

# Intrahepatic Cytokine Expression is Downregulated During HCV/HIV Co-Infection

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HIV co-infection is associated with reduced HCV treatment response rates and accelerated HCV-related liver disease. Cytokines play an important role in regulating hepatic inflammation and fibrogenesis during chronic HCV infection, yet the roles of HIV and/or its therapies on cytokine expression are unknown. Total RNA was extracted from liver biopsies of 12 HCV mono-infected and 14 HCV/HIV co-infected persons. We used real-time PCR to quantify cytokines that contribute to innate and adaptive immune responses, including IFN $\alpha$ , IFN $\gamma$ , TNF $\alpha$ , TGF $\beta$ <sub>1</sub>, IL-2, IL-4, IL-8, IL-10, and IL-12p40. Positive- and negative-strand HCV RNA levels were quantified using a molecular beacon approach. Detection of positive-strand HCV RNA was 100% in both groups; negative-strand HCV RNA was detected in four (33%) HCV mono-infected persons and in nine (64%) HCV/HIV co-infected persons. Median strand-specific HCV RNA levels were not significantly different between the two groups. Detection rates of cytokine mRNAs were lower for the HCV/HIV co-infected group compared to the HCV mono-infected group; the detection rates for TNF $\alpha$ , IL-8, and IL-10 were statistically significant. Overall, cytokine mRNA quantities were lower for HCV/HIV co-infected compared to HCV mono-infected persons, with the exception of TGF $\beta$ <sub>1</sub>. These data suggest that a defect in cytokine activation may occur in HCV/HIV co-infected persons that limits efficient clearance of HCV from the liver. **J. Med. Virol.** 78:202–207, 2006.

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## INTRODUCTION

Because of their shared routes of transmission, co-infection with hepatitis C virus (HCV) and human immunodeficiency virus (HIV) is common, representing 15%–30% of all HIV-infected persons and 5%–10% of all HCV-infected persons [Alter et al., 1999; Sherman et al., 2002]. As AIDS-related morbidity and mortality decrease in large part due to the effectiveness of highly active antiretroviral therapy, HCV has emerged as a major source of morbidity and mortality for these persons [Bica et al., 2001; Tedaldi et al., 2003]. HIV co-infection is associated with significantly reduced HCV treatment response rates [Chung et al., 2004; Torriani et al., 2004] and has an adverse impact on liver fibrosis, HCV RNA levels, and HCV disease progression (reviewed in [Sulkowski and Thomas, 2003]). The mechanisms by which these two viruses interact remain unclear; nonetheless, defining these interactions is critical for the design of more effective HCV treatments for co-infected individuals.

Cytokines, as key immunologic signaling molecules, play a central role in generating immune responses to viral infections through direct inhibition of viral replication and by regulation of the host response. For instance, T-helper type 1 (Th1) cytokines produce a self-limited, acute response to HCV [Jacobson-Brown

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and Neuman, 2001]. Their levels are upregulated in chronic HCV patients compared to uninfected controls and are correlated with histological fibrosis [Napoli et al., 1996]. In contrast, Th2 cytokines inhibit the potentially damaging effects of Th1 cytokines following acute viral infection and are decreased during chronic HCV infection [Spanakis et al., 2002]. Pro-inflammatory cytokines, such as interleukin 8 (IL-8), are associated with severe hepatitis activity, inflammation, and decreased interferon sensitivity [Fukuda et al., 1996; Shimoda et al., 1998; Polyak et al., 2001a,b], while tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) is associated with hepatic inflammation and steatosis [Neuman et al., 2002; Gochee et al., 2003]. The fibrogenic cytokine transforming growth factor  $\beta_1$  (TGF $\beta_1$ ) is also a key regulator of liver fibrosis [Schuppan et al., 2002] and may be upregulated during HIV infection [Pal and Schnapp, 2004]. Fibrogenic cytokines may, nonetheless, be counterbalanced, in part, by antifibrotic cytokines, such as the interferons. Thus, any perturbation of the delicate balance that exists among intrahepatic cytokines could have profound consequences for liver disease, as well as sensitivity to HCV treatment [Fukuda et al., 1996; Masaki et al., 2002; Gochee et al., 2003].

