Induction and Regulation of CXCL10 in Hepatocytes During Hepatitis C Virus Infection

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2013

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Program Authorized to Offer Degree:

Pathobiology

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Abstract

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Chronic Hepatitis C affects an estimated 170 million people worldwide and 4 million in the United States. The pro-inflammatory chemokine CXCL10 is induced by hepatitis C virus (HCV) infection *in vitro* and *in vivo*, and is associated with the outcome of interferon (IFN)-based therapies. Since persistent hepatic inflammation can lead to degenerative liver disease, this work sought to evaluate how innate immune sensors of HCV infection (Toll-like receptor 3 [TLR3] and retinoic acid inducible gene I [RIG-I]) contribute to CXCL10 induction in hepatocytes. CXCL10 mRNA and protein were measured in primary human hepatocytes (PHH) and hepatocyte lines harboring functional or non-functional TLR3 and RIG-I pathways following HCV infection or exposure to receptor-specific stimuli. The contribution of hepatocyte-derived type I and type III IFNs and specific pro-inflammatory transcription factors to CXCL10 induction

were also examined. In this study, PHH and immortalized PH5CH8 hepatocytes were confirmed to express functional TLR3 and RIG-I. Specific activation of TLR3 and RIG-I led to CXCL10 induction in a non-synergistic manner, and Huh7 human hepatoma cells expressing both receptors (TLR3+/RIG-I+ Huh7 cells) 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, suggesting that standard PHH cultures contain non-parenchymal cells (NPCs). Immunodepletion of NPCs eliminated expression of immune and anti-inflammatory markers in PHH cultures, which then showed no IFN requirement for CXCL10 induction during HCV infection. Instead, HCV infection and specific TLR3/RIG-I activation induced binding of NF-κB and IRF3 to the CXCL10 promoter. Together, these data indicate that initial CXCL10 induction in hepatocytes during early HCV infection is independent of hepatocyte-derived type I and type III IFNs, while NPC- and immune cell-derived IFNs contribute to CXCL10 induction during HCV infection in PHH cultures and *in vivo*. Further elucidation of the regulatory pathways controlling CXCL10 induction may reveal novel targets for host-oriented therapies to reduce chronic inflammation, as well as provide insight into the complex and redundant signaling network of the innate immune system.

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ACKNOWLEDGEMENTS

“NOTHING IS IMPOSSIBLE! NOT IF YOU CAN IMAGINE IT!

THAT’S WHAT BEING A SCIENTIST IS ALL ABOUT!”

- Professor Hubert J. Farnsworth, *Futurama*

This dissertation would not have been possible without the support and guidance provided by my mentor, Dr. Stephen Polyak, as well as by Jessica Wagoner, Erica Lovelace, Jacob Bruckner, and other current and former members of the Polyak group. In addition, Derek Thirstrup, Dr. Wesley Smith, and Dr. Isaac Mohar provided technical guidance that was crucial for the success of the project. The project also benefited from the guidance of the members of my thesis committee (Drs. Michael Gale Jr, Richard Darveau, Timothy Rose, and I. Nicholas Crispe) as well as Drs. Hugo Rosen and Young Hahn.

I was also fortunate to have the mental, spiritual, and culinary support of Michael Roberto, Melanie Gasper, Sela Davis, and others, which helped me rally during the last months of this project. And I will be forever grateful for the love and support of my family. For my parents, who always encouraged and enabled me to pursue my passions. And for my brother, who always knows how to make me laugh. And who totally put that smiley-faced antenna cap on the Vibe.

Mom, I hope you liked my dissertation.

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LIST OF ABBREVIATIONS

AP-1: Activator Protein-1

ASC: Apoptosis-associated Speck-like Protein Containing a CARD

BIR: Baculovirus Inhibitor of Apoptosis Protein Repeat

C/EBP-β: CCAAT/enhancer-binding protein-β

CARD: Caspase Recruitment Domain

ChIP: Chromatin Immunoprecipitation

dsRNA: Double-Stranded RNAs

GAG: Glycosaminoglycans

GAS: IFNγ Activation Site

HAU: Hemagglutination Units

HCC: Hepatocellular Carcinoma

HCV: Hepatitis C Virus

HIV: Human Immunodeficiency Virus

HSC: Hepatic Stellate Cell

IFIT1: IFN-induced Protein with Tetratricopeptide Repeats 1

IFN: Interferon

IL: Interleukin

ISG: Interferon Stimulated Gene

IRF: Interferon Regulatory Factor

ISRE: Interferon Stimulated Response Element

JAK: Janus Kinase

KC: Kupffer Cell

LRR: Leucine-Rich Repeat

LSEC: Liver Sinusoidal Endothelial Cell

MAP: Mitogen-Activated Protein

MAVS: Mitochondrial Anti-Viral Signaling

MDA-5: Melanoma Differentiation-Associated Protein 5

MMP: Matrix Metalloproteinase

MOI: Multiplicity of Infection

MyD88: Myeloid differentiation primary response gene 88

NF-κB: Nuclear Factor-κB

NK: Natural Killer

NLR: Nod-like Receptor

NLRP3: NLR-family pyrin-containing protein 3

NOS: Reactive Nitrogen Species

NPC: Non-Parenchymal Cell

NS: Non-Structural

PAMP: Pathogen Associated Molecular Pattern

PCR: Polymerase Chain Reaction

PKR: Protein Kinase R

PHH: Primary Human Hepatocytes

PRR: Pattern Recognition Receptor

RIG-I: Retinoic Acid-Inducible Gene I

RLR: RIG-I-like Receptor

ROS: Reactive Oxygen Species

RSV: Respiratory Syncytial Virus

RT: Reverse Transcription

SeV: Sendai Virus

SRB1: Scavenger Receptor class B Type I

STAT: Signal Transducer and Activator of Transcription

SVR: Sustained Virologic Response

T_c: Cytotoxic T Cell

T_H1: Type I Helper T Cell

TIMP: Tissue Inhibitor of Metalloproteinase

TIR: Toll/IL-1R Homology

TLR: Toll-like Receptor

TNFα: Tumor Necrosis Factor α

TRIF: TIR-domain-containing adapter-inducing IFN-β

VLDL: Very Low-Density Lipoprotein

VSV: Vesicular Stomatitis Virus

CHAPTER 1:

INTRODUCTION AND LITERATURE REVIEW

"IN THE BEGINNING THE UNIVERSE WAS CREATED. THIS HAS MADE A LOT OF PEOPLE VERY ANGRY AND BEEN WIDELY REGARDED AS A BAD MOVE."

- DOUGLAS ADAMS, *THE RESTAURANT AT THE END OF THE UNIVERSE*

OVERVIEW

This chapter presents an overview of the literature relevant to the thesis. Topics include innate immune signaling in response to virus infections, hepatitis C epidemiology and treatment, the hepatitis C virus life cycle, and an overview of the current literature on the chemokine CXCL10. The aims of the thesis are also outlined.

Portions of the “Hepatitis C - the Virus and the Disease“ section of this chapter were reprinted in our review "Molecular Pathways: Hepatitis C Virus, CXCL10, and the Inflammatory Road to Liver Cancer" (*Clinical Cancer Research*, 2013, 19(6): 1347-1352; see Appendix A).

INTRODUCTION

The human immune system has evolved to combat viral infection through the use of diverse, interdependent cell types and a complex network of signaling pathways. However, non-specific components of this immune response can cause bystander damage to host tissue in the process of trying to eliminate infection. During persistent viral infections such as hepatitis C, this bystander damage may cause host morbidity in excess of that caused by direct viral pathogenesis. Therefore, treatment of these chronic diseases must manage both the inflammatory immune response as well as the primary infection in order to prevent disease.

THE INNATE IMMUNE RESPONSE TO VIRUSES

Viral Sensing

The host immune system first detects viral infections through Pathogen Recognition Receptors (PRRs). These innate receptors recognize specific, conserved motifs known as Pathogen Associated Molecular Patterns (PAMPs). Upon PAMP recognition, each PRR activates signaling pathways that lead to transcription of innate immune genes including cytokines, chemokines, and additional PRRs as part of a positive feedback loop (Figure 1.1). PRRs are divided into three structural families: Toll-like Receptors (TLRs), RIG-I-like Receptors (RLRs), and Nod-like Receptors (NLRs).

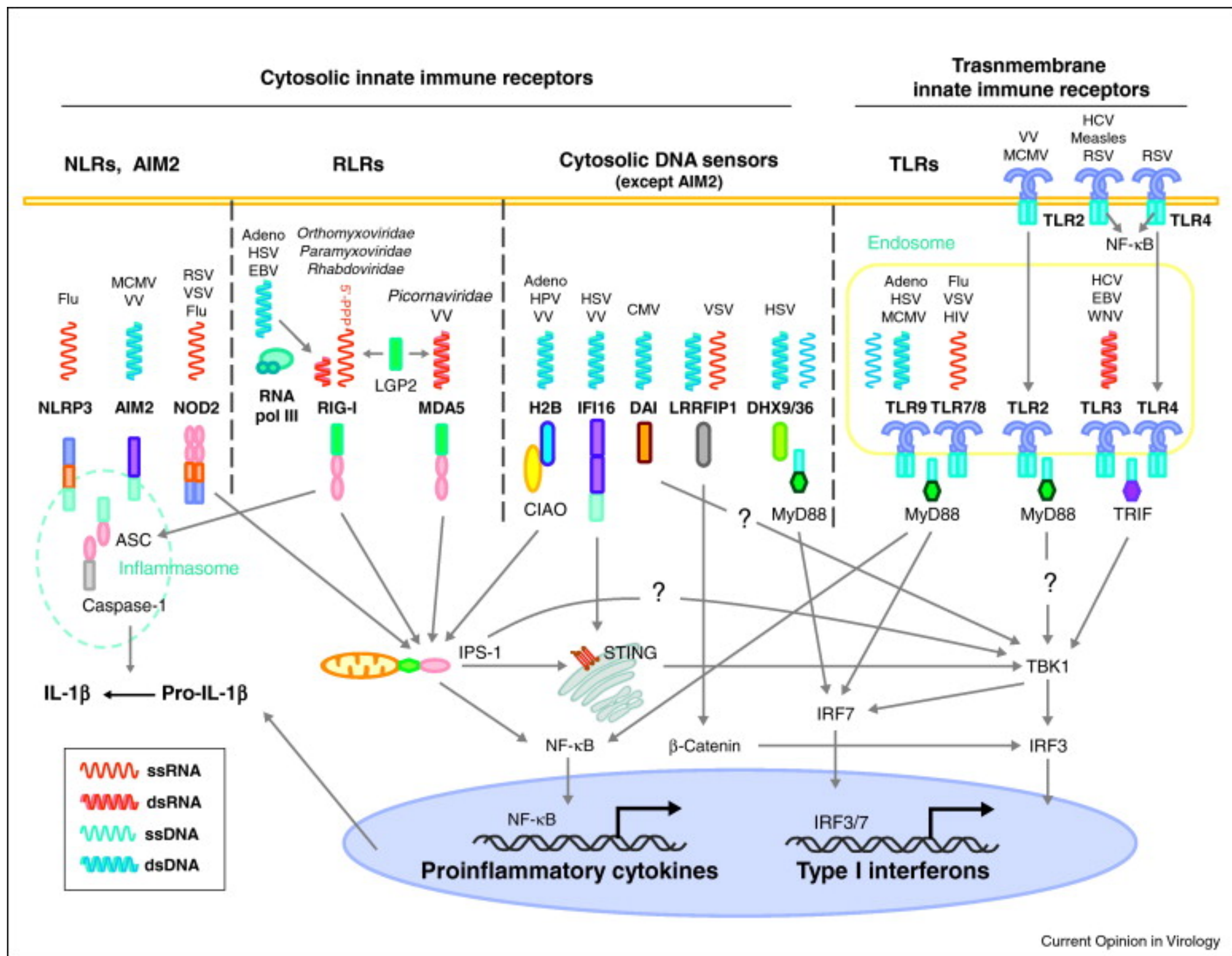

Figure 1.1: Pathogen Recognition Receptors of Viral Infection. *Reprinted from Current Opinion in Virology, 1, Taiki Aoshi, Shohei Koyama, Kouji Kobiyama, Shizuo Akira, Ken J Ishii, "Innate and adaptive immune responses to viral infection and vaccination", 226-232, Copyright (2011), with permission from Elsevier*

Toll-like Receptors - TLRs

TLRs are a class of membrane bound PRRs that were originally discovered in *Drosophila* [1] They are characterized by the presence of leucine-rich repeat (LRR) sequences near their N-terminus as well as a cytoplasmic Toll/IL-1R homology (TIR) domain near the C-terminus [1,2]. Ten unique TLRs have been identified in humans, all of which recognize different characteristic PAMPs (See Table 1.1, Figure 1.1, and [2]). The LRR domains are responsible for this PAMP recognition, while the TIR domain

relays the activation signal to one of two signaling adaptor proteins (Myeloid differentiation primary response gene 88 [MyD88] and/or TIR-domain-containing adapter-inducing interferon-β [TRIF]) [2]. In turn, these adaptors relay the activation signal to various downstream effectors of the anti-viral, pro-inflammatory, and inflammasome response pathways, which are described in more detail below.

Six TLRs are located in the plasma membrane: TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11. These TLRs are known to recognize a wide variety of bacterial PAMPs including lipopolysaccharide (TLR4), flagellan (TLR5), and lipoprotein (TLR2) [2]. However, TLRs located on the plasma membrane have also been implicated in a wide variety of virus infections (Figure 1.1). For example, TLR4-mediated recognition of the surface glycoprotein from Ebola virus and respiratory syncytial virus (RSV) fusion proteins in monocytes leads to the induction of the pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α [3,4]. Monocytes and macrophages also respond to the Core and Non-structural (NS) 3 proteins of hepatitis C virus (HCV) and the hemagglutinin protein of measles virus in a TLR2-dependent manner [5,6].

The remaining TLRs (TLR3 and TLR7-9) are located within endosomal compartments and specialize in the detection of nucleic acids [2]. TLR7/8 and TLR9 are well-characterized sensors of single-stranded (ss) viral RNA and DNA genomes, respectively (See Table 1.1 and ref. [2]). In contrast, TLR3 detects double-stranded (ds) RNA viral genomes or replication intermediates [2,7]. Activation of these TLRs has been documented in a number of different viral infections, including those by HCV (TLR7, TLR3; ref. [7,8]), herpes simplex virus (TLR9; ref. [9]) and human immunodeficiency virus (HIV; TLR7/8; ref. [10]). The exact mechanism by which nucleic acids are

delivered to the endosome for detection remains unclear but appears to be dependent on endosomal acidification following virus or PAMP uptake [11-13]. Viral RNAs and DNAs may also be transported into endosomes through normal cellular autophagy, as was demonstrated for activation of TLR7 during vesicular stomatitis virus (VSV) infection of plasmacytoid dendritic cells [14].

TLR	Location	PAMP	Signaling Adaptor
TLR1	Plasma Membrane	Triacyl lipoprotein	MyD88
TLR2	Plasma Membrane	Lipoprotein	MyD88
TLR3	Endosome	dsRNA	TRIF
TLR4	Plasma Membrane	LPS	MyD88/TRIF
TLR5	Plasma Membrane	Flagellin	MyD88
TLR6	Plasma Membrane	Diacyl lipoprotein	MyD88
TLR7/8	Endosome	ssRNA	MyD88
TLR9	Endosome	CpG DNA	MyD88
TLR10	Endosome	Unknown	MyD88

Table 1.1: Known Human Toll-Like Receptors. Additional details may be found in refs. [2,15].

RIG-I-like Receptors - RLRs

The RLR family of PRRs is named after its prototypical member, retinoic acid-inducible gene I (RIG-I), and also includes melanoma differentiation-associated protein 5 (MDA-5) as well as LGP2 [2]. Similar to TLR3, all RLRs recognize dsRNAs, with longer dsRNAs preferentially activating MDA-5 [16,17]. This makes RLRs key sensors of RNA viral infections. For example, RIG-I specifically recognizes a polyuridine-containing PAMP identified in the 3' non-translated region of the HCV genome [18].

