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Human Immunodeficiency Virus and Hepatitis C infections induce distinct immunologic imprints in peripheral mononuclear cells

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Abstract

Co-infection with Hepatitis C virus (HCV) is present in one-third of all Human Immunodeficiency Virus-infected (HIV) individuals in the United States and is associated with rapid progression of liver fibrosis and poor response to pegylated interferon (IFN) and ribavirin. In this study, we examined gene expression profiles in peripheral blood mononuclear cells (PBMCs) from different groups of individuals who are mono- or co-infected with HIV and HCV. Data showed that HIV and HCV viremia up-regulate genes associated with immune activation and immunoregulatory pathways. HCV viremia is also associated with abnormalities in all peripheral immune cells, suggesting a global effect of HCV on the immune system. Interferon- α -induced genes were expressed at a higher level in PBMCs from HIV-infected individuals. HCV and HIV infections leave distinct profiles or gene expression of immune activation in PBMCs. HIV viremia induces an immune activated state; by comparison, HCV infection induces immunoregulatory and pro-inflammatory pathways that may contribute to progression of liver fibrosis. An aberrant type-I IFN response seen exclusively in HIV-infected individuals could be responsible for the poor therapeutic response experienced by HIV/HCV co-infected individuals receiving interferon- α based current standard of care.

Keywords

immunity; immune activation; microarray; interferon-inducible genes; biomarker

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Conflict of Interest Statement

None of the other authors have any conflicts of interest to report.

Introduction

Chronic co-infection with hepatitis C virus (HCV) is documented in one-third of all HIV-infected persons in the United States, and is associated with increased morbidity and mortality relative to mono-infection with either virus (1,2). Since the advent of antiretroviral therapy (ART) for controlling HIV replication *in vivo*, AIDS-associated opportunistic infections have declined considerably (3). However, recent data suggest an increasing number of HIV-infected individuals are now dying from liver disease (3–5). Moreover, HCV/HIV co-infected individuals see a rapid progression of liver fibrosis to cirrhosis when compared to HCV mono-infected individuals (6). Several adverse effects associated with ART are exacerbated in HCV/HIV co-infected individuals, making it difficult to accomplish adequate virologic control of HIV infection among such individuals (7–12). Additionally, HCV/HIV co-infected individuals have a higher HCV RNA viral load than do HCV mono-infected individuals (13–17). Finally, co-infection with HIV decreases the rates of sustained virologic response (SVR) of HCV and increases the rate of relapses after discontinuation of anti-HCV therapy among those who have achieved an end-of treatment response (ETR) to combination therapy (14–17).

Chronicity of infection with HCV mono-infected individuals is associated with an inconspicuous immune response against the virus (18); in contrast, in HIV mono-infected individuals, the resultant immune response is readily detectable, but is unable to contain HIV replication, leading to establishment of chronic infection (19). The characterization of and relationships between (the immune responses against HCV and HIV in co-infected individuals are not completely understood.

To determine the differential host immune responses to each virus, we employed a DNA microarray study using PBMCs from five different groups of individuals. DNA microarrays has been used previously to study the pathogenesis of HCV (20) and HIV in mono-infected individuals (21). However, such studies failed to define gene expression imprints and/or adequately compare the differentiated gene profiles induced by each of these viruses alone and in co-infection because the studies did not involve direct comparison of the gene expression profiles of HCV and HIV co-infected individuals. In this study, we performed DNA microarray analysis on PBMCs from HIV negative, HIV viremic, HIV aviremic, HCV viremic, and HCV/HIV co-infected individuals to determine the differential gene expression among these groups. To our knowledge this study is the most comprehensive DNA microarray study that involves cross sectional analysis of all 5 control groups. These genetic imprints provide insights into the pathophysiology of chronic HCV infection in those subjects who are co-infected with HIV.

Methods

Study subjects

Peripheral Blood Mononuclear Cells (PBMCs) were obtained by venipuncture from thirty-three subjects belonging to the following clinical categories: HIV negative (n=7), HCV mono-infected viremic (n=7), HIV mono-infected viremic (n=8), HCV/HIV co-infected (n=5), and HIV aviremic (n=6) (Table 1A). All donors signed informed consents approved by the Institutional Review Board (IRB) of the National Institute of Allergy and Infectious Diseases (Bethesda, MD).

Isolation of PBMCs and RNA

PBMCs were isolated from white blood cells by the standard Ficoll-Hypaque Plus (Amersham Biosciences, Uppsala, Sweden) density gradient separation technique. RNA was

isolated using Qiagen mRNA isolation kits (Qiagen, Germantown, MD) following the manufacturer' protocol.

Cell Cultures

Each patient' PBMCs (2×10^6) were incubated in 12-wellplates with complete RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT), enicillin-streptomycin (Invitrogen), and L-glutamine (Invitrogen),. Cultures were incubated at 37° in a 5% CO₂ incubator for up to 48-hours, and supernatants from each culture were collected.

DNA Microarray Analysis

PBMCs were analyzed using Affymetrix U133A 2.0 oligonucleotide arrays according to the protocols specified by the manufacturer (Affymetrix, Santa Clara, CA). A significant analysis of microarray (SAM) algorithm was used to determine the genes that were differentially expressed after an extensive filtering processes (22). Genes with low variability or undetectable expression levels (for the majority of samples) were eliminated from analysis if the Guanosine-Cytosine Robust Multi Array values for these genes were within the interquartile range of <0.263 or a 75th percentile of <5 .

bDNA Multiplex Assay

Validation of DNA microarray data was performed using a novel customized bDNA multiplex assay capable of detecting the expression of 35 genes. The RNA transcripts are released from cells in the presence of Lysis Mixture and hybridized to the Probe Sets. The RNA-Probe Set complexes are captured to their respective Capture Beads through the cooperative hybridization of multiple CEs with the Capture Probes on the Capture Beads during an overnight incubation. Signal amplification is performed by sequential hybridization of the bDNA Amplifier and biotinylated Label Probe. The Streptavidin-conjugated R-Phycoerythrin (SAPE) binds to the biotinylated Label Probe. The Capture Beads are analyzed using a Luminex instrument. The amount of each target RNA present in a sample is quantified by determining the amount of SAPE fluorescence signal and the identity of the beads.

Flow Cytometry

For flow-cytometric analyses, the following combinations of fluorochrome-conjugated antibodies were used: CD3 (allophycocyanin-conjugated [APC]) with CD54 (phosphatidylethanolamine [PE]), CCR2 (PE), CCR7 (PE), CD10 (PE), CD80 (PE), CD86 (PE), CD274 (PE), CX₃CR1 (PE), IGF-1R (PE), and NCR3 (PE). All antibodies and appropriate isotype controls were obtained from BD Biosciences, San Jose, CA. After washing, PBMCs were incubated with appropriate antibodies for 30 min at 4°C. The cells were washed, fixed, and suspended in 1% paraformaldehyde in PBS and flow-cytometric analysis was performed on a FACS Array (BD Biosciences). For subset analysis, a lymphocyte gate and gates uniquely identifying CD3⁺ cells were applied, ~100,000 events were collected, and the frequency of CD3⁺ and CD3⁻ expressing each receptor was analyzed with FlowJo software (TreeStar, Ashland, OR).