Several groups have shown that HCV-specific adaptive immune responses are generally very weak during HIV co-infection, suggesting that HCV may persist as a result of impaired cell-mediated immune responses [Valdez et al., 2000; Lauer et al., 2002; Anthony et al., 2004]. Because cytokine dysregulation is a hallmark of HIV infection [Breen, 2002], HIV co-infection may contribute to this weakened immunologic state by altering the cytokine milieu and/or by providing inappropriate immunologic signals. In contrast, recent data suggest the beneficial effects of ART utilization in HCV/HIV co-infected persons with respect to liver disease progression [Qurishi et al., 2003; Mehta et al., 2005]. To elucidate potential immunologic interactions between HIV and HCV, we retrospectively examined cytokine expression in HCV mono-infected and HCV/HIV co-infected persons to determine how HIV specifically alters the intrahepatic environment observed during chronic HCV infection.

## METHODS

Individuals attending the HIV/HCV co-infection clinics at the Massachusetts General Hospital (Boston, MA) and the Miriam Hospital (Providence, RI) who underwent liver biopsies as part of routine medical care were enrolled in this cross-sectional study. Two centimeter sections of tissue were obtained at biopsy, with ~50% being frozen at  $-80^{\circ}\text{C}$ . Eight patients undergoing liver biopsies for a variety of non-HCV related etiologies were also included as uninfected controls. The Institutional Review Boards of the participating institutions approved this study.

Total RNA was extracted from liver biopsies of 12 HCV mono-infected and 14 HCV/HIV co-infected persons using the High Pure RNA Tissue kit (Roche Diagnostics;

Meylan, France) and eluted in 100  $\mu\text{l}$  of RNase-free water. Cytokines were selected based upon their Th1 (IFN $\gamma$ , TNF $\alpha$ , and IL-2) or Th2 (IL-4 and IL-10) phenotype, as well as their inflammatory (IL-8 and IL-12p40) or fibrogenic properties (IFN $\alpha$  and TGF $\beta_1$ ). Intrahepatic mRNA levels were quantified by real-time PCR (rtPCR) using cytokine specific primer sets designed and purchased from Search-LC (Heidelberg, Germany; www.Search-LC.com). Briefly, 10  $\mu\text{l}$  of RNA was reverse transcribed at  $50^{\circ}\text{C}$  for 1 hr using the Thermoscript Reverse Transcriptase kit (Invitrogen; Carlsbad, CA) and oligo(dT). A denaturing step was performed at  $95^{\circ}\text{C}$  for 5 min. The resulting cDNA was diluted 1:10, and 10  $\mu\text{l}$  were used for amplification. Real-time PCR was carried out with the LC Fast start DNA SYBR Green I kit and the LightCycler apparatus (Roche Diagnostics) according to the protocol provided with the parameter specific kits: 35 amplification cycles, denaturation at  $95^{\circ}\text{C}$ , primer annealing at  $68^{\circ}\text{C}$  with touch-down to  $58^{\circ}\text{C}$ , amplicon extension at  $72^{\circ}\text{C}$ . To control for specificity of the amplification products, a melting curve analysis was performed. Expression levels of each cytokine were calculated using the LightCycler relative quantification software. To control for variations in the amount of liver material obtained and the quality of viral and cellular RNA, all cytokine values were normalized to a housekeeping gene (HKG)—cyclophilin B—using uninfected liver tissue (BD Bioscience; San Jose, CA) as the calibrator according to the following formula:  $[\text{cytokine}_{\text{sample}} \cdot \text{HKG}_{\text{sample}}] / [\text{cytokine}_{\text{calibrator}} \cdot \text{HKG}_{\text{calibrator}}]$ . Therefore, the rtPCR approach utilized here did not determine a copy number of the target cytokine gene; rather, all results were expressed as a normalized ratio with arbitrary units. Use of a commercial liver RNA “calibrator” further served to normalize for the divergent sensitivities of target genes and to provide a constant calibration point between reactions. The limit of detection for each cytokine was one transcript per microliter of cDNA. Only samples with detectable levels of HKG were analyzed further.

