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HCV viremia associates with NK cell activation and dysfunction in anti retroviral HIV/HCV co-infected subjects

E. Papasavvas[1], **L. Azzoni**[1], **X. Yin**[1], **Q. Liu**[1], **J. Joseph**[1], **A. Mackiewicz**[1], **B. Ross**[1], **K. M. Lynn**[2], **J. M. Jacobson**[3], **K. Mounzer**[4], **J. R. Kostman**[5], and **L. J. Montaner**[1]

[1]The Wistar Institute, Philadelphia, PA, USA

[2]Presbyterian Hospital-University of Pennsylvania hospital, Philadelphia, PA, USA

[3]Temple University, Lewis Katz School of Medicine, Philadelphia, PA, USA

[4]Jonathan Lax Immune Disorders Treatment Center, Philadelphia Field Initiating Group for HIV-1 Trials, Philadelphia, PA, USA

[5]John Bell Health Center, Philadelphia Field Initiating Group for HIV-1 Trials, Philadelphia, PA, USA

Abstract

The impact of Hepatitis C virus (HCV) RNA levels on immune status in chronically HCV mono-infected when compared to HIV/HCV co-infected on antiretroviral therapy (ART) remains poorly understood. A total of 78 African American subjects HCV viremic/naïve to HCV treatment (33 HCV genotype 1 mono-infected, 45 HCV genotype 1/HIV co-infected on ART) were studied. Clinical and liver enzymes measurements were performed. Whole blood was analyzed for immune subset changes by flow-cytometry. Peripheral blood mononuclear cells (PBMC) were used for same-day constitutive and *in vitro* Interferon (IFN)-α-induced Signal Transducer and Activator of Transcription (STAT) phosphorylation, K562 target cell lysis and K562 target cell recognition-mediated IFN-γ production. Statistical analysis was done using R (2.5.1) or JMP Pro 11. While both groups did not differ in the level of liver enzymes, HIV/HCV had higher T cell activation/exhaustion, and constitutive STAT-1 phosphorylation compared to HCV. In contrast, $CD4^+FoxP3^+CD25^+$ frequency, IFN-αR expression on NK cells, as well as constitutive and IFN-α-induced direct cytotoxicity were lower in HIV/HCV. Linear regression models further supported these results. Finally, increase in HCV viral load (vl) and $CD4^+$ T cell count had an opposite effect between the two groups on NK cell activity, and T cell activation respectively. HCV viraemia in antiretroviral -treated HIV/HCV co-infection was associated with greater immune activation/exhaustion and NK dysfunction than HCV viral load alone in HCV mono-infection. The more pronounced immune modulation noted in antiretroviral treated HIV co-infected / untreated HCV viremic subjects may impact HCV disease progression and/or response to immunotherapy.

Reprints or correspondence: Dr. Luis J. Montaner, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA, 19104; Tel 215-898-9143; Fax 215-573-7008; montaner@wistar.org.

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Keywords

HCV; HIV/HCV; HCV viral load; NK; T cells

INTRODUCTION

Hepatitis C virus (HCV) infects nearly 200 million people worldwide with over 80% of infected people progressing to chronic infection (1–4). Due to shared routes of transmission, HCV and human immunodeficiency virus (HIV) co-infection is common (5), affecting 60–95% of subjects with parenteral exposure [e.g. intravenous (IV) drug users, blood products recipients]. Sexual transmission of HCV is less common, but may be facilitated by concurrent HIV infection. It is estimated that approximately 4 to 5 million people are living with chronic HIV/HCV co-infection (6).

A number of studies support that HCV viremia is associated with both immune activation and subsequent gradual loss of immune function (7). A number of reports show that in chronic HCV infection HCV-specific cytotoxic T lymphocytes have impaired or exhausted proliferative, cytokine, and cytotoxic effector functions (8–12). Natural killer (NK) cell activity has also been described to be decreased (13) as a result of several mechanisms including direct interaction of the viral protein E2 with surface CD81 on NK cells (14), hepatocyte reduction of type 1 interferon (IFN) production via protein kinase R inactivation (15, 16), greater stabilization of hepatocyte major histocompatiblity complex-I molecules (17), and early inhibition of activation and IFN-γ production by NK cells (14). Other mechanisms of immune dysfunction include decreased dendritic cells (DC) frequencies, impairment of the antigen-presenting function of DCs (18–21), direct impairment of plasmacytoid DC (PDC) function by HCV core protein via increased interleukin-10, and reduced production of interleukin-12, and IFN-α (22, 23).

Similar to HCV infection, HIV infection also leads to increased T cell activation (24), and functional impairment (25), which is partially restored by antiretroviral therapy (ART) (26, 27). The impact of HIV on the natural course of HCV infection is deleterious with higher HCV viral load (vl), higher rate of HCV persistence, and a higher risk of mortality or co-morbidities than HCV mono-infected patients (5, 28). On the other hand, some studies also support a significant effect of HCV infection on the progression of HIV to acquired immune deficiency syndrome-defining illness and related mortality (29–34). HIV/HCV co-infected subjects have been described to retain high levels of immune activation that persist after ART-mediated HIV suppression (35–38), yet the role of HCV viremia in driving persistent immune activation and/or its relationship to innate immune reconstitution after ART remains unknown.

Little is known about the additive effect of ART-treated HIV co-infection and HCV vl on innate and adaptive immune status in subjects with untreated chronic HCV infection. To address these questions, we used freshly obtained blood and peripheral blood mononuclear cells (PBMCs) for the characterization of adaptive and innate cells subsets, activation/exhaustion, and innate signaling and function in HIV/HCV co-infected and HCV mono-infected subjects.

MATERIALS AND METHODS

Participants

33 untreated HCV viremic mono-infected (HCV mono-infected) and 45 ART-treated HIV co-infected / untreated HCV viremic (HIV/HCV co-infected) subjects were studied. Clinical parameters [complete blood count differential, CD4 count, HCV and HIV vl], liver enzymes [alanine aminotransferase, aspartate aminotransferase], and immune markers were assessed in all subjects by Quest Diagnostics (NJ, USA). All participants were chronically infected with HCV, had HCV single or mixed genotype 1, were HCV viremic and naïve to HCV treatment. HCV infection was diagnosed by detection of antibodies (Abs) against HCV and confirmed by two PCR-based determinations of HCV RNA (limit of detection: 43 copies/ml) with time elapsed 4–6 months. Subjects with established non-compensated cirrhosis, current IV drug use or usage of IV drugs within 3 months prior to enrollment, or current alcohol abuse [>40g/day (two drinks/day) or average of >80g/day (4 drinks a day)] anytime in 3 months prior to enrollment were excluded. HIV infection in HIV/HCV co-infected subjects was confirmed by western blot or PCR (limit of detection: 20 copies/ml). Presence of ART treatment at the time of study was necessary for inclusion of HIV/HCV co-infected subjects in the study, yet no minimum time of ART or minimum CD4 T cells/mm^3 were established as criteria for inclusion. Informed, written consent was obtained from all participants. The study was performed according to the World Medical Association Declaration of Helsinki. The study protocol was approved by the Institutional Review Boards of the authors' institutions.

