

Pro-fibrogenic role of alarmin HMGB1 in HIV-HBV co-infection

Short title ('running head') HMGB1 in HIV-HBV coinfection

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ABSTRACT

Objective: Liver disease is accelerated in people living with HIV (PLWH) with hepatitis B (HBV) coinfection. We hypothesised that liver fibrosis in HIV-HBV is triggered by increased hepatocyte apoptosis, microbial translocation and/or HIV/HBV viral products.

Design: Sera from PLWH with HBV coinfection versus from those with HBV only or putative mediators were used to examine the pathogenesis of liver disease in HIV-HBV.

Methods: We applied sera from PLWH and HBV coinfection versus HBV alone, or putative mediators (including HMGB1), to primary human hepatic stellate cells (hHSC) and examined pro-fibrogenic changes at the single cell level using flow cytometry. HMGB1 levels in the applied sera were assessed according to donor fibrosis stage.

Results: Quantitative flow cytometric assessment of pro-fibrogenic and inflammatory changes at the single cell level revealed an enhanced capacity for sera from PLWH with HBV coinfection to activate hHSC. This effect was recapitulated by LPS, HIV-gp120, hepatocyte conditioned-media and the alarmin HMGB1. Induction of hepatocyte cell death increased their pro-fibrogenic potential, an effect blocked by HMGB1 antagonist glycyrrhizic acid. Consistent with a role for this alarmin, HMGB1 levels were elevated in sera from PLWH and hepatitis B coinfection compared to HBV alone and higher in those with HIV-HBV with liver fibrosis compared to those without.

Conclusions: Sera from PLWH and HBV coinfection have an enhanced capacity to activate primary hHSC. We identified an increase in circulating HMGB1 which, in addition to HIV-gp120 and translocated microbial products, drove pro-fibrogenic changes in hHSC, as mechanisms contributing to accelerated liver disease in HIV-HBV.

Key Words: liver; HBV; HIV; fibrosis; hepatic stellate cells; pro-fibrogenic; HIV-HBV

Introduction

People living with human immunodeficiency virus (HIV) (PLWH) with hepatitis B virus (HBV) coinfection have accelerated liver disease and fibrogenesis compared with people living with either alone^[1]. Antiretroviral therapy active against HIV and HBV (HBV-active ART) improves outcomes in PLWH with HBV coinfection, reducing overall and liver-related mortality^[2, 3]. However, hospital utilization, hepatocellular carcinoma (HCC) and mortality remain elevated in PLWH with HBV coinfection compared to those with either HIV or HBV alone on treatment^[1, 4]. Furthermore, liver fibrosis continues to progress in 10-20% of PLWH with HBV coinfection on HBV-active ART^[5, 6] and pathogenesis is poorly understood.

Human hepatic stellate cells (hHSC) are liver-specific pericytes in the Space of Disse, between the liver sinusoids and hepatocytes and are drivers of liver fibrosis^[7-9]. When quiescent, hHSC contain retinoid-rich droplets and are star-shaped with protrusions extending to encircle the sinusoids. Hepatocellular damage leads to hHSC activation and transition to a myofibroblast-like phenotype with increased expression of α -smooth muscle actin (α -SMA) and synthesis and secretion of fibrillar collagens causing scar tissue deposition^[8]. Liver remodelling is a dynamic process of production and resorption of extracellular matrix, with fibrosis resulting from production increasing over resorption^[8, 9]. hHSC also contribute to fibrogenesis by expressing factors that inhibit enzymes stimulating tissue breakdown (e.g. tissue inhibitor matrix metalloproteinase-1 [TIMP-1]). Activated hHSC also exhibit contractility, chemotaxis, proliferation and increased expression of receptors for activating ligands^[9].

In HIV, multiple factors may drive HSC activation. Firstly, CD4⁺ T cells depletion from the gastrointestinal tract occurs early in infection and is associated with increased microbial translocation measured as elevated circulating lipopolysaccharide (LPS) and/or soluble (s)-CD14, consistent with monocyte activation and systemic immune activation^[10, 11]. LPS is a component of the gram-negative bacterial cell wall and ligand for toll like receptor (TLR)-4, expressed on KC and HSC^[12-15]. The acylated amino-terminus of bacterial lipoprotein is recognised by TLR-1/2 (mimicked by synthetic tri-acylated lipoprotein Pam3cys-SerLys4 (Pam3CSK4)), which can also mediate hHSC activation^[9, 14, 16].

In PLWH and hepatitis C (HCV), studies show an association between microbial translocation, immune activation, and liver cirrhosis^[17-20]. In HBV and HCV infection local/systemic inflammation was associated with cirrhosis and predicted progression to end-stage liver disease^[15]. We showed that Pam3CSK4 increased production of CXCL10 from an HBV-transfected cell line, which further increased with HIV infection (VSV-pseudotyped HIV), and both intrahepatic/circulating CXCL10 are associated with liver fibrosis in PLWH with HBV coinfection^[21].

