaustion levels in patients with chronic hepatitis B

Methods

Inhibitory receptors and suppressive cytokines expression in circulating CD4⁺ and CD8⁺ T cells was detected by flow cytometry. The correlations between PD-1 and TIM-3 expression and alanine aminotransaminase (ALT) levels, aspartate aminotransferase (AST) levels and HBV DNA levels were analyzed using GraphPad Prism 7.0.

Results

PD-1 and TIM-3 expression levels were significantly higher on CD4⁺T and CD8⁺T cells from co-infected patients than on those from the HBV patients. In addition, CD4⁺T cells and CD8⁺T cells function was inhibited by *C. sinensis* and HBV coinfection, secreting low levels of IFN- γ , IL-2 and TNF- α . Then, a significant positive correlation was found between the PD-1 and TIM-3 expression levels on T cells and the AST ,ALT levels and HBV DNA levels.

Conclusions

Our current results suggested that *C. sinensis* co-infection could exacerbate T cell exhaustion in patients with chronic hepatitis B. Furthermore, it maybe one possible reason for the weaker response to antiviral therapies and the chronicity of HBV infection in co-infected patients. We must realize that the importance of *C.sinensis* treatment for HBV infected patients. It might provide useful information for the clinical doctors to choose the right treatment plans.

Background

Clonorchiasis, resulted from *Clonorchis sinensis* (*C. sinensis*), is a food-borne

parasitic disease. Over 35 million people are infected by *C. sinensis* in Asia, among

which about 15 million infected people were prevalent in China[1–3]

.One third of them are from Guangdong province[4].*Clonorchis sinensis* is a fish-borne trematode that locates in the bileduct of mammals including humans[5]. Freshwater fish act as the intermediate hosts in which the cercariae penetrate and transform to the metacercariae. Humans become infected by eating raw or undercooked freshwater fish[6]. Clinically, clonorchiasis patients accompany different severity of the symptoms. Most of the infected people are not treated in time because of mild or unspecific symptoms, such as asthenia, nausea,diarrhea, jaundice, hepatomegaly and liver tenderness. So,

Clonorchiasis is a major but surprisingly neglected tropical disease in China. Only about 10% of the infected patients who have been treated in time show obvious acute clinical symptoms in the early infection, such as cholelithiasis, cholangitis and cholecystitis. What's worse, chronic infection can develop to the cirrhosis or cholangiocarcinoma[7–9]. The excretory-secretory products (ESPs) of *C. Sinensis* which cause chemical damage often trigger highly polarized immune responses during chronic infections[10–12]. Meanwhile, our previous research results show that the ESPs could induce immune cells to mainly produce Th2 cytokines such as IL-4, IL-6 and IL-10[13–15].

Hepatitis B virus (HBV) infection is a major public health challenge that may cause severe complications, such as cirrhosis and hepatocellular carcinoma (HCC)[13–15]. Adaptive immune responses against HBV, including CD4 + T and CD8 + T cells, are thought to play a dominant role in determining whether HBV infection is followed by recovery or viral persistence[17–19]. A lot of studies have indicated the essentiality of T cell exhaustion in HBV infection[20–23]. Patients who infected with acute hepatitis B presented progressive high frequency and functional impairment peripheral blood[24]. As the same time, experimentally infected chimpanzees verified the antiviral functions of CD8 + T cells, indicating that CD8 + T cells that were knocked out can delayed the clearance of virus. On contrast, chronic HBV infection often shows weak virus specific CD8 + T cell reactivity and then arises a inability state of T cell which is described as the “exhaustion” [25, 26]. T cell exhaustion was firstly described during chronic lymphocytic choriomeningitis virus (LCMV) infection in mice more than a decade ago as dysfunction of antigen-specific T cells[27, 28]. Since then, T cell exhaustion has been confirmed in plenty of animal models and in humans with chronic viral infections and cancer[27, 28]. T cell exhaustion is characterized by expression of multiple cell surface inhibitory receptors, secretion suppressive cytokines and appearance of immunoregulatory cells, such as programmed cell death-1(PD-1), lymphocyte activation gene-3(LAG-3), T cell immunoglobulin mucin-3 (Tim-3), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), IL-2, IFN- γ and TNF- α [30–34]. HBV can't be cleaned up and continuous replication, then develop into liver cirrhosis and liver cancer. The main reason for the chronicity of HBV infection is virus specific T cell exhaustion. Reversal of these exhausted T cells will pave the way for the development of more effective immunotherapeutic strategies for the treatment of chronic HBV infection.

