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HCV Infection Induces a Unique Hepatic Innate Immune Response Associated With Robust Production of Type III Interferons

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Abstract

BACKGROUND & AIMS—Polymorphisms in the *IL28B* gene have been associated with clearance of hepatitis C virus (HCV), indicating a role for type III interferons (IFNs) in HCV infection. Little is known about the function of type III IFNs in intrinsic antiviral innate immunity.

METHODS—We used in vivo and in vitro models to characterize the role of the type III IFNs in HCV infection and analyzed gene expression in liver biopsy samples from HCV-infected chimpanzees and patients. Messenger RNA and protein expression were studied in HCV-infected hepatoma cell lines and primary human hepatocytes.

RESULTS—HCV infection of primary human hepatocytes induced production of chemokines and type III IFNs, including interleukin (IL)-28, and led to expression of IFN-stimulated genes (ISGs). Chimpanzees infected with HCV showed rapid induction of hepatic type III IFN, associated with up-regulation of ISGs and minimal induction of type I IFNs. In liver biopsy specimens from HCV-infected patients, hepatic expression of IL-28 correlated with levels of ISGs but not of type I IFNs. HCV infection produced extensive changes with gene expression in addition to ISGs in primary human hepatocytes. The induction of type III IFNs is regulated by IFN regulatory factor 3 and nuclear factor κ B. Type III IFNs up-regulate ISGs with a different kinetic profile than type I IFNs and induce a distinct set of genes, which might account for their functional differences.

CONCLUSIONS—HCV infection results predominantly in induction of type III IFNs in livers of humans and chimpanzees; the level of induction correlates with hepatic levels of ISGs. These findings might account for the association among IL-28, level of ISGs, and recovery from HCV infection and provide a therapeutic strategy for patients who do not respond to IFN therapy.

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Keywords

IRF; Cytokine; IL29; Nonresponder

Intrinsic innate immune responses provide a first line of defense against invading pathogens.¹ These early innate immune responses not only blunt the initial spread of infection but also activate the adaptive immune system and other secondary host defense mechanisms.² Production of type I interferons (IFNs) is a critical aspect of this initial response and occurs following the detection of viral pathogen-associated molecular patterns (PAMPs).³ These PAMPs stimulate several cellular pathways, including those involving the intracellular RIG-I-like helicases as well as Toll-like receptors that ultimately lead to an antiviral state.^{4,5}

Hepatitis C virus (HCV) is a single-stranded positive-sense RNA virus that induces strong IFN responses in the liver of infected humans and chimpanzees.^{6–8} Stimulation of the innate immune system and subsequent induction of antiviral genes results from detection of components of the viral genome.⁹ The importance of up-regulation of these antiviral genes by HCV is underscored by the fact that HCV-infected patients exhibiting high levels of hepatic IFN-stimulated genes (ISGs) are less responsive to the current treatment regimen consisting of ribavirin and pegylated IFN- α .⁷

Recently, a new family of IFNs was discovered and designated as type III IFN or IFN- λ (also known as interleukin [IL]-28A/B and IL29).¹⁰ Members of the type III IFN system possess antiviral activities against HCV.¹¹ In addition, polymorphisms surrounding the IL28B (λ 3) predict treatment responses to HCV antiviral therapy as well as spontaneous clearance of the virus.¹² To characterize the role of the type III IFN system in HCV infection, we used several in vitro and in vivo models, including HCV-infected primary human hepatocytes (PHHs), chimpanzees, and humans. In addition, we investigated a well-characterized clinical marker of HCV infection, IP-10 (CXCL10),¹³ to validate our findings. Data presented in this study provide evidence that type III IFN and IP-10 are induced predominantly in hepatocytes by HCV infection and that these genes are likely involved in the antiviral responses to HCV. The consequences of the type III IFN induction by HCV are also explored and put into context with data obtained from chimpanzee and human models of HCV infection.

Materials and Methods

Cells and Virus

Detailed information on the cells used in this study is included in Supplementary Materials and Methods. The HCV JFH1¹⁴ strain was used in cell culture experiments at a multiplicity of infection (MOI) of 0.5 unless otherwise indicated. The HCV JFH1-Luciferase virus was provided by C. Rice (Rockefeller University, New York, NY).

