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Independent, Parallel Pathways to CXCL10 Induction in HCV-Infected Hepatocytes

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Abstract

Background & Aims—The pro-inflammatory chemokine CXCL10 is induced by HCV (hepatitis C virus) infection *in vitro* and *in vivo*, and is associated with outcome of IFN (interferon)-based therapy. We studied how hepatocyte sensing of early HCV infection via TLR3 (Toll-like receptor 3) and RIG-I (retinoic acid inducible gene I) led to expression of CXCL10.

Methods—CXCL10, type I IFN, and type III IFN mRNAs and proteins were measured in PHH (primary human hepatocytes) and hepatocyte lines harboring functional or non-functional TLR3 and RIG-I pathways following HCV infection or exposure to receptor-specific stimuli.

Results—Huh7 human hepatoma cells expressing both TLR3 and RIG-I produced maximal CXCL10 during early HCV infection. Neutralization of type I and type III IFNs had no impact on virus-induced CXCL10 expression in TLR3+/RIG-I+ Huh7 cells, but reduced CXCL10 expression in PHH. PHH cultures were positive for monocyte, macrophage, and dendritic cell mRNAs. Immunodepletion of non-parenchymal cells (NPCs) eliminated marker expression in PHH cultures, which then showed no IFN requirement for CXCL10 induction during HCV infection. Immunofluorescence studies also revealed a positive correlation between intracellular HCV Core and CXCL10 protein expression ($r^2=0.88$, $p<0.001$).

Conclusions—While CXCL10 induction in hepatocytes during the initial phase of HCV infection is independent of hepatocyte-derived type I and type III IFNs, NPC-derived IFNs contribute to CXCL10 induction during HCV infection in PHH cultures.

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Keywords

HCV; TLR3; RIG-I; type I interferon; type III interferon; non-parenchymal cells

INTRODUCTION

Chronic hepatitis C is characterized by hepatic infiltration of pro-inflammatory immune cells [1–3]. Damage to neighboring tissue from this persistent yet ineffective inflammatory response can lead to progressive liver disease over multiple decades [4,5]. The causative agent, HCV (hepatitis C virus), is a positive sense, single-stranded RNA virus that primarily and, in the majority of cases, persistently infects hepatocytes [6]. However, the underlying biological mechanisms of how persistent infection and chronic hepatic inflammation are established remain unclear.

Intrahepatic levels of CXC chemokines lacking the N-terminal Glu-Leu-Arg (ELR) motif (CXCL9, CXCL10, and CXCL11) are elevated in chronic hepatitis C patients and in experimentally infected chimpanzees [1,7]. Additionally, serum and intrahepatic CXCL10 (i.e. IFN (Interferon)-gamma-induced protein 10 [IP-10]) correlates negatively with the outcome of pegylated-IFN /Ribavirin therapy and positively with increased HCV RNA in the plasma of acutely infected HCV patients [8–10]. Intrahepatic production of CXCL10 and other non-ELR chemokines recruits a pro-inflammatory, anti-viral immune response to the liver by activating the chemokine receptor CXCR3 on CD4⁺ T_H1, CD8⁺ T_C, and NK (natural killer) cells [2,3]. These observations suggest that non-ELR CXC chemokines, and specifically CXCL10, help coordinate the persistent hepatic inflammatory response characteristic of chronic hepatitis C.

Induction of CXCL10 and other chemokines in hepatocytes occurs through recognition of conserved PAMPs (pathogen associated molecular patterns) by innate PRRs (pattern recognition receptors) such as TLR3 (Toll-like receptor 3) and RIG-I (retinoic acid inducible gene I). Both TLR3 and RIG-I sense HCV infection [11–14]. RIG-I is a cytoplasmic sensor of double-stranded, 5' tri-phosphate RNAs [15]. Upon PAMP recognition, RIG-I changes conformation and binds the adaptor MAVS (mitochondrial antiviral-signaling protein). TLR3 is found in endosomes and recognizes double-stranded RNAs generated during viral replication [14]. Activated TLR3 binds the adaptor TRIF (TIR-domain-containing adapter-inducing IFN- γ) through its cytoplasmic receptor domain [16,17]. Signaling from MAVS or TRIF activates various transcription factors including IRF-3 (IFN regulatory factor 3), IRF-7, NF- κ B (nuclear factor- κ B) and AP-1 (activator protein 1) [18]. These in turn induce pro-inflammatory cytokines and chemokines as well as type I and type III IFNs [18,19].

IFNs amplify chemokine production through autocrine and paracrine activation of anti-viral and pro-inflammatory pathways. Binding of type I IFNs (IFN α and IFN β) to the IFNAR1/IFNAR2 receptor activates Janus kinases and various STAT (signal transducer and activator of transcription) proteins [20]. These in turn induce ISGs (IFN-stimulated genes) by binding to ISREs (IFN-stimulated response elements) in their promoters [20,21]. Most cells, including hepatocytes, produce type I IFNs as part of the general anti-viral response [20]. HCV infection of hepatocytes also induces type III IFNs (IL-28A, IL-28B, IL-29), which activate STAT-signaling by binding to the IL10R2/IL-28R receptor [20,22,23]. Thus, PRR-activated genes whose promoters contain putative ISREs (including CXCL10) may also respond to hepatocyte-derived IFNs during initial HCV infection [22,24].

