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ORIGINAL RESEARCH

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Expression patterns of immune checkpoint proteins and *Plasmodium falciparum*-induced cytokines in chronic hepatitis B virus-infected and uninfected individuals: A cross-sectional study

Selorm P. Segbefia[1,2] | Diana A. Asandem[3,4] | Abigail Pobee[1] | Bright Asare[1,5] | Ahu Diana Prah[3] | Rawdat Baba-Adam[1,3] | Jones Amo Amponsah[1] | Eric Kyei-Baafour[1] | William van der Puije[1] | Frank Osei[1] | Doreen Teye-Adjei[1] | Seth Agyemang[3] | Theophilus Brenko[1] | Lutterodt Bentum-Ennin[1] | John K. A. Tetteh[1] | Kofi J. H. Bonney[4] | Samuel Asamoah Sakyi[2] | Linda E. Amoah[1,3] | Kwadwo A. Kusi[1,3]

[1]Department of Immunology, NMIMR, College of Health Sciences, University of Ghana, Accra, Ghana

[2]Department of Molecular Medicine, School of Medicine and Dentistry, College of Health Sciences, KNUST, Kumasi, Ghana

[3]Department of Biochemistry, Cell and Molecular Biology, West African Centre for Cell Biology of Infectious Pathogens, College of Basic and Applied Sciences, University of Ghana, Accra, Ghana

[4]Department of Virology, NMIMR, College of Health Sciences, University of Ghana, Accra, Ghana

[5]Department of Animal Biology and Conservation Science, College of Basic and Applied Sciences, University of Ghana, Accra, Ghana

Correspondence

Selorm P. Segbefia, Department of Immunology, Noguchi Memorial Institute for Medical Research (NMIMR), College of Health Sciences, University of Ghana, Accra, Ghana.
Email: ssegbefia@noguchi.ug.edu.gh

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Abstract

Background and Aim: Chronic hepatitis B virus (CHB) infection remains a major public health problem. The American Association for the Study of Liver Diseases (AASLD) 2018 Hepatitis B Guidelines provide that CHB individuals not requiring antiviral therapy yet are monitored to determine the need for antiviral therapy in the future; however, these tests do not include measurement of cytokines and immune cell characterization. This case-control study compared the cytokine and immune checkpoint protein expression profiles between CHB individuals not yet on antiviral treatment and hepatitis B virus (HBV)-negative individuals.

Methods: CD4 and CD8 T cells from CHB and HBV-negative individuals were characterized for immune checkpoint proteins programmed cell death-1 (PD1), T cell Immunoglobulin domain and mucin domain-containing protein 3 (TIM-3), and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (CD152), and a memory marker CXCR3 (CD183) using flow cytometry. Malaria-induced cytokine expression levels were determined by stimulating their blood cells with *Plasmodium falciparum* 3D7 strain antigens (CSP, AMA-1, and TRAP) in whole blood assays, and cytokine levels were measured using a 13-plex Luminex kit.

Results: HBV-negative and CHB individuals had comparable levels of CD4+ and CD8+ T cells. However, a proportion of the CD4+ and CD8+ populations from both groups, which were CXCR3+, expressed PD-1 and CD152. The ability to produce cytokines in response to malaria antigen stimulation was not significantly different between the groups.

Conclusion: These findings support excluding CHB individuals from antiviral therapy at this stage of infection. However, CHB individuals require regular monitoring to determine the need for later antiviral treatment.

KEYWORDS
chronic, cytokines, hepatitis B, immune checkpoint proteins, *Plasmodium falciparum*

1 | INTRODUCTION

Chronic hepatitis B virus (CHB) infection remains a major public health problem in sub-Saharan Africa. In 2019, the World Health Organization (WHO) estimated that 296 million individuals had chronic hepatitis B infection, with 1.5 million new infections each year, with 820,000 deaths.[1] In Ghana, the average prevalence of hepatitis B virus (HBV) infections among adults as of 2019 was estimated to be about 8.36%, based on HBV surface antigen seropositivity. Adolescents had a prevalence of 14.3%.[2]

In chronic infections, such as that of HBV, T cells are exposed to persistent antigen signals. This scenario is often associated with the deterioration of T cell function: a state called "exhaustion." Exhausted T cells express multiple inhibitory receptors such as programmed cell death-1 (PD-1), T cell Immunoglobulin domain, and mucin domain-containing protein 3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), and cytotoxic T lymphocyte antigen 4 (CTLA-4), among others.[3–6] These exhausted T cells lose robust effector functions, such as the production of cytokines. This impairment reduces their capacity to manage the infection, favoring the survival of the pathogen and maintaining persistent infection.

The exhaustion of CD8+ T cells[7] and the chronic production of cytokines by T cells have been reported to account for the failure to get rid of HBV.[8,9] Chronic production of type I Interferons (IFN) promotes immune suppression, hence facilitating viral persistence.[8] Although IFN-α and β signals promote effector CD8+ T cell function during early infection by enhancing viral control and suppression,[10,11] chronic exposure to IFN-α and β can promote T cell dysfunction.[9] Transforming growth factor (TGF)-β and interleukin-10 (IL-10) signaling also facilitate viral persistence by promoting immune suppression.[12] In contrast, interleukin-6 (IL-6) signaling and other inflammatory pathways may be involved in controlling chronic viral infection and the prevention of CD8+ T lymphocyte exhaustion.[8,13] Proinflammatory cytokines such as IL-1β, IL-6, IL-17, IL-22, IFN-α/β, and tumor necrosis factor-α (TNF-α) are believed to contribute to the development of liver cirrhosis and cancer in CHB individuals.[12,14,15]