Whole blood-based phenotypic characterization of immune subsets

To assess adaptive and innate cell subsets, same day whole blood 7-color staining was performed as previously described (39) by using the combinations of directly fluorochrome-conjugated anti-human cell surface monoclonal Abs shown in Supplementary Table 1. All antibodies were from Becton Dickinson (BD) Biosciences (San Diego, CA, USA) except blood dendritic cells antigen (BDCA) 2-allophycocyanin (APC), BDCA4-APC and IgG1-APC which were purchased from Miltenyi Biotec (San Diego, CA, USA). Stainings "a-c" allowed for the assessment of activation/exhaustion markers [CD25, CD38, CD94, CD95, HLA-DR, programmed cell death 1 (PD1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), B and T lymphocyte attenuator (BTLA), CD160] on T cells ($CD3^+CD8^-$, $CD3^+CD8^+$). Stainings "d-h" allowed for the identification of exhaustion [programmed death-ligand (PDL) 1, PDL2, herpes virus entry mediator (HVEM)], costimulatory (CD86) and apoptosis [tumor necrosis factor-alpha-related apoptosis-Inducing ligand (TRAIL)] markers on DC (40) [$BDCA2^+BDCA4^+$ (PDC), and $CD19^-BDCA1^+CD11c^+$ (myeloid DC, MDC)] and monocytes ($CD14^+$). Staining "i" allowed for the assessment of CD81, and CD69 expression on T cells ($CD3^+$), as well as for the assessment of NK cell subsets (41) ($CD3^-CD56^{dim}CD16^-$, $CD3^-CD56^{dim}CD16^+$, $CD3^-CD56^{bright}$, and $CD3^-CD56^-CD16^+$), and of CD81, CD69, and IFN-α receptor (IFN-αR) expression on NK cell subsets.

Briefly, 200 μl of whole blood were incubated for 15 min at room temperature with the appropriate Ab combinations, lysed for 10 min at room temperature with 3 ml of FACS Lysis solution (BD Biosciences), and centrifuged for 5 min at 1200 rpm. Cells were then

washed for 5 min with 3 ml of FACS washing buffer at 1500 rpm, and re-suspended in 200 μl of FACS washing buffer. Regulatory T cells (Tregs, $CD4^+CD25^{hi}FoxP3^+$) staining was performed by using the BD Biosciences FoxP3 staining kit according the manufacturer's instructions. Cells were analyzed on LSRII cytometer (BD Biosciences) by collecting >200000 events. Data were analyzed using FloJo software (Version 8.8.4, Tree Star, Ashland, OR, USA). Gating was originally done on singlets, and then on "live lymphocyte" (for T, NK, and Tregs) or "all live cell" (for DC, and monocytes) gates defined by size and granularity in forward scatter and side scatter. Thresholds were set by isotype-matched negative controls and unstained cells. Results were expressed as mean fluorescent intensity (MFI), percent positive (%) and cells/mm^3.

Assessment of the *in vitro* role of IFN-α on STAT-1 phosphorylation within PBMC cell subsets

To assess constitutive and *in vitro* induced signal transducer and activator of transcription (STAT) phosphorylation, fresh PBMC (2×10^6/ml), isolated from whole blood as previously described by standard Ficollhypaque density gradient centrifugation (39), were stained for: a) CD3-fluorescein isothiocyanate (FITC), CD14-FITC, CD19-APC, CD20-APC, CD16-Pacific Blue, CD56-phycoerythrinCy7 (PECy7), and b) CD14-FITC, BDCA2-APC, BDCA4-APC, CD3-Pacific Blue, or c) corresponding isotypes (IgG1k-FITC, IgG2ak-FITC, IgG1-APC, IgG1k-Pacific Blue, IgG1k-PECy7) for 30 min at 4°C, washed with 1xPBS at 1500 rpm for 5 min and re-suspended in warm 1xPBS. PBMCs were then treated for 10 min at 37°C with media alone, or *in vitro* IFN-α (5000 U/ml, PBL). Cells were then fixed with paraformaldehyde (final concentration 5%) for 10 min at 37°C, washed and permeabillized with PhosFlow buffer (BD Biosciences) for 30 min at RT. Subsequently, PBMCs were washed in FACS washing buffer at 2200 rpm for 10 min, stained with an Ab against phosphorylated (p)-STAT-1 [p-STAT-1-peridinin chloropyll Cy5.5 (PerCP-Cy5.5)] or corresponding isotype IgG2ak-PerCP-Cy5.5 for 1 hr at RT, washed with FACS washing buffer and analyzed in the Cyan cytometer as described above. Staining "a" allowed for the assessment of NK cell subsets (identified as: $Lin3^-CD56^+CD16^+$, $Lin3^-CD56^+CD16^-$, or $Lin3^-CD56^-CD16^+$, with Lin3 consisting of CD3, CD14, CD19, and CD20), while staining "b" allowed for the identification of monocytes ($CD3^-CD14^+$), and PDC ($CD3^-CD14^-BDCA2^+BDCA4^+$). All antibodies were from BD Biosciences except BDCA2-APC, BDCA4-APC and IgG1-APC which were purchased from Miltenyi Biotec. Constitutive STAT-1 phosphorylation for all the above described cell subsets was expressed as MFI of p-STAT-1 in the absence of *in vitro* IFN-α stimulation. *In vitro* IFN-α-induced STAT-1 phosphorylation for all the above described cell subsets was calculated by dividing *in vitro* IFN-α-induced MFI of p-STAT-1 by the constitutive MFI of p-STAT-1.