Secondly, HIV may have direct effects on liver cells that accelerate fibrosis. *In vitro* and *in vivo* HIV can infect hepatocytes^[22, 23], KC^[24], HSC^[25] and CD4⁺ T-cells recruited to the liver during inflammation^[26]. HIV envelope glycoprotein (gp)-120 can bind to CC-chemokines on HSC and trigger inflammation, migration^[27] and pro-fibrogenic gene expression^[28].

Thirdly, increased hepatocyte cell death may drive liver fibrogenesis in HIV. We found a higher level of hepatocyte cell death using terminal dUTP nick-end labelling (TUNEL) in liver from PLWH with HBV coinfection compared with HBV alone^[29]. Similar findings were seen in HIV-HCV co-infection^[30]. Mechanisms for hepatocyte cell death are undefined in HIV-HBV but may include increased tissue necrosis factor (TNF) receptor apoptosis inducing ligand (TRAIL) sensitivity of hepatocytes^[31], increased hepatocyte Fas expression^[30] and binding of HIV gp120^[32].

We used primary hHSC to investigate effects of HIV-HBV on hHSC activation. We demonstrate that hepatocyte cell death, microbial translocation and viral factors all increase hHSC activation leading to accelerated hepatic fibrogenesis in HIV-HBV co-infection. Sera from PLWH with HBV co-infection has an enhanced capacity to drive pro-fibrogenic changes in HSC and contains increased levels of high mobility group box (HMGB)1 correlating with fibrosis; we confirm a functional role for this alarmin in driving profibrotic changes in HSC.

Methods

Study participants

For isolation of primary hHSC, liver was obtained under the Human Tissue Act from healthy liver margins resected for colorectal cancer metastases (The Royal Free Hospital, London, UK). Serum was obtained from healthy volunteers (University College London (UCL)). PLWH with HBV coinfection and those with HBV without HIV were recruited from the HIV-Netherlands-Australia-Thai Research Collaboration (HIV-NAT), Thai Red Cross AIDS Research Centre, Bangkok, Thailand (Supplementary Table 1, <http://links.lww.com/QAD/C719>). Inclusion criteria were HIV antibody positive, HBV surface antigen (HBsAg) and/or HBV DNA positive twice ≥ 6 months apart, ≥ 18 years old and ART naïve. Liver biopsies were scored (single blinded pathologist) according to Metavir classification^[33].

Participants gave written, informed consent. Ethical approval was obtained under Research Ethics Committee Reference: 5472/6743 (tissue), 11/LO/0421 (serum, UCL) and Human Research Ethics Committees in Australia/Thailand Reference: HN178 (HIV-NAT) and 1443345 (The University of Melbourne).

In vitro stimulation of HSC: HBV, HIV proteins and other proteins

hHSC were isolated, expanded, and frozen down in aliquots^[34]. For *in vitro* experiments, aliquots were thawed and cultured in 25/75 cm² tissue flasks in Stellate Cell Medium® (ScienCell Research Laboratories, San Diego, CA) to 80% confluence. Stimulation experiments were carried out in duplicate/triplicate, and conditions repeated in ≥ 2 independent experiments. For hHSC, experiments were performed in ≥ 2 independent hHSC donors. hHSC were stimulated with: 100 IU/ml purified HBsAg recombinant from yeast (HBsAg) or HB core antigen (HBcAg) recombinant from E.coli (HBc145) (Rhein Biotech

GmbH (Dynavax Europe, Dusseldorf, Germany), 10 µg/mL/100 ng/ml HIV gp120 (BaL strain, R5-tropic; Centre for AIDS Reagents, National Institute for Biological Standards and Control, UK repository reference EVA657), recombinant human HMGB1 1-1000 ng/mL (Biolegend, London, UK), TLR4 agonist LPS-EB (Invivogen, San Diego, CA), or TLR1/2 agonist synthetic tri-acylated lipoprotein PC3SK4 (InvivoGen, San Diego, CA).

Hepatocyte Conditioned Media

Hepatocyte-conditioned media was generated using human hepatoblastoma-derived hepatocyte cell line, HepG2 (American Type Culture Collection (ATCC)) or HepG2-derived HepG2.2.15 cells. HepG2.2.15 are transfected with complementary DNA (cDNA) of pregenomic (pg) RNA and mimic the HBV life-cycle including expression of HBV DNA and RNA replicative intermediates, HBsAg and HBeAg production^[35]. Hepatocytes were grown for 72 hours (80% confluence), then culture media was harvested for hHSC stimulation. 300µL hHSC media was replaced with 'hepatocyte-conditioned media' (18 hours).

Immunofluorescence of HepG2.2.15 cells confirmed the presence of HBeAg. Cells permeabilized with 0.1% Triton X-100 (20 minutes) were incubated overnight with primary mouse anti-HBeAg (Abcam, Cambridge, UK, [#ab8637], 1:400) after blocking with 1% BSA. Cells were then stained with secondary goat anti-mouse antibody (1:200), and DAPI (0.5 µg/mL DAPI) for nuclear staining. Imaging was with a Zeiss LSM 700.

