

## HIV infection increases HCV-induced hepatocyte apoptosis

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**Background & Aims:** HCV related liver disease is one of the most important complications in persons with HIV, with accelerated fibrosis progression in coinfecting persons compared to those with HCV alone. We hypothesized that HCV–HIV coinfection increases HCV related hepatocyte apoptosis and that HCV and HIV influence TRAIL signaling in hepatocytes.

**Methods:** We analyzed the effect of HIV in JFH1-infected Huh7.5.1 cells. Apoptosis was measured by Caspase-Glo 3/7 assay and Western blotting for cleaved PARP. TRAIL, TRAIL receptor 1 (DR4), and 2 (DR5) mRNA and protein levels were assessed by real-time PCR and Western blot, respectively. We also investigated activation of caspase pathways using caspase inhibitors and assessed expression of Bid and cytochrome C.

**Results:** We found increased caspase 3/7 activity and cleaved PARP in JFH1 HCV-infected Huh7.5.1 cells in the presence of heat-inactivated HIV, compared to Huh7.5.1 cells infected with JFH1 or exposed to heat-inactivated HIV alone. Both DR4 and DR5 mRNA and protein expression were increased in JFH1-infected cells in the presence of inactivated HIV compared to Huh7.5.1 cells infected with JFH1 or exposed to heat-inactivated HIV alone. Pancaspase, caspase-8, and caspase-9 inhibition blocked apoptosis induced by HCV, inactivated HIV, and HCV plus inactivated HIV. A caspase-9 inhibitor blocked apoptosis induced by HCV, HIV, and HCV–HIV comparably to pancaspase and caspase-8 inhibitors.

HCV induced the activation of Bid cleavage and cytochrome C release. The addition of HIV substantially augmented this induction.

**Conclusions:** Our findings indicate that hepatocyte apoptosis is increased in the presence of HCV and HIV compared to HCV or HIV alone, and that this increase is mediated by DR4 and DR5 up-regulation. These results provide an additional mechanism for the accelerated liver disease progression observed in HCV–HIV co-infection.

**Keywords:** HCV–HIV coinfection; Apoptosis; TRAIL receptor.

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**Abbreviations:** JFH-1, Japanese Fulminant Hepatitis 1; TRAIL, TNF-related apoptosis-inducing ligand; PARP, poly (ADP-ribose)polymerase; TGF- $\beta$ 1, transforming growth factor beta1; DR4, Death receptor 4; DR5, death receptor 5.

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### Introduction

Hepatitis C virus (HCV) infects about 170 million people and is a leading cause of chronic liver disease worldwide [1,2]. It is a major cause of cirrhosis, a significant cause of hepatocellular carcinoma (HCC), and the leading reason for liver transplantation worldwide.

HCV and human immunodeficiency virus (HIV) frequently coexist because of their common routes of transmission, and liver disease caused by HCV has rapidly become one of the most important complications in persons with HIV [3].

Indeed, end-stage liver disease is the most frequent cause of death among HIV-infected hospitalized patients [4]. Although the cause of liver injury in HIV-infected individuals is likely multifactorial (i.e., due to coinfection and medications), a number of reports have documented histological liver abnormalities that occurred solely as a result of HIV infection [5,6]. Moreover, HIV-1 RNA [5] and p24 antigen have been isolated from livers of infected individuals [7], although direct evidence that HIV infects hepatocytes is lacking. These observations suggest that HIV may indirectly result in hepatocellular injury. The hallmark of HIV infection is depletion and exhaustion of the immune system.

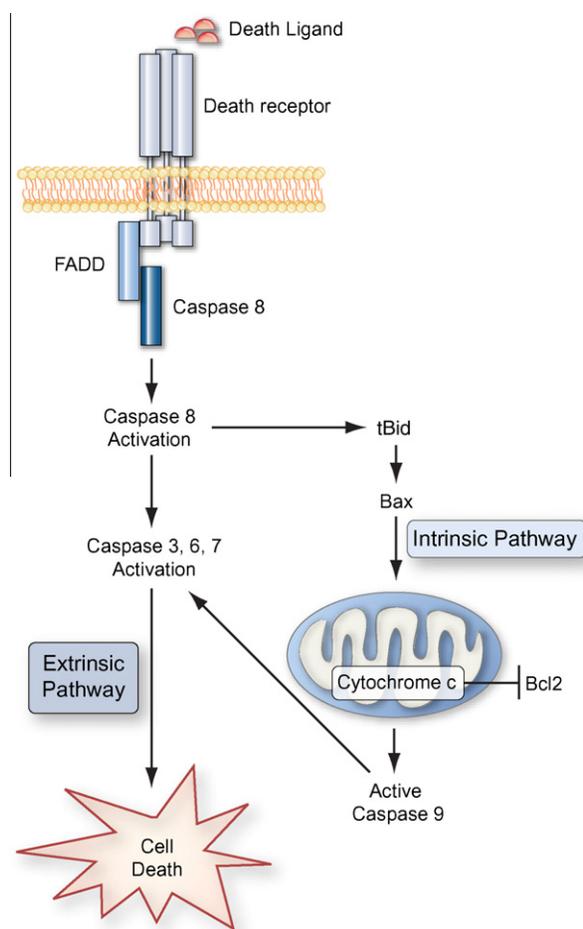
Hepatocyte cell death by apoptosis is emerging as a fundamental component of virtually all acute and chronic liver diseases. The ensuing responses of cell repair, inflammation, regeneration, and fibrosis may all be triggered by apoptosis [8–11]. An increasing body of evidence from both experimental and clinical studies suggests that hepatocyte apoptosis may contribute to liver fibrogenesis. Engulfment of apoptotic bodies by hepatic stellate cells stimulates the fibrogenic activity of these cells and may be one mechanism by which hepatocyte apoptosis promotes fibrosis [12]. Furthermore, recent studies in chronic hepatitis C virus (HCV) infection demonstrate that hepatocyte apoptosis correlates with disease severity [13] and stage of fibrosis in patients with steatosis [14,15].

Death receptors 4 and 5 (DR4 and DR5), also called tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)–R1 and R2, are members of the tumor necrosis factor–receptor



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(TNFR) family [16,17] and receptors for TNF-related apoptosis-inducing ligand (TRAIL) [18,19]. TRAIL selectively induces apoptosis in cancer cells *in vitro* and *in vivo* with little or no toxicity toward normal cells [20–22]. DR4 and DR5 mediate TRAIL-induced apoptosis through the formation of a death inducing signaling complex (DISC) containing the death receptor, adapter proteins such as Fas-associated death domain (FADD), and initiator caspases such as pro-caspase-8 [23] or pro-caspase-10 [24,25]. Consequently, pro-caspase-8 or pro-caspase-10 is activated by autoproteolytic processing, which then cleaves and activates downstream effector caspases, such as caspase-3 (extrinsic pathway). Additionally, the Bcl-2-interacting protein Bid is also cleaved by caspase-8 [26]. Truncated-Bid causes the loss of mitochondrial membrane potential and caspase-9 cleavage, resulting in apoptosis. Thus, truncated-Bid is a mediator that transduces the pro-apoptotic signal from the extrinsic pathway to the intrinsic (mitochondrial) pathway (Fig. 1).



