Association among cytokine profiles of innate and adaptive immune responses and clinical-virological features in untreated patients with chronic hepatitis B

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Abstract

Background

Complete clearance of intracellular viruses depends on effector cells of innate and adaptive immune system. This study aimed to identify the relationship between antiviral cytokines produced by natural killer (NK) and T cells and clinical-virological characteristics in untreated chronic hepatitis B (CHB) patients.

Methods

We measured antiviral cytokines interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-2 (IL-2) produced by T, NK and natural killer T (NKT) cells in a cohort of chronic hepatitis B virus (HBV) infection (CHB). We also correlated these cytokines with clinical-virological characteristics using a linear regression model.

Results

IFN-γ+ and TNF-α+ by CD4+ and CD8+ T cells were significantly higher in immune active (IA) than other phases. Immune tolerant (IT) patients showed the least expression of IFN-γ+ by NK and NKT cells, and TNF-α+ by NK cells. IFN-γ+, TNF-α+ and IL-2+ by CD4+ and CD8+ T cells were comparable between IA and gray zones (GZ) phases. Principal component analysis based on cytokines confirmed that most IT patients significantly differ from inactive carriers (IC) and IA patients, while GZ patients were widely scattered. Multivariate analysis showed both T and NK cells producing IFN-γ+ and TNF-α+, but not IL-2+, had significant association with serum alanine aminotransferase (ALT). Moreover, IFN-γ+ by NKT cells was associated with HBV DNA, while IFN-γ+ by CD4+ and CD8+ T cells had correlation with age.

Conclusion
HBV clinical phases are characterized by distinct cytokines signatures, which showed little relationship to viral features at a single time point in these untreated CHB patients.

Background

Chronic infection with HBV is estimated to affect more than 240 million people worldwide, leading to 620,000 deaths per year [1, 2]. Virus utilizes multifaceted strategies to evade the host surveillance system while the host attempts to prevent and eradicate infection with minimal collateral damage to itself [3, 4]. Initially, the virus must recognize, bind, and enter its target hepatocytes, and migrate to the nucleus. Here its genome is transcribed, translated, and replicated to permit the assembly and export of new virions so the infection can spread to additional susceptible cells [5-7]. The host must be able to recognize the presence of the virus and eliminate it as quickly and efficiently as possible via protective immune response [8-10]. This usually occurs as a stepwise series of events in which the infected cells and the immune system each play a critical role [11].

Depending on the nature of the infecting virus, the infected cells may be triggered by the virus to produce antiviral cytokines that inhibit one or more steps in the HBV life cycle, thereby limiting the extent of the infection [12]. Granulocytes, NK cells and perhaps NKT cells constitute an early line of defense. Although effector cells of the innate and adaptive immune system can destruct the infected cells directly, much of the antiviral potential of these cells reflects their ability to produce antiviral cytokines such as IFN-γ+ and TNF-α+ at the site of infection [13-15]. Indeed, these cytokines can purge virus from infected cells noncytopathically, or control viral infections indirectly, by modulating immune response and by
upregualting antigen processing and display of viral epitopes at the surface of infected cells [3, 16]. Therefore, it is not surprising that a number of viral proteins have the potential to inhibit the antiviral activity of these cytokines [17-20].

IFN-γ+ and TNF-α+ can play a critical role in the control of HBV infection at several levels. First, they can recruit and activate macrophages, NK cells, and T cells to perform their effector functions, including the production of immunoregulatory and antiviral monokines and cytokines. Second, they can polarize the T cell response toward the development of antiviral effector functions needed for effective control of viral infection. Third, they can upregulate antigen processing, transport, and major histocompatibility complex (MHC) expression locally in the infected hepatocytes. Finally, they can exert direct antiviral activity. Besides IFN-γ+ and TNF-α+, IL-2+ also possesses antiviral activity and regulates the cellular immune response in HBV infection.

Although numerous studies have shown host-virus relationship in CHB infection, few studies have attempted to test associations among innate immunity and T cells derived antiviral cytokines and the clinical-virological factors in a treatment-naïve CHB cohort. In this study, we measured antiviral cytokines, including IFN-γ+, TNF-α+ and IL-2+ by T cells, NK cells and NKT cells, respectively, in treatment-naïve CHB patients with different disease phases and analyzed the correlations between these cytokines and clinical characteristics.

Methods

Subjects

Consecutive adult patients with CHB infection observed in the dedicated viral
hepatitis clinic of The Third Affiliated Hospital of Sun Yat-sen University between July 2015 and July 2016 were recruited. Patients were excluded for the following reasons: receiving antiviral treatment (IFN-α or nucleoside analogs) within the previous 6 months; patients with human immunodeficiency virus (HIV), hepatitis C virus, or hepatitis D virus coinfection; and patients with end-stage liver insufficiency, autoimmune disorders, immunosuppressive treatment, cirrhosis, and malignancies. Written informed consent was obtained from all patients. The study was approved by the Institutional Review Board of the Sun Yat-sen University, and it conforms with the provisions of the Declaration of Helsinki.