Some studies had shown that in areas where *C. sinensis* is prevalent, such as guangdong province, we often observe the concurrent infection of HBV and *C. sinensis*. And both *C. sinensis* infection and chronic hepatitis B virus infection can cause liver diseases. There were few studies on the co-infection with HBV and *C. sinensis*. The aim of our study is to evaluate the effect of co-infection with *C. sinensis* on T cell exhaustion levels in patients with chronic hepatitis B. Our results showed that *C. sinensis* co-infection could exacerbate T cell exhaustion in patients with chronic hepatitis B. Furthermore, it maybe one possible reason for the weaker response to antiviral therapies in co-infected patients. We must realize that the importance of *C. sinensis* treatment for HBV infected patients. It might provide useful information for the clinical doctors to choose the right treatment plans.

Methods

Subjects

All patients were hospitalized or followed-up in the Third Affiliated Hospital of Sun Yat-sen University. According to the infection of HBV and *C. sinensis*, the patients were divided into three groups: patients who were mono-infected with HBV surface-antigen (HBsAg)-positive and HBV DNA > 20 IU/mL, patients who were mono-infected with *C. sinensis* eggs-positive and patients who were co-infected with HBsAg-positive, HBV DNA > 20 IU/mL and *C. sinensis* eggs-positive. The exclusion criterion for patients with the following other causes of chronic liver damage were excluded from the study: hepatitis A, C, D and E, HIV, *Schistosoma japonicum*, *Schistosoma mansoni* or other parasites, alcoholic or autoimmune factor. And Patients with diabetes, hematological system diseases, serious heart diseases, pregnant women were also excluded from the study. Twenty-five healthy individuals with matched age and sex were also enrolled as normal controls. We summarize the clinical characteristics obtained for the enrolled subjects in Table 1. The study protocol was approved by the Clinical Research Ethics Committee of the Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, China and informed consent forms were signed by all participants according to the Declaration of Helsinki.

Table 1
Clinical Characteristics of the Study Groups

	HC group (n = 25)	HBV group (n = 24)	C. sinensis group (n = 23)	co-infected group (n = 20)
Age,years (Mean ± SD)	45.48 ± 11.34	43.29 ± 12.35	53.17 ± 11.58	45.90 ± 7.52
Gender(male/female)	19/6	18/6	19/4	18/2
HBsAg(COI)	negative	5390.20 ± 3397.34	negative	4893.50 ± 2892.48
HBV DNA log10 copies/ml(Mean ± SD)	negative	4.73 ± 1.99	negative	5.50 ± 1.92
ALT(U/L)	17.00(8.00– 34.00)	48.50(13.00– 774.00)	45.00(10.00– 296.00)	455.50(18.00– 894.00)abc
AST(U/L)	17.00(10.00– 40.00)	44.50(16.00– 660.00)	39.00(12.00– 172.00)	243.50(20.00– 902.00)abc
TB(μmol/L)	8.30(4.50– 15.80)	21.35(4.70– 478.60)	12.50(4.00– 382.10)	88.60(6.90– 469.50)abc
Data are expressed as the Mean ± SD or median and ranges. AST: aspartate aminotransferase; ALT: alanine aminotransferase; HBV: hepatitis B virus; TB: total bilirubin				
a Statistically significantly different vs. healthy control group				
b Statistically significantly different vs. HBV mono-infected group				
c Statistically significantly different vs. C. sinensis mono-infected group				
A				

