LPS, immune activation and liver abnormalities in HIV-HBV co-infected individuals on HBV-active combination antiretroviral therapy

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Abstract:

We investigated the relationship between microbial translocation, immune activation and liver disease in HIV-HBV co-infection. Lipopolysaccharide (LPS), soluble (s)CD14, CXCL10 and CCL-2 were elevated in HIV-HBV co-infection and declined following HBV-active combination antiretroviral therapy (cART) but CXCL10 remained elevated. No markers were associated with liver disease severity on liver biopsy (n=96) but CXCL10, IL-6, IL-10, TNFα and IFNγ were all associated with elevated liver enzymes on HBV-active cART. Stimulation of hepatocyte cell lines in vitro with IFNγ and LPS induced a profound synergistic increase in production of CXCL10. LPS may contribute to liver disease via stimulating persistent production of CXCL10.
**Introduction:**

There are currently 33 million people infected with HIV and on average 5-10% are co-infected 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 reduced, however, total and liver-related mortality still remains significantly elevated [2].

HIV significantly depletes CD4+ T-cells in the gastrointestinal (GI) 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 co-morbidities [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 limit of normal (ULN) for liver enzymes were; alanine transferase (ALT) 40 IU/ml, aspartate transferase (AST) 37 IU/ml and alkaline phosphatase (ALP) 111 IU/ml (male) and ALT 31 IU/ml, AST 41 IU/ml and ALP 123 IU/ml (female). HIV, hepatitis B and C uninfected controls (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 sCD14 levels was quantified with the Quantikine sCD14 ELISA (R&D Systems) and
all others with a custom multiplex bead array (Millipore). All markers were assessed in patient plasma samples taken at study visits (baseline/pre-cART, 1 year and following at least 3 years and up to 7 years on cART).

*In vitro model of hepatocyte response to IFNg*

Human hepatocyte cell lines HepG2’s and Huh7s (obtained from the ATCC) were treated with 100ng/ml IFNg (Bioscientific) and/or 50ng/ml LPS (Sigma), 50ng/ml TNFa (Peprotech), 100ng/ml IL-10 (eBioscience) and 50ng/ml IL-6 (Biolegend) for 24 hours and CXCL10 detected by ELISA (Qiagen). Cells were harvested in Trizol (Invitrogen) for RNA extraction.

*Quantitative Real Time-PCR (qRT-PCR)*

CXCL10 mRNA was quantified using primers to CXCL10 [6] and the reference gene RLPLO [7] and the comparative cycle threshold delta-delta Ct method.

*Statistical analysis*

Differences in baseline clinical characteristics between patient groups were assessed using a Chi squared test or a Mann-Whitney U test. Comparisons of baseline levels of LPS and immune mediators were assessed with a Kruskal-Wallis test and Dunn’s post test. Differences in LPS, immune mediators and clinical parameters pretherapy and on HBV-active cART in the HIV-HBV co-infected patient group were assessed with a Wilcoxon signed rank test and comparisons between co-infected patients on 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 mono-infected patients and HIV-HBV co-infected patients prior to initiation of therapy. For each outcome, modelling was limited to a single predictor at a time (i.e. unadjusted modelling) to avoid over-fitting 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 post baseline) and subsequent advanced liver disease (metavir score greater than or equal to 3), or abnormal liver enzyme activity (AST or ALT>2x ULN). A repeated measures trend model was preferred to static pairwise comparisons of individual on-therapy time points as it better captures trends in associations between predictor and outcome over time. For this analysis, data was censored at the point where 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, Inc., San Diego, CA).

