Lipopolysaccharide, Immune Activation, and Liver Abnormalities in HIV/Hepatitis B Virus (HBV)–Coinfected Individuals Receiving HBV-Active Combination Antiretroviral Therapy

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We investigated the relationship between microbial translocation, immune activation, and liver disease in human immunodeficiency virus (HIV)/hepatitis B virus (HBV) coinfection. Lipopolysaccharide (LPS), soluble CD14, CXCL10, and CCL-2 levels were elevated in patients with HIV/HBV coinfection. Levels of LPS, soluble CD14, and CCL-2 declined following receipt of HBV-active combination antiretroviral therapy (cART), but the CXCL10 level remained elevated. No markers were associated with liver disease severity on liver biopsy (n = 96), but CXCL10, interleukin 6 (IL-6), interleukin 10 (IL-10), tumor necrosis factor α, and interferon γ (IFN-γ) were all associated with elevated liver enzyme levels during receipt of HBV-active cART. Stimulation of hepatocyte cell lines in vitro with IFN-γ and LPS induced a profound synergistic increase in the production of CXCL10. LPS may contribute to liver disease via stimulating persistent production of CXCL10.

Keywords. HIV/HBV coinfection; microbial translocation; fibrosis.

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There are currently 33 million people infected with human immunodeficiency virus (HIV), and on average 5%–10% are coinfected with hepatitis B virus (HBV) [1]. In the presence of HIV, HBV-related liver disease progression is accelerated, and liver-related mortality is significantly increased [1]. With the introduction of HBV-active combination antiretroviral therapy (cART), liver-related mortality rates have significantly decreased. However, total and liver-related mortality still remains significantly elevated [2].

HIV significantly depletes $CD4^+$ T cells in the gastrointestinal tract, leading to increased microbial translocation [3, 4]. The resultant systemic low-level endotoxemia and chronic immune activation are believed to be major drivers of HIV disease progression and other non-AIDS comorbidities [3]. There is therefore a potential role for microbial translocation and chronic immune activation in HIV/HBV-associated liver disease progression.

METHODS

Patient Details

All patients were recruited at Chulalongkorn University Hospital (Bangkok, Thailand) as described previously [5]. The upper limits of normal (ULNs) for liver enzyme levels in men were as follows: alanine aminotransferase (ALT), 40 IU/mL; aspartate aminotransferase (AST), 37 IU/mL; and alkaline phosphatase (ALP), 111 IU/mL. For women, the ULNs were as follows: ALT, 31 IU/mL; AST, 41 IU/mL; and ALP, 123 IU/mL. Controls without HIV, HBV, or hepatitis C virus (HCV) infection (n = 10) were recruited from University Hospital staff in Bangkok.

Quantification of Lipopolysaccharide (LPS) and Immune Mediators in Plasma and Cell Culture Supernatant

LPS was quantified with the chromogenic limulus amebocyte lysate assay (Lonza) [4]. Plasma soluble CD14 (sCD14) levels were quantified with the Quantikine sCD14 enzyme-linked immunosorbent assay (ELISA; R&D Systems), and levels of all other immune mediators were quantified with a custom multiplex bead array (Millipore). All markers were assessed in patient plasma samples collected at study visits (baseline/before cART initiation, at 1 year, and after at least 3 years and up to 7 years of cART receipt).

In Vitro Model of Hepatocyte Response to Interferon γ (IFN-γ)

Human hepatocyte cell lines HepG2s and Huh7s (obtained from the ATCC) were treated with 100 ng/mL IFN-γ (Bioscientific) and/or 50 ng/mL LPS (Sigma), 50 ng/mL tumor necrosis

factor α (TNF-α; Peprotech), 100 ng/mL interleukin 10 (IL-10; eBioscience), and 50 ng/mL interleukin 6 (IL-6; Biolegend) for 24 hours, and CXCL10 was detected by ELISA (Qiagen). Cells were harvested in Trizol (Invitrogen) for RNA extraction.

