Published in final edited form as:

J Acquir Immune Defic Syndr. 2013 August 15; 63(5): 563–571. doi:10.1097/QAI.0b013e3182909847.

Plasma proteome analysis reveals overlapping, yet distinct mechanisms of immune activation in chronic HCV and HIV infections

Daniela M. Schlatzer¹, Julia M. Sugalski², Yanwen Chen³, Jill Barnholtz-Sloan³, Perica Davitkov², Fred E. Hazlett¹, Nicholas Funderburg², Benigno Rodriguez², Michael M. Lederman², Scott F. Sieg², Mark R. Chance¹, and Donald D. Anthony^{2,4,*}

¹Center for Proteomics and Bioinformatics, Case Western Reserve University, Cleveland, Ohio 44106

²Divisions of Infectious and Rheumatic Diseases, Department of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

³Case Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland, Ohio

⁴Veterans Administration Medical Center, Center for AIDS Research, and University Hospitals of Cleveland, Cleveland OH

Abstract

Background—Human immunodeficiency virus (HIV) infection contributes to accelerated rates of progression of liver fibrosis during hepatitis C virus (HCV) infection, and HCV liver disease contributes to mortality during HIV infection. Although mechanisms underlying these interactions are not well known, soluble and cellular markers of immune activation associate with disease progression during both infections.

Methods—We identified proteins varying in expression across the plasma proteomes of subjects with untreated HIV infection, untreated HCV infection with low AST/platelet ratio-index (APRI), untreated HCV infection with high APRI, HIV-HCV co-infection, and controls. We examined correlations between dysregulated proteins and markers of immune activation to uncover biomarkers specific to disease states.

Results—We observed the anticipated higher frequencies of HLADR+CD38+CD4 and CD8 T-cells, higher serum sCD14 levels, and higher serum IL-6 levels for HCV and HIV infected groups compared to controls. Plasma proteome analysis identified 2,297 peptides mapping to 227 proteins, and quantitative analysis of peptide intensity identified significant changes in 85 proteins across the five groups. Abundance for seven of these proteins was validated by ELISA. Forty-three of these proteins correlated with markers of immune activation, including at least two proteins that may directly drive T-cell activation. As a functional validation, we tested the enzymatic pathway product (lysophosphatidic acid, **LPA**) of one such protein, ENPP2, for ability to activate T-cells *in vitro*. LPA activated T-cells to express CD38 and HLA-DR.

*Address all correspondence and reprint requests to: Donald D. Anthony MD, PhD, Associate Professor of Medicine and Pathology, Biomedical Research Building 1028, Case Western Reserve University, 2109 Adelbert Rd., Cleveland OH, 44106, Ph: 216-368-3540, Fax: 216-368-2034, DDA3@CASE.EDU.

This work was presented as an oral poster at HIV and Liver Disease meeting September 2012.

Conflicts of Interest: There are no conflicts to disclose.

Conclusions—These data indicate elevated levels of ENPP2 and LPA during advanced HCV disease may play a role in exacerbating immune activation during HCV-HIV co-infection.

Introduction

Hepatitis C virus (HCV) is the most common cause of chronic viral hepatitis in the US[1, 2]. The long-term risk of cirrhosis is estimated at 20%, with subsequent increase in risk for liver failure and hepatocellular carcinoma[2, 3]. Due to overlapping modes of transmission, approximately 30% of HIV infected individuals are co-infected with HCV[1, 4]. While in recent years HIV related morbidity and mortality has been reduced with use of antiretroviral therapy (ART), HCV infection remains a cause of increased morbidity and mortality in HCV-HIV co-infected individuals[5-10]. For example, some studies indicate HCV infection is associated with a delay in CD4 T-cell recovery during ART[11, 12]. Additionally, HCV co-infection is associated with lower response to HBV vaccine[13]. HIV infection is also associated with accelerated progression to cirrhosis and liver failure during HCV chronic infection[14, 15]. One possible mechanism underlying virus interactions contributing to immune dysfunction is immune activation. Immune activation is reflected by soluble markers such as IL-6 and soluble CD14 (sCD14), as well as cellular markers such as T-cell HLA-DR and CD38 expression. These markers of immune activation are predictive of disease progression during HIV infection, and recent evidence supports the same for HCV and HCV-HIV co-infection[9, 16-20]. While markers of immune activation are associated with each other, and with disease activity, little is known about common and distinct factors that contribute to immune activation in the setting of HCV compared to HIV infection.

Plasma proteins play a critical role in mediating inflammation and innate immunity. Circulating levels of complement, defense collagens, and cytokines are essential in the immune response, playing roles such as modulation of antigen presentation and T/B-cell maturation/differentiation[21-24]. Moreover, soluble innate immune molecules have emerged as important molecules in HIV and HCV pathogenesis[25, 26]. To gain insight into markers and mediators of immune activation in HCV and HIV infection we examined validated markers of immune activation, and conducted unbiased plasma proteomic profiling, where hundreds of proteins were quantified. The initial statistical assessment of protein dysregulation in the unbiased proteomics experiment was carried out across HCV, HIV and control groups to enhance power. For proteins of interest that passed this statistical threshold, the patient specific protein abundance data were subjected to correlation analysis against specific variables of immune activation to select proteins that had maximal biological and clinical significance. This two-stage approach identified key potential biomarkers of disease that were examined for functional significance in the context of immune activation.

