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Effects of co-infection with *Clonorchis sinensis* on T cell exhaustion levels in patients with chronic hepatitis B

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Research Paper

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Abstract

To investigate the effects of co-infection with *Clonorchis sinensis (C. sinensis)* on T cell exhaustion levels in patients with chronic hepatitis B, we enrolled clinical cases in this study, including the patients with concomitant *C. sinensis* and HBV infection. In this study, we detected inhibitory receptors and cytokine expression in circulating CD4+ and CD8+ T cells by flow cytometry. 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 were significantly inhibited by *C. sinensis* and HBV co-infection compared with HBV single infection, secreting lower levels of Interferon gamma (IFN-γ), Interleukin-2 (IL-2), and TNF-α. Our current results suggested that *C. sinensis* co-infection could exacerbate T cell exhaustion in patients with chronic hepatitis B. PD-1 and TIM-3 could be novel biomarkers for T cell exhaustion in patients with *Clonorchis sinensis* and chronic hepatitis B co-infection. Furthermore, it may be one possible reason for the weaker response to antiviral therapies and the chronicity of HBV infection in co-infected patients. We must realize the importance of *C. sinensis* treatment for HBV-infected patients. It might provide useful information for clinical doctors to choose the right treatment plans.

Introduction

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 are prevalent in China (Botelho *et al.* 2009; Deng *et al.* 2020; Na *et al.* 2020; Wang *et al.* 2018). *C. sinensis* is a fish-borne trematode that locates in the bile duct of mammals, including humans (Young *et al.* 2010). Freshwater fish act as the intermediate hosts in which the cercariae penetrate and transform into the metacercariae. Humans become infected by eating raw or undercooked freshwater fish (Liang *et al.* 2009).

Clinically, clonorchiasis patients exhibit 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 is worse, chronic infection can develop into the cirrhosis or cholangiocarcinoma (Choi *et al.* 2004; Qian *et al.* 2012; Tang *et al.* 2016).

Hepatitis B virus (HBV) infection is a major public health challenge that may cause severe complications, such as cirrhosis and hepatocellular carcinoma (HCC) (Trépo *et al.* 2014). Both *C. sinensis* infection and chronic hepatitis B virus infection can cause liver diseases. So, some studies have shown that the cases of the concurrent infection of HBV and *C. sinensis* often appear in areas where *C. sinensis* is prevalent, especially in the Guangdong province (Chen *et al.* 2012; Yang *et al.* 2014). Our previous study confirmed that co-infected patients presented weaker liver function and higher HBV Deoxyribo Nucleic Acid (DNA) copies, and the presence of *C. sinensis* may aggravate the disease state (Li *et al.* 2016a).

In chronic infections and cancer, T cells show poor effector function and persistent expression of multiple inhibitory receptors. This distinct state is often associated with T cell dysfunction, which is described as 'exhaustion' (Bertoletti A and Naoumov NV 2003; Chisari *et al.* 2010). T cell exhaustion was first described during chronic lymphocytic choriomeningitis virus (LCMV) infection in mice more than a decade ago as dysfunction of antigen-specific T cells (Gallimore *et al.* 1998; Zajac *et al.* 1998). Since then, it has been confirmed in plenty of animal models and humans with chronic viral infections and cancer (Virgin *et al.* 2009).

T cell exhaustion is characterised by the expression of multiple cell surface inhibitory receptors, defective production of some 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), Interleukin-2 (IL-2), Interferon gamma (IFN-γ), and transforming growth factor-α (TNF-α) (Evans *et al.* 2008; Hartzell *et al.* 2020; Liu *et al.* 2016; Wongjitrat *et al.* 2013; Ye *et al.* 2017).

Many studies have illustrated that T cell exhaustion has also been observed in humans during chronic infection diseases, such as HBV, hepatitis C virus (HCV), LCMV, human immunodeficiency virus (HIV), and cancers (Boni *et al.* 2012; Fisicaro *et al.* 2020; Saeidi *et al.* 2018; Ye *et al.* 2015). It has been linked to these chronic infectious diseases. Our study puts attention on T cell exhaustion in HBV infection. HBV cannot be cleaned up, and continuous replication then develops into liver cirrhosis and liver cancer. The main reason for the chronicity of HBV infection is virus-specific T cell exhaustion (Bertoletti and Ferrari 2012; Ferrari 2020; Meng et al., 2019). 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.