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

CXCL10 messenger RNA was quantified using primers to CXCL10 [6], the reference gene RLPLO [7], and the comparative cycle threshold ΔΔCt method.

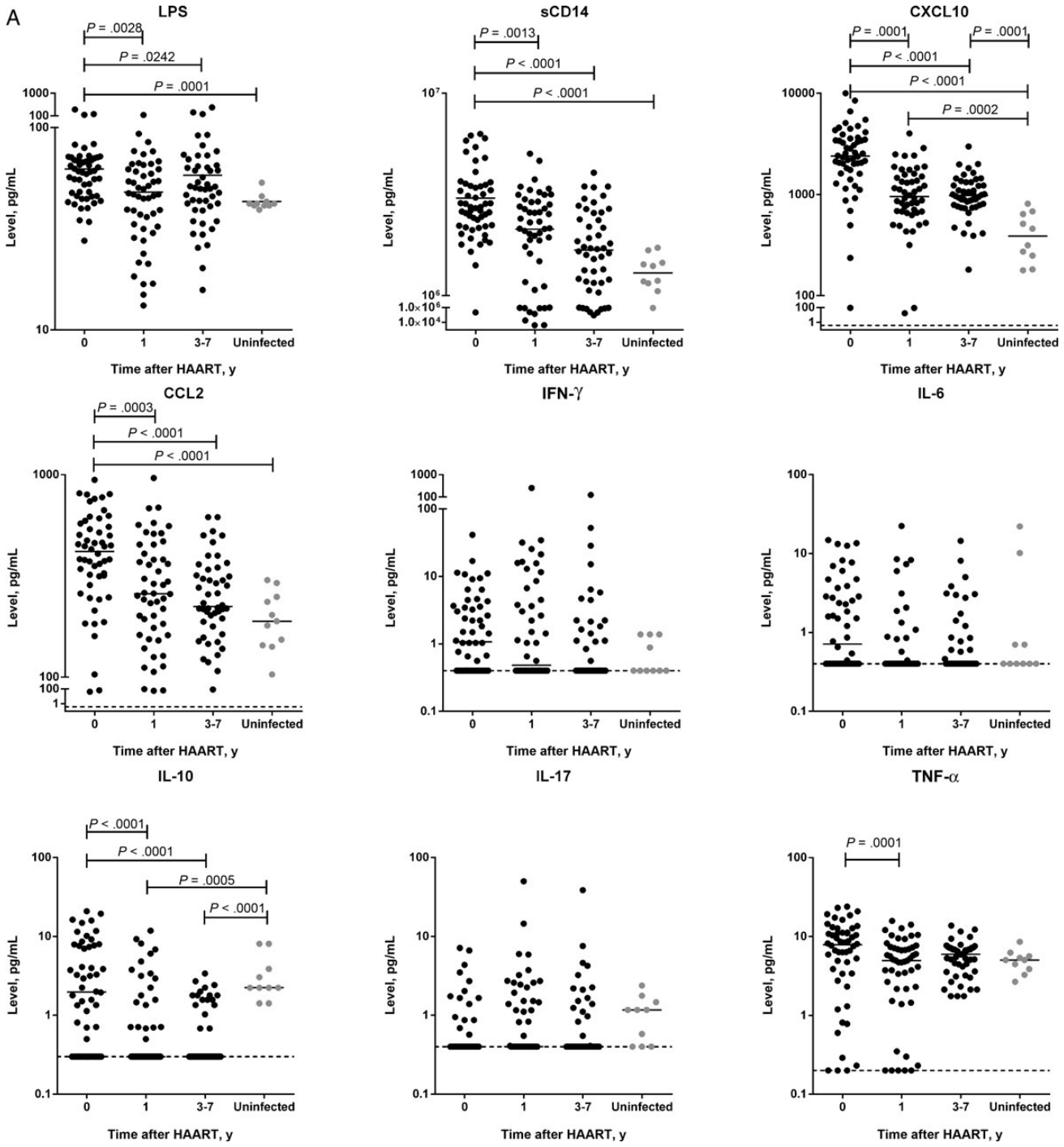

Figure 1. Levels of lipopolysaccharide (LPS) and immune mediators following hepatitis B virus (HBV)–active combination antiretroviral therapy (cART). *A*, Levels of LPS and immune mediators in human immunodeficiency virus (HIV)/HBV–coinfected patients at baseline (black; n = 54) and after 1 year (n = 49) and 5–7 years of HBV-active cART (n = 47) and uninfected controls (grey; n = 10) in patient plasma (pg/mL). Individual dots represent a single patient, and the dotted black line indicates the lower limit of detection. *P* values were calculated by the Wilcoxon signed rank test for comparisons within the HIV/HBV-coinfected group and by the Mann–Whitney *U* test for comparisons between the coinfected and uninfected groups.

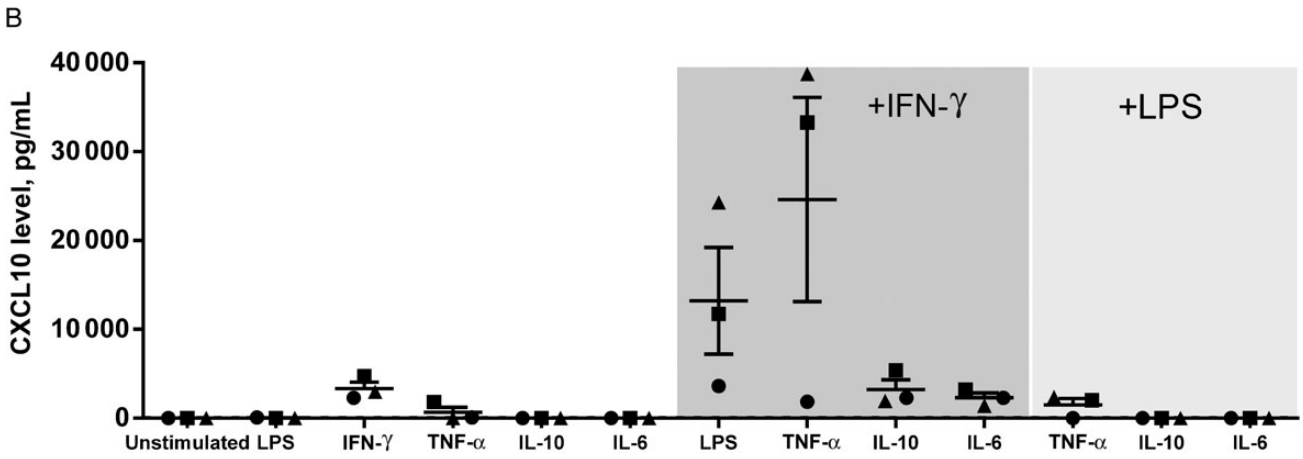