The WHO 2015 and the AASLD 2018 Hepatitis B Guidelines provide that CHB individuals not requiring antiviral therapy yet are monitored to determine the need for antiviral therapy in the future.[16,17] This study, therefore, looked at HBV-specific immune responses in plasma and assessed whether the HBV infection generally suppressed immune responses to other comorbid infections such as *Plasmodium* by stimulating immune cells from HBV-infected and uninfected persons using selected *Plasmodium falciparum* antigens. We further evaluated the levels of cytokines and immune checkpoint proteins among CHB and HBV-negative individuals, as these markers are not included in the routine monitoring tests. This is vital because the inability of individuals to clear the virus suggests a defective immune response. Additionally, host immune responses to the virus-infected hepatocytes, mediated by proinflammatory cytokines, are believed to contribute to liver cell injury. With long-term chronic liver inflammation due to CHB, usually as a result of defective immune response needed for viral clearance, this process may contribute to the development of liver cirrhosis and cancer.[18,19]

2 | MATERIALS AND METHODS

2.1 | Study design and population

This study is part of a larger ongoing study titled "Clinical and Immunopathological Consequences of Hepatitis B and Malaria Co-infections (HEPMAL)," based at the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana. This is the leading, largest, and the most resourced biomedical research facility. Participants for this study were recruited from various parts of the Greater Accra Region, which has a population of about 5,455,692 in 2021 according to the Ghana Statistical Service. The capital city of Ghana (Accra) is located in this region Accra and is one of the most populated and fast-growing metropolises in Africa with an annual growth rate of 4.3%[20] and lies in the coastal savannah region. The prevalence of CHB in this region was 6.5% as of 2021.[21] Samples used in this study were obtained from 60 CHB individuals not meeting the criteria for antiviral therapy and 55 HBV-negative individuals, between April 2021 and December 2021. Samples from CHB individuals were taken at the Gastroenterology Clinic of the Korle Bu Teaching Hospital (KBTH) and the Legon Hospital Public Health Unit, both in Accra, Ghana. KBTH is a leading national referral and tertiary hospital that houses the University of Ghana Medical School. These participants were in the early phases of chronicity, and do not qualify for antiviral therapy because their serial (6-monthly) serum ALT (≤ULN: upper limits of normal or <2 times ULN), viral DNA (<2000 or <20,000 IU/mL) and clinical examination to determine liver fibrosis has been below the cutoff for treatment. Individuals between the ages of 18 and 60, having CHB infection that is verifiable by routine laboratory and clinical methods, not pregnant, not anemic

(hemoglobin [Hb] > 11 g/dL), without other detectable liver infections, and willing to provide written informed consent were included in the study as cases. Age (between 18 and 60 years) and location (within 2 km radius of where the case lives)-matched individuals with no liver infections or other conditions, not pregnant, not anemic (Hb > 11 g/dL), and willing to provide written informed consent were recruited as controls. Generally, individuals who had CHB infections with a viral load >2000 IU/mL that required treatment, or had other chronic inflammatory conditions, were anemic (Hb < 11 g/dL), or had other detectable liver infections or pregnant were excluded from the study.

The sample size for this study was determined using the GPower 3.1.9.7 online software, with the parameters: statistical test (means: Wilcoxon–Mann–Whitney test-two groups), two-tailed, effect size of 0.55, and power ($1 - \beta$) of 0.8. The calculated total sample size was 112, thus, at least 56 each for cases and controls. Therefore, data from 60 CHB and 55 HBV-negative individuals were used for this study (those with complete sociodemographic, flow cytometry, and cytokine data). Individuals who met the inclusion criteria were randomly selected and recruited after written informed consent had been obtained. All experiments were conducted in accordance with the principles of the Belmont Report.

2.2 | Whole blood assays

This assay was used to determine the cytokine secretion from CHB individuals and HBV-negative individuals. Two hundred and fifty microliters (250 µL) of sodium heparin blood was stimulated in 24-well culture plates with 1.25 µg/mL of phytohemagglutinin A (PHA) and 10 µg/mL of apical membrane antigen 1 (AMA-1), circumsporozoite protein (CSP), and thrombospondin-related adhesive protein (TRAP) respectively in an Roswell Park Memorial Institute 1640-based culture medium. Plates were incubated at 37°C, 5% CO_2 humidified incubator for 24 h. Culture supernatants were harvested 24 h after initiation of culture and stored immediately at −80°C until required for analysis in multiplexed assays.

2.3 | Multiplexed measurement of cytokine levels

This was performed on both separated plasma samples as well as on supernatants from whole blood cultures. These samples were analyzed for 13 cytokines (IFN-γ, IFN-α, IFN-β, TNF-α, IL-1β, IL-2, IL-6, IL12p70, IL-4, IL-13, IL-21, IL-17A, and IL-10) by a multiplex bead assay using commercially multiplexed kits, Human ProcartaPlex Mix & Match 13-Plex kit from Invitrogen. Data was acquired using the Luminex MAGPIX analyzer (Luminex Corporation). The comma-separated values (CSV) files containing the mean fluorescent intensities (MFIs) were then uploaded onto an online analysis app (ProcartaPlex app; ThermoFisher Scientific) which then converts the MFIs into concentrations in pg/mL.

2.4 | Peripheral blood mononuclear cells (PBMCs) isolation and cryopreservation

Heparinized whole blood was taken from individuals with CHB infection but have not progressed to the stage where treatment is required, and HBV-negative participants. The PBMCs were isolated with differential centrifugation using Ficoll (density = 1.077 g/mL) (Cytiva; Sweden AB). The cells were counted using the Countess II® machine which relies on the Trypan blue exclusion method. Cells were then resuspended in 90% fetal bovine serum (FBS) and 10% dimethylsulfoxide and stored in 1.5 mL cryotubes at 1×10^6 cells/mL. The cryotubes were then transferred into Mr Frosty™ freezing containers (Thermo Scientific) and stored at −80°C overnight. The cryotubes were then transferred into liquid nitrogen for long-term storage.

