Interferon Lambda Alleles Predict Innate Antiviral Immune Responses and Hepatitis C Virus Permissiveness

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SUMMARY

Hepatitis C virus (HCV) infection can result in viral chronicity or clearance. Although host genetics and particularly genetic variation in the interferon lambda (IFNL) locus are associated with spontaneous HCV clearance and treatment success, the mechanisms guiding these clinical outcomes remain unknown. Using a laser capture microdissection-driven unbiased systems virology approach, we isolated and transcriptionally profiled HCV-infected and adjacent primary human hepatocytes (PHHs) approaching single-cell resolution. An innate antiviral immune signature dominated the transcriptional response but differed in magnitude and diversity between HCV-infected and adjacent cells. Molecular signatures associated with more effective antiviral control were determined by comparing donors with high and low infection frequencies. Cells from donors with clinically unfavorable IFNL genotypes were infected at a greater frequency and exhibited dampened antiviral and cell death responses. These data suggest that early virus-host interactions, particularly host genetics and induction of innate immunity, critically determine the outcome of HCV infection.

INTRODUCTION

The majority of people infected with hepatitis C virus (HCV) develop chronic infection, which can remain asymptomatic for many years, ultimately leading to the development of liver fibrosis, cirrhosis, and hepatocellular carcinoma (Alter and Liang, 2012; Brown, 2005). Interestingly, 20%–30% of people infected with HCV are able to clear the infection and do not progress to chronicity (Thomas et al., 2009). The molecular mechanisms driving this clinical dichotomy remain unknown, in part because of challenges in studying HCV in its native environment, the human hepatocyte in the liver. Low levels of HCV replication and antigen in infected hepatocytes have hampered the identification and isolation of infected cells by antibody staining (Liang et al., 2009). Thus, the transcriptional response to HCV in an infected hepatocyte in patients is not known. Exploring this host response may reveal key pathways that influence clearance versus chronicity and uncover new avenues for enhancing treatment success.

Systems virology approaches generate unbiased data sets, which can be mined to obtain more comprehensive views of virus-host interactions. Initial systems approaches for HCV utilized liver biopsy tissue from infected humans or chimpanzees in which a minority of hepatocytes was infected (estimated 7%–20%) (Bigger et al., 2001, 2004; Liang et al., 2009; Sarasin-Filipowicz et al., 2008; Su et al., 2002). Thus, the transcriptional response in an HCV-infected cell population could not be separated from signals originating from uninfected hepatocytes and other liver cell types. Given the challenges of studying HCV-infected hepatocytes in vivo, cultures of primary human hepatocytes (PHHs) offer an attractive alternative. Like native hepatocytes in the liver, PHHs are polarized, largely terminally differentiated, and can robustly upregulate the innate immune response upon infection (Andrus et al., 2011; Dill et al., 2012; Jilg et al., 2013; Marukian et al., 2011; Metz et al., 2012; Park et al., 2012; Thomas et al., 2012). However, mimicking HCV infection in vivo, PHH infection frequency (2%–30%) and the levels of virus replication are low, preventing viral antigen detection and isolation of uniformly infected cells (Andrus et al., 2011; Liang et al., 2009; Ploss et al., 2010).

In the current study, we have overcome these challenges and succeeded in defining the transcriptional response in HCV-infected hepatocytes nearing single-cell resolution. We coupled an HCV infection-dependent fluorescence relocational (HDFR) reporter (Jones et al., 2010) with laser capture microdissection (LCM) (Espina et al., 2006) to isolate infected and adjacent cells. An antiviral immune signature dominated the transcriptional response but differed in HCV-infected and adjacent cells. By comparing and contrasting data from donors with high and low infection frequencies, we determined the signatures associated with effective antiviral control. In HCV-infected patients, genetic variations at the interferon lambda (IFNL) locus exert a profound effect on spontaneous viral clearance and treatment success (Ge et al., 2009; Heim, 2013; Thomas et al., 2009). We found that hepatocytes from donors with clinically less
favorable IFNL genotypes (IFNL3 rs12979860, minor allele T/T and C/T) were more permissive for HCV infection compared to cells from donors with favorable alleles (IFNL3 rs12979860, major allele C/C). Although the antiviral program was not absent in donors with IFNL minor alleles, responses were neither uniform nor robust. These results highlight the power of studying viral infection in disparate genetic backgrounds and reveal a remarkable convergence with clinical findings. Overall, our study suggests that early virus-host interactions, in particular host genetics and the induction of innate antiviral immunity, play a critical role in determining the outcome of HCV infection.

