⌘ *Author's Choice*

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 4, pp. 2665–2674, January 28, 2011

HIV and HCV Cooperatively Promote Hepatic Fibrogenesis via Induction of Reactive Oxygen Species and NFκB*

Received for publication, July 26, 2010, and in revised form, November 19, 2010 Published, JBC Papers in Press, November 22, 2010, DOI 10.1074/jbc.M110.168286

Wenyu Lin[‡1], Guoyang Wu[‡§1], Shaoyong Li[¶], Ethan M. Weinberg[‡], Kattareeya Kumthip[‡], Lee F. Peng[‡], Jorge Méndez-Navarro[‡], Wen-Chi Chen[‡], Nikolaus Jilg[‡], Hong Zhao[‡], Kaku Goto[‡], Leiliang Zhang[‡], Mark A. Brockman[‖], Detlef Schuppan[¶], and Raymond T. Chung[‡2]

From the [‡]Gastrointestinal Unit, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114, the [§]Hepatobiliary Surgery Department, Zhongshan Hospital, Xiamen University, Fujian, P. R. of China, the [¶]Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215, and the [‖]Simon Fraser University, Burnaby, British Columbia V5A1S6, Canada

HIV/HCV coinfection leads to accelerated hepatic fibrosis progression, with higher rates of cirrhosis, liver failure, and liver death than does HCV mono-infection. However, the profibrogenic role of HIV on hepatocytes and hepatic stellate cells (HSC) has not been fully clarified. We hypothesized that HIV, HCV induce liver fibrosis through altered regulation of the production of extracellular matrix and matrix metalloproteinases. We examined the fibrogenesis- and fibrolysis-related gene activity in LX2 HSC and Huh7.5.1 cells in the presence of inactivated CXCR4 and CCR5 HIV, as well as HCV JFH1 virus. The role of reactive oxygen species (ROS) upon fibrosis gene expression was assessed using the ROS inhibitor. Fibrosis-related transcripts including procollagen α1(I) (CoL1A), TIMP1, and MMP3 mRNA were measured by qPCR. TIMP1 and MMP3 protein expression were assessed by ELISA. We found that inactivated CXCR4 HIV and CCR5 HIV increased CoL1A, and TIMP1 expression in both HSC and Huh7.5.1 cells; the addition of JFH1 HCV further increased CoL1A and TIMP1 expression. CXCR4 HIV and CCR5 HIV induced ROS production in HSC and Huh7.5.1 cells which was further enhanced by JFH1 HCV. The ROS inhibitor DPI abrogated HIV-and HCV-induced CoL1A and TIMP1 expression. HIV and HCV-induced CoL1A and TIMP1 expression were also blocked by NFκB siRNA. Our data provide further evidence that HIV and HCV independently regulate hepatic fibrosis progression through the generation of ROS; this regulation occurs in an NFκB-dependent fashion. Strategies to limit the viral induction of oxidative stress are warranted to inhibit fibrogenesis.

Hepatitis C virus (HCV)[3] is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. HCV and human immunodeficiency virus (HIV) infect ~180 million and 40 million people, respectively, worldwide. Among these, 5 million persons are coinfected with HIV/HCV(1–4). HCV does not have a major impact on HIV disease progression, whereas HIV accelerates HCV-related liver disease (3, 5). HIV infects human CD4 T lymphocytes, macrophages, and dendritic cells, but does not replicate in human hepatocytes. However, both HIV and its envelope gp120 protein have been demonstrated to induce cell signaling within hepatocytes, hepatic stellate cells (HSC) and other immune cells through its interaction with CCR5 or CXCR4 chemokine receptors (4, 6–8). Of note, it has been demonstrated that HIV/HCV coinfection leads to accelerated hepatic fibrosis progression, higher rates of liver failure and death compared with patients with HCV mono-infection, even despite successful control of HIV with antiretroviral therapy (9).

HIV-HCV coinfection induces a significant increase in TGF-β1, a central mediator of liver fibrogenesis, in the liver and serum of patient and in cell culture (4, 8, 10–12). We have demonstrated that HCV increases hepatocytes TGF-β1 expression in HCV replicon cells (13), and that this occurs through the enhanced production of reactive oxygen species (ROS) in HCV JFH1 cell culture (6, 14). Liver fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) components, reduction of ECM-removing matrix metalloproteinases (MMPs), and an up-regulation of tissue inhibitors of MMPs (TIMPs), mainly TIMP-1 (15). However, the profibrogenic role of HIV on uninfected and HCV-infected hepatocytes, and on HSC has not been fully explored. We hypothesize that HIV- and HCV-induced liver fibrosis by altered regulation of the production of extracellular matrix (ECM) and matrix metalloproteinases (MMP) molecules which are unbalanced via the formation of ROS. In this study, we used inactivated HIV (CXCR4 and CCR5) and HCV JFH1 to examine the impact of HIV and HCV on fibrogenesis-related gene activity in the HSC line LX2 and Huh7.5.1 hepatoma cells. We found that HIV and HCV increased the profibrogenic genes procollagen α1(I) and TIMP-1 and inhibited expression of the putatively anti-profibrogenic gene MMP-3 through the induction of ROS and activation of NFκB.

EXPERIMENTAL PROCEDURES

Cell Cultures—Huh7.5.1 human hepatoma cells (16), HCV JFH1-infected Huh7.5.1 cells (17), and HSC LX-2 (18) cells

* This work was supported, in whole or in part, by National Institutes of Health-MGH Center For Human Immunology Pilot/Feasibility Study Grant (to W. L.), and National Institutes of Health Grants AI069939 (to R. T. C.), AI082630 (to R. T. C.), and AI066313U19 (to D. S.).

⌘ *Author's Choice*—Final version full access.

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Gastrointestinal Unit, Warren 1007, MA General Hospital, Boston, MA 02114. Tel.: 617-724-7562; Fax: 617-643-0446; E-mail: rtchung@partners.org.

[3] The abbreviations used are: HCV, hepatitis C virus; CoL1A, procollagen α1(I); ROS, reactive oxygen species; MMP, matrix metalloproteinases; TIMP, tissue inhibitors of MMP; DPI, diphenyliodonium.

TABLE 1
List of primer sequences for real time PCR

Gene name	Forward primer	Reverse primer
Human GAPDH	ACAGTCCATGCCATCACTGCC	GCCTGCTTCACCACCTTCTTG
JFH1-HCV	TCTGCGGAACCGGTGAGTA	TCAGGCAGTACCACAAGGC
Human TIMP-1	TGTTGTTGCTGTGGCTGATAGC	TCTGGTGTCCCCACGAACTT
Human procollagen α1(I)	CAGCCGCTTCACCTACAGC	TCAATCACTGTCTTGCCCCA
Human MMP-3	GTTCCGCCTGTCTCAAGATGA	GGGACAGGTTCCGTGGGTA

were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) medium. The JFH1 cells used in this study were harvested at between days 4–20 post-infection. To monitor the effect of inactivated HIV on TIMP1 and MMP3 protein expression in culture supernatant, cells were cultured in UltraCulture Serum-Free Medium (BioWhittaker, Walkersville, MD) supplemental with 2 mM glutamine (Mediatech, Inc. Herndon, VA).

HIV Stocks—Inactivated HIV supernatant was produced as previously described (8). HIV and control supernatant were heat-inactivated at 56 °C for 30 min. Viral stock concentrations were measured for HIV-1 p24 using the Alliance p24 Antigen ELISA kit (PerkinElmer, Waltham, MA). HIV p24 concentrations are 65 ng/ml for NL4–3 CXCR4-tropic HIV (X4 HIV); and 80 ng/ml for BaL CCR5-tropic HIV (R5 HIV). The cells were incubated in UltraCulture serum-free medium with or without HIV stock supernatant (1:10 diluted for final concentration) for 24 h.

JFH1 HCV—JFH1 RNA was generated and transfected into Huh7.5.1 cells by electroporation as previously reported (16, 17). The JFH1-infected cells used in this study were between day 4 and 20 post-infection.

Protein Sample Preparation—Protein samples for ELISA or Western blot were prepared as previously described (19, 20).

Western Blot—Western blot was performed as previously reported (19, 20). The primary antibodies used for the Western blots included mouse anti-actin (Sigma Life Science and Biochemicals), and phosphorylated and unphosphorylated NFκB (p65) (Cell Signaling Technology, Inc., Danvers, MA).

ELISA—HCV core concentration was measured by Ortho HCV core antigen ELISA (Wako Chemicals USA, Inc., Richmond, VA) (19, 20). The commercially available kits Quantikine Human TIMP-1 Immunoassay (sensitivity, 0.15–10 ng/ml), and Quantikine Human MMP-3 ELISA (sensitivity, 0.15–10 ng/ml) (R&D Systems, Minneapolis, MN) were used according to the manufacturer's protocols to measure TIMP-1, and MMP-3 protein levels.