2.5 | Flow cytometry

Isolated cryopreserved PBMCs of subjects that gave at least 90% viability upon thawing and with a minimum count of 10^6 cells were used. CD4 and CD8 T cells from chronic HBV and HBV-negative individuals were characterized for immune checkpoint molecules by antibody staining for programmed cell death-1 (PD1), T cell immunoglobulin domain, and mucin domain-containing protein 3 (TIM3) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (CD152) or the memory marker CXCR3 (CD183). Antibodies used were purchased from BD Biosciences (Supporting Information S1: Table S3). Staining was performed in Pharm stain buffer (FBS) from BD (Lot 554656) according to the manufacturer's instructions. A BD LSRFortessa X-20 cell analyzer was used for data acquisition.

2.6 | Data handling/statistical analyses

Continuous variables are presented as median (interquartile range [IQR]). Categorical variables are presented as proportions. The participants' ages were compared using the Mann–Whitney *U* test and the χ^2 test was used for categorical variables. Flow cytometry standard (FCS) files from the flow cytometry experiments were analyzed using FlowJo version 10.9.0. The number of CD4+ and CD8+ T-cells that were positive for one, two, three, or four markers (TIM-3, PD-1, CTLA-4, or for CXCR3) were determined using the Boolean gating strategy. These results were then exported as CSV files and analyzed using GraphPad Prism 9.0.0. Luminex data were exported as CSV files for further statistical analysis. Plasma cytokine levels, the number of activated CD4+ and CD8+ T cells, levels of immune checkpoint proteins expression, and the levels of *P. falciparum*-induced cytokine production were compared between the two groups using the Mann–Whitney *U* test. Correlations between the

expression of the immune checkpoint proteins on CD4+ and CD8+ T cells were performed using the Spearman correlation rank test. All statistical tests were two-sided, and all differences were considered statistically significant when test p values were less than 0.05. All statistical analyses were performed with GraphPad Prism 9.0.0.

3 | RESULTS

3.1 | Sociodemographic characteristics of participants

The study involved 115 participants; 60 CHB individuals, who do not yet require antiviral therapy, and 55 HBV-negative individuals. The average age of cases (34.9 years) and controls (37.8 years) were not significantly different. Majority of both CHB and HBV-negative individuals had attended at least senior high school (SHS) (77.1% and 70.9%) and (59.0% and 54.5%) were males respectively. A higher proportion of CHB and HBV-negative individuals (77.1% and 85.5%) were employed respectively and (55.7%) of CHB individuals had normal body weight. The ages, gender, educational, and employment status were comparable among the two groups (Table 1).

3.2 | Chronic HBV-infected individuals had similar plasma cytokine levels as that of HBV-negative individuals

This study compared plasma cytokine levels on a subset of samples; between 27 chronic HBV (not on antiviral therapy) and 32 HBV-negative individuals. Five of the cytokines (IFN-β, IL-12p70, IL-13, IL-17A, and IL-21) assayed were below the detection limit of the assay kit. Appreciable levels of IFN-γ, IL-1β, IL-2, IL-6, IL-10, and TNF-α were produced by both groups (Figure 1B–D,F–H), and these were not significantly different between the two groups. Two of the cytokines (IFN-α and IL-4) were however produced in very minute quantities, and their levels were also not significantly different between the two groups (Figure 1A,E). IL-6 levels were the highest of the cytokines produced by the two groups but were not statistically different between the two groups (Figure 1).

3.3 | Chronic HBV and HBV-negative individuals had similar CD4+ and CD8+ T lymphocytes

To determine the level of CD4+ and CD8+ T lymphocytes, we analyzed CD4+ and CD8+ T lymphocytes in PBMCs from CHB and HBV-negative individuals. The two groups had similar proportions of

TABLE 1 Sociodemographic characteristics of participants.

Variable	Level	Cases (n = 60)	Controls (n = 55)	p Value
Age	Mean (SD)	34.9 (9.3)	37.8 (10.2)	0.11
Gender	Males	35 (59.0)	30 (54.5)	
	Females	25 (41.0)	25 (45.5)	0.63
Level of education	Tertiary	30 (49.2)	22 (40.0)	0.46
	JHS	5 (8.2)	7 (12.7)	0.96
	None	3 (4.9)	4 (7.3)	1
	SHS	17 (27.9)	17 (30.9)	0.73
	Basic	5 (9.8)	5 (9.1)	0.63
Employment status	Formal	30 (49.2)	26 (47.3)	0.42
	Informal	17 (27.9)	21 (38.2)	0.16
	None	13 (23.0)	8 (14.5)	1
BMI	Overweight	15 (24.6)	14 (25.5)	0.19
	Obese	11 (18.0)	16 (29.1)	0.38
	Normal	33 (55.7)	21 (38.2)	0.07
	Underweight	1 (1.6)	4 (7.3)	0.14

Note: Comparison between groups was done using χ^2 test for categorical variables and Mann–Whitney U test for age. $p < 0.05$ was considered statistically significant.

Abbreviations: BMI, body mass index; cases, CHB individuals who do not yet require antiviral therapy; controls, HBV-negative individuals; JHS, junior high school; SHS, senior high school.