Assessment of intracellular IFN-γ cytokine production in NK cells following effector cells/target interactions

To study functional NK-target cell interaction within the context of cell-specific measures on NK subsets, constitutive and target-induced cytokine production (IFN-γ) in the presence or absence of *in vitro* stimulation were measured using flow cytometry. Briefly, fresh PBMC (1×10^6 cells per condition) were incubated with or without *in vitro* IFN-α (5000 U/ml, PBL, Piscataway, NJ, USA) and in the absence or presence of K562 target cells (2×10^5 cells per

condition) for 2 hrs at 37ºC, followed by addition of Brefeldin (10 μg/ml), and further incubation for 16 hrs at 37ºC. At the end of the incubation, cells were stained for 15 min at RT with NK cell surface Ab combinations (Lin3-FITC, CD16-Pacific Blue, CD56-PECy7), washed with 2 ml FACS washing buffer at 1500 rpm for 5 min and fixed/permeabilized for 10 min at RT with 250 μl Cytofix/Cytoperm. After added washes, cells were stained for intracellular IFN-γ-APC or corresponding isotype IgG1k-APC, washed again with 2 ml Perm washing buffer at 1500 rpm for 5 min and re-suspended in 200 μl of FACS washing buffer. All Abs were from BD Biosciences. Cells were analyzed in the LSRII as described above. This staining allowed for the assessment of IFN-γ-producing NK cell subsets (identified as: $Lin3^{-}CD56^{-}CD16^{+}$, $Lin3^{-}CD56^{dim}CD16^{-}$, $Lin3^{-}CD56^{dim}CD16^{+}$, and $Lin3^{-}CD56^{bright}$). Constitutive IFN-γ production was calculated by subtracting the percentage of cells producing IFN-γ constitutively in the absence of targets from the percentage of cells producing IFN-γ constitutively in the presence of targets. *In vitro* induced IFN-γ-production was calculated by subtracting the percentage of cells producing IFN-γ constitutively in the presence of targets from the percentage of cells producing IFN-γ after *in vitro* stimulation with IFN-α in the presence of targets.

Assessment of direct cytotoxicity against a MHC-cell null cancer target cell line

The standard ^{51}Cr release assay was used as previously described to assess constitutive and *in vitro* induced NK cell-mediated cytotoxicity, using fresh PBMC preparations as effectors cells against the tumor derived erythroblastoid MHC-null cell line K562 (42).

Briefly, fresh PBMC were treated for 18 hrs at 37C° with media alone or *in vitro* IFN-α (5000 U/ml, PBL). K562 cells, which served as targets, were labeled with $Na_2{}^{51}CrO_4$ (~ 50μCi) for 1.30 hr at 37C°, washed and re-suspended at a concentration of 1×10^5 cells/ml in media. Effectors and labeled-K562 targets were cultured in triplicate to yield the desired effector:target (E:T) ratios in 0.2 ml volume (usually 50:1, 25:1, 12.5:1, and 6.25:1) in round bottomed 96-well plates and incubated for 4 hrs at 37C°. Percent lysis was determined as [(experimental counts-spontaneous released counts)/(total counts-spontaneous released counts)] × 100. Results were expressed as area under the curve (AUC) for E:T ratios of 50:1, 25:1, 12.5:1 and 6.25:1 for both constitutive and *in vitro* IFN-α-induced NK function.

Statistical Analysis

Data were described as medians, 25^{th} and 75^{th} quartiles. Group comparisons were done by t-test or Wilcoxon rank sum test depending on data distributions. Unadjusted p-values that were less than 0.05 are reported, along with adjusted p-values, based on the approach of Benjamini and Yekutieli (BY), that were less than 0.01. Multivariate linear regression models were used to explore the effect of HCV vl (log10 IU/ml) and HIV co-infection, as well as of $CD4^{+}$ T cell count and HIV co-infection on a subset of the variables with unadjusted p-values <0.05 between the two groups. Analysis was performed using R version 2.5.1 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria), or JMP Pro 11 (SAS Institute, Cary, NC, USA).

RESULTS

Study subjects demographics

Demographic and clinical characteristics of the study subjects are shown in Table 1. A total of 5/45 HIV/HCV co-infected subjects had HIV vl >400 copies/ml suggesting lack of ART-mediated suppression in a minority of the HIV/HCV co-infected subjects. Interestingly, 22/45 HIV/HCV co-infected subjects had $CD4^+$ T cell count <400 cells/mm^3, with only 2/22 having HIV vl >400 copies/ml, suggesting lack of complete immune re-constitution in about 1/2 of the HIV/HCV co-infected subjects, that was not associated with lack of HIV suppression. Finally, no significant difference was observed between the two groups for plasma levels of liver enzymes (alanine aminotransferase, aspartate aminotransferase).

Higher levels of T cell activation/exhaustion and lower levels of Tregs in HIV/HCV co-infected subjects

HIV infection leads to increased T cell activation (24), and functional impairment (25), which is partially restored by ART (26, 27, 43, 44). In equal manner, in HIV/HCV co-infected subjects, we detected higher levels of T cell activation [e.g. MFI of CD38 on $CD8^+$ ($p<0.001$) and $CD8^-$ ($p<0.001$) T cells], together with increased expression of exhaustion marker (45) CD160 on $CD8^+$ T cells ($p=0.007$) when compared to HCV mono-infected subjects. Unadjusted data were also consistent with higher expression of exhaustion markers (i.e. CD160, BTLA/CD160, and CTLA-4) on $CD8^+$ T cells. In contrast, HIV/HCV co-infected subjects had lower frequencies of $CD3^+CD4^+$ T cells ($p<0.001$) and Tregs ($p=0.02$) when compared to HCV mono-infected subjects (Table 2, Fig. 1, Supplementary Fig. 1).

Higher levels of innate activation and constitutive STAT-1 phosphorylation and lower NK function in HIV/HCV co-infected subjects

While total frequency of NK and myeloid cell subsets examined (MDC, PDC, CD14 subsets) did not differ between groups, we document that constitutive STAT-1 phosphorylation was significantly higher on NK cells ($p=0.05$), and monocytes ($p=0.02$) in HIV/HCV co-infected subjects when compared to HCV mono-infected subjects (Table 2, Fig. 2). NK function was assessed by measurement of direct cytotoxicity against K562 target cells and NK cell-associated IFN-γ production following *in vitro* stimulation with IFN-α (Supplementary Fig. 2). HIV/HCV co-infected subjects had lower constitutive ($p=0.04$) and *in vitro* IFN-α-induced direct cytotoxicity against K562 target cells ($p=0.02$) (Table 2, Fig. 2). Consistent with group comparison results obtained after multiple testing adjustment in support of an increased immune activation state in HIV/HCV co-infected subjects, it should be noted that (1) a lower IFN-αR expression on NK cells together with higher expression of CD69 on NK cells, and (2) a higher expression of CD86 (46), and TRAIL (47) on myeloid cells were also detected in HIV/HCV co-infected subjects when compared to HCV mono-infected subjects before multiple testing adjustment (Table 2).

Overall, innate markers of activation and function as listed in Table 2 support the interpretation of higher constitutive STAT-1-mediated activation in both monocyte and NK cells in spite of lower NK cytotoxicity in HIV/HCV co-infected subjects as compared to HCV mono-infected.