Patient Sera

Frozen sera were thawed and added at 1:4 ratio with cell culture media to hHSC (3-6 hours). hHSC were washed twice with 1XPBS, left for 18 hours, detached, washed, and stained for flow cytometry.

Hepatocytes

SuperKiller TRAIL™ (sKT®; Enzo Life Sciences, Exeter, UK), an enhanced soluble human recombinant TRAIL was added at 500ng/mL to HepG2.2.15 or HepG2 cultures in 6-well plates (30 minutes) to trigger apoptosis and cell death. Hepatocytes were washed twice and cultured (12 hours). We confirmed the effect of sKT® by flow cytometry of hepatocytes stained with anti-caspase-3-PE antibody (BD Bioscience, Oxford, UK, [#550821], 1:30) in the presence of fixable cell viability stain (Live/Dead™, Invitrogen, Thermofischer Scientific, Carlsbad, CA, [#L10119], 1.5:1000) to quantify cell death and apoptosis, after FcR blocking (Miltenyi Biotec, Bergisch Gladbach, Germany). TRAIL receptor expression on hepatocytes was confirmed using anti-TRAIL receptor monoclonal antibodies (R&D Systems, TRAIL-R1-PE, [#FAB347P], TRAIL-R2-PE [#FAB6311P], TRAIL-R3-PE [#FAB6302P], TRAIL-R4-PE [#FAB633P]), with Live/Dead™ stain. (Supplementary Figure 3, <http://links.lww.com/QAD/C719>).

Quantification of HMGB1

HMGB1 was measured in serum by ELISA (IBL International GMBH, Hamburg, Germany).

Flow cytometry

LX2/hHSC were detached with trypsin-EDTA (Life Technologies, Thermo Fisher Scientific), re-plated in 24-well plates in DMEM with 10% fetal calf serum at 50,000 cells/well and left 24-48 hours to adhere. HIV or HBV-related proteins, LPS-EB, PC3SK4 or hepatocyte-conditioned media was added. After 12 hours, cells were detached, washed, and stained with viability stain before FcR blocking, then with anti-CD54 (intercellular adhesion molecule (ICAM)1-PacificBlue; Biolegend, London, UK, [#322716], 1:50), CD14-V500 (BD Biosciences, [#561391], 2:50), α SMA-PE; R&D Systems, [#IC1420P], 1.5:50), C-C motif chemokine ligand 2 (CCL2; monocyte chemoattractant protein-1 (MCP1)-FITC; R&D Systems, [#IC27915], 1:50) or CCL2-AF700 (Novus Biologicals, Littleton, CO, [#NBP2-22115AF700], 1:50). For hHSC: IL8-PerCP (Biolegend, [#514606], 2:50), and TIMP1-FITC; R&D Systems, [#IC970F], 2:50) were used. Isotype controls were used. Data show fold change expression compared to cells cultured in media alone.

Similar methods were used for flow cytometry of hepatocytes, to evaluate the presence of TRAIL receptors 1-4 (above) (Supplementary Figure 3, <http://links.lww.com/QAD/C719>).

To confirm/quantify hepatocyte cell death, 12 hours following exposure to sKT®, HepG2/HepG2.2.12 were stained with anti-caspase-3-PE antibody with viability stain or viability stain alone, after FcR blocking.

Data were acquired on a BD Bioscience™ LSR II flow cytometer (Beckton Dickinson, New Jersey, USA) and analysed using FlowJo (Treestar, OR).

Statistical Analysis

Parameters were compared across patient groups with Kruskal-Wallis test and Dunn's post-test for multiple comparisons. For *in vitro* experiments, activation marker expression was presented as fold change MFI relative to baseline and defined as stimulation when the median of replicates was significantly different to 1.0 (Wilcoxon signed-rank test). Paired data was compared by paired t-test. For correlations Spearman's r test was used. A two-sided 5% level of significance was used, without multiple testing adjustment. GraphPad Prism® was used.

Results

Increased activation of primary hHSC by sera from PLWH with HBV coinfection compared to HBV without HIV or individuals not diagnosed with HIV or HBV.

We first aimed to determine if there was a circulating factor in PLWH with HBV co-infection which could directly stimulate activation of hHSC. We compared the response of hHSC to incubation with serum from healthy volunteers, people living with HBV without HIV and with

