Effect of Exposure to Injection Drugs or Alcohol on Antigen-Specific Immune Responses in HIV and Hepatitis C Virus Coinfection

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Background. Ongoing substance use is a potential confounder for immunological studies on hepatitis C virus (HCV), but there is little in the literature regarding the effects of injection drug use (IDU) or alcohol on HCV-specific immune responses. We wanted to determine whether IDU or alcohol affected immune responses in HCV-infected and human immunodeficiency virus (HIV)/HCV coinfected subjects.

Methods. Eight-four subjects with HIV/HCV and 57 with HCV were classified as either injection drug users, drinkers, or nonusers based on questionnaire results. Immune responses were studied with enzyme-linked immunosorbent spot assay for interferon (IFN)–γ, interleukin (IL)–10, and tumor necrosis factor (TNF)–α against HCV proteins Core, NS3, and NS5 and recall antigens.

Results. Subjects with HIV/HCV, in aggregate, had significantly lower HCV-specific IFN-γ and TNF-α responses than subjects with HCV. However, HIV/HCV injection drug users had HCV-specific IFN-γ and IL-10 responses that were similar to those of HCV injection drug users and were significantly higher than in nonusers with HIV/HCV. Conversely, subjects who drank alcohol had similar immune responses to those who were abstinent, among both subjects with HIV/HCV and subjects with HCV.

Conclusions. Studies that examine IFN-γ or IL-10 immune responses in HIV/HCV-coinfected or HCV-infected persons need to consider current IDU. Alcohol, at levels consumed in this cohort, does not appear to have as much of an effect on antigen-specific immune responses.

Hepatitis C virus (HCV) is not considered cytopathic in most cases, and the liver inflammation and progressive fibrosis found in many patients with chronic viremia is believed to be due to an ongoing, ineffectual host immune response to persistent infection. It is paradoxical that HIV infection, an immunocompromised state, is associated with accelerated liver disease, and this suggests that the type of immune response may be as relevant as the quantity of immune response when defining specific immunological profiles that are associated with progressive HCV-related liver disease.

Many persons with HIV/HCV coinfection acquired both infections via parenteral injection drug use (IDU), and ongoing polysubstance abuse is common [1–3]. Persons with HIV/HCV coinfection and heavy alcohol consumption (>50 g/day) are at high risk of severe liver fibrosis [4, 5]. Alcohol modifies cellular and humoral immune responses in cell culture models as well as animal models; studies show an increase in tumor necrosis factor (TNF)–α production or a shift from a type 1 (interferon [IFN]–γ and interleukin [IL]–2) to a type 2 (IL-4 and IL-10) immune response in the presence of alcohol [6–10]. However, little has been reported on the effect of alcohol on HCV-specific cellular immune
responses in humans or about potential immune mechanisms by which alcohol modifies the disease course of HCV-related liver damage.

Similarly, in animal models, chronic morphine treatment leads to decreased IFN-γ and IL-2 and increased IL-4 and IL-5, which appears to be mediated by increasing GATA 3 expression and decreasing T-bet expression, thus shifting T helper cells to a type 2 phenotype [11]. The opioid antagonist, naloxone, can counteract the type 2 shift in cytokine production and increase IFN-γ and IL-2 production [12]. These data are consistent with an endogenous role of opioids in inhibiting excessive inflammatory responses with injury or other pain-inducing processes by augmenting anti-inflammatory type 2 responses and limiting proinflammatory type 1 responses [13, 14]. However, the effect of opiates on antigen-specific immune responses in humans with chronic HCV infection has not been studied.

It is important to understand how substance abuse affects antigen-specific immune responses because these immune responses may affect HCV-related liver disease outcomes. For example, early after acute HCV infection, it has been shown that persons who develop chronic HCV infection but maintain HCV-specific IFN-γ responses have a slower progression of liver fibrosis [15]. We have shown that chronic HCV coinfection who have higher HCV and Candida-specific IFN-γ immune responses are significantly more likely to have lower inflammation and fibrosis scores on liver biopsy [16].