Hepatocytes are a major source of CXCL10 during HCV infection both *in vivo* and *in vitro* [1,14,22,25], and others have shown CXCL10 induction following treatment with IFNs or

various PAMPs [22,26]. However, the combined contribution of PRR stimulation and IFN signaling to CXCL10 induction during the initial stages of HCV infection of hepatocytes has not yet been examined, even though deregulation of these pathways may contribute to the establishment of persistent hepatic infection and inflammation. Therefore, we characterized the contribution of type I IFN, type III IFN, and PRR signaling via TLR3 and RIG-I to CXCL10 induction during acute HCV infection of primary and immortalized hepatocytes. We show that CXCL10 is induced primarily through an IFN-independent pathway following PRR signaling in the HCV-infected hepatocyte *in vitro*, that both TLR3 and RIG-I are required for maximal induction, and that type I and type III IFNs produced by NPCs (non-parenchymal cells) amplify CXCL10 induction in PHH (primary human hepatocyte) preparations.

MATERIALS AND METHODS

Detailed protocols, reagents, and statistics are included in Supplemental Methods.

Cells, Hepatitis C Virus, and PAMPs

Cells, viruses, and PAMPs are described in Supplemental Methods.

Quantitative Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

Quantitative RT-PCR was performed on cDNA derived from cellular mRNA for detection of HCV, CXCL10, IFN- α , IFN- γ , IL-28B, and IL-29. Chemokine and cytokine data are reported as fold change derived from C_t using GAPDH as an endogenous control [27]. Microfluidic high-throughput quantitative RT-PCR was performed using the Fluidigm BioMark HD system (Fluidigm Corporation, South San Francisco, CA). Targets for Fluidigm PCR are listed in Supplemental Table 1.

Luminex Bead Arrays

Samples were tested for CXCL10 using polystyrene Antibody Bead kits (Biosource/Invitrogen) and the Luminex 200 system according to the manufacturer's protocol (Luminex, Austin, TX).

Western Blotting

Cellular protein lysates were run on SDS-PAGE gels and transferred to nitrocellulose membranes for chemiluminescent protein detection using LumiGLO (Cell Signaling Technology, Beverly, MA) according to the manufacturer's protocol.

Type I and Type III IFN Neutralization Assays

Infections were performed in the presence of 2 μ g/ml B18R protein (eBioscience, San Diego, CA) for type I IFN neutralization, or 4 μ g/ml IL-28B/IL-29 neutralizing antibody (R&D Systems; MAB15981) for type III IFN neutralization.

Negative Selection of Primary Hepatocytes

Primary hepatocytes were incubated with biotin-conjugated antibodies against CD45 (R&D Systems; BAM1430), CD68 (i.e. SR-D1; R&D Systems; BAF2040), and CD31 (i.e. PECAM-1; R&D Systems; BAM3567) and MACS anti-biotin-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA) before being applied to a magnetic MACS Cell Separation column (Miltenyi Biotec). Non-adherent cells were collected and plated following the standard culture protocol. Adherent and non-adherent cells were analyzed by microfluidic quantitative RT-PCR.

Immunofluorescence

Cells were cultured on chamber slides (Nunc/ThermoScientific) and infected with HCV (MOI 0.5) as described above for 72 hours or treated with 100 ng/ml IFN γ and 40 ng/ml Tumor Necrosis Factor- α (TNF α) for 24 hours [1]. Brefeldin A (1 μ g/ml; VWR International, Radnor, PA) was added during the last 5 hours of treatment. Cells were fixed, stained for CXCL10 and HCV Core proteins, and analyzed by deconvolution microscopy (see Supplemental Methods).

RESULTS

Maximal CXCL10 induction during early HCV infection requires both TLR3 and RIG-I

After confirming previous reports [22,26] that CXCL10 is induced by TLR3 and RIG-I-specific stimulation (Supplemental Figure 1), we utilized four Huh7-derived hepatoma cell lines that differentially expressed each PRR to study infection (see Supplemental Methods, Supplemental Figure 2A,B). These PRRs were functional (Supplemental Figure 2C and [13]). Differential PRR expression affected permissivity of the cell lines to HCV infection, with TLR3-/RIG-I- cells being the most permissive and TLR3+/RIG-I+ cells being the least permissive (Figure 1A).

During asynchronous, low MOI infection, TLR3+/RIG-I+ cells had the largest induction of CXCL10 at 72 hours after normalization to HCV RNA copy number (Figure 1B). Data were normalized in order to account for variability in cell permissivity to viral replication and thus PAMP exposure. To validate our findings in the absence of normalization, synchronous, high MOI infections were conducted. CXCL10 induction was evaluated at 12 hours post-infection when intracellular HCV RNA was essentially equivalent among the four cell lines. With this approach, TLR3+/RIG-I+ cells again produced the largest CXCL10 mRNA induction (Figure 1C). The data indicate that both TLR3 and RIG-I signaling are required for maximal CXCL10 induction during early HCV infection in hepatocytes.