Figure 1 continued. *B*, CXCL10 concentration (pg/mL) in supernatants from Huh7 cultures stimulated with LPS (50 ng/mL), interferon γ (IFN-γ; 100 ng/mL), tumor necrosis factor α (TNF-α; 50 ng/mL), interleukin 10 (IL-10; 50 ng/mL), and/or interleukin 6 (IL-6; 100 ng/mL; n = 3). The dotted line indicates the lower limit of detection (10 pg/mL). Symbols represent individual experiments. Bars represent the mean and standard error of multiple individual experiments. $P < .05$, by the paired *t* test; no adjustment for multiple testing was performed. Abbreviations: ELISA, enzyme-linked immunosorbent assay; HAART, highly active antiretroviral therapy; IL-17, interleukin 17; sCD14, soluble CD14.

Statistical Analysis

Differences in baseline clinical characteristics between patient groups were assessed using a χ^2 test or a Mann–Whitney *U* test. Comparisons of baseline levels of LPS and immune mediators were assessed with a Kruskal–Wallis test and the Dunn post test. Differences in LPS levels, immune mediator levels, and clinical parameters before therapy and during HBV-active cART in the HIV/HBV-coinfected group were assessed with a Wilcoxon signed rank test, and comparisons between coinfected patients receiving cART and uninfected patients were made with a Mann–Whitney *U* test.