RLRs share a characteristic C-terminal Repressor Domain for binding dsRNA and a central DExD/H-box helicase/ATPase domain whose activity is required for both binding and signal transduction [2,19-21]. RIG-I and MDA-5 also have two tandem N-

terminal caspase recruitment domains (CARDs). Upon PAMP recognition, these CARDs bind to identical domains within the mitochondrial anti-viral signaling (MAVS) adaptor protein in order to activate downstream pro-inflammatory and anti-viral signaling (see below and ref. [2]). In contrast, the CARD-deficient LGP2 has been shown to negatively regulate RIG-I and MDA-5 activation through the sequestration of dsRNAs and through non-CARD binding of MAVS [22-24]. However, LGP2 has also been reported to enhance RIG-I and MDA-5 signaling through its ATPase domain [25], suggesting that cross-regulation among the RLRs is more complex than previously indicated.

Nod-like Receptors - NLRs

Similar to RLRs, NLRs are cytoplasmic receptors that consist of three domains. All NLRs share a central nucleotide-binding domain (i.e. NOD domain) and a C-terminal LRR domain that is responsible for PAMP recognition and binding [2,26]. The third, N-terminal domain found in NLRs varies between family members and is typically either a CARD, a pyrin domain, or baculovirus inhibitor of apoptosis protein repeat (BIR) domain [2]. NLRs possessing pyrin or BIR domains trigger inflammasome signaling by activating caspase-1 (see below and ref. [2,26,27]. Alternatively, NLRs possessing CARDs (NOD1 and NOD2) are typically involved in induction of pro-inflammatory genes [2,26,27].

NLRs are canonically activated by viral DNA and components of bacterial peptidoglycan [2,26]. However, a few studies have shown activation of NLRs by negative sense, ssRNA viruses. For example, NOD2 can be activated by RNA from

RSV, VSV, and influenza virus [28], while NLR-family pyrin-containing protein 3 (NLRP3, i.e. NALP3), reportedly recognizes influenza as well as Sendai virus [27,29,30]. Direct activation of NLRs by the genomes of positive sense RNA viruses (i.e. HCV) has not yet been documented, although activation of NLRP3 by HCV-induced potassium efflux in THP-1 macrophages has recently been reported [31].

Pro-Inflammatory Responses to Viral Infections

The innate immune response following activation of TLRs, RLRs, and NLRs by viral PAMPs falls into one of three categories: the pro-inflammatory response, the anti-viral/interferon response, or the inflammasome response. The pro-inflammatory response to viral infections is in large part mediated by the transcription factor nuclear factor-κB (NF-κB). NF-κB is a heterodimer consisting of RelA, RelB, or c-Rel (all of which possess a C-terminal transactivation domain) and either p50 or p56 [32]. All NF-κB dimers are held in an inactive state by the IκB family of repressor proteins under normal cellular conditions [33,34]. Upon PRR activation, IκB is phosphorylated by the IκB kinase and ubiquitinated by an E3 ubiquitin ligase, causing its dissociation from NF-κB and degradation by the proteasome [35]. This process can be induced by most TLRs and RLRs, as well as NOD1 and NOD2 [2,26].

Following IκB dissociation, activated NF-kB translocates into the nucleus where it binds to the promoters of inflammatory genes such as TNFα, IL-6, CXCL8 (i.e. IL-8), iNOS2, COX2, and pro-IL-1 [26,32,33]. Levels of these cytokines are elevated in a wide variety of inflammatory diseases such as rheumatoid arthritis and asthma in addition to viral infection [36-38]. Production of pro-IL-1 is also key for activation of the

inflammasome (see below and ref. [26]). Additionally, autocrine and paracrine signaling by these pro-inflammatory cytokines leads to increased production of PRRs and NF-kB as part of a positive feedback loop that perpetuates the inflammatory response [32].

PRR-mediated phorphorylation of the JNK and p38 mitogen-activated protein (MAP) kinase families can also lead to the activation of the pro-inflammatory transcription factors Activator Protein-1 (AP-1) and C/EBP-β [39-43]. Although the role of these transcription factors in virus-induced inflammation is less well-characterized, both AP-1 and C/EBP-β induce IL-6 and TNFα in response to direct PRR-stimulation [44]. AP-1 and C/EBP-β also induce CXCL8 secretion in the airways of patients with asthma, leading to excessive infiltration of pro-inflammatory neutrophils during exacerbations [45].

Clearer evidence exists for the role of interferon regulatory factors (IRFs) in the direct transcriptional induction of pro-inflammatory genes following PRR sensing of virus infection. For example, the promoter of CXCL8 can be bound by IRF3 and IRF1 during HCV and RSV infection, respectively [46,47]. IRF3 has also been implicated in the induction of CCL5 and ISG56 during Sendai Virus infection [48,49]. Finally, IL-6 and TNFα production in dendritic cells following TLR3, TLR4, or TLR9 activation has been linked to IRF5 [50]. IRFs bind to IFN-stimulated response elements (ISRE) in the promoters of these target genes. Since ISRE-containing genes also respond to interferon (IFN) signaling (see below), they are often referred to as Interferon Stimulated Genes (ISGs).

Interferon Responses (Type I, II, and III) to Viral Infections

PRR-mediated induction of IFNs and their subsequent autocrine and paracrine induction of ISGs is another prominent aspect of the innate immune response to viral infection. Over 300 IFN-induced ISGs have been identified, including direct inhibitors of virus replication (i.e. Protein Kinase R, 2'-5'-oligoadenylate synthetase 2) as well as genes responsible for the recruitment and activation of immune effector cells [51]. ISG induction is critical for the containment of acute viral infections *in vivo*. Indeed, pegylated-IFNs are used clinically to treat acute as well as chronic HCV and hepatitis B virus infections [52,53].

Multiple different classes of IFNs have been described in the literature [54-56]. Induction of the type I IFNs IFNα (which consists of 13 subtypes) and IFNβ can occur in all nucleated cells and is initiated by IRF7 and IRF3 [2,55]. In contrast, type II IFNs are produced by a subset of immune cells including natural killer (NK) cells, NKT cells, CD8+ cytotoxic T (T_c) cells, and CD4+ type I helper T (T_H1) cells [54,56]. This suggests that type II IFNs comprise a secondary wave of anti-viral responses that is activated once immune cells are recruited from the periphery to sites of infection. Type III IFNs are the least characterized of the three classes of IFNs. Currently, there are three known subtypes: IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B) [55]. Their transcription is induced by a combination of NF-κB, IRF3, and IRF7 [57]. They are thought to work as a first line of defense in a manner similar to type I IFNs, but their production is restricted to a subset of cell types including plasmacytoid dendritic cells and liver tissue [55,57].

All IFNs signal through different heterodimeric receptors. The type I IFN receptor is composed of the IFNAR1 and IFNAR2 subunits [55]. Dimerization of this receptor activates Janus kinases (JAKs) and signal transducers and activators of transcription (STAT) signaling, which results in the formation of the ISGF3 complex [54,55]. ISGF3 then translocates into the nucleus and binds to ISREs within the promoters of ISGs, thereby activating transcription [54]. In contrast, binding of type II IFNs to the IFNGR1/IFNGR2 receptor induces formation of STAT1 homodimers, which bind to IFNγ activation site (GAS) elements in the promoters of target genes [54,56]. Activation of the type III IFN receptor, which consists of the IL10R2 and IL-28Rα subunits, can lead to the formation of both STAT1 homodimers and the ISGF3 complex [55]. Thus, multiple mechanisms exist for the paracrine activation of ISGs during virus infection both at the site of infection and in neighboring cells and tissues.

Inflammasome Responses to Viral Infections

Viral infection can also induce inflammasome activation through a two-part process. First, pro-IL-1β and pro-IL-18 are induced by NF-κB following TLR and/or RLR activation as described above [26]. Secondary signaling by specific NLRs (ex. NRLP3) to the adaptor apoptosis-associated speck-like protein containing a CARD (ASC) leads to the activation of caspase-1, which cleaves pro-IL-1β and pro-IL-18 in their active forms [26,27]. Secreted IL-1β and IL-18 contribute to fever generation and the activation of lymphocytes at the site of viral infection [58,59]. These responses help to perpetuate the anti-viral type I immune responses initiated and recruited by IFN, NF-κB and other pro-inflammatory signaling factors.

CXCL10 – A Central Chemokine for T cell Recruitment

Chemokines are a key factor in the maintenance of these inflammatory innate immune responses and the activation of adaptive immune responses during viral infection. These 8-10 kDa chemotactic cytokines are classified into four groups based on the arrangement of the first two of four conserved cysteine residues: CC, CXC, C, and CX_3C [60]. Some are considered tissue specific, while others are more generally produced. For example, the chemokine CXCL10 (IFN-gamma-induced Protein 10 [IP-10]) is secreted by a wide variety of cell types in response to PRR-sensing of viral infection, including hepatocytes [61,62], lung epithelial cells [63], neurons [64], and pancreatic β-cells [65].

Initially produced as a 10.88 kDA precursor protein with a N-terminal signal peptide, CXCL10 is cleaved to a final size of 8.6 kDa prior to secretion [66]. Once secreted, CXCL10 can form homodimers and bind to glycosaminoglycans (GAG) in the extracellular matrix [67]. These dimers exist in equilibrium near the cell surface with monomeric CXCL10, which diffuses into the periphery and becomes the dominant form found at standard serum concentrations [67,68]. Monomeric CXCL10 in the periphery binds and activates the G-protein coupled CXCR3 receptor on CD4+ T_H1, CD8+ T_c, and NK cells [69-73]. Activation of CXCR3 induces cellular proliferation and chemotaxis towards the site of infection and CXCL10 production [60,74,75].

Due to this role in coordinating type I immune responses and its production by diverse tissues, elevated CXCL10 is found in patients with a wide variety of viral infections. This includes both acute infections, such as H1N1 influenza A [76], and chronic infections such as HIV and hepatitis C [62,77,78]. Elevated intrahepatic and

serum levels of CXCL10 are also predictive of treatment outcome in patients with hepatitis C [79-81]. These observations suggest that CXCL10 is an important player in orchestrating the type I immune response in response to viral infection *in vivo* as well as *in vitro*.

HEPATITIS C - THE VIRUS AND THE DISEASE

Hepatitis C Virus - The Causative Agent of Hepatitis C

HCV is a positive sense ssRNA virus originally isolated in 1989 as the cause of non-A, non-B hepatitis, now known as hepatitis C [82,83]. Six genotypes have been identified to date, with genotype 1 being the most prevalent in the the United States [82,84]. HCV is a member of the *Hepacivirus* genus within the *Flaviviradae*, making it structurally related to West Nile Virus, Yellow Fever Virus, and Dengue Virus [85].

The 9.6 kb HCV genome encodes a polyprotein of 3010 amino acids, which is cleaved into 10 separate products: Core, E1, E2, NS1, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (See Figure 1.3 and ref. [82]). Core, E1, and E2 are structural proteins. Core makes up the viral nucleocapsid that surrounds the ssRNA genome, while E1 and E2 are heavily glycosylated proteins embedded in the viral membrane [82]. E2 mediates viral entry by binding to the receptors CD81, scavenger receptor class B type I (SRB1), claudin-1, and occludin (Figure 1.3 and red. [86]). The very low-density lipoprotein (VLDL) receptor and GAGs have also been implicated in mediating HCV attachment [86,87]. All of these receptors are expressed on hepatocytes, which is the predominant cell type infected by HCV [88]. While HCV infection has also been reported in

macrophages, lymphocytes, and neural tissue, the extent and significance of viral replication within these cells remains unclear [89-92].

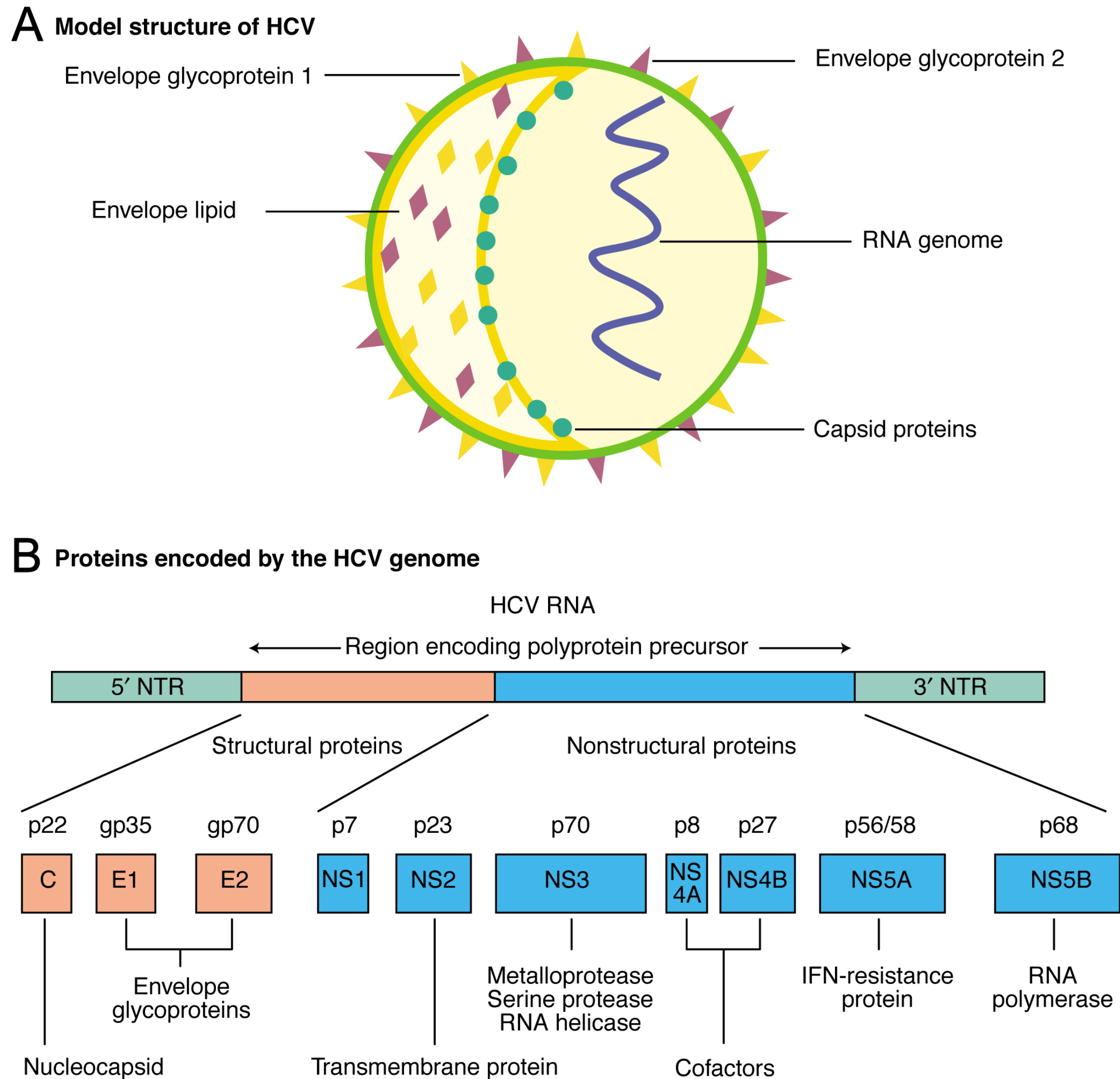

Figure 1.2: Structure of Hepatitis C Virus (HCV). (A) Diagram of an assembled HCV particle. **(B)** Schematic of the HCV genome and encoded proteins. *Reprinted from Expert Reviews in Molecular Medicine, 5, Anzola M, Burgos JJ, "Hepatocellular carcinoma: molecular interactions between hepatitis C virus and p53 in hepatocarcinogenesis", 1-16, Copyright (2003), with permission from Cambridge University Press.*