HIV-HBV, using flow cytometry to quantify response at the single cell level (representative gating strategy **Figure 1A**). There was no difference in proportion of live cells between groups (Supplementary Figure 1A, <http://links.lww.com/QAD/C719>). Stimulation with serum from PLWH with HBV coinfection led to increased expression of multiple hHSC activation markers isolated from two independent donor livers (data depicts median activation levels relative to expression after incubation with media alone), including ICAM-1, α -SMA, CCL2, TIMP-1, IL-8 and Ki67 (**Figure 1B** and **1C**, Wilcoxon rank sum test, Supplementary Figure 1B, <http://links.lww.com/QAD/C719>), reflecting diverse functionality of activated hHSC, including inflammatory and leukocyte chemoattraction and adhesion (ICAM1, CCL2, IL-8), proliferation (Ki67), contractility and fibrogenesis (α -SMA) and inhibition of matrix degradation (TIMP-1)^[36]. Responses of hHSCs (both donors) to stimulation with serum from the three participant groups were significantly different for ICAM-1, α -SMA, CCL2, IL-8, TIMP-1 and Ki67 (Kruskal-Wallis, $p < 0.05$). Higher expression after stimulation with serum from HIV-HBV was seen for all markers, and was statistically significant for ICAM1, α -SMA and CCL2 compared to both HBV and HC sera when tested against batches of hHSC from two different donors (and significantly higher than stimulation with HC sera for all markers in both hHSC donors) (Figure 1B and 1C, Supplementary Figure 1B, <http://links.lww.com/QAD/C719>, Dunn's post-test).

In contrast to consistent changes with sera from PLWH with HBV coinfection, sera from people living with HBV without HIV, or healthy controls (HC), did not result in changes in activation/profibrogenic markers that were preserved between both batches of hHSC (Figure 1, Supplementary Figure 1B, <http://links.lww.com/QAD/C719>).

LPS and HIV gp120, but not HBV proteins, activate hHSC

To investigate viral/microbial factors that could be elevated in the serum of PLWH with HBV coinfection and have potential pro-fibrogenic effects, we stimulated hHSC with microbial products, HIV or HBV proteins (**Figure 2A**). LPS-EB, Pam3CSK4 and HIV gp120 led to increased proliferation (Ki67), fibrogenesis and contractility (α -SMA), mitogenesis and chemotaxis to areas of injury (PDGFR- β) and pro-inflammatory (CCL2, ICAM1) activation of hHSC compared to media alone (**Figure 2B**). Similar findings were observed when assessing activation of hepatic stellate cell line, LX2 (Supplementary Figure 2A, <http://links.lww.com/QAD/C719>).

We stimulated hHSC with HBV viral products using conditioned media from HBV-replicating cell lines or recombinant HBsAg. Unexpectedly, hepatocyte cell conditioned media from both uninfected and HBV-infected hepatoma lines induced hHSC activation (**Figure 2C**). However, no significant augmentation of this was seen using media from the HBV-replicating HepG2.2.15 cell line (**Figure 2C**), in which HBV replication was confirmed by immunofluorescence staining for HBcAg (**Figure 2D**). Supporting the lack of specific effect on hHSC activation by HBV, purified recombinant HBsAg and HBcAg failed to induce hHSC activation when added to hHSC cultures (Supplementary Figure 2B, <http://links.lww.com/QAD/C719>).

Dead/apoptotic hepatocytes activate hHSC in a HMGB1-dependent manner

Given we had previously showed elevated intra-hepatic markers of apoptosis in liver biopsies from PLWH with HBV coinfection compared to HBV alone^[29] and because supernatants from HBV-infected and uninfected hepatocyte cell lines *both* activated hHSC, we sought to determine if hHSC could be activated by apoptotic/dead hepatocytes. To examine the pro-fibrogenic effect of hepatocyte apoptosis/death, we treated HepG2 and HepG2.2.15 cells with sKT® for 30 minutes (**Figure 3A**). HepG2 expressed TRAIL death receptors TR2 and TR4 (Supplementary Figure 3A, <http://links.lww.com/QAD/C719>) and were therefore susceptible to TRAIL-induced apoptosis (anti-caspase-3) and death (live/dead stain) (Supplementary Figure 3B, <http://links.lww.com/QAD/C719>). Staining revealed some HepG2 death at baseline (consistent with triggering of pro-fibrogenic changes noted above) and a marked increase in dead and apoptotic cells following short-term incubation with sKT® (Supplementary Figure 3B). We found a significant increase in markers of hHSC activation including pro-inflammatory (ICAM-1, CCL2), contractility and fibrogenesis (α -SMA), chemotaxis/proliferation (PDGFR- β) and proliferation (Ki67) after stimulation with conditioned media from both HepG2 and HepG2.2.15 cells exposed to sKT®, compared to untreated HepG2/HepG2.2.15 cells (**Figure 3B**). Similar findings were noted with LX2s (Supplementary Figure 3C, <http://links.lww.com/QAD/C719>).

We next evaluated the possibility that pro-fibrogenic activation of hHSC with supernatants from dead/apoptotic hepatocytes may be related to damage associated molecular protein(s) (DAMP), also known as ‘alarmins’. HMGB1 has been shown to be actively secreted during cellular stress and passively released upon cell death^[37], binding to RAGE on hepatic stellate cells to drive pro-fibrogenic changes *in-vitro* and *in-vivo*^[38, 39]. To confirm this, we added recombinant HMGB1 to primary hHSC and found a small but consistent increase in hHSC activation with increasing dose (**Figure 3C**). No synergism was seen in combination with LPS (**Figure 3C**).