Unadjusted logistic regression was used to determine associations between measured parameters and liver disease for HBV monoinfected patients and HIV/HBV-coinfected patients before initiation of therapy. For each outcome, modeling was limited to a single predictor at a time (ie, unadjusted modeling) to avoid overfitting a comparatively small number of outcome events. A repeated-measures logit model was used to investigate unadjusted associations between on-therapy markers (measured at multiple time points after baseline) and subsequent advanced liver disease (defined as a Metavir score of ≥3) or abnormal liver enzyme activity (AST or ALT levels of >2 times the ULN). A repeated-measures trend model was preferred to static pair-wise comparisons of individual on-therapy time points because it better captures trends in associations between predictor and outcome over time. For this analysis, data were censored at the point when the outcome was recorded. A Hosmer-Lemeshow goodness-of-fit test was used to assess overall model fit.

Paired *t* tests were used to compare CXCL10 production by cell lines in response to stimulation. Data were analyzed using Stata, version 12 (StataCorp, College Station, TX) and GraphPad Prism4 (GraphPad Software, San Diego, CA).

RESULTS

Characteristics of Patients at Baseline and During Therapy

The baseline clinical characteristics of HIV/HBV-coinfected and HBV-monoinfected patients are summarized in Supplementary Table 1. Changes in HIV RNA load, HBV DNA load, $CD4^+$ T-cell count, and liver enzyme levels (ALT) in the HIV/HBV-coinfected patients following HBV-active cART have been previously reported [5]. The proportion of HIV/HBV-coinfected patients with an ALT level of > 2 times the ULN during HBV-active ART was 8% (8/97). There was no significant change in ALT, AST, or ALP levels after 5–7 years of therapy.

LPS and Immune Mediators at Baseline

Levels of LPS sCD14, CXCL10, and CCL2 was significantly higher in HIV/HBV-coinfected patients than in either HBV-monoinfected patients or uninfected controls. Levels of IL-6 and TNF-α were significantly elevated in HIV/HBV-coinfected patients, compared with HBV-monoinfected patients, but they were not significantly different from those in uninfected controls. Levels of interleukin 17 (IL-17) were significantly lower in HIV/HBV-coinfected patients, compared with HBV-monoinfected patients, but were not significantly different from those in uninfected controls. There were no significant differences observed in levels of IL-10 or IFN-γ between groups (Supplementary Figure 1).

Table 1. Univariable Associations and Baseline Predictors of Elevated Liver Enzyme Levels