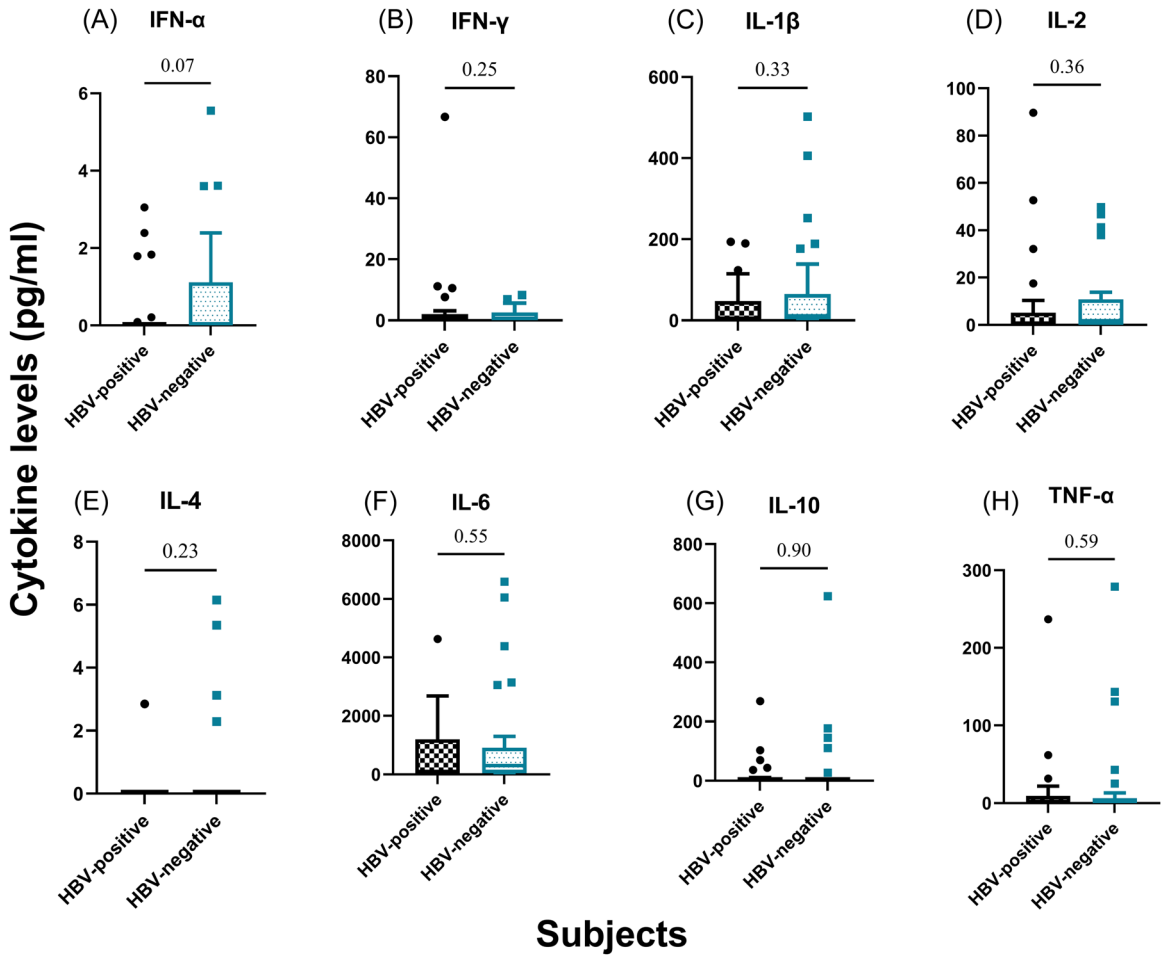

FIGURE 1 Plasma cytokine levels: Levels of 13 plasma cytokines were estimated using a Human ProcartaPlex Mix & Match 13-Plex kit. The graphs display Tukey plots of (A) IFN-α, (B) IFN-γ, (C) IL-1β, (D) IL-2, (E) IL-4, (F) IL-6, (G) IL-10, and (H) TNF-α levels in (pg/mL). A Mann–Whitney *U* test was used to compare the medians between the two groups and the corresponding *p* values are indicated on the graph. All statistical tests were two-tailed. HBV, hepatitis B virus; IFN-α, interferon-α; IL, interleukin; TNF-α, tumor necrosis factor-α.

CD4+ and CD8+ T lymphocytes (Figure 2A,C). Majority of the CD4+ and CD8+ T cells were of the activated phenotype (CD183+) (Figure 2B,D).

3.4 | Circulating CD4+ and CD8+ T lymphocytes in chronic HBV and HBV-negative individuals expressed comparable levels of immune checkpoint proteins

To determine the level of expression of immune checkpoint molecules, we analyzed CD4+ and CD8+ T lymphocytes among CHB and HBV-negative individuals. There were similar proportions of CD4+ and CD8+ T cells, and cells from the two groups expressed comparable levels of inhibitory immune checkpoint molecules (Tables 2 and 3). Majority of the CD4+ and CD8+ T cells were of the activated phenotype (CD183+), which expressed PD-1 and CD152 (Tables 2 and 3). Averagely, more than 75% of the CD4+ and CD8+ T lymphocytes from CHB and HBV-negative individuals did not express the checkpoint markers CD183, PD-1, TIM-3, and CD152 (Tables 2 and 3). We also determine the level of coexpression of immune checkpoint proteins. Increasing number of immune checkpoint proteins coexpression by T lymphocytes correlates with the severity of the exhaustion.[9] Less than 0.5% of total CD4+ or CD8+ T cells expressed TIM-3 and multiple immune checkpoint molecules (Tables 2 and 3) among the CHB and HBV-negative individuals. Significantly, the inverse relationship was observed between the quantity of CD8+ T cells lacking immune checkpoint proteins of interest in CHB and HBV-negative individuals and the activated CD8+ T cells expressing CD183+ and CD152+ ($r = -0.22$, $p < 0.019$), and CD152+CD183+PD1+ only ($r = -0.22$, $p < 0.015$) (Supporting Information S1: Table S1). In contrast, a similar observation did not extend to CD4+ T cells. However, among the two groups, both CD4+ and CD8+ T cells exclusively expressing TIM-3 exhibited a negative correlation with CD152+CD183+PD1+ cells ($r = -0.27$, $p < 0.003$ and $r = -0.20$, $p < 0.035$, respectively) (Supporting Information S1: Tables S1 and S2).