ELISA

Culture supernatants from PBMCs at 48 hours were tested for levels of IL-23A, β 2 microglobulin, TNF, CCL-7, CCL-20, IL-8, and CXCL1 by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN).

Statistical Analysis

ANOVA with Tukey's multiple comparison test was used to compare means of the independent groups. The paired t test with the Bonferroni adjustment for multiple testing was used to compare paired responses.

Results

Differential gene expression profiles in PBMCs of HCV and HIV infected individuals

In order to identify the gene expression profiles induced by both HCV and HIV, we performed DNA microarray analyses using total RNA isolated from fresh PBMCs from the aforementioned five patient groups. There were no significant differences in the 5 groups based on the major demographic characteristics such as sex, age, treatment responses, etc (Table 1A). Using Affymetrix human genome U133A oligonucleotide arrays consisting of probes encompassing over 22,000 genes and a SAM algorithm (22), we identified 1813 differentially expressed genes (Fig 1). The corresponding genes and samples from the individuals were grouped by using hierarchical clustering. Differences in relative levels of gene expression (Z-score) are indicated in color, where red indicates up-regulation and green indicates down-regulation relative to that of corresponding gene expression in controls (Fig 1). The hierarchical analyses classified the genes into six distinct clusters based on differential expression between the five groups. Of these, Cluster 1 consists of 174 genes down-regulated both in HCV and HIV infected individuals (group B and C). Cluster 2 consists of 369 genes up-regulated in HCV-infected individuals (groups B and D). Cluster 3 consists of genes up-regulated in HIV mono-infected individuals (group C and E). Cluster 4 consists of genes up-regulated in only HIV viremic individuals (group C). Cluster 5 consists of 302 genes down-regulated in only HCV mono-infected individuals (group B). Cluster 6 consists of 599 genes up-regulated in only HCV mono-infected individuals (group B). To identify HCV-induced changes in gene expression in PBMCs, we chose to focus our analyses on clusters 2, 5 and 6. In this regard, cluster 2 distinctively showed genes up-regulated by HCV viremia as observed in both HIV negative and positive individuals. Meanwhile, clusters 5 and 6 showed genes that are either down-regulated or up-regulated by HCV viremia alone as observed in HCV mono-infected individuals. Hierarchical clustering analyses indicated that there was a similarity in the transcriptional profile of genes that were differentially expressed in PBMCs of HCV-infected individuals (seen in cluster 2 genes) and also distinct gene expression profiles in HCV mono-infected and HCV/HIV co-infected individuals (seen in cluster 5 and 6 genes). Representative genes that belong to each cluster identified using rigorous literature-mining algorithms and statistical analyses are shown in Fig 1.

Selection of genes for validation of DNA microarray by amplification

To validate our DNA microarray data, we selected genes based on an extensive literature-mining algorithm, significance of microarray analysis data and biology of the disease process (both HIV and HCV). This analytical approach following the biology of response rather than the mere expression levels of genes will help us ascertain our results are driven by the biology (disease process, infection status etc) rather than race, age, sex etc. Although this approach reduces the influence of these variables in interpretation of results, it will certainly not eliminate the influence completely, warranting validation of these results in a larger study. We performed gene amplification from RNA, estimated surface expression of receptors of freshly isolated PBMCs and the levels of secreted proteins in culture supernatants. Gene amplification performed by bDNA analysis of selected genes that were differentially regulated using a custom-designed bDNA array is shown in Fig 2. Two candidate genes OAS1 (2A & B) and MX1 (2 C & D) were selected for validation of gene expression and were consistently reproduced by bDNA assay.

Selection of genes/gene products for validation by microarray flow cytometry and ELISA

For further validation of our DNA microarray analyses at the level of surface expression of proteins, we selected most biologically relevant gene products based on biological relevance (Fig. 3 and Table 1B). From cluster 2, we chose IGF-1R, a cell surface receptor that increases insulin secretion upon stimulation (23); CCR7, which is a chemokine homing receptor of immune cells (24) and NKp30, which is one of the NK activating receptors facilitating killing of infected targets (25). From cluster 5, we examined CCR2 and CX₃CR1, two chemokine receptors involved in inflammatory response and lymphocyte activation (26). From cluster 6, we studied the expression of CD10, CD54, CD80, and CD274. CD10 is a cell surface marker of immature B cells (27); CD54 is involved with cell-cell adhesion and formation of immunological synapses (28); CD80 is a major T-cell co-stimulatory factor (29); and CD274 is a cell surface molecule with a T-cell regulatory function (30). To elucidate the effect of HIV and HCV on the secretory function of PBMCs, we tested the ability of PBMCs to secrete the following proteins, CCL-7, CCL-20, CX₃CL1, IL-8 and TNF- α . CCL-7 (MCP-3) is a chemokine that attracts monocytes to sites of inflammation and regulates macrophage function (24). CCL-20, otherwise called liver activation regulated chemokine (LARC) or macrophage inflammatory protein-3 A (MIP-3 α), is a chemokine that attracts lymphocytes, but is a weaker target for monocytes (31). CX₃CL-1 also known as GRO- α or fractalkine, is a cytokine belonging to the CX₃C chemokine family and is a neutrophil chemo attractant, which is also involved in angiogenesis, inflammation and tissue healing (32). TNF- α and IL-8 are pro-inflammatory cytokines secreted by T cells that mediate chemotaxis and inflammatory responses (33). All these genes formed part of cluster 6, which are up-regulated in HCV-mono-infected individuals.