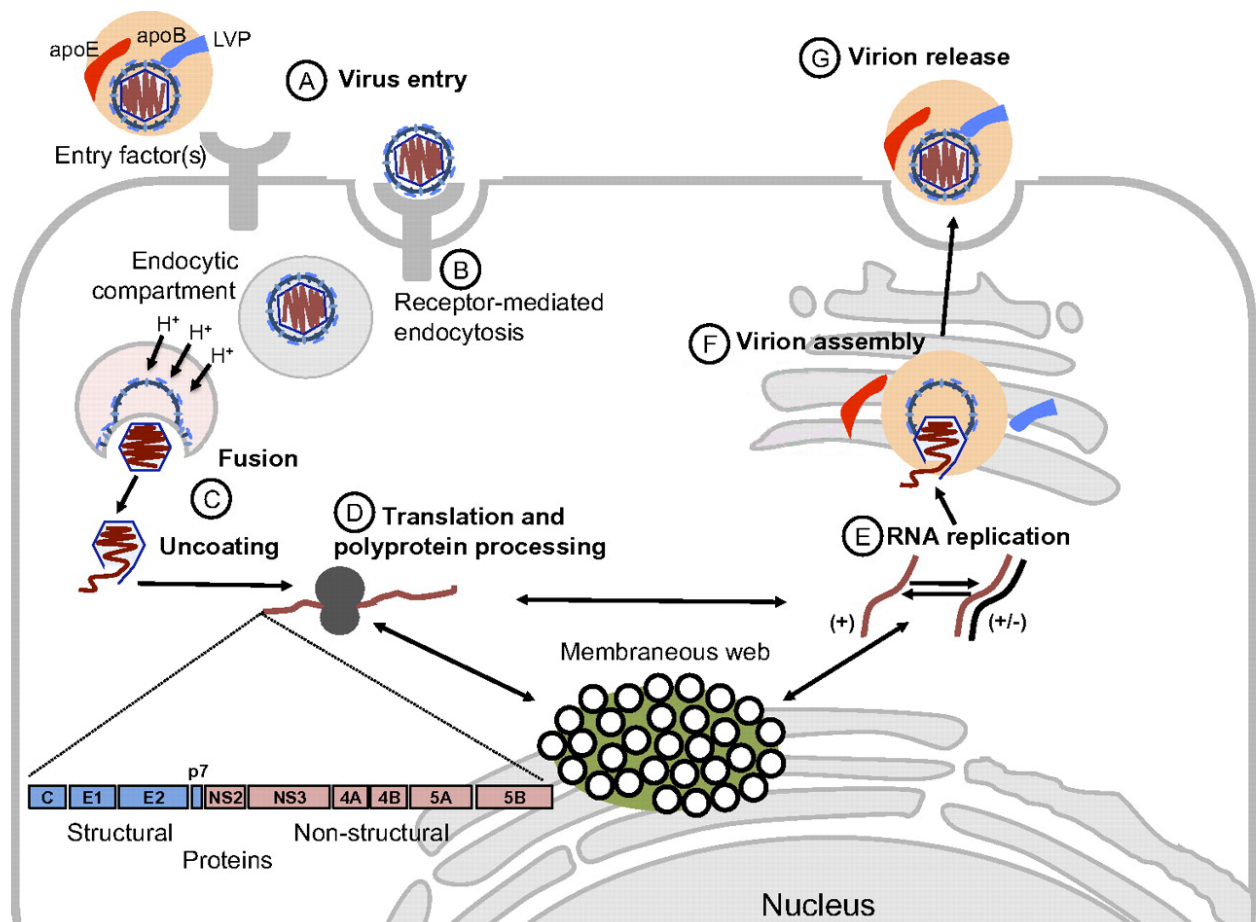

Figure 1.3: **The HCV Life Cycle. (A)** The glycoprotein E2 mediates HCV attachment via CD81, SRB1, claudin-1, and occluding. **(B)** Virus entry occurs via clathrin-mediated endocytosis. **(C)** Acidification of the late endosome leads to the fusion of viral and endosomal membranes, releasing the HCV nucelocapsid and genomic RNA into the cytoplasm. **(D)** Translation of the positive sense HCV genome generates NS2 and other viral proteins, which form a membraneous web on the ER surface. This is the site of further viral protein production, **(E)** replication of the RNA genome by NS5B, and **(F)** virion assembly. **(G)** Non-lytic virion release occurs through LDL secretion pathways. *Adapted from "New advances in the molecular biology of hepatitis C virus infection: towards the identification of new treatment targets", Ploss A, Dubuisson J, 61: i25-i35, Copyright (2012) with permission from BMJ Publishing Group Ltd.*

The HCV Life Cycle

Upon binding to these co-receptors, HCV enters the cell by clathrin-mediated endocytosis (Figure 1.3 and ref. [93]). Acidification-dependent fusion of the viral membrane with the membrane of the endosome then releases the viral nucelocapsid into the cytoplasm [93]. The positive sense HCV RNA genome is then immediately transcribed and translated by the cellular machinery to produce the HCV polyprotein. Cleavage of the polyprotein is mediated by cellular signal peptidases as well as the viral proteases NS2 and NS3/4A [82,86]. The remaining viral proteins congregate with NS4B to form a “membranous web” of vesicles within the endoplasmic reticulum membrane that contain viral proteins, viral RNA, and lipid droplets [86]. Within this web, the viral RNA-dependent RNA polymerase NS5B synthesizes new copies of the viral genome and virus assembly occurs. Viral egress from the cell is hypothesized to occur through the very low density lipoprotein (VLDL) secretion pathway [86]. This process does not result in cell lysis, enabling persistent infection.

Epidemiology of Hepatitis C

An estimated 170 million people are chronically infected by HCV worldwide, with 4 million infected in the United States [94]. As a bloodborne pathogen, HCV is transmitted between patients through transfusions of contaminated blood or through contact with equipment (i.e. needles) that has been contaminated [94]. Transfusion was common method of transmission in the United States before blood screening was implemented in 1992 [94,95]. Currently, the most common method of HCV transmission in the United States is intravenous drug use, but cases of healthcare associated

infections involving contaminated equipment and blood products still occur in countries that lack routine blood screening [95].

Chronic hepatitis C develops in 75-85% of patients exposed to HCV [94]. Only 20-30% of these patients display non-specific symptoms of acute infection, which may include jaundice, dark urine, fatigue, and/or abdominal pain [94]. Symptoms of chronic infection are more characteristic of liver injury, but these take decades to develop since they are the result of cumulative, non-specific inflammatory damage to tissue. For example, deposition of excess collagen as part of the inflammatory hepatic wound healing response can lead to scarring within the liver known as fibrosis [96]. Accumulation of this excess scar tissue leads to the development of cirrhosis in 5-20% of chronic hepatitis C patients, which is characterized by the formation of regenerative nodules that disrupt liver function [97]. Constant cellular turnover during cirrhosis can also result in hepatocellular carcinoma (HCC) [98]. Incidences of HCV-related liver diseases are predicted to increase over the next decade as patients who were unknowingly infected prior to the implementation of blood screening begin to exhibit symptoms [99].

Treatment of Hepatitis C

Prior to the end of 2011, standard of care therapy for hepatitis C that had not yet progressed to cirrhosis or HCC involved co-treatment with pegylated-IFNα and the general anti-viral medication Ribivirin [94]. Therapy is considered successful if a patient achieves a Sustained Virologic Response (SVR), which is defined as undetectable HCV in his or her blood at the end of treatment and after an additional six months [94]. The

likelihood of achieving SVR depends on viral load at the start of treatment as well as the genotype with which a patient is infected [84]. Genotype 1 is the most difficult to treat, with only 25-35% of patients achieving SVR with standard therapy [84]. The likelihood of SVR can be increased in genotype 1 patients through co-administration of NS3/4A protease inhibitors such as Telapravir and Broceprivir [100,101]. These newly FDA-approved protease inhibitors represent the first HCV-specific anti-viral medications on the market, although others are currently in the advanced stages of clinical testing [102].

Other patient factors may also influence the likelihood of achieving SVR. High pre-treatment levels of ISGs, including CXCL10, are correlated with poor treatment outcome [79,80,103]. Genome-wide association studies have also found that the rs12979860 SNP in the gene encoding the type III IFN IL-28B is predictive of spontaneous clearance and treatment response [104,105]. Specifically, patients with a homozygous C/C genotype at this locus are more likely to achieve SVR than patients who are T/T homozygous or C/T heterozygous [80,105]. Interestingly, combining pre-treatment levels of CXCL10 with IL-28B genotype has been shown to improve the ability of these models to predict treatment outcome [80,106]. This suggests that CXCL10 plays a key role in the success or failure of the pro-inflammatory, anti-HCV immune response *in vivo*.

THESIS AIMS

In vivo and *in vitro* evidence indicates that inflammation plays a key role in the pathogenesis of viral infections, including HCV. CXCL10 is a central regulator of this response that is induced following activation of PRR signaling pathways [107]. Hepatocytes, which express PRRs necessary for sensing viral PAMPs, have been implicated as the main source of CXCL10 within the liver during chronic hepatitis C [62,107]. Thus, this work examines the transcriptional regulation of CXCL10 in hepatocytes during early HCV infection using an *in vitro* culturing system. Three areas of research are described:

1) The contribution of PRRs that recognize HCV RNA (TLR3, RIG-I) to CXCL10 induction in hepatocytes in response to PAMPs and early HCV infection.
2) The contribution of hepatocyte- and non-parenchymal cell-derived type I and type III IFNs to CXCL10 induction in hepatocytes during early HCV infection.
3) Identification of the transcription factors responsible for induction of CXCL10 in hepatocytes in response to PAMPs and early HCV infection.

This work provides insight into the complex and redundant signaling pathways of the innate immune system, and may help to uncover how the persistent inflammation that is characteristic of hepatitis C is established within the liver. In turn, this may inform the development of novel, host-oriented treatments for the reduction of excessive inflammation, thereby preventing the development of fibrosis, cirrhosis, and HCC.

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CHAPTER 2:

MATERIALS AND METHODS

“ANY SUFFICIENTLY ADVANCED TECHNOLOGY IS

INDISTINGUISHABLE FROM MAGIC.”

- ARTHUR C. CLARKE

MAMMALIAN CELL CULTURE

All experiments were performed in immortalized human hepatocyte cell lines or primary human hepatocyte cultures maintained at 37°C with 5% CO_2. Human hepatoma Huh7 (TLR3-/RIG-I+) cells were obtained from Apath, LLC and shown previously to lack TLR3 expression [1]. Huh7.5.1 (TLR3-/RIG-I-) cells containing non-functional RIG-I were obtained from Francis Chisari (Scripps Research Institute) [2]. PH5CH8 immortalized hepatocytes were obtained from Noboyuki Kato (Okayama University) [1]. All three cell lines were maintained in DMEM-based Huh7 medium described previously [3]. Additional Huh7 and Huh 7.5 cells obtained from Kui Li (University of Tennessee at Memphis) were stably transduced with a FLAG-TLR3-encoding lentiviral vector (TLR3+/RIG-I+ and TLR3+/RIG-I-cells, respectively) [4]. These were maintained in Huh7 medium supplemented with 2 µg/ml blasticidin for selection. Immortalized cell lines were also submitted to the University of Washington Molecular Virology laboratory for IL-28B genotyping (SNP rs12979860) [5]. All four Huh7-derived cell lines were found to be C/T (heterozygous) at this locus. PH5CH8 cells were C/C (homozygous).

Primary hepatocyte cultures were either obtained cryopreserved from Invitrogen (Carlsbad, CA) or obtained fresh from Dr. Steven Strom (University of Pittsburgh) through the NIH Liver Cell and Tissue Distribution System (LCTDS)). Human hepatocyte cultures from the LTCDS were harvested under an IRB exemption approval from the University of Pittsburgh to Dr. Strom (letter on file) as surgical waste tissue. Hepatocytes received through the NIH LCTDS were supplied in culture with a Matrigel overlay (BD Biociences, San Jose, CA) and were maintained in supplemented Williams E media as previously reported [6]. Cryopreserved hepatocyte cultures were thawed

and maintained using CHEK/CHRM reagents (Invitrogen). Geltrex matrix overlay (Invitrogen) was added to the first 3 cryopreserved hepatocyte cultures but not to the following experiments after it was determined to not affect the results. Cyropreserved lots used in experimentation are described in Table 2.1. Fresh hepatocyte cultures were provided without identifying patient information. Hepatocytes from both sources were maintained on collagen-coated plates.

Lot	Gender	Age (yrs)	Alcohol Use	Drug Use	Smoker	Cause of Death
Hu4196	F	63	No	No	Yes	CVA
Hu8085	F	1	No	No	No	ICH
Hu8089	M	36	Yes	Yes	Yes	Head Trauma
Hu1258	M	73	Yes	No	Yes	-
Hu4227	M	<1	No	No	No	Anoxia

Table 2.1: Cryopreserved Primary Human Hepatocyte Lots Used in Experimentation. Characterization of cryopreserved primary human hepatocytes purchased from CellzDirect (now ADME/Tox, a subsidiary of Life Technologies, Carlsbad, CA) for experiments.

PLASMIDS

A CMV-BL plasmid encoding a constitutively active mutant IRF3 (IRF3-5D) [7] was provided by John Hiscott (McGill University). Firefly luciferase reporter pGL4 plasmids expressing the wild-type, ΔκB1, ΔκB2, ΔAP-1, ΔC/EPB-1, or ΔISRE CXCL10 promoter were provided by David Proud (University of Calgary) [8]. ΔISRE and wild-type CXCL10 constructs were sequenced through SimpleSeq DNA Sequencing (Eurofins MWG Operon [Huntsville, AL] in combination with ThermoFischer Scientific [Rockford, IL]) to verify the ΔISRE mutation and an intact open reading frame for luciferase. The empty vector pcDNA3.1 plasmid was obtained from Invitrogen.

VIRUSES AND INFECTION

JFH-1 HCV (genotype 2a) viral stock preparation and titration were performed as described previously [2,9]. Immortalized and primary hepatocyte cultures were incubated with virus inoculum for 5 hours at MOIs (multiplicities of infection) of 0.05, 0.5, 0.6, 1 or 2 to allow for virus adsorption. Virus inoculum was then removed and replaced with appropriate cell culture medium. Infected hepatocyte cultures were incubated for 6-72 hours.

For specific activation of RIG-I, 100 hemagglutination units (HAU) of Sendai Virus (SeV; Cantrell Stain; Charles River Laboratories [Wilmington, MA]) was diluted in serum-free DMEM and allowed to adsorb to cells for 1 hour. An equal volume of appropriate cell culture medium was then added. Infected cells were incubated for 24 hours.

NUCLEIC ACID PATHOGEN ASSOCIATED MOLECULAR PATTERNS (PAMPs)

Specific activation of RIG-I was also achieved by transfecting cells with 0.5 µg polyI:C (Amersham, now GE Healthcare Life Sciences [Pittsburgh, PA]) complexed with FuGene 6 [Promega, Madison, WI]) or 0.5 µg 5' pU HCV PAMP RNA (provided by Michael Gale Jr. [University of Washington]) complexed to MATra-A magnetofection reagent (PromoKine, Heidelberg, GER). Specific activation of TLR3 was achieved by adding 5 µg/ml polyI:C to cell culture medium. Cells were incubated with all polyI:C and 5' pU HCV PAMP treatments for 24 hours.

REAL TIME REVERSE TRANSCRIPTION (RT)-POLYMERASE CHAIN REACTION (PCR)

mRNA was harvested and extracted from cells using the Qiagen RNeasy Mini Kit (Qiagen, Germantown, MD) and converted into cDNA via the SuperScript III First Strand RT Kit (Invitrogen). Equivalent amounts of RNA were loaded into each cDNA reaction. RNA concentration was determined using Quant-iT RiboGreen RNA Assay kit (Invitrogen).