RESULTS

Isolation of HCV-Infected PHH by LCM

PHH cultures were created from fetal liver of similar gestational age and were similarly differentiated (Figure S1 available online). We employed a fluorescent cellular reporter system (i.e., HDFR; Figure 1A) to identify productively infected cells and isolate small numbers (30 cells in quadruplicate) of mock-infected cells (HDFR expressing, HCV naive), HCV-infected cells (HDFR positive, nuclear RFP), or cells adjacent to infected cells (adjacent cells, HDFR expressing, perinuclear RFP; Figure 1C) via LCM (Figure 1B). HDFR is sensitive and requires active HCV replication for efficient cleavage of the mitochondrial-localized RFP reporter, which then accumulates in the nucleus (Figure S2). Importantly, LCM of discrete populations within a mixed culture yielded minimal cross-contamination of transcriptomes, even when in direct contact (Figure S3). Captured cell lysates were divided for whole transcriptome analysis (20 cells) and qRT-PCR (10 cells) to quantitate HCV genomes in each sample (Figure 1D). As confirmation, sequential photographs were taken throughout the LCM process (Figure 1E). Cells positive for HDFR harbored significantly more HCV genomes than adjacent cells (Figure S4). We pooled time-matched samples of all donors to allow a systems-level analysis of the responses guiding HCV replication and immune responses. Given HCV’s ability to establish chronic infection with multiple mechanisms to subvert innate immune responses, these data unexpectedly indicate that an antiviral program dominates the HCV-induced transcriptional response at very early times after infection.

Distinct Antiviral Programs in Infected and Adjacent Cells

To examine possible differences between HCV-infected and adjacent cells, gene ontology analyses were performed. Antiviral responses in HCV-infected cells followed a distinct temporal order comprised of three phases (Figure 2F). During the initial phase, genes related to the induction of the innate immune response were upregulated in HCV-infected cells (e.g., DDX58, IRF7, STAT1, etc.) along with interferon-stimulated effector genes (ISGs; e.g., IFIT1, OAS2, etc.), leukocyte-recruiting chemokines (CXCL10 and CCL5), and genes related to posttranslational modification and regulation (e.g., ISG15, USP18, etc.). During the second phase, expression of the inducers of innate immunity had for the most part waned, but the expression of effector ISGs (e.g., DDX60, IFI27, IFITM1, etc.) and genes related to antigen presentation (B2M, HLA-B, etc.) increased. The third “constitutive” phase was marked by antiviral genes that were upregulated at all time points (e.g., BST2, HERC6, IFI44, IFI6, and OAS2). While kinetically similar to infected cells, adjacent cells regulated fewer (about half) antiviral genes, with only a handful being uniquely regulated. To demonstrate that fluctuations in the array data were not due to cDNA library defects, beta actin expression in mock, HCV, and adjacent cells was compared to two differentially regulated ISGs, IFIT1 and CCL5 (Figure 2G). Unlike beta actin, IFIT1 was upregulated in HCV-infected and adjacent cells compared to mock. In contrast, significant upregulation of CCL5 was only observed in HCV-infected cells (Figure 2G). These observations were confirmed by qPCR (Figure 2H). Taken together, these data define donor-independent, distinct, and dynamic antiviral responses in HCV-infected and adjacent PHH, information that would have been impossible to obtain without this LCM-driven transcriptomic approach.

Host Responses Associated with More Effective Antiviral Control

Despite infecting PHH with the same virus inoculum and protocol, we observed significant donor-to-donor variation in HCV...
IFN Lambda Alleles Predict HCV Permissiveness

A. Phase contrast and red channel images showing cellular morphology.

B. Schematic of laser capture microdissection (LCM) process.

C. Diagram illustrating the comparison of Viral/Host Phenotypes using microarray and qPCR.

D. Flowchart showing the process from LCM of cells to final analysis.

E. Images showing phase contrast, red channel, post-laser cut, post-capture, and captured sample for Mock, HCV, and Adjacent conditions.