Methods

Study Subjects

Subjects provided written informed consent for phlebotomy under protocols approved by the institutional review boards for human studies at University Hospitals of Cleveland and Cleveland VA Medical Center. Study groups included age-range matched healthy control subjects (n=12), chronic HCV infected (n=24), HIV infected off ART (n=12), and HCV-HIV co-infected subjects (n=4). HCV chronic infected subjects were not on therapy for HCV > 6 months, HCV antibody and RNA positive, and HIV negative by ELISA. HCV infected subjects were categorized by APRI score ($(AST(U/L)/AST\ ULN)/PLT10^9/L \times 100$). APRI score <0.4 is associated with <10% F2-4/4 fibrosis stage and APRI >1.5 is associated with >90% F2-4 during HCV mono-infection[27, 28]. The HCV group was divided into lower (HCV lo, APRI <0.68) and higher (HCV hi, APRI >0.68) than median

overall group APRI score (0.68). HIV infected subjects had positive tests for HIV by ELISA, PCR and Western blot, and were HCV antibody and RNA undetectable. HIV subjects were off HIV therapy at least 3 months. HIV-HCV co-infected subjects had the above combination of above criteria. A verification data set included healthy controls (n=5) and HCV infected (n=30, divided into HCVlo and HCVhi) subjects. Plasma (Potassium EDTA, BD, Franklin Lakes, NJ) and serum (SST, BD) samples were frozen at -70°C until analysis.

Flow cytometric analysis

Peripheral Blood Mononuclear cells (PBMC, 1×10^6) were stained with anti-CD3FITC (clone SK7), anti-CD4PE (clone RPA-T4), anti-CD8APC (clone RPA-T8), anti-CD38PE-Cy7 (clone HB7), and anti-HLA-DRPerCP (clone L243) (BD Bioscience, San Jose, CA) 20 minutes, washed and fixed with 1% paraformaldehyde (Electron Microscopy Science, Hatfield, PA), and analyzed by flow cytometry on a BD LSRII using FACS Diva Software (BD Bioscience). Lymphocytes were identified by forward scatter/side scatter. T-cells were identified by co-expression of CD3 and CD4 or CD8. Proportions of CD3+CD4+ or CD3+CD8+ T-cells expressing CD38 and HLA-DR were quantified.

sCD14, IL-6 and LPS

Serum samples were tested for sCD14 (R&D Systems, Minneapolis, MN) by ELISA, LPS (Lonza, Allendale, NJ) by bioassay, and IL-6 (R&D Systems) by ELISA.

Sample Preparation for Label Free Protein Expression

Plasma samples (40µL) were depleted of the 14 most abundant proteins using a 4.6×100mm multiple affinity removal system (MARS-Hu14, Agilent Technologies, Santa-Clara, CA). The samples were concentrated and buffer exchanged with 50mM Tris-pH-8.8 to a final volume of 100µL using a 5,000 molecular weight cutoff filter (Millipore, Billerica, MA). Protein concentrations were determined by 2D Quant Kit (GE Healthcare Piscataway, NJ). After digestion, samples were adjusted to 25µg/50µL. 20µL of 0.2% Rapigest (Waters, Milford, MA) was added, followed addition of 5mM dithiothreitol, incubation at 80°C 15 minutes, and cooled to room temperature prior to alkylation with 10mM iodacetamide 30 minutes. Proteolytic digestion was performed with bovine trypsin (Promega, Madison, WI) 1:10 (w/w) 18h 37°C.

Liquid Chromatography and Mass Spectrometry

600ng of each sample were analyzed by LC/MS/MS using a Waters NanoAcquity Ultra-high pressure liquid chromatography system (Waters) and a LTQ-Orbitrap Velos mass spectrometer (Thermo, Waltham, MA). The order of sample injections was randomized. Separation and detection of peptides was performed as previously published [29]. LC/MS/MS data were processed using Rosetta Elucidator (Rosetta Biosoftware, Seattle, WA) to generate MS/MS peak lists. These were subsequently searched by Mascot version 2.2.0 (Matrix Science, London, UK). The database used was the human International Protein Index (IPI) (68020 sequences). The criteria for peptide identification were a mass accuracy of 10 ppm and an expectation value of p 0.05. Proteins identified with 2 or more peptides matching the above criteria were considered confirmed assignments while proteins identified with one peptide regardless of the Mascot score were considered tentative assignments. Automated differential quantification of peptides was accomplished with Rosetta Elucidator as previously described [30].

Statistical Analysis

The Rosetta pre-processed data were obtained for the five study groups. Missing values were imputed by the peptide median intensity within each corresponding group. Statistical analysis of peptide intensity difference between the five study groups were carried out using the Wilcoxon-Mann-Whitney test, which assumed each peptide was independent. A false discovery rate (**FDR**) p -value <0.1 was considered significant for any given peptide[31, 32]. Intensities for significant peptides were summed within a protein for the following association and correlation analysis. The potential association between top proteins and immune activation variables was assessed using generalized linear models. A p -value <0.05 was considered significant. Pairwise correlation analysis with immune activation variables was performed by employing the R package “Hmisc”. All statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC) and R2.15.1.

Molecular Network Analysis

Significant peptides (FDR $p<0.1$) identified from the statistical analysis and their corresponding estimated log-ratios were imported into Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA) for molecular network analysis. Once imported, a single log ratio value was generated at the protein level by determining the mean for all peptides identified for a specific protein.