Neutralization of type I or III IFNs does not affect CXCL10 induction during early HCV infection of TLR3+/RIG-I+ Huh7 cells

We also observed low-level IFN γ and IFN α induction during HCV infection of TLR3+/RIG-I+ Huh7 cells (Supplemental Figure 3). Since CXCL10 is a known ISG, and induction was observed in TLR3+/RIG-I+ Huh7 cells treated for 24 hours with IFN γ , IFN α , IL-28B, or IL-29 (Supplemental Figure 4), early paracrine IFN signaling might amplify the CXCL10 response. We therefore neutralized residual IFNs produced during HCV infection of TLR3+/RIG-I+ Huh7 cells and evaluated the effect on CXCL10 induction.

Neutralization of IFN γ and IFN α was achieved by adding recombinant *Vaccinia* virus B18R protein (a soluble type I IFN receptor [28]) to the culture medium following virus adsorption. This treatment did not impact CXCL10 mRNA or protein production at 24 or 48 hours post-infection, but completely abrogated CXCL10 induction by recombinant IFN γ (Figure 2A). Expression of the ISG IFIT1 (IFN-induced protein with tetratricopeptide repeats 1) was also induced by IFN γ (Figure 2B), and eliminated by B18R co-treatment, further confirming neutralization.

Treatment with a pan type III IFN neutralizing antibody (IFN λ) also did not affect CXCL10 production during HCV infection, but did reduce induction following treatment with recombinant IL-28B (Figure 2C). Neutralization also reduced IFIT1 expression following recombinant IL-28B or IL-29 treatment (Figure 2D, Supplemental Figure 5). Simultaneous neutralization of type I and type III IFNs also had no effect on CXCL10 production during virus infection while completely abrogating IFIT1 induction by combined

treatment with IFN α and IL-28B (Supplemental Figure 6). Taken together, these results indicate that neither type I nor type III IFNs produced by TLR3+/RIG-I+ Huh7 cells are necessary for CXCL10 induction during early HCV infection.

HCV infection and paracrine cytokine treatment produce distinct intracellular CXCL10 staining patterns

To confirm that hepatocyte-derived IFNs are dispensable for HCV-mediated CXCL10 induction in these cells, we used immunofluorescence to compare the pattern of CXCL10 induction during infection to the pattern of induction by a known paracrine stimulus: a combination of IFN α and TNF α [1]. As expected for a paracrine stimulus, all cells exposed to IFN α /TNF α were positive for CXCL10 protein (Figure 3A, left). In contrast, infected cells (HCV Core positive) showed much stronger CXCL10 staining than non-infected cells (HCV Core negative; Figure 3A, right). Quantitative analysis of CXCL10 and HCV Core staining was also conducted on a per-cell basis within the HCV-exposed population (n=2145, see Supplemental Methods). HCV Core-positive cells had a significantly higher mean CXCL10 signal than Core-negative cells ($p < 0.001$, Figure 3B). We also observed a direct, positive correlation between the HCV Core and CXCL10 signal intensities ($r^2 = 0.88$, $p < 0.001$, Figure 3C), confirming that the intracellular CXCL10 expression pattern in hepatocyte monoculture is virus-dependent and is distinct from a paracrine IFN stimulus.

Neutralization of type I and type III IFNs reduces CXCL10 induction in PHH cultures

The innate immune response of immortalized hepatoma cells differs from that of the liver parenchyma *in vivo* [23,29]. Indeed, while type III IFN production was undetectable in HCV-infected TLR3+/RIG-I+ Huh7 cells (Supplemental Figure 3), PHH produced both type I and type III IFNs during HCV infection and following TLR3-specific stimulation (Supplemental Figure 7 and [22]). Thus, we examined the IFN requirements for CXCL10 induction during acute HCV infection of PHH cultures.

In contrast to the Huh7 system, neutralization of type I IFNs in PHH culture resulted in 92% and 89% reduction in CXCL10 mRNA and protein production respectively at 24 hours post-HCV infection (MOI 0.2; Figure 4A). CXCL10 protein levels rebounded during type I IFN neutralization at 48 hours post-infection (Figure 4A, right panel). This rebound was not observed in other PHH preparations (See Figure 4E below). Neutralization of type III IFNs in the same PHH culture had no effect on HCV induction of CXCL10 at either 24 or 48 hours (Figure 4B). However, type III IFNs did contribute to CXCL10 induction in other PHH preparations (see Figure 4E below). These data suggest that, despite donor-to-donor variation, both type I and type III IFNs are involved in CXCL10 induction in PHH cultures during early HCV infection.

Residual NPCs in PHH cultures produce type I and type III IFNs that contribute to virus-induced CXCL10 induction

The involvement of type I and type III IFNs in CXCL10 induction during early HCV infection of PHH cultures directly contrasted our results in Huh7 cells, where these IFNs were dispensable for CXCL10 induction. Since NPCs, including KCs (Kupffer cells), LSECs (liver sinusoidal endothelial cells), and hepatic stellate cells, are a known source of type I IFNs and other cytokines in the liver [30], we hypothesized that contaminating NPCs produced IFNs that amplified CXCL10 induction.

