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 (-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).

ACCEPTED

Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

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 co-infection, 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

ACCEPTED

Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

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 co-infection 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].

ACCEPTED

Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

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.

ACCEPTED

Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

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).

Acknowledgements

We are very grateful to all study participants and staff who assisted with sample collection.

ACCEPTED

References

1. Singh KP, Crane M, Audsley J, Avihingsanon A, Sasadeusz J, Lewin SR. **HIV-hepatitis B virus coinfection: epidemiology, pathogenesis, and treatment**. *Aids* 2017; 31(15):2035-2052.

2. Tsai WC, Hsu WT, Liu WD, Sun HY, Chuang YC, Huang YS, et al. **Impact of antiretroviral therapy containing tenofovir disoproxil fumarate on the survival of patients with HBV and HIV coinfection**. *Liver Int* 2019; 39(8):1408-1417.

3. van Welzen BJ, Smit C, Boyd A, Lieveld FI, Mudrikova T, Reiss P, et al. **Decreased All-Cause and Liver-Related Mortality Risk in HIV/Hepatitis B Virus Coinfection**

Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

Coinciding With the Introduction of Tenofovir-Containing Combination Antiretroviral Therapy. *Open Forum Infect Dis* 2020; 7(7):ofaa226.

4. Kouame GM, Boyd A, Moh R, Badje A, Gabillard D, Ouattara E, et al. **Higher Mortality Despite Early Antiretroviral Therapy in Human Immunodeficiency Virus and Hepatitis B Virus (HBV)-Coinfected Patients With High HBV Replication**. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2018; 66(1):112-120.

5. Dezanet LNC, Miailhes P, Lascoux-Combe C, Chas J, Maylin S, Gabassi A, et al. **Profiles of liver fibrosis evolution during long-term tenofovir treatment in HIV-positive patients coinfected with hepatitis B**. *Liver Int* 2021.

6. Sterling RK, King WC, Khalili M, Chung RT, Sulkowski M, Jain MK, et al. **A Prospective Study Evaluating Changes in Histology, Clinical and Virologic Outcomes in HBV-HIV Co-infected Adults in North America**. *Hepatology (Baltimore, Md)* 2021; 74(3):1174-1189.

7. Mederacke I, Hsu CC, Troeger JS, Huebener P, Mu X, Dapito DH, et al. **Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology**. *Nat Commun* 2013; 4:2823.

8. Kisseleva T, Brenner D. **Molecular and cellular mechanisms of liver fibrosis and its regression**. *Nat Rev Gastroenterol Hepatol* 2021; 18(3):151-166.

9. Tsuchida T, Friedman SL. **Mechanisms of hepatic stellate cell activation**. *Nat Rev Gastroenterol Hepatol* 2017; 14(7):397-411.

10. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, et al. **Microbial translocation is a cause of systemic immune activation in chronic HIV infection**. *Nature medicine* 2006; 12(12):1365-1371.

11. Sandler NG, Douek DC. **Microbial translocation in HIV infection: causes, consequences and treatment opportunities**. *Nat Rev Microbiol* 2012; 10(9):655-666.

12. Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nat Med 2007; 13(11):1324-1332.

13. Paik YH, Schwabe RF, Bataller R, Russo MP, Jobin C, Brenner DA. Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. Hepatology (Baltimore, Md) 2003; 37(5):1043-1055.

14. Aoyama T, Paik YH, Seki E. Toll-like receptor signaling and liver fibrosis. Gastroenterol Res Pract 2010; 2010.

ACCEPTED

Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

15. Sandler NG, Koh C, Roque A, Eccleston JL, Siegel RB, Demino M, et al. Host response to translocated microbial products predicts outcomes of patients with HBV or HCV infection. Gastroenterology 2011; 141(4):1220-1230, 1230 e1221-1223.

16. Getachew A, Abbas N, You K, Yang Z, Hussain M, Huang X, et al. SAA1/TLR2 axis directs chemotactic migration of hepatic stellate cells responding to injury. iScience 2021; 24(5):102483.

17. Medrano LM, Garcia-Broncano P, Berenguer J, Gonzalez-Garcia J, Jimenez-Sousa MA, Guardiola JM, et al. Elevated liver stiffness is linked to increased biomarkers of inflammation and immune activation in HIV/hepatitis C virus-coinfected patients. Aids 2018; 32(9):1095-1105.

