Chronic immune activation is a distinguishing feature of liver and PBMC gene signatures from HCV/HIV coinfected patients and may contribute to hepatic fibrogenesis

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Introduction

Chronic HCV infection may lead to severe hepatic injury, including fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Liver disease typically progresses slowly, with severe disease developing over 10–30 years following HCV infection. However, human immunodeficiency virus (HIV) coinfection can profoundly impact the natural history of HCV infection. Several studies have demonstrated that HCV/HIV coinfected patients exhibit increased hepatic inflammation (Di Martino et al., 2001; Martinez-Sierra et al., 2003; Pol et al., 1998), and progress more rapidly to severe liver disease (Graham et al., 2001; Soto et al., 1997) in comparison to HCV-monoinfected patients. Due to the effectiveness of highly active antiretroviral therapy (HAART), the incidence of HIV progression to acquired immune deficiency syndrome (AIDS) has been dramatically decreasing. However, HCV/HIV-coinfected patients are increasingly experiencing morbidity and mortality associated with HCV-induced liver disease.

The mechanisms underlying rapid liver disease progression in HCV/HIV co-infected patients are poorly understood and are likely multifactorial. Contributing factors which may accelerate liver disease progression include HIV-associated immune dysfunction (Gonzalez et al., 2009b; Sandberg et al., 2010), oxidative stress (Baum et al., 2011; Lin et al., 2011; Wanchu et al., 2009), apoptosis (Jang et al., 2011), enhanced HCV replication (Lin et al., 2008), viral modulation of cytokine/chemokine responses (Allison et al., 2009), and HIV infection or activation of resident liver cells such as hepatic stellate cells or Kupffer cells (Tuyama et al., 2010). Unfortunately, no widely accepted experimental model of HCV/HIV coinfeciton is currently available. Therefore, detailed studies of liver disease pathogenesis have been limited, and are restricted to clinical samples.

Previously, we analyzed liver biopsies from HCV mono- and HCV/HIV coinfected patients and identified gene expression signatures indicative of dysregulation of apoptosis, increased immune cell activation, and decreased innate antiviral responses...
compared to normal liver reference samples (Walters et al., 2006). However, a specific intrahepatic signature distinguishing HCV/HIV coinfection from HCV monoinfected patients was not identified. In this study, we utilized core needle liver biopsies and a whole genome microarray with over twice the human transcriptome coverage of those used previously to identify intrahepatic gene signatures specifically associated with HCV/HIV. We also explored previous observations that HCV mono- and HCV/HIV coinfection induce unique immunologic gene expression profiles in peripheral mononuclear blood cells (PBMC) (Kottii et al., 2009). In addition to liver biopsy specimens, PBMCs from the same subjects were evaluated by microarray to investigate how transcriptional programming of immune cells in the periphery might contribute to hepatic inflammation, cellular infiltration, and disease progression.

Expression profiling of clinical specimens can prove challenging due to numerous confounding variables, limitations on sample sizes, and heterogeneous subject characteristics. In an attempt to mitigate some of these challenges, and to expand the scope of our functional analysis, we used our transcriptomic data to construct Bayesian networks in conjunction with published liver (Lamb et al., 2011; Schadt et al., 2008; Zhong et al., 2010) and PBMC gene expression data (Emilsson et al., 2008) obtained from larger independent cohorts. These networks allowed us to identify additional genes in critical pathways functionally associated with HCV/HIV coinfection, enhancing our understanding of the complex biology of liver disease progression in HCV/HIV coinfected patients.