Label Free Protein Expression ELISA Verification

Seven putative plasma biomarkers identified in the label free proteomic analysis as significant and with robust abundance changes across HCV and HIV disease groups were selected for verification via ELISA: Ecotomucleotide pyrophosphatase/phosphodiesterase-2 (ENPP2, R&D Systems); Lectin galactoside-binding soluble-3-binding protein (LGALS3BP, Abnova, Walnut, CA); Intercellular adhesion molecule-1 (ICAM1, Abcam, Cambridge, MA); Vascular cell adhesion molecule-1 (VCAM1, RayBio, Norcross, GA); Insulin-like growth factor binding protein-3 (IGFBP3, RayBio); Insulin-like growth factor acid labile subunit (IGFALS, BioVendor, Chandler, NC); and soluble CD163 (CD163, R&D Systems). All kits have inter and intra assay coefficients of variation of less than 15%.

T-cell activation assay

PBMCs (5×10^5) were stimulated overnight at 37°C using complete RPMI medium+5% human AB serum in absence or presence of 25ug/mL phytohemagglutinin (PHA; Sigma, St. Louis, MO) in 96 well round bottom plates, followed by stimulation with medium or 100μM Oleoyl-L-lysophosphatidic acid (LPA; Sigma; solvated in a 90% chloroform (0.5% final), 5% acetic acid, and 5% methanol solution) for 3 days at 37°C. Stimulated PBMC were collected, washed, and stained with anti-CD3FITC (clone SK7), anti-CD4PE (clone RPA-T4), anti-CD8APC (clone RPA-T8), anti-CD38PE-Cy7 (clone HB7), and anti-HLA-DRPerCP (clone L243) (BD Biosciences). Flow cytometric analysis was performed as described above.

Results

HCV and HIV infection are associated with immune activation

We focused on the plasma proteome in relation to soluble and cellular markers of immune activation during untreated HCV and HIV infection to gain insight into shared and distinguishing factors and pathways of immune activation comparing HCV and HIV infection. We obtained plasma, serum and PBMC samples from persons with untreated chronic HCV infection (lower and higher APRI score subgroups referred to here as HCV lo and HCV hi), untreated HIV infection, HCV-HIV co-infection and healthy controls. Study

group characteristics are shown in Table 1. Age differed across groups, and is reflective of the age range of our clinical cohorts of HCV and HIV infected patients. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), platelet (PLT), albumin (Alb) and total bilirubin (Tbili) differed across cohorts in an expected manner, with higher levels of liver inflammation, lower albumin and PLT levels in the higher APRI HCV group (HCV hi). As expected, proportions of CD4 and CD8 T-cells that are activated (HLA-DR⁺CD38⁺) differed across groups. When intergroup comparisons were performed, proportions of CD4 and CD8 T-cells that express HLADR and CD38 were greater in HIV and HCV hi groups compared to controls (Figure 1A and 1B). Soluble CD14 levels were greater in HCV infected subject groups than in controls (Figure 1C), and serum IL-6 levels were greater in HCV hi and HCV-HIV subject groups than among controls (Figure 1D). These findings are consistent with previous literature[9, 20, 33]. Additionally, though serum LPS levels did not significantly differ among groups (Table 1), the levels were comparable to those described in the literature[18, 20, 34, 35]. When parameters in Table 1 were examined for associations with each other, activated CD4 cell frequencies correlated with activated CD8 cell frequencies in all virally infected groups ($r=0.66$ $p=0.02$, $r=0.61$ $p=0.04$, and $r=0.80$ $p=0.002$ in HCV lo, HCV hi, and HIV groups respectively). Additionally serum ALT correlated with frequencies of activated CD4 cells in the HCV lo group ($r=0.71$, $p=0.01$), while albumin level negatively correlated with frequencies of activated CD8 cells in the HCV hi group ($r=-0.67$ $p=0.02$). Additionally, albumin level negatively correlated with serum IL-6 level in HCV lo, HCV hi and all HCV subjects combined ($r=-0.636$ $p=0.04$, $r=-0.831$ $p=0.001$, and $r=-0.735$ $p=0.0001$ respectively). Serum IL-6 levels also tended to correlate with frequencies of activated CD8 cells in all HCV infected subjects combined ($r=0.413$ $p=0.05$). Within the HIV group LPS levels tended to correlate with sCD14 levels ($r=0.59$ $p=0.05$). Age was found only to inversely correlate with ALT, and only in the HCV lo group ($r=-0.70$, $p=0.01$).

Plasma proteome analysis reveals differences in protein abundance reflecting immune activation comparing HCV and HIV infection

Multiple proteomic profiling techniques are currently available for discovery based protein quantification. Label-free protein expression analysis is a peptide based ('bottom up') proteomic technique that capitalizes on the highly reproducible chromatography and parts-per-million (ppm) mass accuracy available in current liquid chromatography/mass spectrometry (LC-MS) systems[36-39]. Peptide species in these approaches are quantified by ion intensity, while individual peptides are grouped across samples based on precise mass and retention time measurements[40, 41]. The proteomic label free data were processed using Rosetta Elucidator as previously described[42-44]. Peptide and protein assignments were made using peptide and protein teller algorithms[45, 46] in Rosetta Elucidator with a false discovery rate of 1% (Rosetta Biosoftware, Seattle, WA).

Quantification and imputation were performed only on those peptides that passed the above identification thresholds, which yielded 2,297 peptides mapping to 227 proteins. Of those, 360 peptides mapping to 85 proteins differed across groups ($FDR<0.10$). Figure 2 highlights the peptide abundance changes (and ELISA validation) for two example proteins identified from this analysis. Table S1 details all of the peptides and proteins identified. Those that significantly differed in abundance across groups are also indicated. Table S2 contains all pathways that significantly differed among groups.