To assess whether NPCs were present in our PHH cultures, we utilized a panel of 46 chemokine, cytokine, and immune cell lineage markers on a microfluidic quantitative RT-PCR platform (Supplemental Table 1). Eight PHH cultures showed strong baseline expression of cytokines, chemokines (including CXCL10), and immune cell lineage markers

such as CD14, CD209, CD86, EMR1, and MARCO. Expression intensity varied between cultures, suggesting that the level of NPC contamination is different between PHH preparations (Supplemental Figure 8). Samples from TLR3+/RIG+ Huh7 cells were included for comparison, and showed low to non-detectable expression of most markers.

Contaminating NPCs were immunodepleted from PHH cultures using a mixture of streptavidin-conjugated magnetic beads and biotin-conjugated antibodies against pan-CD45 (leukocytes), CD68 (monocytes/macrophages [including KCs]), and CD31 (LSECs) [31–34]. Microfluidic quantitative RT-PCR analysis indicated that following HCV infection, non-depleted PHH cultures (“Normal”) displayed strong induction of markers for dendritic cells (CD209), macrophages (CXCL13), and KCs (CD86), as well as cytokines (IFN- and IL10; Figure 4C). In striking contrast, NPC-depleted PHH cultures (“Depleted”) failed to express these immune cell markers or cytokines following HCV infection. However, both Normal and Depleted cultures showed strong viral induction of CXCL10. Additionally, cells that bound to the magnetic column (“Bound Cells”) expressed several markers characteristic of the monocyte/macrophage lineages (Figure 4D). Bound Cells also showed expression of type I IFNs, suggesting that contaminating NPCs do produce these cytokines in PHH cultures.

The NPC-depleted and non-depleted PHH cultures were then used in IFN neutralization experiments (Figure 4E). As expected for non-depleted (“Normal”) PHH cultures, neutralization of type I IFN reduced CXCL10 mRNA to undetectable levels and reduced CXCL10 protein by 73% during HCV infection. Neutralization of type III IFN in the same culture also reduced induction of CXCL10 mRNA and protein by 42% and 53% respectively. In contrast, HCV-induction of CXCL10 mRNA and protein in Depleted PHH was relatively unaffected by neutralization of either IFN. The data indicate that residual NPCs in PHH preparations produce type I and type III IFNs that amplify CXCL10 induction in HCV-infected hepatocytes. Moreover, NPC removal does not eliminate the ability of PHH to produce CXCL10 during early HCV infection. Thus, in both TLR3+/RIG-I+ Huh7 cells and NPC-depleted PHH, CXCL10 induction during HCV infection is independent of hepatocyte-derived IFNs.

DISCUSSION

Hepatocytes express both TLR3 and RIG-I and produce both type I and type III IFNs *in vivo* [20,22,26]. However, the combined contribution of these innate immune components to induction of the CXCL10-orchestrated inflammatory response during acute HCV infection of hepatocytes has not been previously evaluated. Here we show for the first time that both TLR3 and RIG-I signaling are required for maximal induction of CXCL10 during *in vitro* HCV infection of hepatocytes, and that IFN neutralization does not affect CXCL10 production during HCV infection of Huh7 cells expressing functional TLR3 and RIG-I. A direct, positive correlation between intracellular CXCL10 and viral protein expression was also observed. However, neutralization of type I and, to a lesser extent, type III IFN reduced CXCL10 production during acute HCV infection of PHH cultures. This IFN requirement was abrogated following depletion of NPCs from PHH cultures, consistent with the IFN-independent induction of CXCL10 in Huh7 monoculture. Thus, our study reveals that CXCL10 induction in hepatocytes during the early stages of HCV infection occurs through direct signaling following PRR activation rather than through secondary paracrine signaling of hepatocyte-derived IFNs. This suggests that CXCL10 does not behave as a classical IFN-induced ISG during early HCV infection despite the presence of ISREs in its promoter.

Many studies have shown that IFN-signaling to ISG induction occurs within the liver during acute and chronic HCV infection [35]. Indeed, patients with robust pre-treatment hepatic

ISG expression are less likely to respond to standard IFN-based therapy [36], and PHH generate type I and type III IFN responses following PRR stimulation and during HCV infection *in vitro* (See Supplemental Figure 7 and [22,23,37]). Robust induction of IL-29 mRNA was also observed in serial liver biopsies from chimpanzees with acute HCV infection [37]. However, neutralization of these responses in TLR3+/RIG-I+ Huh7 cells and NPC-depleted PHH cultures failed to impact CXCL10 production during HCV infection (Figures 2 and 4). This suggests that hepatocyte-derived type I and type III IFNs do not play a significant role in CXCL10 production during the initial hepatocyte response to HCV infection, although they may induce expression of other ISGs.