	Before Therapy				During Therapy		HIV/HBV Coinfection (Baseline Predictors) (n = 97)[b]	
	HBV Infection (n = 52)[a]		HIV/HBV Coinfection (n = 54)[a]		HIV/HBV Coinfection (n = 97)[b,c]			
Variable	AST Level	ALT Level	AST Level	ALT Level	AST Level	ALT Level	AST Level	ALT Level
HBV DNA load, log_{10} U/mL								
95% CI	1.22 (1.00–1.50)	1.26 (1.07–1.48)	1.06 (0.85–1.32)	1.52 (1.10–2.20)	1.00 (0.99–1.00)	1.61 (1.04–2.50)	1.14 (0.90–1.45)	1.38 (0.99–1.93)
P	**.023**	**.005**	.594	**.025**	.903	**.034**	.281	.056
HBeAg positivity								
95% CI	2.81 (0.68–11.70)	4.00 (1.02–15.76)	0.23 (0.04–1.3)	0.75 (0.21–2.63)	1.95 (0.063–23.27)	0.78 (0.12–5.12)	0.46 (0.10–2.05)	0.74 (1.98–2.75)
P	.155	**.048**	.095	.649	.598	.796	.309	.650
IL-10 level, pg/mL								
95% CI	1.64[d] (1.00–2.70)	1.65[d] (1.04–2.60)	1.06 (0.93–1.21),	1.02 (0.91–1.14)	1.88 (1.10–3.16)	1.72 (0.95–3.10)	4.53[d] (1.16–17.77)	1.10 (0.99–1.23)
P	**.046**	**.032**	.422	.738	**.017**	.072	**.030**	.085
CXCL10 level, pg/mL								
95% CI	1.11[d] (1.02–1.23)	3.80[d] (1.32–10.95)	0.99 (0.99–1.00)	0.99 (0.96–1.03)	11.35[d] (1.46–88.01)	1.07 (0.96–1.18)	1.00 (0.99–1.00)	1.43 (0.92–2.20)
P	**.022**	**.013**	.836	.772	**.02**	.221	.224	.112
TNF-α level, pg/mL								
95% CI	1.22 (0.93–1.60)	1.31 (0.99–1.74)	1.03 (0.91–1.67)	1.01 (0.91–1.12)	1.41 (1.02–1.94)	1.27 (0.91–1.79)	3.60[d] (1.22–10.64)	1.09 (0.99–1.20)
P	.152	.060	.622	.847	**.039**	.164	**.02**	.088
IL-6 level, pg/mL								
95% CI	1.18 (0.85–1.64)	1.14 (0.83–1.57)	1.16 (0.98–1.38)	1.00 (0.85–1.19)	1.82 (0.74–4.52)	1.33 (0.87–2.02)	8.10[d] (1.77–37.23)	1.09 (0.94–1.26)
P	.330	.411	.084	.984	.195	.193	**.007**	.242
IFN-γ level, pg/mL								
95% CI	1.06 (0.98–1.15)	1.01 (0.99–1.15)	1.09 (0.98–1.21)	1.04 (0.95–1.34)	1.02 (0.99–1.05)	1.02 (0.99–1.04)	3.26[d] (1.33–7.99)	2.27[d] (1.06–4.85)
P	.128	.107	.122	.394	.154	.240	**.010**	**.034**
LPS level, pg/mL								
95% CI	1.00 (0.95–1.06)	1.98 (0.15–25.30)	0.98 (0.94–1.03)	1.00 (0.98–1.03)	1.00 (0.95–1.05)	1.01 (0.97–1.05)	1.01 (0.98–1.04)	0.99 (0.97–1.02)
P	.974	.600	.488	.989	.938	.663	.658	.680
sCD14 level, pg/mL								
95% CI	1.00 (0.99–1.00)	1.33 (0.19–94.36)	0.99 (0.99–1.00)	0.99 (0.99–1.00)	1.00 (0.99–1.00)	0.99 (0.99–1.00)	0.67 (1.96–2.41)	0.99 (0.99–1.00)
P	.977	.896	.240	.187	.833	.866	.539	.134
CCL2 level, pg/mL								
95% CI	1.00 (0.96–1.01)	1.00 (0.99–1.01)	1.00 (0.99–1.01)	1.05 (0.93–1.19)	0.99 (0.99–1.01)	1.00 (0.99–1.01)	2.70 (0.54–13.58)	2.30 (0.63–8.40)
P	.406	.274	.369	.411	.797	.847	.229	.210

Table 1 continued.

Variable	Before Therapy				During Therapy			
	HBV Infection (n = 52)[a]		HIV/HBV Coinfection (n = 54)[a]		HIV/HBV Coinfection (n = 97)[b,c]		HIV/HBV Coinfection (Baseline Predictors) (n = 97)[b]	
	AST Level	ALT Level	AST Level	ALT Level	AST Level	ALT Level	AST Level	ALT Level
IL-17 level, pg/mL								
95% CI	0.98 (0.92–1.05)	0.99 (0.93–1.05)	1.26 (0.82–1.94)	1.16 (0.78–1.72)	1.10 (0.97–1.23)	1.12 (0.96–1.30)	1.41 (0.96–2.06)	1.46 (0.74–2.88)
P	.658	.720	.290	.463	.154	.151	.080	.279

Data are odds ratios (95% confidence intervals); odds ratios represent a 1 unit change, unless otherwise indicated. Statistically significant *P* values are in bold. Levels 2 times the upper limit of normal were considered elevated and were defined in the laboratory in Bangkok, Thailand, as follows: AST, 74 IU/mL (males) and 82 IU/mL (females); and ALT, 80 IU/mL (males) and 74 IU/mL (females).