In order to determine molecular pathways specific for HIV, HCV or co-infected cohorts we performed a separate non-parametric t-test between groups. Significant proteins for each disease group comparison were subsequently incorporated with existing molecular networks and disease specific literature using the IPA software to provide pathways associated with disease. For example, Figure S1 summarizes the major connections in the NF- κ B and

growth factor signaling pathways and illustrates the corresponding proteins identified in the discovery study that significantly differed between HCV and HIV groups. The data suggests activation of NF- κ B signaling across both HIV and HCV disease groups with increases in ICAM1, ICAM2, VCAM and FN1 observed specifically in the proteomics study. On the other hand, alteration in growth factor signaling was specific to HCV lo and HCV hi groups, with lower plasma levels of IGF2, IGFBP3 and IGFALS (Figure S1).

Plasma concentrations were determined for seven proteins associated with activation pathways of interest including Ecotomucleotide pyrophosphatase/phosphodiesterase-2 (ENPP2); Lectin galactoside-binding soluble-3-binding protein (LGALS3BP); Intercellular adhesion molecule-1 (ICAM1); Vascular cell adhesion molecule-1 (VCAM1); Insulin-like growth factor binding protein-3 (IGFBP3); Insulin-like growth factor acid labile subunit (IGFALS); and soluble-CD163 (sCD163). This verified our initial label free measurements, and additionally provided concentrations for the selected proteins. Figure 2 highlights ELISA results and label free peptide intensities for ENPP2 and VCAM1, and Table S3 provides ELISA results for all seven proteins. Overall, these data indicate the plasma proteome analyses provide an accurate and comprehensive view of plasma protein dysregulation across HCV, HIV and co-infected HCV-HIV groups.

Plasma protein levels that are associated with markers of immune activation in HCV and HIV infection selectively differ

We next assessed whether established markers of immune activation (HLA-DR and CD38 expression on CD4 and CD8 T-cells, sCD14, and IL-6) are correlated with changes in plasma protein abundance, for proteins that differed in abundance among groups. We excluded HCV-HIV co-infected subjects due to the small sample size and in one analysis also combined the chronic HCV mono-infected subjects to enhance sample size (n=24). Peptide intensities mapping to 43 proteins significantly correlated ($p < 0.05$) with a parameter of immune activation within one or more disease group (Table 2). As an example, Figure S2 illustrates correlations between protein abundance of C5 and ENPP2 vs. CD4 activation within HCV and HIV groups. Plasma C5 level was positively correlated with activated CD4 T-cell frequency within the HIV ($r = 0.72$, $p = 0.008$) but not HCV groups, while ENPP2 level was associated with activated CD4 T-cell frequencies (HLA-DR⁺CD38⁺) in both HCV and HIV groups ($r = 0.53$ $p = 0.009$, $r = 0.72$ $p = 0.008$). The latter is of interest as ENPP2 abundance was not statistically changed with HIV disease state compared to controls (Figure 2), while ENPP2 does correlate with T-cell activation in this group.

Plasma proteins identified by our analysis may directly contribute to T-cell activation in the setting of HCV and HIV infection

A subset of proteins identified by proteomic analysis that correlated with markers of immune activation were identified as potentially playing a role in modulating immune activation state (ENPP2 and LGALS3BP). In particular ENPP2 is known to contribute to immune modulation[47, 48], and is increased in states of HCV associated liver disease[49, 50]. ENPP2 (aka autotaxin) is an enzyme responsible for production of the lipid mediator lysophosphatidic acid (LPA) and is the major LPA producing enzyme in plasma[51]. LPA binds to select G-protein-coupled-receptors (GPCR) and induces many cellular responses such as T-cell activation[52]. Dendritic cells and T lymphocytes both express LPA receptors and *in vitro* studies indicate LPA can directly activate CD4 T-cells to proliferate[53, 54]. To explore the functional significance of ENPP2, we evaluated whether the product of ENPP2 enzymatic activity, LPA, is capable of T-cell activation characteristic of that present in HIV and HCV infection.

We performed experiments with PBMC from healthy control and HIV infected subjects off ART. Cells were treated with medium alone or the mitogen PHA overnight, followed by treatment with media or LPA for another 3 days. PHA was included in some cultures to mimic conditions of prior cellular activation, and because this condition has been shown to contribute to LPA induced T-cell activity[53, 54]. We observed LPA alone resulted in greater frequencies of activated CD4 T cells expressing HLA-DR and CD38 for all subjects combined (Figure 3A), and this tended to exist within the healthy control and HIV groups when separately analyzed ($p=0.1$ for each). Additionally, when cells were pre-activated with PHA, LPA enhanced CD4 cell activation for all subjects combined (Figure 3A), and for healthy control ($p=0.04$), but not HIV subject ($p=0.6$) subgroups. Similar results were observed with CD8 cells when pre-treated with PHA (Figure 3B) and this held in healthy control ($p=0.04$), and nearly HIV ($p=0.1$) groups when separately analyzed.

Discussion

Our unbiased evaluation of plasma protein abundance coupled to flow cytometric analysis and ELISA analysis of immune activation markers in chronic HCV and HIV infection identified a number of candidate plasma markers that may predict or directly contribute to a state of immune activation during disease. The protein biomarkers of interest included a number of proteins involved in innate immunity as well as proteins that have previously been described to be associated with liver disease stage during chronic HCV infection. Our focus on ENPP2 provided proof of concept that one protein, and its enzymatic pathway product (LPA), identified by our approach may contribute to immune activation. It is noteworthy that this protein was flagged for interest in both HIV and HCV disease only by our “second stage” analysis, where dysregulated proteins were explored for correlations with clinical variables provided by flow cytometry. Given this protein has been previously identified to be associated with advanced liver disease during HCV infection it is plausible that ENPP2 may directly contribute to HCV mediated immune activation enhancement observed during HCV-HIV infection. Though ENPP2 levels were not elevated in HIV infection, the correlation between ENPP2 and T cell activation during HIV infection may suggest tighter ENPP2 regulation of LPA levels in the setting of HIV infection, perhaps due to substrate availability (LPC).