Our data instead suggest that CXCL10 induction in hepatocytes during early HCV infection occurs through direct transcriptional activation of the CXCL10 promoter following TLR3 and RIG-I engagement. The CXCL10 promoter is known to be directly activated by IRFs in non-hepatic cell types following polyI:C exposure or virus infection [38,39]. IRF3 specifically can also induce several other ISGs in response to viral infections [39,40]. This binding can occur independently of type I IFN [39,41], supporting the novel observations reported here regarding HCV induction of CXCL10 in hepatocytes. CXCL10 and other pro-inflammatory factors are also induced by direct NF- κ B activation during HCV infection in Huh7-derived cells [14,42], and binding sites for the pro-inflammatory transcription factors AP-1 and C/EBP β are annotated in the CXCL10 promoter [24,43,44]. Since we observed a linear correlation between HCV Core and intracellular CXCL10 expression (Figure 3), the overall intensity of CXCL10 induction may depend on additive or synergistic binding of these transcription factors.

Transcription factor binding may also depend on which PRRs are actively signaling. As observed in Figure 1B, cells expressing either TLR3 or RIG-I alone exhibit a smaller CXCL10 induction during HCV infection. Figure 1B also shows that TLR3+/RIG-I– Huh7 cells had greater CXCL10 induction during infection than TLR3–/RIG-I+ cells. This suggests that TLR3 activates more potent transcription factors for CXCL10 induction. Indeed, induction of the NF- κ B-dependent inflammatory cytokines TNF- and G-CSF in PHH cultures was more pronounced following stimulation by extracellular polyI:C (a TLR3 PAMP) than by Sendai virus (a RIG-I PAMP) [14]. However, the overexpression of TLR3 in TLR3+/RIG-I– Huh7 cells may also inflate the level of CXCL10 induction above that observed for the endogenously expressed RIG-I [6,12,13]. In either case, CXCL10 induction during early HCV infection may reflect direct co-regulation by anti-viral (IRF3/IRF7) and pro-inflammatory (AP-1/NF- κ B) transcription factors activated by these two PRRs [43]. We are currently evaluating which transcription factors drive HCV-induced CXCL10 transcription in hepatocytes.

While IFNs appear to be dispensable for the initial wave of CXCL10 induction during *in vitro* HCV infection, type I, II, and III IFNs secreted by NPCs as well as by infiltrating immune cells do contribute to CXCL10 induction in hepatocytes during acute and chronic HCV infection *in vivo*. Recombinant type I or type III IFNs moderately induced CXCL10 expression in TLR3+/RIG-I+ Huh7 cells (Supplemental Figure 4), and pegylated-IFN triggers robust intrahepatic ISG expression in patients responding anti-HCV therapy [36]. Indeed, neutralization of type I and type III IFNs during HCV infection in standard PHH cultures substantially reduced CXCL10 production (Figure 4). However, the minimal effect of IFN neutralization during HCV infection in Depleted PHH (Figure 4E) suggests that an IFN-independent, direct signaling pathway is active in hepatocytes and is crucial for intrinsic induction of CXCL10 and potentially other pro-inflammatory genes during early HCV infection. Removal of anti-inflammatory cytokines such as IL-10 by NPC removal (Figure 4C) may also contribute to CXCL10 induction in Depleted PHH cultures. Since hepatocytes are the predominant cell type infected by HCV [45], direct, intrinsic induction

of CXCL10 may be crucial for maintaining the chemokine gradient responsible for recruiting NK cells, CD8+ T_c cells, CD4+ T_H1 cells, and resident NPCs to the site of infection within the liver during acute HCV infection *in vivo* [2,3]. Type II IFN, a potent inducer of CXCL10 in many cells types, is primarily produced by these infiltrating cells and would trigger a secondary wave of CXCL10 induction both intrahepatically and in the periphery [1,46,47]. This may explain why CXCL10 is only first detectable 3–11 weeks after HCV RNA in the plasma of acutely infected HCV patients [10].

Our results thus lead to a revised model of CXCL10 induction during acute HCV infection where initial expression occurs in hepatocytes through direct activation of the CXCL10 promoter by transcription factors activated downstream of PRR signaling. This primary wave of CXCL10 recruits immune effector cells and hepatic NPCs to the site of infection. Secretion of type I, II, and III IFNs by these cells then amplifies the pre-established CXCL10 response during the later stages of acute HCV infection, in addition to directing the development of a pro-inflammatory, anti-viral state within the liver. This IFN-independent (i.e. direct) induction of CXCL10 thus initiates the cycle of inflammation that can lead to progressive liver disease. Indeed, higher levels of intrahepatic CXCL10 have been found in chronic hepatitis C patients with necroinflammation and fibrosis [7]. However, an antagonistic form of CXCL10 that may inhibit migration has also been detected in the plasma of chronic hepatitis C patients [48]. Further research into the relationship between peripheral CXCL10, intrahepatic CXCL10, and hepatic inflammation may be necessary before this pathway can be targeted for development of host-oriented treatments for HCV-related liver disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HCV	Hepatitis C Virus
IFN	Interferon
NK	Natural Killer
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptor
TLR3	Toll-like Receptor 3
RIG-I	Retinoic Acid Inducible Gene I
MAVS	Mitochondrial Antiviral-Signaling protein
TRIF	TIR-domain-containing adapter-inducing IFN-
IRF	Interferon Regulatory Factor