**Fig. 1. Pathways of Cellular Apoptosis.** Death receptor mediates death ligand-induced apoptosis through the formation of a death inducing signaling complex (DISC) containing the death receptor, adapter proteins such as the Fas-associated death domain (FADD), and initiator caspases such as pro-caspase-8 or pro-caspase-10. Consequently, pro-caspase-8 or pro-caspase-10 is activated by autoproteolytic processing, which then cleaves and activates downstream effector caspases, such as caspase-3 (extrinsic pathway). Additionally, the Bcl-2-interacting protein Bid is also cleaved by caspase-8. Truncated-Bid causes the loss of mitochondrial membrane potential and caspase-9 cleavage, resulting in apoptosis. Thus, truncated-Bid is a mediator that transduces the pro-apoptotic signal from the extrinsic pathway to the intrinsic (mitochondrial) pathway.

We hypothesized that HIV coinfection augments hepatocyte apoptosis attributable to HCV and that this represents an additional mechanism of accelerated liver disease in coinfection. We tested this hypothesis using an infectious HCV cell culture model.

## Materials and methods

### Cell cultures and transfection

Huh7.5.1 cells (human hepatocellular carcinoma cells) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). Japanese Fulminant Hepatitis 1 (JFH1) is a known tissue culture infectious strain of HCV. We transfected JFH1 RNA into Huh7.5.1 cells and collected the culture medium at 72 h post-transfection, as described previously [27]. We then inoculated naïve Huh7.5.1 cells with these culture supernatants, and confirmed JFH1 virus infection of newly inoculated cells by real-time PCR and Western blot for HCV RNA and core protein, respectively. NL4-3 is a CXCR4-tropic (T lymphocyte-tropic) HIV strain. BaL is a CCR5-tropic (macrophage-tropic) HIV strain. Heat-inactivated HIV (CXCR4 and CCR5-tropic virus) (56 °C for 30 min) was incubated with Huh7.5.1 cells or JFH1-infected Huh7.5.1 cells for 72 h at 1:4 dilution (11.25 ng/ml p24 for NL4-3; 8.75 ng/ml p24 for BaL). Naïve Huh7.5.1 cells were seeded at a density of  $2 \times 10^5$  cells/well in 6-well plates 24 h before transfection. Infection of JFH1 and incubation with heat-inactivated HIV were performed simultaneously. Subsequently, cells were washed with PBS and collected 72 h later after transfection.

### HIV stocks

Heat-inactivated HIV was prepared as previously described [28]. Laboratory-adapted HIV-1 strains were obtained from the NIH AIDS Research and Reference Reagent Program. A plasmid encoding the CXCR4-tropic virus NL4-3 was used to transfect HEK293T cells, and the supernatant virus was propagated using a CEM-derived T-cell line that expresses CD4, CXCR4, and CCR5 (CEM-GXR) [28]. The CCR5-tropic virus BaL [29] was also propagated using CEM-GXR cells. Viral stocks were assayed for HIV-1 p24 using the Alliance p24 Antigen ELISA Kit (PerkinElmer, Waltham, MA). Previously, we found that the increase in HCV levels mediated by heat-inactivated CCR5-tropic HIV and CXCR4-tropic HIV was abrogated by pre-incubation with neutralizing antibody to CXCR4 and CCR5 [30]. These data provided evidence for the indirect effect of HIV virus on HCV infection signaling through engagement of its co-receptors on the surface of Huh7.5.1 cells rather than through direct infection of hepatocytes.

### Real-time polymerase chain reaction quantification

Total cellular RNA was harvested using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Total cDNA was synthesized by reverse transcription using the GeneAmp RNA PCR Kit (Applied Biosystems, Branchburg, New Jersey). DR4, DR5, TRAIL and  $\beta$ -actin mRNA levels were quantified by real-time PCR. Human DR4 (GenBank#: NM\_003844), Human DR5 (GenBank# NM\_003842) and Human TRAIL (GenBank# NM\_003810) were measured using the following primers: DR4 sense primer CTGAGCAACGACAGCTCGCTGCCAC; DR4 antisense primer-TCAAAGGACACGGCAGAGCCTGTGCCA; DR5 sense primer-GGGAGCCGCTCATGAGGAAGTTGG; DR5 antisense primer-GGCAAGTCTCTCC-CAGCGTCTC; TRAIL sense primer-AATCATCAAGGAGTGGGCATTG; TRAIL antisense primer-ATGACCAGTTCACCATTCCTCAA. Human  $\beta$ -actin sense primer-GCACTCTTCCAGCCTTCTCCT; human  $\beta$ -actin antisense primer AGGTCTTTGGCGGATGTCAC.  $\beta$ -Actin was used as a control for basal RNA levels. DR4, DR5, TRAIL, and  $\beta$ -actin levels were quantified by real-time PCR using the Bio-Rad IQ5 (Bio-Rad Laboratories, Hercules, CA). Finnzymes SYBR green I dye (New England Biolabs, Ipswich, MA) was used for detection, as previously described [31].

### Protein sample preparation

At the time of harvest, cells were washed twice with phosphate-buffered saline, and whole-cell protein samples were extracted with lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 50 mM NaF, 1 mM  $\text{Na}_2\text{VO}_4$ , 1% Triton X-100, 150 mM NaCl, 10  $\mu\text{g}/\text{ml}$  aprotinin, phosphatase inhibitor cocktail, and protease inhibitor cocktail) (Roche Applied Science, Indianapolis, IN).

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### Western blotting

Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) with NuPAGE Novex Bis-Tris precast 4–12% gradient gels (Invitrogen Life Science, Carlsbad, CA) and transferred to nitrocellulose membranes. The primary antibodies used for Western blots were as follows: rabbit anti-cleaved PARP (1:1000)(Cell signaling Technology, Inc., Beverly, MA), mouse anti-DR4 (1:1000) (Abcam Inc., Cambridge, MA), rabbit anti-DR5, rabbit anti-TRAIL, mouse anti-Bid, rabbit anti-cytochrome C (all at 1:1000, Cell signaling Technology, Inc., Beverly, MA), mouse anti-HCV core (1:1000)(Affinity BioReagents Inc., Golden, CO), and mouse anti-actin (1:10,000)(Sigma Life Science and Biochemicals, St. Louis, MO). The secondary antibody was horseradish peroxidase-conjugated ECL donkey anti-rabbit IgG (1:2000) or sheep anti-mouse IgG (1:10,000) (Amersham Biosciences, Piscataway, NJ). Chemiluminescent signals were detected using the ECL Western blotting detection Kit (Amersham Biosciences, Piscataway, NJ). Densitometry was performed using the Universal Hood II (Bio-Rad, Hercules, CA).