ENPP2 (ectonucleotide pyrophosphatase/phosphodiesterase-2) is also known as Autotaxin, or Lysophospholipase-D. It was originally purified as an autocrine motility factor for melanoma cells, and is a key enzyme in lysophosphatidic acid (LPA) synthesis. Downstream effects of LPA include immune modulation, neuropathic pain modulation, platelet aggregation, wound healing, vasopressor activity, and angiogenesis[47, 48]. Additionally, both plasma LPA and ENPP2 levels and activity are increased in chronic HCV infection, and are positively correlated with serum hyaluronic acid, and negatively with PLT count, serum albumin, and prothrombin time[50]. Additionally LPA level correlates with ENPP2 activity[50]. Moreover, stage of fibrosis correlates with serum ENPP2 activity and plasma LPA level[50]. LPA also regulates stellate cell proliferation, though it is unclear if it is a cause or effect of liver injury[55]. In addition, ENPP2 is overexpressed within the liver of HCV infected subjects with hepatocellular carcinoma compared to those without cancer[49]. Here we observed increases in activated CD4 T-cell frequencies when PMBC were stimulated with the enzymatic product of ENPP2, LPA. These data extend prior published data, indicating LPA is capable of contributing to HLA-DR and CD38 expression on T-cells, placing this lipid mediator in a potential causal role for T-cell activation during disease states such as HCV infection with advanced liver disease where elevated ENPP2 and LPA levels are known to exist.

One limitation of this approach is that proteomic analyses do not detect all available plasma proteins due to the wide dynamic range of protein abundance spanning 10 orders of magnitude. Another limitation is that of false discovery due to the small sample size and many comparisons. We tried to minimize this by applying FDR correction[32]. Additionally, we have evaluated ENPP2 and IL-6 levels in the serum of an additional group of healthy controls (n=5) and HCV infected subjects (n=30, divided into HCV low and high APRI subgroups). We verified IL-6 and ENPP2 are both elevated during HCV infection ($p=0.04$ and $p<0.001$ for HCV hi compared to controls, and $p=0.05$ for HCV lo compared to controls for IL6). We also verified ENPP2 correlates with APRI ($r=0.59$, $p=0.001$), AST ($r=0.92$, $p<0.001$), PLT ($r=-0.40$, $p=0.03$), and IL-6 levels ($r=0.37$, $p=0.045$ in all HCV subjects combined, and $r=0.45$ $p=0.08$ in HCV lo APRI subgroup). Additionally, another limitation of our study is that specific proteins of interest may also be removed from the analysis along with abundant protein removal during sample preparation. However, the strength of our approach is that we were able to simultaneously detect and quantify over 200 proteins in 52 patients across 4 different disease groups. Further analyses of markers identified here in other cohorts with well characterized clinical characteristics, including morbidity, will help to better define the role of such proteins in activation pathways and in determining clinical outcome during HCV and HIV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the study subjects for their participation.

Source of Funding: Grant Support: This work was supported National Institute of Health grant P20-DA-026133, NIH R01 DK068361, VA Merit, the CWRU Center for AIDS Research Core facilities (AI 36219), and the Clinical and Translational Science Collaborative of Cleveland, UL1TR000439 from the National Center for Advancing Translational Sciences (NCATS) component of the National Institutes of Health and NIH roadmap for Medical Research

References

1. Dienstag JL, McHutchison JG. American Gastroenterological Association technical review on the management of hepatitis C. *Gastroenterology*. 2006; 130:231–264. quiz 214-237. [PubMed: 16401486]
2. Lauer GM, Walker BD. Hepatitis C virus infection. *N Engl J Med*. 2001; 345:41–52. [PubMed: 11439948]
3. Freeman AJ, Dore GJ, Law MG, Thorpe M, Von Overbeck J, Lloyd AR, et al. Estimating progression to cirrhosis in chronic hepatitis C virus infection. *Hepatology*. 2001; 34:809–816. [PubMed: 11584380]
4. Zylberberg H, Pol S. Reciprocal interactions between human immunodeficiency virus and hepatitis C virus infections. *Clin Infect Dis*. 1996; 23:1117–1125. [PubMed: 8922812]
5. Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, et al. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med*. 1998; 338:853–860. [PubMed: 9516219]
6. Cacoub P, Geffray L, Rosenthal E, Perronne C, Veyssier P, Raguin G. Mortality among human immunodeficiency virus-infected patients with cirrhosis or hepatocellular carcinoma due to hepatitis C virus in French Departments of Internal Medicine/Infectious Diseases, in 1995 and 1997. *Clin Infect Dis*. 2001; 32:1207–1214. [PubMed: 11283811]
7. Monga HK, Rodriguez-Barradas MC, Breaux K, Khattak K, Troisi CL, Velez M, et al. Hepatitis C virus infection-related morbidity and mortality among patients with human immunodeficiency virus infection. *Clin Infect Dis*. 2001; 33:240–247. [PubMed: 11418885]