HIV co-infection affects the relationship between HCV vl and NK cell function

We assessed the effect of HCV vl and HIV co-infection on selected variables using multivariate linear regression models. The results of this analysis are summarized in Table 3 and Fig. 3. Briefly, when the effect of HCV vl and patient group was assessed independently (model 1), of all the variables tested, only CD81 expression on NK cells (Supplementary Fig. 3) was positively associated with HCV vl (p=0.02). The same model indicated no significant effect of HIV co-infection on CD81 expression. On the other hand, consistent with group comparisons described above, HIV co-infection, but not HCV vl, had a significant effect on T cell activation/exhaustion, as CD38 (p<0.0001), CTLA-4 (p=0.04), and CD160 (p<0.0001) expression on $CD8^+$ T cells were higher, while Tregs levels (p=0.0002) were lower in HIV/HCV co-infected subjects than in HCV mono-infected subjects (Table 3, Fig. 3). HIV co-infection had also an effect on NK cell activity, as CD69 (p=0.04) and p-STAT-1 expression (p=0.002) were higher, while IFN-αR expression (p=0.01) and IFN-α-induced direct cytotoxicity (p=0.02) were lower in HIV/HCV co-infected subjects than in HCV mono-infected subjects (Table 3, Fig. 3). Models assessing the effect of $CD4^+$ T cell count showed that HIV co-infection, but not $CD4^+$ T cell count, had a significant effect on T cell activation/exhaustion, with CD38 (p=0.001), BTLA (p=0.03), and CD160 (p=0.01) expression on $CD8^+$ T cells being higher, and Tregs levels (p=0.01) being lower in HIV/HCV co-infected subjects than in HCV mono-infected subjects (Supplementary Table 2, Supplementary Fig. 4).

The effect of HIV co-infection on the association between the markers described above and HCV vl was assessed by adding an interaction term to the already described models. Significant interactions were found for NK cells-associated variables, but not for T cells. More precisely, the model suggests that the association between HCV vl and NK cells expression of CD69 (p=0.03), constitutive STAT-1 phosphorylation (p=0.04) and frequency of IFN-α-induced IFN-γ^+ cells (p=0.008) is significantly different between HIV/HCV co-infected and HCV mono-infected subjects (Table 3, Fig. 3). In contrast, addition of interaction term in the model assessing the effect of HIV co-infection on the association between the markers described above and $CD4^+$ T cell count showed that of the variables tested only the association between $CD4^+$ T cell count and CD38 expression on $CD8^+$ T cells was significantly different between HIV/HCV co-infected and HCV mono-infected subjects. More precisely, increasing $CD4^+$ T cell count was associated with low levels of CD38 expression in HIV/HCV co-infected in contrast to HCV mono-infected (Supplementary Table 2, Supplementary Fig. 4), possibly as a result of ART-mediated immune reconstitution.

Taken together, these findings support that increasing HCV viremia is associated with a greater degree of NK cell activation and dysfunction in ART-treated HIV co-infected / untreated HCV subjects than in untreated HCV viremic mono-infected subjects.

DISCUSSION

We assessed the effects of HCV viremia in ART-treated HIV/HCV co-infected versus HCV mono-infected subjects where ethnic distribution, HCV genotype, liver enzymes, and a lack of de-compensating disease are comparable between subjects. Based on the deleterious

effect of HIV on the natural course of HCV infection, we expected to observe greater immune activation and dysfunction in HIV/HCV co-infected subjects, with HCV vl levels playing a determinant role in this immunodeficiency. We confirmed this hypothesis showing that HCV viremia associates with greater NK cell activation and dysfunction in HIV/HCV co-infected than in HCV mono-infected subjects, yet we found no difference on the association between HCV vl and T cell activation between groups.

HIV/HCV co-infected subjects have been reported to have high levels of immune activation, particularly T cell activation (i.e. CD38, HLA-DR), even in the presence of suppressive ART (35–38). In this study, we confirmed higher levels of T cell activation, and also observed higher levels of markers of T cell exhaustion in ART-treated HIV/HCV co-infected subjects, as noted by the higher expression of CD160 and other exhaustion markers on $CD8^+$ T cells. Linear regression models further supported these results by showing an effect of patient group but not of HCV vl on T cell activation/exhaustion markers, with HIV/HCV co-infected subjects having higher levels of T cell activation/exhaustion. These results are overall in agreement with the findings by Feuth et al (48) suggesting an increased exhaustion in HCV viremic / HIV co-infected subjects on ART, despite the fact that in our study, subjects with established non-compensated cirrhosis were excluded.

Recent studies have shown that Treg cell activity is increased in patients with chronic HCV infection when compared to those who clear infection (49–51) and that Tregs contribute to HCV persistence by suppressing the proliferation and IFN-γ production of HCV-specific $CD8^+$ T cells (8, 49, 52). There is limited and conflicting information available on Treg cells in HIV/HCV co-infection (53, 54). Consistent with Roe et al (54) who showed lower Tregs levels in ART untreated HIV/HCV co-infection when compared to HCV mono-infection, our observations on ART-treated HIV/HCV co-infected subjects showed lower Tregs frequency when compared to HCV mono-infected, suggesting the possible presence of an association between Tregs frequency and HIV co-infection maintained even after ART. This finding was supported by linear regression models showing an effect of patient group, but not of HCV vl, on lower Tregs levels in HIV/HCV co-infected subjects.

In both HCV and HIV infection a quantitative and qualitative impairment of DC subsets have been reported, which is partially restored in HIV-infected subjects after therapy (18–23, 27, 54–56). In our study, myeloid and NK cells were detected to have higher levels of constitutive STAT-1 phosphorylation. While a higher stringency of analysis (adjusted p values) did not detect a difference in the frequency of DC subsets or the expression of co-stimulatory or inhibitory molecules on MDC or PDC between study groups, unadjusted p values did suggest the potential for higher expression of CD86 and TRAIL in HIV/HCV co-infected subjects.

IFN-αR is involved in anti-HCV responses as noted by the positive association of its expression on NK cells and a favorable response to Peg-IFN-α/RBV (57, 58). We show that in ART-treated HIV/HCV co-infection a lower IFN-αR expression may still indicate a lack of innate immune reconstitution by a sustained state of chronic NK activation with lower functionality. Group comparisons and linear regression models suggested lower expression of IFN-αR on NK cells together with higher constitutive STAT-1 phosphorylation and lower

cytotoxic potential in HIV/HCV co-infected subjects irrespective of HCV vl. We interpret that the latter is consistent with the retention of an activated, yet dysfunctional ("exhausted") NK response in the presence of HCV viremia. Although no difference was observed between study groups for the expression of CD81 on NK cells, we do show that HCV vl was associated to an increase in CD81 expression irrespective of patient group. The direct interaction of the HCV protein E2 with surface CD81 on NK cells (14) has been suggested to be one of the mechanisms resulting in the decreased NK cell activity observed during HCV infection (13), while our data show that in the context of HCV viremia HIV co-infection-associated additional mechanisms may contribute to greater NK dysfunction. More precisely, HCV vl showed an opposite effect in both groups for NK cell activation, constitutive STAT-1 phosphorylation and IFN-α-induced IFN-γ production, suggesting an increase in NK activation and loss of NK function with HCV vl in the context of HIV/HCV co-infection. Further studies with greater numbers in each group are needed to confirm these findings. Higher STAT-1 phosphorylation levels in NK cells together with decreased IFN-αR expression raises the hypothesis that this dysfunction may help explain prior reports of lower HCV sustained responses in HIV/HCV co-infected when compared to HCV mono-infected when an older approach to HCV therapy was taken by using pegylated-IFN-α / ribavirin (59, 60).