Positive- and negative-strand HCV RNA levels were quantified using a previously described strand-specific molecular beacon method that amplifies a highly conserved region of the 5' untranslated region [Komurian-Pradel et al., 2004]. Briefly, RNA was reverse transcribed with the Thermoscript Reverse Transcriptase kit using either the RC21 primer (5'-CTC CCG GGC CAC TCG CAA GC-3') or the tag-RC1 primer (5'-GGC CGT CAT GGT GGC GAA TAA GTC TAG CCA TGG CGT TAG TA-3') for the positive-strand and the negative-strand assay, respectively. The reaction was then treated with 20 U of RNaseOut. Real-time PCR was carried out with 2  $\mu\text{l}$  of cDNA, 2 pmol of molecular beacon (5'-FAM GCTAGC ATT TGG GCG TGC CCC CGC IAG A GCTAGC DABCYL-3') and 5 pmol of RC1 (5'-GTC TAG CCA TGG CGT TAG TA-3') and RC21 primers for positive-strand amplification or with 5 pmol of Tag (5'-GGC CGT CAT GGT GGC GAA TAA-3') and RC21 primers for negative-strand amplification. The reaction was carried out with the LC Fast start DNA Master

hybridization probes and the LightCycler apparatus. The PCR protocol consisted of an initial denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C, primer annealing at 55°C, and amplicon extension at 72°C, with a temperature transition rate of 20°C/sec. Quantification was carried out using an external standard curve generated as described previously [Komurian-Pradel et al., 2001]. The lower limit of detection for this assay was 10 copies of positive-strand HCV RNA and 100 copies of negative-strand HCV RNA per reaction.

Cytokine and HCV RNA detection rates were compared using Fisher's exact test. Cytokine mRNA quantities were compared using the Wilcoxon rank sum test with the lowest rank imputed when cytokine levels were undetectable. Spearman's correlation test was used to investigate the linear relationship between virologic and clinical variables and cytokine levels. All *P*-values were two-sided, and only *P*-values less than 0.05 were considered statistically significant. As these were exploratory analyses, no adjustments were made for multiple comparisons.

## RESULTS

The HCV mono-infected and HCV/HIV co-infected groups did not differ with respect to gender, HCV genotype, ALT or AST level, fibrosis score, or hepatic activity index (Table I). Antiretroviral therapy (ART) utilization in the HCV/HIV co-infected group was as

follows: at the time of liver biopsy, nine were being treated with ART, while five were not treated. ART regimens included one protease inhibitor (PI) + 2 nucleoside reverse transcriptase inhibitors (NRTIs) (*N* = 2); 2 PIs + ≥2 NRTIs (*N* = 3); 3 NRTIs (*N* = 1), and 1 non-nucleoside reverse transcriptase inhibitor (NNRTIs) + 3 NRTIs (*N* = 2). The ART regimen was not reported for one individual. At the time of biopsy, none of the 26 individuals enrolled in the study were receiving any HCV treatment. Median CD4 cell count and HIV RNA level were 282 cells/mm<sup>3</sup> and 2.6 log<sub>10</sub> copies/ml, respectively, in the HCV/HIV co-infected group. There was a trend towards higher serum HCV viral loads in the HCV/HIV co-infected group compared to the HCV mono-infected group (median levels: 6.31 vs. 5.83 log<sub>10</sub> copies/ml; *P* = 0.09).

Detection of intrahepatic positive-strand HCV RNA was 100% in both groups. Intrahepatic negative-strand HCV RNA—a replication intermediate indicative of active viral replication—was detected in 4 of 12 (33%) and 9 of 14 (64%) persons from the HCV mono-infected and HCV/HIV co-infected groups, respectively, although this difference was not statistically significant (*P* = 0.19). The median positive-strand HCV RNA level was 5.5 and 5.8 log<sub>10</sub> copies of HCV/μg of RNA for the HCV mono-infected and HCV/HIV co-infected groups, respectively (*P* = 0.14) (Fig. 1). The median negative-strand HCV RNA level was not defined for the HCV mono-infected group and was 3.96 log<sub>10</sub> copies of HCV/μg of RNA for the HCV/HIV co-infected

TABLE I. Study Cohort Characteristics

	HCV mono-infected ( <i>N</i> = 12)	HCV/HIV co-infected ( <i>N</i> = 14)	<i>P</i> -value
Gender			
Female	1	2	NS <sup>a</sup>
Male	11	12	
HCV genotype			NS <sup>a</sup>
1	7	8	
2	1	2	
3	2	1	
Unknown	2	3	
Median ALT	104	110	NS <sup>b</sup>
Median AST	90	94	NS <sup>b</sup>
Median serum HCV RNA (log <sub>10</sub> copies/ml)	5.83	6.31	0.09 <sup>b</sup>
Fibrosis score			NS <sup>c</sup>
0	2	3	
1–2	5	5	
3	1	2	
4	2	1	
5–6	2	3	
Median	2	2	
Hepatic activity index			NS <sup>c</sup>
0–2	3	4	
3–4	5	4	
≥5	4	6	
Median	3.5	4	

NS, not significant.