HCV infection has a global effect on peripheral blood immune competent cells

To investigate the effect of ongoing HCV replication on various peripheral blood immune competent cells, we performed flow cytometry analysis for the expression of surface molecules whose genes were differentially regulated in the DNA microarray analysis. As shown in Figure 4, HCV replication has profound effects on B cells, NK cells, and on antigen presenting cells. B cells from HCV-monoinfected individuals expressed a significantly higher percentage of CD10⁺ immature B cells in their peripheral blood when compared to that seen in the other groups (14.2 \pm 0.8% (Group B) vs. 7.1 \pm 0.3% (Group A), 8.2 \pm 1.1% (Group C), 3.2 \pm 0.1% (Group D), and 3.9 \pm 0.3%; $p < 0.03$ for Group B versus the others). A significantly higher proportion of NK cells from HCV monoinfected and HIV/HCV co-infected individuals expressed the natural cytotoxicity receptor 3 (NCR3 or NKp30) on their surface compared to the other 3 groups (3 \pm 0.4% (Group A), 9.8 \pm 0.3% (Group B), 2.6 \pm 0.4% (Group C), 8.7 \pm 0.7% (Group D), and 1.8 \pm 0.2% (Group E); $p < 0.04$ for Groups B and D vs Groups A and C and $p < 0.03$ for Groups B and D vs Group E). The percentage of cells expressing CD80 in the peripheral blood of HCV mono-infected subjects was significantly higher compared to that seen with the other 4 groups (5.1 \pm 0.6% (Group A), 14.3 \pm 0.9% (Group B), 4.8 \pm 0.8% (Group C), 5.1 \pm 0.7% (Group D), and 4.4 \pm 0.5% Group E; p value < 0.03 for Group B vs the others). The levels of expression of CD274 (PD-L1) were not significantly different among the groups (data not shown).

Higher levels of markers of hepatic injury were secreted by PBMCs of individuals with chronic hepatitis C infection

Several non-invasive markers of liver fibrosis have been identified in individuals with chronic hepatitis B and C infection (34). We selected markers of liver fibrosis CX₃CL1 and IGF-R1 from cluster 6 and 2 whose gene expressions were also up-regulated in groups B and D (HCV mono-infected and HCV/HIV co-infected subjects) (Fig 3). CX₃CL1 and its receptor CX₃CR1 have been found to be associated with liver injury and have been

suggested to be a marker of liver disease (35). When we measured the levels of CX₃CL1 expression in the supernatants of PBMCs, the cells from HCV mono-infected subjects produced significantly higher levels of CX₃CL1 than did cells from other 4 groups (Fig 5A; 276 ± 18 pg/mL (Group A), 2870 ± 420 pg/mL (Group B), 1020±180 pg/mL (Group C), 950± 110 pg/mL (Group D), and 180 ±30 pg/mL (Group E); p <0.02 for Group B vs Groups C and D and p<0.01 for Group B vs Groups A and E). IGF-R1 expression (Mean Fluorescent Intensity) was significantly higher among HCV mono-infected and HCV/HIV co-infected subjects than that of the other groups (72 ± 3% (Group A), 198± 9% (Group B), 82 ± 8% (Group C), 202±12% (Group D), and 87± 5% (Group E); p value < 0.03 for Groups B and D vs others. These results also validate the respective gene expression pattern observed with DNA microarray analysis.

PBMCs from HCV mono-infected subjects secrete higher levels of pro-inflammatory cytokines

Since both HCV and HIV are chronic viral infections that could result in immune activation and pro-inflammatory responses, we measured the levels of pro-inflammatory cytokines whose genes were differentially expressed among the groups in the DNA microarray analysis (Cluster 6, Fig 3). The levels of CCL-7 (MCP-3) in the culture supernatants of PBMCs from HCV mono-infected subjects were significantly increased compared to those of the other 4 groups (Fig 6A; 90 ± 9 pg/mL (Group A), 2190±650 pg/mL (Group B), 150 ± 15 pg/mL (Group C), 240 ± 22 pg/mL (Group D), 75 ± 5 pg/mL (Group E); p< 0.001 between Group B vs the others). The levels of CCL-20 (MIP-3α) were also significantly elevated in the culture supernatants of PBMCs from HCV mono-infected subjects compared to those of the other 4 groups (Fig 6B; 10 ± 1 pg/mL (Group A), 51 ± 8 pg/mL (Groups B), 2 ± 0.1 pg/mL (Group C), 48 ± 9 pg/mL (Group D), 1.8 ± 0.1 pg/mL (Group E); p <0.01 between Group B vs Groups A and D and p<0.005 for Group B vs Groups C and E). These results consistently validate our DNA microarray data.

HIV-infected subjects express higher levels of IFIG than do HCV mono-infected or normal subjects

We had observed previously that interferon inducible gene (IFIG) expression in PBMCs is significantly higher in HCV/HIV co-infected individuals who fail to respond to treatment (36). Furthermore, we demonstrated that non-responders to IFN-α therapy failed to induce IFIG expression even after exogenous IFN-α treatment (manuscript in preparation). It is unclear whether HCV/HIV co-infected individuals have a higher level of IFIG expression when compared to HCV mono-infected individuals. Therefore, we examined the microarray data for IFIG expression in all 5 groups (Fig 7, A through F) and measured the expression of 25 IFIG by bDNA multiplex. Our results showed that HIV mono-infected individuals had significantly higher expression of IFIG genes than did the other groups (p value <0.05).

Discussion

In this study, we demonstrate that HCV and HIV infections induce distinct immunological profiles in PBMCs as determined by gene expression. While HCV infection leads to the expression of genes that reflect a pro-inflammatory immune response, mainly on non-T cells. HIV infection induces an immune activation profile involving CD4⁺ and CD8⁺ T cells. Liver activation-specific inflammatory markers were induced in PBMCs of both HCV mono- and HIV/HCV co-infected individuals. HCV infection induces activation of all peripheral immune cells reflecting a global effect of chronic HCV replication on the immune system. Moreover, the IFIG expression, which is highly predictive of the therapeutic response to IFN-α based therapy, is expressed at a higher level in HIV-infected individuals,

re-iterating a mechanistic role for type-I IFN baseline hyper-expression *in vivo* in HCV/HIV co-infected individuals who fail to clear HCV with IFN- α therapy.

Several studies have demonstrated that HIV infection induces a state of immune activation, which is responsible in part for the immunopathogenesis of the disease (37). HIV viremia has been shown to induce cellular activation as demonstrated by serum markers (38), activation of B cells (39), NK cells (25), pDCs (40), CD4⁺ (41) and CD8⁺ T cells (42). Although analysis of HIV-induced gene expression profiles in PBMCs was not the primary objective in this study, our results largely confirmed the activation of peripheral T cells in HIV-infected viremic subjects, when compared to those who were aviremic or HIV seronegative. These changes were most remarkable among CD8⁺ T cells, which showed over expression of genes coding for surface markers, such as CD38, HLA DR, CD25, as well as granzyme and perforin genes, which represent an activated peripheral CD8 response to ongoing HIV replication (data not shown).