F. Graphs showing the logarithm of genomes per 10 cells at 1dpi, 3dpi, and 7dpi for Mock, HCV, and Adjacent conditions.

Legend:
- *: Significant difference
- : Limit of quantitation

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infection frequency (Figure 2A). Given that host antiviral responses dominated, we hypothesized that gene signatures associated with more effective antiviral control would emerge from comparing donors with disparate infection frequencies. We created two groups: high donors and low donors, each comprised of the three donors with the highest and lowest infection frequencies based on the following criteria. Low donors had the lowest infection frequencies on 1 dpi and/or the lowest frequencies on 3 dpi, while high donors had the highest frequencies on 1 dpi and/or 3 dpi (Figures 3A and 4B). An inverse relationship is seen when comparing the two groups (Figure 3A) with respect to the number of significantly regulated genes (Figure 3B). Despite a significantly higher infection frequency at 1 dpi, high donors regulated only about half as many genes as low donors (Figure 3B). The number of antiviral genes upregulated in low donors exceeded that of high donors in both number and percentage (Figure 3C), indicating that a more robust antiviral response might be determining differences in infection frequency.

Ingenuity gene ontology analyses defined the responses associated with more effective viral control. Compared to high donors, the antiviral response in low donors was increased in magnitude and complexity with inducers of the innate immune response (DDX58, TRIM25, STAT1, etc.) and 14 other antiviral genes uniquely upregulated (Figure 3D). Additionally, low donors showed unique regulation of 10 antiviral genes in adjacent cells, whereas high donors uniquely regulated none. These data are consistent with the idea that more robust antiviral responses in adjacent cells may limit cell permissiveness to virus infection and spread. Pathway analysis predicted the functional consequences of the transcripational response (Figure 3E). The biological function RNA virus replication was among the top five most significantly affected functions associated with low donors. The associated Z score, a predictor of functional effect, suggested that the suite of associated genes would reduce RNA virus replication to a greater degree than those associated with high donors. Thus, the virologic, transcriptomic, and bioinformatic data converge, defining responses associated with lower infection frequency and more effective viral control.

Linkage of IFNL Variation to PHH Infection Frequency and Host Response

SNPs at or near the IFNL3 (interleukin-28B [IL-28B]) locus (e.g., rs12979860) strongly associate with HCV clearance and treatment response, but the underlying mechanisms responsible remain unclear (Ge et al., 2009; Naggie et al., 2012; Thomas et al., 2009; Urban et al., 2010). More recently, polymorphisms at an IFNL lambda locus, IFNL4, were shown to have similar predictive value, thus galvanizing the importance of IFNL in HCV infection (Figure 4A) (Prokunina-Olsson et al., 2013). We genotyped all of our donor cells (n = 22) and confirmed the strong linkage disequilibrium (Prokunina-Olsson et al., 2013) between IFNL3 and IFNL4 SNPs, with zygosity of major and minor IFNL3 and IFNL4 alleles in perfect agreement (Figure 4B).

To examine the importance of the IFNL locus on HCV infection outcome in PHH, we stratified our data by IFNL genotype. Significantly more cells were infected in donors with unfavorable genotypes (minor alleles) as compared to favorable genotypes (major alleles) (Figure 5A; p < 0.05). While examining genotype-specific host responses, we observed that the numbers of significantly regulated genes increased stepwise from homozygous major (favorable) to homozygous minor (unfavorable) alleles in both HCV-infected and adjacent cells (Figure 5B). People with unfavorable genotypes chronically infected with HCV typically have higher baseline levels of ISGs (Urban et al., 2010). We observed a similar trend with the highest absolute number of regulated antiviral genes seen in minor allele donors (Figures 5C and 5E). In contrast, the fraction of the total response dedicated to the antiviral response among homozygous donors was the highest in major allele donors (Figure 5C; IFNL3 T/T: HCV 32%, ADJ 15% versus IFNL3 C/C: HCV 41%, ADJ 49%). In pathway analyses similar to those in Figure 3E, “viral infection” was among the top three most significantly affected biological functions. Even though minor allele donors regulated more genes within this pathway (number of regulated genes per condition: HCV C/C = 22, HCV T/T = 38, ADJ C/C = 14, ADJ T/T = 43), the Z scores predicted a similar negative effect on viral infection (Z scores: HCV C/C = −3.6, HCV T/T = −4.3, ADJ C/C = −3.3, ADJ T/T = −3.7) (Figure 5D). Additionally, IFNL major allele donors regulated fewer cellular pathways in addition to the antiviral response as compared to minor allele donors, thus demonstrating that major allele donor responses were predominantly antiviral and more focused (Figure 5F).