Of the 244 individuals eligible for participation in this study, 15 were excluded because of missing values, leaving 229 patients available for analysis. The classification and denomination of the patients with CHB in this work were based exclusively on serologic and biochemical parameters in accordance with published international treatment guidelines as follows: (1) immune tolerant (IT) phase: normal ALT level, HBV DNA elevated, typically > 1 million IU mL-1, hepatitis B e antigen (HBeAg) positive; (2) IA phase: elevated ALT level, HBeAg positive and HBV DNA > 20,000 IU mL-1, or HBeAg negative and HBV DNA > 2,000 IU mL-1; (3) IC: normal ALT level, antibody to hepatitis B e antigen (HBeAb) positive, and low HBV DNA level; (4) GZ: ALT and HBV DNA levels do not fall into the same traditionally characterized phases [21]. Blood was also obtained from age-matched non-HBV infected healthy controls (n = 17, p =0.77 and 0.98 for age and sex respectively, compared with CHB patients). Information on the demographics (age range, sex distribution, family history, vertical transmission, infection time), HBV markers (HBeAg, HBV DNA, hepatitis B surface antigen [HBsAg]), ALT, HBV genotypes was listed in Table 1.
**Cell-surface and intracellular cytokines staining and flow cytometry analysis**

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples using Ficoll density gradients according to the manufacturer’s instructions. Isolated PBMCs were stained for surface markers, fixed, permeabilized with IntraPreReagent (Beckman Coulter, Fullerton, CA), and further stained with antibodies directed against intracellular markers. Leukocytes were stimulated with Leukocyte Activation Cocktail (BD Pharmingen, San Diego, CA) at 37°C for 4 h prior to the intracellular staining using the manufacturer’s staining protocol. Anti-human mAbs against PE-CF594-CD3, APC-CD4, V450-CD8, FITC-CD56, FITC-IFN-γ, PE-IL-2, PE-TNF-α with corresponding isotype-matched controls were purchased from BD Biosciences (San Jose, CA, USA). Data were acquired on a Gallios instrument (Beckman Coulter, Brea, CA) and analysed with FlowJo software (Ashland, OR).

**Clinical and serologic parameters**

Upon recruitment, patient serum was tested for HBsAb, HBeAb, HBeAg using commercial kits (Abbott Laboratories, North Chicago, IL). Quantitative HBsAg (qHBsAg) (dynamic range from 0.05 to 52,000IU/ml) and HBsAb levels were measured with the Elecsys HBsAg II Quant reagent kits (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions. Serum HBV DNA level was measured by Roche COBAS Ampliprep/COBAS TaqMan HBV Test v2.0 (dynamic range from 20 to 1.7E + 08 IU/mL, Roche Molecular Diagnostics, Branchburg, NJ). Level of fibrosis was defined by liver stiffness measurement ( Fibros- can, Echosens, Paris, France). Genotyping of HBV was carried out by polymerase chain
reaction-restriction fragment length polymorphism of the surface gene of HBV.

Briefly, the extracted DNA was amplified for the fragment of the HBV genome between nucleotide positions 256 and 796. The polymerase chain reaction products were subsequently treated with restriction enzymes. After incubation, the samples were run on a 3% agarose gel and stained by ethidium bromide. Six genotypes (A-F) of HBV were identified by the restriction patterns of DNA fragments. Unclassified genotype was defined as an unpredictable or atypical restriction pattern.

**Statistical analysis**

We compared two patient groups using the Mann-Whitney test for continuous variables and the \( \chi^2 \) test for categorical variables. We explored the association between continuous variables using a linear regression model, Pearson correlation or Spearman correlation. For the cluster analysis, we used the principle component analysis to separate the sample into four clusters. All the other statistical tests were performed using R software version 3.2.2. Statistical significance was set to 0.05.

**Results**

**Baseline characteristics of the study population**

To study viral and immune correlation in the different CHB disease phases, we carefully selected a homogeneous cohort of untreated chronic HBV infected patients, without any other comorbidities, attending our outpatient clinic. To rule out the impact of advanced liver fibrosis on any identified immune parameter, patients with advanced fibrosis (F2 fibrosis or higher) were excluded. Typical for natural history of CHB patients, IT patients were youngest among the patient cohort. Owing to the stringent definition criteria, differences in age, ALT and HBV
DNA levels were observed between clinical phases. Unlike some recent reports, qHBsAg level in this study was higher in GZ patients than IT patients [22, 23] (Table 1).

**Cytokines profiles in CHB patients with different stages**

To investigate whether CHB patients beyond current treatment criteria are characterized by a state of defective antiviral response, we analysed the expression profiles of three major effector cytokines, IFN-γ+, TNF-α+ and IL-2+, produced by innate and adaptive immunity. The representative dot plots and gating strategies of flow cytometry analysis for T cells, NK and NKT cells derived cytokines were shown in Supplementary Figure 1. We first analysed their T cells derived cytokines profiles and compared them with those in healthy controls. As expected, both frequencies of IFN-γ+ and TNF-α+ by CD4+ and CD8+ T cells were statistically significant higher in IA patients than IT patients. Similar result was found in frequency of IL-2+ by CD4+ T cells in current CHB patient cohort. There were no significant different distributions of these cytokines profiles found among IA, IC and GZ patient (Figure 1A).

We also measured the frequencies of cytokines by NK and NKT cells in current CHB cohort. As expected, statistically significant differences were observed in frequencies of NK and NKT cells secreting IFN-γ+, with progressive decrease level from patients of IA, IC, GZ, and to IT. The differences between the frequencies of IFN-γ+ and TNF-α+ by NKT and NK cells, respectively, in the patients of IA and GZ were not statistically significant. However, the frequencies of IFN-γ+ produced by
NKT, NK cells and TNF-α⁺ produced by NK cells of patients in IA phase were all higher than those in IT phase (P = 0.004 and 0.0008 for IFN-γ⁺ by NKT and NK cells in IA vs IT; P = 0.004 for TNF-α⁺ by NK cells in IA vs IT, respectively, Figure 1B).