### Poly (ADP-ribose) polymerase assay

PARP, a 116 kDa nuclear poly (ADP-ribose) polymerase, appears to be involved in DNA repair in response to environmental stress. This protein can be cleaved by many ICE-like caspases *in vitro* and is one of the main cleavage targets of caspase-3 *in vivo*. In human PARP, the cleavage occurs between Asp214 and Gly215, which separates the PARP amino-terminal DNA binding domain (24 kDa) from the carboxy-terminal catalytic domain (89 kDa). PARP helps cells to maintain their viability; cleavage of PARP facilitates cellular disassembly, and serves as a marker of cells undergoing apoptosis [32].

### Caspase 3/7 activity assay

The Caspase-Glo® 3/7 Assay provides a homogeneous luminescent assay that measures caspase-3/7 activity. Naïve Huh7.5.1 cells were seeded at a density of  $10^4$  cells/well (100  $\mu$ l of DMEM with 10% FBS) in 96-well plates, 24 h before transfection. Infection of JFH1 and incubation with heat-inactivated HIV were performed simultaneously. Caspase-Glo 3/7 reagent (Promega Corporation, Madison, WI) was then added to each well, 72 h after transfection. The contents of the plate were gently mixed using a plate shaker at 300–500 rpm for 30 s. The cells were then lysed at room temperature for 1 h. Luminescence activities (relative caspase 3 and 7 activities) were measured with a BioTek Synergy 2 Microplate Reader (Winooski, VT). We measured caspase-3/7 activities as a function of JFH1 concentration using the Caspase-Glo® 3/7 Assay. We found that this assay sensitively detected apoptosis at very low concentrations of JFH1 (data not shown). Because of its sensitivity, we used caspase-3/7 activity as an apoptotic marker for this study.

### Caspase inhibitors

Cells were preincubated with caspase inhibitors (pan-caspase inhibitor: Z-VAD-FMK (20  $\mu$ M), caspase-8 inhibitor: Z-IETD-FMK (20  $\mu$ M), caspase-9 inhibitor: Z-LEHDFMK (20  $\mu$ M); all from R&D Systems) for 6 h prior to the caspase-Glo 3/7 assay. Caspase inhibitors were subsequently diluted in DMSO before use.

### siRNA and transfection

To further explore the relationship between apoptosis and TRAIL receptor expression, we performed RNAi to knock down TRAIL receptor (DR4 and DR5) expression. The siRNAs were transfected into cells using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA). The siRNAs used for gene knockdown were as follows: On-Target SMARTpool DR4 siRNA, On-Target SMARTpool DR5 siRNA (Dharmacon, Inc., Lafayette, CO). siGENOME Non-Targeting siRNA #1 (Dharmacon, Inc., Lafayette, CO) was used as a negative control for siRNA-mediated knockdown. The knockdown of each gene was confirmed by real-time PCR and the extent of apoptosis was confirmed by Caspase-Glo 3/7 assay.

### Statistics

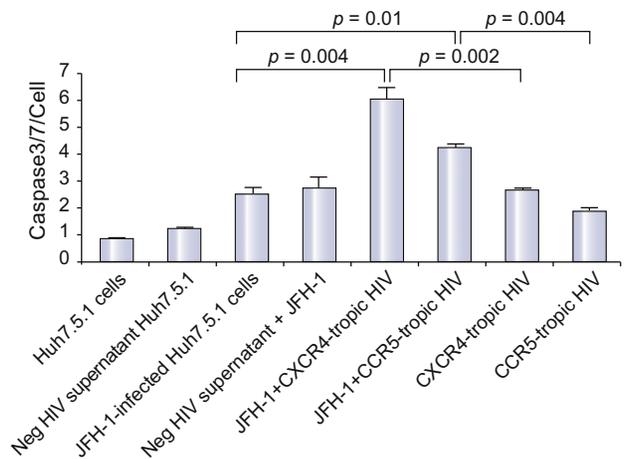
Data analysis was carried out using paired *t*-test. Data were expressed as mean  $\pm$  SD of at least three sample replicates, unless stated otherwise.

## Results

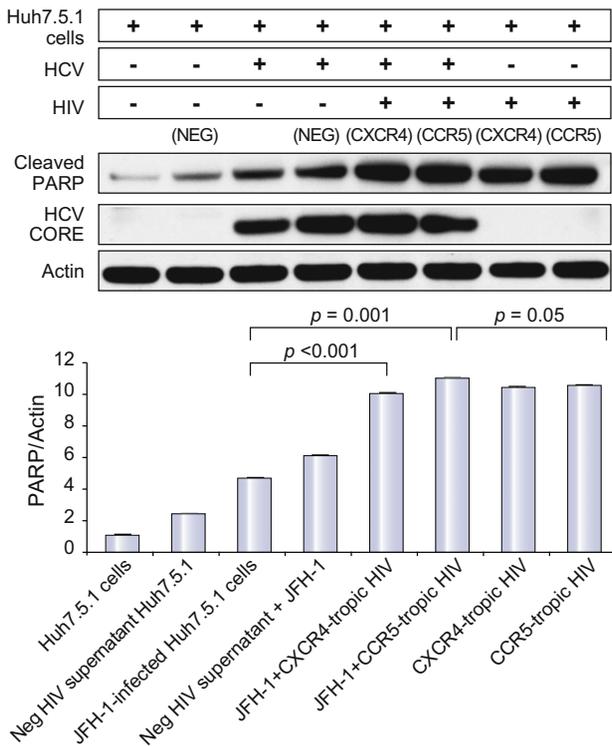
### Inactivated HIV increases apoptosis in JFH1-infected Huh7.5.1 cells

To assess whether HCV and HIV induce apoptosis in hepatocytes, we performed a quantitative caspase activity assay and Western blot in Huh7.5.1 cells, JFH1-infected Huh7.5.1 cells, Huh7.5.1 cells grown in the presence of inactivated HIV (CCR5- and CXCR4-tropic strains), and JFH1-infected Huh7.5.1 cells grown in the presence of inactivated HIV (referred to as JFH1-infected/inactivated-HIV treated Huh7.5.1 cells). Using the Caspase-Glo 3/7 assay, we examined the apoptotic effects of JFH1 HCV and heat-inactivated HIV on Huh7.5.1 cells.