Virological and Biochemical Assessments

Biochemical assessments was measured using automated biochemical techniques (Hitachi 7600, Tokyo, Japan). The serum HBsAg level was detected using the electrochemiluminescence immunoassay kit for the COBAS-e801 system (Roche Diagnostics, Mannheim, Germany), with a positive result recorded as COI > 1.00. The serum levels of HBV DNA were determined by real-time PCR with a lower detection limit of 20 IU/mL (Roche Diagnostics, Mannheim, Germany)

Peripheral Blood Mononuclear Cells Isolation

Peripheral Blood Mononuclear Cells(PBMCs) were from fresh heparinized blood (5 mL) collected from each group patient and isolated by Ficoll-Hypaque density gradient centrifugation (HaoYang Biological Manufacture, Tianjin, China). PBMCs were cultured at 1×10⁶ cells/mL in RPMI 1640 (Invitrogen Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen Gibco).

Determination of PD-1,TIM-3,LAG-3 and CTLA-4 expression by flow cytometry

The PBMCs were washed twice with PBS buffer and incubated with Anti-CD3-PE-cy7(Bioscience, USA), anti-CD4-APC(Bioscience), anti-CD8-FITC (Bioscience), anti-PD-1-PerCP-cy5.5(Bioscience), anti-Tim-3-PE(Bioscience), anti-LAG-3-A1eXa Fluor Φ 647(Bioscience), and anti-CTLA-4-BV421(Bioscience) for surface staining at room temperature for 30 min in the dark. IgG isotype control antibodies were used as the negative controls.Staining cells were analyzed with a BD FACS Aria II analyzer (BD Biosciences) and data was analyzed with FlowJo version 10 for Windows(Tree Star Inc, USA).

Determination of intracellular cytokine release by flow cytometry

The PBMCs were stimulated with a cell stimulation cocktail (1:500, eBioscience, USA). After 4-6h of incubation, anti-CD3-PE-cy7(Bioscience, USA), anti-CD4-APC(Bioscience) and anti-CD8-FITC (Bioscience) was used for cells staining at room temperature for 30 min in the dark. After permeabilization and fixation, the cells were washed twice with PBS buffer and incubated with anti-IFN- γ -Alexa Fluor 647(Biolegend), anti-IL-2-BV42(Biolegend) and anti-TNF- α -PE(Biolegend) at room temperature for 30 min in the dark. IgG isotype control antibodies were used as the negative controls.Staining cells were analyzed with a BD FACS Aria II analyzer (BD Biosciences) and data was analyzed with FlowJo version 10 for Windows(Tree Star Inc, USA).

Statistical analysis

All data of continuous variables were presented as the mean values $\pm$ standard error or median and ranges. Differences in continuous variables were performed by independent-samples T test or ANOVA for comparison with more than two groups. The Wilcoxon rank sum test was used for non-parametric data. The correlations between the PD-1,TIM-3, LAG-3 and CTLA-4 expression levels and the ALT, AST, TB, HBsAg and HBV DNA levels were analyzed in the co-infected group by Pearson's correlation analysis. The data were analyzed using GraphPad Prism 7.0. P values < 0.05 were considered statistically significant.

Results

Demographic and clinical characteristics of the study subjects

The enrolled subjects in this study were classified into four groups: co-infected group(n = 20), HBV group(n = 24), C. sinensis group(n = 23), and HC group(n = 25). Age, gender, liver function, HBsAg and serum HBV DNA concentrations were demonstrated in Table 1.The co-infected group showed higher levels of ALT, AST and TB than the C. sinensis group and the HC group. Especially, the levels of ALT, AST and TB in the co-infected group was significantly higher than that in the HBV group (p < 0.05, respectively)

.However, no difference in the HBV DNA log copies and HBsAg was found in the co-infected group and in the HBV group ($P > 0.05$). In brief, these data indicate that *C. sinensis* may weak liver function and aggravate the disease state in patients with chronic hepatitis B. Eventually, *C. sinensis* and HBV coinfection may lead to the chronicity of HBV infection.

Expression of inhibitory receptors on the surface of peripheral blood CD4 + T and CD8 + T cells from the co-infected group, HBV group and *C. sinensis* group and the HC group.