<sup>a</sup>Fisher's exact test.

<sup>b</sup>Wilcoxon rank sum test.

<sup>c</sup>Exact Kruskal–Wallis test.

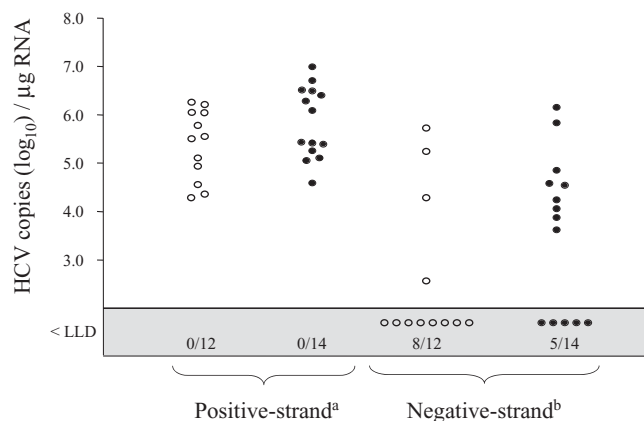


Fig. 1. Strand-specific HCV RNA levels. HCV mono-infected (white); HCV/HIV co-infected (black). The lower limit of detection (LLD) is 10 copies of negative-strand HCV RNA and 100 copies of positive-strand HCV RNA per reaction. **a:**  $P=0.14$  comparing the median HCV RNA levels for the two groups. **b:**  $P=0.23$  comparing the median HCV RNA levels for the two groups.

group ( $P=0.23$ ). These findings are consistent with those of other studies demonstrating lower intrahepatic quantities of negative-strand HCV RNA relative to positive-strand HCV RNA [Laskus et al., 1998; Komurian-Pradel et al., 2004]. As expected, neither positive- nor negative-strand HCV RNA was detected in the livers of uninfected controls (data not shown).

Due to the limited biopsy material available for analysis, we selected 10 cytokines for further study based upon their Th1/Th2 phenotype or their putative role in hepatic inflammation and/or fibrosis. The highly sensitive real-time PCR approach utilized here can detect as little as one transcript per microliter of cDNA; thus, both cytokine detection rates and quantities were measured. Detection rates for individual cytokine mRNAs were lower for the HCV/HIV co-infected group compared to the HCV mono-infected group (Table II). For the two infection groups, detection rates were as follows: IFN $\alpha$  (1/12 in the HCV mono-infected group vs. 0/14 in the HCV/HIV co-infected group), IFN $\gamma$  (6/12 vs. 2/14), TNF $\alpha$  (10/12 vs. 5/14), TGF $\beta_1$  (12/12 vs. 13/14),

TABLE II. Intrahepatic Cytokine mRNA Detection Rates

Cytokine	HCV mono-infected	HCV/HIV co-infected	P-value
IFN $\alpha$	8% (1/12)	0% (0/14)	NS
IFN $\gamma$	50% (6/12)	14% (2/14)	NS
TNF $\alpha$	83% (10/12)	36% (5/14)	0.02
TGF $\beta_1$	100% (12/12)	93% (13/14)	NS
IL-2	50% (6/12)	14% (2/14)	NS
IL-4	Not detected	Not detected	
IL-8	83% (10/12)	29% (4/14)	0.008
IL-10	67% (8/12)	7% (1/14)	0.003
IL-12p40	Not detected	Not detected	

NS, not significant.