Human Collagen Type I Competitive ELISA—To measure human collagen type I protein levels in supernatants, a human collagen I competitive ELISA was developed based on purified human collagen type I and a mono-specific rabbit antibody as previously described (21, 22). Briefly, triple helical collagen type I was coated onto 96-well plates (1 μg, 100 μl/well) overnight. The plates were washed with PBS twice and then blocked with 2% BSA. After washing three times, coated plates were dried by air and then stored at −20 °C until use. A pretested amount of primary antibody was added to standard (10–0.01 μg/ml) or culture samples in separate tubes and incubated at room temperature on shaker for 1 h. The primary antibody diluted with culture media alone served as negative control (no inhibitor present). Primary antibody incubated with standard or samples was transferred to the collagen type I-coated 96-well plates and incubated at room temperature for 1 h. After washing with PBS, HRP-conjugated goat anti-rabbit IgG (Rockland Immunochemicals Inc., Gilbertsville, PA) was added to each well and incubated at RT for 1h, followed by addition of TMB (3,3′,5,5′-tetramentylbenzidine) for 10 min, and reaction quenching with 1 N HCl. The absorbance was measured at 450 nm using an ELISA plate reader (Molecular Devices, MN), with subtraction of the absorption of 3 blanks incubated with the HRP-conjugated goat anti-rabbit IgG. The standard curve was generated with 4-parameter fit (SoftMax Pro 4.0) (Molecular Devices, MN).

Patients—Liver biopsies from six patients were analyzed for mRNA levels for CoL1A, TIMP1, and MMP3. Laboratory and clinical data were collected from medical records at the time of liver biopsy. Liver biopsies were stored in RNAlater solution (Applied Biosystems/Ambion, Austin, TX). The study protocol was approved by the MGH Institutional Review Board.

RNA Sample Preparation—Cell lystates or liver biopsies were collected using the QIA shredder kit (Qiagen, Valencia, CA). Total cellular RNA was harvested using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.

qPCR—Total cDNA was synthesized by reverse transcription using random primers by the Applied Biosystems High-Capacity cDNA Reverse Transcription Kits (Invitrogen, Carlsbad, CA). CoL1A, TIMP1, MMP3, and GAPDH were quantified by qPCR as previously described (19). The primer sequences are listed in Table 1. Each gene mRNA level was normalized to GAPDH to calculate a fibrosis gene/GAPDH arbitrary unit (fold).

ROS Measurements and Cell Viability Assay—Cells (10,000 cell/well) were seeded in 100 μl of 10% FBS DMEM in 96-well clear bottom white assay plates overnight (14 h). Huh7.5.1 or JFH1 cells were incubated with 10 μl of heat-inactivated HIV supernatant for 24 h. LX2 cells were incubated with 10 μl of heat-inactivated HIV supernatant and 10 μl of JFH1 supernatant for 24 h. The negative control supernatant was obtained from Huh7.5.1 cells grown for 3 days in 10% FBS DMEM (DMEM). Diphenyliodonium (DPI) (ROS inhibitor) (20 μM final concentration) (EMD Chemicals, Inc., Gibbstown, NJ) was added to the appropriate wells for 3 h. ROS levels were measured as previously described (23–25). Briefly, the cells were incubated with 10 μM carboxy-derivative of fluorescein (carboxy-H2DCFDA) (ROS dye) (Invitrogen, Carlsbad, CA) in warm PBS for 1 h according to the manufacturer's protocol. ROS levels were assessed through the measurement of fluorescence at an excitation of 485 nm and an emission of 528

TABLE 2

JFH1 infects Huh7.5.1 cells, but not HSCs

To determine the binding or replication activity of JFH1 HCV in HSCs or Huh7.5.1 cells, 200,000 cells/well were seeded in 1 ml of medium in 12-well plates for 12 h. JFH1 stock virus (100 μl) was added to the correspondent well for 1 min, 10 min, 1 hr, 24 h, and 96 hrs. The JFH1 supernatants were harvested, and the cells washed twice with PBS, followed by analysis of the cell lysates using the HCV core protein ELISA. HCV core protein content (pg/ml) is expressed as means and STDs from three sample replicates.

	HCV core			HCV cor	
HSC+JFH1	Ave	STD	Huh7.5.1+JFH1	Ave	STD
	pg/ml	*pg/ml*		*pg/ml*	*pg/ml*
1 min	63.4	5.0	1 min	43.5	3.8
10 min	217.2	18.9	10 min	217.5	10.2
60 min	653.6	24.9	60 min	786.6	41.3
24 h	1088.1	87.4	24 hr	4540.7	315.7
48 h	1044.0	71.3	48 hr	29144.7	1568.6
96 h	906.1	40.9	96 hr	43902.5	3012.5

nm. Cell viability was monitored through the Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). Fluorescent microscopy was utilized to view ROS production. ROS/Cell Viability Arbitrary Unit (fold) was calculated by normalizing ROS level to cell viability.

siRNA and Transfection—To determine the specific molecular pathways of HIV and HCV regulated fibrosis-related gene expression, we performed RNAi to knock down NFκB gene expression. siRNAs were transfected into cells using HiPer-Fect Transfection Reagent (Qiagen, Valencia, CA). The siRNAs used for gene knock-down were as follows: SignalSilence NFκB p65 siRNA kit (Cat. 6536) which including (NFκB p65 siRNA I, product 6261, siRNA duplex sequences: 5′-GCCCUAUCCCUUUACGUCA-3′, and 5′-UGACGUAAAGGGAUAGGGC-3′), and (NFκB p65 siRNA II, product 6534, siRNA duplex sequences: 5′-GCUGAAGUGCAUCCAAAGG-3′, and 5′-CCUUUGGAUGCACUUCAGC-3′) (Cell Signaling Technology, Inc.). SignalSilence Control siRNA (product 6568, siRNA duplex sequences: 5′-CGUACGCGGAAUACUUCGA-3′, and 5′-UCGAAGUAUUCCGCGUACG-3′) (Cell Signaling Technology, Inc.) were used as negative controls for siRNA transfection. Knockdown of NFκB protein expression was confirmed by Western blot.

Statistics—Data analysis was carried out using a 2-tailed Student's *t* test with pooled variance. Data are expressed as mean ± S.D. of at least three sample replicates, unless stated otherwise.

RESULTS

JFH1 Does Not Replicate in Human Stellate Cells—To test the interaction and infectivity of JFH1 in HSC and Huh7.5.1 cells, we incubated JFH1 supernatant with HSC or Huh7.5.1 cells. We found that HCV core levels in JFH1-incubated HSC cells increased from 63.4 ± 5.0 pg/ml at 1 min to 217.2 ± 18.9 pg/ml, 653.6 ± 24.9 pg/ml, 1088.1 ± 87.4 pg/ml at 10 min, 60 min, and 24 h, respectively. However, longer periods of incubation (48 h and 96 h) did not further increase HCV core level (1044.0 ± 71.3 pg/ml, and 906.1 ± 40.9 pg/ml, respectively), suggesting that JFH1 did not replicate in HSC cells (Table 2). In contrast, we found that HCV core expression in JFH1-infected Huh7.5.1 cells increased 5.8-, 37-, and 55.9-fold from 786.6 ± 41.3 pg/ml at 60 min of incubation to 4540.7 ± 315.7 pg/ml, 29144.7 ± 1586.6 pg/ml, and 43902.5 ± 3012.5 pg/ml at 24, 48, and 96 h, respectively (Table 2), confirming JFH1 HCV replication in Huh7.5.1 cells.

HIV Increases Procollagen α1(I) mRNA and Protein Expression in HSC; and mRNA Expression in Huh7.5.1, and JFH1 Cells—To explore the effects of HIV on procollagen α1(I) (CoL1A) gene and protein expression in LX-2 HSC, Huh7.5.1, and JFH1-infected Huh7.5.1 cells, we performed qPCR to measure the level of CoL1A gene activity in cell and collagen type I protein level in supernatant in cell incubated with inactivated HIV infection supernatants. We found that heat-inactivated X4 HIV or R5 HIV enhanced CoL1A mRNA expression in HSC by 2.03 ± 0.36-fold ($p = 0.01$), and 1.93 ± 0.22-fold ($p = 0.003$) respectively, compared with cells treated with medium alone (Fig. 1*A*). Incubation of HSCs with JFH1 HCV supernatant modestly increased CoL1A mRNA expression by 1.32 ± 0.21-fold ($p = 0.09$) compared with untreated HSC cells. Incubation of HSCs with either X4 HIV or R5 HIV plus JFH1 further significantly enhanced CoL1A mRNA expression by 2.24 ± 0.22 fold ($p = 0.001$), and 2.30 ± 0.23-fold ($p = 0.001$), respectively, compared with HSCs alone (Fig. 1*A*). In line with the CoL1A mRNA data, we found that X4 HIV or R5 HIV enhanced collagen type I protein levels in HSC from 0.34 ± 0.04 μg/ml to 1.94 ± 0.26 μg/ml, and 1.60 ± 0.17 μg/ml respectively, compared with cells treated with medium alone (Fig. 1*B*). Incubation of HSCs with JFH1 HCV supernatant increased collagen type I protein levels to 2.02 ± 0.23 μg/ml. Incubation of HSCs with either X4 HIV or R5 HIV plus JFH1 further increased collagen type I protein levels to 7.85 ± 0.58 μg/ml, and 6.67 ± 0.38 μg/ml, respectively, compared with HSCs alone (Fig. 1*B*). We found that Huh7.5.1 cells express some CoL1A mRNA. However, the qPCR threshold cycle (Ct) value in CoL1A mRNA is roughly 10-fold higher in Huh7.5.1 cell (Ct range was between 27–30) than which in HSC cell (Ct range was between17–20). We also found that CXCR4 and CCR5 HIV enhanced CoL1A mRNA expression in Huh7.5.1 cells by 1.59 ± 0.14-fold ($p = 0.01$), and 1.46 ± 0.19-fold ($p = 0.004$), respectively, compared with untreated Huh7.5.1 cells (Fig. 1*C*). JFH1 HCV infection increased CoL1A mRNA expression by 1.54 ± 0.26 fold ($p = 0.04$) in Huh7.5.1 cells. Again, X4 or R5 HIV further increased CoL1A mRNA expression in JFH1-infected Huh7.5.1 cells by 3.70 ± 0.73-fold ($p = 0.003$), and 4.68 ± 0.39-fold ($p < 0.001$), respectively, compared with uninfected Huh7.5.1 cells. In contrast, HIV negative control supernatant had no effect on CoL1A gene expression in Huh7.5.1 or JFH1-infected cells (Fig. 1*C*). Collagen type I protein levels in uninfected or JFH1-infected Huh7.5.1 cells were below the reliable range of ELISA detection (data not shown).