The aim of our study was 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 may be one possible reason for the weaker response to antiviral therapies in co-infected patients. We must realize the importance of *C. sinensis t*reatment for HBV-infected patients. It might provide useful information for clinical doctors to choose the right treatment plans.

Materials and methods

Subjects

All patients were hospitalised or followed up at the Third Affiliated Hospital of Sun Yat-sen University. According to the infection of HBV and C. sinensis, the enrolled subjects were divided into four 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, healthy individuals with matched age and sex as normal controls.

The inclusion criteria for normal controls were negative for both HBsAg and *C. sinensis* eggs. In addition, mono-infected patients with HBV and co-infected patients with HBV and *C. sinensis* were prescribed antiviral drugs, entecavir (ETV, 0.5 mg once daily) only. Neither mono-infected with *C. sinensis* nor co-infected patients with HBV and *C. sinensis* were treated with anthelmintic treatment.

The exclusion criterion for all the enrolled subjects 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, or alcohol. And Patients with autoimmune diseases, diabetes, hematological system diseases, serious heart diseases, or pregnant women were also excluded from the study. Twenty-five healthy individuals with matched age and sex were also enrolled as normal controls.

We summarised 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.

Virological and biochemical assessments

Biochemical assessments were 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 cut-off index (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)

Eggs of C. sinensis per gram of feces count

Eggs of *C. sinensis* per gram of feces were determined by a sodium hydroxide digestion method in mono-infected and co-infected with *C. sinensis* patients. The specific process of sodium hydroxide digestion is as follows: 1 g feces is put into a centrifugal sedimentation tube containing 5 ml of 10% sodium hydroxide solution, then we stir it fully, and digest it for 24 hr. We suck 0.075 ml for smear and count the eggs in the whole piece under the microscope, and then multiply it by 100.

Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were from fresh heparinised blood (5 ml) collected from each group of patients and isolated by Ficoll-Hypaque density gradient centrifugation

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	5,390.20 ± 3,397.34	Negative	4,893.50 ± 2,892.48
HBV DNA log10 copies/ml (Mean ± SD)	Negative	4.73 ± 1.99	Negative	5.50 ± 1.92
Clonorchis sinensis eggs (n/g)	Negative	Negative	200(100–1,200)	100(100–300)
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; COI: cut-off index; DNA: Deoxyribo Nucleic Acid; HBV: hepatitis B virus; HC: healthy control; TB: total bilirubin; SD: Standard Deviation; a: statistically significantly different versus healthy control group; b: statistically significantly different versus HBV mono-infected group; c: statistically significantly different versus *C. sinensis* mono-infected group.

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(Haoyang Biological Manufacture, Tianjin, China). Cell viability was analysed by trypan blue staining and automated counting of live and dead cells. We ensured that the viability of PBMCs was more than 90% after isolation. PBMCs were cultured at 1×106 cells/ml in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen Gibco, New York, 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 a Phosphate Buffered Saline (PBS) buffer and incubated with Anti-CD3-PE-Cy7 (eBioscience, California, USA), anti-CD4-APC (eBioscience, California, USA), anti-CD8-FITC (eBioscience, California, USA), anti-PD-1-PerCP-Cy5.5 (eBioscience, California, USA), anti-Tim-3-PE (eBioscience, California, USA), anti-LAG-3-A1eXa Fluor®647 (eBioscience, California, USA), and anti-CTLA-4-BV421 (eBioscience, California, USA) for surface staining at room temperature for 30 min in the dark. IgG isotype control antibodies were used as the negative controls. Stained cells were analysed with a BD FACS Aria II analyser (BD Biosciences, New Jersey, USA), and data were analysed with FlowJo version 10 for Windows (Tree Star Inc., California, USA).