Reagents

HCV PAMP RNA was generated from a plasmid containing the 3' and 5' untranslated regions of HCV that was previously reported.¹⁵ Anti-HCV E2 antibody (AP33) was described previously.¹⁶ Additional reagents are included in Supplementary Materials and Methods.

RNA Interference, Microarray Analysis, and Quantitative Polymerase Chain Reaction

Detailed protocols for these methods are described in Supplementary Materials and Methods. Intracellular and extracellular copy numbers of HCV RNA were determined by realtime quantitative reverse-transcription polymerase chain reaction (qPCR) as described previously.¹⁷

Patient Antiviral Therapy and Liver Biopsies

Patients with chronic hepatitis C, genotype 1, underwent percutaneous liver biopsy, off treatment, in a research protocol as described in Supplementary Materials and Methods. Liver biopsy specimens from chimpanzee 1 (CH6412),¹⁸ chimpanzee 2 (CH10273),¹⁷ and chimpanzee 3 (CH10274)¹⁹ were described previously with additional information included in Supplementary Materials and Methods.

Statistical Analysis

Please see Supplementary Materials and Methods.

Results

Expression of IL-28 in Chimpanzee and Human Models of HCV Infection

HCV infection in both humans and chimpanzees induces high levels of ISGs in the infected liver, but the direct stimulus for ISG induction remains unclear.^{6,7} To explore the mechanism of this ISG induction, we studied liver biopsy specimens from chimpanzees obtained before and after HCV infection. Gene expression analysis using qPCR showed a robust up-regulation of IL-28 but minimal type I or II IFN in the liver of these animals early following HCV infection (Figure 1A). The induction of IL-28 corresponded to increased levels of ISGs, including ISG15 and IFIT1. To further explore this relationship, we examined liver biopsy specimens obtained from 19 HCV-infected patients. Because preinfection biopsy samples were not available from these HCV-infected patients, expressions of various ISGs and all 3 types of IFN were quantified by qPCR and analyzed statistically for correlation. Comparisons between 3 types of IFNs and ISG15 or IFIT1 levels showed a highly significant correlation between IL-28 expression and these ISG levels but not type I or II IFNs (Figure 1B and C and Supplementary Figure 1A), although the IFN- α analysis may show some correlation without an outlier.

Robust Induction of IL-28, IP-10, and Other Cytokines in Response to Viral PAMPs

Because IL-28 displayed significant induction in the liver of chimpanzees following HCV infection, we next examined the induction of IL-28 in cultured hepatocyte models. HCV RNA is believed to be the predominant stimulator of the IFN response,⁹ so we initially used viral nucleic acid mimetics. To closely model hepatocytes that are present in the liver in vivo, we used PHH cultures. As expected, treatment of PHHs with poly(I:C) resulted in induction of IFN- β at the mRNA and protein levels (Supplementary Figure 1B). However, the levels of IFN- β produced were much lower than those seen in other cell types treated with poly(I:C).²⁰ In contrast, a robust induction of IL-28 at the mRNA and protein levels was observed following treatment with poly(I:C). Similar effects were seen on the mRNA and protein levels of IP-10, a gene whose expression correlates to treatment response in HCV-infected patients.¹³ To substantiate our findings, we determined the effect of transfected poly(I:C) on antiviral responses in the HepG2 using the same panel of genes and observed similar results (Figure 2A).

Induction of IL-28 Depends on the IRF3 and Nuclear Factor κ B Signaling Pathways

Type III IFNs are up-regulated in response to viral stimuli through the RIG-I-like helicase and Toll-like receptor pathways, similar to type I IFNs.²¹ To determine the cellular signaling molecules important for the type IL-28 induction in hepatocytes, we used RNA interference to block components of these pathways. HepG2 cells were treated with small interfering RNAs (siRNAs) targeting IPS-1, IRF-3, NEMO, or TRIF (TICAM1) and then stimulated with poly(I:C). As shown in Figure 2*B–D*, IPS-1, IRF-3, and NEMO were necessary for the full induction of IL-28 in response to transfected viral PAMPs.