18. Kooij KW, Wit FW, van Zoest RA, Schouten J, Kootstra NA, van Vugt M, et al. Liver fibrosis in HIV-infected individuals on long-term antiretroviral therapy: associated with immune activation, immunodeficiency and prior use of didanosine. Aids 2016; 30(11):1771-1780.

19. Marquez M, Romero-Cores P, Montes-Oca M, Martin-Aspas A, Soto-Cardenas MJ, Guerrero F, et al. Immune activation response in chronic HIV-infected patients: influence of Hepatitis C virus coinfection. PLoS One 2015; 10(3):e0119568.

20. Sacchi P, Cima S, Corbella M, Comolli G, Chiesa A, Baldanti F, et al. Liver fibrosis, microbial translocation and immune activation markers in HIV and HCV infections and in HIV/HCV co-infection. Dig Liver Dis 2015; 47(3):218-225.

21. Singh K, Zerbato J, Zhao W, Baat S, Deleage C, Tennakoon S, et al. Intrahepatic CXCL10 is strongly associated with liver fibrosis in HIV-Hepatitis B co-infection. PLoS pathogens 2020; Accepted for publication.

22. Cao YZ, Dieterich D, Thomas PA, Huang YX, Mirabile M, Ho DD. Identification and quantitation of HIV-1 in the liver of patients with AIDS. Aids 1992; 6(1):65-70.

23. Iser DM, Warner N, Revill PA, Solomon A, Wightman F, Saleh S, et al. Coinfection of hepatic cell lines with human immunodeficiency virus and hepatitis B virus leads to an increase in intracellular hepatitis B surface antigen. Journal of virology 2010; 84(12):5860-5867.

24. Hufert FT, Schmitz J, Schreiber M, Schmitz H, Racz P, von Laer DD. Human Kupffer cells infected with HIV-1 in vivo. Journal of acquired immune deficiency syndromes 1993; 6(7):772-777.

25. Tuyama AC, Hong F, Saiman Y, Wang C, Ozkok D, Mosoian A, et al. Human immunodeficiency virus (HIV)-1 infects human hepatic stellate cells and promotes collagen I and monocyte chemoattractant protein-1 expression: implications for the

ACCEPTED

Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

pathogenesis of HIV/hepatitis C virus-induced liver fibrosis. Hepatology (Baltimore, Md) 2010; 52(2):612-622.

26. Crane M, Oliver B, Matthews G, Avihingsanon A, Ubolyam S, Markovska V, et al. Immunopathogenesis of hepatic flare in HIV/hepatitis B virus (HBV)-coinfected individuals af**ter the initiation of HBV-active antiretroviral therapy**. *J Infect Dis* 2009; 199(7):974-981.

27. Bruno R, Galastri S, Sacchi P, Cima S, Caligiuri A, DeFranco R, et al. gp120 modulates the biology of human hepatic stellate cells: a link between HIV infection and liver fibrogenesis. Gut 2010; 59(4):513-520.

28. Salloum S, Holmes JA, Jindal R, Bale SS, Brisac C, Alatrakchi N, et al. Exposure to human immunodeficiency virus/hepatitis C virus in hepatic and stellate cell lines reveals cooperative profibrotic transcriptional activation between viruses and cell types. Hepatology (Baltimore, Md) 2016.

29. Iser DM, Avihingsanon A, Wisedopas N, Thompson AJ, Boyd A, Matthews GV, et al. Increased intrahepatic apoptosis but reduced immune activation in HIV-HBV co-infected patients with advanced immunosuppression. Aids 2011; 25(2):197-205.

30. Macias J, Japón MA, Sáez C, Palacios RB, Mira JA, García-García JA, et al. Increased hepatocyte fas expression and apoptosis in HIV and hepatitis C virus coinfection. J Infect Dis 2005; 192(9):1566-1576.

31. Babu CK, Suwansrinon K, Bren GD, Badley AD, Rizza SA. HIV induces TRAIL sensitivity in hepatocytes. PLoS One 2009; 4(2):e4623.