Taken together, these results indicate that a certain number of CHB patients beyond the current treatment guidelines, particularly, patients in GZ phase, still produce antiviral cytokines.

**Distribution of distinct cytokine profiles in CHB with different disease phases**

Because clinical-virological features from patients with CHB had association with TNF-α⁺, IFN-γ⁺ and IL-2⁺ by T cells and NK cells, respectively, we then assessed whether their combined evaluation could be used to identify maturation of an efficient antiviral response to therapy in individual treatment-naïve CHB patient. We firstly investigated the correlation among current 3 pairs of T-cell subsets cytokines and 2 pairs of NK and NKT cell cytokines. The overall correlation among these 10 cytokines was shown in Figure 2A. After correlation analysis, expressions of 6 cytokines (CD4_IFN-γ⁺, CD4_IL-2⁺, CD8_TNF-α⁺, CD8_IL-2⁺, NK_IFN-γ⁺, NKT_TNF-α⁺) were selected to construct an IA-similar cytokines profiles. The assumption was that acquisition of an IA-similar cytokines profiles could reflect a vigorous response to antiviral therapy. A threshold was thus established as shown by the mean value found in IA patients plus one standard deviation for the above selected parameters, and calculation of their expressions in individual patient was conducted to compare with each matched threshold. Individual cytokines distribution profile was
distinguished according to the altered number of applicable parameters beyond the threshold for all patients. A profile with all the applicable parameters altered was assumed to reflect immune active response to therapy whereas the one with no applicable parameters altered was predicted to be associated with an awakening response to therapy. IA and IC with active immune response to HBV showed a prevalent expression of more inflammatory patterns with 6 and 4 altered applicable parameters respectively. In contrast, IT patients showed an immune depletive pattern with only 2 altered application parameters. GZ patients instead showed an intermediate behavior with 5 altered applicable parameters, as a likely result of the transition from an immune depletive to an inflammatory pattern of typical IA patients (Figure 2B). Based on Spearman’s rank correlation analysis, CD4_TNF-α+ , CD8_IL-2+ and NK_IFN-γ+ were selected to be the representative cytokines in current CHB cohort for principal component analysis, which further confirmed that most IT patients significantly differed from IA and IC patients (red, blue and black circles, respectively), both of who clustered homogeneously and an intermediate distribution was observed for GZ patients who were widely scattered (green circles, Figure 2C).

**Association among T-cell secreting cytokines and correlation with clinical-virological characteristics**

The linear regression analysis was used to examine the association between T cells producing cytokines and clinical-virological parameters. Univariate analysis revealed that positive HBeAg, higher levels of ALT and HBV DNA were associated with increased level of CD4+ T cells secreting TNF-α+. Older age and higher ALT
level were associated with more proportion of IFN-γ⁺ by CD4⁺ T cells, while IL-2⁺ by CD4⁺ T cells was linked to the increased HBV genotypes. After adjusting for other confounding factors, multivariate analysis revealed that both higher ALT level and older age were significantly associated with increased IFN-γ⁺ and TNF-α⁺ produced by CD4⁺ T cells (Table 2). Univariate analysis of relationship between CD8⁺ T cells derived cytokines and clinical-virological factors showed age and ALT were associated with both TNF-α⁺ and IFN-γ⁺ by CD8⁺ T cells, respectively. Multivariate analyse indicated older age and high ALT were still associated with increased IFN-γ⁺, while only ALT was significantly related to the higher TNF-α⁺ by CD8⁺ T cells. There was no statistically significant association between IL-2⁺ by CD8⁺ T cells and viral parameters (Table 2).

Therefore, IFN-γ⁺ by either CD4⁺ or CD8⁺ T cells had significantly associated with older age and higher ALT, while TNF-α⁺ from these T cells subsets was associated with ALT.

**Association among NK and NKT-cell secreting cytokines and correlation with clinical-virological characteristics**

Similarly, the linear regression analysis was used to examine the association between clinical-virological factors and NK or NKT-cell expressing cytokines in current CHB cohort. Univariate analysis on the NK-cell cytokines profiles showed more frequencies of IFN-γ⁺ and TNF-α⁺ were correlated with higher ALT level, while only TNF-α⁺ was also associated with HBV DNA. Multivariate analysis also showed
similar results regarding the association between cytokines and ALT (Table 3). We also detected cytokines produced by a subset of T cells that express NK cell markers, NKT cells. HBeAg, ALT, and HBV genotype were found to be associated with NKT secreting TNF-α+ via univariate analysis (Table 4).

In summary, multivariate analysis from 10 clinical-virological parameters and 10 cytokines implied that ALT was statistically significant association with 8 cytokines from T cells and NK cells. Age and HBV DNA had association with 2 cytokines, while gender was correlated with only one cytokine frequency (supplementary figure 2).

**Discussion**

Published data indicate adaptive responses to HBV infection are efficient and induced immediately after active virus replication begins due to the poor induction of innate immunity [14]. While other studies show innate immunity may acquire a key role in dictating the course of HBV infection because of T-cell impairment [24]. These controversial issues imply that a synergic and coordinated role of all cellular components may contribute to the disease status and clinical outcome of CHB. In this scenario, our observational study, involving 229 treatment-naïve CHB subjects falling in different disease phases with detailed cytokines profiles by T cells, NK cells, and NKT cells, adds to the existing knowledge a series of novel and important pieces of information.