To further characterize genes involved in the production of IL-28 in HCV cell culture models, we studied the Huh7 cell line and its derivative, Huh7.5.1, because these cells support HCV infection and replication to high titers (Supplementary Figure 2*A*).¹⁴ The Huh7.5.1 cell line has a known mutation in the RIG-I gene that results in a dysfunctional mutant protein²² (Supplementary Figure 2*A*). To assess the role of the RIG-I gene in induction of IL-28, we compared gene induction in response to poly(I:C) in both Huh7 and 7.5.1 cells. As expected, the Huh7.5.1 cell line showed minimal IFN- β induction, while a modest IFN- β induction was observed in the Huh7 cells in response to poly(I:C) transfection (Supplementary Figure 2*B*). A more robust induction of IL-28 was observed in Huh7 cells but not in Huh7.5.1 cells, supporting a role of RIG-I in the induction of IL-28.

Although the Huh7 cell line has an intact RIG-I pathway, JFH1 infection (Supplementary Figure 2*C*) resulted in minimal to no up-regulation of ISG15 or IFIT1 (Supplementary Figure 2*D*), as reported previously in Huh7.5 cells.²³ Similarly, we infected CD81-transduced HepG2 cells that support HCV entry and low-level infection,²⁴ but we were unable to detect any ISG induction (Supplementary Figure 2*E*). We therefore used PHHs to characterize the innate immune response to HCV cell culture (HCVcc). In these cells, IL-28 production was observed early (6 hours) following poly(I:C) transfection with detection of IL-28 protein by enzyme-linked immunosorbent assay (ELISA) in the cell culture supernatant (Supplementary Figure 3*A*). We also observed production of IP-10 and IFN- β both at the messenger RNA (mRNA) and protein levels, although the level of IFN- β mRNA induction was much lower than that of IL-28. Other ISGs like IFIT1 and ISG15 were also up-regulated following poly(I:C) transfection. IL29, another member of the type III IFN family, was also significantly induced by poly(I:C) (Supplementary Figure 3*B*). Next, to address the HCV-specific antiviral responses, an in vitro transcribed RNA containing the 5' and 3' untranslated region of the HCV genome was used as the HCV PAMP⁹ to stimulate cells and was found to have similar effects on gene expression (Figure 3*A*).

IL-28 and IP-10 Are Induced by HCV Infection in PHH Culture

Despite the induction of a diverse set of ISG mRNAs in the livers of human and chimpanzee models of HCV infection,^{6,7} cell culture models of HCV infection have not shown similar up-regulation of ISGs.²³ To study this further, we infected PHHs with the JFH1 virus and detected marked changes in gene expression, including induction of ISGs. Both IL-28 and IP-10 were markedly induced at the mRNA and protein levels in response to the virus (Figure 3*B*). Treatment with IFN- α resulted in up-regulation of IP-10 but not IL-28, showing that IL-28 is not strongly stimulated by IFN- α (Figure 3*B*). Interestingly, type I IFNs (IFN- β and IFN- α 1) were not induced at the RNA (Figure 3*C*) and protein (data not shown) level, showing differential stimulation of IL-28, IP-10, and type I IFNs by HCVcc infection in PHHs. In addition, a slight decrease from the basal levels of type I IFNs was observed at early time points, suggesting that JFH1 may interfere with constitutive low-level expression of type I IFNs believed to be important in antiviral defenses.²⁵ In addition, we were able to detect gene induction of many ISGs, including IFIT1 and ISG15, as well as protein ISGylation by JFH1 in these experiments (Figure 3*D* and *E*). Intracellular and

extracellular virus levels from these experiments are shown in Supplementary Figure 4A and B. Up-regulation of IL-28 and IP-10 was also dependent on the MOI because higher MOI resulted in more robust gene induction (Supplementary Figure 4C–F). Similar to the result obtained using poly(I:C) in HepG2 cells (Figure 2A), induction of IL-28 by JFH1 in PHHs was dependent on IRF3 and NEMO (Figure 3F).

To confirm the importance of viral infection in this system, we tested the effects of HCV-blocking antibodies and viral inhibitors. As shown in Figure 4A and B, administration of a neutralizing antibody targeting the viral glycoprotein E2 resulted in a diminution of production of IL-28 and IP-10 and lower levels of intracellular viral RNA. Similarly, pretreatment of cells with a blocking antibody targeting CD81 (Figure 4C) resulted in significantly reduced IL-28 and IP-10 production by the virus. Figure 4D shows that administration of IFN- α and the antiviral molecule 2'-C-methylcytidine were able to block IL-28 protein production as well as limit viral replication (Figure 4E) in infected PHHs.