HIV Increases TIMP1 mRNA and Protein Expression in HSC, Huh7.5.1, and JFH1-infected Huh7.5.1 Cells—Heat-inactivated X4 HIV or R5 HIV enhanced HSC TIMP-1 mRNA expression by 2.01 ± 0.36-fold ($p = 0.01$), and 2.07 ± 0.37-fold ($p = 0.01$), respectively, compared with medium-only treated HSC cells (Fig. 1*D*). This was accompanied by an increase of secreted TIMP-1 protein from 50.3 ± 5.5 ng/ml in medium-treated HSC cell to 147.3 ± 10 ng/ml ($p = 0.001$) in X4 HIV and 151.1 ± 17.4 ng/ml ($p = 0.001$) in R5 HIV (Fig.

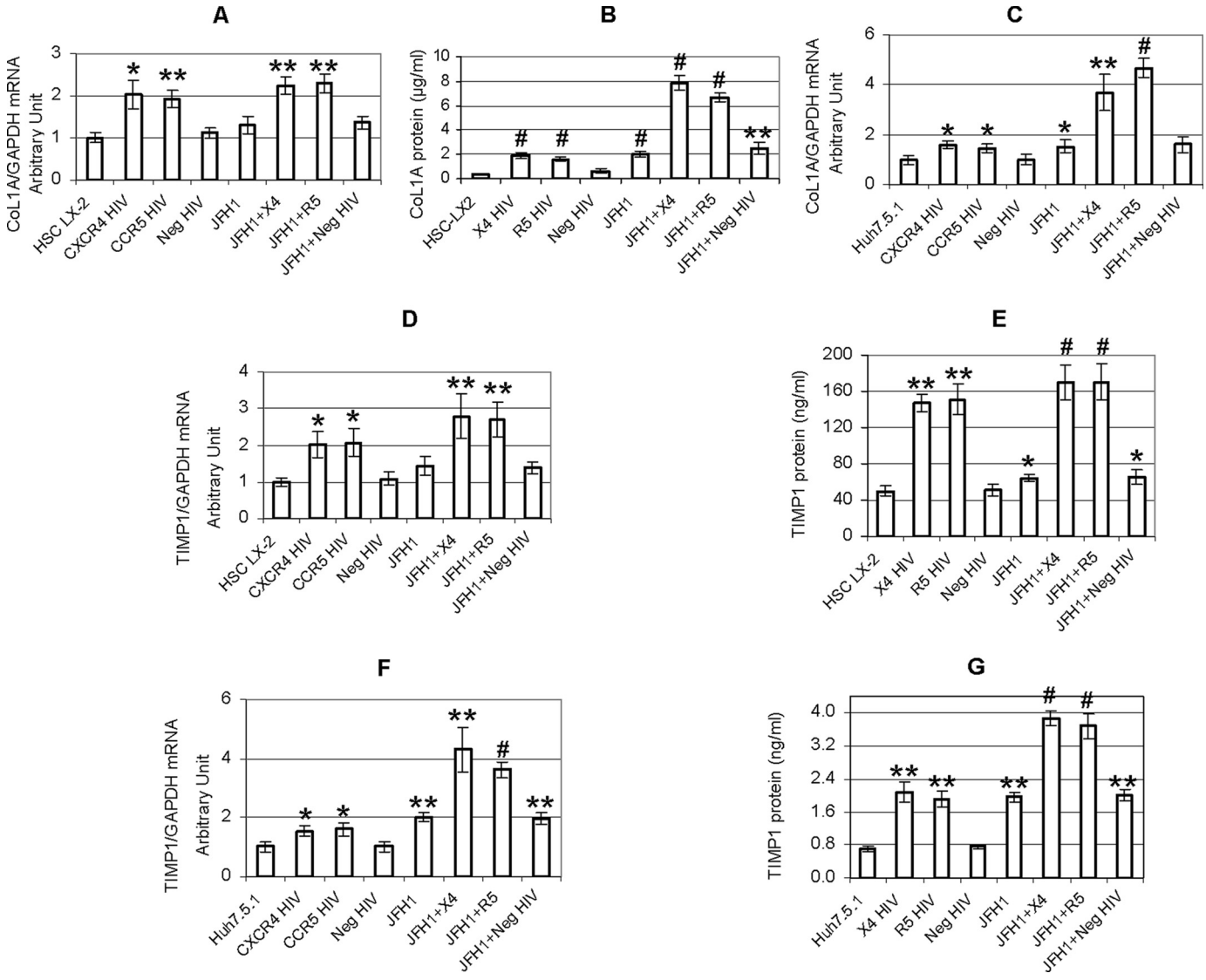

FIGURE 1. **HIV increased CoL1A and TIMP1 expression in HSC, Huh7.5.1, and JFH1 cells.** HSC cells were incubated with heat inactivated CXCR4 HIV (X4 HIV), CCR5 HIV (R5 HIV), or HIV-uninfected supernatant with or without JFH1 virus for 24 h. Huh7.5.1- or JFH1-infected cells were incubated with heated X4 HIV, R5 HIV, or Neg HIV supernatant for 24 h. The supernatants were collected for collagen type I protein and TIMP1 ELISA, and total RNA was harvested to quantitate mRNA levels of CoL1A and TIMP1 by qPCR. CoL1A mRNA or TIMP1 levels were normalized to GAPDH mRNA yielding arbitrary units (fold). Results are expressed as mean ± STDs from three experiments. *, $p < 0.05$; **, $p < 0.01$; and #, $p < 0.001$ for comparison of indicated treatment and HSC-LX2 or Huh7.5.1. *A*, HIV induced CoL1A mRNA expression in HSC cells. *B*, HIV and HCV increased collagen type I protein levels in HSC cells. *C*, HIV and HCV induced CoL1A mRNA expression in Huh7.5.1 cells. *D*, HIV and HCV induced TIMP-1 mRNA expression in HSC cells. *E*, HIV and HCV increased TIMP-1 protein levels in HSC cells. *F*, HIV and HCV induced TIMP-1 mRNA expression in Huh7.5.1 cells. *G*, HIV and HCV increased TIMP-1 protein levels in Huh7.5.1 cells.

1*E*). HSC cells incubated with JFH1 HCV supernatant exhibited minimal increases in TIMP-1 mRNA expression (1.32 ± 0.21-fold, $p = 0.9$) compared with medium-treated HSC cells, while HSC cells incubated with X4 HIV or R5 HIV combined with JFH1 HCV supernatant showed further significant up-regulation of TIMP-1 mRNA expression by 2.24 ± 0.22-fold ($p = 0.001$), and 2.30 ± 0.23-fold ($p = 0.001$), respectively, compared with medium-treated HSC cells (Fig. 1*D*). Incubation with JFH1 HCV supernatant increased TIMP-1 protein expression in HSC cells to 64.7 ± 4.7 ng/ml ($p = 0.03$) compared with medium treated HSC cells (50.3 ± 5.5 ng/ml). Incubation of HSC cells with X4 HIV or R5 HIV alone or plus JFH1 HCV supernatant further significantly induced TIMP-1 protein expression to 169.8 ± 19.6 ng/ml ($p < 0.001$), and 170.3 ± 20.2 ng/ml ($p < 0.001$) respectively, compared with medium-treated HSC cells (Fig. 1*E*) or JFH1-infected cells. TIMP-1 mRNA expression was up-regulated by X4 HIV or R5 HIV in Huh7.5.1 cell by 1.55 ± 0.17-fold ($p = 0.015$), and 1.61 ± 0.23-fold ($p = 0.021$), respectively, compared with medium-treated Huh7.5.1 cells, while JFH1 HCV infection increased TIMP-1 mRNA expression by 2.02 ± 0.16-fold ($p = 0.002$). X4 and R5 HIV further induced TIMP-1 mRNA expression in JFH1 cell by 4.32 ± 0.76-fold ($p = 0.002$), and 3.62 ± 0.28-fold ($p < 0.001$), respectively, compared with medium-treated Huh7.5.1 cells (Fig. 1*F*). Equally, X4 HIV and R5 HIV significantly increased TIMP-1 protein levels to 2.08 ± 0.25 ng/ml ($p = 0.001$), and 1.91 ± 0.19 ng/ml ($p = 0.001$) respectively, compared with medium-treated Huh7.5.1 cells (0.71 ± 0.06 ng/ml). In Huh7.5.1 cells, Neg HIV control had no significant effect on TIMP-1 protein levels in Huh7.5.1

cells. In line with the mRNA data, JFH1 mono-infection also increased TIMP1 cytokine level to 1.97 ± 0.13 ng/ml (p = 0.001) in Huh7.5.1 cells. X4 HIV or R5 HIV further significantly enhanced TIMP-1 protein levels to 3.87 ± 0.17 ng/ml ($p < 0.001$), and 3.68 ± 0.31 ng/ml ($p < 0.001$), respectively, compared with medium or JFH1-treated Huh7.5.1 cells (Fig. 1*G*).