To gain a better understanding of the host responses unique to homozygous IFNL alleles, we performed Ingenuity pathway analysis (IPA) on genes unique to each genotype. The top network associated with the 32 genes unique to HCV-infected cells of major allele donors was cell death and survival, while the antimicrobial response led in minor allele donors (Figure 5E). Of the genes shared between genotypes, the antiviral program was dominant in both HCV-infected (69%) and adjacent (72%) cells. The numbers of significantly regulated antiviral genes unique to minor allele donors (HCV, 13; ADJ, 15) exceeded that of major allele donors (HCV, 8; ADJ, 3) (Figure 5E), but when expressed as a fraction of the overall host response, this trend was reversed (HCV C/C 25%, T/T 16%; ADJ C/C 20%, T/T 9%) (Figure 5E). While the magnitude of antiviral gene induction was for the most part similar, six genes (IFI6, IFIT1, IFIT2, IFIT3, OAS1, and RSAD2) in minor allele donors were upregulated more than...
Figure 2. Systems Virology Reveals Unique Signatures Associated with HCV-Infected and Adjacent Cells

(A) HCV infectious virus production in PHHs (left). Cells were infected at a similar moi. Titers in culture supernatants were determined by TCID50 (donor n: 1, 3 dpi = 19, 7 dpi = 17). Each time point represents the 24 hr accumulation of virus. The dashed line indicates the limit of detection. HCV infection frequency is shown (right). Each dot represents the average infection frequency per donor (donor n: 1, 3 dpi = 22, 7 dpi = 20).

(B) The numbers of significantly regulated genes (ANOVA, Benjamini Hochberg MTC, FDR 0.05, fold change cutoff of 2) in HCV infected and adjacent cells. Donor n per time point: 8 (1 dpi), 3 (3 dpi) and 4 (7 dpi).

(C) The numbers of antiviral response genes induced in HCV infected and adjacent cells.

(D) Microarray data for the IFNL1 gene.

(E) IFNL1 secreted in culture supernatants from donors 4720, 5679, 5728, 5123, 5686, and 3868. Select cultures were treated with HCV replication inhibitor 2' CMA.

(F) The temporal order of the antiviral response in HCV-infected and adjacent cells (ADJ). The absence of a colored box indicates that fold change did not exceed the 2-fold cutoff.

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Stochasticity of the Host Response Is Associated with Host Genotype and Infection Frequency

In our previous array analyses analyzed by ANOVA, each quadruplicate sample was averaged for each gene, and if the variation was excessive the data were discarded. Thus, biologically significant data could be missed in the pursuit of statistical significance. Figure 6 displays the viral load and host response in each quadruplicate sample of 20 mock, HCV, or adjacent cells for each donor profiled on 1 dpi, organized from left to right with decreasing infection frequency. Similar to the data in Figure 1F, HDFR-positive cells were enriched for HCV genomes as compared to mock and adjacent cells (Figure 6A). The expression of beta actin via microarray or qRT-PCR was found to be uniform for each donor, suggesting that fluctuations in genes of interest were not due to random sample variation (Figure 6B). No correlation was observed between the levels of HCV RNA and ISG expression (IFI15, CCL5, and BST2) on a per sample basis. Importantly, the ISG array data for each sample were confirmed by qRT-PCR (Figure 6C). We then investigated the relationship between donor infection frequency and ISG expression in each LCM sample. In contrast to lower infection frequency donors, high infection frequency donors (47/28, HFTR) failed to consistently upregulate IFIT1 in HCV-infected and adjacent cells (Figure 6C). For lower infection frequency donors, CCL5 expression was fairly uniform in HCV-infected cells but intermittent in adjacent cells, a trend not observed in high infection frequency donors (Figure 6C). Similarly, qRT-PCR measurement of BST2 gene expression in HCV-infected and adjacent cells was more common in lower infection frequency donors. Together, these data demonstrate that (1) ISG expression can be compartmentalized (e.g., CCL5) in specific populations (HCV-infected cells) within a complex mixture, (2) the uniformity of ISG gene expression in HCV-infected and adjacent cells varies from donor to donor, and (3) the uniformity of ISG expression correlates with the level of infection frequency.