HCV Infection Results in a Distinct Pattern of Gene Expression

Having validated the up-regulation of IL-28 and IP-10 at the mRNA and protein levels, we next examined the global gene expression profile following HCVcc infection of PHHs. In addition, we compared HCVcc-induced changes in gene expression with those observed following treatment with IL28B. We identified genes that showed significant changes using a cutoff of $P < .05$ and fold change greater than 2.0 (Figure 5A and B) as well as with a false discovery rate cutoff of 0.05 (data not shown). In HCVcc-infected samples, many genes were affected, with a similar number of genes being up-regulated and down-regulated (Figure 5A). As expected, approximately 70% of the genes up-regulated by IL28B were also up-regulated following HCVcc infection (Figure 5A). The results shown in the heat map of Figure 5B illustrate the broad range of changes in the transcriptome that occur following HCVcc infection of PHHs.

Using pathway analysis, we identified various biological processes that were overrepresented by genes whose expression levels were significantly affected following IL28B treatment and HCVcc infection (Figure 5C). IL28A and IP-10 (CXCL10) were among the top 5 genes up-regulated, ranked by fold induction, in the HCVcc-infected cells (Figure 5D). Figure 5E shows the top 10 genes, ranked by fold induction, that were significantly induced by HCVcc but minimally by IL28B. Using additional microarray data, including analysis of PHHs treated with transfected poly(I:C), the IL28A, IL29, and CCL5 (RANTES) genes were shown to be predominantly induced by HCVcc but minimally by the IFNs (Figure 5E and F).

ISG Up-regulation Is a Result of Type III IFN Production Following HCV Infection

The results presented previously show that PHHs can produce antiviral cytokines in response to HCVcc. Unexpectedly, hepatocytes produce mainly type III IFNs in response to HCVcc. Consequently, production of these cytokines by infected hepatocytes may be a more likely stimulus for the high ISG levels readily observed in the livers of HCV-infected chimpanzees and humans. To examine this scenario further, kinetic analysis of gene induction following poly(I:C) stimulation in HepG2 cells was performed. qPCR analysis showed that IL-28 mRNA was rapidly up-regulated (<3 hours) after treatment and was subsequently followed by induction of prototypical ISGs such as IFIT1 and ISG15 (Figure 6A). IFN- β and IFN- α 1 were not up-regulated at these early time points (Figure 6A) but were up-regulated later (data not shown).

To determine if the ISG induction was a result of type III IFN production and subsequent receptor-mediated signaling, experiments were conducted using blocking antibodies against

the type III IFNs. We specifically used blocking antibodies targeting both the type III IFN receptor and the IL-28 protein to limit IL-28-stimulated gene induction in HepG2 cells, and we found that this combination was most effective (Supplementary Figure 5A). Experiments subsequently performed using Transwell inserts further showed that IL-28 produced following poly(I:C) transfection contributes to ISG induction in HepG2 cells (Supplementary Figure 5B). In addition, Figure 6B and C and Supplementary Figure 5C show the ability of these blocking antibodies to specifically limit IL28B and not type I IFN induction of ISGs in PHHs. We next performed similar Transwell experiments with HCVcc infection of PHHs. Figure 6D and E show that these blocking antibodies limit ISG induction in cells plated on Transwell membranes exposed to media of JFH1-infected PHHs producing IL-28. However, these blocking antibodies had little effect on the replication status of infected cells (Supplementary Figure 5D), suggesting that the endogenously induced type III IFN may not be capable of curing HCV infection, although the level of IL-28 in the culture supernatant can reach nanograms per milliliter, which is well within the effective concentration of IL-28. Interestingly, blocking type III IFN enhanced the antiviral activity of type I IFN (Figure 6F).