IL-2 (6/12 vs. 2/14), IL-8 (10/12 vs. 4/14), and IL-10 (8/12 vs. 1/14). IL-4 and IL-12p40 mRNAs were not detected in any livers tested. These differences in detection rates between the two groups reached statistical significance for TNF $\alpha$  ( $P=0.02$ ), IL-8 ( $P=0.008$ ), and IL-10 ( $P=0.003$ ). For each cytokine, detection rates in the HCV mono-infected group were very similar to those of the uninfected control group (data not shown).

We also quantified intrahepatic cytokine mRNA expression in these two groups (Table III). It is important to note that cytokine quantities were not absolute values; rather, the particular real-time PCR approach utilized here compared cytokine levels relative to that of healthy liver tissue used as a calibrator. Moreover, the quantity and quality of each biopsy sample was normalized to a housekeeping gene. Due to the low detection rates in the HCV/HIV co-infected group, medians were often not defined for this group; instead, the 75th percentiles were reported. Overall, median levels were lower for the HCV/HIV co-infected group compared to the HCV mono-infected group for all cytokines measured with the notable exception of TGF $\beta_1$ . The median levels of IFN $\gamma$  (0.1 in the HCV mono-infected group vs. not defined in the HCV/HIV co-infected group,  $P=0.045$ ), IL-8 (38.5 vs. not defined,  $P=0.005$ ), and IL-10 (7.0 vs. not defined,  $P=0.011$ ) mRNA were significantly lower in the HCV/HIV co-infected group. We also found a modest increase in TGF $\beta_1$  levels associated with HCV/HIV co-infection (2.7 in the HCV mono-infected group vs. 3.9 in the HCV/HIV co-infected group), although this difference did not achieve statistical significance.

We were also interested in examining the relationships among virologic and clinical variables. Among the HCV/HIV co-infected group, positive-strand HCV RNA levels in the liver were not correlated with CD4 cell count or HIV RNA levels (data not shown). Similarly, among the most common cytokines detected in the co-infected group—IL-8, TNF $\alpha$ , and TGF $\beta_1$ —intrahepatic levels were not correlated with fibrosis score, hepatic activity index, CD4 cell count, HIV RNA levels, or HCV RNA levels in the liver (data not shown), although our ability to detect important correlations is likely limited

TABLE III. Intrahepatic Cytokine mRNA Quantities

Cytokine	HCV mono-infected	HCV/HIV co-infected	P-value
IFN $\gamma$	0.1 (21.4)	ND (ND)	0.045
TNF $\alpha$	14.6 (18.4)	ND (43.7)	NS
TGF $\beta_1$	2.7 (5.1)	3.9 (7.1)	NS
IL-2	1.8 (15.0)	ND (ND)	NS
IL-8	38.5 (1576.2)	ND (1.5)	0.005
IL-10	7.0 (59.8)	ND (ND)	0.011

Results are expressed as relative units as described in the Methods section. The limit of detection for each cytokine is one transcript per microliter of cDNA. ND indicates that the quantile is not defined because of low detection rates. Numbers in parentheses indicate the 75th percentile. NS, not significant.

by the number of available liver biopsies. However, hepatic activity index and fibrosis score were positively correlated ( $P = 0.002$ ). The low detection rates of HCV negative-strand RNA precluded further analysis of this variable.

## DISCUSSION

Although the role of HCV in HIV disease progression remains controversial, HIV co-infection clearly accelerates progression of HCV-related fibrosis and liver disease [Darby et al., 1997; Soto et al., 1997; Benhamou et al., 2001]. Moreover, there appears to be a delicate balance between the beneficial and detrimental effects of ART on the liver that requires further study [Sulkowski, 2004].

While HCV-specific humoral and cellular immune responses may occur during HCV infection, viral persistence nonetheless occurs in the majority of persons. Thus, it is reasonable to assume that persistent HCV replication may result from inadequate or ineffective HCV-specific immune responses and/or viral proteins that promote immune evasion (reviewed in [Freeman et al., 2001]). Moreover, these very immune responses, while unable to achieve viral clearance, may actually contribute to progressive hepatic injury through induction of pro-inflammatory and/or pro-fibrogenic mediators. As a profound perturbation of the cytokine environment is common during HIV infection, these processes may be further altered during HCV/HIV co-infection. Nonetheless, the intrahepatic cytokine milieu has not been adequately investigated during HCV/HIV co-infection.