HIV Suppresses MMP-3 Gene and Protein Expression in HSC Cells—X4 HIV or R5 HIV suppressed MMP-3 mRNA expression to 0.70 ± 0.06-fold (p = 0.008) and 0.75 ± 0.06-fold (p = 0.01), respectively, compared with medium-treated HSC cells, while incubation of HSCs with JFH1 HCV supernatant did not affect MMP-3 mRNA expression. X4 HIV or R5 HIV each combined with JFH1 HCV supernatant did not further suppress MMP-3 mRNA expression compared with HIV alone treated HSCs (Fig. 2*A*). X4 HIV and R5 HIV significantly down-regulated MMP-3 protein secretion to 14.1 ± 1.37 ng/ml (p = 0.005) and 13.99 ± 1.22 ng/ml (p = 0.005), respectively, compared with medium treated HSC (26.15 ± 3.56 ng/ml). However, MMP-3 protein secretion was not further suppressed in HSC incubated with JFH1 HCV supernatants (Fig. 2*B*). MMP-3 protein in uninfected or JFH1-infected Huh7.5.1 cells was below the limits of detection (data not shown).

HIV/HCV Co-infected and HCV Infected Increase CoL1A and TIMP1 mRNA and Decrease MMP3 mRNA Levels in Human Liver Tissue—To determine whether HIV/HCV co-infected and HCV infected have effects on liver fibrosis *in vivo*, we measured mRNA levels in liver biopsy from non-infected, HCV infected, and HIV/HCV co-infected patients. HIV alone data were not available due to the lack of availability of HIV mono-infected liver biopsy material. We found the following transcript mRNA levels for procollagen α1 (CoL1A) and TIMP-1: HIV/HCV co-infected > HCV infected > non-infected; and for MMP3: HIV/HCV co-infected < HCV infected < non-infected (Fig. 3, *A*–*C*). These data indicate that the results from the cell culture model are consistent with patient liver tissue samples, confirming that HIV/HCV co-infection and HCV infection produce a milieu that accelerates hepatic fibrosis.

HIV Increases ROS Production in HSC, Huh7.5.1, and JFH1 Cells—ROS production has been suggested to induce liver fibrogenesis (13, 15). We have previously shown that HCV replication induces ROS production in both JFH1 and HCV replicon cells. To determine whether HIV has an effect on ROS generation, we monitored ROS production in HSC, Huh7.5.1, and JFH1-infected Huh7.5.1 cells incubated with inactivated HIV supernatants. We found that X4 HIV and R5 HIV significantly induced ROS production by 1.62 ± 0.12

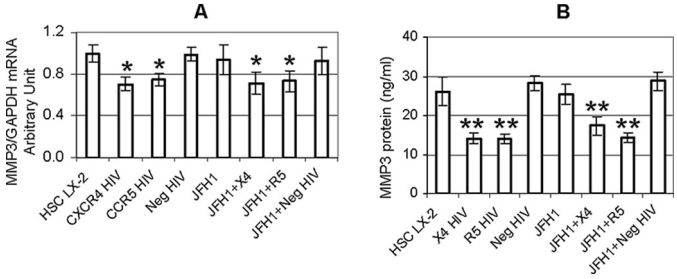

FIGURE 2. **HIV inhibits MMP-3 expression in HSC, Huh7.5.1, and JFH1 cells.** HSC, Huh7.5.1, or JFH1 cells were incubated with heated X4 HIV, R5 HIV, or HIV-negative with/without JFH1 HCV for 24 h. The supernatants were collected for ELISA, and total RNAs were harvested for qPCR analysis. *, $p < 0.05$; **, $p < 0.01$ for comparison of indicated treatment and HSC-LX2. *A*, HIV inhibited MMP-3 mRNA expression in HSCs. *B*, HIV blocked MMP-3 protein level in HSCs supernatants.

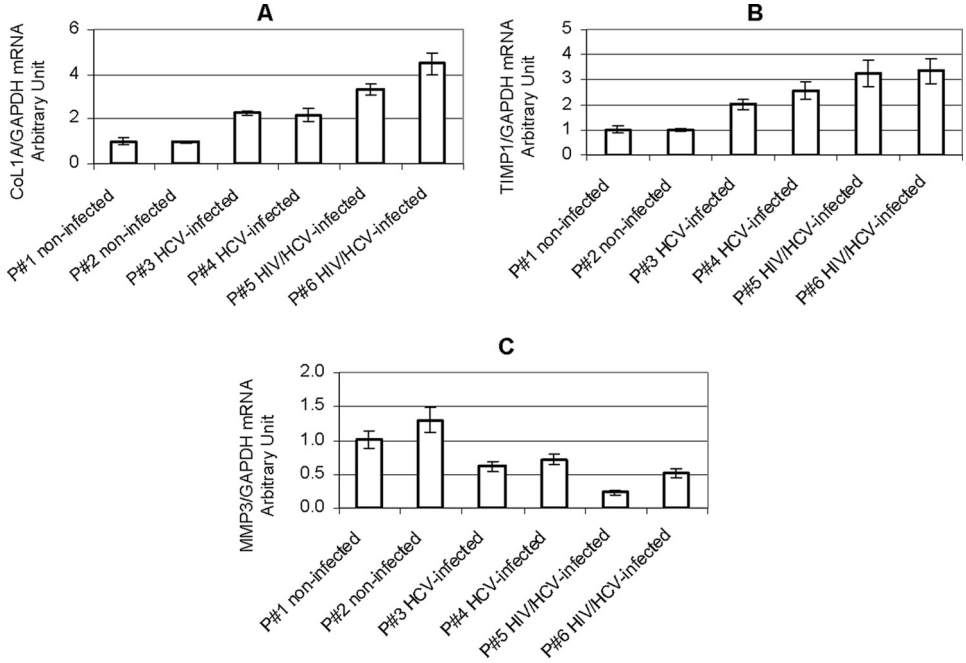

FIGURE 3. **HIV/HCV co-infected and HCV infected increase CoL1A, TIMP1 mRNA and decrease MMP3 mRNA levels in human liver biopsy tissue.** Patients 1 and 2 were not HIV- and HCV-infected (non-infected); patients 3 and 4 were HCV-infected; patients 5 and 6 were HIV/HCV-co-infected. *A*, HIV/HCV and HCV infection increase CoL1A mRNA expression. *B*, HIV/HCV and HCV infection increase TIMP1 mRNA expression. *C*, HIV/HCV and HCV infection decrease MMP3 mRNA expression.

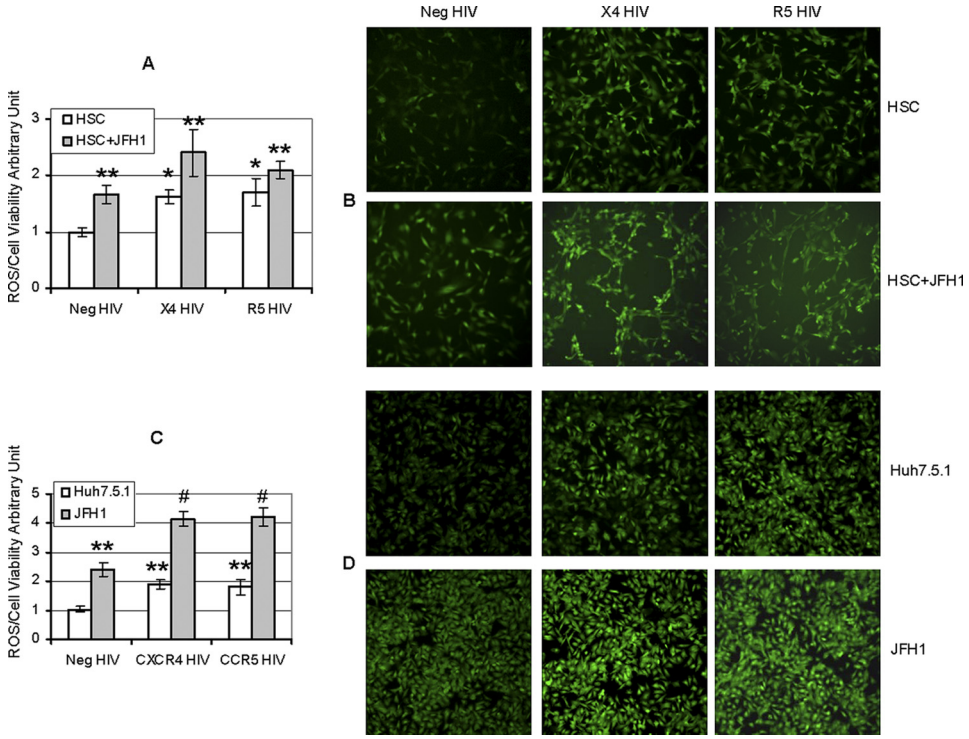

FIGURE 4. **HIV increased ROS production in HSC, Huh7. 5.1, and JFH1 cells.** Cells were incubated with X4 HIV, R5 HIV, or Neg HIV supernatant for 24 h. ROS fluorescence was measured by using ROS dye. Cell viability was monitored by the Cell Viability Assay. ROS level was normalized by cell viability to calculate the ROS/Cell Viability Arbitrary Unit (fold). ROS data represent mean ± S.D. ($n = 4$). ROS fluorescent images in live cells were monitored by fluorescent microscopy. *, $p < 0.05$; **, $p < 0.01$; and #, $p < 0.001$ for comparison of indicated treatment and Neg HIV. *A*, HIV and HCV enhanced ROS production in HSC cells. *Empty bar*: HSC cell, *Gray bar*: HSC incubated with JFH1 HCV. *B*, ROS fluorescence images in live HSC cells. *C*, HIV and HCV increased ROS production in Huh7.5.1 cells. *Empty bar*: Huh7.5.1 cells, *gray bar*: JFH1-infected Huh7.5.1 cells. *D*, ROS fluorescence images in live Huh7.5.1 and JFH1 cells.