We found a divergent ability of circulating T and NK cells to produce cytokines in CHB patients with different disease phases. Frequencies of half of the tested cytokines (CD4_IFN-γ+, CD4_TNF-α+, NK_IFN-γ+, NK_TNF-α+, NKT_TNF-α+) were
significantly higher in IA patients than other 3 groups, suggesting more inflammatory lesion in the liver in this patient group. Notably, levels of other cytokines (CD8_IFN-γ⁺, CD8_TNF-α⁺, CD4_IL-2⁺, CD8_IL-2⁺, NK_TNF-α⁺) in GZ patients were comparable to those in IA patients, implying that this proportion of patients who were not strictly recommended to the treatment preserved T-cell and NK-cell cytokines functions. These findings along with previous data from young people in IT phase [25] reflected the inadequacy of ALT and HBV DNA levels in the assessment of disease activity [26, 27].

In agreement with previously studies [28, 29], both T and NK cells producing IFN-γ⁺ and TNF-α⁺ were positively correlated with ALT levels, highlighting a possible role of these cytokines in maintaining liver inflammation. However, cytokines from T cells were not increased in patients with higher or lower HBV DNA level. This association of antiviral cytokine response with higher ALT value but not with lower HBV DNA level might be interpreted that the rate of clearance of HBV-infected cells is not the principal determinant if steady-state HBV DNA level exists during chronic infection. Moreover, IFN-γ⁺ by T cells and NKT cells had statistically significant association with age and HBV DNA. The results were consistent with other reports that IFN-γ⁺ plays a prominent role on the clinical pathogenesis by recruiting inflammatory immune cells [30, 31]. Nevertheless, none of the immune cytokines by T and NK cells was significantly associated with HBsAg, HBeAg and other demographic characteristics of patients. A trend was seen toward a positive relationship between HBeAg level and IFN-γ⁺ and TNF-α⁺ by CD4⁺ T cells in current cohort, however, this did not achieve statistical significance (Table 2).
The frequency of IL-2+ by T cells was increased in patients with genotype C although their correlation was not significantly (P = 0.067). Genotype C has been considered to be associated with more inflammation, high fibrosis and cirrhosis in numerous studies [32]. Thus, whether patients with higher IL-2+ linked to the higher clonal hepatocyte population that may lead to HCCs needs to be further identified. IL-2+ has essential roles in key functions of immune system, primary via its direct effects on T cells. However, we failed to find significant associations of IL-2+ with IFN-γ+ and TNF-α+ by T cells in current study. These negative data suggest that IL-2+ may not require to activate CD4+ and CD8+ T cells, whereas it may contribute to the regulatory T cells (Tregs) [33-35]. A recent report demonstrated the potential of IL-2+ to enhance Tregs therapy in autoimmune disease [36, 37]. On the other hand, inconsistent with report of inverse correlation found between cytokines by T and NK cells in CHB [24], our data showed the absence of association regarding IFN-γ+ secreting function between CD8+ T and NK cells. Therefore, T and NK cells may exert an additive effect to produce antiviral cytokines.

The detailed analysis of the noncytolytic control of viral infection derived from CHB patients with different disease phases included a total of 10 cytokines by the innate and adaptive immune response. Although at some instances significant differences emerged between the clinical-virological characteristics and cytokines profiles, it became clear that no single parameter was enough to sufficiently distinguish the disease phase from each other based on antiviral cytokine activity. Thus, we performed correlation analysis and principle component analysis with these 10
cytokines to further improve the classification of individuals with different disease phases (Figure 2). Using these methods, IT patents can clearly be separated from patients of IA and IC patients. Principle component analysis also revealed that some of GZ patients and other 3 groups differed in cytokines expressions. In particular, some were found to be scatter within other groups. This finding could in part explain a considerable proportion of GZ patients with high levels of inflammatory cytokines. They seemed neither to be an immune tolerate or inactive state, instead, they were more close to the immune active status and may be beneficial from antiviral therapy.

Conclusions

In conclusion, the current analysis considered clinical, virological, laboratory, and immunological information gathered at a single time point for each subject, and therefore represented only a snapshot in time during the long course of a chronic disease. If immune-mediated liver injury is cumulative, the liver histology would be expected to depend on both the level of HBV specific immune responses and the duration of infection. Variation in the duration of infection in the study cohort would therefore constitute a confounding factor in our analysis that we detected some immunological associations with disease despite this statistical bias likely strengthens our conclusions and necessitates cautious interpretation of the negative data. Longitudinal evaluation of disease progression and evolution of immune and viral responses in this and other cohorts should provide additional information critical to a more complete understanding of virus-host dynamics in chronic HBV infection.
Abbreviations

NK: natural killer

CHB: chronic hepatitis B

IFN-γ: interferon-gamma

TNF-α: tumor necrosis factor-alpha

IL-2: interleukin-2

NKT: natural killer T

PCA: Principal Component Analysis

IA: immune active

IT: immune tolerant

IC: inactive carriers

GZ: gray zones

ALT: alanine aminotransferase

HBV: hepatitis B virus

MHC: major histocompatibility complex

HBeAg: hepatitis B e antigen

HBeAb: hepatitis B e antigen

HBsAg: hepatitis B surface antigen

PBMCs: Peripheral blood mononuclear cells

qHBsAg: Quantitative hepatitis B surface antigen

Declarations

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Authors’ contributions
YG, YL contributed equally to this work. YG designed and performed experiments and drafted manuscript. YL performed experiments, analyzed data and generated figures. QZ analyzed data and generated figures. ZH performed experiments. YB performed experiments. JL collected samples and patients information. LG collected samples and patients information. YLH performed experiments. YW collected samples. YHH advice on study design, supervised experiments and data analysis, critical review of manuscript, provided funding.