Results

Subject characteristics

Demographic and clinical characteristics of the subjects are shown in Table 1. In total, 10 HCV monoinfected and 13 HCV/HIV coinfected patients were recruited in this study. The two groups were well-matched for age, ethnic background, body mass index (BMI), duration of HCV infection, alcohol use, serum ALT levels, HCV genotype, HCV viral load, and Batts–Ludwig liver disease stage. The HCV/HIV coinfected group was predominantly male (92%) and demonstrated significantly lower CD4 T cell counts (mean 451 versus 1120, \( p = .0014 \)) compared to the HCV monoinfected group. Also, the majority of coinfected patients (77%) received anti-retroviral treatment, which likely contributed to the lack of detectable HIV RNA in 63% of the subjects. Both groups were naive to HCV therapy. Seventy percent of the subjects reported abstinence from alcohol use for at least one year prior to enrollment. We also performed a correlation analysis to assess which clinical parameters might confound our gene expression results, and identified HIV status and consequent CD4 cell counts as the only parameters significantly influencing gene expression (data not shown). It is likely that the correlation with CD4 T cell counts reflects the lower counts observed in the HIV-positive subjects. Due to the limited sample size, a multivariate analysis was not performed. We specifically did not find any association between gene expression and other HIV-specific covariates such as serum HIV quantitation (suppressed or not) and the use of antiretroviral therapy, though we note that in this study, these patient subgroups are too small to perform a statistically robust correlation analysis.

Common transcriptional signatures in liver and PBMC samples from HCV/HIV coinfected patients

Using a two-way ANOVA approach, a molecular signature common to liver and PBMC samples from coinfected patients was identified. A total of 467 upregulated and 338 downregulated differentially expressed genes (DEG; \( p < .01 \)) were identified in both samples (Fig. 1A; Table S1). Common differential expression in both liver and PBMC may indicate that these pathways are comparably regulated separately in both tissues, and/or that many PBMC have migrated into and substantially contribute to the overall gene expression profile in liver. Functional analysis was performed on the 805 DEG common to both tissues using Ingenuity Pathway Analysis (IPA). Many of the top functional categories among the upregulated genes were associated with inflammation, indicating that distinct mechanisms may drive progression of hepatic inflammation in coinfected patients. Among 84 upregulated genes associated with inflammatory and immunological responses and disease (Table S1), we observed DEG associated with components of complement, chemokines,

Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV-negative (n=10)</th>
<th>HIV-positive (n=13)</th>
<th>( P ) value</th>
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<tr>
<td>Age</td>
<td>47.2 ± 7.5</td>
<td>42.6 ± 7.8</td>
<td>.18</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>60</td>
<td>92</td>
<td>.06</td>
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<tr>
<td>Race (%)</td>
<td></td>
<td></td>
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<tr>
<td>Caucasian</td>
<td>90</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>10</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Native American</td>
<td>0</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>HCV duration (years)</td>
<td>24.5 ± 7.8</td>
<td>22.0 ± 11.0</td>
<td>.57</td>
</tr>
<tr>
<td>Alcohol use in lifetime (gm/d)</td>
<td>24.1, 40.7</td>
<td>17.0, 46.9</td>
<td>.80</td>
</tr>
<tr>
<td>Alcohol use in last 6 months (gm/d)</td>
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<td>0, 9</td>
<td>.45</td>
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<tr>
<td>ALT (U/L)</td>
<td>92 ± 50</td>
<td>68 ± 36</td>
<td>.23</td>
</tr>
<tr>
<td>HCV genotype (%)</td>
<td>80</td>
<td>77</td>
<td>.86</td>
</tr>
<tr>
<td>HCV RNA level (log 10)</td>
<td>6.2 ± .5</td>
<td>6.3 ± 1.0</td>
<td>.60</td>
</tr>
<tr>
<td>Antiretroviral therapy (%)</td>
<td>NA</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>HIV RNA level (log 10)*</td>
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<td></td>
</tr>
<tr>
<td>HIV RNA level, % undetectable (&lt; 400 copies/mL)</td>
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<td></td>
</tr>
<tr>
<td>CD4 cell count (cells/μL)</td>
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<td>451 ± 292</td>
<td>.0014</td>
</tr>
<tr>
<td>HCV disease grade (0–4)*</td>
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<td>1.8 ± .8</td>
<td>.05</td>
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<tr>
<td>HCV disease stage (0–4)*</td>
<td>2.1 ± .3</td>
<td>2.1 ± 1.0</td>
<td>.9</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SD unless stated otherwise. NA, not applicable; IQR, interquartile range.

* In those with detectable virus.

* Batts–Ludwig scoring system.
and antigen presentation and T cell activation (Fig. 1B). The presence of gene expression changes associated with immune activation and migration may indicate enhanced trafficking of activated immune effector cells from the periphery to the liver in HCV/HIV coinfect ed patients.