A strength of our study is that all assays were performed on fresh blood or freshly isolated PBMCs, thereby eliminating any influence of cryopreservation on immune cells and markers of activation/exhaustion, signaling and function. Another strength is the homogeneity of the study groups limiting the secondary effects of ethnicity or non-compensated cirrhosis. On the other hand, the lack of HCV specific T cell responses restricting our findings as non-HCV specific is a limitation of our study. However, it has been suggested that non-specific activation may affect disease progression, as liver infiltrates are largely composed of HCV non-specific T cells (61, 62). Another limitation of our study is the lack of an HIV ART-treated / HCV uninfected control group to address direct differences in ART-mediated immune reconstitution with HIV/HCV co-infected subjects in the presence of HCV viremia. However, while the association between $CD4^+$ T cell count and T cell activation was significantly different between the two groups, no association between $CD4^+$ T cell count and NK markers was observed in either group suggesting that the level of $CD4^+$ T cell immune reconstitution did not affect the HCV viremia effects on NK cells noted here. Future longitudinal studies should address the impact of HCV vl on ART-mediated immune reconstitution, where pre-ART $CD4^+$ T cell count is matched between HIV/HCV co-infected and HIV mono-infected groups.

Overall, in HIV ART-treated / HCV viremic co-infected subjects, we found evidence to support higher levels of activation on T, NK and myeloid cells together with lower levels of IFN-αR expression on NK cells and NK cell direct cytotoxicity than in HCV mono-infected subjects. Importantly, HCV vl was found to have opposite effects on NK activation and function between groups suggesting the presence of greater detrimental effects for HCV vl on NK cells in HIV/HCV co-infected subjects despite ART. This data show a greater state of immune dysfunction or lack of functional innate immune reconstitution in HIV/HCV co-infected subjects when compared to HCV mono-infected, which may impact HCV disease

progression, comorbidities, and/or response to therapy between HCV mono-infected and HIV/HCV ART-treated co-infected subjects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DISCLOSURES

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Abbreviations

HCV	hepatitis C virus
HIV	human immunodeficiency virus
IV	intravenous
NK	natural killer
IFN	interferon
DC	dendritic cells
PDC	plasmacytoid DC
ART	antiretroviral therapy
vl	viral load
PBMCs	peripheral blood mononuclear cells
Abs	antibodies
BD	becton dickinson
BDCA	blood dendritic cells antigen
APC	allophycocyanin
PD1	programmed cell death 1
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
BTLA	B and T lymphocyte attenuator

PDL	programmed death-ligand
HVEM	herpes virus entry mediator
TRAIL	tumor necrosis factor-alpha-related apoptosis-inducing ligand
MDC	myeloid DC
IFN-αR	IFN-α receptor
Tregs	regulatory T cells
MFI	mean fluorescent intensity
%	percent positive
STAT	signal transducer and activator of transcription
FITC	fluorescein isothiocyanate
PECy7	phycoerythrinCy7
p-STAT-1	phosphorylated-STAT-1
PerCP-Cy5.5	peridinin chlorophyll Cy5.5
E	T, effector:target
AUC	area under the curve
BY	Benjamini and Yekutieli

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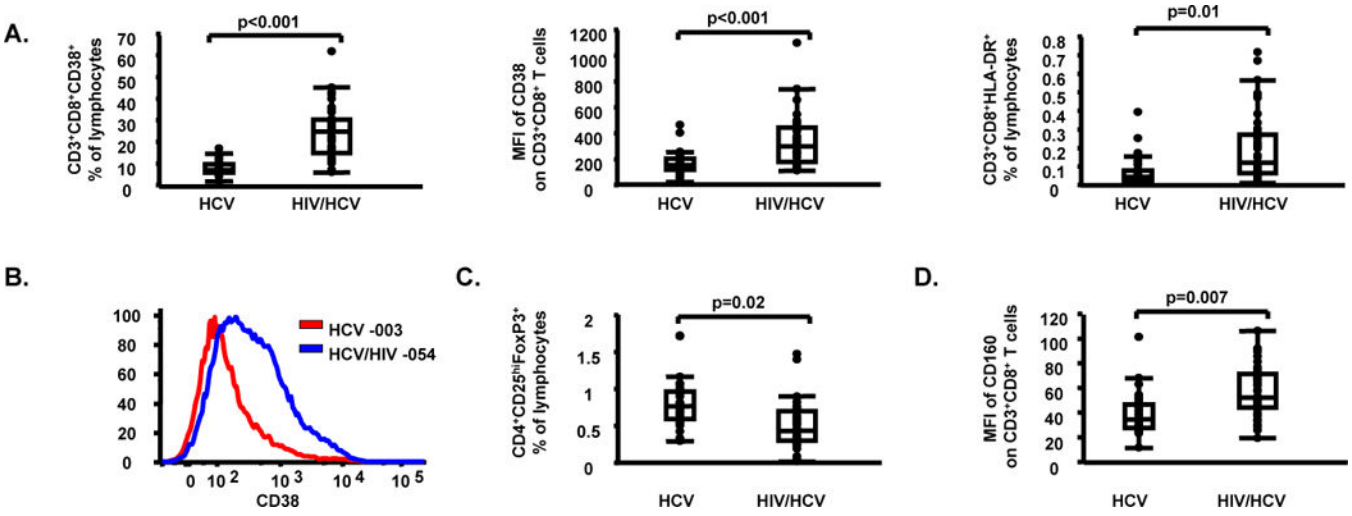

Fig. 1.

Higher levels of T cell activation and lower levels of Tregs in HIV/HCV co-infected subjects when compared to HCV mono-infected subjects. (A) $CD3^+CD8^+CD38^+$ percentages (%) of lymphocytes, mean fluorescent intensity (MFI) of CD38 on $CD3^+CD8^+$ T cells, and $CD3^+CD8^+HLA\text{-}DR^+$ % of lymphocytes in HCV mono-infected and HIV/HCV co-infected subjects; (B) MFI of CD38 on $CD3^+CD8^+$ T cells in one representative HCV mono-infected subject [subject 003, MFI CD38 on $CD3^+CD8^+$ T cells = 156 (red line)] and one representative HIV/HCV co-infected subject [subject 054, MFI of CD38 on $CD3^+CD8^+$ T cells = 360 (blue line)] are shown; (C) Tregs % of lymphocytes in HCV mono-infected and HIV/HCV co-infected subjects; (D) MFI of CD160 on $CD3^+CD8^+$ T cells, in HCV mono-infected and HIV/HCV co-infected subjects. Data in panels (A), (C) and (D) are shown as inter-quartile box plots with median and outliers for each group, and significant (<0.1) P values.