The frequencies of CD4 + T and CD8 + T cells with surface expression of the inhibitory receptors PD-1, TIM-3, LAG-3 and CTLA-4 were evaluated in the co-infected group, HBV group, *C. sinensis* group and HC group using flowcytometry. Compared with HC group, the percentage of PD-1 + CD4 + T cells, PD-1 + CD8 + T cells, TIM-3 + CD4 + T cells, TIM-3 + CD8 + T cells, LAG-3 + CD8 + T cells, CTLA-4 + CD4 + T cells and CTLA-4 CD8 + T cells were all significantly increased in both HBV group and Co-infected group (all $P < 0.002$, Fig. 1), while no significant difference was found in the LAG-3 on the CD4 + T cells. (Fig. 1D). Significantly higher frequencies of PD-1 + CD4+, PD-1 + CD8+, TIM-3 + CD4 + and TIM-3 + CD8 + cells were observed in the co-infected group than in the HBV group ($P = 0.000$, $P = 0.045$, $P = 0.006$, and $P = 0.010$, respectively). No difference was found in the LAG-3 and CTLA-4 expression levels (Fig. 1D,E,H,I). This result demonstrated that high PD-1 and TIM-3 expression in the co-infected group could exacerbate T cell exhaustion in patients with chronic hepatitis B.

Difference in suppressive cytokines production between the CD4 + T and CD8 + T cells from the Co-infected group, HBV group and *C. sinensis* group and the HC group.

The percentages of CD4 + T and CD8 + T cells with suppressive cytokine expression, such as IL-2, IFN- γ , and TNF- α were assessed in the four group by flow cytometry.

Compared with HC group, the percentage of IFN- γ , IL-2 and TNF- α in both the CD4 + T cells and CD8 + T cells were all significantly decreased in both HBV group and co-infected group (all $P < 0.001$, Fig. 2), and lower IL-2 expression levels were detected in both the CD4 + T cells and CD8 + T cells obtained from the co-infected group than those in the CD4 + T cells and CD8 + T cells obtained from the HBV group ($P = 0.004$, $P = 0.016$, Fig. 2B,2E). The co-infected group showed lower levels of IFN- γ and TNF- α in the CD8 + T cells than the HBV group ($P = 0.032$, $P = 0.038$, Fig. 2F,2G), while no significant difference was found in the IFN- γ and TNF- α in the CD4 + T cells. (Fig. 2C,2D). These findings suggested that CD4 + T cells and CD8 + T cells function was inhibited by *C. sinensis* and HBV coinfection, secreting low IFN- γ , IL-2 and TNF- α .

Correlation between clinical indicators and the surface expression of the inhibitory receptors expression levels on T cells

Since PD-1 and TIM-3 were highly expressed by CD4 + T cells and CD8 + T cells in co-infected group relative to the expression levels in the HBV group, we analyzed the association between the PD-1 and TIM-3 expression levels on T cells and the serum