Our retrospective analysis was limited by the relatively small number of patients with available biopsy material, and by the lack of a large ART-naïve comparison group. Thus, caution must be taken when extrapolating our results to larger HCV/HIV co-infected populations. Given the cross-sectional nature of our study, we were not able to investigate HCV-specific immune responses present in the liver. Nonetheless, this is one of the first studies to comprehensively compare multiple intrahepatic regulators of the immune response, inflammation, and fibrosis in HCV mono-infected and HCV/HIV co-infected individuals. Surprisingly, we found that cytokine mRNA detection rates, particularly for  $\text{TNF}\alpha$ , IL-8, and IL-10, were lower among HCV/HIV co-infected persons. Given the highly sensitive rtPCR approach utilized to amplify cytokine mRNAs, it is highly unlikely that undetectable cytokine values were due to small sample quantity or quality as the housekeeping gene, cyclophilin B, was routinely amplified from all samples.

With the notable exception of  $\text{TGF}\beta_1$ , cytokine mRNA levels were also reduced significantly in HCV-positive persons co-infected with HIV. We speculate that reduction of pro-inflammatory (but not pro-fibrogenic) cytokines may be one mechanism by which antiretroviral therapy successful slows the progression of liver disease in HCV/HIV co-infected persons. Moreover, the net

suppression of intrahepatic cytokines may lead to an imbalance between pro-fibrogenic and anti-fibrogenic cytokines, thus favoring liver fibrosis and HCV replication in co-infected persons. Our finding of increased  $\text{TGF}\beta_1$  mRNA levels among HCV/HIV co-infected persons is intriguing as  $\text{TGF}\beta_1$  is a potent inducer of fibrogenesis in the effector cells of hepatic fibrosis (e.g., activated hepatic stellate cells and myofibroblasts). However, fibrogenesis proceeds more quickly when additional pro-fibrogenic stimuli are present (reviewed in [Schuppan et al., 2002]). Thus, HIV co-infection may further contribute to liver fibrosis in HCV-positive persons by inducing  $\text{TGF}\beta_1$  expression. Furthermore,  $\text{TGF}\beta_1$  production significantly reduces the  $\text{IFN}\gamma$  response of  $\text{CD8}^+$  cells to viral infection [Garba et al., 2002], suggesting an important mechanism by which HIV, through increased  $\text{TGF}\beta_1$  and decreased  $\text{IFN}\gamma$  expression, may promote HCV persistence.

While we also found a higher detection level of negative-strand HCV RNA in the HCV/HIV co-infected group compared to the HCV mono-infected group, negative-strand was detected less frequently than positive-strand in both groups. We cannot exclude the possibility of low levels replication of HCV below the assay's limit of detection. Nonetheless, these findings are consistent with other studies that have demonstrated 1–2 log lower quantities of negative-strand HCV RNA compared to positive-strand HCV RNA [Laskus et al., 1998; Komurian-Pradel et al., 2004].

Collectively, our data suggest that a defect in cytokine activation may occur in HCV/HIV co-infected persons. While we are unaware of other studies that have comprehensively measured qualitative and quantitative differences in intrahepatic expression of a variety of cytokines associated with HCV mono-infection compared to HCV/HIV co-infection, one other study has measured intrahepatic interferon levels during HCV mono-infection and HCV/HIV co-infection [Abbate et al., 2004]. The authors found that  $\text{IFN}\gamma$  was not detectable during co-infection. Furthermore,  $\text{IFN}\alpha$  and  $\text{IFN}\beta$  levels were higher in livers with less fibrosis, suggestive of a protective role for these cytokines against fibrosis [Abbate et al., 2003]. In the current study, we found a decrease in  $\text{IFN}\gamma$  detection rates and mRNA levels associated with co-infection. However, we also found very low detection rates of  $\text{IFN}\alpha$  (1 of 26 livers tested) regardless of HIV infection status, in contrast to the previous study. These differences could be explained by the use of HCV therapies in our population and/or differing degrees of immunosuppression among HCV/HIV co-infected persons. Further study is warranted to determine the impact of anti-retroviral therapy on intrahepatic cytokine levels and its correlation with reconstitution of an effective HCV-specific immune response. Additional studies of cytokine production by specific liver-derived cell types in persons that control HIV without therapy versus those with HIV suppression while on therapy are also necessary.

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