Real-time qPCR was performed on the Viia7 Real-Time PCR machine utilizing TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA). TaqMan FAM-MGB Gene Expression assays (Applied Biosystems) were used to amplify JFH-1 HCV, CXCL10, CCL4, IFN-α2, IFN-β, IL-28B, and IL-29 transcripts. All chemokine and cytokine data are reported as fold change derived from ΔΔCt using GAPDH as an endogenous control [10]. Absolute quantification of JFH-1 HCV genomes was achieved using a standard curve (0-10^7 copies JFH-1 HCV plasmid provided by Takaji Wakita [National Institute of Infectious Diseases]) to generate JFH-1 RNA copy numbers based on cycle threshold.

Microfluidic real-time RT-PCR on was performed using a BioMark 48.48 Dynamic- Array and the Fluidigm BioMark HD system (Fluidigm Corporation, South San Francisco, CA). cDNA was pre-amplified using Bio-X-Act Short Mix (Bioline, Taunton, MA) and the resultant reactions were run in microfluidic real-time qPCR using TaqMan Universal PCR Master Mix (Applied Biosystems). TaqMan FAM-MGB Gene Expression assays were used in both protocols. Targeted genes are listed in Table 2.2. Data are reported for each target gene both as raw Cts and as fold change over untreated cells.

Gene	Classification	Gene	Classification
CCL2	Chemokine/Cytokine	CSF1R	Lineage (Macrophage)
CCL5	Chemokine/Cytokine	EMR1	Lineage (Macrophage)
CXCL9	Chemokine/Cytokine	CCR2	Lineage (Monocyte)
CXCL10	Chemokine/Cytokine	CD14	Lineage (Monocyte)
CXCR3	Chemokine/Cytokine	CX3CR1	Lineage (Monocyte)
IFNA1	Chemokine/Cytokine	FCGR3A/B	Lineage (Monocyte)
IFNAR2	Chemokine/Cytokine	ITGAM	Lineage (Monocyte)
IFNB	Chemokine/Cytokine	CIITA	M1 Macrophage Phenotype
IFNG	Chemokine/Cytokine	IL6	M1 Macrophage Phenotype
IL1A	Chemokine/Cytokine	IL12B	M1 Macrophage Phenotype
IL1B	Chemokine/Cytokine	MARCO	M1 Macrophage Phenotype
IL10	Chemokine/Cytokine	NOS2	M1 Macrophage Phenotype
IL12A	Chemokine/Cytokine	PTGS2	M1 Macrophage Phenotype
IL12RB1	Chemokine/Cytokine	SOCS3	M1 Macrophage Phenotype
IL18	Chemokine/Cytokine	ARG1	M2 Macrophage Phenotype
IL18BP	Chemokine/Cytokine	CXCL13	M2 Macrophage Phenotype
IL23A	Chemokine/Cytokine	IRF4	M2 Macrophage Phenotype
IL29	Chemokine/Cytokine	KLF4	M2 Macrophage Phenotype
TNF	Chemokine/Cytokine	SOCS2	M2 Macrophage Phenotype
TNFSF9	Chemokine/Cytokine	CLEC4f	Kupffer Cell Phenotype
TNFSF10	Chemokine/Cytokine	CD86	Kupffer Cell Phenotype
CD209	Lineage (DC)	IDO1	Leukocyte Phenotype
CD8a	Lineage (T)	GAPDH	Reference
ITGAX	Lineage (DC)	HPRT	Reference

Table 2.2: Genes Profiled By Fluidigm Microfluidic Real-Time qPCR Array. Forty-eight unique genes were selected to characterize infiltrating and resident immune cell markers as well as cytokine responses in primary hepatocyte cultures exposed to JFH-1 HCV infection. Gene names are based on the HUGO Gene Nomenclature Committee classification and represent their official name.

LUMINEX BEAD ARRAYS

Supernatants of treated cells were collected and infectious supernatants inactivated with 1% Triton-X. Samples were centrifuged at 14,000 RPM prior to storage at -80°C. Samples were tested for CXCL10, CXCL8, CCL4, and CCL11 using polystyrene Luminex Antibody Bead kits (Biosource/Invitrogen) and the Luminex 200 system according to the manufacturer's protocol (Luminex, Austin, TX).

WESTERN BLOTTING

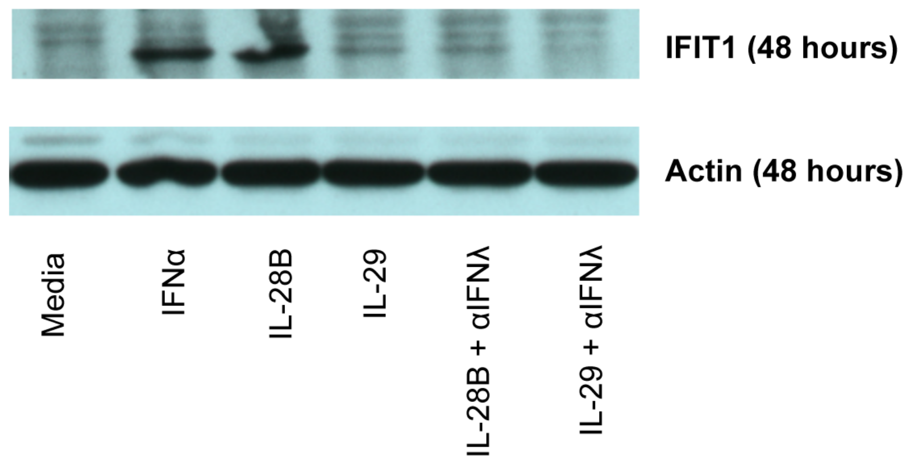
Whole cell lysates were collected in RIPA buffer and protein was quantified using BCA Protein Assay (Pierce/ThermoFischer Scientific). Proteins were run on Novex Tris-Glycine 4-20% gels (Invitrogen). For detecting TLR3 and RIG-I expression, cytoplasmic lysates in RIPA buffer were run on NuPAGE Bis-Tris 4-12% gels (Invitrogen). Following transfer to a nitrocellulose membrane, target proteins were detected with the following antibodies: rabbit anti-TLR3 [4], rabbit anti-RIG-I (provided by Tadaatsu Imaizumi) [11], rabbit anti-IFIT1 (Pierce), goat anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA). HCV proteins were detected using random, de-identified HCV-infected patient serum as previously described [12]. HRP-conjugated secondary antibodies were purchased from Pierce and Santa Cruz Biotechnology.

siRNA-MEDIATED KNOCKDOWN

ON-TARGET*plus* siRNA SMART pools targeting TLR3 and RIG-I, siGLO Lamin A/C siRNA and siGENOME Non-Targeting siRNA Pool 2 were purchased from Dharmacon (ThermoFischer Scientific). 100 nM siRNAs were transfected into PH5CH8 cells plated at 250,000 cells/ml using MATra-A. 5' pU HCV PAMP and exogenous polyI:C treatments (see above) were added 24 hours post-transfection. Supernatants and cellular RNA were harvested 48 hours post-transfection as described above for Luminex Bead Arrays and real-time RT-PCR respectively.

INTERFERON NEUTRALIZATION ASSAYS

Cells were cultured with or without HCV (MOI 0.5) for 5 hours to allow virus adsorption. Infectious media was then replaced with either normal media, media containing 2 μg/ml B18R protein (eBioscience, San Diego, CA) for type I IFN neutralization, or media containing 4 μg/ml IL-28B/IL-29 neutralizing antibody (R&D Systems, Minneapolis, MN; MAB15981) for type III IFN neutralization. IL-28B/IL-29 neutralizing antibody was tested for effectiveness against IL-28B and IL-29 (Figure 2.1). Twenty ng/ml pegylated-IFN-α2 (Pegasys©; Genentech, South San Francisco, CA) and 1 ng/ml recombinant IL-28B (R&D Systems) served as positive controls for type I and type III IFN induction respectively, and were added 30 minutes after the addition of the neutralization agents. Whole cell protein lysates, supernatants, and mRNA were harvested 24 and 48 hours post-infection and analyzed as described above.



<u>Figure 2.1:</u> IL-28B/IL-29 Neutralizing Antibody is Effective Against Both IL-28B and IL-29. IFIT1 protein was induced at 48 hours following treatment with 1 ng/ml IL-28B or IL-29. This induction was diminished when 4 ng/ml pan type III IFN neutralizing antibody (αIFNλ) was co-administered with either IFN treatment.

IMMUNOFLUORESCENCE

Cells were cultured on 8-chamber well slides (Nunc/ThermoFischer Scientific) and infected with JFH-1 HCV (MOI 0.5) as described above for 72 hours or treated with 100 ng/ml IFNγ in combination with 40 ng/ml Tumor Necrosis Factor-α (TNFα) for 24 hours [13]. Brefeldin A (VWR International, Radnor, PA) was added at 1 μg/ml during the last 5 hours of infection to prevent export of secreted proteins. Cells were fixed in 4% paraformaldehyde and blocked with 3% normal bovine serum (Jackson ImmunoResearch, West Grove, PA) prior to immunostaining. Primary antibodies included mouse anti-HCV Core (Pierce/ThermoFischer Scientific) and goat anti-CXCL10 (R&D Systems). Secondary antibodies included sheep anti-mouse conjugated to Dy488 (Jackson ImmunoResearch) and bovine anti-goat HRP secondary antibody (Santa Cruz Biotechnology). CXCL10 signal was further amplified using the PerkinElmer TSA-Plus Biotin Kit (PerkinElmer, Waltham, MA) combined with Cy3-

labeled streptavidin (Jackson ImmunoResearch). Nuclei were stained with DAPI and mounted using ProLong Gold Anti-Fade Reagent (Invitrogen). Slides were visualized with a 3i Marianas widefield deconvolution microscope at 40x magnification under a 10x ocular and full frame 1392x1040 pixel dimensions (Intelligent Imaging Innovations, Ringsby, CT).

CXCL10 and HCV Core protein abundance was quantified with CellProfiler (Version r11710; www.cellprofiler.org) [14]. A four-class image classifier was then constructed for unbiased quantitative scoring on a single cell basis with CellProfiler Analyst (www.cellprofiler.org), where the abundance of cytoplasmic CXCL10 in each individual cell is quantified as a function of its HCV Core abundance. CellProfiler then quantified the direct relationship of HCV Core to CXCL10 in each individual cell by first identifying nuclei as primary objects using the DAPI image channel. Cytoplasmic CXCL10 immunostaining was then designated as the secondary object image channel. HCV Core protein immunostaining was designated as the tertiary object channel, which is defined as the cytoplasmic area bounded by each cells' previously defined membrane and nuclear compartments. A four-class image classifier was then generated with the aid of a gentle boosting-based supervised machine learning classifier module in CellProfiler Analysis r1123011246 using the previously generated CellProfiler measurement SQLite database [15]. Classification rules scored each cell as a member of one of four expression classes (Table 2.3).

Expression Class	HCV Core Signal	CXCL10 Signal	Notes
HCV Core+/CXCL10+	High	High	Expected of virus-induced signaling
HCV Core-/CXCL10+	Low	High	Expected of paracrine signaling
HCV Core+/CXCL10-	High	Low	Not expected
HCV Core-/CXCL10-	Low	Low	Expected of uninfected cell

Table 2.3: CellProfiler Expression Classes for Quantifying HCV Core and CXCL10. Cells were sorted into one of four expression classes based on intracellular abundance of HCV Core and CXCL10. Quantification of immunofluorescent signal was performed with CellProfiler (Version r11710; www.cellprofiler.org). Classification rules were developed in CellProfiler Analyst (www.cellprofiler.org).

IMMUNODEPLETION OF NON-PARENCHYMAL CELLS

Freshly thawed primary human hepatocytes were combined 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 incubated for 10 minutes at 4°C. Cell/antibody complexes were then incubated with MACS anti-biotin-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA) for 15 minutes before being applied to a magnetic MACS Cell Separation column (Miltenyi Biotec) for separation. Column was washed 3 times with PHH Plating Media and all fractions were collected. These non-adhered cells were spun down, re-suspended, and plated following standard culture protocols (see Supplemental Materials and Methods). The column was then removed from the magnet and washed with PHH Plating Media once more to isolate adherent cells. Fluidigm real-time RT-PCR panel (see above) was run on pre-depletion, post-depletion, and adhered cell mRNA samples to assess efficiency of NPC removal.

LUCIFERASE ASSAYS

For PAMP-based activation, 50 ng of each CXCL10 Firefly luciferase reporter plasmid (see above) was transfected into cells using MATra-A. After 24 hours, exogenous polyI:C or transfected 5' pU HCV PAMP was added to designated wells as described above. PBS and 100 ng/ml IFNγ in combination with 40 ng/ml TNFα were also added as negative and positive controls respectively. Luciferase activity was read after an additional 24 hours using BriteLite reagent (PerkinElmer). For JFH-1 HCV-based activation, CXCL10 plasmids were transfected into cells using X-treme Gene 9 (Roche, Indianapolis, IN). Cells were then infected 48 hours post-transfection with JFH-1 HCV (MOI 1) or treated with IFNγ/TNFα or PBS as above. Luciferase activity was read after an additional 24 hours using BriteLite reagent. Cell viability in each well during infection was assessed via Cell Titer Fluor (Promega) and used to normalize luciferase readings.

For direct activation via IRF3, 50 ng of IRF3-5D plasmid or pcDNA3.1 was co-transfected with 50 ng of the wild-type or ΔISRE CXCL10 reporter plasmid into cells using MATra-A. Immediately after transfection 2 μg/ml B18R, 4 μg/ml IL-28B/IL-29 neutralizing antibody, or PBS was added to the culture media. Luciferase activity was read after an additional 24 hours using BriteLite reagent and were normalized for cell viability as above.

CHROMATIN IMMUNOPRECIPITATION (ChIP)

$6.6x10^6$ cells were plated in 10 cm dishes in standard Huh7 media. After 24 hours cells were mock infected, infected with SeV for 6 hours, or infected with JFH-1 HCV for 12 or 18 hours (MOI 0.6; see above for protocol). Chromatin was harvested following formaldehyde-fixation and sheared by sonication using a probe-Ultrasonic Homogenizer (Cole-Parmer Instrument Co; 4710 series). An aliquot from each sheared chromatin sample was then purified via ethanol precipitation and quantified using Quant-iT PicoGreen DNA Assay kit (Invitrogen). This sample was also run on a 1% TAE gel alongside the 1KB-plus DNA ladder (Invitrogen) to assess shearing efficacy.

ChIP was performed using the ChIP Express Chromatin Immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 15 ug of each chromatin preparation was incubated overnight with Protein G-coated magnetic beads in combination with polyclonal rabbit anti-IRF3 sera (Active Motif) or normal rabbit sera (Jackson ImmunoResearch) at equivalent concentrations. Protein-chromatin complexes were eluted off magnetic beads after several washes and the formaldehyde-induced cross-links reversed. Proteins were then degraded by Proteinase K to produce purified chromatin fragments. Additional DNA from each sheared chromatin sample that was not immunoprecipitated ("Input DNA") was also processed to remove bound proteins.

Chromatin fragments were PCR amplified using Phusion Hot Start II High-Fidelity DNA Polymerase (ThermoFischer Scientific) and primers directed against the ISRE region of the CXCL10 promoter (Forward: 5'-TGGATTGCAACCTTTGTTTTT-3'; Reverse: 5'-GTCCCATGTTGCAGACTCG-3'). Input DNA samples and a no template

reaction were included as positive and negative controls respectively. Reactions were run for 35 cycles using a Veriti 96-well Thermocycler (Applied Biosystems) and the cycling parameters suggested by the manufacturer for the Phusion DNA polymerase (Melting Temperature: 64°C). PCR products were resolved on a 1% TBE agarose gel and visualized using a GelDoc (BioRad, Hercules, CA). Bands were quantified using ImageJ (Version 1.46; http://rsb.info.nih.gov/ij/).

STATISTICAL METHODS

All data reported for immortalized cell lines is representative of results from at least two independent experimental repeats are presented as mean ± standard deviation. Experiments utilizing primary hepatocytes were repeated using cells from at least two donors, and representative data are presented as mean ± standard deviation. Statistical analysis of Luminex protein and luciferase reporter data was predominantly conducted in Prism (GraphPad Software, Inc) using standard Student's t tests for determining significance. Chemokine and cytokine fold change data from real-time RT-PCR experiments was presented as ranges of fold changes, with overlapping ranges indicating non-significance.