($p = 0.003$) and 1.70 ± 0.24 ($p = 0.01$)-fold in HSC, respectively, compared with control (Neg HIV) treated cells (Fig. 4*A*). Although JFH1 HCV did not replicate in HSC cells (Table 2), we found that HSC cells incubated with HCV JFH1 supernatant also increased ROS production by 1.69 ± 0.14 ($p = 0.003$)-fold compared with HSC cells incubated with uninfected Huh7.5.1 supernatant (Fig. 4*A*). X4 HIV or R5 HIV combined with JFH1 HCV supernatant further enhanced ROS production by 2.40 ± 0.42 ($p = 0.002$) and 2.09 ± 0.15 ($p = 0.001$) fold, respectively, compared with untreated HSC cells (Fig. 4*A*). Furthermore, we observed that X4 HIV or R5 HIV supernatant added to Huh7.5.1 cells significantly induced ROS production by 1.88 ± 0.17 ($p = 0.002$) and 1.81 ± 0.25 ($p = 0.008$)-fold, respectively, compared with Neg HIV supernatant-treated Huh7.5.1 cells (Fig. 4*C*). We confirmed that JFH1 infection increased ROS production by 2.35 ± 0.29-fold ($p = 0.001$) in Huh7.5.1 cells compared with uninfected controls (Fig. 4*C*) (6). X4 HIV or R5 HIV further increased ROS production by 4.15 ± 0.26 ($p < 0.001$) and 4.2 ± 0.33 ($p < 0.001$)-fold, respectively, in JFH1-infected Huh7.5.1 cells compared with uninfected Huh7.5.1 cells (Fig. 3*C*). To visualize intracellular ROS activity, we obtained ROS fluorescent images in live cells that confirmed X4 and R5 HIV induced higher levels of ROS activity in HSC, Huh7.5.1, and JFH1-infected Huh7.5.1 cells than seen in Neg HIV controls (Fig. 4, *B* and *D*).

HIV and HCV Induced TIMP1 and CoL1A Gene Expressions are ROS-dependent—To assess whether HIV and HCV induction of TIMP1 and CoL1A gene expression are linked to ROS production, we used DPI, a potent inhibitor of ROS formation. DPI efficiently blocked HIV- and HCV-induced ROS enhancement in HSC, Huh7.5.1, and JFH1 cells (Fig. 5, *A* and *D*). DPI also inhibited HIV- and HCV-induced enhancement of TIMP-1 and CoL1A mRNA expressions in HSC-, Huh7.5.1-, and JFH1-infected Huh7.5.1 cells (Fig. 5, *B* and *C*, *G* and *H*). We found that DPI had no effect on HSC cell viability (Fig. 5*B*), and moderately decreased cell viability in Huh7.5.1- and JFH-infected Huh7.5.1 cells (Fig. 5*F*). Theses data indicate that HIV and HCV regulation of TIMP1 and CoL1A gene expression occur through ROS production.

HIV- and HCV-induced NFκB Phosphorylation Is ROS-dependent—To explore the effects of HIV and HCV on NFκB activity, we monitored NFκB phosphorylation by Western blot. We found that HIV and HCV each increased NFκB phosphorylation in HSC (Fig. 6*A*) and Huh7.5.1 cells (Fig. 6*B*). We also observed that DPI blocked HIV- and HCV-induced NFκB phosphorylation in HSC and Huh7.5.1 cells (Fig. 6, *A* and *B*), indicating that HIV and HCV activate NFκB via ROS production.

HIV- and HCV-induced TIMP1 and CoL1A Expressions Enhancement Are Abrogated by NFκB siRNA—To determine whether NFκB participates in HIV- and HCV-mediated enhancement of TIMP1 and CoL1A expression, we performed siRNA-mediated knockdown of NFκB in both HSC and Huh7.5.1 cells. We found that both HIV- and HCV-induced TIMP1 mRNA and protein, and CoL1A mRNA expressions were inhibited by NFκB-specific siRNA in HSC (Fig. 7, *A*–*C*) and Huh7.5.1 cells (Fig. 8, *A*–*C*). Western blotting confirmed

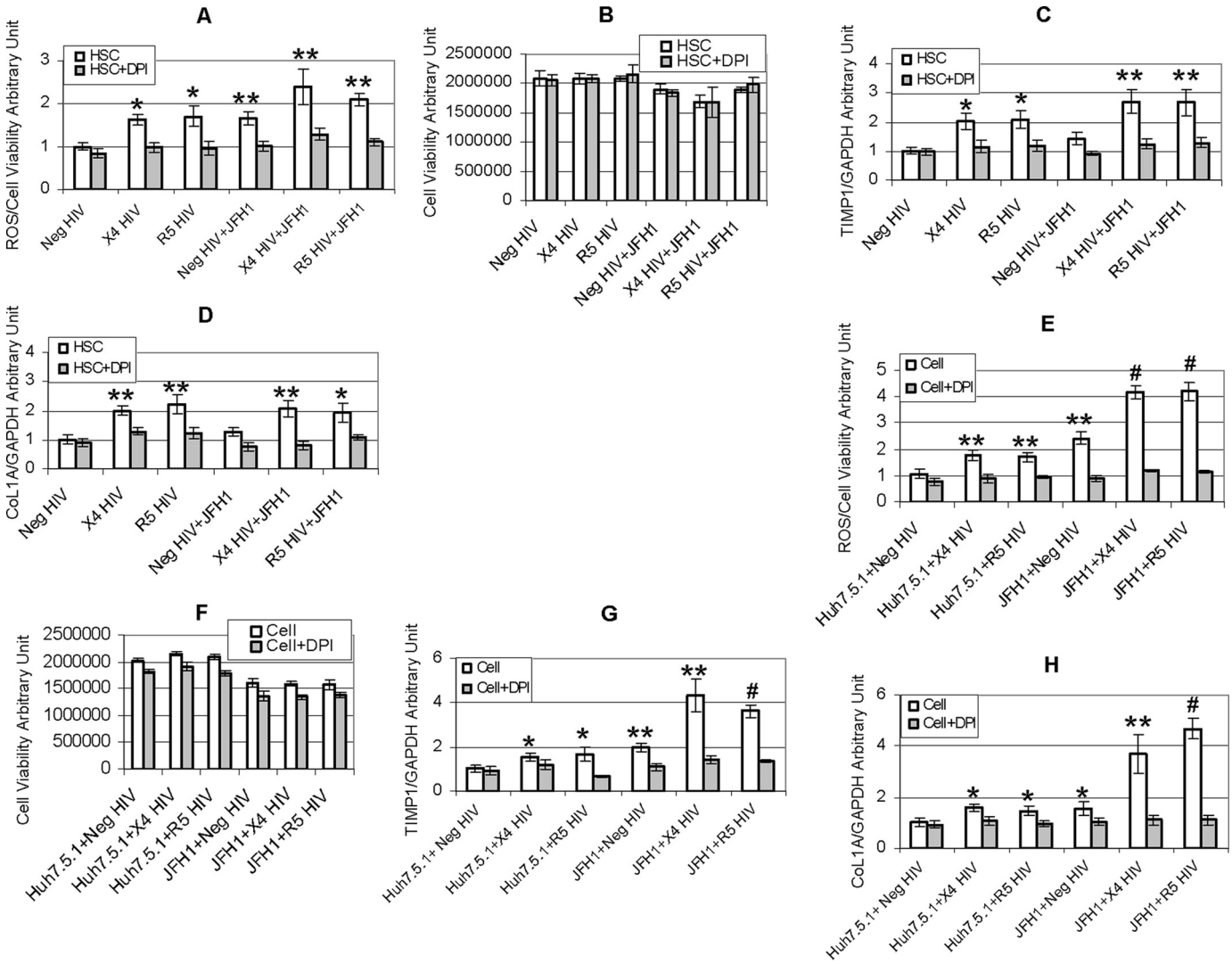

FIGURE 5. **ROS inhibition blocks HIV and HCV induced TIMP-1 and CoL1A mRNA expression.** Cells were incubated with HIV supernatant for 21 h. The cells were then treated with or without ROS inhibitor DPI (20 μM) for 3 h. ROS levels were normalized to cell viability. TIMP1 and CoL1A mRNA level were measured by qPCR. *, $p < 0.05$; **, $p < 0.01$; and #, $p < 0.001$ for comparison of indicated treatment and Neg HIV. DPI blocked the X4 HIV, R5 HIV and/or JFH1 HCV generated ROS production in HSC cells (*A*), and in Huh7.5.1 cells (*E*). DPI had no effect on HSC cell viability (*B*). HCV reduced cell viability in JFH1-infected Huh7.5.1 cell, and DPI moderately decreased cell viability in Huh7.5.1 and JFH1-infected Huh7.5.1 cells (*F*). DPI completely blocked HIV and JFH1 HCV induced TIMP-1 mRNA in HSC cells (*C*), and in Huh7.5.1 cells (*G*). DPI efficiently blocked HIV and JFH1 HCV induced CoL1A mRNA in HSC cells (*D*), and in Huh7.5.1 cells (*H*).

that NFκB protein expression was knocked down by NFκB siRNA in both cell lines (Figs. 7*D* and 8*D*). These data indicate that HIV- and HCV-induced TIMP1 and procollagen α1(I) production is dependent on NFκB activation. However, we found that HIV- and HCV-mediated ROS production was not inhibited by NFκB siRNA in HSC and Huh7.5.1 cells (Fig. 9, *A* and *B*), indicating that NF-κB activation lies downstream of ROS production.