Ethics approval and consent to participate
We have read and complied with the policy of the journal on ethical consent, as stated in the Guide to Authors. The study was approved by the Institutional Review Board of the Sun Yat-sen University, and the work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and Uniform Requirements for manuscripts submitted to Biomedical journals. Written informed consent was obtained from all patients. The privacy rights of human subjects has always been observed.
Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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**Tables**

**Table 1 Clinical-virological characteristics of patients included in the study**

| Characteristics | IT (n = 17) | IA (n = 120) | IC (n = 20) | GZ (n = 72) | HC (n=1) |
|-----------------|------------|-------------|------------|------------|---------|
| Age, years, median (quartile) | 25 (24, 26) | 29 (25, 33.25) | 32 (28.75, 37) | 31.5 (26, 38.25) | 27(25.5, |
| Gender | | | | | |
| Male, n (%) | 12 (70.6) | 77 (64.2) | 17 (85) | 55 (76.4) | 12 (70.6) |
| Female, n (%) | 5 (29.4) | 43 (35.8) | 3 (15) | 17 (23.6) | 5 (29.4) |
| ALT, U/L, median (quartile) | 20.6 (18.3, 22.4) | 20.8 (19.1, 22.5) | 22.3 (21.6, 23.8) | 21.484 (19.8, 23.4) | 16(12.1) |
| Fibroscan, Kpa median (quartile) | 4.9 (4.2, 5.4) | 5.3 (4.3, 6.5) | 4.4 (4.0, 5.3) | 4.8 (4.4, 5.4) | 4.6(4.0~) |
| HBV DNA, Log IU/ml, median (quartile) | 8.2 (8.2, | 7.7 (5.0, 8.2) | 2.2 (1.6, 3.1) | 3.3 (2.1, 4.3) | 2.2 (1.6, 3.2) |
| HBeAg status | Negative, n (%) | Positive, n (%) | Missing, n (%) |
|--------------|----------------|----------------|--------------|
|              | 0 (0)          | 41 (34)        | 20 (100)     |
|              | 17             | 78 (65)        | 0 (0)        |
|              | 0 (0)          | 1 (1)          | 0 (0)        |

| HBeAb status | Negative, n (%) | Positive, n (%) | Missing, n (%) |
|--------------|----------------|----------------|--------------|
|              | 17             | 70 (58.3)      | 1 (5)        |
|              | 0 (0)          | 47 (39.2)      | 19 (95)      |
|              | 0 (0)          | 3 (2.5)        | 0 (0)        |

| qHBsAg, Log IU/ml, median (quartile) | 4.6 (4.5, 4.7) |
|                                     | 4.0 (3.3, 4.7) |
|                                     | 2.9 (2.0, 3.2) |
|                                     | 3.2 (2.3, 3.6) |

| HBsAb status | Negative, n (%) | Positive, n (%) | Missing, n (%) |
|--------------|----------------|----------------|--------------|
|              | 15             | 106 (88)       | 20 (100)     |
|              | 2 (12)         | 14 (12)        | 0 (0)        |

| HBV genotype | C, n(%) | B, n (%) | N, n (%) | O, n (%) | Missing, n(%) |
|--------------|---------|----------|---------|---------|--------------|
|              | 2(12)   | 12       | 0 (0)   | 1 (6)   | 2(11)        |
|              | 30(25)  | 74 (62)  | 2 (2)   | 8 (1)   | 6(5)         |
|              | 3(15)   | 8 (40)   | 9 (45)  | 0 (0)   | 0(0)         |
|              | 15(21)  | 27 (38)  | 23 (32) | 23 (32) | 1(1)         |

| HBV genotype: Other included C+D, B+D, B+C, D; N, not detected |
| IT, immune tolerant; IA, immune active; IC, inactive carrier; GZ, gray zones; ALT, alanine aminotransferase; HBeAb, antibody to HBV e antigen; HBeAg, HBV e antigen; HBsAb: antibody to hepatitis B surface antigen; HBsAg, hepatitis B surface antigen. |