Both JFH1 infection and inactivated HIV increased caspase 3/7 activity in Huh7.5.1 cells compared to uninfected controls (Fig. 2) ( $p < 0.01$  for each). Furthermore, JFH1-infected/inactivated-HIV, both CCR5- and CXCR4-tropic strains, produced a greater than 2-fold increase of caspase 3/7 activity compared to either JFH1-infected or inactivated-HIV Huh7.5.1 cells (Fig. 2) ( $p < 0.05$  for each). Western blot for cleaved PARP demonstrated that both JFH1-infected and inactivated-HIV Huh7.5.1 cells increased cleaved PARP expression compared to negative controls (Fig. 3) ( $p < 0.01$  for each); the expression of cleaved PARP was further increased in JFH1-infected/inactivated-HIV Huh7.5.1 cells ( $p < 0.05$  for each). From these experiments, we demonstrate that HIV increases HCV-induced hepatocyte apoptosis, and that HIV indirectly promotes hepatocyte apoptosis.



**Fig. 2. Increased caspase 3/7 activity in JFH1-infected, heat-inactivated HIV-treated Huh7.5.1 cells compared to JFH1-infected or HIV-treated Huh7.5.1 cells alone.** To assess the effects of HCV and HIV infection on apoptosis, 10,000 cells/well of Huh7.5.1 cells were added to 96-well plates in 100  $\mu$ l of 10% FBS DMEM, 24 h before transfection. Transfection of JFH1 and incubation with heat-inactivated HIV were performed simultaneously. Caspase-Glo 3/7 reagent was added to each well 72 h later. Each treatment was performed in triplicate. Caspase 3/7 activity was increased by over 2-fold in JFH1-infected, heat-inactivated HIV-treated Huh7.5.1 cells compared to JFH1-infected or HIV-treated Huh7.5.1 cells ( $p < 0.05$  for each). Y axis refers to caspase 3/7 activity per cell. Lane #1 Huh7.5.1, #2 Huh7.5.1 + negative supernatant HIV, #3 JFH1, #4 JFH1 + negative supernatant HIV, #5 JFH1 + CXCR4 tropic HIV, #6 JFH1 + CCR5 tropic HIV, #7 CXCR4 tropic HIV, #8 CCR5 tropic HIV.



**Fig. 3. Expression of cleaved PARP was increased in JFH1-infected, heat-inactivated HIV-treated Huh7.5.1 cells compared to JFH1-infected or HIV-treated Huh7.5.1 cells alone.** To assess apoptosis in HCV and HIV co-infected Huh7.5.1 cells, we monitored cleaved PARP, HCV core, and  $\beta$ -actin levels by Western blot and corresponding densitometry. We confirmed that expression of cleaved PARP was increased in JFH1-infected, heat-inactivated HIV-treated Huh7.5.1 cells compared to JFH1-infected or HIV-treated Huh7.5.1 cells alone ( $p < 0.05$  for each). Lane #1 Huh7.5.1, #2 Huh7.5.1 + negative supernatant HIV, #3 JFH1, #4 JFH1 + negative supernatant HIV, #5 JFH1 + CXCR4-tropic HIV, #6 JFH1 + CCR5-tropic HIV, #7 CXCR4-tropic HIV, #8 CCR5-tropic HIV.

**Increased expression of TRAIL receptor 1 and 2 is observed in HCV-infected Huh7.5.1 cells in the presence of HIV compared to HCV-infected or HIV-treated Huh7.5.1 cells**

To further assess the molecular mechanisms of apoptosis induced by these viruses, we examined known mediators of apoptotic signaling, specifically TRAIL and TRAIL receptor 1, 2 (DR4, DR5). We first measured levels of TRAIL receptor 1 (DR4), 2 (DR5), and TRAIL using real-time PCR. We found that DR4 and DR5 mRNA levels were increased in HCV-infected Huh7.5.1 cells in the presence of heat-inactivated HIV compared to Huh7.5.1 cells infected with JFH1 or exposed to heat-inactivated HIV alone (Fig. 4A and B). DR5 was significantly increased in JFH1-infected, heat-inactivated HIV-treated Huh7.5.1 cells compared to JFH1 or heat-inactivated HIV-treated Huh7.5.1 cells alone ( $p < 0.05$ ) [1.23-fold (vs HCV), 2.41-fold (vs HIV)] and DR4 was moderately increased ( $p = 0.05$ ) (1.37-fold (vs HCV), 2.48-fold (vs HIV)). In the case of TRAIL, mRNA levels were decreased in the presence of HCV compared to Huh7.5.1 cells and HIV-incubated Huh7.5.1 cells (Fig. 4C). For further evaluation of TRAIL signaling, we performed Western blot for DR4, DR5, and TRAIL. As shown in Fig. 5, DR4 and DR5 induction was observed in HCV-infected Huh7.5.1 cells in the presence of heat-inactivated HIV compared to either JFH1-infected or heat-inactivated HIV-treated Huh7.5.1 cells

[DR4 ( $p < 0.01$ ) (2.02-fold (vs HCV), 1.80-fold (vs HIV))] [DR5 ( $p < 0.01$ ) (1.55-fold (vs HCV), 1.50-fold (vs HIV))]. Protein expression of TRAIL was decreased in the presence of HCV. These results suggest that HIV increases HCV-induced hepatocyte apoptosis, and that this increase in apoptosis is induced by up-regulation of the TRAIL receptors DR4 and DR5.

*HCV-HIV induced apoptosis is dependent on caspases within the mitochondrial pathway*

To further delineate the intracellular apoptotic pathway modulated by HCV and HIV, we investigated the activation of caspase pathways during apoptosis using several caspase inhibitors. We performed Caspase-Glo 3/7 assay after pre-incubation with several caspase inhibitors. The pan-caspase inhibitor zVAD-fmk decreased HCV, HIV, or HCV-HIV-induced caspase 3/7 activity by over 4-fold ( $p < 0.01$ ). A caspase-8 inhibitor blocked HCV, HIV, or HCV-HIV-induced apoptosis to the same degree as zVAD-fmk. In addition, a caspase-9 inhibitor blocked apoptosis induced by HCV, HIV, or HCV-HIV comparably to pancaspase or caspase-8 inhibition (Fig. 6). Taken together, these data indicate that HCV, HIV, or HCV-HIV-induced apoptosis are dependent on both extrinsic caspases and caspases related to the mitochondrial pathway.

*Analysis of Bid cleavage and cytochrome C levels*

To confirm the activation of the mitochondrial pathway upon HCV-HIV infection of Huh7.5.1 cells, we performed Western blot of the mitochondrial apoptosis activating protein Bid and cytochrome C. While HCV alone induced the activation of Bid cleavage and cytochrome C release, addition of HIV substantially augmented this induction (3.5-fold for BID, 1.6-fold for cytochrome C, Fig. 7). These data confirm that HCV-HIV-related apoptosis utilizes the mitochondrial pathway.