Unlike HIV, HCV primarily infects and replicates in human hepatocytes (43). Although several studies have shown that HCV can be detected in non-hepatic cells and tissues, maximal replication is sustained only in primary hepatocytes (44). Recent studies also have shown that chronic HCV infection does not lead to the extent of immune activation of T cells seen in HIV infected subjects (43). Our results also show increased expression of certain genes and expression of various receptors on NK cells (NKp30), B cells (CD10), and pDCs (CD80) in PBMCs from HCV-infected subjects, when compared with HIV-infected or HIV seronegative subjects. This increased expression of NKp30 on NK cells suggests a level of activation of NK cells in chronically viremic HCV mono-infected subjects. Future functional studies should confirm whether this increased expression of a natural cytotoxicity receptor may result in enhanced cytotoxic capacity of NK cells from HCV viremic individuals or whether the over expression of this receptor merely represents a state of aberrant activation or anergy of NK cells from HCV viremic subjects. HCV-infected subjects had a significantly increased proportion of CD10⁺ immature B cells when compared to normal controls. Expansion of CD10⁺ B cells have been described in other chronic persistent viral infections such as HIV and is thought to represent an accelerated release of immature B cells as a result of IL-7 that is responding to the CD4⁺ T cell lymphopenia (39). In this regard, it is possible that chronic HCV infection also results in B cell dysfunction and might explain to some extent the defective humoral immune responses previously described in chronic HCV-infected subjects (45). Finally, PBMCs from HCV mono-infected subjects express higher levels of the T cell co-stimulatory molecule, CD80 when compared to controls. The functional significance of the over expression of this molecule on antigen presenting cells is not completely understood, yet it may represent chronic activation of antigen presenting cells as a result of the persistence of HCV antigens.

Several inflammatory cytokines have been linked to liver damage and the recruitment of effector cells to the liver. There are several markers of liver injury and dysfunction that are elevated in peripheral blood. Expression of CX₃CL1 was increased in individuals infected with HCV when compared to those who do not have underlying liver disease. Recent studies have suggested that fractalkine (CX₃CL1) recruits CX₃CR1-expressing monocytes to the liver that may participate in the process of hepatic inflammation, in agreement with the pro-inflammatory role of fractalkine in other conditions of inflammation (35). These findings reiterate that CX₃CL1/CX₃CR1 interactions do play a significant role in inducing the hepatic inflammation seen in chronic HCV infection. Our results indicate that fractalkine expression is elevated in the peripheral blood cells of individuals with chronic HCV infection and suggest that this parameter be useful as a marker of liver fibrosis/injury, when validated in larger studies. Furthermore, metabolic abnormalities, such as insulin resistance, diabetes, hyperlipidemia are much more common in HCV-infected individuals than in the

other groups (37,40). The expression of IGF-1R on cells from HCV-infected individuals was higher than that in the other patient groups. As demonstrated in our study, PBMCs of HCV mono-infected individuals produce increased levels of several proinflammatory cytokines. These results suggest a role for these cytokines in recruiting effector inflammatory cells to the liver and also serve as a non-invasive marker of liver fibrosis. Both HCV mono and HIV/HCV co-infected individuals have increased serum levels of markers of liver fibrosis compared to seronegative HIV individuals and HIV-infected individuals without liver disease. These findings, when validated in larger studies will definitively reiterate their role as possible non-invasive markers of liver fibrosis.

In summary, some immune markers such as NKp30 and IGF-R1 seem to be up-regulated among patients with HCV with or without HIV co-infection, some others seem to be selectively up-regulated in HCV mono-infected subjects (CD10, CD80, CCL-7 CCL20 and CX3CL1). It is plausible that HIV infection may have resulted in regulating the expression of some of these receptors. Additionally, it is also possible that the liver disease staging might have influenced the levels of some of these cytokines as well. Further studies are warranted to address whether HIV infection specifically interferes with the effects of chronic HCV infection on immune cells.

Our previous studies have described that high IFIG expression is the single most important predictor of therapeutic non-response to IFN-based treatment for HCV (36). This study demonstrates higher expression of IFIG expression among HIV-infected individuals than HCV mono-infected individuals; suggesting HIV infection is the major driving factor in turning on a type-I IFN signature gene expression. Although our study does not involve follow up analysis of treatment responses of all HCV-infected patients, this is the only study that has compared HCV mono-infected subjects to HIV/HCV co-infected subjects with respect to IFIG expression. The results are highly suggestive that HIV co-infection drives type-I IFN signature gene expression, which could result in blunting of responsiveness to exogenous IFN therapy. A recent study has suggested that increased IFIG expression was seen in the liver of HCV mono-infected patients who were IFN-non-responders than those who achieved SVR (46). However, this study did not examine the hepatic IFIG expression, nor did select out patient population based on treatment response. However, a comparative study to look at hepatic and peripheral IFIG expression among HCV-monifected patients is warranted to see if there is a strong correlation between the two compartments.

In summary, our study offers a comprehensive analysis of the differential regulation of host immune responses in HCV and HIV-infected subjects. The results show that both chronic viral infections have distinct immunological profiles that are consistent with the pathogenesis of the disease process. Future studies will be focused on identifying the specific mechanisms involved in the interactions of HIV and HCV that contribute to the establishment of chronicity and the accelerated progression of liver disease seen in co-infected individuals.

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Abbreviations

HCV	Hepatitis C virus
ART	antiretroviral therapy

RNA	ribonucleic acid
HIV	human immunodeficiency virus
ETR	end of treatment response
AIDS	acquired immune disease syndrome
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
ELISA	Enzyme linked immunosorbent assay
FACS	fluorescent-activated cell sorting
IRB	Institutional Review Board
SAM	significant analysis of microarray
SAPE	Streptavidin-conjugated R-Phycoerythrin
CCR	CC chemokine receptor
PE	phosphatidylethanolamine
APC	allophycocyanin-conjugated
CCL	CC chemokine ligand
IL	interleukin
GRO	growth regulated oncogene
MCP	macrophage inflammatory proteins
MCP	monocyte chemotactic protein
CXCR	CXC chemokine receptor
TNF	tumor necrosis factor
CXCL	CXC chemokine ligand
IFN	interferon
IFIG	interferon inducible genes

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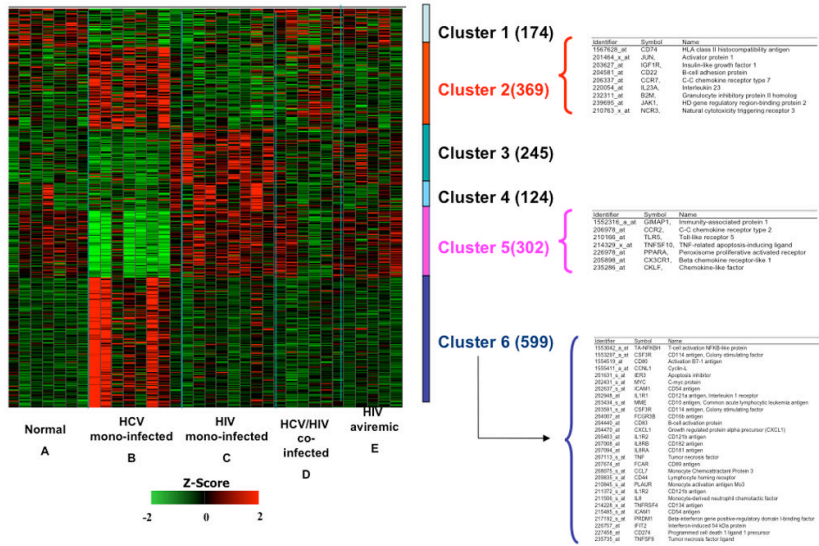