**Table 2. Factors associated with CD4+ T cells secreting cytokines in the univariate and multivariate linear regression analysis**
| Variable                        | CD4_IFN-γ | CD4 |
|--------------------------------|-----------|-----|
|                                | Univariate (raw effects) | Multivariate (adjusted effects) | Univariate (raw effects) |
|                                | B (95% CI) | P   | Adjusted B (95% CI) | Adjusted P | B (95% CI) | P   |
| Age (≥ 25 vs < 25 years)       | 4.26 (1.15, 7.36) | 0.008 | 4.09 (0.94, 7.24) | 0.011 | 5.47 (-0.99, 11.93) | 0.009 |
| Gender (Male vs Female)        | 2.35 (-0.6, 5.3) | 0.118 | 2.38 (-0.55, 5.31) | 0.110 | 5.48 (-0.55, 11.52) | 0.075 |
| Family History (Yes vs No)     | 0.56 (-2.14, 3.26) | 0.684 | 0.55 (-2.41, 3.51) | 0.714 | 1.96 (-3.57, 7.48) | 0.485 |
| Vertical Transmission (Yes vs No) | -1.34 (-5, 2.33) | 0.472 | -1.42 (-5.41, 2.57) | 0.483 | 2.05 (-3.57, 9.57) | 0.591 |
| Infection Time (≥ 20 vs < 20 years) | -0.2 (-4.01, 3.6) | 0.917 | -1.88 (-5.74, 1.98) | 0.336 | 0.85 (-6.95, 8.64) | 0.831 |
| Log HBsAg                      | 0.60 (-1.54, 2.75) | 0.577 | 0.79 (-1.35, 2.93) | 0.466 | 0.004 (1.77, 21.58) | 0.999 |
| Log HBV DNA                    | 0.09 (-0.45, 0.63) | 0.741 | 0.99 (-2.09, 0.11) | 0.078 | 1.45 (0.36, 2.54) | 0.010 |
| HBeAg (Positive vs Negative)   | 1.18 (-1.51, 3.87) | 0.387 | 4.30 (-0.10, 8.70) | 0.055 | 7.18 (1.77, 12.58) | 0.010 |
| ALT (< 2 ULN is the reference group) | | | | | 14.41 (7.64, 21.19) | 0.000 |
| ≥ 2 & < 5 ULN                  | 6.16 (2.76, 9.56) | 0.000 | 6.77 (3.08, 10.46) | 0.000 | 14.41 (7.64, 21.19) | 0.000 |
| ≥ 5ULN                         | 3.89 (-0.34, 8.12) | 0.072 | 3.82 (-0.79, 8.44) | 0.104 | 13.2 (4.77, 21.64) | 0.002 |
Genotype (Genotype C is the reference group)

|   | Univariate (raw effects) | Multivariate (adjusted effects) | Univariate (adjusted effects) |
|---|--------------------------|-------------------------------|-----------------------------|
|   | B (95% CI) | P     | Adjusted B (95% CI) | Adjusted P | Adjusted B (95% CI) | Adjusted P |
| B  | -0.84 (4.2, 2.53) | 0.623 | -0.14 (3.42, 3.13) | 0.930 | -4.03 (10.79, 2.73) | 0.241 |
| N  | -0.85 (5.43, 3.73) | 0.715 | -2.39 (7.83, 3.06) | 0.388 | -9.19 (18.39, 0.01) | 0.050 |
| O  | -5.27 (10.98, 0.45) | 0.071 | -3.46 (9.08, 2.17) | 0.226 | -16.2 (27.68, -4.72) | 0.006 |

Significant values are shown in boldface. B: Unstandardized Coefficients.

ALT, alanine aminotransferase; HBeAg, HBV e antigen; HBsAg, hepatitis B surface antigen;

Table 3. Factors associated with CD8+ T cells secreting cytokines in the univariate and multivariate linear regression analysis

| Variable                   | CD8_IFN-γ Univariate (raw effects) | CD8 Univariate (raw effects) |
|----------------------------|-----------------------------------|------------------------------|
|                            | B (95% CI) | P     | Adjusted B (95% CI) | Adjusted P | Adjusted B (95% CI) | Adjusted P |
| Age (≥ 25 vs < 25 years)  | 8.08 (1.79, 14.36) | 0.012 | 6.92 (0.41, 13.43) | 0.037 | 8.2 (1.04, 15.37) | 0.025 |
| Gender (Male vs Female)   | 5.25 (-0.69, 11.2) | 0.083 | 3.36 (-2.7, 9.41) | 0.275 | 5.41 (-1.35, 12.17) | 0.116 |
| Family History (Yes vs No)| -1.42 (-6.86, 4.02) | 0.607 | -3.27 (-9.39, 2.86) | 0.293 | -0.56 (-6.75, 5.62) | 0.858 |
|                                | B                          | N                          | O                          |
|--------------------------------|----------------------------|-----------------------------|-----------------------------|
| Vertical Transmission (Yes vs  | -0.84 (-8.24, 6.57)        | 4.76 (-2.88, 12.4)         | -2.35 (-7.78, 3.08)        |
| No)                            | 0.824 (0.001)              | 0.220 (-2.08, 0.2)         | 0.394 (-3.78, 1.21)        |
| Infection Time (≥ 20 vs < 20   | 1.82 (3.81, 17.75)         | 0.97 (-2.88, 3.08)         | 3.87 (-7.78, 12.97)        |
| years)                         | 0.663 (4.87, 20.13)        | 0.810 (-4.35, 0.2)         | 0.402 (-5.24, 12.97)       |
| Log HBsAg                      | -0.2 (-4.62, 4.23)         | 0.931 (-4.35, 0.2)         | 0.54 (-1.37, 1.12)         |
| -0.021 (-0.033, -0.009)        | 0.001 (-4.35, 0.2)         | 0.829 (-4.37, 5.44)        | 0.849 (-7, 8.94)           |
| Log HBV DNA                    | -0.054 (-0.087, -0.021)    | 0.001 (-4.35, 0.2)         | 0.662 (-7.54, 4.81)        |
| HBeAg (Positive vs Negative)   | -3.57 (-10.39, 3.24)       | -2.42 (-9.2, 4.36)         | 4.93 (-4.61, 14.48)        |
| ALT (< 2 ULN is the reference  | 10.78 (3.81, 17.75)        | 12.5 (4.87, 20.13)         | 12.97 (12.08, 15.31)       |
| group)                         | 0.003 (4.87, 20.13)        | 0.002 (4.18, 19.99)        | 15.31 (12.08, 15.31)       |
| ≥ ≥ 2 & < 5 ULN                | 12.08 (4.18, 19.99)        | 0.274 (-4.38, 15.31)       | 15.31 (12.08, 15.31)       |
| ≥ 5ULN                         | 12.08 (4.18, 19.99)        | 0.274 (-4.38, 15.31)       | 15.31 (12.08, 15.31)       |
| Genotype (Genotype C is the    | -3.57 (-10.39, 3.24)       | -10.05 (-12.99, 5.56)      | -8.44 (-20.01, 3.13)       |
| reference group)               | -0.302 (4.36, 4.25)        | -0.080 (-21.31, 1.22)      | 0.152 (-18.92, 4.32)       |
| B                              | -6.27 (-16.8, 4.25)        | -6.65 (-21.77, 4.47)       | -4.83 (-12.56, 2.9)        |
| N                              | 0.481 (-16.8, 4.25)        | 0.800 (-21.77, 4.47)       | 0.216 (-12.56, 2.9)        |
| O                              | -3.24 (-16.8, 4.25)        | 4.36 (-21.77, 4.47)        | 4.32 (2.9)                 |
| 0.302 (-16.8, 4.25)            | 0.481 (-16.8, 4.25)        | 0.800 (-21.77, 4.47)       | 0.216 (-12.56, 2.9)        |
| -6.27 (-16.8, 4.25)            | 0.481 (-16.8, 4.25)        | 0.800 (-21.77, 4.47)       | 0.216 (-12.56, 2.9)        |
| 0.481 (-16.8, 4.25)            | 0.481 (-16.8, 4.25)        | 0.800 (-21.77, 4.47)       | 0.216 (-12.56, 2.9)        |
| -6.27 (-16.8, 4.25)            | 0.481 (-16.8, 4.25)        | 0.800 (-21.77, 4.47)       | 0.216 (-12.56, 2.9)        |