8. Backus LI, Phillips BR, Boothroyd DB, Mole LA, Burgess J, Rigsby MO, et al. Effects of hepatitis C virus coinfection on survival in veterans with HIV treated with highly active antiretroviral therapy. *J Acquir Immune Defic Syndr*. 2005; 39:613–619. [PubMed: 16044016]
9. Kovacs A, Karim R, Mack WJ, Xu J, Chen Z, Operskalski E, et al. Activation of CD8 T cells predicts progression of HIV infection in women coinfecting with hepatitis C virus. *J Infect Dis*. 2010; 201:823–834. [PubMed: 20151840]
10. Limketkai BN, Mehta SH, Sutcliffe CG, Higgins YM, Torbenson MS, Brinkley SC, et al. Relationship of liver disease stage and antiviral therapy with liver-related events and death in adults coinfecting with HIV/HCV. *JAMA*. 2012; 308:370–378. [PubMed: 22820790]
11. Greub G, Ledergerber B, Battegay M, Grob P, Perrin L, Furrer H, et al. Clinical progression, survival, and immune recovery during antiretroviral therapy in patients with HIV-1 and hepatitis C virus coinfection: the Swiss HIV Cohort Study. *Lancet*. 2000; 356:1800–1805. [PubMed: 11117912]
12. Sulkowski MS, Moore RD, Mehta SH, Chaisson RE, Thomas DL. Hepatitis C and progression of HIV disease. *Jama*. 2002; 288:199–206. [PubMed: 12095384]
13. Gandhi RT, Wurcel A, Lee H, McGovern B, Shopis J, Geary M, et al. Response to Hepatitis B Vaccine in HIV-1-Positive Subjects Who Test Positive for Isolated Antibody to Hepatitis B Core Antigen: Implications for Hepatitis B Vaccine Strategies. *J Infect Dis*. 2005; 191:1435–1441. [PubMed: 15809901]
14. Benhamou Y, Bochet M, Di Martino V, Charlotte F, Azria F, Coutellier A, et al. Liver fibrosis progression in human immunodeficiency virus and hepatitis C virus coinfecting patients. The Multivirc Group. *Hepatology*. 1999; 30:1054–1058. [PubMed: 10498659]
15. Graham CS, Baden LR, Yu E, Mrus JM, Carnie J, Heeren T, et al. Influence of human immunodeficiency virus infection on the course of hepatitis C virus infection: a meta-analysis. *Clin Infect Dis*. 2001; 33:562–569. [PubMed: 11462196]
16. Anthony DA, et al. Baseline levels of soluble CD14 and CD16+56- NK cells are negatively associated with response to IFN/Ribavirin therapy during HCV-HIV co-infection. *Journal of Infectious Disease*. 2012 epub ahead of print.
17. Gonzalez VD, Landay AL, Sandberg JK. Innate immunity and chronic immune activation in HCV/HIV-1 co-infection. *Clin Immunol*. 2010; 135:12–25. [PubMed: 20100670]
18. Sandler NG, Wand H, Roque A, Law M, Nason MC, Nixon DE, et al. Plasma levels of soluble CD14 independently predict mortality in HIV infection. *J Infect Dis*. 2011; 203:780–790. [PubMed: 21252259]
19. Giorgi JV, Hultin LE, McKeating JA, Johnson TD, Owens B, Jacobson LP, et al. Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J Infect Dis*. 1999; 179:859–870. [PubMed: 10068581]
20. Sandler NG, Koh C, Roque A, Eccleston JL, Siegel RB, Demino M, et al. Host response to translocated microbial products predicts outcomes of patients with HBV or HCV infection. *Gastroenterology*. 2011; 141:1220–1230. 1230 e1221–1223. [PubMed: 21726511]
21. Perrin-Cocon LA, Villiers CL, Salamero J, Gabert F, Marche PN. B cell receptors and complement receptors target the antigen to distinct intracellular compartments. *J Immunol*. 2004; 172:3564–3572. [PubMed: 15004157]
22. Cretin FC, Serra VA, Villiers MB, Laharie AM, Marche PN, Gabert FM. C3b complexation diversifies naturally processed T cell epitopes. *Mol Immunol*. 2007; 44:2893–2899. [PubMed: 17320958]
23. Barrionuevo P, Beigier-Bompadre M, Ilarregui JM, Toscano MA, Bianco GA, Isturiz MA, et al. A novel function for galectin-1 at the crossroad of innate and adaptive immunity: galectin-1 regulates monocyte/macrophage physiology through a nonapoptotic ERK-dependent pathway. *J Immunol*. 2007; 178:436–445. [PubMed: 17182582]
24. Fraser DA, Tenner AJ. Directing an appropriate immune response: the role of defense collagens and other soluble pattern recognition molecules. *Curr Drug Targets*. 2008; 9:113–122. [PubMed: 18288962]