**Identification of hepatic signatures of coinfection**

We also sought to identify significant hepatic DEG characteristic of HCV/HIV coinfect ed patients. Using one-way ANOVA ($p < .05$, fold change $> 1.15$ in at least 7 samples), we identified
transcriptional signatures in the coinfected patient cohort using microarray data from liver samples only (Fig. 2A; Table S2). Functional analysis by IPA demonstrated that in liver samples from coinfected patients, over 250 genes related to infectious disease and immune responses were upregulated, including 67 specifically related to HIV infection. Many of these genes are

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Fig. 2. Compartmental gene signatures distinguishing HCV/HIV coinfected patients. (A) Heatmap of 3039 significantly differentially expressed genes in liver or PBMC identified by one-way ANOVA ($p < .05$, fold change $> 1.15$ in at least 7 patients) and separated by K-means clustering. (B) IPA network of intrahepatic signature showing directly connected molecules associated with inflammation and immunity.
functionally similar to those identified in the common signature, and are associated with chemotaxis and cellular migration, including various chemokines, integrins (ITGAD, ITGAV, ITGAV7, ITGAV2), actin and tubulin cytoskeletal components (ACTB, ACTG, TUBA1A, TUBA1C, TUBA4A, TUBG1), and multiple Ras-like homolog members and related genes [RHOA, RHOB, RHOD, ROHQ, Rho-related C3 botulinum toxin 1, rho family small GTP binding protein 1 (RAC1)] involved in the recruitment of circulating immune cells to the liver. Many DEG associated with T cell activation processes, including human leukocyte antigens (HLA-DQA1, HLA-DQB1) that mediate antigen presentation, molecules associated with dendritic cell (DC) maturation (CD209), and T cell receptor signaling machinery (CD3, CD8A) were upregulated. This suggests that the T cells infiltrating the liver in coinfected patients may be transcriptionally distinct from T cells of HCV monoinfected patients, and may have a more activated phenotype due to chronic HIV infection. The substantial overlap (286 genes) between the hepatic signature and the signature common to both liver and PBMC (identified using two-way ANOVA) supports an important contributing role for infiltrating immune cells within the livers of coinfected patients.

We further observed the upregulation of genes functionally linked to hepatic stellate cell (HSC) activation including the Rho family GTPases, actins, myosin light chain 6 (MYL6), and cell division cycle 42 (CDC42), a major regulator of cytoskeletal family GTPases, actins, myosin light chain 6 (MYL6), and cell immune cells within the livers of coinfected patients. The substantial overlap (286 genes) between the hepatic signature and the signature common to both liver and PBMC (identified using two-way ANOVA) supports an important contributing role for infiltrating immune cells within the livers of coinfected patients.

Identification of peripheral signatures of coinfection

In the PBMC compartment, functional analysis using IPA revealed that many of the upregulated genes were associated with immune induction and immunological disease. Specifically, 271 genes associated with inflammatory responses, antigen presentation, and humoral immune responses, as well as hematological, immunological, and inflammatory disease were significantly upregulated (Table S3). Genes within this group include chemoattractant and apoptotic induction were upregulated. This contrasts with the downregulation of genes associated with inhibition of apoptosis (such as baculovirus IAP repeat-containing 5 [BIRC5]) and cell cycle progression (such as p21-activated kinase 7 [PAK7]) and kinesin family member 11 [KIF11]) and is consistent with a report that HSC apoptosis is antirectoredly associated with HCV-induced fibrogenesis. Taken together, these findings suggest that in HCV/HIV coinfected patients, increased migration of activated immune effector cells likely contributes to increased immune infiltration. In this turn may lead to increased HSC activation, hastening collagen deposition and fibrogenesis and possibly contributing to more rapid liver disease progression in HCV/HIV coinfected patients.