Significant values are shown in boldface. B: Unstandardized Coefficients.

ALT, alanine aminotransferase; HBeAg, HBV e antigen; HBsAg, hepatitis B surface antigen.
antigen;

Table 4. Factors associated with NK cells secreting cytokines in the univariate and multivariate linear regression analysis

| Variable                          | NK_IFN-γ |                   |                   |
|-----------------------------------|----------|-------------------|-------------------|
|                                  | Univariate (raw effects) | Multivariate (adjusted effects) |
|                                  | B (95% CI) | P | Adjusted B (95% CI) | AdjB (95% CI) |
| Age (≥ 25 vs < 25 years)          | 3.36 (-5.44, 12.16) | 0.452 | 3 (-5.91, 11.9) | 0.507 |
| Gender (Male vs Female)           | 0.32 (-7.92, 8.56) | 0.939 | -1.23 (-9.52, 7.06) | 0.507 |
| Family History (Yes vs No)        | -0.82 (-8.3, 6.66) | 0.829 | -3.98 (-12.36, 4.4) | 0.507 |
| Vertical Transmission (Yes vs No) | -1.64 (-11.81, 8.53) | 0.750 | 0.51 (-10.79, 11.8) | 0.769 |
| Infection Time (≥ 20 vs < 20 years) | 6.21 (-4.28, 16.7) | 0.244 | 4.06 (-6.85, 14.97) | 0.769 |
| Log HBsAg                         | -0.66 (-6.60, 5.27) | 0.825 | -0.72 (-6.78, 5.33) | 0.769 |
| Log HBV DNA                       | 0.024 (-0.001, 0.048) | 0.58 | -2.84 (-5.95, 0.28) | 0.814 |
| HBeAg (Positive vs Negative)      | 2.61 (-4.85, 10.07) | 0.490 | 8.29 (-4.17, 20.75) | 0.190 |
| ALT (< 2 ULN is the reference group) |                   |                   |                   |
| ≥ 2 & < 5 ULN                     | 16.73 (7.3, 26.15) | 0.001 | 17.63 (7.19, 28.07) | 0.056 |
| ≥ 5ULN                           | 11.45 (-0.27, 24.72) | 0.056 | 11.66 (-1.4, 24.72) | 0.056 |
Genotype (Genotype C is the reference group)

| Variable | Univariate (raw effects) | Multivariate (adjusted effects) | Univariate (raw effects) | Multivariate (adjusted effects) |
|----------|--------------------------|-------------------------------|--------------------------|-------------------------------|
| B        | -6.57                    | 0.159                         | 5.2 (-14.47, 4.08)       |                               |
|          | (-15.74, 2.6)            |                               |                          |                               |
| N        | -10.05                   | 0.114                         | -15.6 (0.114)            |                               |
|          | (-22.53, 2.43)           |                               | (-31.01, -0.19)          |                               |
| O        | -22.02                   | 0.006                         | -20.06 (0.006)           |                               |
|          | (-37.59, -6.46)          |                               | (-35.96, -4.15)          |                               |

Significant values are shown in boldface. B: Unstandardized Coefficients.