Figure 1. Clustering of differentially expressed genes in PBMCs from 5 groups (See Methods section)

Levels of gene expression were assayed using Affymetrix Human Genome U133A chips. A total of 1,813 differentially expressed genes were identified. Genes were grouped using K-means clustering, and samples were grouped by hierarchical clustering. Differences in relative levels of gene expression (Z-score) are indicated in color, where red indicates up-regulation and green indicates down-regulation relative to that of corresponding gene expression in controls. The numbers in parentheses indicate the number of genes in each cluster. Some genes are listed twice because certain probes recognize multiple regions of a single gene. Cluster 1 consists of genes down-regulated both in group B and C individuals. Cluster 2 consists of genes up-regulated in groups B and D individuals. Cluster 3 consists of genes up-regulated in group C and E. Cluster 4 consists of genes up-regulated only in group C individuals. Cluster 5 consists of genes down-regulated only in group B individuals. Cluster 6 consists of genes up-regulated only in group B individuals. Select biologically relevant genes of Clusters 2, 5 and 6 are shown on the right.

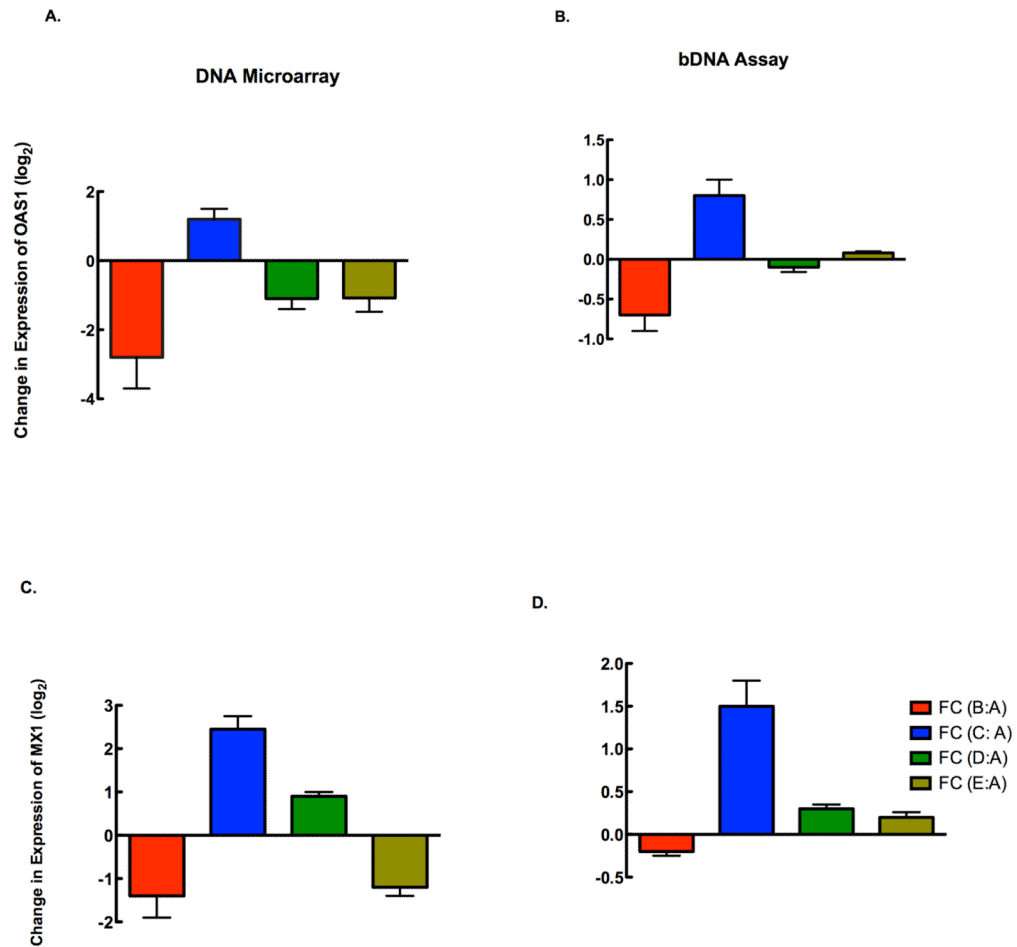


Figure 2. Validation of DNA microarray data by bDNA analysis

DNA microarray expression of IFIG genes was validated using a customized bDNA assay. The expression of two representative IFIGs by DNA microarray (left) and bDNA assay (right) for the two gene candidates OAS1 (A,B) and MX1 (C,D) indicate that HIV-infected individuals have a higher level of IFIG expression overall than the other groups of individuals. Fold change of gene expression is calculated as the change within a group compared to that observed in normal volunteers. The expression of both OAS1 and MX1 were significantly different between Group C and others by microarray ($p < 0.03$, and $p < 0.02$ respectively) and bDNA ($p < 0.04$ and $p < 0.01$ respectively).

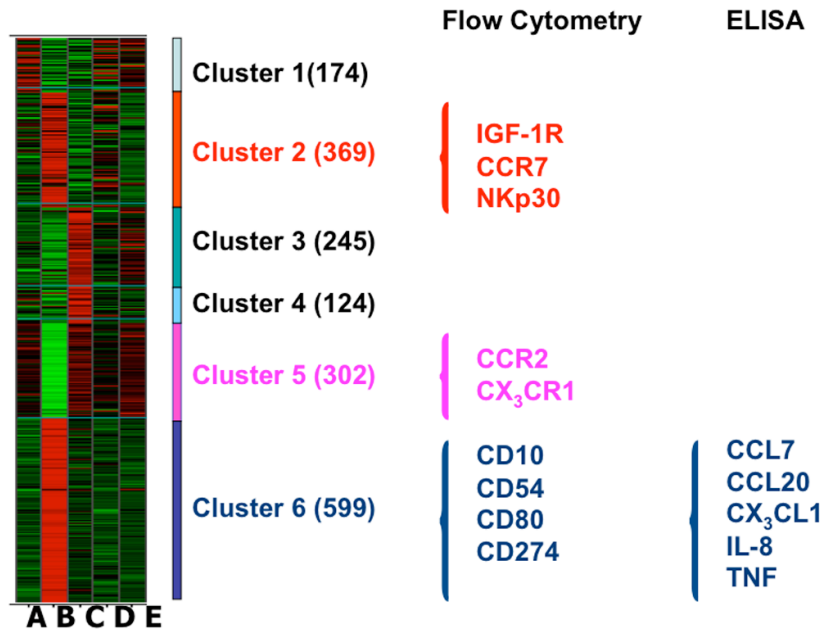


Figure 3. Average differential gene expression profiles of the five groups of individuals
 Using either flow cytometry (surface expression) or ELISA (secreted proteins) selected genes were validated based on their biological relevance (following literature-mining algorithms). Clusters 2, 5 and 6 were selected based on the identification of cell surface receptors and secreted proteins most relevant to HCV infection status.