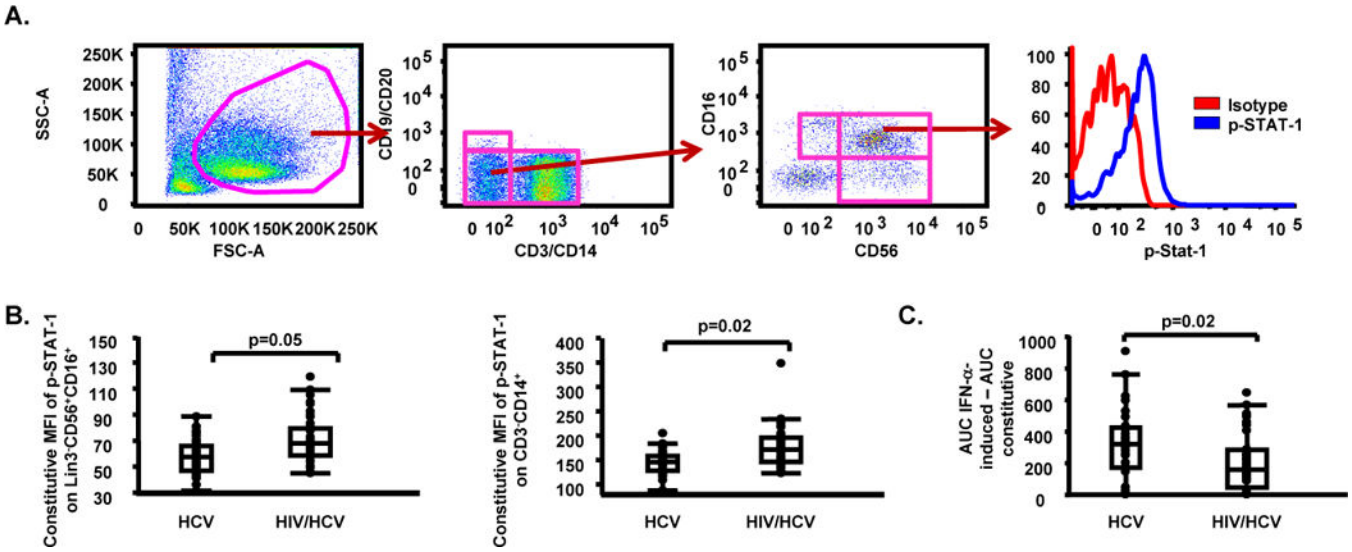

Fig. 2.

Higher levels of constitutive STAT-1 phosphorylation and lower levels of *in vitro* IFN-α-induced direct cytotoxicity against K562 target cells in HIV/HCV co-infected subjects when compared to HCV mono-infected subjects. (A) Gating approach for phosphorylated (p)-STAT-1 on NK cell subsets [(isotype (red line), p-STAT-1 (blue line)]; (B) Constitutive MFI of p-STAT-1 on $Lin3^{-}CD56^{+}CD16^{+}$, and $CD3^{+}CD14^{+}$ cells in HCV mono-infected and HIV/HCV co-infected subjects; (C) *In vitro* IFN-α-induced area under the curve (AUC) - constitutive AUC for effector:target (E:T) ratios of 50:1, 25:1, 12.5:1 and 6.25:1 in HCV mono-infected and HIV/HCV co-infected subjects. Data in panels (B) and (C) are shown as inter-quartile box plots with median and outliers for each group, and significant (<0.1) P values.