DISCUSSION

Studying HCV in its native environment, the human hepatocyte, is technically challenging. Low infection frequencies, low levels of viral antigen, and high autofluorescence are a few of the challenges that have prevented the identification, isolation, and transcriptional profiling of HCV-infected cells in vivo. HCV infection is most often diagnosed during the chronic phase. Hence, we lack an understanding of the early responses in the human liver during the first few days of acute infection. This void has been addressed to some extent by experimental infection of chimpanzees (Bigger et al., 2001, 2004; Su et al., 2002). Similar to our data, the antiviral response (i.e., IFI16, IFIT1, IFITM1, ISG15, MX1, etc.) dominates at early times (2 dpi) in the livers of chimpanzees destined to resolve acute HCV infection (Bigger et al., 2001, 2004). However the origin of this signature, whether from infected hepatocytes, noninfected hepatocytes, nonparenchymal cells, or inflammatory cells, is not clear. This is unlikely to be addressed in the future, given the new restrictions on chimpanzee research (Kaiser, 2013) and the inability to obtain human liver samples during early acute infection. Despite this, systems biology approaches such as the one described here can provide a unique and unbiased insight into the initial battle between virus and host.
PHHs are arguably the most physiological in vitro model of HCV pathogenesis, but the challenges that complicate in vivo studies (i.e., low infection frequency, low levels of viral antigen, etc.) are also encountered with PHHs (Andrus et al., 2011; Marukian et al., 2011; Ploss et al., 2010). By coupling an HCV-dependent fluorescent cellular reporter with LCM, we successfully isolated and transcriptionally profiled HCV-infected PHHs. Induction of the innate antiviral program was the hallmark of the early host response to HCV in PHHs. Our data stand in stark contrast to those gleaned from the profiling of HCV-infected cancer cell lines where cell cycle, apoptosis, oxidative stress, lipid metabolism, and, to a lesser extent, host defense were chiefly

![Figure 3. Host Responses Associated with More Effective Viral Control](image)

(A) Infection frequency in high (donors 4728, HFTR, and 5737) and low (donors 5123, 5780, 5763) infection frequency donors. Each dot represents the average infection frequency per donor. Asterisks indicate statistical significance as determined by t test (p < 0.005).

(B) Significantly regulated genes in HCV-infected and adjacent cells at 1 dpi.

(C) Antiviral response genes in HCV-infected and adjacent cells at 1 dpi.

(D) Venn diagram comparing all significantly regulated genes or antiviral response genes for HCV-infected and adjacent cells and heatmap of antiviral response genes. White boxes indicate that the gene did not exceed the 2-fold change cutoff.

(E) IPA for the biological function RNA virus replication. The –log(p value) (orange bars) measures the statistical strength of the submitted gene list. The numbers of associated genes are listed above each bar. The Z score (purple bars) predicts the functional effect of the gene list. Negative Z scores indicate a reduction in function. ADJ refers to adjacent cells throughout.
affected (Blackham et al., 2010; Walters et al., 2009). Since the hepatoma cell lines used for these studies have multiple functional abnormalities (Li et al., 2005; Sainz et al., 2009), the dramatic differences with PHHs are not surprising and highlight the dangers of extrapolating data derived from commonly used cancer cell lines to initial virus and host interactions in vivo. Rather, our data are reminiscent of the transcriptional profile of HCV-infected PHHs reported by Thomas et al., where the induction of a subset of antiviral genes (IFIT1, CXCL10, IFI44L, CCL5, etc.) was observed, but the contribution of infected or bystander cells to this response was not determined (Thomas et al., 2012). In contrast, we were able to measure the transcriptional response in uniformly infected PHHs over time, thus defining transcriptional response kinetics at very early times after infection. In addition, we determined the gene signatures associated with more effective antiviral control through the comparison of responses from donors with high and low infection frequencies. Although many of the genes associated with antiviral response in low infection frequency donors have been reported to have anti-HCV activities (Schoggins et al., 2011), a number of others represent potential anti-HCV genes.