For population-based analysis of immunofluorescence data, an F-test was used to estimate the fit of the least squares regression. Due to differences in variance, a Welch's two-sample t test was used to determine if differences between CXCL10 signal in HCV positive and HCV negative cells was significant. All tests were performed in R (www.r-project.org).

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CHAPTER 3:

EXPRESSION AND FUNCTIONALITY OF TOLL-LIKE RECEPTOR 3 (TLR3) AND RETINOIC ACID INDUCIBLE GENE I (RIG-I) IN HEPATOCYTES AND HEPATOCYTE CELL LINES

"A PROCESS CANNOT BE UNDERSTOOD BY STOPPING IT. UNDERSTANDING MUST MOVE WITH THE FLOW OF THE PROCESS, MUST JOIN IT AND FLOW WITH IT."

- FRANK HERBERT, *DUNE*

OVERVIEW

This chapter explores the extent of Toll-like Receptor 3 (TLR3) and Retinoic Acid Inducible Gene - I (RIG-I) expression and functionality in primary human hepatocytes (PHH) and hepatocyte-derived cell lines. Specifically, it investigates whether treatment with TLR3- and RIG-I-specific Pathogen Associated Molecular Patterns (PAMPs) leads to induction of cytokines and chemokines, including CXCL10. Our results indicate that PHH and hepatocyte-derived cell lines are capable of producing pro-inflammatory and anti-viral responses following treatment with PAMPs. While variations in induction intensity were observed between cell types, responses were shown to be TLR3- and RIG-I specific. These experiments demonstrate that cell culture systems that are commonly used to investigate elements of hepatitis C virus (HCV) infection *in vitro*, and which were used in experiments described in later chapters, contain functional versions of the pathways being investigated.

Portions of this chapter's Results section have been submitted to the *Journal of Hepatology* as supplemental material in the revised manuscript entitled "Independent, Parallel Pathways to CXCL10 Induction in HCV-Infected Hepatocytes".

INTRODUCTION

Innate immune signaling within hepatocytes represents the first line of defense for the host against hepatitis C virus (HCV) infection. Activation of these pathways can trigger non-specific immune effector mechanisms (ex. oxidative stress) as well induce the production of chemokines and cytokines to respectively recruit and activate leukocytes (NK cells, macrophages, dendritic cells etc) and lymphocytes (ex. T and B cells) at the site of infection within the liver [1,2]. Activation of these crucial signaling pathways depends upon recognition of conserved HCV motifs known as pathogen-associated molecular patterns (PAMPs) by innate pattern recognition receptors (PRRs) [2,3]. TLR3 and RIG-I are two PRRs expressed by hepatocytes that are known to recognize HCV PAMPs *in vitro* [4-6].

TLR3 recognizes double-stranded RNAs (dsRNAs) found within the endosome. These PAMPs are generated during replication of RNA viruses like HCV and are thought to re-localize to the endosome through autophagy [5]. Alternatively, dsRNAs found outside of the cell can activate TLR3 after being taken up via endocytosis [7]. N-terminal leucine-rich repeat (LRR) sequences within TLR3 bind dsRNAs and induce dimerization. The TLR3 homodimer then binds to the cytosolic TIR-domain-containing adapter-inducing interferon-β (TRIF) through its cytoplasmic Toll/Interleukin-1 receptor (TIR) domains [7]. The exclusive use of TRIF makes TLR3 unique among the TLRs, which generally signaling through a different adaptor known as myD88 [8]. However, signaling from TRIF triggers a signaling cascade that leads to induction of various transcription factors including Interferon Regulatory Factor (IRF)-3, IRF-7, NF-kB and

AP-1 [8,9]. IRFs in turn induce production of anti-viral type I and type III IFNs while NF-kB and AP-1 induce pro-inflammatory cytokines [8].

Similarly, RIG-I is a cytoplasmic sensor of double-stranded RNAs or 5' tri-phosphate RNAs containing poly-U or poly-A motifs [10,11]. Saito *et al* identified the highly conserved polyU/UC-containing 3' UTR of the hepatitis C virus (HCV) genome as the main PAMP recognized by RIG-I in the context of a 5' tri-phosphate [10]. The HCV 3' UTR is bound by basic residues within the C-terminal Repressor Domain in conjunction with the central DExD/H-box helicase/ATPase domain [11,12]. The helicase function of the DExD/H-box domain causes RIG-I to translocate along the bound RNA, inducing a conformational change that exposes the tandem N-terminal Caspase Recruitment Domains (CARDs) in an ATP-dependent manner [12-14]. This activated RIG-I then dimerizes, and the dimerized CARDs bind identical domains found in the mitochondrial-bound adaptor MAVS (Mitochondrial Antiviral-Signaling protein). MAVS is a membrane-bound protein typically localized to the mitochondria, although it has been reportedly found anchored in other membrane-bound vesicles within the cytoplasm (e.g. peroxisomes) [15]. Activation of MAVS also leads to the activation of IRF3, NF-κB and other pro-inflammatory and anti-viral transcription factors, triggering the induction of innate immune genes as described above. It has also been suggested that CARD binding of unanchored K63-polyubiquitin chains in the presence of RNA PAMP and ATP further enhances this signaling [16].

Due to the overlapping and broad-reaching signaling capacity of these PRRs, both TLR3 and RIG-I play a central role in raising the initial innate immune response against HCV. However, HCV has several mechanisms for inhibiting their signaling and

allowing persistence of the infection. The most predominant of these is the degradation of both TRIF and MAVS by the HCV NS3/4a protease, which prevents the downstream induction of pro-inflammatory and anti-viral genes as described above. Despite this negative regulation, intrahepatic and serum levels of the PRR-induced pro-inflammatory chemokine CXCL10 are elevated in hepatitis C patients who are refractory to pegylated interferon-α (IFNα)/Ribavirin therapy [17-19]. Understanding how TLR3 and RIG-I signaling specifically contributes to CXCL10 induction within the hepatocyte during the early phases of HCV infection may uncover the mechanism for this paradoxical phenomenon (i.e. CXCL10 induction during NS3/4a blockade of TLR3 and RIG-I signaling) and reveal host-oriented targets for novel hepatitis C therapies.

While the importance of these signaling pathways in CXCL10 induction has been previously demonstrated in hepatocyte-like progenitor cells, TLR3 and RIG-I signaling to CXCL10 induction has yet to be examined in cell culture systems amenable to hepatitis C virus (HCV) infection [20]. Additionally, the full functionality of these signaling pathways has not yet been comparatively evaluated between immortalized and primary hepatocytes. Therefore, we sought to comparatively evaluate the expression and signaling capacity of both the TLR3 and RIG-I pathways in several immortalized hepatocyte cell lines as well as in PHH and examine the roles these PRRs play in CXCL10 induction in these cells. The intention was to establish the signaling capacity of these cells for additional *in vitro* comparison studies of HCV infection and CXCL10 induction. Herein, we demonstrate that both TLR3 and RIG-I are expressed by PHH as well as by a variety of immortalized hepatocyte cell lines, but that their capacity to induce production of chemokines and cytokines varies significantly between cell types.

We also demonstrate that CXCL10 is inducible in immortalized and primary hepatocytes following activation of TLR3 and RIG-I, that this activation is specific, and that synergistic induction of CXCL10 is limited in cell types without PRR overexpression.

MATERIALS AND METHODS

Detailed protocols, reagents, and statistics are included in Chapter 2 (pg 29).

Cells

Human hepatoma Huh7 (TLR3-/RIG-I+) cells were obtained from Apath, LLC [21]. Huh7.5.1 (TLR3-/RIG-I-) cells were obtained from Francis Chisari (Scripps Research Institute) [22]. Additional Huh7 and Huh 7.5 cells obtained from Kui Li (University of Tennessee at Memphis) were stably transduced with a FLAG-TLR3-encoding lentiviral vector (TLR3+/RIG-I+ and TLR3+/RIG-I-cells, respectively) [23]. PH5CH8 immortalized hepatocytes were obtained from Noboyuki Kato (Okayama University) [21]. Primary hepatocyte cultures were either obtained cryopreserved from Invitrogen (Carlsbad, CA) or obtained fresh from Dr. Steven Strom (University of Pittsburgh) through the NIH Liver Cell and Tissue Distribution System (LCTDS)). Human hepatocyte cultures from the LTCDS were harvested under an IRB exemption approval from the University of Pittsburgh to Dr. Strom (letter on file) as surgical waste tissue. All cells were maintained in culture as described in Chapter 2 (see pg 30).

Pathogen Associated Molecular Patterns (PAMPs)

Specific activation of RIG-I was achieved by infecting cells with 100 hemagglutination units (HAU) of Sendai Virus (SeV; Cantrell Stain; Charles River Laboratories [Wilmington, MA]) according to the protocol detailed in Chapter 2 (pg 32), by transfecting cells with 0.5 µg polyI:C (Amersham, now GE Healthcare Life Sciences [Pittsburgh, PA]), or by transfecting cells with 0.5 µg 5' pU HCV PAMP RNA (provided by Michael Gale Jr. [University of Washington]). Specific activation of TLR3 was achieved by adding 5 µg/ml polyI:C to cell culture medium. Cells were incubated with all polyI:C and 5' pU HCV PAMP treatments for 24 hours.

Real-time Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

Real-time RT-PCR was performed on cellular mRNA using TaqMan Gene Expression Assays (Applied Biosystems, Calsbad, CA) for detection of CXCL10, CCL4, IFN-α2, IFN-β, IL-28B, and IL-29 transcripts. Chemokine and cytokine data are reported as fold change derived from ΔΔCt using GAPDH as an endogenous control [24].

Luminex Bead Arrays

Supernatants of treated cells were collected and centrifuged at 14,000 RPM prior to storage at -80°C. Samples were tested for CXCL10, CCL4, CCL11, and CXCL8 using polystyrene Luminex Antibody Bead kits (Biosource/Invitrogen) and the Luminex 200 system according to the manufacturer's protocol (Luminex, Austin, TX).

Western Blotting

Cytoplasmic lysates were collected in RIPA buffer and run on NuPAGE Bis-Tris 4-12% gels (Invitrogen). Target proteins were detected with the following antibodies: rabbit anti-TLR3 [23], rabbit anti-RIG-I (provided by Tadaatsu Imaizumi) [25], goat anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA). HRP-conjugated secondary antibodies were purchased from Pierce and Santa Cruz Biotechnology.

siRNA-Mediated Knockdown

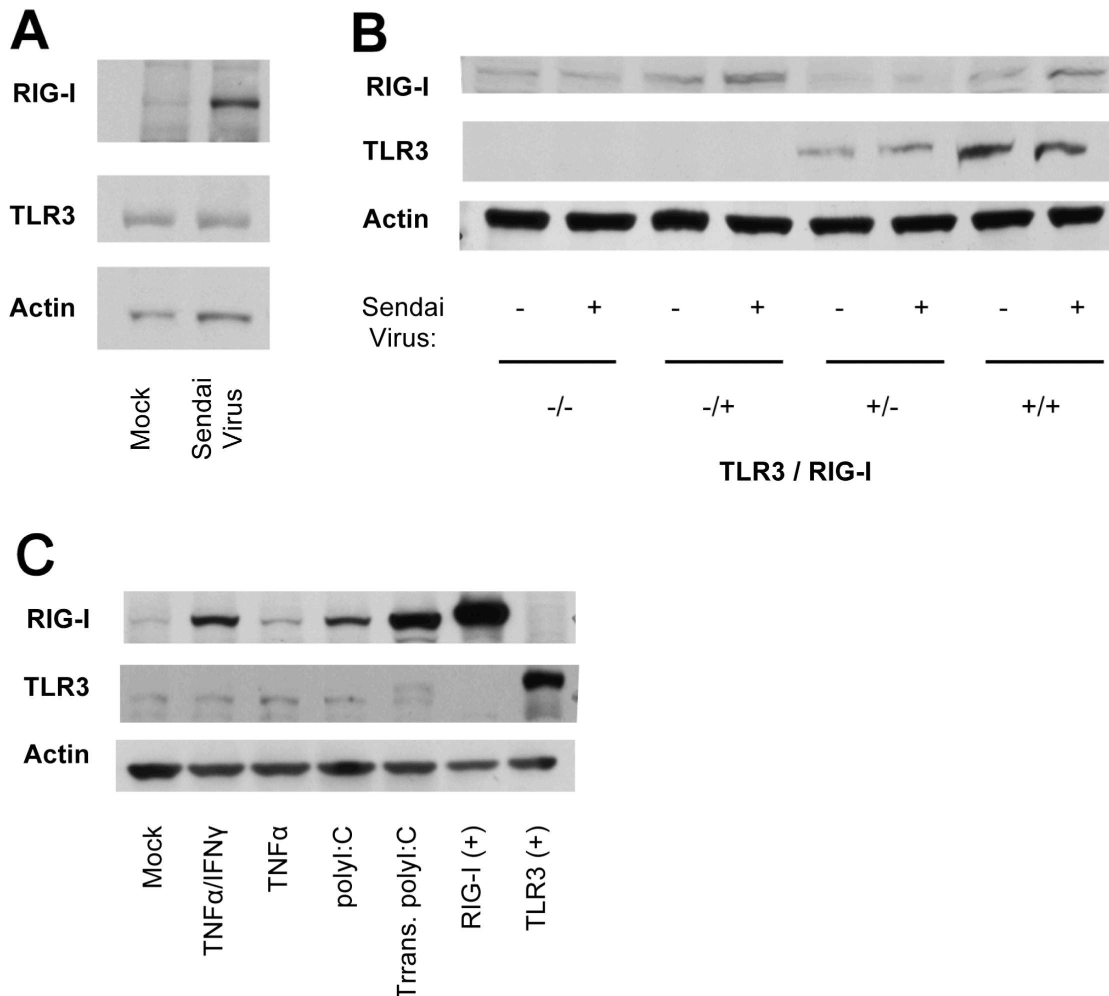
ON-TARGET*plus* siRNA SMART pools targeting TLR3 and RIG-I, siGLO Lamin A/C siRNA and siGENOME Non-Targeting siRNA Pool 2 were purchased from Dharmacon (ThermoFischer Scientific). 100 nM siRNAs were transfected into PH5CH8 cells using MATra-A. 5' pU HCV PAMP and exogenous polyI:C treatments (see above) were added 24 hours post-transfection. Supernatants and cellular RNA were harvested 48 hours post-transfection as described above for Luminex Bead Arrays and real-time RT-PCR respectively.

RESULTS

TLR3 and RIG-I Expression and Functionality in Hepatocyte Cultures

The TLR3 and RIG-I signaling pathways are attractive targets for studying the early and immediate-early innate immune response to HCV infection because they are known to sense HCV PAMPs and are targeted for inhibition by viral proteins during chronic infection [2,26,27]. In order to evaluate the potential capacity for TLR3 and RIG-I signaling in culture systems commonly used for *in* vitro HCV research, we compared

the endogenous protein expression level of both PRRs in these systems. Whole cell lysates prepared from PHH and from hepatocyte-derived immortalized cell lines were probed for TLR3 and RIG-I via Western Blot (Figure 3.1). Expression of both receptors was detected in PHH, with RIG-I expression increasing following 24-hour exposure to a known viral RIG-I PAMP (Sendai Virus [SeV]; Figure 3.1A). Receptor expression in a series of Huh7-derived hepatoma cell lines correlated with their declared differential expression of endogenous RIG-I and transduced FLAG-TLR3 (Figure 3.1B; [23]). The faint RIG-I bands detectable in the two RIG-I-negative cell lines derived from the functionality inactive form of RIG-I expressed by these cells [22]. Indeed, the intensity of this band does not increase following SeV infection, unlike in PHH or in the two RIG-I-positive cell lines. Expression of TLR3 does not appear to be affected by SeV infection in these cells.