Results:

Characteristics of patients at baseline and on therapy

The baseline clinical characteristics of HIV-HBV co-infected and HBV mono-infected patients are summarised in Supplementary Table 1. Changes in HIV RNA, HBV DNA, CD4 count and liver enzymes (ALT) in the HIV-HBV co-infected patients following HBV-active cART have been previously reported [5]. The proportion of HIV-HBV co-infected patients with ALT > 2x ULN on HBV-active ART was 8% (8/97). There was no significant change in ALT, AST or ALP 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 co-infection than either HBV mono-infection or uninfected controls. Levels of IL-6 and TNFa were significantly elevated in HIV-HBV co-infection compared to HBV mono-infection, however, they were not significantly different to uninfected controls. Levels of IL-17 were significantly lower in HIV-HBV co-infection compared to HBV mono-infection but were not
significantly different to uninfected controls. There were no significant differences observed in levels of IL-10 or IFNγ between groups (Supplementary Figure 1).

**LPS and immune mediators on therapy**

Changes in LPS and immune mediators are all shown in Figure 1. Levels of LPS, sCD14 and CCL2 in HIV-HBV co-infection significantly declined following HBV-active cART and after 3-7 years were no different from uninfected controls. Levels of CXCL10 declined significantly following one year of therapy, however, remained significantly higher than uninfected controls. Levels of IL-10 significantly declined following one and 5-7 years therapy to below that of uninfected controls. TNFα declined following one year but were not significantly different to uninfected controls. There was no evidence of change in IL-6, IL-17 or IFNγ with cART.

**Associations between markers of immune activation and abnormal liver function**

In treatment naive HBV mono-infected patients, AST >2x ULN was significantly associated with increasing HBV DNA, IL-10 and CXCL10 (Table 1) while ALT >2x ULN was significantly associated with the same parameters as well as HBeAg positivity. In treatment naive HIV-HBV co-infection, only ALT >2x ULN was significantly associated with increased HBV DNA. When we examined HIV-HBV co-infected patients on HBV-active cART, we found that >2xULN AST was significantly associated with increased IL-10, CXCL10 and TNFα; and >2xULN ALT was associated only with increased HBV DNA (Table 1).

We then examined baseline predictors of elevated liver enzymes in HIV-HBV co-infected patients on HBV-active cART, and found that AST>2xULN was significantly associated with increased baseline IFNγ, IL-6, TNF-α and IL-10; and an ALT>2xULN was associated with increased baseline IFNγ. There were no significant associations with the Metavir score
in any of the analyses of HIV-HBV co-infected patients (data not shown). All analyses were univariate given the low frequency of the end points of elevated ALT and AST.

In vitro production of CXCL10 from hepatocytes

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

IFNg and TNFa 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 IFNg resulted in a synergistic increase in CXCL10. This synergistic response was not observed with LPS and either TNFa, IL-10 or IL-6 (Figure 1). We confirmed these findings by ELISA and RT-PCR in Huh7 and a second hepatocyte cell lines, HepG2 (supplementary Figure 2).

Discussion:

This is the first study to evaluate the relationship between LPS, markers of immune activation and liver disease severity in HIV-HBV co-infection. We found elevated levels of LPS sCD14, CXCL10 and CCL2 in HIV-HBV co-infection compared to HBV mono-infection and uninfected controls pre-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 prior to or following HBV-active cART. However, elevated AST was significantly associated with CXCL10, IL-6, IL-10, TNFa and IFNg. Finally, we showed that production of CXCL10 by hepatocytes could potentially be driven by a synergistic response to LPS and IFNg.

Elevated levels of LPS has been demonstrated in HIV infection and occurs 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 co-infection [8, 9], while two other studies have been unable to demonstrate an association [10, 11]. LPS levels have also been shown to be elevated in HCV and HBV mono-infection compared to
uninfected controls and sCD14 was associated with markers of liver activity such as AST [12]. However, this study combined patients with HBV and HCV 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 enzymes in HIV-HBV co-infection, nor did we find an elevation in LPS in HBV mono-infected 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 co-infected and HBV mono-infected respectively) than other studies (53% in a cohort of HIV-HCV co-infected patients [9] and 86% in HBV mono-infection [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 in a univariable analysis in HBV mono-infected patients and in co-infected patients off and on therapy. We were unable to assess whether HBV DNA was independently associated with elevated ALT on therapy as too few patients had a detectable outcome of elevated ALT (8%). However, the data suggests that full suppression of HBV DNA to undetectable levels should remain a priority to reduce liver inflammation.