Our long-term goal has been to define the immunological alterations in cellular immune responses to HCV that may impact on liver disease progression. However, because many of our subjects with HIV/HCV have a history of IDU, we sought to determine whether current substance abuse affects the results of our standard immunologic assays. Therefore, this study was designed to determine whether current IDU or any alcohol exposure was associated with alterations in HCV-specific immune responses and, if so, whether the effects were the same in HCV-infected and HIV/HCV-coinfected subjects. We examined HCV-specific and recall antigen responses in subjects who consumed alcohol or were active injection drug users and who were HCV-infected or HIV/HCV-coinfected, compared with responses in subjects who did not use injection drugs or consume alcohol, to determine the relative impact of HIV status and alcohol or injection drug exposure on cellular immune responses.

SUBJECTS, MATERIALS, AND METHODS

Subjects. Subjects were recruited from the Boston Medical Center Hepatitis C, HIV, and Related Morbidity (CHARM) cohort, a prospective natural history cohort that includes demographics, alcohol and drug use histories, clinical and laboratory information, and collection of peripheral blood mononuclear cells (PBMCs) every 12 months. All subjects who were HCV-antibody positive by ELISA and had detectable HCV RNA completed a questionnaire regarding drug and alcohol use. Those who had PBMCs collected simultaneously for immune assays were included in the cohort from which subjects for this study were selected. Injection drug users were defined as subjects who had injected heroin in the last month before collection of PBMCs and denied any alcohol use in the past year. Drinkers were defined as subjects who had consumed any alcoholic beverages in the last month and denied any IDU in the past year. Subjects who both drank alcohol and were injection drug users were excluded. All subjects completed the Alcohol Use Disorders Identification Test (AUDIT) questionnaire, and subjects with scores ≥8 were considered to have hazardous drinking [17, 18]. We also used the alcohol consumption ranges in AUDIT to define “heavy drinkers” as subjects who consumed 3 or more drinks 4 or more times a week or at least 6 drinks in one sitting. “Nonusers” were defined as subjects who denied alcohol consumption and IDU for at least 1 year; for the present analysis, a subgroup of nonusers were randomly selected and matched by HIV status to the drinker cohort (the larger subject group). This study was reviewed and approved by the Institutional Review Board of Boston University Medical Center and Beth Israel Deaconess Medical Center, human experimentation guidelines of the respective institutions were followed in the conduct of this research, and all subjects provided written, informed consent.

Recombinant HCV proteins. The recombinant HCV proteins used were derived from HCV genotype 1b and included Core protein (aa 1–115) and nonstructural (NS) proteins NS3 (aa 1007–1534) and NS5 (aa 2622–2868) at 1 µg/mL (Mikrogen).

Enzyme-linked immunosorbent spot (ELISPOT) assay. ELISPOT assays were performed on viable, cryopreserved PBMCs. One hundred microliters of primary monoclonal antibody (Mab) (anti–IFN-γ or anti–IL-10 [Mabtech] or anti–TNF-α [BD Pharmingen]) was added to 96-well polystyrene plates (Millipore) at a concentration of 10 µg/mL. Positive control wells contained 0.05% Tween20. Biotin-conjugated secondary MAbs were included in the cohort from which subjects for this study were selected. Injection drug users were defined as subjects who had injected heroin in the last month before collection of PBMCs and denied any alcohol use in the past year. Drinkers were defined as subjects who had consumed any alcoholic beverages in the last month and denied any IDU in the past year. Subjects who both drank alcohol and were injection drug users were excluded. All subjects completed the Alcohol Use Disorders Identification Test (AUDIT) questionnaire, and subjects with scores ≥8 were considered to have hazardous drinking [17, 18]. We also used the alcohol consumption ranges in AUDIT to define “heavy drinkers” as subjects who consumed 3 or more drinks 4 or more times a week or at least 6 drinks in one sitting. “Nonusers” were defined as subjects who denied alcohol consumption and IDU for at least 1 year; for the present analysis, a subgroup of nonusers were randomly selected and matched by HIV status to the drinker cohort (the larger subject group). This study was reviewed and approved by the Institutional Review Board of Boston University Medical Center and Beth Israel Deaconess Medical Center, human experimentation guidelines of the respective institutions were followed in the conduct of this research, and all subjects provided written, informed consent.