<u>Figure 3.1:</u> TLR3 and RIG-I Expression in Immortalized and Primary Human Hepatocytes. (A) PHH and **(B)** four Huh7-hepatoma derived cell lines that differentially express TLR3 and RIG-I expressed these PRRs at baseline and following SeV infection (100 HAU; 24 hours). **(C)** PH5CH8 immortalized hepatoctes expressed TLR3 and RIG-I at baseline and following 24 hour stimulation with cytokines (TNFα/IFNγ and TNFα) and exogenously-added or transfected polyI:C (polyI:C and Trans. polyI:C). Lysates from cells expressing constitutively active TLR3 (TLR3 (+)) and RIG-I (RIG-I (+)) were included as positive controls.

TLR3 and RIG-I were also faintly detected in PH5CH8 immortalized hepatocytes (Figure 3.1C). Unlike Huh7-derived cell lines, the PH5CH8 cell line endogenously expresses functional copies of both PRRs [21]. RIG-I expression was strongly induced in these cells following transfection of polyI:C (a RIG-I PAMP), treatment with exogenous polyI:C added to the culture media (a TLR3 PAMP), or treatment with a

combination of TNFα and IFNγ for 24 hours. The molecular weight of these bands was comparable to the positive control band produced by cells transiently transfected with a plasmid that constitutively expressed RIG-I (Figure 3.1C, Lane 6). In contrast, TLR3 expression appeared to only slightly increase with 24 hour TNFα or polyI:C treatment in these cells. The molecular weight of these bands also appears to be slightly less than that of the positive control band (Figure 3.1C, Lanes 3,5, and 7). This small discrepancy is likely due to fact that the positive control band was derived from cells transiently expressing a plasmid encoding a FLAG-tagged TLR3, while the other bands were produced from cells expressing endogenous, untagged TLR3. Thus, we observed TLR3 and RIG-I expression in several different hepatocyte-derived culture systems used to study HCV infection *in vitro*.

We next evaluated whether the forms of TLR3 and RIG-I expressed in these cells could induce innate immune signaling following activation (Figure 3.2). To determine the capacity for general pro-inflammatory chemokine induction in immortalized hepatocytes, supernatants were collected from Huh7-derived cells expressing both TLR3 and RIG-I (TLR3+/RIG-I+ Huh7 cells) and PH5CH8 cells following 24 hours of SeV infection or treatment with exogenous-added polyI:C and run on the multiplex Luminex bead array platform to detect secreted CXCL8, CCL4, and CCL11 (Figure 3.2A,B). TLR3+/RIG-I+ Huh7 cells had high background expression of CXCL8, with polyI:C treatment further inducing expression ($p<0.01$; Figure 3.2A). Significant CCL4 expression was observed following both polyI:C treatment and SeV infection in these cells ($p<0.01$). In PH5CH8 cells, only SeV infection induced CCL4 expression ($p<0.01$) while both SeV infection

and polyI:C treatment induced CXCL8 expression above background levels (p<0.01 and p<0.05 respectively; Figure 3.2B). CCL11 was not detected in either cell type (p>0.05).

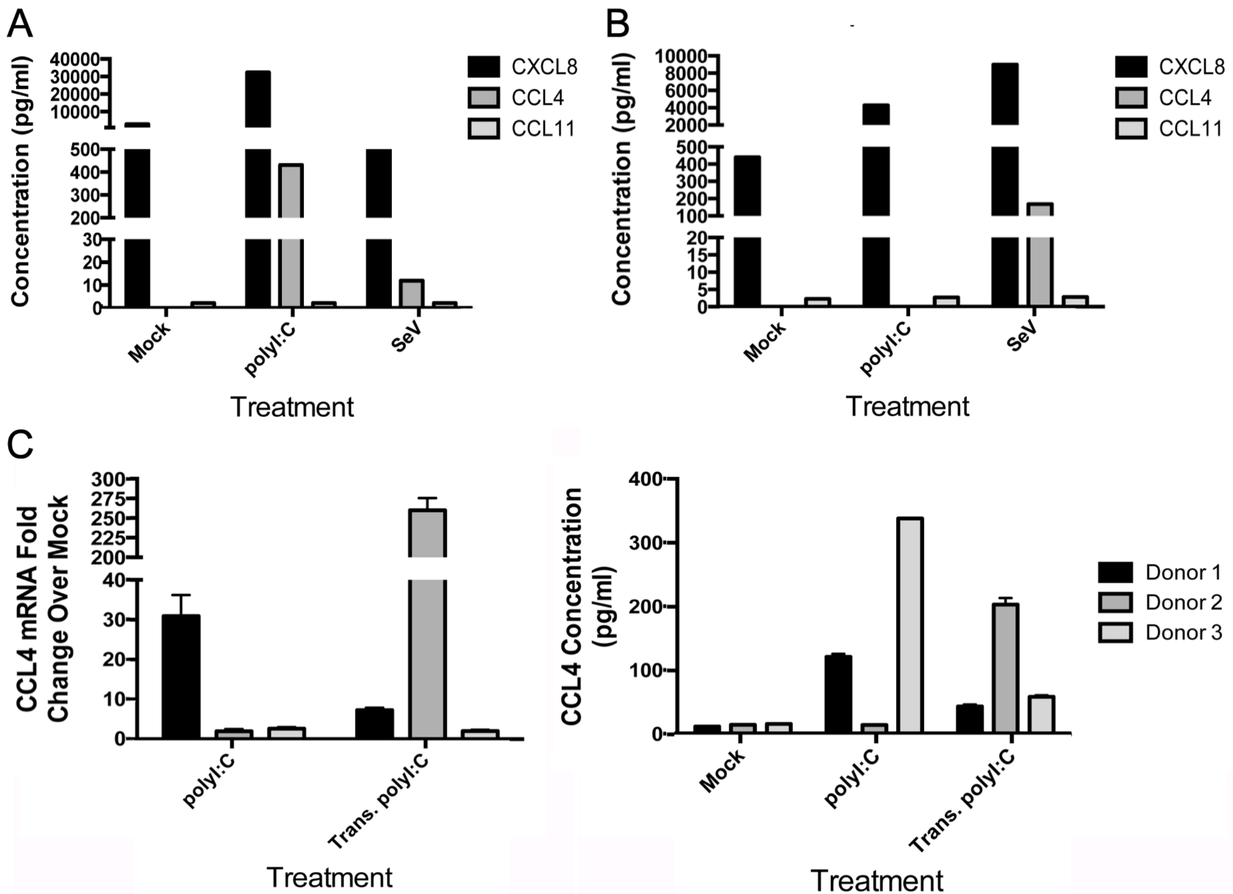
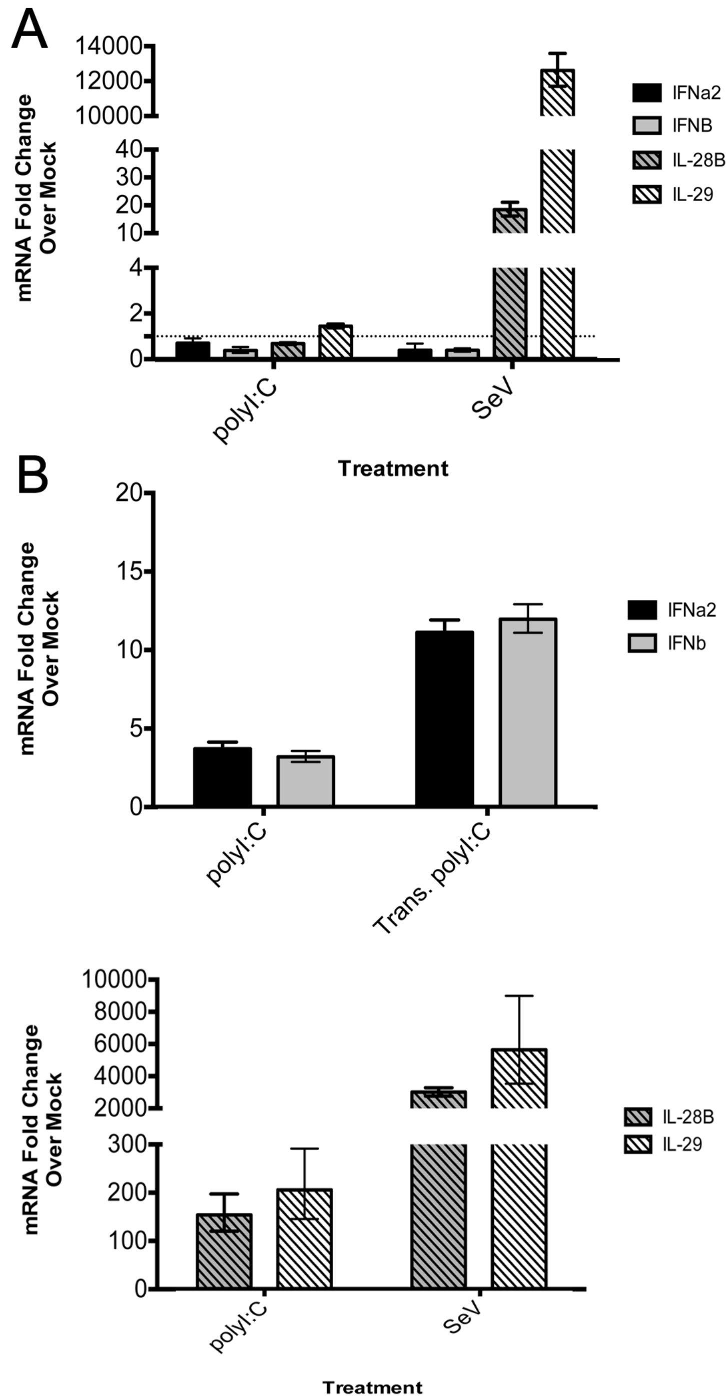


Figure 3.2: General Chemokine Induction Following Specific Activation of TLR3 and RIG-I. High levels of CXCL8 and CCL4 were secreted by **(A)** TLR3+/RIG-I+ Huh7 cells and **(B)** PH5CH8 cells following exposure to exogenously-added polyI:C (5 µg/ml) or SeV infection (100 HAU) for 24 hours. **(C)** CCL4 mRNA (left) and protein (right) induction was detected in PHH isolated from 3 independent donors following exposure exogenously-added or transfected polyI:C. Intensity of induction appeared to be donor-dependent.

Since CCL4 had the cleanest induction profile in immortalized cells, we also used it to evaluate chemokine induction in PHH. CCL4 production was measured in PHH isolated from three different donors 24 hours following treatment with exogenous polyI:C

or following its transfection into the cytoplasm (Figure 3.2C). Induction of CCL4 mRNA (Figure 3.2C, Left) and secreted protein (Figure 3.2C, Right) was observed in 2 out of 3 donors. Donor 1 produced maximal CCL4 in response to treatment with polyI:C, while maximum CCL4 was detected in Donor 2 following transfection of polyI:C. CCL4 protein was also detected in Donor 3 following transfection of polyI:C, although the corresponding mRNA was below the limit of detection for our real-time RT-PCR assay.

We also sought to evaluate the level of IFN production by immortalized and primary hepatocytes following TLR3 and RIG-I activation (Figure 3.3). Type I IFN production was measured in TLR3+/RIG-I+ Huh7 cells and PHH following exposure to exogenous polyI:C, SeV infection, or transfected polyI:C for 24 hours. Type III IFN production was measured following exogenous polyI:C treatment and SeV infection. Total cellular mRNA was harvested and real-time RT-PCR was used to detect induction of IFNα2, IFNβ, IL-28B (IFNλ3), and IL-29 (IFNλ1). In TLR3+/RIG-I+ Huh7 cells, both IL-28B and IL-29 were highly induced following SeV infection while IL-29 was marginally induced following polyI:C treatment (Figure 3.3A). Neither PAMP induced type I IFN production in these cells. In contrast, all four IFNs were detected in PHH following treatment with either TLR3 or RIG-I PAMPs (Figure 3.3B). Thus, while the profile of induced genes differs between primary and immortalized hepatocytes, all of the cell culture systems we evaluated are capable of generating an innate immune response following specific TLR3 and RIG-I activation.



<u>Figure 3.3:</u> Type I and III Interferon Induction Following Specific Activation of TLR3 and RIG-I. (A) TLR3+/RIG-I+ Huh7 cells produced detectable type III IFN but not type I IFN responses following treatment with exogenously-added polyI:C (5 µg/ml) or SeV infection (100 HAU) for 24 hours. **(B)** PHH generated a type I IFN response following 24 hour treatment with exogenously-added or transfected polyI:C (top) and a type III IFN response following treatment with exogenously-added polyI:C or SeV infection (100 HAU) for 24 hours.

CXCL10 Induction Following Treatment with TLR3 and RIG-I PAMPs

Hepatocytes from both humans and chimpanzees with chronic hepatitis C have been shown to produce CXCL10, and intrahepatic pre-treatment levels of this chemokine are elevated in patients who do not respond to pegylated IFN-α/Ribavirin therapy [17-19,28,29]. Therefore, we hypothesized that CXCL10 would also be produced by PHH in response to TLR3 and RIG-I PAMPs as part of the innate immune response.

Total cellular mRNA (Figure 3.4A, left) and supernatants (Figure 3.4A, right) were harvested from PHH obtained from two different donors following exposure to exogenous polyI:C or transfected polyI:C for 24 hours and analyzed for CXCL10 induction. As predicted, both donors showed significant induction in both CXCL10 mRNA and secreted protein following treatment with either PAMP. The mRNA response from Donor 1 was equivalent between the two PAMPs, while transfected polyI:C induced greater protein production. Conversely, exogenous polyI:C treatment yielded higher induction of both CXCL10 mRNA and secreted protein in Donor 2. Such donor-to-donor variation was also observed in the production of CCL4 (Figure 3.2C).