Determination of intracellular cytokine release by flow cytometry

The PBMCs were stimulated with a cell stimulation cocktail (eBioscience, California, USA), which is a cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 hr, followed by the addition of the protein transport inhibitor cocktail (eBioscience, California, USA), a cocktail of Brefeldin A and monensin, at 2 hr before detection. After 6 hr of incubation, anti-CD3-PE-Cy7 (eBioscience, California, USA), anti-CD4-APC (eBioscience, California, USA), and anti-CD8-FITC (eBioscience, California, USA) were used for cell staining at room temperature for 30 min in the dark. Intracellular staining for cytokines was performed with a Fixation/Permeabilization Kit (BD Biosciences, New Jersey, USA) and after permeabilisation and fixation, the cells were washed twice with a PBS buffer and incubated with anti-IFN-γ-Alexa Fluor 647 (Biolegend, California, USA), anti-IL-2-BV42 (Biolegend, California, USA), and anti-TNF-α-PE (Biolegend, California, USA) at room temperature for 30 min in the dark. IgG isotype control antibodies were used as the negative controls. Staining cells were analysed with a BD FACS Aria II analyser (BD Biosciences, New Jersey, USA), and data were analysed with FlowJo version 10 for Windows (Tree Star Inc., California, USA).

Statistical analysis

All data of continuous variables were presented as the mean values ± standard error or median and ranges. Differences in continuous variables were performed by one-way Analysis of Variance (ANOVA) for comparison with more than two groups. The Wilcoxon rank sum test was used for non-parametric data. The data were analysed 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 are demonstrated in Table 1. The co-infected group showed higher levels of aspartate aminotransferase (ALT), alanine aminotransaminase (AST), and total bilirubin (TB) than the *C. sinensis* group and the HC group. Especially, the levels of ALT, AST, and TB in the co-infected group were 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 weaken liver function and aggravate the disease state in patients with chronic hepatitis B. Eventually, *C. sinensis* and HBV co-infection 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 flow cytometry. Compared with the 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 the HBV group and *C. sinensis* group (all $P < 0.005$, Figure 1), while no significant difference was found in the LAG-3 on the CD4+ T cells (Figure 1b). 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 ($P = 0.000$, $P = 0.017$, $P = 0.001$, and $P = 0.002$, respectively). No difference was found in the LAG-3 and CTLA-4 expression levels 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 (Figure 1). 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 cytokines production between the CD4+ T and CD8+ T cells in the co-infected group, HBV group and C. sinensis group, and the HC group

The percentages of CD4+ T and CD8+ T cells with cytokine expression, such as IL-2, IFN-γ, and TNF-α, were assessed in the four groups by flow cytometry. Compared with the 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 the HBV group and co-infected group (all $P < 0.01$, Figure 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 ($P = 0.006$, $P = 0.033$, Figure 2b, 2c). The co-infected group showed lower levels of IFN-γ and TNF-α in the CD8+ T cells than the HBV group ($P = 0.047$, $P = 0.046$, Figure 2c), while no significant