HCV pathogenesis is a complex process, the outcome of which is affected by viral genotype, host genetics, innate and adaptive immunity, and communication among hepatocytes and nonparenchymal cells. For decades, prevailing dogma posited that HCV chronicity is driven, at least in part, by the ability of the virus to successfully antagonize or inactivate various innate antiviral defenses (Horner and Gale, 2013). Here, we demonstrate that HCV is able to complete its replication cycle in cells with competent innate immunity, with an antiviral program induced in infected cells of almost every donor tested. These data suggest that HCV does not necessarily have to completely suppress innate immunity to establish chronic infection; suppression just long enough to produce progeny capable of infecting another target cell may be sufficient. Another explanation, yet to be tested in PHHs, is that HCV may not need to block innate immune transcriptional responses, but rather evades antiviral effectors at the level of translation. In this scenario, supported by studies in hepatoma cells, HCV alters the infected cell by activating PKR and imposing a selective block on translation of antiviral gene transcripts (Garaigorta and Chisari, 2009).

In addition to viral effects, host genetics play a significant role in the establishment of chronic infection. Host variation in the IFN locus is associated with spontaneous clearance of HCV and type I IFN-based treatment success (Ge et al., 2009; Thomas et al., 2009). Although the underlying mechanisms determining these IFNL-dependent, clinical outcomes remain unknown, our results are consistent with two non-mutually exclusive hypotheses: (1) donors with clinically favorable genotypes have heightened ability to respond to infection or (2) donors with clinically favorable genotypes are able to generate more effective antiviral responses. We observed a significant enrichment in cell death-related genes in cells from donors with IFNL major alleles. Recent clinical studies report a higher basal ISG expression in noninfected livers of patients with IFNL3 major alleles (Raglow et al., 2013), and chronically infected major allele patients exhibit increased liver necroinflammation and poorer clinical outcomes as compared to patients with minor alleles (Noureddin et al., 2013). Taken together, these data collectively suggest that innate genotype-dependent differences could make hepatocytes more poised to respond to infection through activation of cell death and/or antiviral programs. The recent discovery of IFNL4 has provided insight into the effect of host genetics on the quality of the host response to HCV. IFNL4 gene expression is intricately linked to a specific mutation (TT/-G, rs368234815), and minor alleles are strongly associated with poor clinical outcomes in HCV-infected patients (Bibert et al., 2013; Prokunina-Olsson et al., 2013). IFNL4 expression is thought to increase basal ISG expression and induce an IFN refractory state, thus decreasing the efficacy of IFN treatment (Prokunina-Olsson et al., 2013). However, this has not been conclusively demonstrated. Similar to these clinical observations, we observed a correlation between IFNL (both IFNL3 and IFNL4) genotype, HCV infection frequency, and the quality of the antiviral response. This convergence reinforces the utility of our in vitro model for studying aspects of HCV pathogenesis relevant to those seen on the organismal level. As compared to minor allele donors, we show that PHHs from donors with major IFNL alleles have a more focused and uniform antiviral response, especially within nonproductively infected adjacent cells. Communication between HCV-infected and adjacent cells of major allele donors may trigger a more robust innate response, limiting viral spread. Alternatively, adjacent cells may represent a population that has encountered virus, mounted effective control, and aborted productive infection. In contrast, the less focused, more sporadic host antiviral response of IFNL minor allele donors suggests a pervasive dysregulation or response deficit.

Given the complexities of studying hepatotropic infectious diseases in humans, in vitro cultures of PHHs serve to bridge the gap between the laboratory and the clinic. While PHHs are arguably the most biologically and clinically relevant in vitro system by which to study liver biology, the cells from each donor are...
Figure 5. Genetic Variation in IFNL Is Associated with Increased HCV Infection Frequency and Distinct Host Responses
(A) Infection frequency for each IFNL3 (rs12979860) genotype. Each dot represents the average frequency per donor. Statistical significance was determined by ANOVA (p < 0.05).
(B) Significantly regulated genes on 1 dpi. Donor n: IFNL3 C/C = 2, C/T = 4, T/T = 2.
genetically unique. Rather than shudder and recoil from host genetic variability, it should be embraced. These data highlight the power and utility of studying viral infections in PHHs of disparate genetic backgrounds and reveal a remarkable convergence with clinical findings. Overall, our study suggests that early virus-host interactions, in particular host genetics and the induction of innate antiviral immunity, play a critical role in determining the outcome of HCV infection. Further work using PHH donors of disparate genetic backgrounds will continue to provide unique insights into the effect of host genetics on HCV infection but should also serve as a fruitful approach for the study of other clinically important human hepatotropic pathogens, such as hepatitis B virus (HBV), hepatitis A virus (HAV), hepatitis E virus (HEV), yellow fever virus, and malaria.