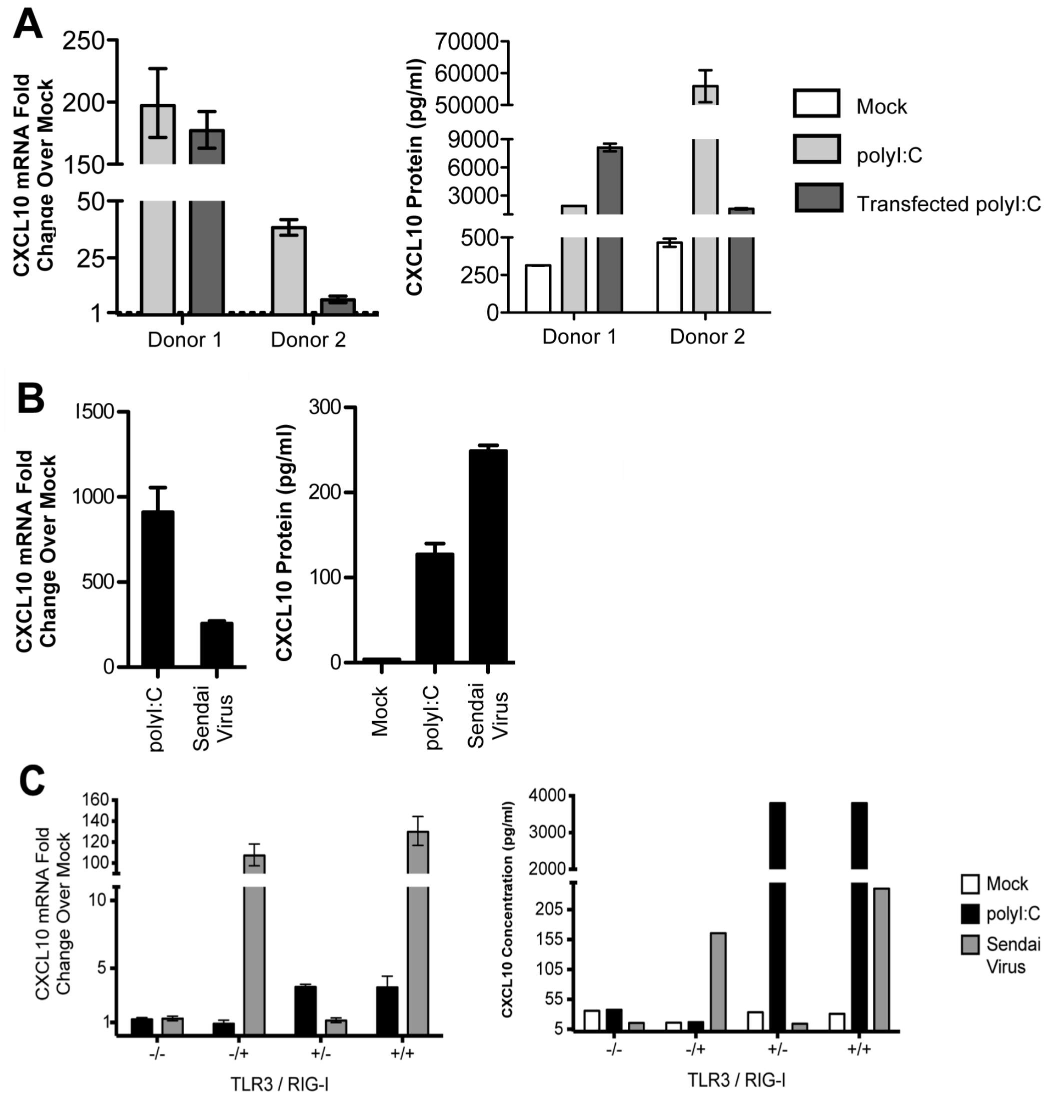
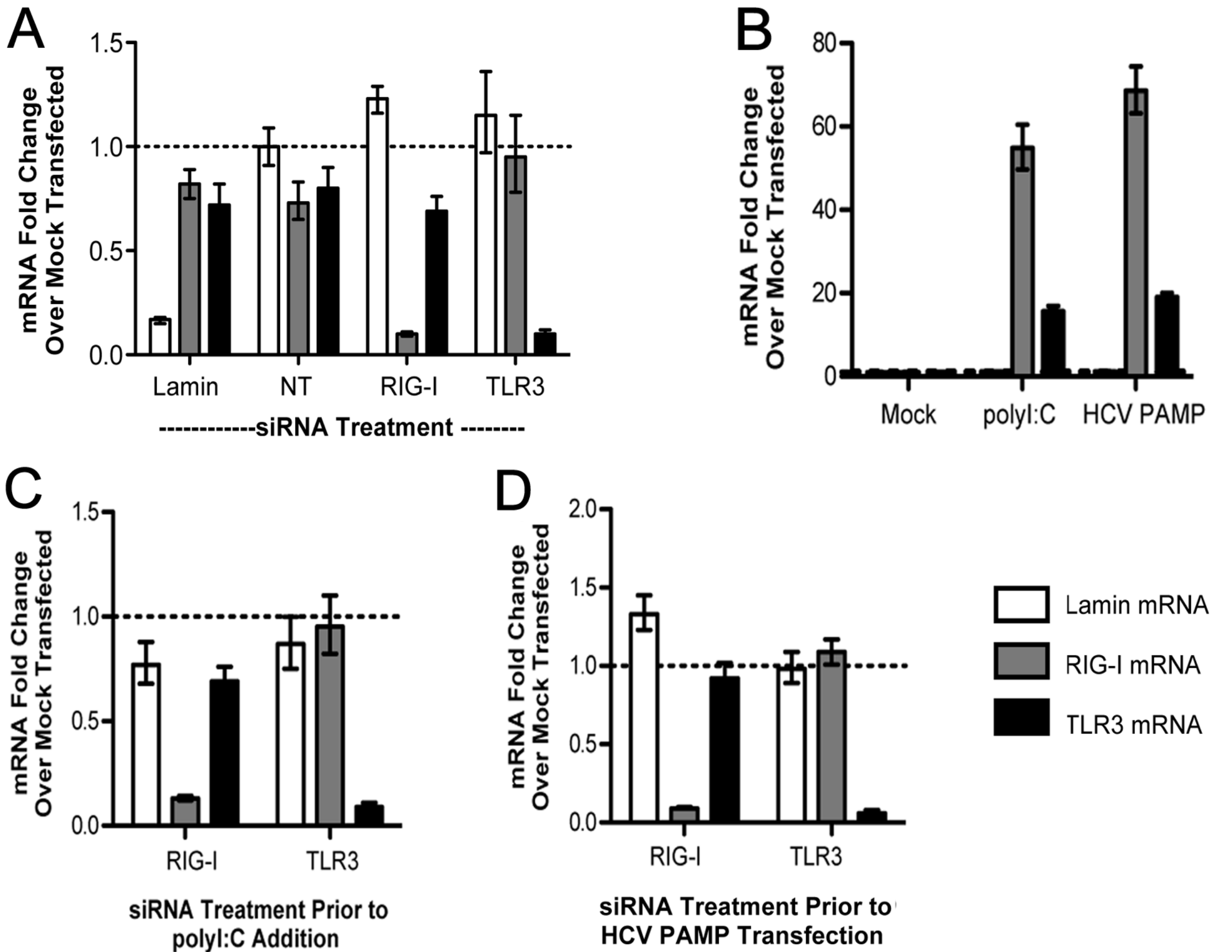


Figure 3.4: CXCL10 Induction Following Specific Activation of TLR3 and RIG-I. (A) PHH from two independent donors produced CXCL10 mRNA (left) and protein (right) following treatment with exogenously-added or transfected polyI:C for 24 hours. CXCL10 mRNA and protein were also induced in **(B)** PH5CH8 immortalized hepatocytes and **(C)** four Huh7-hepatoma derived cell lines that differentially express TLR3 and RIG-I following treatment with exogenously-added polyI:C or SeV infection (100 HAU) for 24 hours. CXCL10 expression to these stimuli among the Huh7-derived cell lines was PRR dependent.

For comparison we also examined CXCL10 induction following TLR3 and RIG-I activation in the four differentially expressing Huh7 cells lines and the immortalized PH5CH8 cell line. In concordance with our previous findings, both SeV infection and

treatment with exogenous polyI:C yielded induction of CXCL10 mRNA (Figure 3.4B, left) and secreted protein (Figure 3.4B, right) after 24 hours in PH5CH8 cells. These stimuli also induced CXCL10 mRNA and protein induction in the Huh7-derived cells lines in a receptor-dependent manner (Figure 3.4C). Thus, CXCL10 is induced in both PHH and hepatocyte-derived cell lines following activation of TLR3 and RIG-I.

To further confirm the specificity of this activation, we treated PH5CH8 cells with TLR3 or RIG-I PAMPs in the presence of siRNAs targeting each receptor. A 90% reduction of both PRRs was achieved (represented as a fold change in gene expression of 0.10 [range: 0.12-0.08] and 0.10 [0.11-0.09] over mock transfected cells for TLR3 and RIG-I respectively; Figure 3.5A) despite upregulation of both receptors during treatment with exogenous polyI:C or the RIG-I specific 5' pU HCV PAMP (Figure 3.5B-D). TLR3 knockdown reduced polyI:C induction of CXCL10 mRNA by 63% (fold change of 2601.54 [3070.21-2204.42] vs. 7065.73 [7422.72-6725.92] for mock transfected cells; Figure 3.6A). A 41% reduction in the CXCL10 response to polyI:C was also observed during RIG-I knockdown, possibly reflecting the 30% off-target reduction of TLR3 expression (see Figure 3.5A). RIG-I knockdown also resulted in a 54% reduction in the CXCL10 response to the 5' pU HCV PAMP (fold change of 2018.03 [2251.75-18080.57] vs. 4426.93 [4779.4-4100.46] for mock transfected cells; Figure 3.6B). TLR3 knockdown had no effect on the response to the HCV PAMP. These data indicate that our TLR3- and RIG-I-directed PAMPs are specifically activating their target PRRs, supporting their role in CXCL10 induction in hepatocytes.



<u>**Figure 3.5:**</u> **Efficacy of TLR3 and RIG-I Knockdown in PH5CH8 Cells. (A)** TLR3 and RIG-I expression were knocked down in PH5CH8 cells to 10% of baseline 48 hours following transfection of 100 nM siRNA against either PRR. Parallel transfection with 100 nM of a pool of non-targeting siRNA ("NT") served as a negative control while transfection with 100 nM siRNA targeting Lamin A/C ("Lamin") served as a positive control. **(B)** Treatment with exogenously-added polyI:C (5 μg/ml) or transfected HCV PAMP (.5 μg) leads to induction of RIG-I and TLR3 mRNA after 24 hours. However, RIG-I and TLR3 expression in PH5CH8 cells following knockdown remains reduced 24 hours after **(C)** treatment with exogenously-added polyI:C or **(D)** transfection with the HCV PAMP treatment.

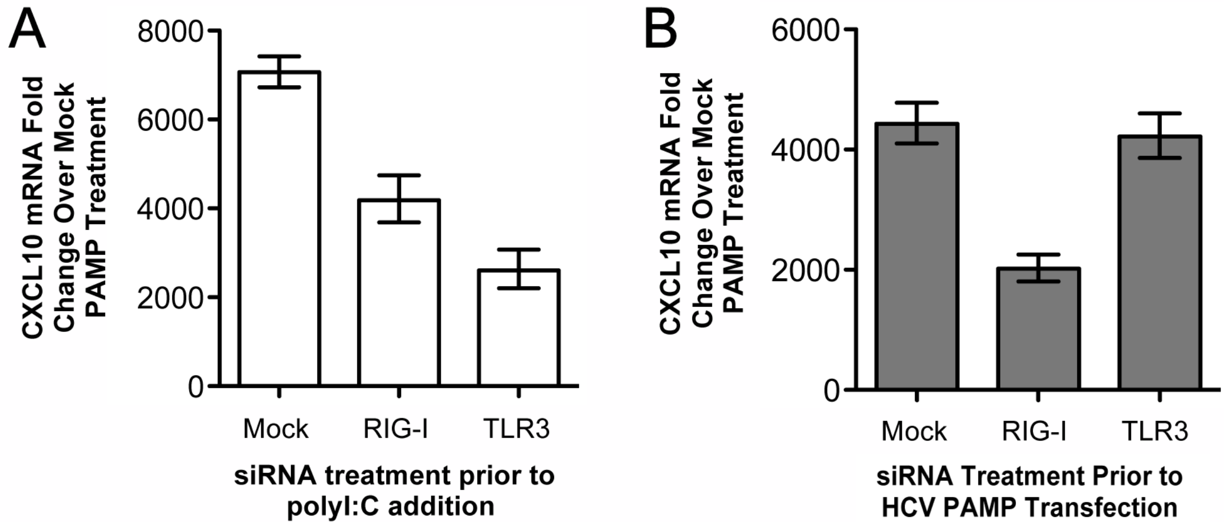
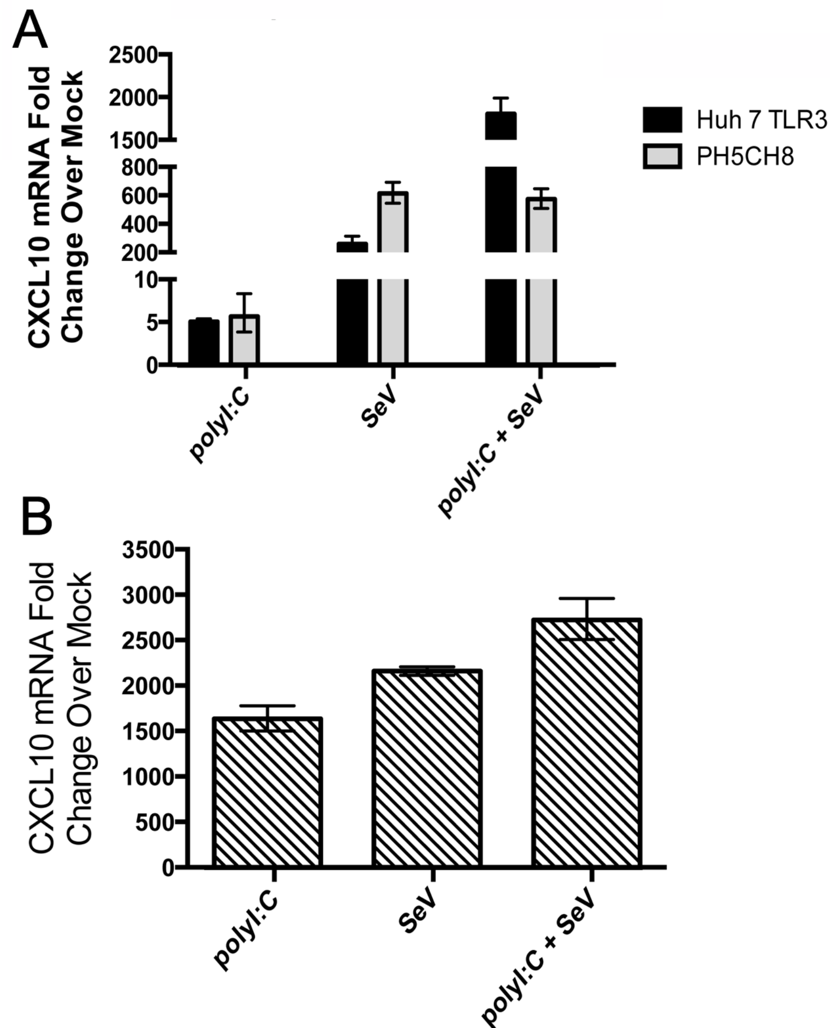


Figure 3.6: Effect of TLR3 and RIG-I Knockdown on CXCL10 Induction Following Specific Activation. (A) CXCL10 mRNA production in PH5CH8 cells in response to 24 hour polyI:C treatment was reduced 63% following knockdown of TLR3 expression. A 41% reduction in the CXCL10 response was also observed during RIG-I knockdown. **(B)** CXCL10 mRNA production in PH5CH8 cells 24 hours after transfection with the 5' pU HCV PAMP was reduced 54% following knockdown of RIG-I expression. No effect was observed following TLR3 knockdown.

TLR3 and RIG-I Synergism During CXCL10 Induction

The TLR3 and RIG-I signaling pathways activate many of the same downstream effectors, including MAP Kinases, IRFs, and NF-kB [8]. This generates the potential for additive or synergistic induction of target innate immune genes such as CXCL10. To investigate this possibility, we compared CXCL10 induction in TLR3+/RIG-I+ Huh7 cells, PH5CH8 cells, and PHH following exposure to SeV infection, exogenous polyI:C treatment, or both PAMPs for 24 hours. An additive effect of the combined treatment was observed in TLR3+/RIG-I+ Huh7 cells, with CXCL10 mRNA fold induction being 10x and 400x higher with both PAMPs than with SeV and exogenous polyI:C respectively (Figure 3.7A, dark bars). This effect was not observed in PH5CH8 cells (Figure 3.7A, light bars) or PHH (Figure 3.7B), where the strength of the combined

treatment was equivalent to that of SeV infection alone. However, all three cells types were still capable of producing CXCL10 in response to each PAMP treatment. This provides further evidence that CXCL10 is induced as part of the innate immune response following specific activation of TLR3 and RIG-I in hepatocytes.



<u>Figure 3.7:</u> TLR3 and RIG-I Synergism in CXCL10 Induction is Cell Type Specific. (A) Simultaneous treatment with exogenously-added polyI:C (5 μg/ml) and SeV infection (100 HAU) for 24 hours lead to higher CXCL10 mRNA induction in TLR+/RIG-I+ Huh7 cells, but not PH5CH8 cells, than either treatment alone. **(B)** Simultaneous treatment also did not lead to higher CXCL10 mRNA induction in PHH.