Subjects. Subjects were recruited from the Boston Medical Center Hepatitis C, HIV, and Related Morbidity (CHARM) cohort, a prospective natural history cohort that includes demographics, alcohol and drug use histories, clinical and laboratory information, and collection of peripheral blood mono-
In total, we examined 84 subjects with hazardous drinking (15/34 subjects with HIV/HCV and 10/19 injected heroin 5 or more times per week). Forty-six percent of groups (data not shown). Ninety-four percent of the IDU group were significantly higher), compared with nonusers. In contrast, subjects with HCV had significantly higher TNF-α responses were also higher in HCV monoinfection.

Next, we compared antigen-specific immune responses in all subjects with IDU (the HIV/HCV and HCV groups combined) and all drinkers to all nonusers to determine whether there were any overall effects of IDU or alcohol on immune responses (table 2). The IDU group had broadly higher IL-10 responses to both HCV proteins and Candida as well as numerically higher TNF-α responses (HCV NS5-4 and Candida responses were significantly higher), compared with nonusers. In contrast, drinkers had only higher IL-10 responses to NS5 and Candida, compared with nonusers.

We then determined whether the immune response differences seen in subjects with HIV/HCV versus subjects with HCV
(figure 1) were affected by IDU. We compared responses to summed HCV proteins and Candida between (1) the HIV/HCV IDU group and the HIV/HCV nonusers group, (2) the HIV/HCV IDU group and the HCV IDU group; (3) the HIV/HCV nonusers group and the HCV nonusers group; and (4) the HCV IDU group and the HCV nonusers group. As shown in figure 2, the HIV/HCV IDU group had significantly higher IFN-γ secretion to summed HCV proteins than the HCV/HCV nonusers group, whereas the HCV-specific IFN-γ responses were not different between the HIV/HCV IDU and HCV IDU groups. In contrast, the HCV nonusers group had significantly higher HCV-specific IFN-γ responses than the HIV/HCV nonusers group. When we examined IFN-γ responses to Candida, we found that both the HIV/IDU and HCV nonusers groups had significantly higher responses than the HIV/HCV IDU group and HIV/HCV nonusers group, respectively.

When we examined IL-10 responses, we found that the HIV/HCV IDU group had significantly higher IL-10 secretion to summed HCV proteins than the HIV/HCV nonusers group, whereas the HCV IDU group had similar IL-10 responses to summed HCV proteins, compared with the HCV nonusers group. In contrast, the HCV IDU group had significantly higher IL-10 secretion to Candida, compared with the HCV nonusers group. There were no significant differences in TNF-α secretion to summed HCV proteins, whereas both the HIV/HCV nonusers group and the HCV IDU group had significantly higher TNF-α secretion to Candida than the HCV nonusers group.

The HIV/HCV IDU group had significantly lower CD4+ T cell counts than the HIV/HCV nonusers group, and it has been described in the HIV literature that advanced immunosuppression is associated with a shift to a type 2–like response [25]. To evaluate whether the higher HCV-specific IL-10 responses in the HIV/HCV IDU group were actually a function of lower CD4+ T cell counts as opposed to IDU, we examined the likelihood of having an HCV-specific IL-10 response in subjects with CD4+ T cell counts <359 cells/mm³ (the median value for the HIV/HCV IDU and nonusers groups), compared with >359 cells/mm³. The associations between HCV-specific IL-10 responses in the HIV/HCV IDU group and the HIV/HCV nonusers group were similar in low versus high CD4+ T cell count strata; thus, these responses were more likely related to IDU than to the degree of immunosuppression (data not shown).