*HCV-HIV induced apoptosis is decreased by DR4 and DR5 siRNA*

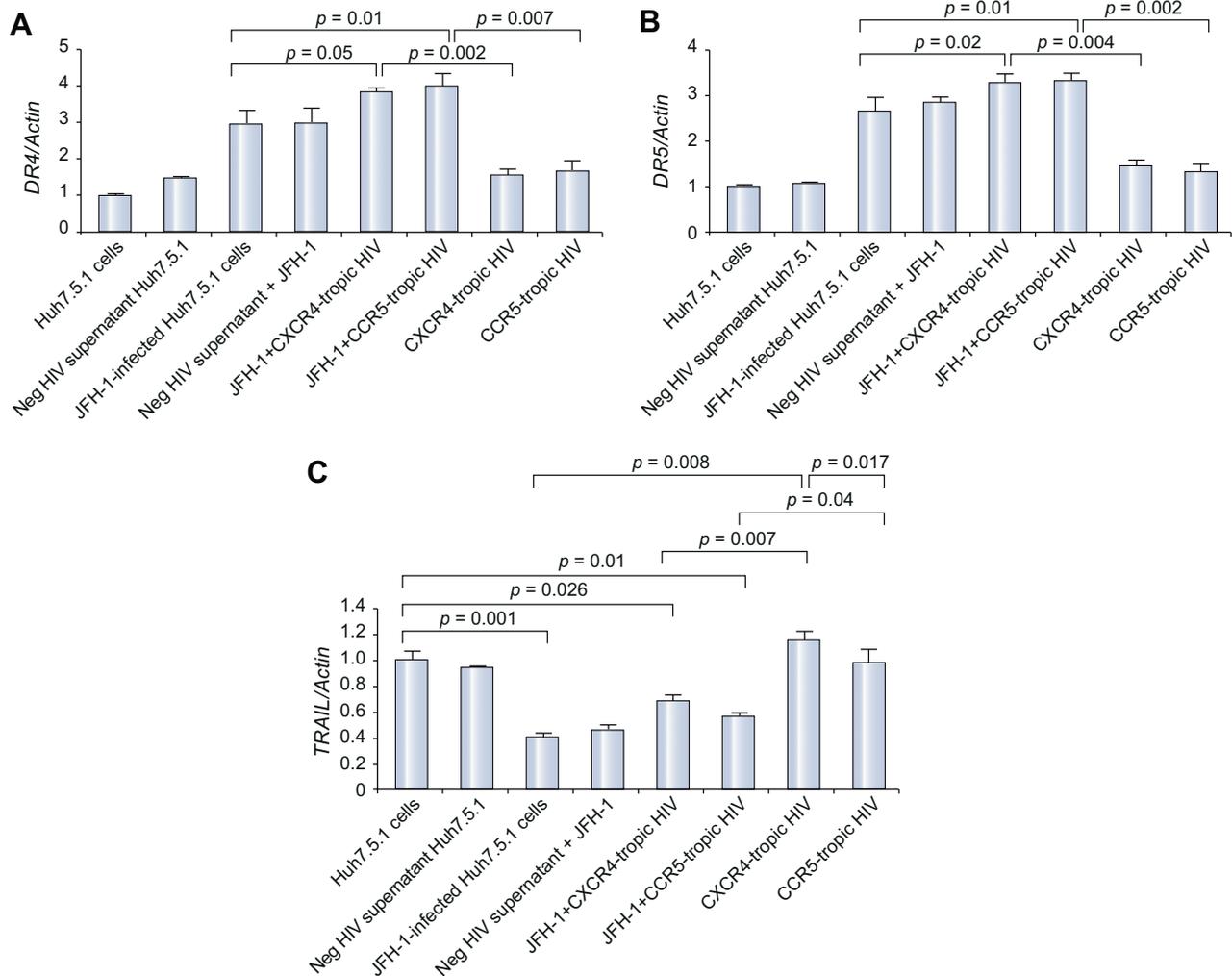
To demonstrate that HCV and HIV-induced increases in apoptosis are mediated by DR4 and DR5, we performed siRNA-mediated knockdown of DR4 and DR5 (Fig. 8). We found that HCV-HIV-induced apoptosis was blocked by DR4- and DR5-specific siRNAs, but not by negative control siRNAs (Fig. 8A). These data confirm that HCV-HIV-induced apoptosis is dependent on DR4 and DR5 up-regulation. The presence of residual apoptosis that is not eliminated by DR4 or DR5 siRNAs in the presence of HIV or HIV-HCV infected cells, raises the possibility that additional non-DR4, -DR5 pathways contribute to apoptosis.

## Discussion

Fibrogenesis is a relatively late event in chronic liver injury, and occurs as a consequence of activation of hepatic stellate cells (HSC) and excessive deposition of extracellular matrix, under conditions of persistent inflammation.

The first phase of liver injury, however, independent of the etiology, is almost always characterized by increased hepatocyte apoptosis. Since for many years apoptosis has been considered a mechanism of cell death not associated with an inflammatory