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B virus e antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; IFN-γ, interferon γ; IL-6, interleukin 6; IL-10, interleukin 10; IL-17, interleukin 17; LPS, lipopolysaccharide; sCD14, soluble CD14; TNF-α, tumor necrosis factor α.

[a] *P* values were calculated by unadjusted logistic regression.

[b] *P* values were calculated by an unadjusted repeated measures logit model.

[c] Time variable after baseline associations.

[d] Units are log-transformed.

LPS and Immune Mediators During Therapy

Changes in levels of LPS and immune mediators are all shown in Figure 1. Levels of LPS, sCD14, and CCL2 in HIV/HBV-coinfected patients significantly declined following HBV-active cART and after 3–7 years were no different from those in uninfected controls. Levels of CXCL10 declined significantly following 1 year of therapy but remained significantly higher than those in uninfected controls. Levels of IL-10 significantly declined after 1 and 5–7 years therapy to below those of uninfected controls. TNF-α levels declined after 1 year but were not significantly different from those in uninfected controls. There was no evidence of changes in IL-6, IL-17, or IFN-γ levels with cART.

Associations Between Markers of Immune Activation and Abnormal Liver Function

In treatment-naive HBV-monoinfected patients, an AST level of > 2 times the ULN was significantly associated with increasing HBV DNA load, IL-10 level, and CXCL10 level (Table 1), whereas an ALT level of > 2 times the ULN was significantly associated with the same parameters, as well as with HBeAg positivity. In treatment-naive HIV/HBV coinfection, only an ALT level of > 2 times the ULN was significantly associated with an increased HBV DNA load. When we examined HIV/HBV-coinfected patients during HBV-active cART, we found that an AST level of > 2 times the ULN was significantly associated with increased IL-10, CXCL10, and TNF-α levels and that an ALT level of > 2 times the ULN was associated only with increased HBV DNA (Table 1).

We then examined baseline predictors of elevated liver enzyme levels in HIV/HBV-coinfected patients during HBV-active cART and found that an AST level of > 2 times the ULN was significantly associated with increased baseline IFN-γ, IL-6, TNF-α, and IL-10 levels and that an ALT level of >2 times the ULN was associated with increased baseline IFN-γ levels. There were no significant associations with the Metavir score in any of the analyses of HIV/HBV-coinfected patients (data not shown). All analyses were univariate, given the low frequency of the end points of elevated ALT and AST levels.

In Vitro Production of CXCL10 From Hepatocytes

Given the persistent elevation of CXCL10 during cART, we next determined the effect of LPS and immune mediators on production of CXCL10 from hepatocytes in vitro.

IFN-γ and TNF-α induced a significant increase in CXCL10 in Huh7 cells. IL-10, IL-6, and LPS alone did not (Figure 1). The combination of both LPS and IFN-γ resulted in a synergistic increase in CXCL10. This synergistic response was not observed with LPS and either TNF-α, IL-10, or IL-6 (Figure 1). We confirmed these findings by ELISA and real-time qPCR in Huh7 cells and a second hepatocyte cell line, HepG2 (Supplementary Figure 2).

DISCUSSION

This is the first study to evaluate the relationship between LPS, markers of immune activation, and liver disease severity during HIV/HBV coinfection. We found elevated levels of LPS, sCD14, CXCL10, and CCL2 in HIV/HBV-coinfected patients, compared with HBV monoinfected patients and uninfected controls, before therapy. Following HBV-active cART, LPS and most markers of immune activation returned to levels of uninfected controls, with the exception of CXCL10. There was no association between liver disease and levels of LPS or sCD14, either before or after initiation of HBV-active cART. However, an elevated AST level was significantly associated with CXCL10, IL-6, IL-10, TNF-α, and IFN-γ levels. Finally, we showed that production of CXCL10 by hepatocytes could potentially be driven by a synergistic response to LPS and IFN-γ.