Type I and III IFNs Have Potent Anti-HCV Activities but Show Distinct Effects on Gene Expression

To further study the role of type III IFNs in HCV infection, we examined the antiviral response and gene induction in Huh7.5.1 cells. Using an HCV-expressing luciferase virus (JFH1-Luc), all 3 type III IFNs displayed dose-dependent anti-HCV activities, achieving approximately an 80% reduction in virus luciferase production at the highest doses. In comparison, IFN- α elicited more than a 95% reduction in virus luciferase production (Supplementary Figure 6A). When Huh7.5.1 cells infected with JFH1-Luc were treated with IL28B and IFN- α and followed up over the course of 3 days, both cytokines showed sustained antiviral activities over the duration of treatment (Supplementary Figure 6B). Similar antiviral effects were observed following infection using the wild-type JFH1 virus based on quantification of intracellular and extracellular HCV RNA levels (Supplementary Figure 6C and D). Among the type III IFNs, IL28B appears to be the most potent in this cell culture system, consistent with a previous study.¹¹

We next examined the effects of type I and III IFNs on ISG induction. When compared with IFN- α , IL28B showed distinct gene induction kinetics on the ISGs RSAD2 and IFI44 in PHHs (Supplementary Figure 6E) and in hepatoma cell lines (Supplementary Figure 6F), consistent with a previous study in Huh7 cells.¹¹ IFN- α induced the greatest level of gene induction after 6 hours of treatment followed by a subsequent decline. In contrast, IL28B treatment resulted in a gradual increase in mRNA levels between 12 and 24 hours. Although IL28B showed a more prolonged induction of ISGs such as RSAD2 and IFI44 by qPCR (Supplementary Figure 6E) and microarray analysis (Figure 7A), IFN- α displayed more potent antiviral activity than IL28B (Supplementary Figure 6A–D). Despite the rapid decline of ISG levels, the antiviral activity of IFN- α was maintained for 72 hours following treatment (Supplementary Figure 6B). To study this further, additional analyses were performed on the microarray data from PHHs treated with these cytokines and Figure 7B shows that although many genes show similar changes in expression levels after treatment with either of these cytokines, many genes were also differentially regulated. Using pathway analysis, we identified various biological processes that were overrepresented by genes whose expression levels were significantly affected following either IL28B or IFN- α treatment (Figure 7C). Supplementary Figure 7 displays the number of genes up-regulated following IFN- α treatment with comparison to genes induced by both IL28B and HCV. A model is presented in Figure 7D summarizing the results of these experiments.

Discussion

In patients and chimpanzees infected with HCV, a robust antiviral response can be detected in the liver. A published study had previously investigated and characterized HCVcc infection of PHHs.²⁶ However, to our knowledge, this study represents the first extensive characterization of the intrinsic innate immune response in adult PHHs following in vitro infection by HCVcc. We reproducibly show the production of IL-28 protein at the nanogram level in response to HCV nucleic acid PAMP and infectious HCVcc, and the produced IL-28 is biologically functional. In contrast, we have been unable to detect robust production of IFN- α and IFN- β proteins from the infected PHHs. These results are unexpected given that the induction of the type III IFNs depends on similar signaling molecules as the type I IFNs.²¹ Several previous studies have described minimal type I IFN mRNA induction in cell culture and primate models of HCV infection.^{6,8,23} However, these studies did not specifically examine the up-regulation of type III interferons following acute HCV infection. Based on this study, we hypothesize that hepatocytes have an intrinsically enhanced ability to produce type III IFNs in response to viral stimuli in comparison to that seen with type I IFNs. Consequently, the type III IFN system is functionally the predominant antiviral pathway induced in hepatocytes.

We also explored changes in the transcriptome following infection with HCVcc in PHHs. When compared with cells treated with IL28B, it is apparent that in addition to stimulating ISGs, HCVcc up-regulates and down-regulates the expression levels of many more genes. The gene list presented in Figure 5E shows up-regulation of the type III IFNs in response to HCVcc and places them among the most stimulated genes that are not themselves strong ISGs, underscoring their importance in the earliest antiviral response. Pathway analysis of the microarray data was also informative as additional insight was gained into the effects of both HCVcc and IL28B on the transcriptome. In HCVcc-infected cells, one of the novel findings is that genes involved in chemotaxis were highly induced second only to the type III IFN response. Thus, in response to HCV, the hepatocytes trigger the IFN response to mitigate virus replication, while chemokine genes such as IP-10 are up-regulated to recruit immune cells from the surrounding liver parenchyma and circulation.