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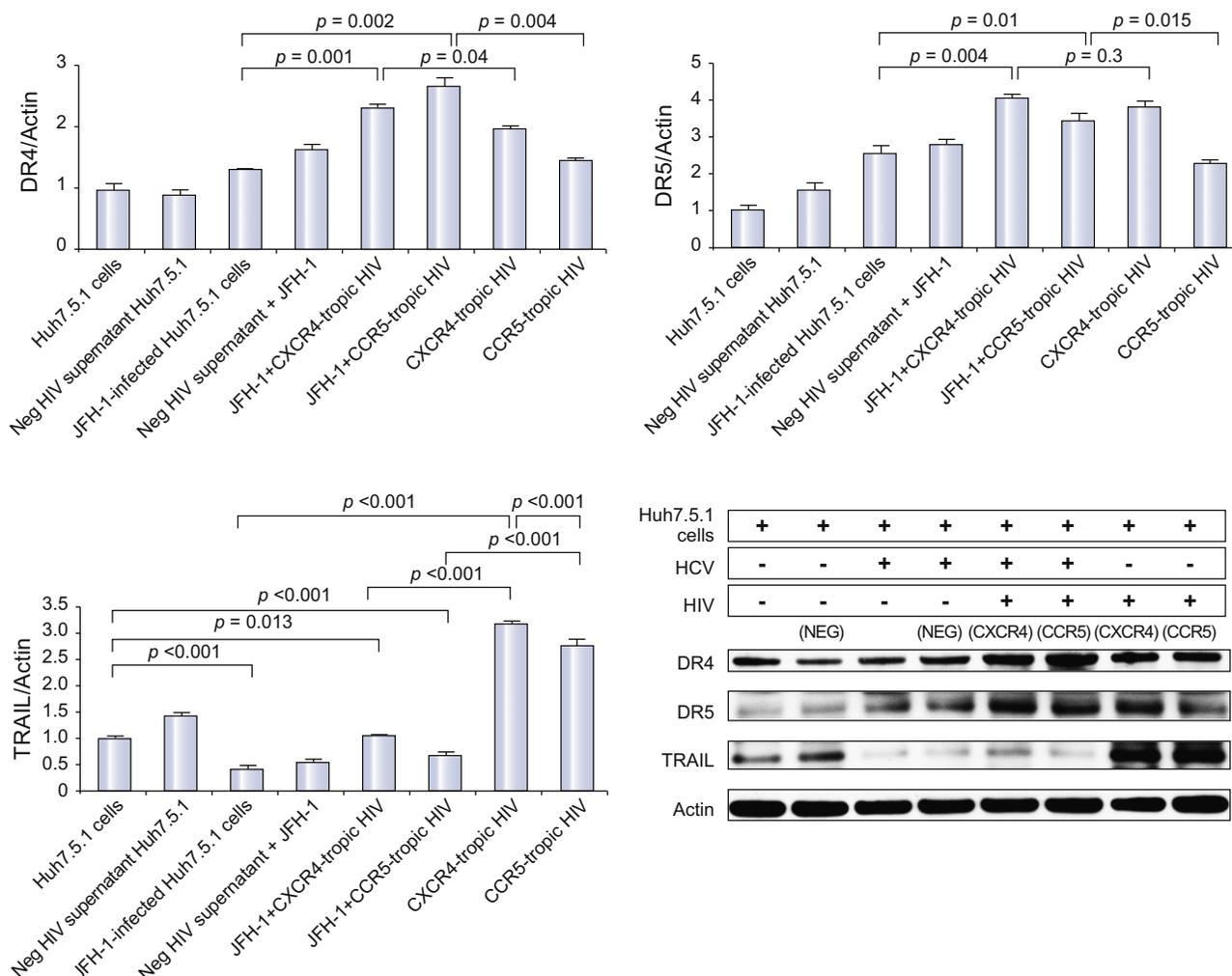
**Fig. 4. Increased DR4 and DR5 mRNA levels in HCV-infected Huh7.5.1 cells in the presence of heat-inactivated HIV compared to JFH1-infected or HIV-treated Huh7.5.1 cells.** Total cellular RNA was harvested, and DR4 (A), DR5 (B), TRAIL (C) and  $\beta$ -actin mRNA levels were quantified by real-time PCR. Each treatment was performed in triplicate. DR4, DR5, and TRAIL levels were normalized to  $\beta$ -actin levels to calculate DR4, DR5, and TRAIL/actin arbitrary units. Real-time PCR demonstrates increased relative DR4 and DR5 mRNA levels in HCV-infected, HIV-treated Huh7.5.1 cell compared to JFH1-infected or HIV treated Huh7.5.1 cells. DR5 mRNA levels were significantly increased in HCV-infected Huh7.5.1 cells in the presence of heat-inactivated HIV compared to JFH1-infected or HIV-treated Huh7.5.1 cells ( $p < 0.05$ ) and DR4 mRNA was moderately increased ( $p = 0.05$ ). In the case of TRAIL, mRNA levels were decreased in the presence of HCV. Lane #1 Huh7.5.1, #2 Huh7.5.1 + negative supernatant HIV, #3 JFH1, #4 JFH1 + negative supernatant HIV, #5 JFH1 + CXCR4-tropic HIV, #6 JFH1 + CCR5 tropic HIV, #7 CXCR4-tropic HIV, #8 CCR5-tropic HIV.

response, these two phases have not been linked until recently [14].

Because of their shared routes of transmission, co-infection with HCV is increasingly a major cause of morbidity and mortality among HIV<sup>+</sup> persons [4]. For instance, it is well established that HIV<sup>+</sup>/HCV<sup>+</sup> persons experience more progressive liver fibrosis and higher rates of cirrhosis, liver failure, and death than HIV<sup>-</sup>/HCV<sup>+</sup> persons [33–38] despite successful control of HIV with antiretroviral therapy.

The mechanisms underlying the accelerated evolution of CHC in HCV/HIV coinfection are not known. We have previously shown [30] that the addition of heat-inactivated HIV indirectly regulates HCV replication and, ultimately, the fibrogenic actions of HCV, by augmenting TGF- $\beta$ 1 expression in HCV-infected hepatocytes. Furthermore, this increase in HCV replication was abrogated by pre-incubation with neutralizing antibody to CXCR4

and CCR5. These data, thereby, provided evidence for an indirect effect of HIV virus signaling through engagement of its co-receptor on the surface of Huh7.5.1 cells rather than through the infection itself. Another possible explanation for accelerated fibrogenesis is enhanced hepatocyte apoptosis. In the current study, we now extend our previous findings to demonstrate that heat-inactivated HIV can also augment HCV-mediated apoptosis. The observed increased apoptosis in JFH1-infected, heat-inactivated HIV-treated Huh7.5.1 cells, compared to JFH1 or heat-inactivated HIV-treated Huh7.5.1 cells alone, may contribute to more progressive liver fibrosis in HCV–HIV coinfecting patients. Because we also found that HIV alone can moderately induce hepatocyte apoptosis, these data suggest that HIV predisposes the liver to a multitude of second hits, including HCV and HBV co-infection, both of whose natural history are accelerated in HIV-infected persons.



**Fig. 5. Increased DR4 and DR5 protein expression in HCV-infected Huh7.5.1 cells in the presence of heat-inactivated HIV compared to JFH1-infected or HIV-treated Huh7.5.1 cells.** Protein lysates were harvested for Western blot for DR4, DR5 and TRAIL. Corresponding densitometry was performed. We found that protein expression of DR4 and DR5 was increased in JFH1-infected, heat-inactivated HIV-treated Huh7.5.1 cells compared to JFH1 or HIV-treated Huh7.5.1 cells alone ( $p < 0.01$  for each). Protein expression of TRAIL was decreased in the presence of HCV. Lane#1 Huh7.5.1, #2 Huh7.5.1 + negative supernatant HIV, #3 JFH1, #4 JFH1 + negative supernatant HIV, #5 JFH1 + CXCR4-tropic HIV, #6 JFH1 + CCR5-tropic HIV, #7 CXCR4-tropic HIV, #8 CCR5-tropic HIV.

Several studies have postulated an autocrine TRAIL-dependent apoptosis of hepatocytes [39,40]. Other studies suggest that a TRAIL autocrine loop does not play a major role in TRAIL-induced sensitization [41]. HCV-dependent up-regulation of TRAIL and apoptosis induction in a novel hepatoma cell line have been described [42]. Others [43] have described that HIV selectively up-regulates TRAIL-R2 expression in hepatocytes and confers an acquired sensitivity to TRAIL-mediated apoptosis.