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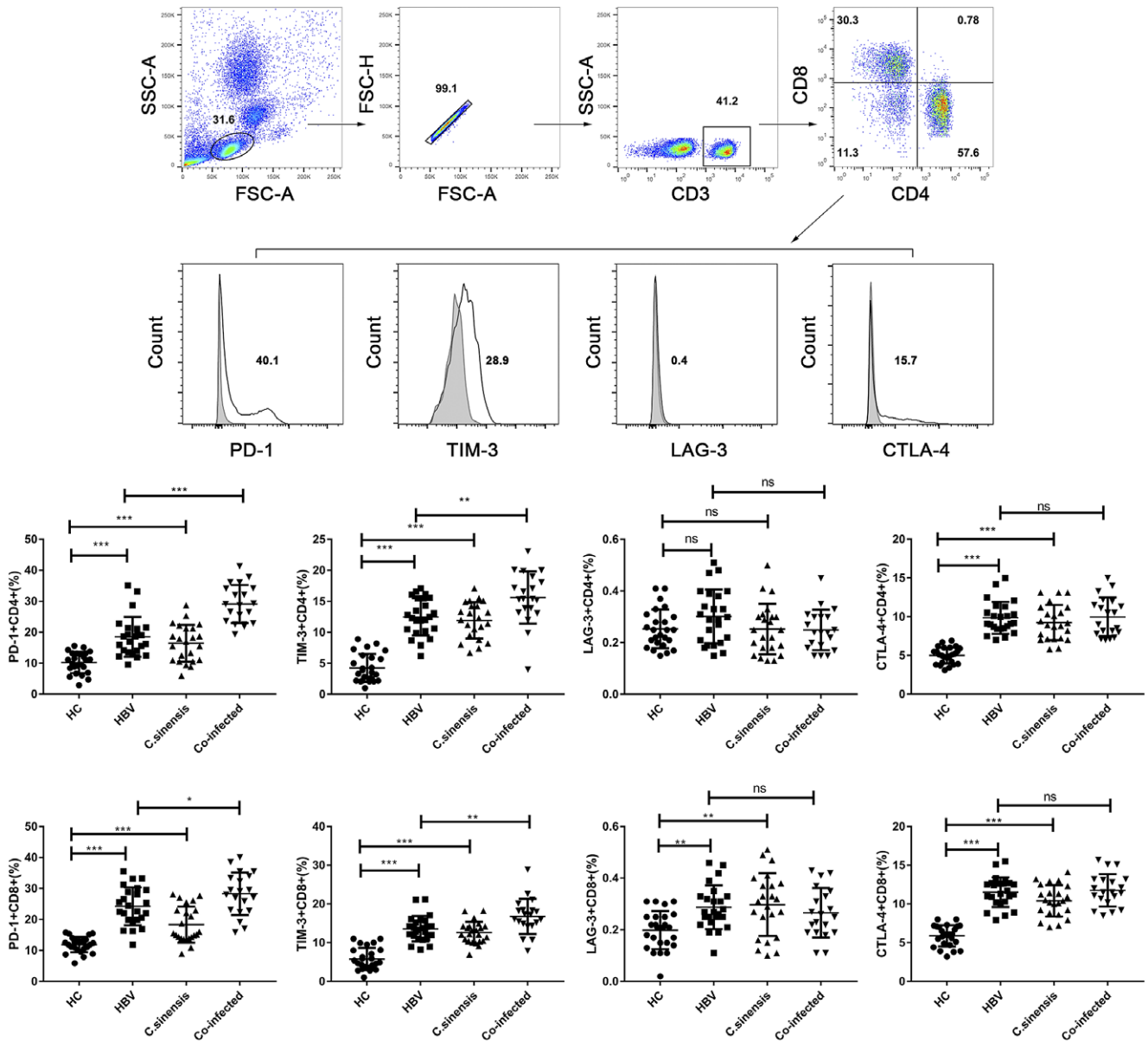

Figure 1. Gating strategy and expression profiles of PD-1, TIM-3, LAG-3, and CTLA-4 in CD4+ T and CD8+ T cells in the co-infected group, HBV group, *C. sinensis* group, and HC group. (A) The gating strategies and representative results of PD-1, TIM-3, LAG-3, and CTLA-4 expression in CD4+ T and CD8+ T cells. (B) The percentages of PD-1, TIM-3, LAG-3, and CTLA-4 in CD4+ T cells expression from co-infected group (n = 20), HBV group (n = 24), *C. sinensis* group (n = 23), and HC group (n = 25). (C) The percentages of PD-1, TIM-3, LAG-3, and CTLA-4 in CD8+ T cells expression from the four groups. Data show the means ± SD. Asterisks indicate statistically significant differences between two groups, as measured by one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001).

difference was found in the IFN-γ and TNF-α in the CD4+ T cells. (Figure 2b). These findings suggested that the CD4+ T cells and CD8+ T cells function was inhibited by *C. sinensis* and HBV co-infection, secreting low IFN-γ, IL-2, and TNF-α upon stimulation with a cell stimulation cocktail and the protein transport inhibitor cocktail separately.

Discussion

T cell exhaustion is considered as a general characteristic of chronic viral infections and cancer, such as HBV (Wherry 2011). A large amount of studies have confirmed that T cell exhaustion has also been observed in humans during chronic infection diseases (Nebbia *et al.* 2012; Wang *et al.* 2019a; Wang *et al.* 2019b). As a consequence, the immunoregulation of T cell exhaustion in chronic HBV infection will provide different therapeutic targets and molecular controls to treat chronic HBV infection.

C. sinensis infection can evolve into a sustained chronic condition. Because trematode locates in the bile duct of the host, it can lead to several chronic diseases, such as periductal liver fibrosis, cholangitis, and even hepatic cirrhosis or cholangiocarcinoma. *C. sinensis* infection was also closely related to T cell exhaustion. Our previous study investigated the relationship between HBV infection and *C. sinensis* infection, and the presence of *C. sinensis* may aggravate the disease state. In this study, we provided further evidence that *C. sinensis* co-infection could exacerbate T cell exhaustion in patients with chronic hepatitis B.