Next, we examined the effect of exposure to alcohol on cellular immune responses in the group of drinkers compared with the same nonusers group to which we had compared the IDU group (figure 3). In contrast to findings in figure 1, the HIV/HCV drinkers group had similar HCV-specific IFN-γ responses to the HCV drinkers group, whereas the HIV/HCV nonusers group had significantly lower IFN-γ responses than the HCV nonusers group. However, similar to figure 1, we
Figure 1. Box and whisker plots demonstrating nos. of spot-forming cells (sfc)/10^6 peripheral blood mononuclear cells (PBMCs) in HIV/hepatitis C virus (HCV; white bars) vs. HCV groups (gray bars) for interferon (IFN)-γ (top panels), interleukin (IL)-10 (middle panels), and tumor necrosis factor (TNF)-α (bottom panels). Responses to individual HCV antigens (Core, NS3, and NS5) are shown in left panels, and summed HCV-specific (sum of responses to HCV proteins Core, NS3, and NS5), as well as recall antigen responses, are shown in right panels. The box represents the 25th, 50th (middle line), and 75th percentile values, whereas the lower and upper lines represent the 10th and 90th percentile values, respectively. Note the different scales for each panel. Significant differences between HIV/HCV and HCV groups (P < .05, Mann-Whitney U test) are indicated.

We found that the HCV drinkers and nonusers groups had significantly higher IFN-γ secretion to Candida than the HIV/HCV drinkers and nonusers groups, respectively. HCV-specific and Candida responses between the HIV/HCV drinkers group and the HIV/HCV nonusers group were also not significantly different for IFN-γ, IL-10, or TNF-α secretion. There were no significant differences in any immune responses between the HCV drinkers group and the HCV nonusers group.

When we examined the immune responses in hazardous drinkers (AUDIT scores ≥ 8) versus nonusers, we found similar patterns of immune responses as when all drinkers were examined, although numerical differences were still not significant (data not shown). We then compared the subgroup of heavy drinkers (n = 13) with the nonusers and found that heavy drinkers had significantly higher IL-10 to NS5 (as found when all drinkers were examined; table 2) and TNF-α secretion to...
Table 2. Comparisons of immune responses in injection drug users or drinkers vs. nonusers.

<table>
<thead>
<tr>
<th>Cytokine, antigen</th>
<th>Injection drug users (n = 35)</th>
<th>Drinkers (n = 53)</th>
<th>Nonusers (n = 53)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IFN-γ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Core</td>
<td>0 (0–3) [9]</td>
<td>0 (0–4) [17]</td>
<td>0 (0–1) [9]</td>
</tr>
<tr>
<td>NS3</td>
<td>0 (0–3) [11]</td>
<td>1 (0–1) [8]</td>
<td>0 (0–3) [6]</td>
</tr>
<tr>
<td>NS5</td>
<td>0 (0–1) [6]</td>
<td>1 (0–1) [4]</td>
<td>0 (0–1) [6]</td>
</tr>
<tr>
<td>Sum-HCV</td>
<td>4 (1–7)</td>
<td>3 (0–11)</td>
<td>1 (0–7)</td>
</tr>
<tr>
<td>Candida</td>
<td>156 (41–281) a [86]</td>
<td>45 (7–148) [74]</td>
<td>43 (5–153) [66]</td>
</tr>
<tr>
<td>Tetanus</td>
<td>11 (0–105) [51]</td>
<td>1 (0–12) [30]</td>
<td>3 (1–11) [26]</td>
</tr>
</tbody>
</table>

| **IL-10**         |                             |                  |                  |
| Core              | 5 (0–25) a [40]             | 0 (0–13) [26]    | 0 (0–5) [21]     |
| NS3              | 4 (0–21) [37]               | 0 (0–4) [11]     | 0 (0–21) [34]    |
| NS5              | 9 (0–52) b [46]             | 3 (0–16) a [34]  | 0 (0–4) [21]     |
| Sum-HCV           | 43 (8–106) b                | 7 (0–44)         | 0 (0–65)         |
| Candida           | 468 (56–1061) a [91]        | 226 (85–500) a [91] | 93 (61–302) [89] |
| Tetanus           | 24 (3–66) [66]              | 7 (0–73) [43]    | 1 (0–76) [38]    |