32. Vlahakis SR, Villasis-Keever A., Gomez Timothy S., Bren, Gary D., Paya Carlos V. Human Immunodeficiency Virus-Induced Apoptosis of Human Hepatocytes via CXCR4. Journal of Infectious Diseases 2003; 188:6.

33. Bedossa P, Poynard T, group M. An algorithm for the grading of activity in chronic hepatitis. Hepatology (Baltimore, Md) 1996; 24:289 - 293.

34. Cossarizza A, Chang HD, Radbruch A, Abrignani S, Addo R, Akdis M, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). Eur J Immunol 2021; 51(12):2708-3145.

35. Ladner SK, Otto MJ, Barker CS, Zaifert K, Wang GH, Guo JT, et al. Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. Antimicrob Agents and Chemotherapy 1997; 41(8):1715-1720.

36. Tacke F, Weiskirchen R. Update on hepatic stellate cells: pathogenic role in liver fibrosis

ACCEPTED

Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

and novel isolation techniques. Expert Rev Gastroenterol Hepatol 2012; 6(1):67-80.

37. Gaskell H, Ge X, Nieto N. High-Mobility Group Box-1 and Liver Disease. Hepatol Commun 2018; 2(9):1005-1020.

38. Ge X, Arriazu E, Magdaleno F, Antoine DJ, Dela Cruz R, Theise N, et al. High Mobility Group Box-1 Drives Fibrosis Progression Signaling via the Receptor for Advanced Glycation End Products in Mice. Hepatology (Baltimore, Md) 2018; 68(6):2380-2404.

39. Arriazu E, Ge X, Leung TM, Magdaleno F, Lopategi A, Lu Y, et al. Signalling via the osteopontin and high mobility group box-1 axis drives the fibrogenic response to liver injury. Gut 2017; 66(6):1123-1137.

40. Gwak GY, Moon TG, Lee DH, Yoo BC. Glycyrrhizin attenuates HMGB1-induced hepatocyte apoptosis by inhibiting the p38-dependent mitochondrial pathway. World J Gastroenterol 2012; 18(7):679-684.

41. Mollica L, De Marchis F, Spitaleri A, Dallacosta C, Pennacchini D, Zamai M, et al. Glycyrrhizin binds to high-mobility group box 1 protein and inhibits its cytokine activities. Chem Biol 2007; 14(4):431-441.

42. Girard JP. A direct inhibitor of HMGB1 cytokine. Chem Biol 2007; 14(4):345-347.

43. Albayrak A, Uyanik MH, Cerrah S, Altas S, Dursun H, Demir M, et al. Is HMGB1 a new indirect marker for revealing fibrosis in chronic hepatitis and a new therapeutic target in treatment? Viral immunology 2010; 23(6):633-638.

44. Del Corno M, Cappon A, Donninelli G, Varano B, Marra F, Gessani S. HIV-1 gp120 signaling through TLR4 modulates innate immune activation in human macrophages and the biology of hepatic stellate cells. J Leukoc Biol 2016; 100(3):599-606.

45. Hong F, Saiman Y, Si C, Mosoian A, Bansal MB. X4 Human immunodeficiency virus type 1 gp120 promotes human hepatic stellate cell activation and collagen I expression through interactions with CXCR4. PLoS One 2012; 7(3):e33659.

46. Yang H, Wang H, Chavan SS, Andersson U. High Mobility Group Box Protein 1 (HMGB1): The Prototypical Endogenous Danger Molecule. Mol Med 2015; 21 Suppl 1:S6-S12.

47. Shi X, Yu L, Zhang Y, Liu Z, Zhang H, Liu P, et al. Glycyrrhetinic acid alleviates hepatic inflammation injury in viral hepatitis disease via a HMGB1-TLR4 signaling pathway. Int Immunopharmacol 2020; 84:106578.

48. Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D, et al. High mobility group box 1 protein interacts with multiple Toll-like receptors. Am J

ACCEPTED

Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

Physiol Cell P*hysiol* 2006; 290(3):C917-924.

49. Zhang Z, Lin C, Peng L, Ouyang Y, Cao Y, Wang J, et al. High mobility group box 1 activates Toll like receptor 4 signaling in hepatic stellate cells. Life Sci 2012; 91(5-6):207-212.