In this study, we addressed the functional role of the type III IFNs by determining if their production was responsible for the ISG induction following HCV infection as observed in HCV-infected chimpanzee and human liver biopsy specimens. We showed that the type III IFNs are a more likely candidate than the type I IFNs. Our data show that type III IFNs, produced by hepatocytes, may be the main driver of ISG induction following HCV infection. However, we cannot rule out a role that infiltrating peripheral blood mononuclear cells may play in producing IFNs in chronically HCV infected liver. Additional studies will have to be performed to determine if IFNs produced by peripheral blood mononuclear cells or other cell types may contribute to ISG induction in vivo. It is also interesting that the type III IFNs, in addition to inducing well-known ISGs, activate a rather distinct set of genes in PHHs from the type I IFNs, raising an intriguing possibility that the signaling pathways beyond receptor engagement may not be identical. Others have studied gene expression changes following treatment with type I and III IFNs in Huh7.5 cells and reported a more prolonged ISG induction by type III IFN.¹¹ We observed similar results in PHHs. These data also support the involvement of type III rather than type I IFN in the induction of ISGs in HCV-infected liver because of the ability of type III IFNs to induce a prolonged upregulation of ISG mRNA as compared with type I IFN.

It is interesting to note that certain genes, like SOCS1, that function to inhibit JAK-STAT activation by type I IFN²⁷ are highly and persistently induced by type III but not type I IFNs. These differences could explain why a high expression of ISGs may contribute to

nonresponse to treatment with IFN- α . To examine this hypothesis in HCV-infected PHHs, we tested the effect of blocking type III IFN on the anti-HCV action of type I IFN. We indeed observed a significant increase in the IFN- α antiviral activity in the presence of blocking antibodies to type III IFN. This observation raises the intriguing possibility that nonresponders may be sensitized to the antiviral action of type I IFN through concomitant treatment with type III IFN blocking antibodies.

In conclusion, our data show robust hepatic type III IFN and other cytokine responses in several models of HCV infection. The antiviral action of type III IFN, however, may be insufficient to result in HCV clearance. The continued induction of type III IFN, with its distinct functional effects from that of type I IFN as discussed previously, may render hepatocytes relatively resistant to further type I IFN action, potentially explaining the paradoxical association of high ISG levels (marker of type III IFN production) and nonresponse to IFN- α treatment. A proposed model of the IFN response and treatment effects in HCV infection is shown in Figure 7D. In HCV-infected humans, cytokine responses to virus are likely determined by numerous factors including those of the host, like the IL28B genotype, and those encoded by the virus (eg, viral genotype). The interplay between the virus and host and the subsequent clinical manifestations seen following acute and chronic infection may ultimately be determined by the initial host response. Although not directly addressed in our studies, we believe that any functional role of the polymorphisms in the IL28B gene locus, in HCV responses, may be identified using these systems. More extensive analyses in a large collection of PHHs with well-defined IL-28 genotypes utilizing multiple HCV genotype strains for infection will need to be undertaken. Further characterization of cytokine responses to HCV using appropriate models will add crucial insight into the pathogenesis of HCV infection and possibly provide innovative targets for therapeutic development against hepatitis C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

ELISA	enzyme-linked immunosorbent assay
HCVcc	hepatitis C virus cell culture
IFN	interferon
IL	interleukin
ISG	interferon-stimulated gene

MOI	multiplicity of infection
PAMP	pathogen-associated molecular pattern
PHH	primary human hepatocyte
qPCR	quantitative reverse-transcription polymerase chain reaction
siRNA	small interfering RNA

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Figure 1.

Induction of IL-28 and ISGs in liver biopsy specimens of HCV-infected chimpanzees and humans. (A) qPCR analysis of IL-28, IFN- α 1, IFN- β , IFN- γ , ISG15, and IFIT1 expression levels in chimpanzees before and after infection. The postinfection biopsies were performed shortly after infection (<4 weeks; see Materials and Methods for specific information on each animal). Correlation of (B) ISG15 and (C) IFIT1 mRNA levels with IL-28, IFN- α 1, IFN- β , and IFN- γ expression, as determined by qPCR, in livers of patients ($n = 19$) with chronic hepatitis C genotype 1 infection. Dashed lines represent log-log regression. P value represents significance of the Spearman correlation coefficient (ρ). r^2 = goodness of fit of the regression line.