ALT levels, AST levels, TB levels, the HBSAg levels and the HBV DNA levels among the co-infected patients. Our results demonstrated that there were significant positive correlations between the levels of ALT and PD-1 and TIM-3 expression on CD4+ (PD-1 + CD4+: $r = 0.696$, $P = 0.001$, TIM-3 + CD4+: $r = 0.600$, $P = 0.005$, Fig. 3A,C) and CD8 + T cells (PD-1 + CD8+: $r = 0.498$, $P = 0.026$, TIM-3 + CD8+: $r = 0.575$, $P = 0.008$, Fig. 3E, G) among the co-infected patients. A positive correlation was observed between PD-1 and TIM-3 expression and the serum AST level on CD4+ (PD-1 + CD4+: $r = 0.716$, $P = 0.000$, TIM-3 + CD4+: $r = 0.524$, $P = 0.018$ Fig. 3B, D) and CD8 + T cells (PD-1 + CD8+: $r = 0.628$, $P = 0.003$, TIM-3 + CD8+: $r = 0.564$, $P = 0.010$, Fig. 3F, H) among the co-infected patients. Meanwhile, there was a significant positive correlation between HBV DNA levels and PD-1 and TIM-3 expression on CD4+ (PD-1 + CD4+: $r = 0.514$, $P = 0.020$, TIM-3 + CD4+: $r = 0.501$, $P = 0.025$, Fig. 3Q, R) and CD8 + T cells (PD-1 + CD8+: $r = 0.527$, $P = 0.017$, TIM-3 + CD8+: $r = 0.539$, $P = 0.014$, Fig. 3S, T) among the co-infected patients. However, no association was found between the PD-1 and TIM-3 expression and TB levels on CD4+ (PD-1 + CD4+: $r = 0.086$, $P = 0.718$, TIM-3 + CD4+: $r = 0.176$, $P = 0.457$, Fig. 3I,J) and CD8 + T cells (PD-1 + CD8+: $r = 0.131$, $P = 0.582$, TIM-3 + CD8+: $r = 0.222$, $P = 0.347$, Fig. 3K,L) and there was no correlation between PD-1 expression and HBSAg levels on CD4+ (PD-1 + CD4+: $r = -0.023$, $P = 0.923$, TIM-3 + CD4+: $r = -0.089$, $P = 0.708$, Fig. 3M,N) and CD8 + T cells (PD-1 + CD8+: $r = -0.198$, $P = 0.403$, TIM-3 + CD8+: $r = 0.179$, $P = 0.451$, Fig. 3O,P) in the co-infected patients. These data suggested that the inhibitory receptors PD-1 and TIM-3, highly expressed by CD4 + T cells and CD8 + T cells from the co-infected group, were related to the degree of hepatic injury and high viral replication.

Discussion

In our study, we investigated the relationship between HBV infection and *C. sinensis* infection and further provided the evidence that *Clonorchis sinensis* co-infection could exacerbate T cell exhaustion in patients with chronic hepatitis B.

In this study, we focused on the exhausted T cells, especially those in patients with co-infection *C. sinensis* and HBV. Actually, T cell exhaustion results in inhibition of host immune responses, which is a status of gradual T cell dysfunction that arises during chronic infections[35]. So immunoregulation is centrally involved. The main performances can be grouped into three categories: cell surface inhibitory receptors, cell factors and immunoregulatory cell types[36]. Our data showed that the cell surface inhibitory receptors such as PD-1, TIM-3, LAG-3 and CTLA-4 levels were significantly higher expressed by CD8 + T cells in CHB group than in HC group. CD4 + T cells expressed higher PD-1, TIM-3 and CTLA-4 levels, not including LAG-3, in CHB group than in HC group. In addition, we also analyzed the PD-1, TIM-3, LAG-3 and CTLA-4 expression levels on both CD4 + T cells and CD8 + T cells among the *C. sinensis* mono-infected patients. These inhibitory receptors were higher expressed in the *C. sinensis* group relative to the expression levels in the HC group. On the other hand, lower IFN- γ , IL-2 and TNF- α expression levels were detected in both the CD4 + T cells and CD8 + T cells from both CHB group and *C. sinensis* group than in the T cells obtained from the HC group. Our results suggest that the two infectious factors of hepatitis B and *C. sinensis* would eventually lead to the T cell exhaustion.

Hepatitis B virus (HBV) is the most prevalent virus that leads to liver inflammation and injury. The ability to clear HBV after infection is associated with a strong specific T-cell response, and during chronic infections the function of exhausted T cells is often characterized with varying degrees of damage[37, 38]. *C. sinensis* always activate greatly polarized immune responses, especially during chronic infection. During HBV infection and clearance, It is well known that CD8 + T cell play a key role. However, CD4 + T cells are the key factor regulating on the cellular CTL response to HBV [39]. According to previous research, the lack of CD4 + T cells was the main cause of CD8 + T cell exhaustion[40]. Like CD8 + T cells, specific CD4 + T cells also lose effector function during chronic viral infection[11–14]. Therefore longer persistent infection or loss of help from CD4 + T cells leads to more severe T cell exhaustion[41]. Although T cell exhaustion CD4 + T cells have remained important, few researches about CD4 + T cell exhaustion in chronic HBV-infected patients are mentioned[42, 43]. These may be the reason that our study focused on both CD4 + T cells and CD8 + T cells.