25. Tarr AW, Urbanowicz RA, Ball JK. The role of humoral innate immunity in hepatitis C virus infection. *Viruses*. 2012; 4:1–27. [PubMed: 22355450]
26. Huber M, Trkola A. Humoral immunity to HIV-1: neutralization and beyond. *J Intern Med*. 2007; 262:5–25. [PubMed: 17598812]
27. Shaheen AA, Myers RP. Diagnostic accuracy of the aspartate aminotransferase-to-platelet ratio index for the prediction of hepatitis C-related fibrosis: a systematic review. *Hepatology*. 2007; 46:912–921. [PubMed: 17705266]
28. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology*. 2003; 38:518–526. [PubMed: 12883497]
29. Schlatzer DM, Dazard JE, Dharsee M, Ewing RM, Ilchenko S, Stewart I, et al. Urinary protein profiles in a rat model for diabetic complications. *Mol Cell Proteomics*. 2009; 8:2145–2158. [PubMed: 19497846]
30. Schlatzer DM, Dazard JE, Ewing RM, Ilchenko S, Tomcheko SE, Eid S, et al. Human biomarker discovery and predictive models for disease progression for idiopathic pneumonia syndrome following allogeneic stem cell transplantation. *Mol Cell Proteomics*. 2012; 11:M111–015479. [PubMed: 22337588]
31. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B*. 1995; 57:289–300.
32. Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100:9440–9445. [PubMed: 12883005]
33. Valdez H, Anthony D, Farukhi F, Patki A, Salkowitz J, Heeger P, et al. Immune responses to hepatitis C and non-hepatitis C antigens in hepatitis C virus infected and HIV-1 coinfecting patients. *Aids*. 2000; 14:2239–2246. [PubMed: 11089611]
34. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med*. 2006; 12:1365–1371. [PubMed: 17115046]
35. Jiang W, Lederman MM, Hunt P, Sieg SF, Haley K, Rodriguez B, et al. Plasma levels of bacterial DNA correlate with immune activation and the magnitude of immune restoration in persons with antiretroviral-treated HIV infection. *J Infect Dis*. 2009; 199:1177–1185. [PubMed: 19265479]
36. Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat Biotechnol*. 2006; 24:971–983. [PubMed: 16900146]
37. Veenstra TD, Conrads TP, Hood BL, Avellino AM, Ellenbogen RG, Morrison RS. Biomarkers: mining the biofluid proteome. *Mol Cell Proteomics*. 2005; 4:409–418. [PubMed: 15684407]
38. Zhao Y, Lee WN, Xiao GG. Quantitative proteomics and biomarker discovery in human cancer. *Expert Rev Proteomics*. 2009; 6:115–118. [PubMed: 19385938]
39. Paczesny S, Krijanovski OI, Braun TM, Choi SW, Clouthier SG, Kuick R, et al. A biomarker panel for acute graft-versus-host disease. *Blood*. 2009; 113:273–278. [PubMed: 18832652]
40. Wang W, Zhou H, Lin H, Roy S, Shaler TA, Hill LR, et al. Quantification of proteins and metabolites by mass spectrometry without isotopic labeling or spiked standards. *Anal Chem*. 2003; 75:4818–4826. [PubMed: 14674459]
41. Chelius D, Bondarenko PV. Quantitative profiling of proteins in complex mixtures using liquid chromatography and mass spectrometry. *J Proteome Res*. 2002; 1:317–323. [PubMed: 12645887]
42. Neubert H, Bonnert TP, Rumpel K, Hunt BT, Henle ES, James IT. Label-free detection of differential protein expression by LC/MALDI mass spectrometry. *J Proteome Res*. 2008; 7:2270–2279. [PubMed: 18412385]
43. Chan EY, Sutton JN, Jacobs JM, Bondarenko A, Smith RD, Katze MG. Dynamic host energetics and cytoskeletal proteomes in human immunodeficiency virus type 1-infected human primary CD4 cells: analysis by multiplexed label-free mass spectrometry. *J Virol*. 2009; 83:9283–9295. [PubMed: 19587052]
44. Weng L, Dai H, Zhan Y, He Y, Stepaniants SB, Bassett DE. Rosetta error model for gene expression analysis. *Bioinformatics*. 2006; 22:1111–1121. [PubMed: 16522673]

45. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem.* 2003; 75:4646–4658. [PubMed: 14632076]
46. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem.* 2002; 74:5383–5392. [PubMed: 12403597]
47. Mutoh T, Chun J. Lysophospholipid activation of G protein-coupled receptors. *Subcell Biochem.* 2008; 49:269–297. [PubMed: 18751915]
48. Shimizu T. Lipid mediators in health and disease: enzymes and receptors as therapeutic targets for the regulation of immunity and inflammation. *Annu Rev Pharmacol Toxicol.* 2009; 49:123–150. [PubMed: 18834304]
49. Cooper AB, Wu J, Lu D, Maluccio MA. Is autotaxin (ENPP2) the link between hepatitis C and hepatocellular cancer? *J Gastrointest Surg.* 2007; 11:1628–1634. discussion 1634-1625. [PubMed: 17902023]
50. Watanabe N, Ikeda H, Nakamura K, Ohkawa R, Kume Y, Aoki J, et al. Both plasma lysophosphatidic acid and serum autotaxin levels are increased in chronic hepatitis C. *J Clin Gastroenterol.* 2007; 41:616–623. [PubMed: 17577119]
51. Nakanaga K, Hama K, Aoki J. Autotaxin--an LPA producing enzyme with diverse functions. *J Biochem.* 2010; 148:13–24. [PubMed: 20495010]
52. Moolenaar WH. Bioactive lysophospholipids and their G protein-coupled receptors. *Exp Cell Res.* 1999; 253:230–238. [PubMed: 10579925]
53. Georas SN. Lysophosphatidic acid and autotaxin: emerging roles in innate and adaptive immunity. *Immunol Res.* 2009; 45:229–238. [PubMed: 19184538]
54. Rubinfeld J, Guo J, Sookrung N, Chen R, Chaicumpa W, Casolaro V, et al. Lysophosphatidic acid enhances interleukin-13 gene expression and promoter activity in T cells. *Am J Physiol Lung Cell Mol Physiol.* 2006; 290:L66–74. [PubMed: 16199434]
55. Watanabe N, Ikeda H, Nakamura K, Ohkawa R, Kume Y, Tomiya T, et al. Plasma lysophosphatidic acid level and serum autotaxin activity are increased in liver injury in rats in relation to its severity. *Life Sci.* 2007; 81:1009–1015. [PubMed: 17850827]

Figure 1. Immune activation is present during chronic HCV and HIV infections
Panel A) Proportion of CD3⁺ CD4⁺ T lymphocytes which are CD38⁺HLA-DR⁺. **Panel B)** Proportion of CD3⁺CD8⁺ T lymphocytes which are CD38⁺HLA-DR⁺. **Panel C)** Serum sCD14. **Panel D)** Serum IL-6. Statistically significant p values (<0.05) are shown for pair wise comparisons.