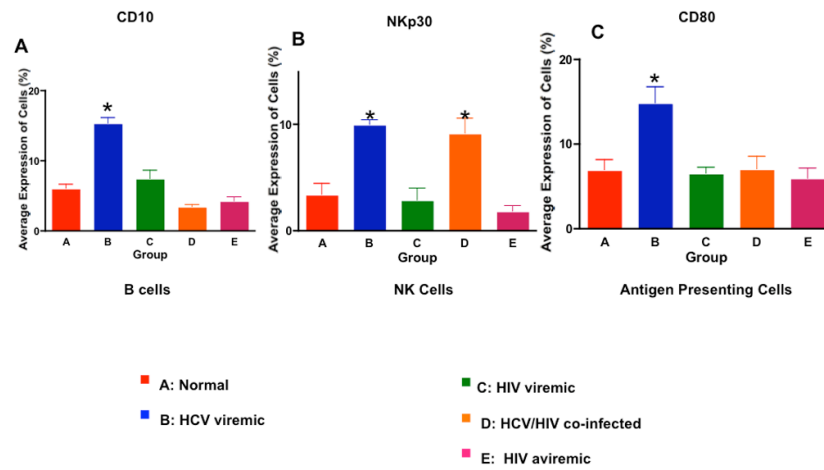


Figure 4. HCV Infection has a Global Effect on Immune Cells

A. The percentage of CD10⁺ immature B cells in the peripheral blood measured by flow cytometry from 5 individuals in the HCV mono-infected group was significantly higher than that of the other 4 groups ($p < 0.03$). B. The percentage of NKp30⁺ cells among NK cells in the peripheral blood was measured by flow cytometry from the 5 groups. HCV-infected (both mono and HIV co-infected) individuals had a significantly higher percentage of NKp30⁺ NK cells than that of the other groups ($p < 0.04$). C. The percentage of CD80⁺ cells among CD3⁻ in HCV mono-infected individuals was significantly higher than that of the other 4 groups ($p < 0.03$).

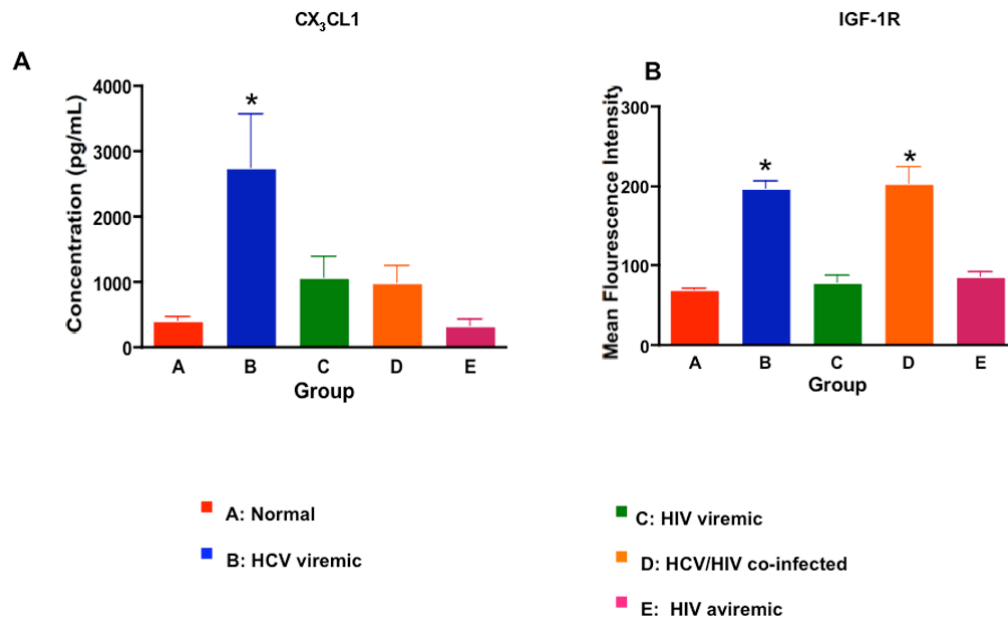


Figure 5. Chronic HCV Infection is Associated with Markers of Liver Injury

A. The levels of CX₃CL1 present in the supernatants of PBMCs from the 5 groups were determined by ELISA. PBMCs from HCV mono-infected individuals secreted significantly higher levels of CX₃CL1 than did the other 4 groups ($p < 0.02$). **B.** Mean Fluorescent Intensity (MFI) of IGF-1R expression on PBMCs showed that HCV-infected (both mono and HIV co-infected) individuals had significantly higher expression of IGF-1R than did the other 3 groups ($p < 0.02$).

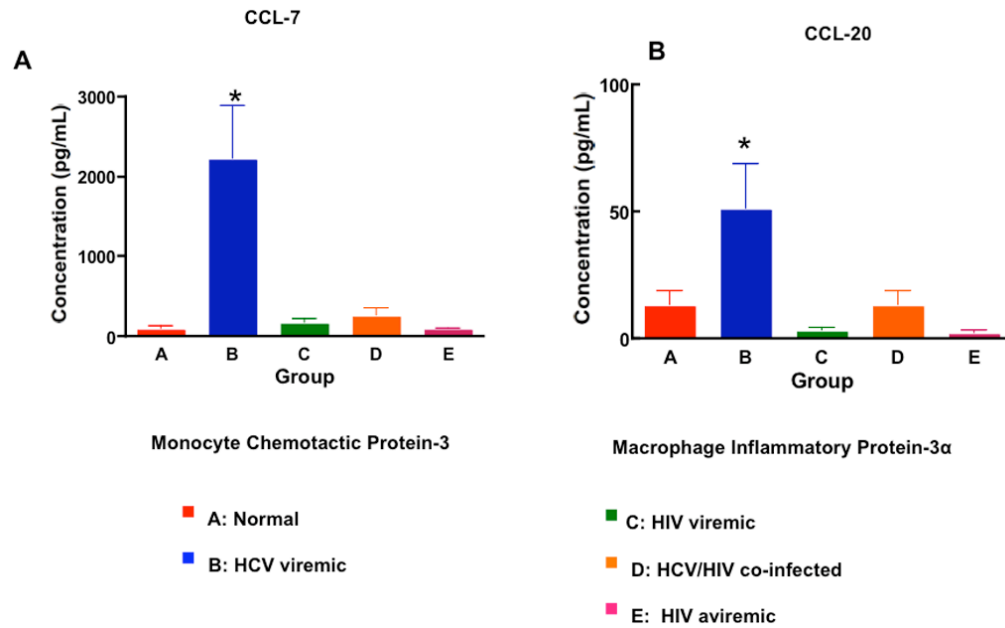


Figure 6. Chronic HCV infection is associated with increased levels of pro-inflammatory chemokines