DISCUSSION

HIV/HCV-coinfected patients show an accelerated progression to cirrhosis, liver failure, and liver-related death compared with HCV-monoinfected patients (3, 4, 8). However, the putative profibrogenic role of HIV upon (HCV-infected) hepatocytes and HSCs has not been carefully explored. In a previous study, we found that inactivated HIV or its envelope glycoprotein gp120 were capable of up-regulating TGF-β1 expression in Huh7.5.1- and JFH1-infected Huh7.5.1 cells (8). Our previous work had suggested that alterations in the circulating and intrahepatic cytokine environment that accompany HIV infection, particularly the increased levels of the profibrogenic cytokine TGF-β1, may contribute to the accelerated liver fibrosis observed in HIV-HCV-coinfected persons (8). HCV infection increases the generation of ROS (14) (26). HCV replication has been demonstrated to increase TGF-β1 production through the generation of ROS in an HCV subgenomic replicon, as well as in the JFH1 infectious model (14) (6, 13). HCV protein expression has also been shown to induce oxidative stress in a TGF-β1-dependent manner (27). TGF-β1 is a potent inducer of extracellular matrix synthesis. It enhances the production of many ECM proteins and down-regulates their MMP-dependent degradation by increasing TIMP expression, and induces production of

type I collagen by HSCs (28). TGF-β1 can also stimulate ROS production, which further contributes to the progression of liver fibrosis (28, 29). TGF-β1 has also been shown to induce liver fibrosis through Kupffer cell-mediated HSC activation and induction of collagen I and TIMP1 expression (30). It has been demonstrated that TGF-β1 stimulates vascular endothelial growth factor (VEGF) through the p38 mitogen-activated protein kinase (p38 MAPK)-dependent pathway, and leads to induction of type I collagen synthesis in murine mesangial cells (31, 32). ROS are natural byproducts of normal oxygen metabolism that have important roles in cell signaling. ROS are predominantly produced in the mitochondria via the electron transport chain through endoplasmic reticulum (ER) stress, which is induced by viral infection (33–35). HCV and other viruses can induce ROS, resulting in significant damage to cell structures, as well as induction of apoptosis and fibrosis, as has been shown in both this report and previous studies (14) (6, 25, 36, 37). We found that both HIV and HCV enhance ROS production in HSC and Huh7.5.1 cells. However, the impact of excess ROS on activation of fibrogenic signaling has not been well characterized. Hepatocytes are the predominant cells in the liver, while activated HSC are the main effector cells of fibrogenesis (15, 38, 39). Liver fibrosis is the product of excessive accumulation of ECM components that is not matched by removal of ECM by proteases, such as fibrolytic MMPs, and the overproduction of mainly TIMP-1 (15, 38, 39). In this study, we investigated the mechanism by which HIV and HCV cooperatively induce fibrosis-related gene and protein expression in HSC and Huh7.5.1 cells. We found that JFH1 HCV infection significantly induced CoL1A and TIMP1 activity in Huh7.5.1 cells. The finding of low level of CoL1A expression in Huh7.5.1 cells is in line with several studies that demonstrate epithelial-mesenchymal transition phenotypes in both hepatocytic cell lines and cultures hepatocytes *in vitro* (40), but this phenotype apparently does not exist *in vivo* (41, 42). Interestingly, we also found that JFH1 HCV induced profibrogenic activity in HSCs, despite the fact that JFH1 did not

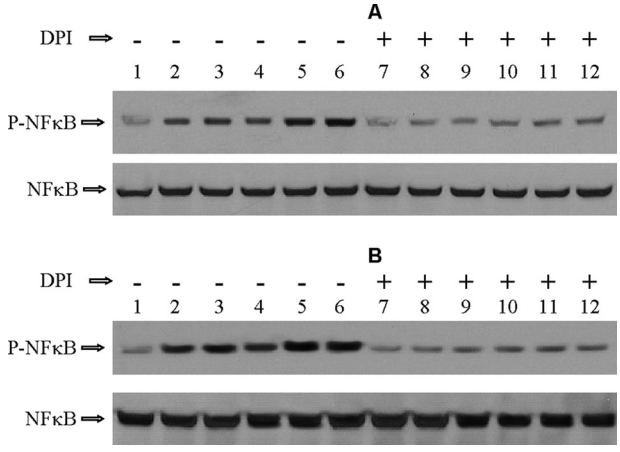

FIGURE 6. **ROS inhibition blocks HIV and HCV induced NFκB phosphorylation.** Cells were incubated with Neg HIV, X4 HIV, or R5 HIV with or without JFH1 HCV for 21 h. The cells were then treated with or without ROS inhibitor DPI (20 μM) for 3 h. Unphosphorylated and phosphorylated NFκB levels were monitored by Western blot. *A*, ROS inhibitor DPI blocked NFκB phosphorylation in HSC cells. HIV and JFH1 HCV activated NFκB phosphorylation (*lanes 2–6*) compared with Neg HIV (*lane 1*), DPI blocked HIV and JFH1 HCV induced NFkB phosphorylation (*lanes 7–12*). *Lanes 1* and *7*, Neg HIV; *2* and *8*, X4 HIV; *3* and *9*, R5 HIV; *4* and *10*, Neg HIV+JFH1; *5* and *11*, X4 HIV+JFH1; *6* and *12*, R5 HIV+JFH1. *B*, DPI blocked NFκB phosphorylation in Huh7.5.1 and JFH1 cells. *Lanes 1* and *7*, Neg HIV+Huh7.5.1; *2* and *8*, X4 HIV+Huh7.5.1; *3* and *9*, R5 HIV+Huh7.5.1; *4* and *10*, Neg HIV+JFH1; *5* and *11*, X4 HIV+JFH1; *6* and *12*, R5 HIV+JFH1.

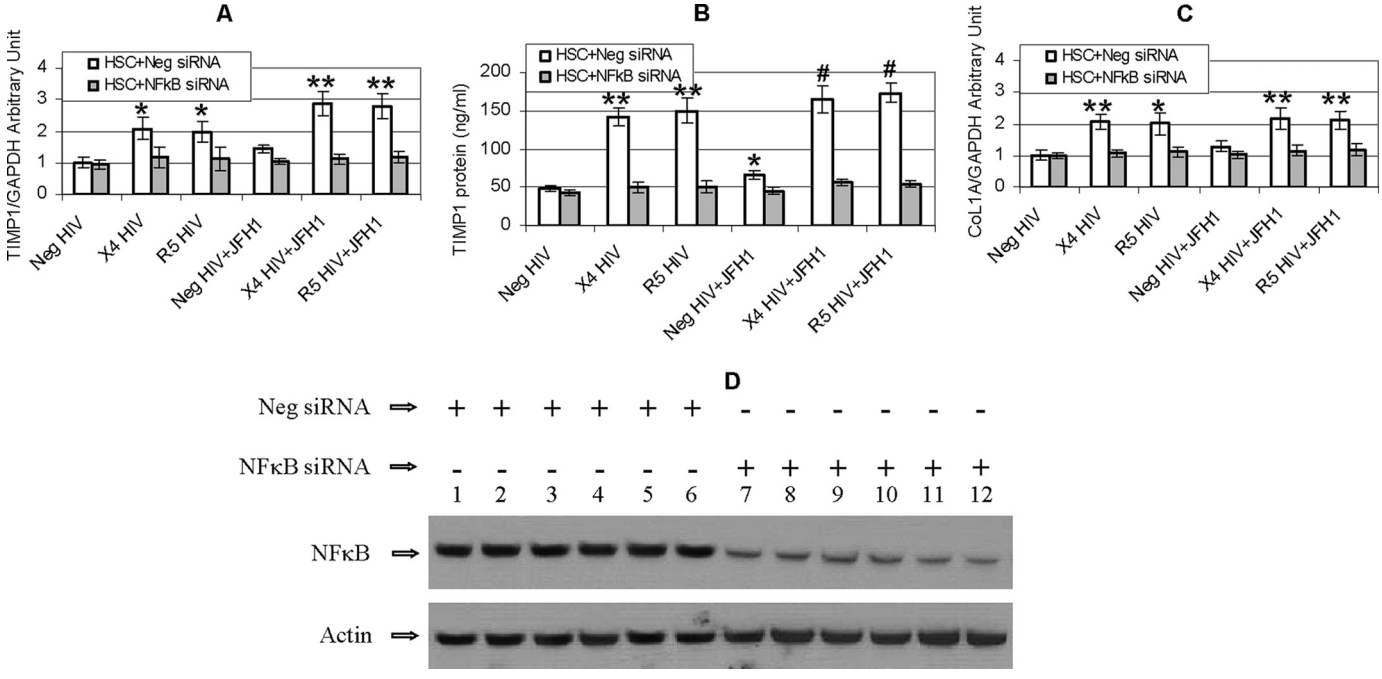

FIGURE 7. **NFκB siRNA blocks HIV and HCV activated TIMP1 and CoL1A expression in HSC cells.** NFκB siRNA was transfected into cells for 24 h, Neg siRNA was used as transfection control. The siRNA-transfected cells were then incubated with X4 HIV, R5 HIV, Neg HIV, or JFH1 supernatants for another 24 h. *, $p < 0.05$; **, $p < 0.01$; and #, $p < 0.001$ for comparison of indicated treatment with negative control siRNA in presence of Neg HIV. *A*, NFκB siRNA inhibited HIV and JFH1 HCV induced TIMP-1 mRNA in HSC cells. *B*, NFκB siRNA blocked HIV and JFH1 HCV induced TIMP-1 protein in HSC cells. *C*, NFκB siRNA inhibited HIV= and JFH1 HCV-induced CoL1A mRNA in HSC cells. *D*, NFκB siRNA knocked down NFκB protein expression in HSC cells. *Lanes 1* and *7*, Neg HIV; *2* and *8*, X4 HIV; *3* and *9*, R5 HIV; *4* and *10*, Neg HIV+JFH1; *5* and *11*, X4 HIV+JFH1; *6* and *12*, R5 HIV+JFH1.