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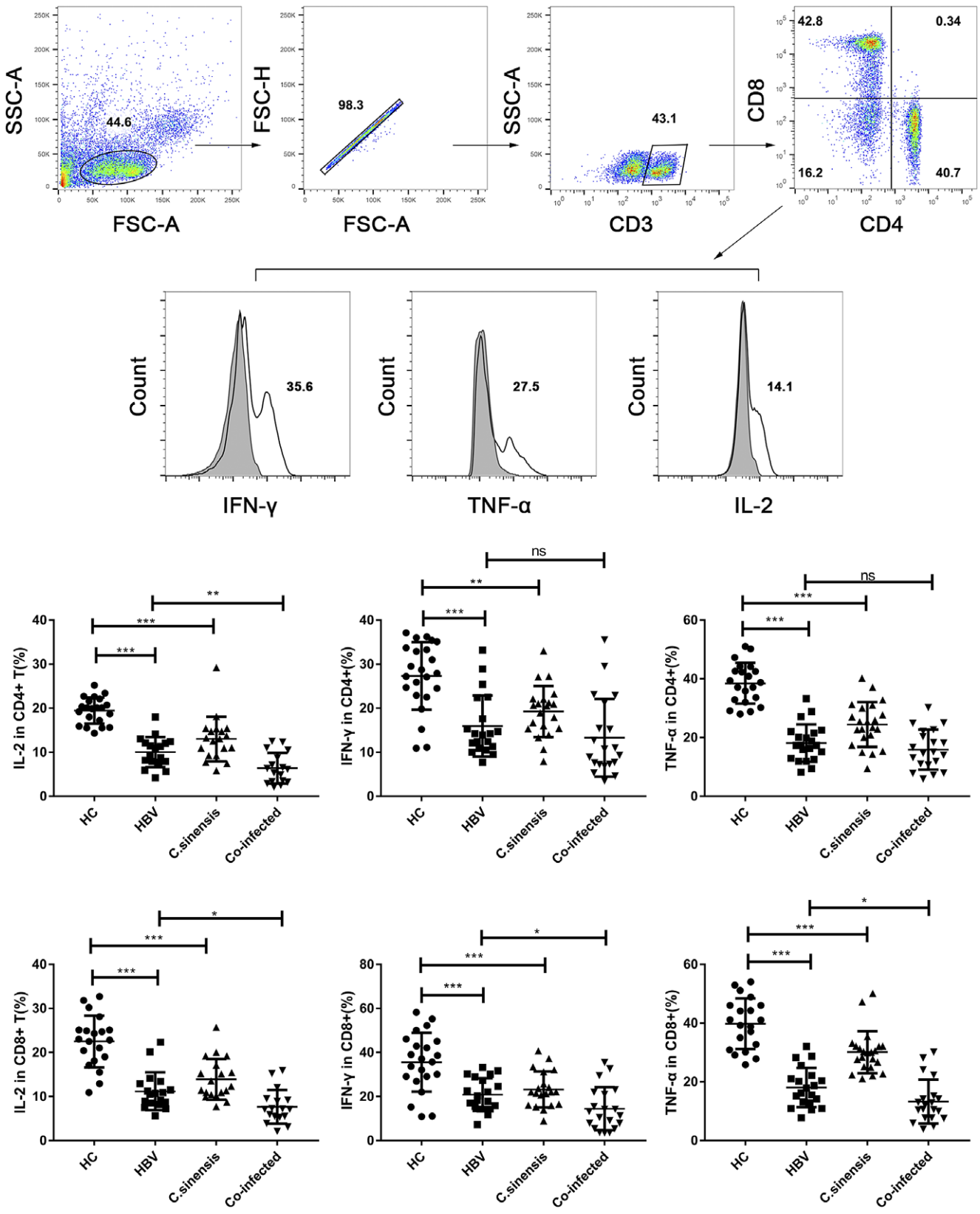

Figure 2. Gating strategy and expression profiles of IL-2, IFN-γ, and TNF-α in CD4+ T and CD8+ T cells in the co-infected group, HBV group, *C. sinensis* group, and HC group. (A) The gating strategies and representative results of IL-2, IFN-γ, and TNF-α expression in CD4+ T and CD8+ T cells. (B) The percentages of IL-2, IFN-γ, and TNF-α expression in CD4+ T cells expression from the co-infected group (n = 20), HBV group (n = 24), *C. sinensis* group (n = 23), and HC group (n = 25). (C) The percentages of IL-2, IFN-γ, and TNF-α expression in CD8+ T cell expression from the four groups. Data show the means ± SD. Asterisks indicate statistically significant differences between two groups, as measured by one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001).