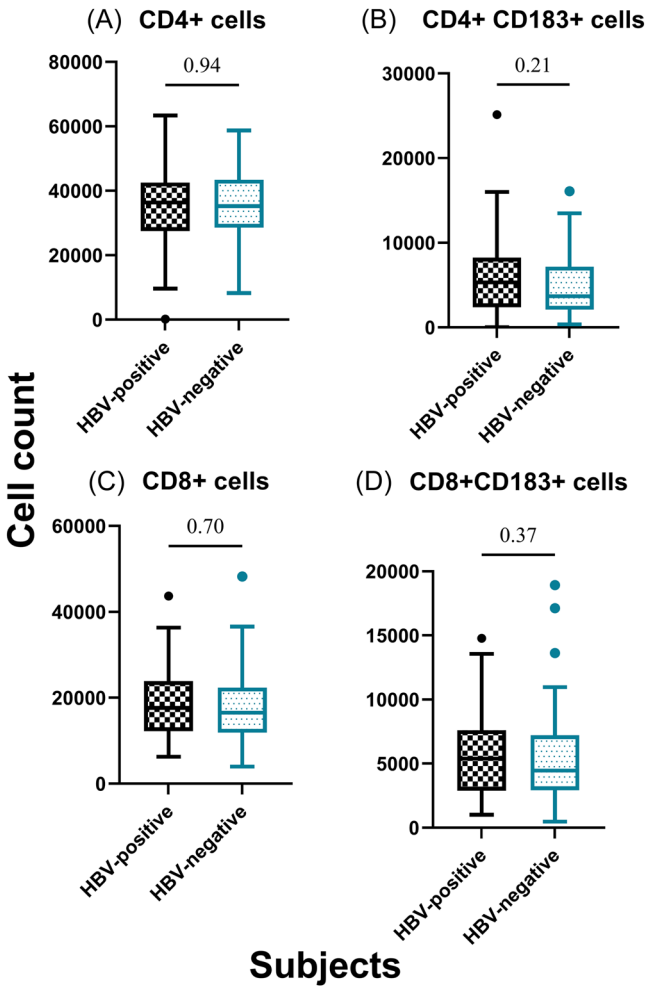

FIGURE 2 CD4+ and CD8+ T cell counts were similar in chronic HBV (black) and HBV-negative (sea blue) individuals. (A)–(D) PBMCs were analyzed by flow cytometry. The graphs display Tukey plots the number of CD4+, CD8+ T cells and the number of these cells that were CD183+. A Mann–Whitney *U* test was used to determine if the number of CD4+, CD8+ T cells, and those expressing CXCR3 (CD183+) were different between the two groups. All statistical tests were two-tailed. CXCR3, C-X-C motif chemokine receptor 3; HBV, hepatitis B virus; PBMCs, peripheral blood mononuclear cells.

3.5 | Chronic HBV-infected and HBV-negative individuals exhibited similar ex vivo cytokine expression patterns

To confirm the findings from the phenotypic characterization of CD4+ and CD8+ cells from the flow cytometry assay, we stimulated the participants' (27 chronic HBV and 32 HBV-negative individuals) blood cells with three *Plasmodium* antigens (CSP, AMA-1, and TRAP) in whole blood assays. This was done to determine whether the cells from the CHB individuals were as immunologically functional as the HBV-negative individuals. We used the production of cytokines as an outcome measure for this immunologic functionality. Our results indicated that there was no statistically significant difference between the CHB (not on antiviral therapy) and HBV-negative individuals in the ability to produce cytokines (Figure 3 and Figure S1). Also, IL-10 levels were comparable between the two groups (Figure 3B and Figure S1B).

4 | DISCUSSION

In CHB infection, the inability of the immune system to eradicate the virus causes the infection to progress to the chronic forms. However, in CHB individuals who do not meet the criteria for antiviral therapy, it is unclear whether their CD4+ and CD8+ T cells are in the process of becoming exhausted or anergic. This study therefore sought to determine whether the CD4+ and CD8+ T cells of these CHB individuals are fully functional compared to the functional status of uninfected persons. First, their CD4+ and CD8+ T lymphocytes were characterized for the presence of CXCR3, CTLA-4, TIM-3, and PD-1, as the coexpression of CTLA-4, TIM-3, and PD-1 and the loss of the memory marker CXCR3 (CD183), signify the degree of exhaustion.[9] This exhaustion may account for their inability to clear the HBV in the acute infection stage. In a study by Kurachi et al.,[22] which aimed to determine how CXCR3 influences the differentiation of antigen-specific CD8+ T cells using mouse models, it was shown that high levels of CXCR3 expression were found on memory CD8+ HBV-specific T cells. This indicates that the levels of CXCR3 reflect the functional capacity of effector or memory CD8+ T cells, with lower levels expressed on exhausted CD8+ T cells.[8,22] Contrary to our expectation, the cell characteristics were similar in both the CHB and HBV-negative individuals, and both groups had comparable levels of functional CD4+ and CD8+ T cells (Figure 2).