Overexpression of REG1A and REG3A secreted proteins

In addition to evaluating the global DEG signature associated with HCV/HIV coinfection, we sought to identify molecules highly expressed by the liver which may be candidates for evaluation as potential therapeutic or diagnostic targets. From DEG in the intrahepatic signature, we observed that regenerating islet-derived 1 alpha and 3 alpha (REG1A and REG3A) were among the most highly upregulated in a subset of 7 patients. REG1x and REG3x are both secreted growth factors associated with pancreatic islet cell regeneration, which were previously linked to a number of gastrointestinal cancers, including hepatocellular carcinoma. By assessing REG1x and REG3x levels in patient plasma specimens by immunoblot (Fig. 3), we observed that HCV/HIV patients exhibited substantially higher levels of circulating REG1x species compared to monoinfected individuals. In particular, a higher molecular weight form of REG1x corresponding to either a precursor
form or a heavily glycosylated protein, was consistently expressed at higher levels. Secreted Reg3γ levels were not significantly different between the two patient conditions, although Reg3γ was detectable in all tested samples. This suggests that the higher molecular weight form of circulating Reg1γ may be a candidate for evaluating hepatic disease progression by a non-invasive, blood-based study, and warrants further evaluation.

Expanding co-infected liver and PBMC signatures by a network approach

The cross-sectional design of this study restricted detection of liver and PBMC signature DEG to one time point. To identify additional genes which may contribute to liver disease progression in the coinfected tissues, we adopted a Bayesian network modeling approach to predict interactions between significant DEG in our study and those in networks built from previously published large data sets. Bayesian networks represent statistical dependencies between genes (“nodes”) as “edges,” which are calculated by multiple expression measurements. Leveraging networks generated from much larger datasets can increase the robustness of biological findings from a smaller dataset by further exploring the interrelated connectivity of DEG found in lower abundance.

Combined replicates of liver signature sequences were used as the input to Bayesian networks built from three human liver cohorts including 400 normal samples (Schadt et al., 2008), 272 samples each of hepatocellular carcinoma (HCC) and adjacent normal livers (Lamb et al., 2011), and 950 livers from obese subjects (Zhong et al., 2010). Out of the 2442 liver signature sequences, 924 genes were present in the three liver networks but only those subnetworks with at least 3 nodes (290 genes) were maintained. In subnetworks meeting these criteria, we expanded along one edge to identify additional connected nodes and obtain the network shown in Fig. 4A with 2544 genes.

We performed functional analysis of this liver network by using the Gene Annotator Module of the Merck Target Gene Information (TGI) Network Analysis and Visualization (NAV) tool, which finds related gene sets based on functional annotation, and ranks significance by calculating an expectation (E) value. Our analysis identified highly enriched pathways involved in chemokine signaling, dendritic cell maturation, interaction between innate and adaptive immunity, T cell receptor signaling, pattern recognition receptor-related pathway, NK cell signaling, and hepatic cholestasis/fibrosis (Table S4). This suggests that the genes added by the network analysis are related to cross-talk between the innate and adaptive immune system, and the interface between these different immune pathways may play an important role in HCV/HIV coinfection.

We identified similar functional categories and pathways using IPA. Some of these overlap with those already identified in Fig. 1B whereas others are novel pathways found to be associated with this liver network. Using IPA, we generated a subnetwork using the genes annotated as involved in communication between innate and adaptive immune cells (Fig. 4B). This subnetwork includes additional genes related to induction of innate antiviral responses (multiple Toll-like receptors; TLR) and inflammation (tumor necrosis factor alpha; TNFα), as well as induction of adaptive immunity via antigen presentation (HLA alleles) and T cell activation (CD3, CD8).

We built a PBMC network model using the similar approach of inputting 2479 combined replicate signature sequences to the deCODE blood Bayesian network built from about 1000 samples (Emilsson et al., 2008). We applied similar criteria as the liver network, maintaining only 131 genes (out of 561 present in the deCODE network) in sub-networks with at least 3 nodes and grew them one edge out to obtain a network with 1117 genes. Annotation of genes in this PBMC network indicates an association with T and B cell signaling, interaction between dendritic and natural killer cells, and communication between innate and adaptive immune cells (Table S4). These results are consistent with findings shown in Fig. 1B, and suggest that PBMC from coinfected patients exhibit the differential regulation of genes bridging innate and adaptive immunity.