We then tested if Glycyrrhizin, a well-defined HMGB1 antagonist^[40-43], could inhibit the pro-fibrogenic effect of dead/apoptotic hepatocyte-conditioned media. Glycyrrhizin abrogated the augmented activation of primary hHSC resulting from stimulation with sKT®-treated hepatocyte conditioned media (**Figure 3D**).

HMGB1 levels are significantly elevated in sera from PLWH with HBV coinfection, and are higher in those with fibrosis

We examined whether HMGB1 might be a mediator increased in serum in HIV-HBV coinfection, in line with the increase in hepatocyte apoptosis. Sera were analysed for HMGB1 by ELISA. We found increased HMGB1 levels in sera of PLWH with HBV coinfection compared with either HBV alone or without HIV or HBV ($p < 0.001$, $p < 0.0001$ (Dunn’s post-test) respectively, $p < 0.001$, Kruskal-Wallis) (**Figure 3E**). HMGB1 levels were higher amongst PLWH with HBV coinfection with greater than stage 0 fibrosis on liver biopsy compared with those without ($p = 0.04$, Dunn’s post-test, $p < 0.001$, Kruskal-Wallis), (**Figure 3F**). HMGB1

levels were also higher amongst PLWH with HBV coinfection with fibrosis compared with people living with HBV without HIV with fibrosis ($p=0.0063$, Dunn's post-test) (**Figure 3F**).

HMGB1 levels were not associated with gender, age, HBeAg (Supplementary Figure 4A-C, <http://links.lww.com/QAD/C719>) serum ALT or HBV viral load (Supplementary Figure 4D-E, <http://links.lww.com/QAD/C719>). Taken together, our data reveal that an alarmin capable of mediating pro-fibrogenic changes in hHSC resulting from damaged hepatocytes, is elevated in the serum of PLWH with HBV coinfection, and that levels are associated with liver disease severity in PLWH with HBV coinfection.

Discussion

We found that serum from PLWH with HBV coinfection not on ART led to greater activation of hHSC than sera from age-matched untreated people with HBV without HIV as well as HC.

We investigated these findings *in vitro* using hHSC and the LX2 cell line and demonstrate by flow cytometry an increase in multiple markers of HSC activation with HIV gp120 and LPS, extending previous studies in which gp120 increased markers of hHSC activation measured from bulk populations using ELISA, qRT-PCR or Western blot^[44, 45]. The potential relevance of gp120 as a pro-fibrogenic stimulus is supported by its detection within liver sinusoids in livers from individuals with HIV-HCV^[45].

We demonstrated an increase in pro-fibrogenic and inflammatory markers of hHSC activation following exposure to conditioned media from dying/apoptotic hepatocytes, which was abrogated by addition of HMGB1 antagonist glycyrrhizic acid. We found that serum HMGB1 was higher in PLWH with HBV coinfection compared to HBV without HIV or HC and was higher in PLWH with HBV coinfection with fibrosis compared to those without. Together these data are consistent with a model whereby increased hepatocyte cell death in HIV-HBV coinfection can drive HSC activation via HMGB1. Although our model detected a relatively modest effect of HMGB1 on HSC, occurring at high concentrations compared to serum levels, in the hepatic microenvironment, HMGB1 levels may likely be higher. Furthermore, HMGB1 may activate hHSC in combination with factors such as products of microbial translocation and HIV gp120^[14, 45].

HMGB1 is a nuclear protein that acts as a structural chromatin binding factor, associated with cellular DNA^[46]. It is passively released by necrotic or damaged cells and actively secreted by activated monocytes and macrophages after translocation into the cytoplasm during cellular stress^[39]. It binds with high affinity to the receptor for advanced glycation end products (RAGE) on hHSC, increasing collagen-I deposition^[38, 39]. In hepatocytes and Kupffer cells, HMGB1 signalling through TLR2/4/9 mediates pro-inflammatory activity in the liver in a mouse model and was abrogated by the HMGB1 inhibitor glycyrrhetinic acid^[47]. hHSC express TLRs, especially TLR4 and evidence of their direct activation by HMGB1 and inhibition with blocking agents including glycyrrhizin-related substances has been reported^[48-50].

A role for HMGB1 is emerging in the pathogenesis of liver disease of various etiologies including alcohol-related, HCV, NAFLD and schistosomiasis [51-53]. In people living with HCV, liver HMGB1 correlated with fibrosis stage [39]. HMGB1 is elevated in serum of PLWH monoinfection^[54, 55] or people living with HBV^[55, 56], however HMGB1 levels in HBV have not been consistently associated with fibrosis or liver damage^[56, 57]. HMGB1 played a key role in intrahepatic recruitment of inflammatory cells into the liver in a transgenic mouse model, leading to amplification of cytotoxic T cell associated necroinflammation, that was attenuated by glycyrrhizin^[58]. Both glycyrrhizinic/glycyrrhetinic acid are obtained from extracts of liquorice and commonly taken in herbal preparations for liver diseases including hepatitis in Asia, especially Japan. [41]

We propose that hepatocyte cell death and HMGB1 are important drivers of HSC activation, and therefore liver disease, in HIV-HBV co-infection. First, in a case-controlled observational study we demonstrated hepatocyte cell death (using dUTP nick-end labelling (TUNEL)) was increased in PLWH with HBV coinfection compared with those with HBV without HBV^[29]. Second, others demonstrated that HIV (and gp120) may contribute to increased hepatocyte death^[31, 59, 60]. We now demonstrate that HMGB1 is elevated in HIV-HBV co-infection compared to HBV mono-infection or HC and activation of primary hHSC by dying HBV-replicating hepatocyte cell line supernatants is HMGB1 dependent.