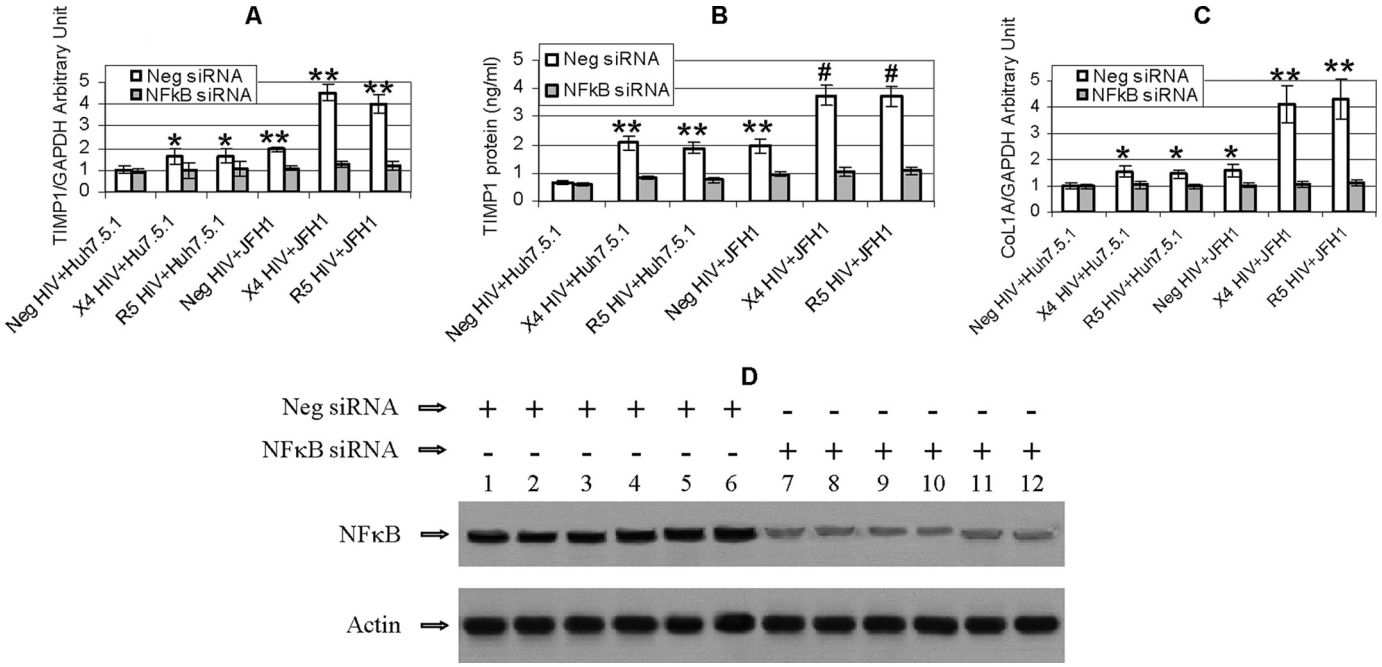

FIGURE 8. **NFκB siRNA blocks HIV and HCV activated TIMP1 and CoL1A expression in Huh7. 5.1 and JFH1 cells.** NFκB siRNA was transfected into Huh7.5.1 or JFH1-infected Huh7.5.1 (Day 1 postinfection) cells for 24 h, Neg siRNA was used as transfection control. The siRNA-transfected cells were then incubated with X4 HIV, R5 HIV, or Neg HIV supernatants for another 24 h. *, $p < 0.05$; **, $p < 0.01$; and #, $p < 0.001$ for comparison of indicated treatment with negative control siRNA in presence of Neg HIV. *A*, NFκB siRNA inhibited HIV- and JFH1 HCV-induced TIMP1 mRNA in Huh7.5.1 cells. *B*, NFκB siRNA blocked HIV- and JFH1 HCV-induced TIMP-1 protein in Huh7.5.1 cells. *C*, NFκB siRNA inhibited HIV- and JFH1 HCV-induced CoL1A mRNA in Huh7.5.1 cells. *D*, NFκB siRNA knocked down NFκB protein expression in Huh7.5.1 cells. *Lanes 1* and *7*, Neg HIV; *2* and *8*, X4 HIV; *3* and *9*, R5 HIV; *4* and *10*, Neg HIV+JFH1; *5* and *11*, X4 HIV+JFH1; *6* and *12*, R5 HIV+JFH1.

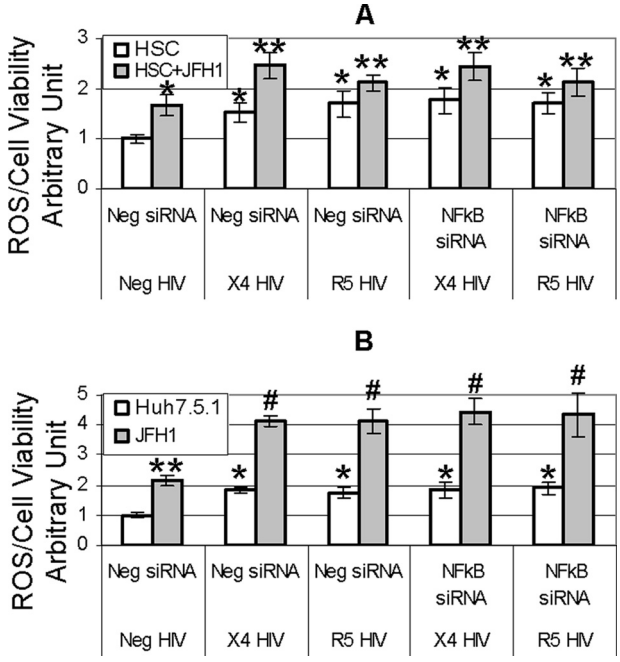

FIGURE 9. **NFκB siRNA does not affect HIV and HCV-induced ROS production.** HSC, Huh7.5.1, or JFH1 cells were transfected with Neg siRNA or NFκB siRNA for 24 h. The siRNA-transfected cells were incubated with Neg HIV, X4 HIV, or R5 HIV with or without JFH1 supernatant for another 24 h. ROS fluorescence was measured by using ROS dye. Cell viability was monitored by Cell Viability Assay. ROS level was normalized by cell viability to calculate the ROS/Cell Viability Arbitrary Unit (fold). ROS data represent mean ± S.D. ($n = 4$). *, $p < 0.05$; **, $p < 0.01$; and #, $p < 0.001$ for comparison of indicated treatment with negative control siRNA in presence of Neg HIV. *A*, NFκB knock down has no effect on X4 HIV, R5 HIV and JFH1 HCV-enhanced ROS production in HSC cells. *B*, NFκB knock down has no effect on X4 HIV or R5 HIV-induced ROS production in Huh7.5.1 and JFH1 cells.

replicate in these cells. In support of this finding, it has been previously shown that binding of the HCV envelope protein E2 can up-regulate MMP2 through E2/CD81 interactions in HSC cells (43). X4 HIV and R5 HIV have been demonstrated to enter and infect activated HSC LX2 and primary HSCs, inducing procollagen I expression and secretion of monocyte chemoattractant protein 1 (MCP-1) (7, 44). We have now demonstrated that HIV and HCV can individually and jointly induce the profibrogenic genes procollagen α1(I) and TIMP1, as well as reduce the potentially antifibrogenic expression of MMP3. We also demonstrate that the induction by HIV and HCV of fibrogenic gene expression in HSC and Huh7.5.1 cells is ROS-dependent. Activation of ROS downstream of the NFκB pathway plays major roles in both regulating the immune response to infection and in profibrogenic activation. This is in line with NFκB being one of the major signal-transducers activated in response to viral infection (6, 45). It has been reported that multiple families of viruses, including HIV, HCV, HBV, HTLV-1, EBV, and influenza virus activate NFκB, which subsequently promotes expression of over 100 target genes (46). In this study, we demonstrate that HIV and HCV regulate fibrosis-related genes through ROS induction and activation of the NFκB pathway. However, in this study and in a previous report (14), we found that siRNA to NFκB did not significantly affect JFH1 replication in Huh7.5.1 cells. Our results provide the first reported mechanistic data on how HIV and HCV may cooperatively drive hepatic fibrogenesis, thereby broadening our understanding of the mechanisms underlying liver disease in patients coinfected with HIV/HCV. It would now appear that strategies to limit HIV/HCV induc-

tion of oxidative stress and inhibition of NFκB activation are warranted to slow or block hepatic fibrosis.