Elevated levels of LPS have been demonstrated in HIV infection and occur secondary to depletion of $CD4^+$ T cells in the gut [3]. Two previous studies have shown microbial translocation to be significantly associated with liver fibrosis in HIV-HCV coinfection [8, 9], while 2 other studies have been unable to demonstrate an association [10, 11]. LPS levels have also been shown to be elevated in HCV- and HBV-monoinfected patients, compared with uninfected controls, and sCD14 was associated with markers of liver activity, such as AST [12]. However, this study combined patients with HBV and HCV infection into one group so it was not possible to determine the effects of each virus [12]. In our study, we found no correlation between LPS or sCD14 and liver disease defined on biopsy or by elevated liver enzyme levels during HIV/HBV coinfection, nor did we find an elevation in LPS levels in HBV-monoinfected patients, compared with uninfected controls. This might be explained by the fact that the frequency of patients with severe liver disease was lower (26% and 7% for HIV/HBV-coinfected and HBV-monoinfected patients, respectively) than in other studies (53% in a cohort of HIV/HCV-coinfected patients [9] and 86% in HBV-monoinfected patients [12]). A higher prevalence of cirrhosis and associated impairment of LPS clearance may potentially account for the observed correlation between LPS and liver fibrosis in previous studies, and in the absence of cirrhosis it is likely there is minimal effect on LPS clearance.

As expected, we found HBV DNA was significantly associated with elevated ALT levels in a univariable analysis among HBV-monoinfected patients and in coinfected patients receiving or not receiving therapy. We were unable to assess whether HBV DNA was independently associated with elevated ALT levels during therapy because too few patients had a detectable outcome of elevated ALT levels (8%). However, the data suggest that full suppression of the HBV DNA load to undetectable levels should remain a priority to reduce liver inflammation.

In HIV/HBV-coinfected patients, multiple markers of immune activation were clearly associated with abnormal liver function in univariate analyses. CXCL-10, IL-6, IL-10, and IFN-γ have all been associated with liver damage in previous studies [13, 14]. We were particularly interested in the role of CXCL10, because it was the only inflammatory marker with levels that remain elevated in HIV/HBV-coinfected patients after prolonged cART. Both primary human hepatocytes and hepatic cell lines can be induced to produce CXCL10 in response to IFN-γ, to the combination of IFN-γ and TNF-α, or to LPS when cocultured with peripheral blood mononuclear cells (PBMCs) in vitro [15]. We demonstrated that in the absence of coculture with PBMCs, although LPS alone had no effect, a combination of LPS and IFN-γ induced a synergistic increase in CXCL10 production, compared with IFN-γ alone. Therefore, in the setting of HIV and depletion of $CD4^+$ T cells in the gut, LPS could be mediating liver damage by potentiating production of chemoattractant CXCL10 from hepatocytes.

This study had several limitations. First, the cohort was relatively small. However, the cohort is quite unique, with recruitment from a single site, genotype C as the predominant HBV genotype, advanced immunosuppression, and prolonged control of HIV and HBV. Second, we were only able to quantify immune activation markers in the periphery, although intrahepatic events may be important. Third, this was a noninterventional observational study, so causation cannot be extrapolated from our findings. Finally, as only a small proportion of patients showed evidence of severe liver disease, our study was underpowered to look at correlations with liver disease severity or progression based on biopsy findings. This question could potentially be answered using a noninvasive marker of liver disease, such as Fibroscan, which is currently underway.

In summary, we have demonstrated that levels of markers of microbial translocation and immune activation were significantly elevated in HIV/HBV coinfection and that levels of most markers, except CXCL10, normalize following prolonged suppressive HBV-active cART. There was no association between levels of LPS and levels of any markers of liver disease in HIV/HBV coinfection. However, in vitro studies suggest that LPS may work synergistically with IFN-γ to increase CXCL10 production, which can contribute to intrahepatic inflammation.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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