A limitation was that participants were unmatched and differed in proportion who were HBeAg positive, and HBV viral load was higher in HIV-HBV co-infection, although none of these were identified as relevant mechanistic co-factors. We did not test sera from people with HIV only and therefore are unable to determine whether increased pro-fibrotic changes in HIV-HBV are due to HIV infection or to an interaction between HIV and HBV. We used apoptotic hepatoma cell lines rather than primary hepatocytes as an experimental source of HMGB1. The death of non-hepatocyte cells may also contribute to higher circulating HMGB1 in HIV-HBV compared to HBV mono-infection, with a potential contribution from ongoing high rates of CD4⁺ T cell turnover in untreated HIV infection^[61]. Increased HMGB1 levels associated with products of microbial translocation may be important in HIV-infection. LPS stimulates HMGB1 release from macrophages and hepatocytes via TLR4 dependent signalling pathways^[62]. In sepsis with high levels of LPS, hepatocytes are the main source of circulating HMGB1^[63]. The source of HMGB1 in HIV or HIV-HBV co-infection remains unclear and warrants further examination. Glycyrrhizin blocks the effects of HMGB1, however also has a wide range of antioxidant and anti-inflammatory actions that may be relevant [40-43, 64]. Future studies with additional HMGB1 blockers or knockdown are needed to consolidate our findings regarding the mechanistic link between serum elevation of alarmin HMGB-1 and the capacity of blockade to abrogate, and recombinant HMGB-1 to recapitulate, its profibrogenic effects.

The major strength of this study was that we assessed primary hHSC as well as a stellate cell line thoroughly at the single cell level with a range of output markers and linked findings with mechanistic experiments targeting HMGB1 as well as with changes observed directly from serum of PLWH with HBV coinfection.

In conclusion, we demonstrated that serum from PLWH with HBV coinfection led to greater activation of hHSCs than that from people with HBV and HC, and could be partially recapitulated by HIV gp120, LPS and HMGB1. We found that conditioned media from hepatoma cell lines with enhanced cell death increased hHSC activation and that this could be attenuated with addition of glycyrrhizic acid, an HMGB1 inhibitor. HMGB1 levels were significantly greater in PLWH with HBV coinfection compared with age-matched people with HBV without HIV and HC and importantly, were higher in those with fibrosis. Our findings are consistent with a model that dead and dying cells in PLWH with HBV coinfection may contribute to liver disease pathogenesis through HSC activation, and this is mediated by the alarmin HMGB1.

Abbreviations

Hepatitis B (HBV), primary human hepatic stellate cells (hHSC), human immunodeficiency virus (HIV) and hepatitis B virus (HIV-HBV), Antiretroviral therapy (ART), hepatocellular carcinoma (HCC), α -smooth muscle actin (α -SMA), tissue inhibitor matrix metalloproteinase-1 (TIMP-1), lipopolysaccharide (LPS-EB) toll like receptor (TLR)-4, Pam3Cys-SerLys4 (Pam3CSK4), HIV-hepatitis C (HIV-HCV), tissue necrosis factor (TNF) receptor apoptosis inducing ligand (TRAIL), HBV surface antigen (HBsAg), SuperKiller TRAIL™ (sKT®), high mobility group box 1 protein (HMGB1), complementary DNA (cDNA) of pregenomic (pg) RNA, fetal calf serum (FCS), intercellular adhesion molecule (ICAM)1, C-C motif chemokine ligand 2 (CCL2).

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Figure 1: Stimulation with serum from PLWH with HBV coinfection results in increased activation of primary human hepatic stellate cells. **a)** Representative sequential gating strategy to identify primary human hepatic stellate cells (hHSC) by flow cytometry, identifying cells based on forward (FSC-A) and side scatter (SSC-A), followed by the exclusion of dead cells and assessment of protein expression for each individual marker (e.g. α -SMA) in comparison to a matched isotype control. Representative histogram plots **b)** and summary data **c)** depicting the fold increase in protein expression for a panel of markers on primary hHSC isolated from resected liver tissue, after stimulation with serum from healthy volunteers (grey; n=29), people living with HBV (blue; n=29) and PLWH with HBV coinfection (red; n=32; mean fluorescence intensity; MFI). hHSC activation was assessed by expression of markers of activation relative to incubation with media alone (markers assessed: ICAM-1, α -SMA, CCL2, TIMP-1, IL-8 and Ki67). Each dot represents a serum sample used, bars represent mean and error bars represent \pm S.E.M. *p*-values were determined using Kruskal-Wallis test (ANOVA) with Dunn's multiple comparisons test marked with asterisks where significant; **p*<0.05; ***p*<0.01. Human hepatic stellate cells (hHSC); forward scatter (FSC-A) and side scatter (SSC-A); C-C motif chemokine ligand 2 (CCL2); intercellular adhesion molecule 1 (ICAM-1); alpha smooth muscle actin (α -SMA).