50. He Q, Fu Y, Ding X, Li D, Wang Z, Tian D, et al. Correction: High-mobility group box 1 induces endoplasmic reticulum stress and activates hepatic stellate cells. Lab Invest 2019; 99(3):439-442.

51. Ni YA, Chen H, Nie H, Zheng B, Gong Q. HMGB1: An overview of its roles in the pathogenesis of liver disease. J Leukoc Biol 2021; 110(5):987-998.

52. Vannier AGL, Wardwell B, Fomin V, PeBenito A, Wolczynski N, Piaker S, et al. Serum HMGB1 associates with liver disease and predicts readmission and mortality in patients with alcohol use disorder. Alcohol 2021; 95:37-43.

53. Zhong H, Gui X, Hou L, Lv R, Jin Y. From Inflammation to Fibrosis: Novel Insights into the Roles of High Mobility Group Protein Box 1 in Schistosome-Induced Liver Damage. Pathogens 2022; 11(3).

54. Nowak P, Barqasho B, Sonnerborg A. Elevated plasma levels of high mobility group box protein 1 in patients with HIV-1 infection. Aids 2007; 21(7):869-871.

55. Li J, Wang FP, She WM, Yang CQ, Li L, Tu CT, et al. Enhanced high-mobility group box 1 (HMGB1) modulates regulatory T cells (Treg)/T helper 17 (Th17) balance via toll-like receptor (TLR)-4-interleukin (IL)-6 pathway in patients with chronic hepatitis B. Journal of viral hepatitis 2014; 21(2):129-140.

56. Inkaya AC, Demir NA, Kolgelier S, Sumer S, Demir LS, Ural O, et al. Is serum high-mobility group box 1 (HMGB-1) level correlated with liver fibrosis in chronic hepatitis B? Medicine (Baltimore) 2017; 96(36):e7547.

57. Wang LW, Chen H, Gong ZJ. High mobility group box-1 protein inhibits regulatory T cell immune activity in liver failure in patients with chronic hepatitis B. Hepatobiliary Pancreat Dis Int 2010; 9(5):499-507.

58. Sitia G, Iannacone M, Müller S, Bianchi ME, Guidotti LG. Treatment with HMGB1 inhibitors diminishes CTL-induced liver disease in HBV transgenic mice. J Leukoc Biol 2007; 81(1):100-107.

59. Vlahakis SR, Villasis-Keever A, Gomez TS, Bren GD, Paya CV. Human immunodeficiency virus-induced apoptosis of human hepatocytes via CXCR4. J Infect Dis 2003; 188(10):1455-1460.

ACCEPTED

Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

60. Balasubramanian A, Ganju RK, Groopman JE. Hepatitis C virus and HIV envelope proteins collaboratively mediate interleukin-8 secretion through activation of p38 MAP kinase and SHP2 in hepatocytes. J Biol Chem 2003; 278(37):35755-35766.

61. Sachsenberg N, Perelson AS, Yerly S, Schockmel GA, Leduc D, Hirschel B, et al. Turnover of **CD4+ and CD8+ T lymphocytes in HIV-1 infection as measured by Ki-67 antigen**. *J Exp Med* 1998; 187(8):1295-1303.

62. Chen G, Li J, Ochani M, Rendon-Mitchell B, Qiang X, Susarla S, et al. Bacterial endotoxin stimulates macrophages to release HMGB1 partly through CD14- and TNF-dependent mechanisms. J Leukoc Biol 2004; 76(5):994-1001.