Based on the viral profile (HBV DNA levels) serum alanine aminotransferase, and clinical parameters of our participants, these CHB individuals can be described to belong to immune-active and inactive CHB groups, as described by the WHO 2015 and AASLD 2018 guidelines. These individuals do not meet the criteria for antiviral therapy based on lab and clinical parameters. This may imply that these individuals are immunologically able to control the virus and prevent it from increasing to levels that can cause serious disease. Having comparable levels of CD4+ and CD8+ T cells to that of HBV-negative individuals meant their immune cells were still fully functional and not exhausted in these early stages of chronicity. Also, the observation that few inhibitory molecules were expressed may be normal as functional effector T lymphocytes transiently express inhibitory receptors when activated.[23–25] We found that the majority of the CD4+ and CD8+ T lymphocytes were of the activated phenotype (CD183+) (Figure 2), which usually expressed low levels of PD-1 and CD152 (Tables 2 and 3) (Supporting Information S1: Table S1). This is corroborated by the reports of Araki et al.[25] and Zhang et al.,[26] where PD-1 expression was rapidly upregulated upon T cell activation, and moderate levels were seen to persist in effector memory CD8+ T cells of healthy individuals.[27]

TIM-3 is an inhibitory receptor, treatment with anti-TIM-3 and anti-PD-1 restored effector T cell function.[28] In acute HBV infection, CD8+ T lymphocytes transiently express TIM-3, however, this is

TABLE 2 Expression of immune checkpoint proteins on CD4+ T lymphocytes in chronic HBV and HBV-negative individuals.

Cells	HBV-positives	HBV-negatives	Difference: H–L	95.05% CI of difference	*p* Value
CD4+ T cells (all negative)					
Median (IQR)	26,420 (20,147–31,846)	28,481 (22,432–36,704)	−1906	−5256 to 1724	0.31
Mean ranks	54.95	61.33			
CD4+CD152+ cells					
Median (IQR)	491 (29.5–923.5)	434 (20–967)	2	−178 to 178	0.91
Mean ranks	58.33	57.65			
CD4+PD1+ cells					
Median (IQR)	1701 (1307–2522)	1809 (1163–2873)	−48	−474 to 359	0.82
Mean ranks	57.30	58.76			
CD4+TIM3+ cells					
Median (IQR)	9 (5–18.5)	10 (3–17)	1	−2 to 4	0.60
Mean ranks	59.58	56.27			
CD4+CD152+PD1+ cells only					
Median (IQR)	27 (2.25–58)	25 (2–61)	0	−8 to 12	0.86
Mean ranks	58.53	57.43			
CD4+CD152+CD183+ cells only					
Median (IQR)	42 (5.25–149)	37 (3–108)	−3	−24 to 7	0.43
Mean ranks	60.34	55.45			
CD4+CD183+TIM3+ cells only					
Median (IQR)	1 (0–2)	1 (0–2)	0	0 to 0	0.64
Mean ranks	59.33	56.55			
CD4+CD152+CD183+PD1+ cells only					
Median (IQR)	6.5 (2–31)	7 (1–25)	0	−2 to 4	0.61
Mean ranks	59.51	56.35			

Note: Median cell counts were compared between cases and controls using Mann–Whitney *U* test. $p < 0.05$ was considered statistically significant.
Abbreviations: Cases, CHB individuals who do not as yet require antiviral therapy; Controls, HBV-negative individuals; CI, confidence interval; HBV, hepatitis B virus; H–L, Hodges–Lehmann estimator; IQR, interquartile range; PD1, programmed cell death-1; TIM-3, T cell immunoglobulin domain and mucin domain-containing protein 3.

rapidly downregulated after viral clearance, while it is highly expressed in CHB infection. This is believed to contribute to the persistence of HBV infection.[28,29] Additionally, in our study, less than 0.5% of total CD4+ and CD8+ T lymphocytes expressed TIM-3 (Tables 2 and 3), and multiple immune checkpoint proteins (Tables 2 and 3). This finding is in agreement with the reports of Wu et al.,[29] where TIM-3 expression on CD4+ and CD8+ T cells decreased in CHB. Notably, only a very few participants had very few of their CD4+ or CD8+ T cells expressing double or triple positive immune checkpoint proteins (CTLA-4, TIM-3, and PD-1). This further supports our position that these cells may be of the effector phenotype. Other studies have shown that, during chronic infections, PD-1 expression is substantially higher than observed on functional effector or memory CD8+ T cells.[9,30]

Further, cytokines have been found to regulate viral replication and aid the cure of HBV by targeting different stages of the virus.[31] However, the pathophysiology of HBV has been linked to cytokines, providing evidence that immune-modulating treatment can be effective.[12,14] Interleukin 1β (IL-1β) enhances IFN-α treatment response. In hepatic cell lines, IL-4 suppressed the replication and expression of HBV. IL-6 inhibits HBV entry and transcription through the downregulation of sodium taurocholate cotransporting polypeptide.[12,32] Interleukin 2 (IL-2) promotes host immune activity, IL-12 enhances the antiviral properties of HBV-specific T lymphocytes and combination treatment with IL-12 favors HBV clearance. Interleukin 21 also enhances HBV-specific CD8+ T lymphocyte effects by enhancing both cytolytic and noncytolytic pathways, IL-17 suppresses HBV replication in a noncytopathic manner and IFN-α, which

TABLE 3 Expression of immune checkpoint proteins on CD8+ T lymphocytes in chronic HBV and HBV-negative individuals.