ALT, alanine aminotransferase; HBeAg, HBV e antigen; HBsAg, hepatitis B surface antigen;

Table 5. Factors associated with NKT cells secreting cytokines in the univariate and multivariate linear regression analysis

| Variable                | NKT_IFN-γ         | NKT_TNF-α         |
|-------------------------|-------------------|-------------------|
|                         | Univariate (raw  | Multivariate (adj |
|                         | effects)          | usted effects)   |
|                         | Univariate (raw  | Multivariate (adj |
|                         | effects)          | usted effects)   |
| B (95% CI)              |                  |                  |
| Age (≥ 25 vs < 25 years)| 8.58 0.06        | 7.6 0.10         |
|                         | (-0.4, 1)        | (-1.6, 5)        |
|                         |                  |                  |
| Gender (Male vs Female) | 3.26 0.45        | 1.42 0.74        |
|                         |                  |                  |

30
|                              | Score 1  | Score 2  | Score 3  | Score 4  | Score 5  | Score 6  |
|------------------------------|----------|----------|----------|----------|----------|----------|
| Female)                      | -5.2 0  | -7.1 3  | -3.2 7  | -2.7 0  | -9.4 -13.4 5  | -11.87 0  |
| Family History (Yes vs No)   | -1.7 0.65| -4.7 0.27| 0.84 0.82| -3.0 0.44| -9.4 -13.4 5  | -11.87 0  |
| Vertical Transmission        | -1.2 0.81| 2.99 0.61| 2.62 0.61| 3.87 0.47| -11.73 9 6  | 13.45 4  |
| Infection Time (≥ 20 vs < 20 years) | 2.34 0.67| -2.5 0.65| 0.67 0.38| 0.45 0.93| -8.53 7 6  | 13.21 5  |
| Log HBsAg                    | 0.24 0.93| 0.15 0.96| 2.0 0.50| -1.3 0.64| -5.8 8 2  | -6.1 2  |
| Log HBV DNA                  | -0.0 0.03| -3.8 0.01| 0.04 0.00| -2.0 0.18| -0.0 3 8  | -0.6 0.07 |
| HBeAg (Positive vs Negative) | -0.5 0.88| 9.48 0.14| 7.79 0.03| 10.6 0.07| -8.2 8 1  | -8.2 1  |

31
ALT (< 2 ULN is the reference group)

|       |       |       |       |       |       |
|-------|-------|-------|-------|-------|-------|
|       |   ≥ 2 & < 5 ULN |       |       |       |       |
|       |   17.4 0.00 |   21.4 0.00 |   22.1 0.00 |   22.2 0.00 |       |
|       |   (7.6 1) |   (10. 0) |   1 0 |       |       |
|       |   (6. 62) |   (13. 1) |   27.1 |       |       |
|       |   5) |   7) |   31.0 |       |       |
|       |       |       |       |       |       |
|       |   ≥ 5ULN |       |       |       |       |
|       |   9.89 0.11 |   13.4 0.05 |   20.8 0.00 |   18.9 0.00 |       |
|       |   (-2.2 0) |   1 1 |   9 0 |       |       |
|       |   5. 5, |   (-0.0 9.7) |       |       |
|       |   22.0 8, |       |       |       |
|       |   2) |   26.8 32.0 |   31.3 |       |
|       |   9) |   2) |       |       |

Genotype (Genotype C is the reference group)

|       |       |       |       |       |       |
|-------|-------|-------|-------|-------|-------|
|       |   B |       |       |       |       |
|       |   -5.7 0.24 |   -3.9 0.41 |   -11. 0.07 |   -5.6 0.20 |       |
|       |   1 4 |       |       |       |       |
|       |   3 9 |   45 4 |       |       |       |
|       |   7 7 |       |       |       |       |
|       |   15. (-13. |   -24. 03) |       |       |
|       |   36. 51, |       |       |       |
|       |   52, |       |       |       |
|       |   3.93 5.65 |       |       |       |
|       |   1.13 |       |       |       |
|       |   3.17 |       |       |       |
|       |   ) |       |       |       |
|       |   ) |       |       |       |
|       |   ) |       |       |       |
|       |   N |       |       |       |       |
|       |   -4.7 0.47 |   -13. 0.09 |   -17. 0.02 |   -11. 0.14 |       |
|       |   5 5 |   52 5 |   77 7 |       |       |
|       |   02 0 |       |       |       |       |
|       |   17. (-29. |   -33. 46) |       |       |
|       |   88, |       |       |       |
|       |   71, |       |       |       |
|       |   8.37 2.39 |       |       |       |
|       |   -2.0 |       |       |       |
|       |   3.67 |       |       |       |
|       |   ) |       |       |       |
|       |   ) |       |       |       |
|       |   8) |       |       |       |
|       |   ) |       |       |       |
|       |   O |       |       |       |       |
|       |   -13. 0.11 |   -9.8 0.23 |   -8.2 0.08 |   -11. 0.12 |       |
|       |   33 0 |   7 6 |   2 1 |       |       |
|       |   74 8 |       |       |       |       |
|       |   -29. (-26. |   -17. 46) |       |       |
|       |   69, |       |       |       |
|       |   89, |       |       |       |
|       |   3.04 6.54 |       |       |       |
|       |   1.03 |       |       |       |
|       |   3.42 |       |       |       |

Significant values are shown in boldface.
Figures
Figure 1

Cytokines profiles of inner and adaptive immune responses from naïve CHB patients. (A) Expressions of cytokines IFN-γ +, TNF-α + and IL-2 + by CD4+ and CD8+ T cells derived from the indicated patient groups were measured. The levels were compared to those of healthy controls. (B) Expressions of cytokines IFN-γ +, TNF-α + by NK and NKT cells derived from the indicated patient groups were measured. The levels were compared to those of healthy controls. (C) The levels were compared to those of healthy controls. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2

Distribution of distinct cytokines profiles in CHB with different disease phases. (A)

Supplementary Files

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