**EXPERIMENTAL PROCEDURES**

**Human Subjects**

All protocols involving the use of human tissue were reviewed and exempted by the Rockefeller University Institutional Review Board.

**Generation of HCVcc**

Cell culture-derived HCV (HCVcc) for a genotype 2a recombinant J6/JFH Clone2 (HCV Clone 2) was generated through electroporation of a human hepatoma cell line, Huh-7.5, with in vitro transcribed HCV genomic RNA as described (Marukian et al., 2008; Walters et al., 2009). HCVcc stocks were collected in serum-free media containing Dulbecco’s modified Eagle’s medium (DMEM), 0.15% BSA (Fraction V, Sigma), penicillin/streptomycin (Gibco), 0.1 mM nonessential amino acids (Gibco), and 20 mM HEPES (Gibco), concentrated 25-fold (Amicon Ultralclf 100K), and stored at –80°C. Mock-transfected control supernatants were generated by electroporation of Huh-7.5 cells in the absence of RNA and treated similarly to those of HCVcc.

**Isolation and Culture of Enriched Human Fetal Hepatocytes**

Coded fetal livers (17–20 weeks gestation) were procured through Advanced Bioscience Resources (ABR) or the Human Fetal Tissue Repository of the Albert Einstein College of Medicine (AECOM). Enriched primary human fetal hepatocyte cultures (PHHs) were prepared as previously described (Andrus et al., 2011). PHHs were seeded on collagen-coated multiwell plates (BD BioCoat) or collagen-coated (0.01% calf, Sigma) polyethylene napthalate (PEN) LCM slides (Molecular Machines and Industries) at 50,000–100,000 cells/cm² in William’s E medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (HyClone), 2 mM L-glutamine (Invitrogen), 1× ITS Plus (BD Biosciences), and antibiotics. At 24 hr after plating, cells were washed with William’s E medium and then maintained in Hepatocyte Defined Medium (HDM; BD Biosciences) with medium exchanges every other day.

**HCV Live-Cell Reporter**

To monitor HCV infection in live cells, lentivirus expressing an HCV-dependent fluorescence recolorization (HDFR) reporter was employed (Jones et al., 2010; Marukian et al., 2011). PHHs were transduced with PPs 1 day after plating as described (Andrus et al., 2011). At 48 hr after transduction and just prior to HCV infection, 70–90% of cells expressed HDFR in all donors.

**Infection of PHHs with HCVcc, Quantitation of Infectious Virus, and Viral Genomes**

At 3 days after PHH plating, culture medium was removed, and HCVcc or mock-transfected supernatants were added at a multiplicity of infection (moi) of 10–12. After 6 hr at 37°C, virus was removed, cultures were rinsed 5–8 times with William’s E, and HDM was added. To monitor infectious virus production after infection, culture medium was harvested and monolayers were thoroughly rinsed (3–6 times) to reduce infectious virus to background levels. Virus titer in culture supernatants was determined by 50% tissue culture infectious dose (TCID50) assay in Huh-7.5 cells (Lindenbach et al., 2005). To measure infection frequency, the total number of HDFR-positive cells was divided by the total number of transduced cells in photographs of 3–5 random 20× objective fields. Images were scored, and select images were blinded and confirmed by a second person. To demonstrate that HDFR translocation is dependent on virus replication, a replicase inhibitor, 2′-C-methyladenosine (2′CMA, 2 μM), or vehicle (DMSO) was added 2 hr prior to HCV infection and then maintained during and after infection. HCV genomic RNA in LCM sample lysate or extracted total cellular RNA (QIAGEN RNeasy) was quantitated via qRT-PCR (EraGen MultiCode RTx, EraGen Biosciences) with primers against the 3′ UTR and a synthetic RNA standard to determine genome copy number.