| **TNF-α**         |                             |                  |                  |
| Core              | 22 (0–134) [53]             | 1 (0–132) [47]   | 0 (0–131) [36]   |
| NS3              | 15 (0–288) [53]             | 5 (0–117) [45]   | 0 (0–162) [44]   |
| NS5              | 10 (0–224) a [50]           | 26 (0–177) [53]  | 0 (0–36) [35]    |
| Sum-HCV           | 210 (30–1206)               | 138 (0–558)      | 87 (0–424)       |
| Candida           | 2132 (1318–2572) b [97]     | 1430 (345–1738) [82] | 880 (412–1728) [83] |
| Tetanus           | 123 (18–1043) [82]          | 55 (0–343) [57]  | 34 (0–491) [58]  |

**NOTE.** Data are median nos. of spot-forming cells (sfc)/10^6 peripheral blood mononuclear cells (PBMCs) (interquartile range) (percentage of subjects who had at least 10 sfc/10^6 PBMCs for each antigen). Injection drug users and drinkers were each compared with nonusers. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

a P < .05, Mann-Whitney U test.

b P < .01, Mann-Whitney U test.

NS5 than nonusers, but all other comparisons were not significantly different (data not shown).

More subjects in the HIV/HCV drinkers group had HIV loads <400 copies/mL than in the HIV/HCV nonusers group. Stratified odds ratios were compared for the likelihood of having an HCV-specific or Candida immune response with IFN-γ, IL-10, or TNF-α between the HIV/HCV drinkers and nonusers groups, by HIV load level (stratified by <400 copies/mL or >400 copies/mL). There was no increased likelihood of immune responses associated with being a drinker in the virologically well-controlled group versus the detectable group; so, immunological responses were not a function of the degree of HIV virological control (data not shown). Of note, estimated duration of HCV infection was also not associated with immune responses for any group in this cohort (data not shown).

In summary, the significantly lower IFN-γ HCV-specific responses found when all subjects with HIV/HCV, in aggregate, were compared with all subjects with HCV seem driven by the much lower responses found in the HIV/HCV nonusers group, because the HIV/HCV IDU group and the HIV/HCV drinkers group had similar frequencies of cytokine secretion as the HCV IDU group and HCV drinkers group, respectively. Overall, IDU appeared to affect HCV-specific IFN-γ and IL-10 responses more than alcohol exposure did in this cohort.

**DISCUSSION**

We have shown that the impact of HIV status on cellular immune responses differs by the cytokine response being tested. IFN-γ secretion was most affected by HIV status, with subjects with HIV/HCV having lower frequency responses for nearly all antigens tested than subjects with HCV alone. Most other studies have not shown a significant difference in HCV-specific IFN-γ secretion between HIV/HCV-infected and HCV-infected groups, although in these studies response frequencies were low in both groups, cohorts were smaller, and current substance use histories were not taken into account [20, 22–24]. One study, using proteins and peptides to stimulate both CD4+ and CD8+ T cells, also showed lower aggregate IFN-γ T cell responses in HIV/HCV-infected versus HCV-infected groups [19]. This finding is of interest because, in a different cohort, we have shown that persons with HIV/HCV coinfection who...
Figure 2. Box and whisker plots demonstrating nos. of spot-forming cells (sfc)/10^6 peripheral blood mononuclear cells (PBMCs) in the HIV/hepatitis C virus (HCV) injection drug use (IDU) group (white bars), the HIV/HCV nonusers group (striped bars), the HCV IDU group (gray bars), and the HCV nonusers group (hatched bars) for interferon (IFN)–γ (top panels), interleukin (IL)–10 (middle panels), and tumor necrosis factor (TNF)–α (bottom panels) in response to summed HCV proteins (responses to Core, NS3, and NS5 were summed; left panels) and Candida (right panels). Note the different scales for each panel. Statistical comparisons were made between the HIV/HCV IDU group and the HIV/HCV nonusers group, the HIV/HCV IDU group and the HCV IDU group, the HCV IDU group and the HCV nonusers group, and the HIV/HCV nonusers group and the HCV nonusers group. Significant differences (*P* ≤ .05, Mann-Whitney U test) are indicated.