As we known, it will be chronic HBV infection, when the virus can't be cleaned up and continuous replication, then develop into liver cirrhosis and liver cancer. The main reason for the chronicity of HBV infection is virus specific T cell exhaustion[24]. *C. sinensis* co-infection could exacerbate T cell exhaustion in patients with chronic hepatitis B. Our results were consistent with this findings. Our data showed that PD-1 and TIM-3 levels were significantly higher expressed on CD4 + T cells and CD8 + T cells in the co-infected group than those in the HBV group. At the same time, IFN- γ , IL-2 and TNF- α expression levels were significantly lower detected in the CD8 + T cells from co-infected group than those in the CD8 + T cells obtained from the HBV group. But in CD4 + T cells, only IL-2 expression levels were significantly lower detected from co-infected group than those from the HBV group. This is consistent with the previous notion that in co-infected patients the efficacy of anti-viral treatment was better in patients who were prescribed with entecavir and praziquantel than entecavir alone[44]. This result further indicated that the efficacy of HBV antiviral treatment was related to the removal worms in co-infected patients. One possible reason for the weaker response to antiviral therapies in co-infected patients was the T cell exhaustion.

Many cell surface inhibitory receptors can coregulate T cell exhaustion. T cells during chronic infection in animal models and in humans can also express PD-1, LAG-3, TIM-3, CTLA-4 and many other inhibitory receptors[10–12]. The pattern of inhibitory receptor expressed on the T cell can substantially affect the severity of dysfunction[45]. Our results are consistent with these findings. Furthermore, the recovery of function is increased considerably by simultaneous block of the PD-1 pathway, LAG-3 pathway, CTLA-4 pathway and TIM-3 pathway[46–48]. Our data showed that only PD-1 and TIM-3 levels were significantly higher expressed on T cells in the co-infected group than those in the HBV group. No difference was found in the LAG-3 and CTLA-4 expression levels. Further investigations are required to address this point. It may be due to the small number of these cases.

T cells mainly secrete suppressive cytokines such as IL-2, IFN- γ and TNF- α to eradicate viruses and parasites causing intracellular infections and play an important role in cellular immunity[49]. Furthermore,

we sought to understand the T cell exhaustion, as measured by changes in the IFN- γ , IL-2 and TNF- α secretion.

These findings suggested that CD4 + T cells and CD8 + T cells function was inhibited by C. sinensis and HBV coinfection, secreting lower IFN- γ , IL-2 and TNF- α , and these suppressive cytokines expression levels were significantly lower detected in the CD8 + T cells from co-infected group than those in the CD8 + T cells obtained from the HBV group. Obviously, C.sinensis has more effect on CD8 + T cells from co-infected group.

Moreover, we observed that a significant positive correlation between the levels of PD-1 and TIM-3 expression either on CD4 and CD8 T cells with the conventional markers for hepatic injury. This result demonstrated that the upregulation of PD-1 and TIM-3 expression were positively correlated with the level of hepatic injury and high viral replication. Because both ALT and AST are the laboratory common indicators used in monitoring the progression of hepatic injury[50], the conclusions of this correlation further suggest that PD-1 and TIM-3 may be some novel indicators indirectly reflecting the persistence of chronic HBV and C. sinensis coinfection.

Conclusions

In summary, this study is the first to provide strong evidence that C. sinensis co-infection could exacerbate T cell exhaustion in patients with chronic hepatitis B. C. sinensis and HBV coinfection may lead to the chronicity of HBV infection, and C. sinensis may play a role in the unresponsiveness to antiviral therapy in co-infected patients. We must realize that the importance of C.sinensis treatment for HBV infected patients.It might provide useful information for the clinical doctors to choose the right treatment plans.