In HIV-HBV co-infected patients, multiple markers of immune activation were clearly associated with abnormal liver function in univariate analyses. CXCL-10, IL-6, IL-10 and IFNg have all been associated with liver damage in previous studies [13, 14]. We were particularly interested in the role of CXCL10 given it was the only inflammatory marker that remain elevated in HIV-HBV co-infected patients after prolonged cART. Both primary human hepatocytes and hepatic cell lines can be induced to produce CXCL10 in response to IFNg, the combination of IFNg and TNFa or to LPS when co-cultured with peripheral blood...
mononuclear cells (PBMC) in vitro [15]. We demonstrated that in the absence of co-culture with PBMC, although LPS alone had no effect, a combination of LPS and IFNg induced a synergistic increase in CXCL10 production as compared to IFNg 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, it is important to note that the cohort is quite unique with recruitment from a single site, predominantly genotype C, advanced immunosuppression and prolonged control of HIV and HBV. Second, we were only able to quantify immune activation markers in the periphery while intrahepatic events may be important. Third, this was a non-interventional 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. This question could potentially be answered using a non-invasive marker of liver disease such as Fibroscan, which is currently underway.

In summary, we have demonstrated that markers of microbial translocation and immune activation were significantly elevated in HIV-HBV co-infection and most markers, except CXCL10, normalize following prolonged suppressive HBV-active cART. There was no association between LPS and any markers of liver disease in HIV-HBV co-infection. However, in vitro studies suggest that LPS may work synergistically with IFNg to increase CXCL10 production, which can contribute to intrahepatic inflammation.
Conflict of interest: None to declare

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Meetings at which work has previously been presented:

21st Conference of the APASL, Bangkok, Thailand, 2011 and the 6th International workshop on HIV and Hepatitis, Tel Aviv, Israel, 2010.

List of abbreviations:
References:


Figure legends

Figure 1. Levels of LPS and immune mediators following HBV-active cART.

a) Levels of LPS and immune mediators in HIV-HBV co-infection at baseline (black, n=54) and following 1 year (n=49) and 5-7 years HBV-active cART (n=47) and uninfected controls (grey, n=10) in patient plasma (pg/ml). Individual dots represent a single patient, dotted black line indicates the lower limit of detection. P values; Wilcoxon signed rank for comparisons within the HIV-HBV co-infected group and Mann-Whitney U test between co-infected and uninfected. b) CXCL10 concentration (pg/ml) in supernatants from Huh7 cultures stimulated with LPS (50ng/ml), IFNg (100ng/ml), TNFa (50ng/ml), IL-10 (50ng/ml) and/or IL-6 (100ng/ml ; n=3). Dotted line: LLOD (10pg/ml). Symbols represent individual experiments. Bars represent the mean and standard error of multiple individual experiments. P<0.05, Paired T-test, no adjustment for multiple testing was performed.
a) plasma levels of LPS and immune mediators

- LPS
- sCD14
- CXCL10
- IFNg
- IL-6
- IL-10
- IL-17
- TNFα

b) Huh7 ELISA

- CXCL10 (pg/ml)

- Unstimulated LPS
- IFNg
- TNFα
- IL-10
- IL-6
- LPS
- TNFα
- IL-10
- IL-6
Table 1: Univariable associations and baseline predictors of elevated liver enzymes (>2x ULN).