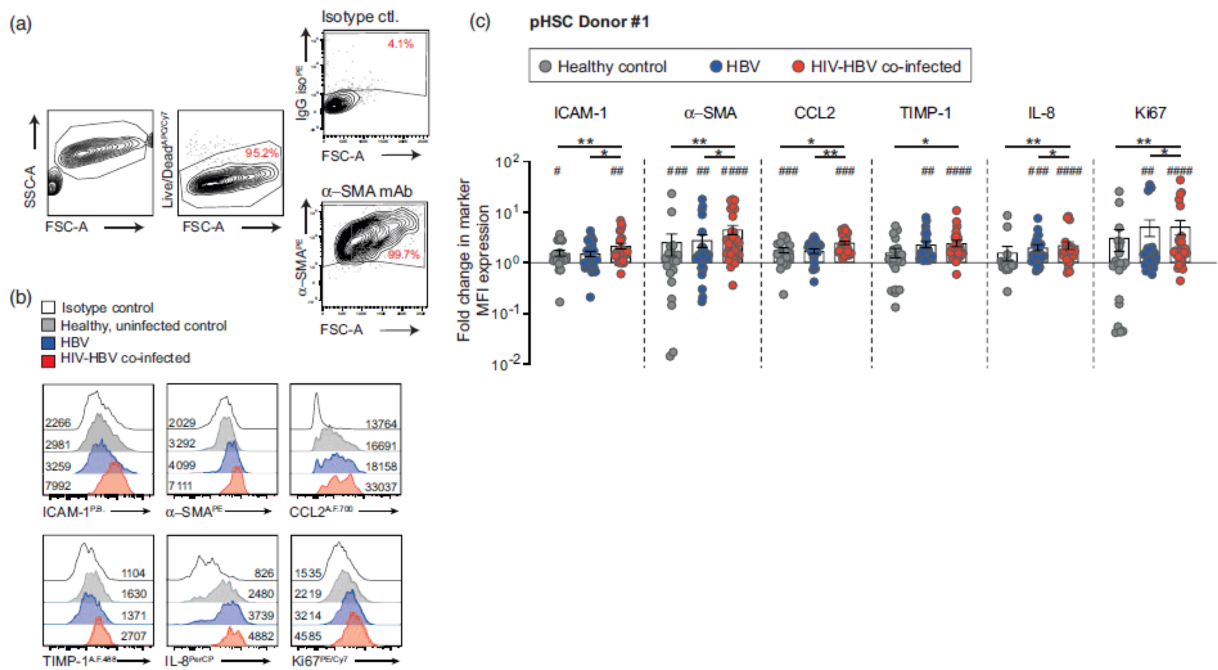
63. Deng M, Tang Y, Li W, Wang X, Zhang R, Zhang X, et al. The Endotoxin Delivery Protein HMGB1 Mediates Caspase-11-Dependent Lethality in Sepsis. Immunity 2018; 49(4):740-753.e747.

64. Leite CDS, Bonafe GA, Carvalho Santos J, Martinez CAR, Ortega MM, Ribeiro ML. The Anti-Inflammatory Properties of Licorice (Glycyrrhiza glabra)-Derived Compou**nds in Intestinal Disorders**. *Int J Mol Sci* 2022; 23(8).

ACCEPTED

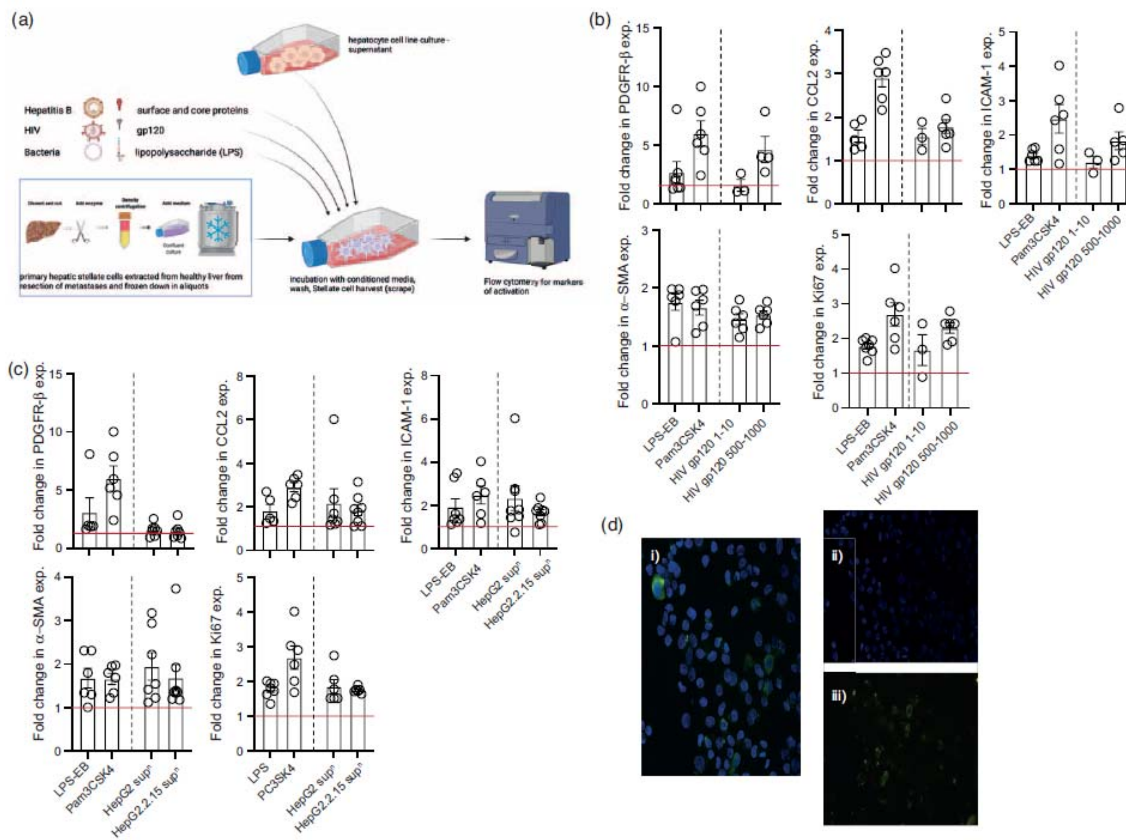
Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