A. The levels of CCL-7 (Monocyte Chemotactic Protein -3) present in the supernatants of PBMCs from the 5 groups were quantified by ELISA. PBMCs from HCV mono-infected individuals secreted significantly higher levels of CCL-7 than did the other 4 groups ($p < 0.001$). B. The levels of CCL-20 (Macrophage Inflammatory Protein-3 α) present in the supernatants of PBMCs from HCV mono-infected individuals were significantly higher than those of the other 4 groups ($p < 0.01$).

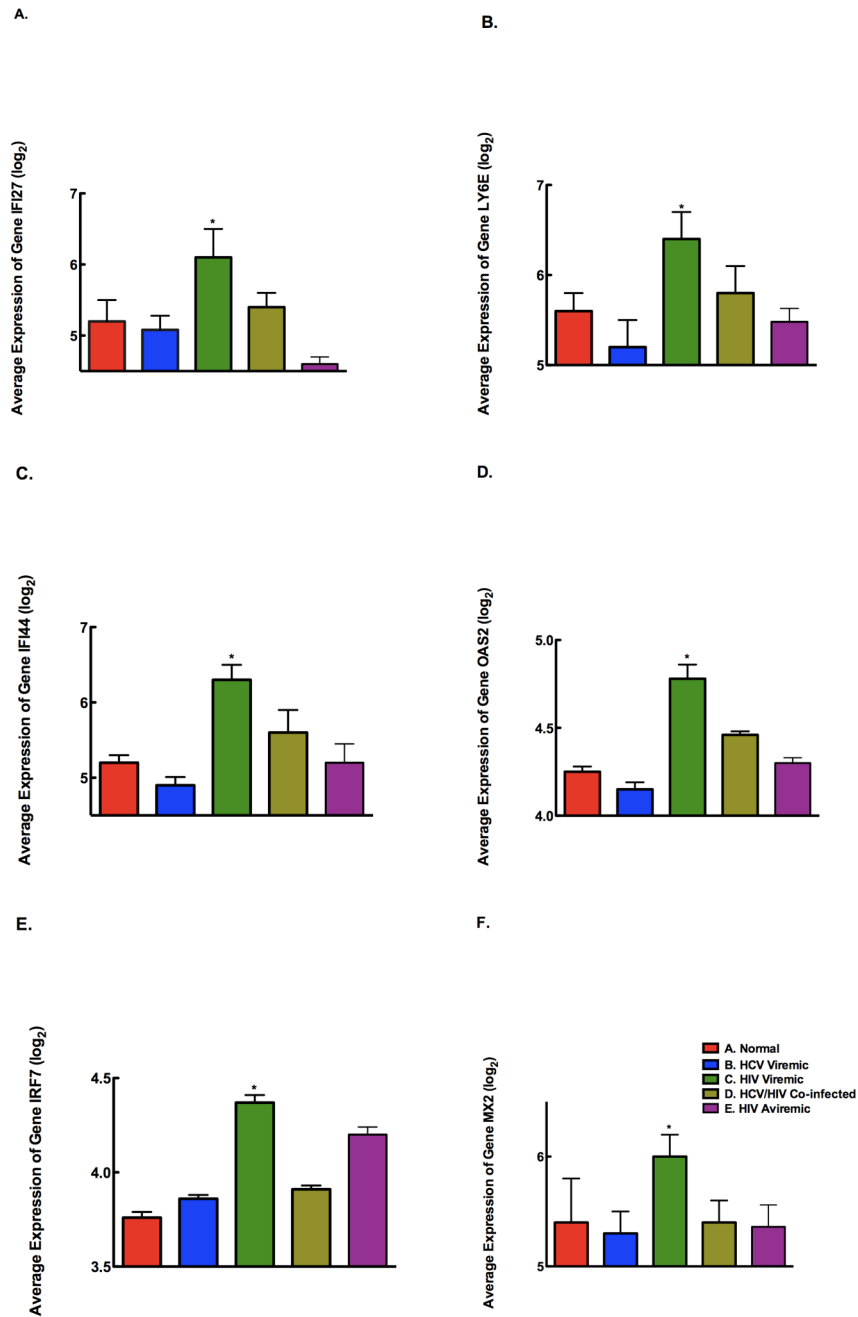


Figure 7. HIV-infection results in increased expression of IFIG that is not seen in normal individuals and HCV-infected individuals

The gene expression profile of several differentially regulated genes was measured using a customized bDNA assay. IFIG genes were found to be up-regulated in HIV viremic individuals compared to other groups of individuals. ($p < 0.05$). Panel A: IFI27 gene expression was significantly higher in PBMCs from Group C compared to Group A ($p < 0.04$), Group B ($p < 0.035$), Group D ($p < 0.048$), and Group E ($p < 0.034$). Panel B: LY6E gene expression was significantly higher in PBMCs from Group C compared to Group A ($p < 0.02$), Group B ($p < 0.01$), Group D ($p < 0.04$), and Group E ($p < 0.015$). Panel C: IFI44 gene expression was significantly higher in PBMCs from Group C compared to Group A

($p < 0.038$), Group B ($p < 0.02$), Group D ($p < 0.04$), and Group E ($p < 0.038$). Panel D: OAS2 gene expression was significantly higher in PBMCs from Group C compared to Group A ($p < 0.02$), Group B ($p < 0.005$), Group D ($p < 0.03$), and Group E ($p < 0.028$). Panel E: IRF7 gene expression was significantly higher in PBMCs from Group C compared to Group A ($p < 0.042$), Group B ($p < 0.039$), Group D ($p < 0.04$), and Group E ($p < 0.044$). Panel F: MX2 gene expression was significantly higher in PBMCs from Group C compared to Group A ($p < 0.02$), Group B ($p < 0.018$), Group D ($p < 0.022$), and Group E ($p < 0.02$).