<table>
<thead>
<tr>
<th></th>
<th>Pre- therapy (n=52) (^a)</th>
<th>On therapy (n=97) (^b)</th>
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<tbody>
<tr>
<td></td>
<td>HBV</td>
<td>HIV-HBV</td>
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<tr>
<td></td>
<td></td>
<td>(baseline predictors)</td>
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<tr>
<td></td>
<td>HBV DNA (\log_{10}) U/mL</td>
<td>1.22(1.00-1.50)</td>
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<td></td>
<td></td>
<td>(p=0.023)</td>
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<td></td>
<td>HBeAg+</td>
<td>2.81(0.68-11.70)</td>
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<td></td>
<td></td>
<td>(p=0.155)</td>
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<tr>
<td></td>
<td>IL-10 pg/ml</td>
<td>1.64(1.00-2.70), (p=0.046^*)</td>
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<td></td>
<td></td>
<td>(p=0.048)</td>
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<tr>
<td></td>
<td>CXCL10 pg/ml</td>
<td>1.11(1.02-1.23), (p=0.022^*)</td>
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<tr>
<td></td>
<td></td>
<td>(p=0.013^*)</td>
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<tr>
<td></td>
<td>TNF(\alpha) pg/ml</td>
<td>1.22(0.93-1.60), (p=0.152)</td>
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<tr>
<td></td>
<td></td>
<td>(p=0.060)</td>
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<tr>
<td></td>
<td>IL-6 pg/ml</td>
<td>1.18(0.85-1.64), (p=0.330)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p=0.411)</td>
</tr>
<tr>
<td></td>
<td>IFN(\gamma) pg/ml</td>
<td>1.06(0.98-1.15), (p=0.128)</td>
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<tr>
<td></td>
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<td>(p=0.107)</td>
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<tr>
<td></td>
<td>LPS pg/ml</td>
<td>1.00(0.95-1.06), (p=0.974)</td>
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<tr>
<td></td>
<td></td>
<td>(p=0.600)</td>
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<tr>
<td></td>
<td>sCD14 pg/ml</td>
<td>1.00(0.99-1.00), (p=0.977)</td>
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<td></td>
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<td>(p=0.896)</td>
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</table>

\(^a\) HIV-HBV patients on combination antiretroviral therapy and hepatitis B treatment; \(^b\) HIV-HBV patients on combination antiretroviral therapy, hepatitis B treatment, and baseline predictors.
<table>
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<tr>
<th>CCL2 pg/ml</th>
<th>1.00(0.96-1.01)</th>
<th>1.00(0.99-1.01)</th>
<th>1.00(0.99-1.01)</th>
<th>1.05(0.93-1.19)</th>
<th>0.99(0.99-1.01)</th>
<th>1.00(0.99-1.01)</th>
<th>2.70(0.54-13.58)</th>
<th>2.30(0.63-8.40)</th>
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<tbody>
<tr>
<td>p</td>
<td>0.406</td>
<td>0.274</td>
<td>0.369</td>
<td>0.411</td>
<td>0.797</td>
<td>0.847</td>
<td>0.229</td>
<td>0.210</td>
</tr>
<tr>
<td>IL-17 pg/ml</td>
<td>0.98(0.92-1.05)</td>
<td>0.99(0.93-1.05)</td>
<td>1.26(0.82-1.94)</td>
<td>1.16(0.78-1.72)</td>
<td>1.10(0.97-1.23)</td>
<td>1.12(0.96-1.30)</td>
<td>1.41(0.96-2.06)</td>
<td>1.46(0.74-2.88)</td>
</tr>
<tr>
<td>p</td>
<td>0.658</td>
<td>0.720</td>
<td>0.290</td>
<td>0.463</td>
<td>0.154</td>
<td>0.151</td>
<td>0.080</td>
<td>0.279</td>
</tr>
</tbody>
</table>

2x ULN defined in the laboratory in Bangkok as AST 74 IU/ml (male), 82 IU/ml (female), ALT 80 IU/ml (male), 74 IU/ml (female). Odds ratio’s (95% confidence interval). P value, a unadjusted logistic regression b unadjusted repeated measures logit model, significant p values are italicised. OR values represent a 1 unit change unless marked with an asterisk (*) where units are log transformed.

# Time variable post baseline associations.