Discussion

Understanding disease pathogenesis in HCV/HIV coinfected patients presents numerous challenges, one of which is discerning how distinct tissue compartments infected with two unique pathogens contribute separately and in concert to liver disease progression. Our data suggest that the chronic immune activation characteristic of HIV infection and progression to AIDS (Deeks et al., 2004; Hazenberg et al., 2003) is a distinguishing feature of the host response to HCV/HIV coinfection. We observed substantial overlap (104 DEG, 24 of which are associated with immune activation and differentiation) between both the individual liver and PBMC compartments and the signature common to both tissues. This further supports the notion that increased immune and inflammatory activity are present in both the liver and the periphery of coinfected patients and are likely important drivers of disease pathogenesis. The enhanced expression of inflammatory genes in both the common and individual compartment signatures suggests that HIV infection causes both circulating leukocytes, and those infiltrating the liver, to be more activated than those from HCV monoinfected patients. Consequently, the pro-inflammatory functions of these cells may enhance pathogenic processes and progression of fibrosis and HCV-induced liver injury via immune effector functions, cytokine and chemokine secretion, increased migration, and activation of HSC and other cellular mediators of fibrogenesis.

Another effect of HCV-infected infiltrating immune cells on HCV-infected hepatic cells may be to stimulate expression of genes with as yet undescribed roles in liver disease progression. The growth factors Reg1γ and Reg3γ have not been associated with HCV or HIV infection or pathogenesis. However, their apparent role in HCC tumorigenesis and metastasis (Cavard et al., 2006; Yuan et al., 2005), as well as our data indicating that these proteins are secreted, merits further evaluation of these proteins in a larger patient cohort as serum biomarkers with prognostic or diagnostic utility.

Recent systems biology approaches incorporating high-throughput data types, such as genome-wide DNA genotyping and RNA expression profiling, has allowed the construction of various types of genetic networks. Characterizing networks that underlie complex phenotypes such as clinical disease can provide more comprehensive understanding of the disease pathophysiology and in turn identify or validate disease susceptibility genotypes (Chen et al., 2008; Derry et al., 2010; Emilsson et al., 2008; Schadt et al., 2008; Yang et al., 2009). In the current study, the gene signatures we obtained were from a single time point (at which disease progression could be heterogeneous) and from a relatively small number of patients. In an effort to address these constraints and expand potential biological coverage, we used coinfected liver and PBMC gene signatures to build Bayesian statistical causal networks based on published large liver and blood data sets (Emilsson et al., 2008; Lamb et al., 2011; Schadt et al., 2008; Zhong et al., 2010). From these networks, we obtained greater enrichment based on functional annotation for the resulting liver network (Fig. 4A and Table 2) and the PBMC network (Fig. 4B and Table 2) compared to our functional analysis using the significant DEG identified in this study alone. These expanded networks
solidified the pathway connections between genes in both the HCV/HIV coinfected liver and PBMC molecular signatures, and provided greater insight into functionally related genes that might also be significant for liver disease pathogenesis in a larger population of HCV/HIV coinfected patients.

NK cells and DCs mediate both effector and regulatory functions in innate immunity and the induction of adaptive responses (Altfeld et al., 2011; Gonzalez et al., 2010; Kim and Chung, 2009); however, the HCV/HIV coinfected liver signature did not capture these functions as significantly associated with coinfection (Fig. 1B). The
expanded liver network, however, did identify significant associations with NK and DC functions (Table 2). Innate immunity-associated genes present in the network, but not in the co-infected liver signature, include TLR1, TLR2, TLR4, and TLR8. TLR signaling provides a critical link between innate and adaptive immunity in numerous viral infections. In chronic HCV infection, reduced TLR responsiveness is associated with liver dysfunction (Chung et al., 2011) and chronic inflammation consequent to reduced induction of adaptive immune responses (Dental et al., 2012; Yonkers et al., 2011). Furthermore, aberrant TLR signaling has been implicated in T cell activation defects in HIV infection (Yonkers et al., 2011a), indicating that these molecules may be important mediators of disease progression. Additionally, follow up studies should determine the clinical utility of potential biomarkers such as Reg1α to identify patients at risk of accelerated severe liver injury.