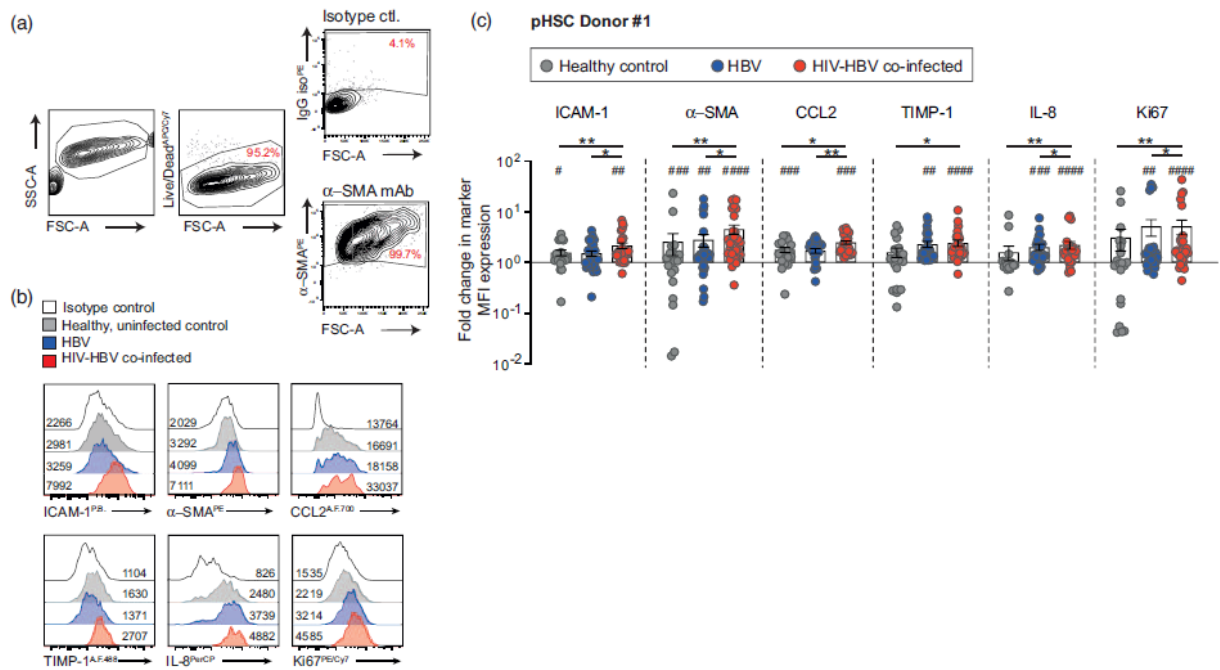


Figure 2: HIV gp120 and products of microbial translocation activate human hepatic stellate cells. **a)** Schematic depicting the experimental model. Primary hHSC were cultured to ~80% confluence prior to stimulation using technical replicates/condition for multiple, independent hHSC donors (n=3-8). Primary hHSC were stimulated with **b)** LPS-EB, Pam3CSK4, or HIV gp120 at two doses (1-10 μ M or 500-1000 μ M) and **c)** LPS-EB, Pam3CSK4 or hepatoma cell line-conditioned media (HepG2 or HepG2.2.15), analysed by flow cytometry for markers of hHSC activation (PDGFR- β , CCL2, ICAM-1, α -SMA and Ki67). Each dot represents a study participant; bars represent mean and error bars represent \pm S.E.M. *p*-values were determined using a two-tailed Wilcoxon *t* Test and marked with asterisks where significant; **p*<0.05; ***p*<0.01. **d)** Fluorescence microscopy of HepG2.2.15 cell line stained with anti-HBcAg antibodies, i. Superimposed images (ii.) and (iii). (nuclear staining and anti-HBcAb) ii DAPI (nuclear) in blue indicating cells, iii. HBcAg in yellow/green. Human hepatic stellate cells (HSC); lipopolysaccharide (LPS-EB); Pam3CysSerLys4 (Pam3CSK4); HIV envelope glycoprotein (gp)120; platelet-derived growth factor receptor beta (PDGFR- β); C-C motif chemokine ligand 2 (CCL2); intercellular adhesion molecule 1 (ICAM-1); alpha smooth muscle actin (α -SMA).