**Laser Capture Microdissection of HCV-Infected Primary Human Hepatocytes**

HCV or mock-infected LCM slides were rinsed extensively to remove virus from the culture surface, fixed with 100% ethanol (EtOH), and immediately used for LCM on the MMI CellCut system (Molecular Machines and Industries). For each donor, 30 cells of the following populations were captured in quadruplicate: mock-infected cells (perinuclear RFP) from mock-infected slides, HCV-infected cells (nuclear RFP) from HCV-infected slides, and cells adjacent (adjacent cells) to HCV-infected cells (perinuclear RFP) from HCV-infected slides. Adjacent cells were between 0 and 10 cell body widths away from an HCV-infected cell (nuclear RFP). For documentation of the process, digital photos were taken throughout. Each population was collected in a separate tube and placed on ice until LCM was completed. Cells were directly lysed on the capture surface with 3 μl of Direct Lysis Buffer (NuGEN) stored at –80°C until processing.

**Transcriptome Amplification and Microarray Analysis**

The transcriptome of each captured population was linearly amplified using OneDirect (NuGEN) according to protocol. For each amplification, a no-template control (lysis buffer) and a positive control (250 pg Universal Human Reference RNA, Agilent Technologies) were included. The cDNA generated via the no-template control did not produce a measureable signal via microarray. Amplified products were biotin labeled (NuGEN, Encore BiotinLI Module) and hybridized (48°C) to Illumina Whole-Genome Expression BeadChips (Illumina, HumanHT-12 V4 0 R2) according to protocol. Similar lots of microarray chips were used for each donor. Array data were analyzed using GeneSpring 11. Significantly regulated genes were determined by ANOVA, with a Benjamini-Hochberg multiple testing correction (MTC) and a false discovery rate (FDR) of 5%, comparing HCV-infected or adjacent cells to a time-matched mock. Only genes with a fold change greater than 2-fold over mock were considered for analysis. IPA software was employed to determine (Andrus et al., 2011). At 48 hr after transduction and just prior to HCV infection, 70–90% of cells expressed HDFR in all donors.
the affected pathways and functions modulated for each data set. The degree to which genes associated with a given function were fulfilled by the submitted list was determined in Ingenuity (−log p value). The functional effect of the submitted gene list was determined by the IPA Z score algorithm. Negative Z scores indicate a reduction in function. A gene list comprised of 538 genes titled antiviral genes was created by merging Ingenuity’s antiviral response, interferon signaling, and activation of IRF by cytosolic pattern recognition receptor lists with the ISG list reported in Schoggins et al. (2011).

To confirm the trends seen in the microarray data, relative gene expression in OneDirect cDNA libraries was determined by SYBR Green qPCR (Applied Biosystems) on a Roche 480 LightCycler. With the exception of beta actin (ACTB), primers were designed for all genes using the Roche Assay Design Center (Table S2). Expression levels were normalized to a housekeeping gene, beta actin, and donor and time-matched mock-infected samples using the comparative Ct method developed by Schmittgen and Livak (2008).

IFNL1 ELISA
IFNL1 secretion was measured in select donors (4720, 5679, 4278, 5123, 5686, 3868) via ELISA (eBioscience). Cultures were either mock infected or...
infected with HCV in the presence or absence of 2°CMA as described above. Culture supernatants representing a 24 hr accumulation of IFNL1 were harvested on the indicated days and stored at −80°C until analysis.

**Donor Genotyping**

DNA was extracted from cell pellets using QIAGEN DNeasy Blood and Tissue Kit or Trizol (Invitrogen). For IFNL3 (rs12979860) genotyping, a 145 bp amplicon was generated using primers F-GCTTGTCTGTACTGAACCA and R-GGCTCGGTTCAAGCATCAC. For SLC5A2 (rs16891982) genotyping, PCR was performed using primers F-ACCACCTGATTCCAAQGAAG and R-CCTCAACAGCCTCAATCTC. For IFNL4 (rs368234815), PCR was performed using F-GAAGCGGTATTGGGACC and R- GCCGTGCGACAAG CAGAGT. All amplicons were directly sequenced to determine genotype.

**Statistical Analysis**

Statistical analysis of virological data was performed with GraphPad Prism 4. Specific tests are noted in figure legends.

**ACCESS NUMBERS**

The NCBI GEO accession number for the microarray data reported in this paper is GSE54648.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.01.007.

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