Figure 2. Peptide intensity based quantification of plasma proteome identifies proteins that differ in plasma level among groups

Panels A and C: intensity of individual peptides that correspond to ENPP2 and VCAM across groups. **Panels B and D** plasma protein level of ENPP2 and VCAM across groups by ELISA. Pairwise comparisons for ELISA P values were determined by control vs each HCV lo, HCV hi, HIV or HCV/HIV groups

Figure 3. LPA dependent CD4 cell activation

500,000 PBMC from 5 healthy controls or 3 untreated HIV infected subjects were cultured overnight in the absence or presence of PHA, followed by 3 days of culture in media or 100uM LPA. Proportion of $CD3^+ CD4^+$ (Panel A) and $CD3^+ CD8^+$ (Panel B) T cells that express of HLA DR and CD38 are shown. Statistical analysis shown is comparing indicated conditions for all 8 subjects combined. Comparisons between presence and absence of LPA in PHA pre-treated healthy control CD4 and CD8 activation were also significant ($P = 0.04$ and $P = 0.04$, not shown).

Table 1

Study subject characteristics

	Healthy Control	HCV lo	HCV hi	HIV	HCV/HIV*	p-value
Number of Subjects	12	12	12	12	4	N/A
Age (years)	47 (6)	56 (5)	58 (6)	31 (10)	55 (4)	<0.0001
HCV (IU/mL)		1,566,408 (1,678,146)	1,029,395 (1,089,721)		4,245,000 (5,516,771)	0.5
Current HIV level (c/mL)				38,151 (36,163)	8,411 (13,072)	0.07
Current CD4+ (cells/uL)				500 (157)	580 (105)	0.3
AST (IU/mL)		38 (13)	123 (73)	33 (27)	64 (46)	0.0002
ALT (IU/mL)		43 (20)	115 (79)	51 (48)	59 (38)	0.005
PLT ($10^3/\text{mm}^3$)		259 (61)	167 (63)	241 (46)	277 (80)	0.008
Alb (g/dL)		4.0 (0.3)	3.4 (0.6)	3.8 (0.5)	3.8 (0.6)	0.04
Tbili (mg/dL)		0.7 (0.2)	1.1 (0.4)	0.5 (0.2)	0.5 (0.2)	0.0001
APRI Score [†]		0.36 (0.17)	1.82 (1.16)	0.34 (0.30)	0.60 (0.54)	<0.0001
Serum LPS (pg/mL)	28.0 (37.7)	27.0 (61.3)	51.4 (47.7)	33.2 (53.6)	26.5 (31.8)	0.40

Clinical characteristics are represented as the mean (Standard Deviation) for each group. P value represents results of non-parametric Anova comparison across control, HCV lo, HCV hi, and HIV groups

* (HCV-HIV group excluded from comparison due to small sample size).

[†] APRI: Aspartate transaminase (AST) to platelet (PLT) ratio index [(AST/45)/PLT]*100

Table 2

Proteome identified proteins correlated with markers of immune activation.

Proteins	CD4 Activation					CD8 Activation					sCD14					Serum IL-6				
	Ctrl	HCV lo	HCV hi	All HCV	HIV	Ctrl	HCV lo	HCV hi	All HCV	HIV	Ctrl	HCV lo	HCV hi	All HCV	HIV	Ctrl	HCV lo	HCV hi	All HCV	HIV
APCS				-												+		-	-	
B2M	-								+				+							
C2							+												+	
C3															+					
C5					+					+									+	
C7																		+	+	
sCD14				+														+	+	+
sCD163					+									+					+	
CD44					+								+					+	+	
CD5L							+		+									+	+	
CRA beta														+				+	+	
DEFA3								+	+									+		
EFEMP1				+														+	+	
ENPP2				+														+	+	
F2	-																			
F5																		+		
FCGBP		+		+					+										+	
FCGR3A						-			+					+				+	+	
FGA					+															
FGB																+		+	+	
FLJ55673									+							+			+	
FLJ60487				+										+				+	+	
FN1				+																
HBD			+																	
HGFAC																	-	-	-	
HPX																				

Proteins	CD4 Activation					CD8 Activation					sCD14					Serum IL-6				
	Ctrl	HCV lo	HCV hi	All HCV	HIV	Ctrl	HCV lo	HCV hi	All HCV	HIV	Ctrl	HCV lo	HCV hi	All HCV	HIV	Ctrl	HCV lo	HCV hi	All HCV	HIV
ICAM1				+	+									+				+		
IGF2				-																
IFGALS	-			-													-		-	
IGFBP3				-	-														-	
ITIH3					+														+	
ITIH4				+														+	+	
LCP1																		+	+	
LGALS3BP				+	+				+			+	+						+	
LILRA3																+				
LUM				+													+		+	
PROC	-																	-	-	
QSOX1				+													+	+	+	
RBP4					-														-	
SERPINA11						-														
SERPINF1													+	+		+		+	+	
SERPING1		+																+	+	
VCAM1				+														+	+	
VWF				+	+													+	+	

Expression of proteome identified proteins (n=85) that differed across groups (HCV lo, HCV hi, HIV, controls) were evaluated for correlation with markers of immune activation (CD4 activation, CD8 activation, sCD14, IL6) within each group (HCV lo, HCV hi, HIV, control, as well as all HCV combined). The + denotes a significant (p<0.05) positive correlation while the – denotes a significant negative correlation.