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 ± 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).



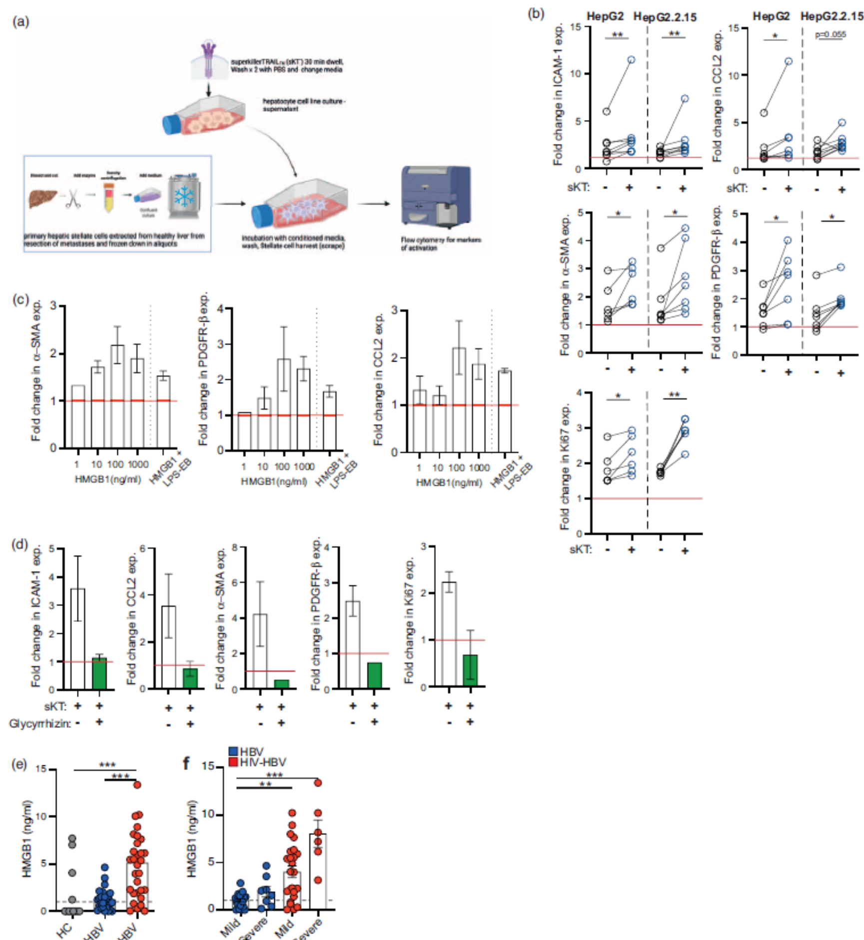
Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

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 µM or 500-1000 µM) 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, -SMA and Ki67). Each dot represents a study participant; bars represent mean and error bars represent ± 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).



Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

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 ± 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).



Copyright © 2022 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.