Cells	HBV-positives	HBV-negatives	Difference: H–L	95.05% CI of difference	*p* Value
CD8+ T cells (all negative)					
Median (IQR)	11,165 (7007–14,539)	6530 (6530–15,751)	309.5	−1628 to 2416	0.73
Mean ranks	59.03	56.87			
CD8+CD152+ cells					
Median (IQR)	777 (50.75–1243)	697 (41–1426)	17	−159 to 373	0.58
Mean ranks	59.67	56.18			
CD8+PD1+ cells					
Median (IQR)	386 (202–646.3)	464 (220–730)	−20	−141 to 82	0.68
Mean ranks	56.77	59.35			
CD8+TIM3+ cells					
Median (IQR)	8.5 (4–16.5)	9 (4–17)	0	−4 to 3	0.77
Mean ranks	57.13	58.95			
CD8+CD183+PD1+ cells only					
Median (IQR)	136.5 (67.25–223.3)	103 (57–149)	21.5	−9 to 57	0.14
Mean ranks	62.43	53.17			
CD8+CD152+PD1+ cells only					
Median (IQR)	7 (1.25–18.75)	4 (1–14)	1	−1 to 4	0.51
Mean ranks	59.96	55.86			
CD8+CD183+CD152+ cells only					
Median (IQR)	241 (15.25–470)	191 (12–453)	5	−51 to 100	0.60
Mean ranks	59.58	56.28			
CD8+CD152+CD183+PD1+ cells only					
Median (IQR)	5.5 (1–18.75)	6 (1–12)	1	−1 to 4	0.33
Mean ranks	60.90	54.84			

Note: Median cell counts were compared between cases and controls using Mann–Whitney *U* test. $p < 0.05$ was considered statistically significant.
Abbreviations: Cases, CHB individuals who do not as yet require antiviral therapy; Controls, HBV-negative individuals; CI, confidence interval; HBV, hepatitis B virus; H–L, Hodges–Lehmann estimator; IQR, interquartile range; PD1, programmed cell death-1; TIM-3, T cell immunoglobulin domain and mucin domain-containing protein 3.

is used as the current standard treatment of HBV, exerts both direct host immunomodulation and antiviral effects.[10] IFN-γ HBV-specific producing T cells are associated with viral clearance.

To determine the extent to which our participants produce these cytokines, we quantified them using unstimulated plasma. Our results showed that the levels of type I IFNs, IL-6, and IL-10 were similar between the two groups. Notably, the type I IFNs were mostly undetectable, while IL-6, crucial for a robust CD8+ T cell response, was the most highly expressed among the cytokines produced by both groups (Figure 1). This may be due to its pleiotropic role in the human body.[33–36] Also, the levels of other cytokines (IL-1β, IL-2, IL-10, and TNF-α) were also comparable between the two groups. This supports the findings of Ribeiro et al.[37] where cytokine levels were not significantly different between CHB (without antiviral therapy) and healthy individuals. The levels of cytokines recorded in this study also support the decision of the caregivers not to put these individuals on antiviral therapy as their immune cells exhibit similar functionality as that of HBV-negative individuals and may have the ability to control the viral levels.

We also used the production of cytokines as a proxy to assess immune cell functionality in the presence of malaria antigens. Blood cells were stimulated with three *P. falciparum* (3D7) antigens—CSP, AMA-1, and TRAP in whole blood assays. Our results indicated that there was no significant difference between CHB and HBV-negative individuals in their ability to produce cytokines following stimulation with malaria antigens. Additionally, when compared to the unstimulated samples, both groups produced comparable higher levels of IL-10, IL-6, TNF-α, and IL-1β, suggesting that the cells from these CHB