Figure 2.

Preferential induction of IL-28 by viral mimetics in hepatocytes is mediated by the IRF3 and nuclear factor κ B signaling pathways. (A) Analysis of IL-28, IFN- β , IP10, and RSAD2 mRNA levels by qPCR and protein production of IL-28, IFN- β , and IP10 by ELISA in HepG2 cells following transfection with poly(I:C) (6 μ g/mL for 24 hours). qPCR analysis of IL-28 mRNA levels in HepG2 cells first treated with siRNAs for 3 days targeting (B) IPS-1, (C) IRF3, (D) NEMO, and (E) TRIF and then transfected with or without poly(I:C) (6 μ g/mL for 24 hours). Quantification of siRNA-mediated specific gene suppression is shown in the *right panel*. Results are representative of more than three experiments. Nontargeting siRNA represents control. N.D., not detected.

Figure 3.

Robust induction of IL-28 and ISGs by viral mimetics and following HCV infection of PHHs. (A) Analysis of IL-28, IFN- β , and IP10 mRNA levels by qPCR and ELISA in PHHs following transfection with poly(I:C) (6 μ g/mL for 24 hours) or HCV PAMP RNA (6 μ g/mL for 24 hours). PHHs infected with JFH1 virus (MOI of 5) for 12, 24, and 36 hours or treated with IFN- α (100 U/mL) for 12 hours. (B) Analysis of IL-28 and IP10 mRNA levels by qPCR and protein production by ELISA. qPCR analysis of (C) IFN- β and IFN- α 1 as well as (D) IFIT1 and ISG15 mRNA expression. (E) Western blot analysis of ISG15 and β -actin on PHHs infected with JFH1 virus (MOI of 5) for 24 and 48 hours. (F) qPCR analysis of IL-28 mRNA levels in PHHs first treated with siRNAs for 3 days targeting IRF3 and NEMO and then infected with or without JFH1 (MOI of 5) for 36 hours. Quantification of siRNA-mediated specific gene suppression is shown in the *right panels*. Results are representative of more than three experiments. Nontargeting siRNA represents control. N.D., not detected.

Figure 4.

Blocking of IL-28 induction following HCV infection in PHHs by anti-E2 and CD81 antibodies and antiviral molecules. (A) Quantification of IL-28 and IP10 protein production by ELISA in PHHs with or without pretreatment with anti-E2 antibodies (10 μ g/mL) for 1 hour and subsequently infected with JFH1 (MOI of 5) for 52 hours or following transfection with poly(I:C) (6 μ g/mL for 24 hours). (B) qPCR analysis of intracellular HCV RNA 52 hours after infection with JFH1 (MOI of 5) in PHHs alone or pretreated with anti-E2 antibodies (10 μ g/mL) for 1 hour or treated with IFN- α (100 U/mL) for 48 hours. (C) Quantitation of IL-28 and IP10 protein production by ELISA in PHHs with or without pretreatment with anti-CD81 antibodies (10 μ g/mL) for 1 hour and subsequently infected with JFH1 (MOI of 5) for 24 and 48 hours. (D) Quantitation of IL-28 protein production by ELISA in PHHs transfected with HCV-PAMP RNA (6 μ g/mL for 24 hours) or poly(I:C) (6 μ g/mL for 24 hours) or infected with JFH1 (MOI of 5) for 52 hours alone or treated with IFN- α (100 U/mL) or 2-methylcytidine (20 μ mol/L) for 48 hours. (E) qPCR analysis of intracellular HCV RNA 52 hours after infection with JFH1 (MOI of 5) in PHH alone or pretreated with CD81 antibodies (10 μ g/mL) for 1 hour or treated with 2-methylcytidine (20 μ mol/L) or IFN- α (100 U/mL) for 48 hours. Numbers above bars represent HCV RNA copies $\times 10^6$. Results are representative of two experiments. N.D., not detected.