had higher IFN–γ responses to HCV antigens or Candida had significantly lower fibrosis and inflammatory scores on liver biopsy [16]. Because many subjects in the present cohort were active substance users and did not have liver biopsies performed, we cannot perform a similar analysis for this study. However, we can speculate that the overall lower IFN–γ responses seen in HIV/HCV-coinfected subjects may be related to the accelerated fibrosis progression seen clinically [26]. Few studies have compared HCV-specific IL-10 responses in HIV/HCV-infected versus HCV-infected groups. One study
found that women with HIV/HCV had low but similar IL-10 responses to HCV and HIV antigens compared with HCV-monoinfected subjects, although HIV/HCV-coinfected women had significantly lower IL-10 to CMV antigens, compared with HIV- or HCV-monoinfected women [24]. We found that secretion of IL-10 to HCV or recall antigens was not significantly different between the HIV/HCV-infected and HCV-infected groups. With overall depressed IFN-γ responses, the HIV/HCV-coinfected subjects in this cohort have a relative shift to type 2–like responses, as has been seen in other HIV cohorts with and without other coinfections [25, 27]. This immune state has been associated with an increased rate of liver fibrosis pro-
progression in HCV/schistosomiasis coinfection and has been associated with poorer response to IFN-based treatment of HCV infection [15, 28].

Based on previous animal models of the effect of opiates on immune responses, we had hypothesized that subjects who use infection drugs would have a shift to lower IFN-γ and higher IL-10 responses than nonusers, regardless of HIV status. When we examined all injection drug users in aggregate, we did find broadly higher IL-10 responses in injection drug users compared with nonusers. Unexpectedly, the subset of the IDU group with HIV/HCV coinfection had significantly higher IFN-γ and IL-10 HCV-specific responses than the HIV/HCV nonusers group, whereas comparable HCV-specific responses did not differ between the HCV IDU group and the HCV nonuser group. It is unknown how this pattern of cytokine production may affect progression of HCV-related liver disease, but this will be examined in more detail in future prospective studies.

We had also hypothesized that drinkers, regardless of HIV status, would have lower IFN-γ secretion than nonusers because of data supporting a shift to a type 2–like immune response with alcohol exposure, although previous studies have not examined HCV-specific cellular immune responses [10, 29, 30]. Surprisingly, in the present study, alcohol had little effect on antigen-specific IFN-γ, IL-10, or TNF-α immune responses when the HCV drinkers group was compared with the HCV nonusers group. In addition, drinkers with HIV/HCV had similar HCV-specific IFN-γ responses, compared with drinkers with HCV, whereas nonusers with HIV/HCV had significantly lower IFN-γ responses, compared with nonusers with HCV. Therefore, these data do not support a strong shift to type 2 cellular immune responses in the presence of alcohol consumption.

For this study, our aim was to determine whether usual patterns of alcohol consumption would affect our immune response assays. In fact, we found few immune response differences between drinkers and nonusers. However, a limitation of the present study is the lack of quantified daily measurements of alcohol consumption. Therefore, we cannot determine whether there is a threshold effect of alcohol consumption on immune responses. Approximately 25% of our subjects were considered heavy drinkers based on categories of alcohol consumption, and there was a suggestion that, in this small group, there may be higher HCV-specific IL-10 and TNF-α secretion, at least for NS5. This question would need to be addressed in cohorts with higher rates of heavy alcohol use.

In conclusion, antigen-specific IFN-γ immune responses are broadly diminished in persons with HIV/HCV coinfection, and HCV-specific TNF-α responses are also weaker in subjects with HIV/HCV, compared with subjects with HCV. This effect is modified by IDU and, to a lesser extent, by alcohol consumption, for which we found a relative increase in immune responses in substance users with HIV/HCV, compared with nonusers with HIV/HCV. Because most studies examining cellular immune responses in HIV/HCV and HCV focus on IFN-γ as the representative T cell effector function, these results suggest that IDU may be a confounding variable in these analyses. Future studies should examine mechanisms of action of IDU on HCV-specific immune responses as well as the effects of immune response alterations on HCV-related liver disease.

References