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During HBV infection and clearance, it is well known that CD8+ T cells play a key role. However, CD4+ T cells are the key factor regulating on the cellular cytotoxic T lymphocyte (CTL) response to HBV because they are needed for the development of the optimal effector CTL and for the generation and maintenance of functional memory CTL (Fisicaro *et al.* 2018; Wherry 2011). According to previous research, the lack of CD4+ T cells was the main cause of CD8+ T cell exhaustion (Trautmann *et al.* 2014). Like CD8+ T cells, specific CD4+ T cells also lose effector function during chronic viral infection. Therefore, longer persistent infection or loss of help from CD4+ T cells leads to more severe T cell exhaustion (Wherry and Ahmed, 2004). Although T cell exhaustion of CD4+ T cells is important, little research has been conducted on CD4+ T cell exhaustion in chronic HBV-infected patients (Antoine *et al.* 2014). This may be the reason that our study focused on both CD4+ T cells and CD8+ T cells.

Actually, T cells exhibited progressive and gradual exhaustion during persistent chronic infection (Jackson *et al.* 2013), and it is characterised by an upregulation in the expression of inhibitory molecules, such as PD-1, TIM-3, LAG-3, and CTLA-4. All the results from our study were in accordance with the previous reports. Our data showed that the cell surface inhibitory receptors, such as PD-1, TIM-3, LAG-3, and CTLA-4, levels were expressed significantly higher by CD8+ T cells in the HBV group than in the HC group. CD4+ T cells expressed higher PD-1, TIM-3, and CTLA-4 levels, not including LAG-3, in the HBV group than in the HC group. In addition, we also analysed 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 expressed higher in the *C. sinensis* group relative to the expression levels in the HC group. Our results provide strong evidence that the two infectious factors of hepatitis B and *C. sinensis* would eventually lead to T cell exhaustion.

The numbers and types of inhibitory receptors are closely related to the degree of T cell exhaustion (Nguyen and Ohashi, 2015; Yoshio et al., 2016). Many studies have demonstrated the relationship between the extent of T cell exhaustion and the severity of infection (Streeck *et al.* 2008; Wherry *et al.* 2003a, 2005). In this study, we focused on the exhausted T cells, especially those in patients with co-infection *C. sinensis* and HBV. *C. sinensis* co-infection could exacerbate T cell exhaustion in patients with chronic hepatitis B. Our data showed that PD-1 and TIM-3 levels were expressed significantly higher on CD4+ T cells and CD8+ T cells in the co-infected group than those in the HBV group. Furthermore, the recovery of T cell function is increased considerably by simultaneous block of the PD-1 pathway, LAG-3 pathway, CTLA-4 pathway, and TIM-3 pathway (Jin *et al.* 2010; Kaufmann *et al.* 2007; Nakamoto *et al.* 2009). Meanwhile, the findings from our study showed only PD-1 and TIM-3 levels were significantly increased on the surface of exhausted T cells in the co-infected group than those in the HBV group.

Among all the inhibitory receptors expressed on the surface of exhausted T cells, PD-1 was considered as one of the best-known inhibitory receptors (Bengsch *et al.* 2014). The axis of PD-1 and its ligand seems to be a major inhibitory receptor pathway in T cell exhaustion. Many studies confirmed that in vivo blockade of PD-1 leads to a substantial improvement in virus-specific $CD8^+$ T cell responses and also enhancement of B cell responses. Therefore, blocking the PD-1 pathway could be a major immunotherapeutic strategy for achieving immunological control of diseases in humans (Brahmer *et al.* 2010; Velu *et al.* 2009). Recently, it was reported that Tim-3 had a non-redundant role in chronic infected patients similar to PD-1 because Tim-3 and PD-1 may be expressed on overlapping subsets of T cells, and Tim-3 was treated as another inhibitory marker of exhausted T cells during chronic infection. The high expressions of Tim-3 levels were correlated with the state of CD8+ T cell exhaustion, induction of T cell dysfunction, and suppression of natural killer cells (Golden-Mason et al., 2009; Jones et al., 2008). In addition, our data showed that 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 cell exhaustion leads to a weakened or suppressed host immune response, which is a status of gradual T cell dysfunction that arises during chronic infections (Schietinger and Greenberg 2014). So, immunoregulation is centrally involved.