Table 1

Table 1A. Demographics and immune profiles of Study Participants: Immune profiling was performed by flow cytometry for all individuals except HCV mono-infected individuals. HIV and HCV viral load had a lower limit of detection of 50 copies/mL and 615 IU/mL respectively. Group A consisted of healthy individuals seronegative for HIV and HCV; group B included HCV mono-infected viremic individuals; group C constituted HIV mono-infected viremic individuals; group D contained HCV/HIV co-infected individuals that were HCV viremic and HIV aviremic, and group E consisted of HIV mono-infected, but aviremic individuals. For Group E individuals, antiretroviral therapy (ART) consisted of at least one HIV protease inhibitor and/or one non-nucleoside reverse transcriptase inhibitor and two reverse transcriptase inhibitors.

Table 1B. Functional Characteristics of all the genes selected for validation in the study

Group	Start Date	Age	Gender	Race	Risk	HCV genotype	TCD4	CD4 (%)	HIV VL	HCV VL	HCV Treatment Response
A	10/27/05	41	F	White	N/A	N/A	737	55	N/A	N/A	N/A
A	10/18/05	34	M	White	N/A	N/A	1225	43	N/A	N/A	N/A
A	10/26/05	56	F	White	N/A	N/A	606	59	N/A	N/A	N/A
A	10/26/05	37	F	Black	N/A	N/A	490	44	N/A	N/A	N/A
A	10/25/05	42	M	White	N/A	N/A	863	46	N/A	N/A	N/A
A	10/17/05	46	F	White	N/A	N/A	726	47	N/A	N/A	N/A
A	10/20/05	39	M	Hispanic	N/A	N/A	352	37	N/A	N/A	N/A
B	10/31/05	51	M	White	IVDU	1b			N/A	2,500,000	NR
B	11/9/05	53	F	White	IVDU	1a			N/A	473000	RELAPSER
B	10/24/05	51	M	Black	IVDU	1			N/A	441,000	SVR
B	10/26/05	45	M	White	IVDU	1a			N/A	3,820,000	RELAPSER
B	11/9/05	42	M	White	IVDU	2			N/A	10,900,000	SVR
B	11/2/05	59	F	Black	IVDU	2b			N/A	7,810,000	SVR
B	10/26/05	70	M	White	IVDU	1b			N/A	3,830,000	SVR
C	12/13/05	41	M	White	MSM	N/A	290	22	29576	N/A	N/A
C	12/19/05	20	M	Black	HETERO	N/A	588	27	168629	N/A	N/A
C	12/2/05	42	M	White	MSM	N/A	337	36	32602	N/A	N/A
C	12/13/05	36	F	Black	HETERO	N/A	415	17	1964	N/A	N/A
C	11/10/05	34	M	Hispanic	MSM	N/A	356	21	23457	N/A	N/A
C	12/1/05	33	M	Hispanic	MSM	N/A	318	14	112697	N/A	N/A
C	12/1/05	59	F	Black	HETERO	N/A	507	27	12648	N/A	N/A
C	12/19/05	23	M	Hispanic	HETERO	N/A	15	2	108507	N/A	N/A

Table 1A

Group	Start Date	Age	Gender	Race	Risk	HCV genotype	TCD4	CD4 (%)	HIV VL	HCV VL	HCV Treatment Response
D	12/22/05	49	M	Black	IVDU	1a	1233	45	121	>7,692,310	Naïve
D	11/28/05	40	M	Black	MSM	1a	1460	44	49	4,976,400	Viral breakthrough
D	11/16/05	51	M	Black	IVDU	1b	727	30	49	3945420	NR
D	12/8/05	49	F	Black	IVDU	1a	794	25	49	1054510	Naïve
D	12/8/05	55	M	Black	MSM	1b	1008	43	49	9504730	NR
E	11/16/05	44	M	White	MSM	N/A	745	31	49	N/A	N/A
E	11/28/05	41	M	Black	HETERO	N/A	258	16	49	N/A	N/A
E	6/20/05	45	F	Hispanic	HETERO	N/A	202	10	49	N/A	N/A
E	11/16/05	36	F	Hispanic	HETERO	N/A	462	22	76	N/A	N/A
E	11/22/05	51	M	White	MSM	N/A	362	32	49	N/A	N/A
E	12/20/05	44	M	Hispanic	MSM	N/A	464	33	296	N/A	N/A

Table 1B

Name of Gene	Function	Relationship with Disease	Relevance in this study
CD10	Surface marker for immature B cells	Upregulated in HCV	Global effect of HCV replication on B cells
NKp30	NK activating Receptor	Upregulated in HCV	Increased in chronic HCV and HCV/HIV
CD80	Major T-Cell co-stimulatory factor	Upregulated in HCV	Global effect of HCV replication on T cells
CX3CL1	Neutrophil chemo attractant involved in angiogenesis, inflammation and tissue healing	Upregulated in HCV	Increased in chronic HCV
IGF-1R	Cell surface receptor that increases insulin secretion upon stimulation	Upregulated in HCV	Increased in chronic HCV and HCV/HIV
CCL-7	Monocyte chemotaxis	Upregulated in HCV	Increased in chronic HCV
CCL-20	Lymphocyte chemotaxis	Upregulated in HCV	Increased in chronic HCV
APOBEC3A	mRNA editing enzyme	Upregulated in HIV	HIV-induced type-I IFN response
APOBEC3G	Interferes with replication of retroviruses	Upregulated in HIV	HIV-induced type-I IFN response
TRIM5	Innate immune defense against retroviruses	Upregulated in HIV	HIV-induced type-I IFN response
EIF2AK2	Viral defense, IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
GIP3	Regulation of apoptosis, IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
IFI27	Impacts cellular apoptosis, IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
IFI44	Impacts cellular apoptosis, IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
IFI171	IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response

Table 1B

Name of Gene	Function	Relationship with Disease	Relevance in this study
IFIT3	IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
IFITM1	IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
IFITM3	IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
IFNA2	IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
IFNB	IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
IFNG	IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
IRF7	Transcriptional activation of virus-inducible cellular genes, IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
ISG15	Ubiquitin like modifier for innate defense, IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
ISG20	Ubiquitin like modifier for innate defense, IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
LY6E	IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
MX1	Responsible for antiviral state against influenza virus infection, IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
MX2	Responsible for antiviral state against influenza virus infection, IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
OAS1	Viral RNA degradation and the inhibition of viral replication, IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
OAS2	Viral RNA degradation and the inhibition of viral replication, IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
PLSCR1	Responsible for the translocation of phospholipids, IFN-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
PPIA	Involved in T cell activation, IFN-inducible	Upregulated in HIV	HIV-induced type-I IFN response
SP110	Transcriptional coactivator, Ifn-inducible gene	Upregulated in HIV	HIV-induced type-I IFN response
STAT1	IFN-signaling	Upregulated in HIV	HIV-induced type-I IFN response