Materials and methods

Study subjects and specimen collection

Approval for this study was obtained from the University of Washington Human Subjects Division, and experiments were performed in compliance with legal, institutional, and ethical standards for human subjects research. All samples were obtained under informed consent.

Ten subjects infected with HCV and 13 subjects coinfected with HCV and HIV were included in this study. Exclusion criteria included chronic hepatitis B infection, alcohol use > 1 drink per day in women and > 2 drinks per day in men, and acute infection of any type. An experienced hepatopathologist (L.V.T.) blinded to all clinical data including HIV status reviewed all liver biopsies. Liver fibrosis stage was assessed using the Batts–Ludwig system: F0, no fibrosis; F1, portal fibrosis; F2, periportal fibrosis with rare bridges; F3, bridging/septal fibrosis; or F4, cirrhosis (Batts and Ludwig, 1995). All samples included were deemed adequate for analysis.

Table 2
Pathways and genes identified for expanded networks.

<table>
<thead>
<tr>
<th>TGI NAV pathways</th>
<th>Expectation</th>
<th>Set</th>
<th>Genes identified in network</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokine signaling</td>
<td>1.587E – 16</td>
<td>189</td>
<td>CCL4;CXCR4;GNB5;PRKCB;AKT2;PRXX;CCR1;CXCR3;CCL4;NGN1;PLCB2;CCL11;PIK3R1;CCL4;GK5;FOXO3;STAT3;CCL2;CRK;RAC2;PRKCB;ELM01;CXCL1;IL8;CCL19;CCL5;DOCK2;CCL15;CXCR2;ADCY7;ITK;CCL16;PIK3CG;PIK3CA;CD42;CXR5;PRKACB;CCL23;CXC15;ROCK1;PRKCB;CCL4;MHC;CCL5;BNV;VGB;MBF2X1;CCL3;CCL12;GNQ2;CC3;RACK1;PREX1;GRK4;CCL13;SHC1;CXCR1;ADCY1</td>
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<tr>
<td>Dendritic cell maturation</td>
<td>4.411E – 14</td>
<td>148</td>
<td>IL6;CD86;REL;CD247;CD83;C10;A2;FGER1;AKT2;HLA–DRB1;STAT4;TLR2;TREM5;TRGV1;PIK3R1;FGCGR2B;HLA–DMB;TLR4;IL18;TYROBP;RAC2;PRK3CB;TNFRSF13B;HLA–DQB1;PIK3CB;CD13;REL;NFKBIE;HLA–DRA;FGCGR3A;TNF;HLA–B;PIK3CG;PRKCI;FGR1B;CD3D;TNFRSF11B;CD58;IL1B;LY75;HLA–DRB3;FGCGR2A;JRF8</td>
</tr>
<tr>
<td>Communication between innate and adaptive immune cells</td>
<td>6.31E – 12</td>
<td>68</td>
<td>IL6;CD86;CCL4;CD247;CD83;FGER1;HLA–DRB1;TLR2;TRGV9;TLR4;IL18;CD4;IL8;CCL5;CCL15;HLA–DRBS1;CD8A;CD3E;TLR1;HLA–DRA;TNF;CD3D;TLR8;IL1B;CCL3;HLA–DRB3;TNFRSF13B;CD8B;IFNG;CD28</td>
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<td>iCOS–iCOSL signaling in T helper cells</td>
<td>2.962E – 11</td>
<td>100</td>
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<tr>
<td>Natural killer cell signaling</td>
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<td>PBMC network</td>
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<td>Altered T cell and B cell signaling in rheumatoid arthritis</td>
<td>.002</td>
<td>80</td>
<td>CD86;FGER1;TNFSF11;TLR2;FAS;CCL21;CD40L;HLA–DMA;IL23A;LTB;TLR5;IL1B;IFNG;CD28</td>
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<td>Crosstalk between dendritic cells and natural killer cells</td>
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<td>Role of macrophages fibroblasts and endothelial cells in rheumatoid arthritis</td>
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<td>294</td>
<td>PRSS53;LRP1;HP;PRKCH;FN1;TNFSF11;TLR2;CALM3;CREB5;PDGFD;TCF7;SFRP4;NFATC2;CCL5;LTB;PRKCA;MYC;IL6ST;KRAS;GNAQ;CAMK4;IL32;TLR5;OSM;IL1B;CSAR1;MAP2K3;LEF1</td>
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staging. At the time of liver biopsy, 2–3 mm of liver tissue was stored in RNALater (Life Technologies, Mountain View, CA) at 4 °C overnight, and then stored at –80 °C until RNA processing. Whole blood was obtained by venipuncture within 30 day of the liver biopsy (typically within one week of the biopsy). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation (Ficoll-Paque Plus; GE Healthcare Biosciences, Piscataway, NJ) within 24 h of blood draw, and 2 × 10⁶ freshly isolated PBMC were pelleted and resuspended in Trizol (Life Technologies, Carlsbad, CA) prior to flash freezing in liquid nitrogen. Samples were stored at –80 °C until RNA was prepared according to the manufacturer’s instructions. Samples were extracted according to manufacturer’s protocol and resuspended in RNase-free water prior to use in microarray experiments.