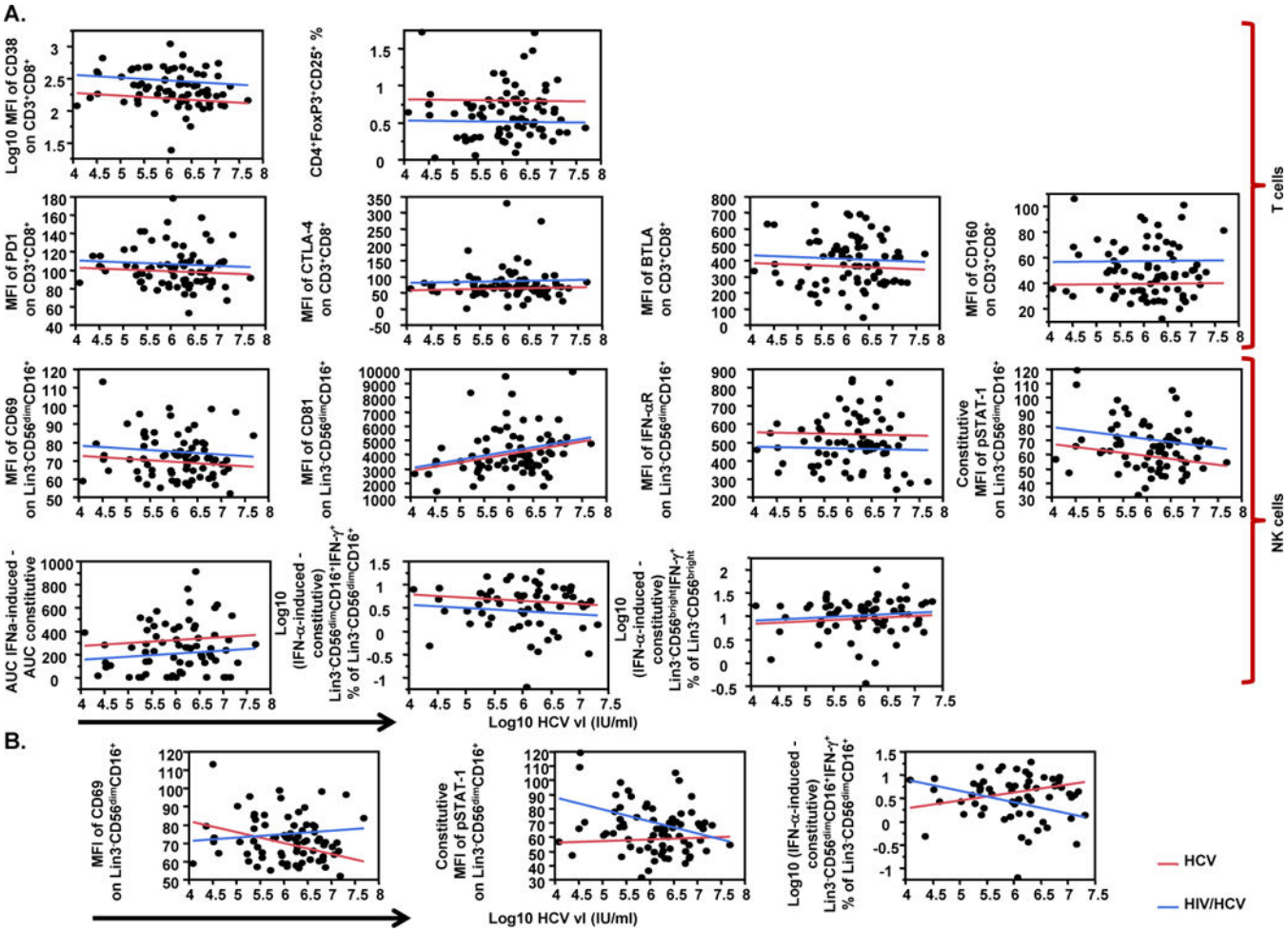

Fig. 3.

Multivariate linear regression models of HCV vl and patient group as predictors of immune variables levels. (A) Effect of HCV vl (log10 IU/ml) and patient group (HCV or HIV/HCV) as independent predictors (model 1) on T cell markers of activation/exhaustion [MFI of CD38 on $CD3^+CD8^+$, $CD4^+FoxP3^+CD25^+$ percentage (%) of lymphocytes, MFI of PD1 on $CD3^+CD8^+$, MFI of CTLA-4 on $CD3^+CD8^+$, MFI of BTLA on $CD3^+CD8^+$, MFI of CD160 on $CD3^+CD8^+$] (top panel), and NK cell markers of activation and function {MFI of CD69 on $Lin3^-CD56^{dim}CD16^+$, MFI of CD81 on $Lin3^-CD56^{dim}CD16^+$, MFI of IFN-αR on $Lin3^-CD56^{dim}CD16^+$, constitutive MFI of phosphorylated (p) STAT-1 on $Lin3^-CD56^{dim}CD16^+$, *in vitro* IFN-α-induced direct cytotoxicity against K562 target cells [expressed as *in vitro* IFN-α-induced area under the curve (AUC) - constitutive AUC for effector:target (E:T) ratios of 50:1, 25:1, 12.5:1 and 6.25:1], (*in vitro* IFNα-induced - constitutive) $Lin3^-CD56^{dim}CD16^+IFN\text{-}\gamma^+$ % of $Lin3^-CD56^{dim}CD16^+$, and (*in vitro* IFNα-induced - constitutive) $Lin3^-CD56^{bright}IFN\text{-}\gamma^+$ % of $Lin3^-CD56^{bright}$} (bottom panel); (B) Effect of HCV vl (log10 IU/ml) and patient group (HCV or HIV/HCV) including the addition of interaction term (model 2) on MFI of CD69 on $Lin3^-CD56^{dim}CD16^+$, constitutive MFI of pSTAT-1 on $Lin3^-CD56^{dim}CD16^+$, and (*in vitro* IFNα-induced - constitutive) $Lin3^-CD56^{dim}CD16^+IFN\text{-}\gamma^+$ % of $Lin3^-CD56^{dim}CD16^+$ NK cells. Data in

panels (A) and (B) are shown as plots of each variable (axis y) against log10 HCV vl (IU/ml) (axis x). Lines in plots represent the perfect fit for each group of patients (red line for HCV mono-infected and blue line for HIV/HCV co-infected subjects).

Table 1

Subjects characteristics

	HCV	HIV/HCV
Total number of subjects	33	45
Gender *	23M, 10F	28M, 16F, 1M to F
Age (years), median (25^{th}, 75^{th} quartiles)	54 (50, 59.5)	50 (46, 54)
Race	33 African American	45 African American
Ethnicity	33 Non-Hispanic	45 Non-Hispanic
Log10 HCV vl (IU/ml), median (25^{th}, 75^{th} quartiles)	6.29 (5.9, 6.74)	6.08 (5.42, 6.51)
HIV vl (copies/ml), median (25^{th}, 75^{th} quartiles)	N/A †	48 (48, 73)
$CD4^+$ T cell count (cells/mm^3), median (25^{th}, 75^{th} quartiles)	963 (743.5, 1272.5)	412 (232.5, 563.5)

* Gender: M:Male, F:Female.

† N/A:Not Applicable.

Table 2

T cell activation/exhaustion, and NK and myeloid cells activation and function in HCV and HCV/HCV subjects before and after multiple testing adjustment

Category	Variable Name	HCV Median (25th, 75th quartile)	N (HCV)	HIV/HCV Median (25th, 75th quartile)	N (HIV/HCV)	P (HCV vs HIV/HCV)	P (BY*: HCV vs HIV/HCV)
T cells	$CD3^+CD4^+$ %	40.2 (36.1, 47.2)	33	18.7 (13.7, 25.3)	44	<0.001	<0.001
	$CD3^+CD8^+$ %	24 (19.5, 27.5)	33	45 (38.9, 51.6)	44	<0.001	<0.001
T cell activation	$CD3^+CD8^+CD38^+$ %	7 (5.9, 9.4)	33	24.8 (14.9, 30)	44	<0.001	<0.001
	$CD3^+CD8^+HLADR^+$ %	0 (0, 0.1)	33	0.1 (0.1, 0.3)	44	<0.001	0.01
	$CD3^+CD8^-CD38^+$ %	15.9 (10.5, 17.7)	33	11 (7.2, 14.7)	44	0.03	ns[f]
	$CD3^+CD8^+CD38^+$ % of $CD3^+CD8^+$	34.6 (27.1, 43.7)	33	55.8 (38.1, 68.5)	44	<0.001	<0.001
	$CD3^+CD8^-CD38^+$ % of $CD3^+CD8^-$	36.6 (28.4, 40.6)	33	54.6 (45.8, 63)	44	<0.001	<0.001
	$CD3^+CD8^-$HLA-DR^+ % of $CD3^+CD8^-$	0.3 (0.2, 0.5)	33	0.8 (0.4, 1.1)	44	0.02	ns
	MFI of CD38 on $CD3^+CD8^+$	156 (118, 201)	33	299.5 (178, 444)	44	<0.001	<0.001
	MFI of HLA-DR on $CD3^+CD8^+$	46.8 (41.9, 51.4)	33	50.4 (42.3, 62.3)	44	0.04	ns
	MFI of CD38 on $CD3^+CD8^-$	166 (131, 206)	33	338 (259, 418)	44	<0.001	<0.001
	MFI of HLA-DR on $CD3^+CD8^-$	44.6 (40.3, 51.2)	33	52.6 (45.9, 64.1)	44	0.009	ns
Tregs	$CD4^+FoxP3^+CD25^+$ %	0.8 (0.6, 0.9)	33	0.4 (0.3, 0.7)	44	<0.001	0.02
T cell exhaustion	$CD3^+CD8^+BTLA^+CD160^+$ % of $CD3^+CD8^+$	0.5 (0.1, 1.2)	33	1.5 (0.5, 3.6)	44	0.01	ns
	MFI of CD160 on $CD3^+CD8^-$	24.4 (20.2, 26.9)	33	29.1 (22.3, 33.8)	44	0.01	ns
	MFI of CD160 on $CD3^+CD8^+$	35 (27.9, 46.6)	33	52.7 (44.5, 71)	44	<0.001	0.007
	MFI of CTLA-4 on $CD3^+CD8^+$	63.4 (44.1, 72.5)	33	69.3 (59.8, 91.1)	44	0.02	ns
Myeloid cells activation/apoptosis	MFI of CD86 on $CD3^-CD14^+$	325 (287,364)	33	333.5 (306.8, 389)	44	0.03	ns
	MFI of CD86 on $CD19^-BDCA1^+CD11c^+$	279 (261, 296)	33	296.5 (267, 321.5)	44	0.004	ns
	MFI of TRAIL on $CD3^-CD14^+$	53.1 (28.8, 69)	33	61.4 (50.2,80.4)	44	0.004	ns