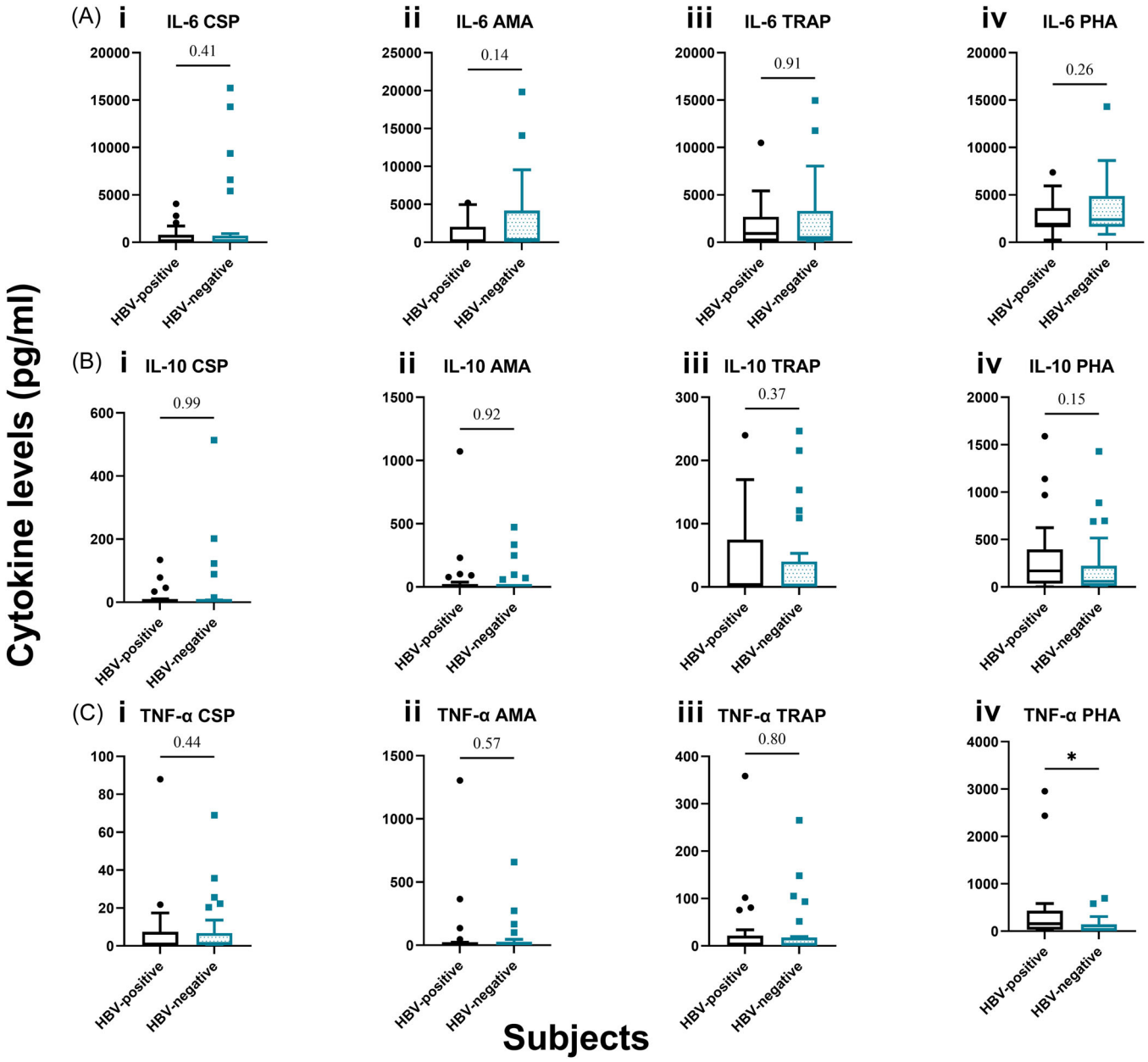

FIGURE 3 Ex vivo production of cytokines by chronic HBV and HBV-negative individuals. Levels of *Plasmodium falciparum* antigen-induced cytokines were estimated using a Human ProcartaPlex Mix & Match 13-Plex kit. The graphs display a Tukey box and whiskers plot of (A) IL-6, (B) IL-10, and (C) TNF-α levels in (pg/mL). A Mann–Whitney test was used for comparison between the two groups and the corresponding *p* values are indicated on the graph. All statistical tests were two-tailed. AMA-1, apical membrane antigen 1; CSP, circumsporozoite protein; HBV, hepatitis B virus; IL, interleukin; TNF-α, tumor necrosis factor-α; PHA, phytohaemagglutinin; TRAP, thrombospondin-related adhesive protein.

individuals are as immunologically functional as the HBV-negative individuals (Figure 3 and Figure S1). These results are supported by our findings from the flow cytometry analyses and are further corroborated by the reports of Kennedy et al.,[38] where cytokine production as a measure of T cell function was preserved in individuals with an immune-tolerant CHB disease. However, they used HBV peptides and a younger population (median age of 17–25 years).

5 | CONCLUSION

A limitation of this study is our inability to include samples from advanced CHB cases for comparison with the early CHB cases. This could have given additional insight into immune responses to CHB in general and added to the existing knowledge on immunity to HBV infection. This notwithstanding, the current data on patients with early CHB is important and demonstrates a clear difference between

this group and advanced CHB cases, not just in terms of the clinical presentation, but also in the underlying immune responses.

In summary, there was no significant difference between CHB and HBV-negative individuals in their ability to produce cytokines. CD4+ and CD8+ T cells from both groups showed similar patterns of immune checkpoint protein expression. These findings support the practice at liver clinics where individuals in the early stages of chronic HBV infection are kept off antiviral therapy and monitored periodically to see if they require treatment in the future.

AUTHOR CONTRIBUTIONS

Selorm P. Segbefia: Conceptualization; data curation; formal analysis; methodology; validation; writing—original draft; writing—review and editing. **Diana A. Asandem**: Data curation; formal analysis; investigation; writing—review and editing. **Abigail Pobee**: Data curation; investigation; writing—review and editing. **Bright Asare**: Data curation; investigation; writing—review and editing. **Ahu Diana Prah**: Formal analysis; investigation; writing—review and editing. **Rawdat Baba-Adam**: Data curation; investigation; writing—review and editing. **Jones Amo Amponsah**: Formal analysis; investigation; writing—review and editing. **Eric Kyei-Baafour**: Formal analysis; investigation; writing—review and editing. **William van der Puije**: Formal analysis; investigation; writing—review and editing. **Frank Osei**: Data curation; investigation; writing—review and editing. **Doreen Teye-Adjei**: Data curation; investigation; writing—review and editing. **Seth Agyemang**: Formal analysis; investigation; writing—review and editing. **Theophilus Brenko**: Data curation; formal analysis; investigation; writing—review and editing. **Lutterodt Bentum-Ennin**: Data curation; formal analysis; writing—review and editing. **John K. A. Tetteh**: Formal analysis; investigation; writing—review and editing. **Kofi J. H. Bonney**: Funding acquisition; investigation; methodology; supervision; validation; writing—review and editing. **Samuel Asamoah Sakyi**: Supervision; writing—review and editing. **Linda E. Amoah**: Funding acquisition; investigation; supervision; writing—review and editing. **Kwadwo A. Kusi**: Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; validation; writing—original draft; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

ETHICS STATEMENT

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Scientific and Technical Committee, NMIMR. Ethical approval was sought from both the NMIMR Institutional Review Board with Federalwide Assurance number FWAA00001824(NMIMR-IRB/046/19-20) and the Ethics Review Committee of the Korle-Bu Teaching Hospital, Accra (KBTH-IRB/00024/2020).

TRANSPARENCY STATEMENT

The lead author Selorm P. Segbefia affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

ORCID

Selorm P. Segbefia http://orcid.org/0000-0001-5795-8314
Eric Kyei-Baafour http://orcid.org/0000-0001-6423-1885
Samuel Asamoah Sakyi http://orcid.org/0000-0001-5168-4762

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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