Liver and PBMC network construction

Networks were constructed as described previously (Zhu et al., 2004, 2008) and were visualized using the Target Gene Information (TGI) Network Analysis and Visualization (NAV) desktop application developed at Rosetta Inpharmatics. We utilized previously published data sets for liver (Lamb et al., 2011; Schadt et al., 2008; Zhong et al., 2010) and PBMC (Emilsson et al., 2008) network constructions. To construct liver and PBMC networks based on HCV/HIV coinfected gene signatures, the two gene signatures were loaded into the database as described in the results section and the TGI NAV tool was used to extract all edges from this network involving the included signature genes of interest.

The TGI NAV tool allows rapid, real-time, graphical analysis of pathway network models built from a comprehensive and fully integrated set of public and proprietary interaction databases available through a back-end central database. In addition, it supports experimentally generated systems biology data such as the statistical associations and causal relationships described in the manuscripts cited above and applied in the current study.

Microarray platform

Samples were amplified and labeled using a custom automated version of the RT/IVT protocol and reagents provided by Affymetrix. Hybridization, labeling and scanning were completed following the manufacturer’s recommendations for Merck Affymetrix Human Custom Arrays 1.0 containing 43,737 unique probe sequences (Affymetrix, Santa Clara, CA). Sample amplification, labeling, and microarray processing were performed by the Rosetta Inpharmatics Gene Expression Laboratory in Seattle, WA (acquired by Merck and Co., Whitehouse Station, NJ). Expression data were loaded into the Resolver (Rosetta proprietary software database) for transformation, normalization, and error modeling.

Microarray data analysis

Microarray data from each individual liver or PBMC sample was compared back to a reference pool composed of all corresponding samples of liver or PBMC, respectively, from HCV monoinfected subjects. HCV/HIV-coinfected gene sets were established based on their statistically significant up- or down-regulation (absolute fold change ≥ 1.15 and ANOVA or combined p-value ≤ 01 in at least 7 samples) when compared to the reference pool. Signature genes from each tissue were identified and compared. Unsupervised clustering methods, including hierarchical and k-means clustering, were performed to identify genes with similar expression patterns. Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA) software was used for functional analysis of significant genes identified by ANOVA.

Immunoblotting

Patient plasma samples were diluted in M-PER (Thermo Scientific, Rockford, IL) and protein concentration was quantified by BCA assay (Thermo Scientific) per the manufacturer’s instructions. A Criterion TGX Any kD Tris-glycine polyacrylamide gel (Bio-Rad, Hercules, CA) was loaded with 20 μg protein per sample, run using standard SDS-PAGE methods, and transferred to a PVDF membrane. Membranes were probed with anti-Reg1α (Abcam, Cambridge, MA) and anti-Reg3α (Abcam) primary antibodies, followed by peroxidase-conjugated donkey anti-rabbit secondary (Jackson ImmunoResearch Labs, West Grove, PA), and detected using ECL Plus reagent (GE Healthcare Biosciences). There is no protein reported to be constitutively secreted at similar levels in plasma or serum samples which could act as a loading control, therefore protein loading per well was normalized by loading equal quantities of protein per lane.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virology.2012.04.011.

References