Figure 5. Changes in gene expression following HCV infection are mediated by IFN-dependent and independent pathways. Microarray analysis of PHHs treated with IL28B (25 ng/mL for 24 hours) and JFH1 (MOI of 5 for 36 hours). (A) Venn diagrams displaying the number of probe sets up-regulated and down-regulated (>2.0-fold change) following treatment. (B) Heat maps generated using microarray data from 3 replicates. (C) GeneGo networks that are significantly up-regulated or down-regulated by IL28B and JFH1. (D) Top 10 genes ranked by fold induction following treatment with IL28B and JFH1. (E) Top 10 genes ranked by fold induction following JFH1 infection whose expression levels were not significantly up-regulated by IL28B. (F) Fold induction of genes up-regulated by JFH1 that are also induced by poly(I:C) (6 μg/mL) but minimally induced by IL28B (25 ng/mL) or IFN-α (10U/mL), by microarray analysis, for the indicated durations of treatment. Data from 3 replicates.

Figure 6.

Autocrine and paracrine IL-28 production contributes to ISG induction following poly(I:C) treatment and HCV infection. (A) qPCR analysis of IL-28, IFN- α 1, IFN- β , RSAD2, IFIT1, and ISG15 mRNAs in HepG2 cells following transfection of poly(I:C) (6 μ g/mL) for 0.75, 1.5, 2, 3, and 12 hours. In the following experiments, antibodies against IL28A (15 μ g/mL) and IL28RA (20 μ g/mL) or equal concentrations of immunoglobulin G were used. (B) qPCR analysis of RSAD2 mRNA level in PHHs treated with IFN- α (100 U/mL) for 12 hours with or without pretreatment with antibodies. (C) qPCR analysis of RSAD2 mRNA levels in PHHs treated with IL28B (25 ng/mL) for 24 hours with or without pretreatment with antibodies. (D) PHHs were plated on bottom wells and infected with JFH1 virus (MOI of 5). The virus was then removed after 4 hours and PHHs plated on Transwell membranes were then placed into the wells for coculturing. The cocultures were treated with antibodies. qPCR analysis of IL-28 mRNA levels of cells in the bottom wells (*left*) and Transwells (*right*) was determined 48 hours later. (E) qPCR analysis of RSAD2, ISG15, and IFIT1 expression levels in PHHs, plated on Transwell membranes (*top panels*), exposed to media from cells infected with JFH1 (MOI of 5, *bottom panels*), and treated with antibodies. (F) qPCR analysis of intracellular HCV RNA levels 52 hours after infection with JFH1 (MOI of 5) in PHH alone or treated with IFN- α (100 U/mL) and antibodies for 48 hours. *Numbers above bars* represent HCV RNA copies $\times 10^6$. Results are representative of 2 experiments.

Figure 7. Differential effects of type III IFN and IFN- α on gene expression and model of HCV infection and subsequent innate immune responses in hepatocytes. (A) Top 42 probe sets, ranked by fold induction, following treatment with IL28B (25 ng/mL) and IFN- α (10 U/mL) for the indicated duration. Data from 3 replicates. (B) Venn diagrams displaying the numbers of probe sets up-regulated (>2.0 -fold change) following the indicated treatment time points. For the 6h + 24h Venn diagrams, probe sets up-regulated (>2.0 -fold change) at either 6- and 24-hour time points were combined for each treatment and then compared. (C) GeneGo networks that are significantly up-regulated by IL28B and/or IFN- α using the 6h + 24h probe set lists. (D) This proposed model highlights the role of type III IFNs in intrinsic antiviral innate immunity to HCV in the liver. (1) HCV infects hepatocytes, resulting in the detection of viral RNA by the RIG-I-like helicases and/or Toll-like receptors and leading to activation of IRF3 and nuclear factor (NF) κ B. They translocate to the nucleus binding to the promoters of virus-stimulated genes (VSGs), resulting in the production of type III IFNs. (2) Type III IFNs are secreted and bind to its cognate receptor, leading to the up-regulation of ISGs. (3) In a subset of chronically infected patients, type III IFNs continually induce genes that block IFN- α from further up-regulating ISGs, leading to nonresponse to exogenous IFN. (4) Blocking type III IFN up-regulation of genes may render nonresponders sensitive to the antiviral activity of exogenous type I IFN by allowing it to up-regulate antiviral ISGs.