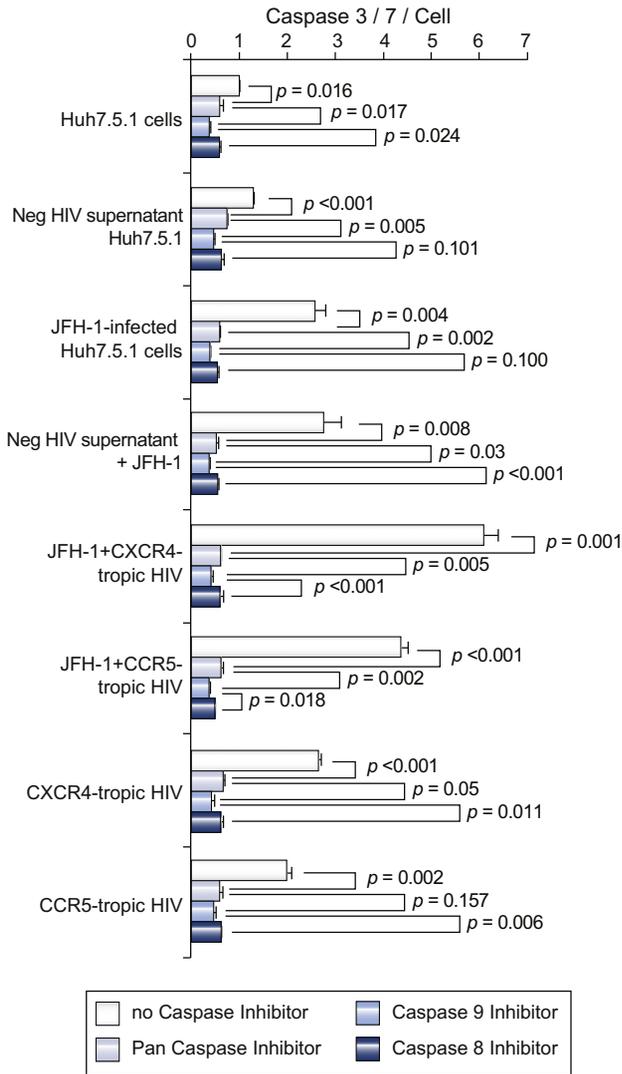
In the present study, we report that HCV-infected, HIV-treated Huh7.5.1 cells express higher levels of TRAIL receptor (DR4 and DR5), and apoptosis than do HCV or HIV-treated Huh7.5.1 cells alone. However, TRAIL was decreased in the presence of HCV, suggesting that an autocrine loop is not operative. Rather, our data suggest either that increased DR4 or DR5 expression or sensitivity may compensate for decreases in TRAIL release or that additional death receptor pathways contribute to this decreased release. The finding that circulating TRAIL levels are increased in HIV mono-infection [44] compared to no infection or HCV

mono-infection suggests another basis for the predisposition of the HIV-infected liver to enhanced injury from other sources.

We investigated the involvement of caspase pathways using several caspase inhibitors. Lan et al. [41] previously demonstrated that the mitochondrially-activated caspase pathway is triggered in HCV infection by the use of selective caspase inhibitors. We also sought to assess the operative pathways in HCV-HIV co-infection using these inhibitors. Our results indicate that the HCV-HIV mediated apoptosis signal is transduced from DR4 and DR5 through activation of caspase 8, which in turn activates the mitochondrial signaling pathway, whose involvement was confirmed by our demonstration of Bid cleavage and cytochrome C activation after HCV and HIV coinfection. Furthermore, because a caspase-8 inhibitor was equally as effective as pancaspase and caspase-9 inhibitors, our data imply that TRAIL-mediated apoptosis engages both the caspase-8 and the mitochondrial pathways.

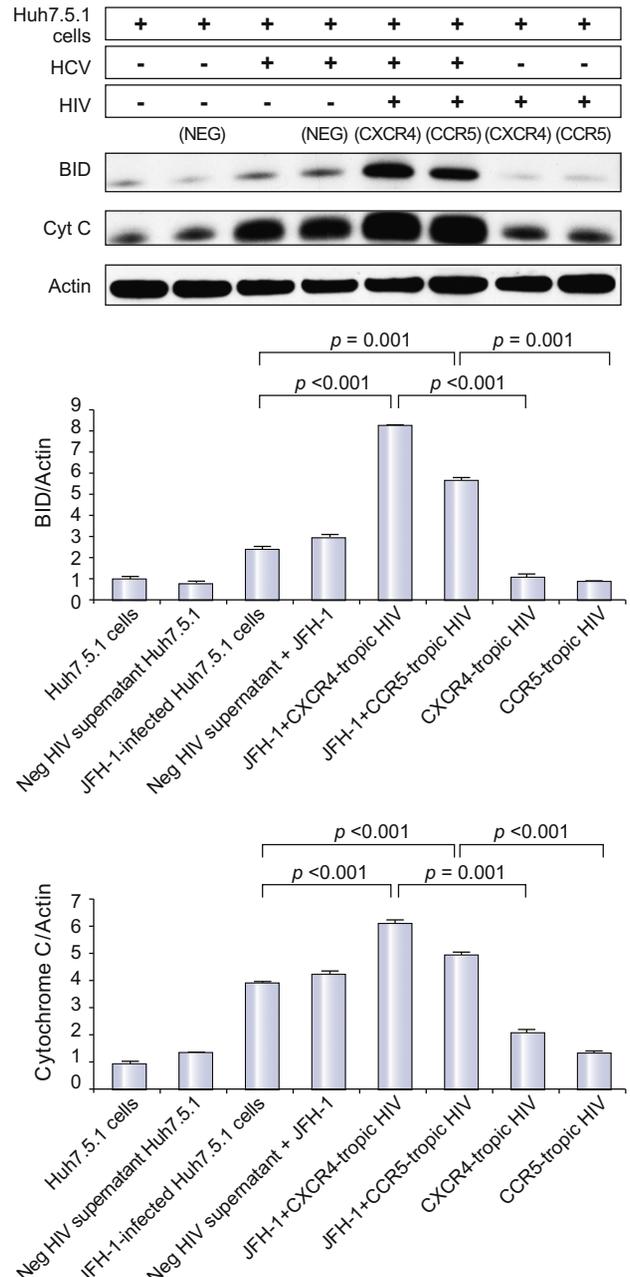
Using an infectious tissue culture model, we provide evidence that HCV and HIV infection increases hepatocyte apoptosis

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**Fig. 6. HCV-HIV induced apoptosis is dependent on caspases related to the mitochondrial pathway.** Naïve Huh7.5.1 cell and HCV and/or HIV treated Huh7.5.1 cells were incubated with 20  $\mu$ M of a variety of caspase inhibitors in 96-well plates (10,000 cell/well) for 6 h prior to Caspase-Glo 3/7 assay. Inhibitors used included the pan-caspase inhibitor Z-VAD-FMK, the caspase 8 inhibitor Z-LETD-FMK, and the caspase 9 inhibitor Z-LEHDFMK. The pan-caspase inhibitor zVAD-fmk decreased HCV, HIV, or HCV-HIV induced caspase 3/7 activity by over 4-fold ( $p < 0.01$ ). The caspase 8 inhibitor blocked HCV, HIV, or HCV-HIV induced apoptosis to the same degree as that inhibited by zVAD-fmk. In addition, a caspase 9 inhibitor significantly blocked apoptosis induced by HCV, HIV, or HCV-HIV comparably to the pancaspase and caspase 8 inhibitors. The y axis refers to caspase 3/7 activity per cell. Lane #1 Huh7.5.1, #2 Huh7.5.1 + negative supernatant HIV, #3 JFH1, #4 JFH1 + negative supernatant HIV, #5 JFH1 + CXCR4 tropic HIV, #6 JFH1 + CCR5 tropic HIV, #7 CXCR4-tropic HIV, #8 CCR5-tropic HIV.