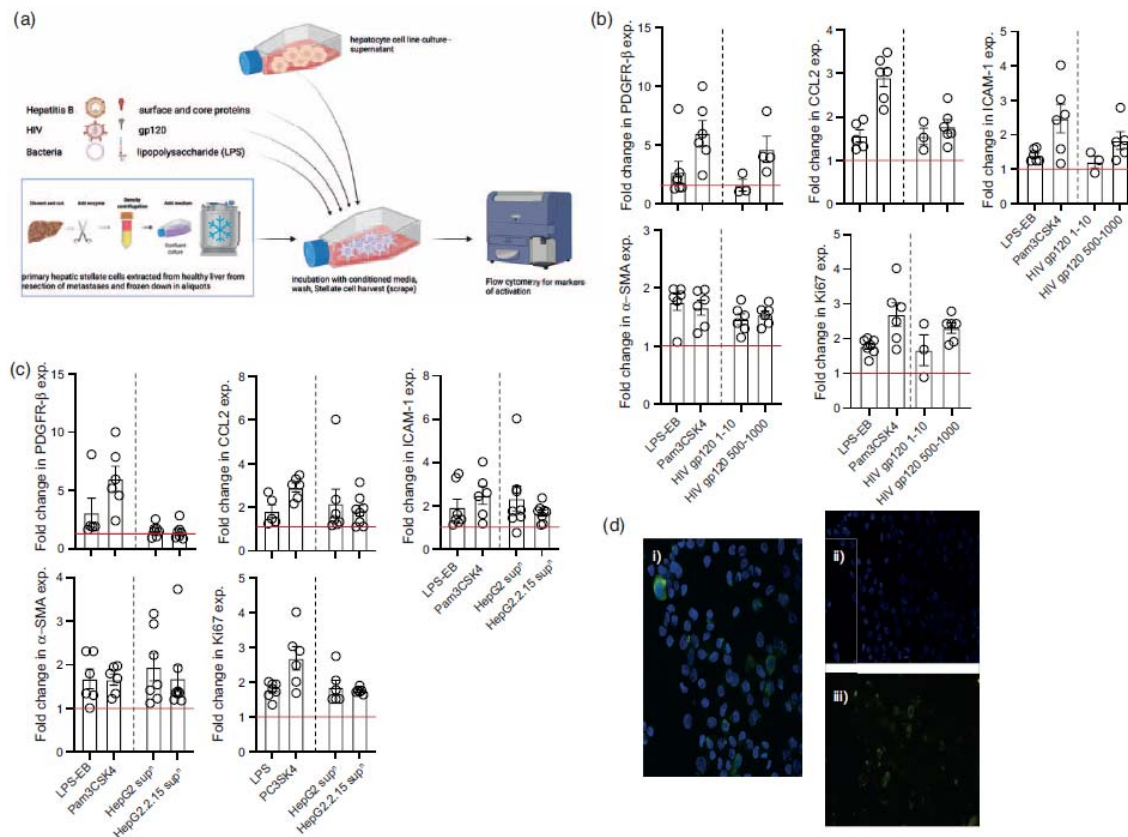


Figure 3: Dead/apoptotic hepatocytes activate human hepatic stellate cells in an HMGB1-dependent manner. **a)** Schematic depicting the experimental model. hHSC were cultured to ~80% confluence prior to stimulation. Stimulation experiments were carried out using technical replicates for multiple, independent hHSC donors (n=8). Primary hHSC were stimulated with **b)** hepatoma cell-line derived media (HepG2 or HepG2.2.15) cultured in the presence or absence of superkillerTRAIL (sKT®) or vehicle control (DMSO) and analysed by flow cytometry, **c)** in the presence of increasing concentrations of recombinant human high mobility group box 1 protein (HMGB1), and with HMGB1 (100ng/mL) in combination with LPS-EB (50ng/mL and **d)** the addition of Glycyrrhizin (n=2) compared to media alone, analysed by flow cytometry for markers of hHSC activation (ICAM-1, CCL2, α -SMA, PDGFR- β , and Ki67). **e)** Summary data depicting the serum concentration of HMGB1 (as measured by ELISA) for independent cohorts of healthy controls (n=12), people living with HBV without HIV (n=28) and PLWH with HBV coinfection (n=30). **f)** HMGB1 levels by liver fibrosis stage (Metavir staging on liver biopsy) in HIV-HBV and HBV. Each dot represents a study participant, bars represent mean and error bars represent \pm S.E.M. *p*-values were determined using a two-tailed Wilcoxon *t* Test or a Kruskal-Wallis test (ANOVA) with Dunn's multiple comparisons test marked with asterisks where significant; **p*<0.05; ***p*<0.01, ****p*<0.001, *****p*<0.0001. Human hepatic stellate cells (hHSC); superkillerTRAIL (tumour necrosis factor (TNF)-related apoptosis-inducing ligand; sKT®); high mobility group box 1 protein (HMGB1), platelet-derived growth factor receptor beta (PDGFR- β); C-C motif chemokine ligand 2 (CCL2); intercellular adhesion molecule 1 (ICAM-1); alpha smooth muscle actin (α -SMA).