The main performances are not only the previous inhibitory receptors but also the inhibitory cytokines (Wherry and Kurachi, 2015). T cells mainly secrete 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 (Shaw *et al.* 2018). We sought to understand the T cell exhaustion, as measured by changes in the IFN-γ, IL-2, and TNF-α secretion. The data demonstrated that lower IFN-γ, IL-2, and TNF-α expression levels were detected in both the CD4+ T cells and CD8+ T cells from both the CHB group and *C. sinensis* group than in the T cells obtained from the HC group. The results suggest that the two infectious factors of hepatitis B and *C. sinensis* would eventually lead to T cell exhaustion. At the same time, IFN-γ, IL-2, and TNF-α expression levels were detected as significantly lower in the CD8+ T cells from the 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 detected as significantly lower from the co-infected group than those from the HBV group. Obviously, *C. sinensis* has more effect on cytokines levels in the CD8+ T cells from the co-infected group. Significantly, during T cell exhaustion, the downregulation of secretion of IFN-γ, IL-2, and TNF-α occurs in a hierarchical manner. It was a process with decreasing IL-2 secretion first, followed by the loss of IFN-γ and TNF-α production (Crawford and Wherry 2009; Wherry *et al.* 2007). Many researchers put their attention on IL-2. Previous studies have highlighted that IL-2 promotes the formation of effector CD8+ T cells, and supplementing IL-2 can reverse T cell exhaustion and recover T cell proliferation (Bachmann *et al.* 2007; Pipkin *et al.* 2010; Schwartz 2003). According to these studies and our study, it is reasonable to believe that IL-2 is the most important cytokine to prevent T cell exhaustion.

Patients with chronic HBV infections are usually characterised by a population of exhausted T cells, which have weak virus-specific T cell responses, impeding the clearance of the virus and recovery from hepatitis. Continuously high viral load and high antigen levels may contribute to liver injury and inflammation and the exhaustion of HBV-specific T cells. So, there is a close relationship between liver damage and T cell exhaustion, as our experimental data also confirm this point.

In recent years, there has been more and more research that shows the mechanisms explaining the exhausted T cells in patients who develop chronic HBV infection (Wherry 2011; Bertoletti and Gehring 2006). These mechanisms for the development of T cell exhaustion were not only observed in chronic HBV infection but also have been confirmed in chronic parasitic infection. It has been proposed that some mechanisms may contribute to the dysfunction of T cells, such as continuously high viral load and high antigen

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levels, suppressive cytokines including IFN-γ and TNF-α, and dendritic cells, which were able to lead to a progressive exhausted T cell function (Goncalves *et al.* 2010; Karp *et al.* 1993).

Overall, the present research on the mechanism of T cell exhaustion is based almost entirely on viral models. Although some mechanisms have been proven in some parasitic diseases, the significance of these molecules during T cell exhaustion needs to be studied more thoroughly in chronic parasitic models. Our study may show that the interaction between *C. sinensis* and hepatitis B virus may exacerbate T cell exhaustion in patients with chronic hepatitis B. Considering that chronic *C. sinensis* and hepatitis B virus co-infection can have a potentially additive effect on T cell exhaustion, we should pay more attention to the T cell exhaustion caused by *C. sinensis* infection in the co-infection patients. Overall, our research provides direction for understanding the mechanisms of T cell exhaustion and for developing effective immunotherapy strategies for these diseases.

Our study may suggest that *C. sinensis* co-infection could exacerbate T cell exhaustion in patients with chronic hepatitis B. This is consistent with the previous notion that in co-infected patients, the efficacy of antiviral treatment was better in patients who were prescribed with entecavir and praziquantel than entecavir alone. This result further indicated that the efficacy of HBV antiviral treatment was related to the removal of worms in co-infected patients. One possible reason for the weaker response to antiviral therapies in co-infected patients could therefore be exacerbated T cell exhaustion in this group.

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 co-infection 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 realise the importance of *C. sinensis* treatment for HBV-infected patients. PD-1 and TIM-3 might be used as novel biomarkers for T cell exhaustion in patients with *Clonorchis sinensis* and chronic hepatitis B co-infection.

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