NF- B	Nuclear Factor- B
AP-1	Activator Protein-1
STAT	Signal Transducer and Activator of Transcription
ISG	Interferon Stimulated Gene
ISRE	Interferon Stimulated Response Element
MOI	Multiplicity of Infection
TNF	Tumor Necrosis Factor
PHH	Primary Human Hepatocytes
IFIT1	IFN-induced protein with tetratricopeptide repeats 1
NPCs	Non-parenchymal cells
KCs	Kupffer cells
LSECs	Liver sinusoidal endothelial cells

REFERENCES

- [1]. Helbig KJ, Ruszkiewicz A, Lanford RE, Berzsenyi MD, Harley HA, McColl SR, et al. Differential expression of the CXCR3 ligands in chronic hepatitis C virus (HCV) infection and their modulation by HCV in vitro. *J. Virol.* 2009; 83:836–846. [PubMed: 18987152]
- [2]. Larrubia J-R, Calvino M, Benito S, Sanz-de-Villalobos E, Perna C, Pérez-Hornedo J, et al. The role of CCR5/CXCR3 expressing CD8+ cells in liver damage and viral control during persistent hepatitis C virus infection. *J. Hepatol.* 2007; 47:632–641. [PubMed: 17560677]
- [3]. Wang J, Holmes TH, Cheung R, Greenberg HB, He X-S. Expression of chemokine receptors on intrahepatic and peripheral lymphocytes in chronic hepatitis C infection: its relationship to liver inflammation. *J. Infect. Dis.* 2004; 190:989–997. [PubMed: 15295707]
- [4]. Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* 2006; 7:R100. [PubMed: 17076895]
- [5]. Aoyama T, Paik Y-H, Seki E. Toll-like receptor signaling and liver fibrosis. *Gastroenterol Res Pract.* 2010:2010.
- [6]. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, et al. Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:9294–9299. [PubMed: 15939869]
- [7]. Zeremski M, Petrovic LM, Chiriboga L, Brown QB, Yee HT, Kinkhabwala M, et al. Intrahepatic levels of CXCR3-associated chemokines correlate with liver inflammation and fibrosis in chronic hepatitis C. *Hepatology (Baltimore, Md.).* 2008; 48:1440–1450.
- [8]. Darling JM, Aerssens J, Fanning G, McHutchison JG, Goldstein DB, Thompson AJ, et al. Quantitation of pretreatment serum interferon- γ -inducible protein-10 improves the predictive value of an IL28B gene polymorphism for hepatitis C treatment response. *Hepatology (Baltimore, Md.).* 2011; 53:14–22.
- [9]. Askarieh G, Alsiö A, Pugnale P, Negro F, Ferrari C, Neumann AU, et al. Systemic and intrahepatic interferon-gamma-inducible protein 10 kDa predicts the first-phase decline in hepatitis C virus RNA and overall viral response to therapy in chronic hepatitis C. *Hepatology (Baltimore, Md.).* 2010; 51:1523–1530.
- [10]. Zeremski M, Hooker G, Shu MA, Winkelstein E, Brown Q, Jarlais Des DC, et al. Induction of CXCR3- and CCR5-associated chemokines during acute hepatitis C virus infection. *J. Hepatol.* 2011; 55:545–553. [PubMed: 21256906]
- [11]. Sumpter R, Loo Y-M, Foy E, Li K, Yoneyama M, Fujita T, et al. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J. Virol.* 2005; 79:2689–2699. [PubMed: 15708988]