Category	Variable Name	HCV Median (25th, 75th quartile)	N (HCV)	HIV/HCV Median (25th, 75th quartile)	N (HIV/HCV)	P (HCV vs HIV/HCV)	P (* BY: HCV vs HIV/HCV)
Myeloid cells activation/apoptosis	MFI of TRAIL on $BDCA2^+BDCA4^+$	75.5 (66.7,86.3)	33	86.5 (77,94.7)	44	0.02	ns
NK cell activation	MFI of CD69 on ǂ $Lin3^-CD56^{dim}CD16^+$	70.2 (59.8, 73.7)	32	72.8 (65, 83.8)	43	0.04	ns
	MFI of CD69 on $Lin3^-CD56^{bright}$	75.2 (70, 80)	32	82.4 (72.2, 90.7)	43	0.04	ns
IFN-αR on NK cells	MFI of IFN-αR on $Lin3^-CD56^{dim}CD16^+$	526 (456, 627.5)	32	480 (367, 520.5)	43	0.01	ns
	MFI of IFN-αR on $Lin3^-CD56^{dim}CD16^-$	465.5 (427, 560.2)	32	410 (353, 475.5)	43	0.02	ns
Signaling (Constitutive STAT-1)	MFI of p-STAT-1 on $Lin3^-CD56^+CD16^+$	57.7 (47.2, 66)	33	68 (59.3, 78.7)	43	<0.001	0.05
	MFI of p-STAT-1 on $Lin3^-CD56^+CD16^-$	101 (76.1, 110)	33	116 (93.6, 136.5)	43	0.006	ns
	MFI of p-STAT-1 on $Lin3^-CD56^-CD16^+$	57.4 (45.1, 70.2)	33	68.8 (60.1, 88.5)	43	0.002	ns
	MFI of p-STAT-1 on $CD3^-CD14^+$	145 (129, 157)	33	171 (145.5, 194)	43	<0.001	0.02
	MFI of p-STAT-1 on $BDCA2^+BDCA4^+$	116 (83.9, 135)	33	134 (113, 151)	43	0.01	ns
NK cell direct cytotoxicity	AUC§ Constitutive	1080.3 (631.9, 1716.2)	29	889.9 (401.2, 1188.2)	40	0.04	N/A¶
	AUC IFNα-induced - AUC constitutive	320.1 (173.4, 419)	29	157.5 (73.7, 277.6)	40	0.02	N/A

*BY: Benjamini and Yekutieli adjusted p.

ǂns: non-significant.

ǂLin3: CD3, CD14, CD19, CD20.

§AUC: Area under the curve.

¶N/A: not applicable.

Table 3

Linear models using HCV vl (log10 copies/ml) and patients group as predictors of immune variables

Response	Terms ▸	HCV vl		Group*	
	Model ▾	Estimate	p	Estimate	p
Log10 MFI of CD38 on $CD3^+CD8^+$	1	−0.04	ns′	−0.14	<0.0001
$CD4^+FoxP3^+CD25^+$ %	1	−0.008	ns	0.14	0.0002
MFI of PD1 on $CD3^+CD8^+$	1	−1.99	ns	−3.85	ns
MFI of CTLA-4 on $CD3^+CD8^+$	1	2.83	ns	−11.48	0.04
MFI of BTLA on $CD3^+CD8^+$	1	−11.22	ns	−23.75	ns
MFI of CD160 on $CD3^+CD8^+$	1	0.36	ns	−8.86	<0.0001
MFI of CD69 on $Lin3^-CD56^{dim}CD16^+$	1	−1.65	ns	−2.78	0.04
MFI of CD81 on $Lin3^-CD56^{dim}CD16^+$	1	590.17	0.02	−98.78	ns
MFI of IFN-αR on $Lin3^-CD56^{dim}CD16^+$	1	−5.03	ns	39.33	0.01
Constitutive MFI of p-STAT-1 on $Lin3^-CD56^+CD16^+$	1	−4.18	ns	−5.92	0.002
AUC IFNα-induced - AUC constitutive	1	26.11	ns	58.39	0.02
Log10 (IFNα-induced - constitutive) $Lin3^-CD56^{dim}CD16^+IFN\text{-}\gamma^+$ % of $Lin3^-CD56^{dim}CD16^+$	1	−0.07	ns	0.11	ns
Log10 (IFN-α-induced - constitutive) $Lin3^-CD56^{bright}IFN\text{-}\gamma^+$ % of $Lin3^-CD56^{bright}$	1	0.06	ns	−0.03	ns

Response	Terms ▸	HCV vl		Group		HCV vl: Group	
	Model ▾	Estimate	p	Estimate	p	Estimate	p
MFI of CD69 on $Lin3^-CD56^{dim}CD16^+$	2	−2.04	ns	−2.7	ns	−3.93	0.03
Constitutive MFI of p-STAT-1 on $Lin3^-CD56^+CD16^+$	2	−3.64	ns	−6.01	0.001	4.78	0.04
Log10 (IFNα-induced - constitutive) $Lin3^-CD56^{dim}CD16^+IFN\text{-}\gamma^+$ % of $Lin3^-CD56^{dim}CD16^+$	2	−0.03	ns	0.1	ns	0.21	0.008

*Group: HCV mono-infected, HIV/HCV co-infected.

′ns: non-significant