Acknowledgments—We thank these investigators and institutes for supplying the reagents listed here: Drs. Ralf Bartenschlager and Takaji Wakita (infectious HCV virus JFH1 DNA construct); Dr. Francis Chisari (Huh7.5.1 cell line); and Dr. Scott L. Friedman (HSC LX-2 cells). The following reagents were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program: HIV pNL4–3 from Dr. Malcolm Martin, HIV BaL virus from Dr. Suzanne Gartner, Dr. Mikulas Popovic, and Dr. Robert Gallo.

REFERENCES

1. Sherman, K. E., Peters, M., and Koziel, M. J. (2007) *Hepatology* **45,** 1566–1577
2. Koziel, M. J., and Peters, M. G. (2007) *N. Engl. J. Med.* **356,** 1445–1454
3. Kim, A. Y., and Chung, R. T. (2009) *Gastroenterology* **137,** 795–814
4. Rotman, Y., and Liang, T. J. (2009) *J. Virol.* **83,** 7366–7374
5. Pascual-Pareja, J. F., Caminoa, A., Larrauri, C., González-García, J., Montes, M. L., Díez, J., Grande, M., and Arribas, J. R. (2009) *Aids* **23,** 971–975
6. Bruno, R., Galastri, S., Sacchi, P., Cima, S., Caligiuri, A., DeFranco, R., Milani, S., Gessani, S., Fantuzzi, L., Liotta, F., Frosali, F., Antonucci, G., Pinzani, M., and Marra, F. (2010) *Gut* **59,** 513–520
7. Hong, F., Tuyama, A., Lee, T. F., Loke, J., Agarwal, R., Cheng, X., Garg, A., Fiel, M. I., Schwartz, M., Walewski, J., Branch, A., Schecter, A. D., and Bansal, M. B. (2009) *Hepatology* **49,** 2055–2067
8. Lin, W., Weinberg, E. M., Tai, A. W., Peng, L. F., Brockman, M. A., Kim, K. A., Kim, S. S., Borges, C. B., Shao, R. X., and Chung, R. T. (2008) *Gastroenterology* **134,** 803–811
9. Macías, J., Berenguer, J., Japón, M. A., Girón, J. A., Rivero, A., López-Cortés, L. F., Moreno, A., González-Serrano, M., Iribarren, J. A., Ortega, E., Miralles, P., Mira, J. A., and Pineda, J. A. (2009) *Hepatology* **50,** 1056–1063
10. Blackard, J. T., Kang, M., Sherman, K. E., Koziel, M. J., Peters, M. G., and Chung, R. T. (2006) *J. Interferon Cytokine Res.* **26,** 834–838
11. Choi, S. H., and Hwang, S. B. (2006) *J. Biol. Chem.* **281,** 7468–7478
12. Choi, J., and Ou, J. H. (2006) *Am. J. Physiol. Gastrointest. Liver Physiol.* **290,** G847–851
13. Schulze-Krebs, A., Preimel, D., Popov, Y., Bartenschlager, R., Lohmann, V., Pinzani, M., and Schuppan, D. (2005) *Gastroenterology* **129,** 246–258
14. Lin, W., Tsai, W. L., Shao, R. X., Wu, G., Peng, L. F., Barlow, L. L., Chung, W. J., Zhang, L., Zhao, H., Jang, J. Y., and Chung, R. T. (2010) *Gastroenterology* **138,** 2509–2518, 2518 e2501
15. Schuppan, D., Krebs, A., Bauer, M., and Hahn, E. G. (2003) *Cell Death Differ.* **10,** Suppl. 1, S59–S67
16. Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T., and Chisari, F. V. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 9294–9299
17. Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Kräusslich, H. G., Mizokami, M., Bartenschlager, R., and Liang, T. J. (2005) *Nat. Med.* **11,** 791–796
18. Xu, L., Hui, A. Y., Albanis, E., Arthur, M. J., O'Byrne, S. M., Blaner, W. S., Mukherjee, P., Friedman, S. L., and Eng, F. J. (2005) *Gut* **54,** 142–151
19. Lin, W., Choe, W. H., Hiasa, Y., Kamegaya, Y., Blackard, J. T., Schmidt, E. V., and Chung, R. T. (2005) *Gastroenterology* **128,** 1034–1041
20. Lin, W., Kim, S. S., Yeung, E., Kamegaya, Y., Blackard, J. T., Kim, K. A., Holtzman, M. J., and Chung, R. T. (2006) *J. Virol.* **80,** 9226–9235
21. Schuppan, D., Cantaluppi, M. C., Becker, J., Veit, A., Bunte, T., Troyer, D., Schuppan, F., Schmid, M., Ackermann, R., and Hahn, E. G. (1990) *J. Biol. Chem.* **265,** 8823–8832
22. Casini, A., Ceni, E., Salzano, R., Milani, S., Schuppan, D., and Surrenti, C. (1994) *Life Sci.* **55,** 1311–1316
23. Miura, K., Taura, K., Kodama, Y., Schnabl, B., and Brenner, D. A. (2008) *Hepatology* **48,** 1420–1429
24. Otani, K., Korenaga, M., Beard, M. R., Li, K., Qian, T., Showalter, L. A., Singh, A. K., Wang, T., and Weinman, S. A. (2005) *Gastroenterology* **128,** 96–107
25. Zhu, Z., Wilson, A. T., Mathahs, M. M., Wen, F., Brown, K. E., Luxon, B. A., and Schmidt, W. N. (2008) *Hepatology* **48,** 1430–1439
26. Choi, J., Lee, K. J., Zheng, Y., Yamaga, A. K., Lai, M. M., and Ou, J. H. (2004) *Hepatology* **39,** 81–89
27. Boudreau, H. E., Emerson, S. U., Korzeniowska, A., Jendrysik, M. A., and Leto, T. L. (2009) *J. Virol.* **83,** 12934–12946
28. Gressner, A. M., Weiskirchen, R., Breitkopf, K., and Dooley, S. (2002) *Front Biosci.* **7,** d793–807
29. Liu, R. M., Choi, J., Wu, J. H., Gaston Pravia, K. A., Lewis, K. M., Brand, J. D., Mochel, N. S., Krzywanski, D. M., Lambeth, J. D., Hagood, J. S., Forman, H. J., Thannickal, V. J., and Postlethwait, E. M. (2010) *J. Biol. Chem.* **285,** 16239–16247
30. Wang, J., Leclercq, I., Brymora, J. M., Xu, N., Ramezani-Moghadam, M., London, R. M., Brigstock, D., and George, J. (2009) *Gastroenterology* **137,** 713–723
31. Wang, L., Kwak, J. H., Kim, S. I., He, Y., and Choi, M. E. (2004) *J. Biol. Chem.* **279,** 33213–33219
32. Kim, S. I., Kwak, J. H., Zachariah, M., He, Y., Wang, L., and Choi, M. E. (2007) *Am. J. Physiol. Renal Physiol.* **292,** F1471–F1478
33. Machida, K., Cheng, K. T., Lai, C. K., Jeng, K. S., Sung, V. M., and Lai, M. M. (2006) *J. Virol.* **80,** 7199–7207
34. Benali-Furet, N. L., Chami, M., Houel, L., De Giorgi, F., Vernejoul, F., Lagorce, D., Buscail, L., Bartenschlager, R., Ichas, F., Rizzuto, R., and Paterlini-Bréchot, P. (2005) *Oncogene* **24,** 4921–4933
35. Okuda, M., Li, K., Beard, M. R., Showalter, L. A., Scholle, F., Lemon, S. M., and Weinman, S. A. (2002) *Gastroenterology* **122,** 366–375
36. Ito, K., Hirao, A., Arai, F., Takubo, K., Matsuoka, S., Miyamoto, K., Ohmura, M., Naka, K., Hosokawa, K., Ikeda, Y., and Suda, T. (2006) *Nat. Med.* **12,** 446–451
37. Urtasun, R., Cubero, F. J., Vera, M., and Nieto, N. (2009) *Gastroenterology **136,** 1410-* **1422,** e1411–1414
38. Friedman, S. L. (2008) *Gastroenterology* **134,** 1655–1669
39. Schuppan, D., and Afdhal, N. H. (2008) *Lancet* **371,** 838–851
40. Taura, K., Miura, K., Iwaisako, K., Osterreicher, C. H., Kodama, Y., Penz-Osterreicher, M., and Brenner, D. A. (2010) *Hepatology* **51,** 1027–1036
41. Milani, S., Herbst, H., Schuppan, D., Hahn, E. G., and Stein, H. (1989) *Hepatology* **10,** 84–92
42. Milani, S., Herbst, H., Schuppan, D., Kim, K. Y., Riecken, E. O., and Stein, H. (1990) *Gastroenterology* **98,** 175–184
43. Mazzocca, A., Sciammetta, S. C., Carloni, V., Cosmi, L., Annunziato, F., Harada, T., Abrignani, S., and Pinzani, M. (2005) *J. Biol. Chem.* **280,** 11329–11339
44. Tuyama, A. C., Hong, F., Saiman, Y., Wang, C., Ozkok, D., Mosoian, A., Chen, P., Chen, B. K., Klotman, M. E., and Bansal, M. B. (2010) *Hepatology* **52,** 612–622
45. Popov, Y., Patsenker, E., Bauer, M., Niedobitek, E., Schulze-Krebs, A., and Schuppan, D. (2006) *J. Biol. Chem.* **281,** 15090–15098
46. Hiscott, J., Kwon, H., and Génin, P. (2001) *J. Clin. Invest.* **107,** 143–151