- [12]. Li K, Chen Z, Kato N, Gale M, Lemon SM. Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. *J. Biol. Chem.* 2005; 280:16739–16747. [PubMed: 15737993]
- [13]. Wang N, Liang Y, Devaraj S, Wang J, Lemon SM, Li K. Toll-like receptor 3 mediates establishment of an antiviral state against hepatitis C virus in hepatoma cells. *J. Virol.* 2009; 83:9824–9834. [PubMed: 19625408]
- [14]. Li K, Li NL, Wei D, Pfeffer SR, Fan M, Pfeffer LM. Activation of chemokine and inflammatory cytokine response in hepatitis C virus-infected hepatocytes depends on Toll-like receptor 3 sensing of hepatitis C virus double-stranded RNA intermediates. *Hepatology (Baltimore, Md.)*. 2012; 55:666–675.
- [15]. Saito T, Owen DM, Jiang F, Marcotrigiano J, Gale M. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. *Nature*. 2008; 454:523–527. [PubMed: 18548002]
- [16]. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*. 2001; 413:732–738. [PubMed: 11607032]
- [17]. Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, et al. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J. Immunol.* 2002; 169:6668–6672. [PubMed: 12471095]
- [18]. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell*. 2010; 140:805–820. [PubMed: 20303872]
- [19]. Osterlund PI, Pietilä TE, Veckman V, Kotenko SV, Julkunen I. IFN regulatory factor family members differentially regulate the expression of type III IFN (IFN-lambda) genes. Multiple values selected. 2007; 179:3434–3442.
- [20]. Ank N, West H, Paludan SR. IFN-lambda: novel antiviral cytokines. *J. Interferon Cytokine Res.* 2006; 26:373–379. [PubMed: 16734557]
- [21]. Aaronson DS, Horvath CM. A road map for those who don't know JAK-STAT. *Science*. 2002; 296:1653–1655. [PubMed: 12040185]
- [22]. Thomas E, Gonzalez VD, Li Q, Modi AA, Chen W, Nouredin M, et al. HCV infection induces a unique hepatic innate immune response associated with robust production of type III interferons. *Gastroenterology*. 2012; 142:978–988. [PubMed: 22248663]
- [23]. Marukian S, Andrus L, Sheahan TP, Jones CT, Charles ED, Ploss A, et al. Hepatitis C virus induces interferon- and interferon-stimulated genes in primary liver cultures. *Hepatology (Baltimore, Md.)*. 2011; 54:1913–1923.
- [24]. Spurrell JCL, Wiehler S, Zaheer RS, Sanders SP, Proud D. Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2005; 289:L85–95. [PubMed: 15764644]
- [25]. Harvey CE, Post JJ, Palladinetti P, Freeman AJ, Ffrench RA, Kumar RK, et al. Expression of the chemokine IP-10 (CXCL10) by hepatocytes in chronic hepatitis C virus infection correlates with histological severity and lobular inflammation. *J. Leukoc. Biol.* 2003; 74:360–369. [PubMed: 12949239]
- [26]. Maire M, Parent R, Morand A-L, Alotte C, Trépo C, Durantel D, et al. Characterization of the double-stranded RNA responses in human liver progenitor cells. *Biochem. Biophys. Res. Commun.* 2008; 368:556–562. [PubMed: 18258184]
- [27]. Biosystems, A. Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR. 2008. p. 1-70. Available from: http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042380.pdf
- [28]. Alcamí A, Symons JA, Smith GL. The vaccinia virus soluble alpha/beta interferon (IFN) receptor binds to the cell surface and protects cells from the antiviral effects of IFN. *J. Virol.* 2000; 74:11230–11239. [PubMed: 11070021]
- [29]. Preiss S, Thompson A, Chen X, Rodgers S, Markovska V, Desmond P, et al. Characterization of the innate immune signalling pathways in hepatocyte cell lines. *J. Viral Hepat.* 2008; 15:888–900. [PubMed: 18673429]

- [30]. Broering R, Wu J, Meng Z, Hilgard P, Lu M, Trippler M, et al. Toll-like receptor-stimulated non-parenchymal liver cells can regulate hepatitis C virus replication. *J. Hepatol.* 2008; 48:914–922. [PubMed: 18362039]
- [31]. Trowbridge IS, Thomas ML. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu. Rev. Immunol.* 1994; 12:85–116. [PubMed: 8011300]
- [32]. Holness CL, Simmons DL. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. *Blood.* 1993; 81:1607–1613. [PubMed: 7680921]
- [33]. Kinoshita M, Uchida T, Sato A, Nakashima M, Nakashima H, Shono S, et al. Characterization of two F4/80-positive Kupffer cell subsets by their function and phenotype in mice. *J. Hepatol.* 2010; 53:903–910. [PubMed: 20739085]
- [34]. Huebert RC, Jagavelu K, Liebl AF, Huang BQ, Splinter PL, LaRusso NF, et al. Immortalized liver endothelial cells: a cell culture model for studies of motility and angiogenesis. *Lab. Invest.* 2010; 90:1770–1781. [PubMed: 20644520]
- [35]. Heim MH. Innate immunity and HCV. *J. Hepatol.* 2013; 58:564–574. [PubMed: 23063572]
- [36]. Sarasin-Filipowicz M, Oakeley EJ, Duong FHT, Christen V, Terracciano L, Filipowicz W, et al. Interferon signaling and treatment outcome in chronic hepatitis C. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:7034–7039. [PubMed: 18467494]
- [37]. Park H, Serti E, Eke O, Muchmore B, Prokunina-Olsson L, Capone S, et al. IL-29 is the dominant type III interferon produced by hepatocytes during acute hepatitis C virus infection. *Hepatology (Baltimore, Md.).* 2012; 56:2060–2070.
- [38]. Veckman V, Osterlund P, Fagerlund R, Melén K, Matikainen S, Julkunen I. TNF-alpha and IFN-alpha enhance influenza-A-virus-induced chemokine gene expression in human A549 lung epithelial cells. *Virology.* 2006; 345:96–104. [PubMed: 16253303]
- [39]. Collins SE, Noyce RS, Mossman KL. Innate cellular response to virus particle entry requires IRF3 but not virus replication. *J. Virol.* 2004; 78:1706–1717. [PubMed: 14747536]
- [40]. DeFilippis VR, Robinson B, Keck TM, Hansen SG, Nelson JA, Früh KJ. Interferon regulatory factor 3 is necessary for induction of antiviral genes during human cytomegalovirus infection. *J. Virol.* 2006; 80:1032–1037. [PubMed: 16379004]
- [41]. Wu C, Ohmori Y, Bandyopadhyay S, Sen G, Hamilton T. Interferon-stimulated response element and NF kappa B sites cooperate to regulate double-stranded RNA-induced transcription of the IP-10 gene. *J. Interferon Res.* 1994; 14:357–363. [PubMed: 7897255]
- [42]. Wagoner J, Austin M, Green J, Imaizumi T, Casola A, Brasier A, et al. Regulation of CXCL-8 (interleukin-8) induction by double-stranded RNA signaling pathways during hepatitis C virus infection. *J. Virol.* 2007; 81:309–318. [PubMed: 17035306]
- [43]. Kawai T, Akira S. Toll-like Receptor and RIG-1-like Receptor Signaling. *Annals of the New York Academy of Sciences.* 2008; 1143:1–20. [PubMed: 19076341]
- [44]. Lu Y-C, Kim I, Lye E, Shen F, Suzuki N, Suzuki S, et al. Differential role for c-Rel and C/EBPbeta/delta in TLR-mediated induction of proinflammatory cytokines. *J. Immunol.* 2009; 182:7212–7221. [PubMed: 19454718]
- [45]. Reynolds GM, Harris HJ, Jennings A, Hu K, Grove J, Lalor PF, et al. Hepatitis C virus receptor expression in normal and diseased liver tissue. *Hepatology (Baltimore, Md.).* 2008; 47:418–427.
- [46]. Dill MT, Makowska Z, Duong FHT, Merkofer F, Filipowicz M, Baumert TF, et al. Interferon-stimulated genes, but not USP18, are expressed in livers of patients with acute hepatitis C. *Gastroenterology.* 2012; 143:777–86. e1–6. [PubMed: 22677194]
- [47]. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* 2004; 75:163–189. [PubMed: 14525967]
- [48]. Casrouge A, Decalf J, Ahloulay M, Lababidi C, Mansour H, Vallet-Pichard A, et al. Evidence for an antagonist form of the chemokine CXCL10 in patients chronically infected with HCV. *J. Clin. Invest.* 2011; 121:308–317. [PubMed: 21183794]