Abbreviations

C. Sinensis: *Clonorchiasis Sinensis*; PD-1: Programmed death-1; TIM-3: T cell immunoglobulin domain and mucin domain-3 ; LAG-3: lymphocyte activation gene-3; CTLA-4: Cytotoxic T-Lymphocyte Associated Protein 4; ALT: alanine aminotransaminase; AST: aspartate aminotransferase; TB: total bilirubin; PBMCs: Peripheral Blood Mononuclear Cells; ESPs: excretory-secretory products ; HBV: Hepatitis B virus

Declarations

Acknowledgements

We are thankful to all volunteer who participated in this study.

Authors' contributions

HD designed the study and wrote the manuscript. ML, YW and YF collected the samples and performed the experiments. MS analyzed and interpreted the results. BH reviewed and edited the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by grants from the National Natural Science Foundation of China (81902082,81802403).

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The study protocol was approved by the Clinical Research Ethics Committee of the Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, China (permission number 0204501) and informed consent forms were signed by all participants according to the Declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Gating strategy and expression profiles of PD-1, TIM-3, LAG-3 and CTLA-4 in CD4+T and CD8+T cells of in the co-infected group, HBV group, *C. sinensis* group and HC group. (A) The gating strategies and representative results of PD-1, TIM-3, LAG-3 and CTLA-4 expression in CD4+T and CD8+T cells. (B-I) The percentages of PD-1+CD4+ (B), TIM-3+CD4+ (C), LAG-3+CD4+ (D), CTLA-4+CD4+ (E), PD-1+CD8+ (F), TIM-3+CD8+ (G), LAG-3+CD8+ (H), CTLA-4+CD8+ (I) T cells expression from co-infected group, HBV group, *C.*

sinensis group and HC group. Data show the means±SD. Asterisks indicate statistically significant differences between Two groups, as measured by paired, two-tailed Student's t-test (*p <0.05, ** p <0.01, *** p <0.001)

Figure 2

Gating strategy and expression profiles of IL-2, IFN-γ, and TNF-α in CD4+T and CD8+T cells of in the co-infected group, HBV group, C. sinensis group and HC group. (A) The gating strategies and representative

results of IL-2, IFN- γ , and TNF- α expression in CD4+T and CD8+T cells. (B-G) The percentages of IL-2 in CD4+(B), IFN- γ in CD4+(C), TNF- α in CD4+(D), IL-2 in CD8+(E), IFN- γ in CD8+(F), TNF- α in CD8+(G) T cells expression from co-infected group, HBV group, C. sinensis group and HC group. Data show the means $\pm$ SD. Asterisks indicate statistically significant differences between Two groups, as measured by paired, two-tailed Student's t-test (*p <0.05, ** p <0.01, *** p <0.001)

Figure 3

Correlation between the percentages of PD-1+ and TIM-3+ on the CD4+T cells and CD8+T cells and the levels of ALT, AST, TB, HBsAg and HBV DNA in the co-infected patients. Relationship between the serum ALT levels and the percentages of PD-1+CD4+(A), TIM-3+CD4+(C), PD-1+CD8+(E), TIM-3+CD8+(G) T cells expression in the co-infected patients; The relationship between the serum AST levels and the percentage of PD-1+CD4+(B), TIM-3+CD4+(D), PD-1+CD8+(F), TIM-3+CD8+(H) T cells expression in the co-infected patients; The relationship between the TB levels and the percentages of PD-1+CD4+(I), TIM-3+CD4+(J), PD-1+CD8+(K), TIM-3+CD8+(L) T cells expression in the co-infected patients; The relationship between the HBsAg levels and the percentages of PD-1+CD4+(M), TIM-3+CD4+(N), PD-1+CD8+(O), TIM-3+CD8+(P) T cells expression in the co-infected patients; The relationship between the HBV DNA levels and the percentages of PD-1+CD4+(Q), TIM-3+CD4+(R), PD-1+CD8+(S), TIM-3+CD8+(T) T cells expression in the co-infected patients.

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