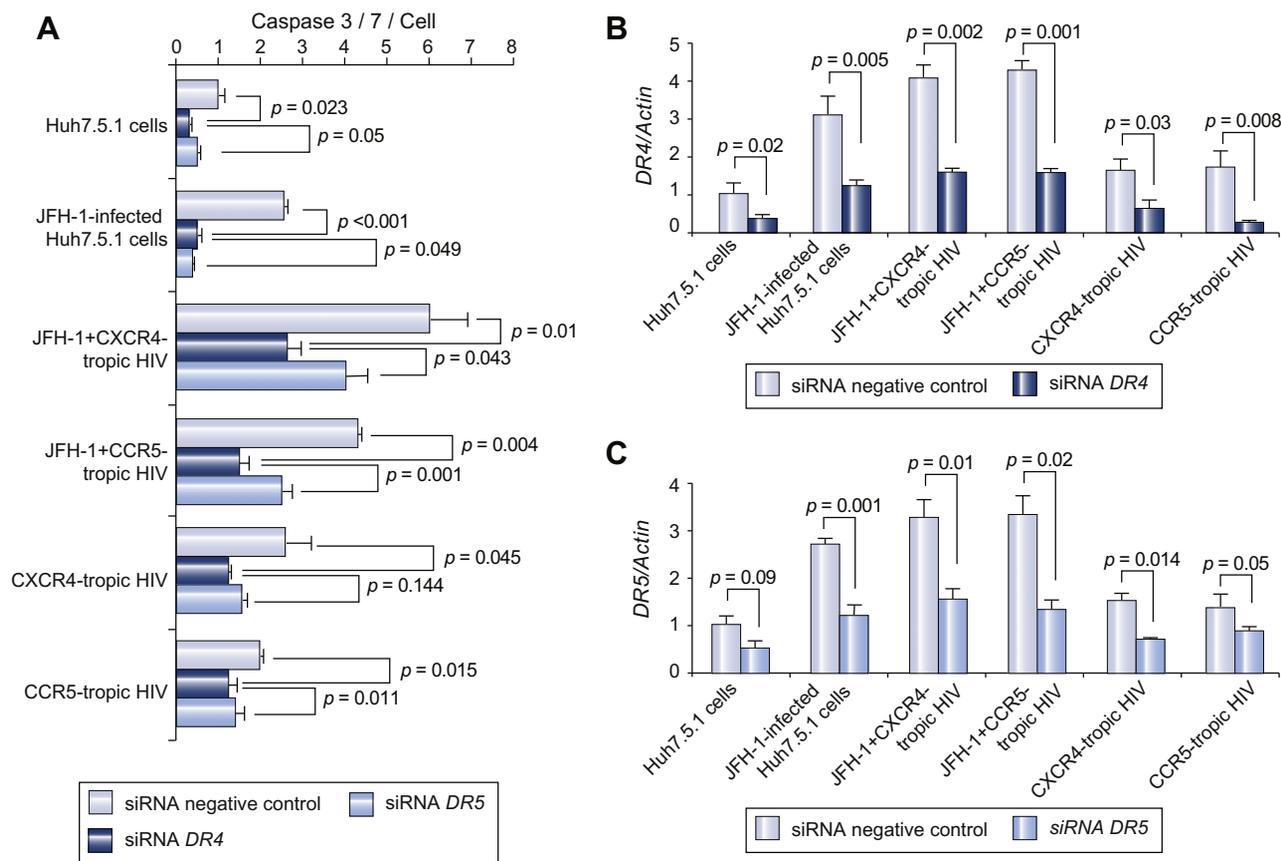
compared to HCV or HIV alone. We propose a model in which HCV and HIV each induces TRAIL receptors (DR4 and DR5), which in turn activate caspase 8 and the mitochondrial pathway. Our data further provide an additional plausible mechanism for the accelerated disease progression observed in HCV-HIV co-infection. Efforts to specifically inhibit this apoptotic program may be warranted to retard liver disease progression in co-infected persons.



**Fig. 7. Analysis of Bid cleavage and Cytochrome C levels.** Protein lysates were harvested for Western blot analysis of the mitochondrial apoptosis activating protein Bid and cytochrome C. Corresponding densitometry was performed. While HCV alone induced the activation of Bid cleavage and cytochrome C release, addition of heat-inactivated HIV substantially augmented this induction (3.47-fold for BID, 1.60-fold for cytochrome C by densitometry). Lane #1 Huh7.5.1, #2 Huh7.5.1 + negative supernatant HIV, #3 JFH1, #4 JFH1 + negative supernatant HIV, #5 JFH1 + CXCR4-tropic HIV, #6 JFH1 + CCR5-tropic HIV, #7 CXCR4-tropic HIV, #8 CCR5-tropic HIV.

### Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.



**Fig. 8. Effects of DR4 and DR5 knockdown on HCV-HIV induced apoptosis.** The indicated siRNAs were transfected into HCV-HIV infected Huh7.5.1 cells in 96-well plate. The siRNAs used for gene knockdown included DR4-, DR5-targeted, or negative control siRNAs. Real-time PCR assay was performed to measure DR4 and DR5 expression levels in these siRNA-transfected cells. (B and C) DR4 and DR5 mRNA levels were decreased by over 2-fold in DR4- and DR5-targeted siRNA-transfected cells compared to negative siRNA-transfected cells. (A) Caspase-Glo 3/7 staining revealed that HCV-HIV induced apoptosis was suppressed by nearly 2-fold in DR4- and DR5-targeted siRNA-transfected cells compared to negative control siRNA-transfected cells. Lane #1 Huh7.5.1, #2 JFH1, #3 JFH1 + CXCR4-tropic HIV, #4 JFH1 + CCR5-tropic HIV, #5 CXCR4-tropic HIV, #6 CCR5-tropic HIV.

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