Fig. 1. Both RIG-I and TLR3 Are Required for Maximal CXCL10 Induction During HCV Infection of Huh7 Cells

(A) HCV RNA varies with PRR expression in Huh7-derived cells at 72 hours post-infection (MOI 0.05). (B) Huh7 cells expressing both TLR3 and RIG-I produce maximal CXCL10 mRNA (left) and protein (right) following HCV infection (72 hours, MOI 0.05) after normalization to HCV RNA copy number. (C) Huh7 cells expressing both TLR3 and RIG-I produce maximal CXCL10 mRNA during HCV infection at MOI 2. Except for TLR3 negative/RIG-I negative cells, viral loads were equivalent across cell lines (12 hours post-infection, $p > .01$).

Fig. 2. IFN Neutralization Does Not Affect CXCL10 Induction in HCV-infected TLR3+/RIG-I+ Huh7 Cells

(A) Neutralization of type I IFNs with B18R does not impact CXCL10 mRNA (left) or protein (right) induction during HCV infection (MOI .5), but (B) negates IFN α 2 induction of IFIT1 mRNA (left) and protein (right). (C) Addition of a pan-type III IFN neutralizing antibody (α IFN) does not impact CXCL10 mRNA (left) or protein (right) at 24 or 48 hours after HCV infection (MOI .5), but (D) reduces virus and IL-28B induction of IFIT1 mRNA (left) and protein (right).

Fig. 3. HCV Infection and Paracrine IFN /TNF Treatment Yield Distinct Intracellular CXCL10 Staining Patterns

(A) CXCL10 signal clusters with viral protein in HCV-infected TLR3+/RIG-I+ cells (72 hours, MOI 0.5; right), whereas IFN- /TNF treated cells (left) show homogenous CXCL10 staining within the culture. Staining: CXCL10 (orange), HCV Core (green), Nuclei (Blue). (B) A positive correlation was observed between total CXCL10 signal and total HCV Core signal in each cell within a HCV-infected population ($r^2 = 0.88$, $n=2145$, $p<.001$). (C) Cells positive for HCV Core protein have significantly higher levels of CXCL10 protein expression than those cell which are negative ($p<.001$).

Fig. 4. IFNs Are Not Required For HCV-Induction of CXCL10 in NPC-Depleted PHH Cultures
HCV-induced CXCL10 mRNA (left) and protein (right) is reduced by (A) type I but not (B) type III IFN neutralization in a PHH culture (MOI 0.2). (C) NPC-depleted PHH cultures (“Depleted”) induce fewer cytokines and immune markers during HCV infection than standard PHH cultures (“Normal”). (D) NPCs removed during immunodepletion (Bound Cells) express type I IFNs and immune markers. Displayed genes represent only detected transcripts. (E) HCV-induced CXCL10 mRNA (top) and protein (bottom) in Depleted PHH was unaffected by IFN neutralization in comparison to Normal PHH responses.