DISCUSSION

Although TLR3 and RIG-I had been previously implicated in CXCL10 induction in hepatocyte-like progenitor cells, we characterized the importance of these pathways in PAMP-mediated induction of CXCL10 in PHH and several hepatocyte-derived immortalized cell lines commonly used to study HCV infection *in vitro*. We observed that PHH and immortalized PH5CH8 hepatocytes express TLR3 and RIG-I endogenously, and that the Huh7-derived hepatoma cell line can be used to examine the behavior of these PRRs through differential expression of each receptor. All of the cell types examined produced CXCL10 and other chemokines when stimulated with PRR-specific PAMPs. The specificity of these inductions was confirmed by siRNA-knockdown. PHH and Huh7-derived cells that express both TLR3 and RIG-I (TLR3+/RIG-I+ Huh7 cells) also produced type III IFNs in response to these stimuli, while only PHH saw significant induction of type I IFNs. Finally, although synergistic signaling to CXCL10 induction was observed between TLR3 and RIG-I in TLR3+/RIG-I+ Huh7 cells, this phenomenon was not observed in PH5CH8 cells or PHH. Taken together, these results indicate that TLR3 and RIG-I signaling is intact in a wide variety of *ex vivo* hepatocytes and hepatocyte-derived cell lines used for studying HCV infection *in vitro*, and that activation of these PRRs by various PAMPs leads to the robust induction of CXCL10.

Despite the overlap between the signaling pathways downstream of TLR3 and RIG-I described in Chapter 1, additive or synergistic signaling did not appear to play a role in CXCL10 induction. In fact, additive signaling was only observed in the TLR3+/RIG-I+ Huh7 cell line (Figure 3.7A, dark bars). TLR3 overexpression in TLR3+/RIG-I+ Huh7 cells may explain this cell-line specific observation, as high-level

constitutive signaling may overcome the innate immune evasion mechanisms of Sendai Virus, the stimulus used to activate RIG-I. Sendai Virus possesses two accessory proteins that are known to interfere with anti-viral cellular responses. Protein C has been shown to blockade the JAK/STAT pathway as well as prevent apoptosis, while protein V can prevent translocation of IRF3 into the nucleus and the subsequent activation of IFNs and ISGs in mice [30-32]. Increased activation of IRF3 by the overexpressed TLR3 in TLR3+/RIG-I+ Huh7 cells could potentially overwhelm these inhibition mechanisms and thus render the cells more sensitive to polyI:C in the presence of Sendai Virus. This suggests that additive and synergistic signaling to CXCL10 might be observed in PH5CH8 cells or PHH if a non-TLR3 impairing stimulus was used for RIG-I activation (e.g. the 5' pU HCV PAMP). Donor-to-donor variation in PRR expression may also influence how synergism affects expression of CXCL10 and other chemokines in PHH.

Intracellular additive and synergistic signaling has been observed between other PRRs. For example, simultaneous treatment of mouse bone-marrow derived macrophages with polyI:C and PAMPs that activated myD88-dependent TLR signaling (peptidoglycan-associated lipoprotein [TLR2], flagellin [TLR5], and CpG [TLR9]) yielded a synergistic increase in TNFα and IL-6 that was replicated *in vivo* in mice [33]. Pre-treatment of mouse RAW264.7 cells with myD88-activating PAMPs was also found to enhance TLR3-induced IFNβ production [34]. The use of synergistic combinations of TLR ligands has even been suggested as a way to enhance vaccine-induced T cell responses [35,36]. However, simultaneous treatment with polyI:C and exogenous IFNβ did not reproduce the synergistic effect observed between TLR3 and myD88-dependent

TLR signaling [33], suggesting that this mechanism of PRR cross-talk lies within the cell and does not involve an external secondary pathway.

One potential mechanism for this intracellular PRR cross-talk could involve the use of alternate signaling adaptors such as the inflammasome adaptor apoptosis-associated speck-like protein containing a CARD (ASC). Binding of ASC to RIG-I has been shown to induce caspase-1-inflammasome activation [37]. Additionally, ASC has been shown to induce pro-inflammatory genes through activation of AP-1, NF-κB, MAPK, and STAT3 [38,39]. This binding likely occurs through shared CARD domains in a manner similar to the formation of the RIG-I/MAVS complex [39]. Synergism in pro-inflammatory gene induction may also be implemented by transcription factors at the promoter level, as IRF5 has been shown to mediate synergistic induction between TRIF- and myD88-signaling TLRs in mice [40].

Low-level CXCL10 induction in the absence of PRR stimulation (Figure 3.4) additionally suggests the presence of alternate signaling pathways that lead to constitutive expression of this chemokine in both PHH and Huh7-derived cells. Indeed, Huh7-derived cells have been shown to express high baseline levels of the chemokine CXCL8 (Figure 3.2 and [41]). *In vivo*, liver-expressed chemokine (i.e. CCL16) is also constitutively present in human hepatocytes and plasma [42]. CCL16 binds to multiple chemokine receptors and induces chemotaxis in monocytes, T cells, and eosinophils [42,43]. Constitutive expression of these molecules could contribute to the generation of the immune privileged state within the liver known as liver tolerance [44]. While such homeostatic involvement of CXCL10 in this process could help explain its importance in

determining patient outcomes in hepatitis C, this has not yet been demonstrated experimentally.

Overall, we have shown that primary human hepatocytes and hepatocyte-derived cell lines express TLR3 and RIG-I and are suitable systems for evaluating the signaling of these PRRs *in vitro*. Investigation of these pathways could generate new insight into the extent of redundancy and synergism within the hepatic innate immune signaling system. Further examination may also reveal new targets for host-oriented therapies for hepatitis C and other inflammation-mediated liver diseases. Therefore, subsequent chapters of this work seek to utilize the systems characterized here to investigate the downstream signaling pathways and transcriptional mechanisms involved in CXCL10 induction within hepatocytes during HCV infection.

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CHAPTER 4:

INTERFERON-INDEPENDENT INDUCTION OF CXCL10 IN HEPATOCYTES DURING EARLY HEPATITIS C VIRUS INFECTION

“POWER RESIDES WHERE MEN BELIEVE IT RESIDES.

IT’S A TRICK. A SHADOW ON THE WALL. AND OFTTIMES, A

VERY SMALL MAN CAN CAST A VERY LARGE SHADOW.”

- VARYS, *GAME OF THRONES*

OVERVIEW

This chapter examines the role of type I and type III interferons in the induction of CXCL10 in hepatocytes during hepatitis C virus infection. Type I and type III interferons are potent activators of anti-viral genes and are induced following TLR3 and RIG-I activation. Thus, we sought to determine whether the CXCL10 induction we observed previously (described in Chapter 3) was due to secondary interferon signaling or to a direct induction mechanism following PRR activation. Herein, we show that CXCL10 mRNA and protein are induced in an interferon-independent manner by Huh7-derived hepatoma cells and primary hepatocytes during early hepatitis C virus infection. Our data further suggest that type I and type III interferons produced by non-parenchymal cells and infiltrating adaptive immune cells contribute to CXCL10 production during acute and chronic infection *in vivo*. However, interferon-mediated induction of CXCL10 only appears to occur after the initial wave of CXCL10 induction in hepatocytes recruits these cells to the site of infection. Viral interference with CXCL10 production in hepatocytes may therefore alter the delicate balance of inflammatory signals during early infection and allow for viral persistence. Further understanding of the pathways that control CXCL10 induction may help identify therapeutic targets for controlling excessive and damaging liver inflammation during hepatitis C.

A revised manuscript derived from this chapter has been re-submitted to the *Journal of Hepatology* under the title "Independent, Parallel Pathways to CXCL10 Induction in HCV-Infected Hepatocytes". Supplementary materials from this manuscript were presented in Chapter 3.

INTRODUCTION

Chronic hepatitis C is characterized histologically by infiltration of pro-inflammatory immune effector cells into the liver [1-4]. Damage to neighboring tissue from this persistent yet ineffective inflammatory response can lead to progressive liver disease over multiple decades [5,6]. The causative agent, hepatitis C virus (HCV), is a positive sense, single-stranded RNA virus of the *Flaviviridae* that primarily infects hepatocytes [7,8]. Current therapies seek to limit severe disease by suppressing viral load using a combination treatment of pegylated-interferon-α (IFNα)/Ribavirin and HCV-specific protease inhibitors [9]. However, the underlying biological mechanisms of how chronic hepatic inflammation is 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 [1,10]. Additionally, serum and intrahepatic CXCL10 (i.e. IFN (Interferon)-gamma-induced protein 10 [IP-10]) correlates negatively with the outcome of pegylated-IFNα/Ribavirin therapy and positively with increased HCV RNA in the plasma of acutely infected HCV patients [11-13]. 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+ type I helper T (T_H1) cells, CD8+ cytotoxic T (T_c) cells, and natural killer (NK) cells [2,4]. While these immune cells are normally required to contain and eliminate acute viral infection, CXCR3+ CD8+ T_c cells have been identified among intrahepatic immune cells in chronic hepatitis C patients [2-4]. These observations suggest that ELR-negative CXC

chemokines, and in particular CXCL10, play a role in coordinating the persistent hepatic inflammatory response characteristic of chronic hepatitis C.

Induction of CXCL10 and other chemokines in non-immune cells such as hepatocytes occurs through recognition of conserved pathogen associated molecular patterns (PAMPs) by innate pattern recognition receptors (PRRs) such as Toll-like receptor 3 (TLR3) and retinoic acid inducible gene I (RIG-I). Both TLR3 and RIG-I sense acute HCV infection [14-17]. RIG-I is a cytoplasmic sensor of double-stranded, 5' tri-phosphate RNAs containing poly-U or poly-A motifs [18]. In contrast, TLR3 is found in endosomes and recognizes double-stranded RNAs generated during viral replication [17]. As described in Chapter 1, signaling from TLR3 and RIG-I leads to the activation of transcription factors including IFN regulatory factor 3 (IRF3), IRF-7, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) [19]. These in turn induce pro-inflammatory cytokines and chemokines (via NF-κB and AP-1) as well as type I and type III IFNs (via IRF3 and IRF7) [19,20].

Type I and type III IFNs amplify chemokine production through autocrine and paracrine activation of anti-viral and pro-inflammatory pathways. As described in Chapter 1, type I IFNs (IFNα and IFNβ) activate various STAT (signal transducer and activator of transcription) proteins and the ISGF3 signaling complex [21]. These in turn induce anti-viral IFN-stimulated genes (ISGs) by binding to IFN-stimulated response elements (ISREs) in their promoters [21,22]. Most cells, including hepatocytes, are capable of producing type I IFNs as part of the general anti-viral response [21]. HCV infection in hepatocytes induces type III IFNs (IL-28A, IL-28B, IL-29), which also activate STAT-signaling [21,23,24]. Thus, PRR-activated inflammatory genes whose

promoters contain putative ISREs (including CXCL10) may also respond to regulation by hepatocyte-derived IFNs during initial HCV infection [23,25].

Hepatocytes are a major source of CXCL10 during HCV infection both *in vivo* and *in vitro* [1,17,23,26], and previous studies have shown CXCL10 induction following treatment with IFNs or various PAMPs [23,27]. However, the combined contribution of PRR stimulation and IFN signaling to CXCL10 induction during the initial HCV infection of hepatocytes has not yet been examined, even though deregulation of these pathways may contribute to the establishment of persistent inflammation within the liver. Therefore, in this study we characterized the contribution of type I IFN, type III IFN, and PRR signaling via TLR3 and RIG-I to CXCL10 induction during early HCV infection of primary and immortalized hepatocytes. We show that CXCL10 is induced primarily through an IFN-independent, direct transcriptional activation 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 non-parenchymal cells (NPCs) amplify CXCL10 induction in primary hepatocyte preparations.

MATERIALS AND METHODS

Detailed protocols, reagents, and statistics are included in Chapter 2 (pg 29).

Cells and Virus

Human hepatoma Huh7 (TLR3-/RIG-I+) cells were obtained from Apath, LLC [15]. Huh7.5.1 (TLR3-/RIG-I-) cells were obtained from Francis Chisari (Scripps Research Institute) [8]. Huh7 and Huh 7.5 cells stably transduced with a FLAG-TLR3 (TLR3+/RIG-

I+ and TLR3+/RIG-I-cells, respectively) [16] were obtained from Kui Li (University of Tennessee at Memphis). PH5CH8 immortalized hepatocytes were obtained from Noboyuki Kato (Okayama University) [15]. Primary hepatocyte cultures were either obtained cryopreserved from Invitrogen (Carlsbad, CA) or obtained fresh from Dr. Steven Strom (University of Pittsburgh) through the NIH Liver Cell and Tissue Distribution System (LCTDS)). Human hepatocyte cultures from the LTCDS were harvested under an IRB exemption approval from the University of Pittsburgh to Dr. Strom (letter on file) as surgical waste tissue. All cells were maintained in culture as described in Chapter 2 (see pg 30).

JFH-1 HCV viral stock preparation and titration were performed as described previously. Cell cultures were infected at MOIs (multiplicities of infection) of 0.05, 0.5, or 2 using the protocol described in Chapter 2 (pg 32) [8,28]. Cells were harvested at 6-72 hours post-infection.

Real-time Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

Standard real-time RT-PCR using the Viia7 Real-Time PCR system (Life Technologies, Carlsbad, CA) was performed on cellular mRNA for detection of HCV, CXCL10, IFN-α2, IFN-β, IL-28B, and IL-29 transcripts. Chemokine and cytokine data are reported as fold change derived from ΔΔCt using GAPDH as an endogenous control [29]. Absolute quantification of JFH-1 HCV genomes was conducted as previously described using 0-10^7 copies JFH-1 HCV plasmid DNA as a standard curve [30]. Micro fluidic high-throughput real-time RT-PCR was performed on PHH for detection of chemokines, cytokines, and immune cell markers using the Fluidigm BioMark HD

system (Fluidigm Corporation, South San Francisco, CA). Targets for Fluidigm PCR are listed in Table 2.2 (Chapter 2, pg 34).

CXCL10 Luminex Bead Arrays

Supernatants were collected and centrifuged at 14,000 RPM prior to storage at -80°C. Infectious supernatants were inactivated with 1% Triton-X-100. Samples were tested for CXCL10 using polystyrene Luminex Antibody Bead kits (Biosource/Invitrogen) and the Luminex 200 system according to the manufacturer's protocol (Luminex, Austin, TX).

Western Blotting

Whole cell lysates were run on Tris-Glycine 4-20% gels (Invitrogen). Target proteins were detected with the following antibodies: rabbit anti-IFIT1 (Pierce), goat anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA). HCV proteins were detected using random, de-identified HCV-infected patient serum as previously described [31]. HRP-conjugated secondary antibodies were purchased from Pierce and Santa Cruz Biotechnology.

Type I and Type III IFN Neutralization Assays

Cells were cultured with or without HCV (MOI 0.5 for TLR3+/RIG-I+ Huh7 cells or MOI 0.2 for primary hepatocytes). After virus adsorption infectious media was replaced with either normal media, media containing 2 μg/ml B18R protein (eBioscience, San Diego, CA) for type I IFN neutralization, or media containing 4 μg/ml IL-28B/IL-29

neutralizing antibody (R&D Systems, Minneapolis, MN; MAB15981) for type III IFN neutralization. Twenty ng/ml pegylated-IFN-α2 (Pegasys©; Genentech, South San Francisco, CA) and 1 ng/ml recombinant IL-28B (R&D Systems) served as positive controls and were added 30 minutes after the addition of the neutralization agents. Protein, supernatants, and mRNA were harvested 24 and 48 hours post-infection and analyzed as described above.

Immunofluorescence

Cells were cultured on 8-well chamber slides (Nunc/ThermoScientific) and infected with HCV (MOI 0.5) as described above for 72 hours or treated with 100 ng/ml IFNγ combined with 40 ng/ml Tumor Necrosis Factor-α (TNFα) for 24 hours [1]. Brefeldin A (VWR International, Radnor, PA) was added at 1 μg/ml during the last 5 hours of infection. Cells were fixed in 4% paraformaldehyde and stained for CXCL10 and HCV Core proteins. Nuclei were stained with DAPI. Slides were visualized with a 3i Marianas widefield deconvolution microscope (Intelligent Imaging Innovations, Ringsby, CT). CXCL10 and HCV Core protein abundance was quantified with CellProfiler (Version r11710; www.cellprofiler.org) [5].

Negative Selection of Primary Hepatocytes

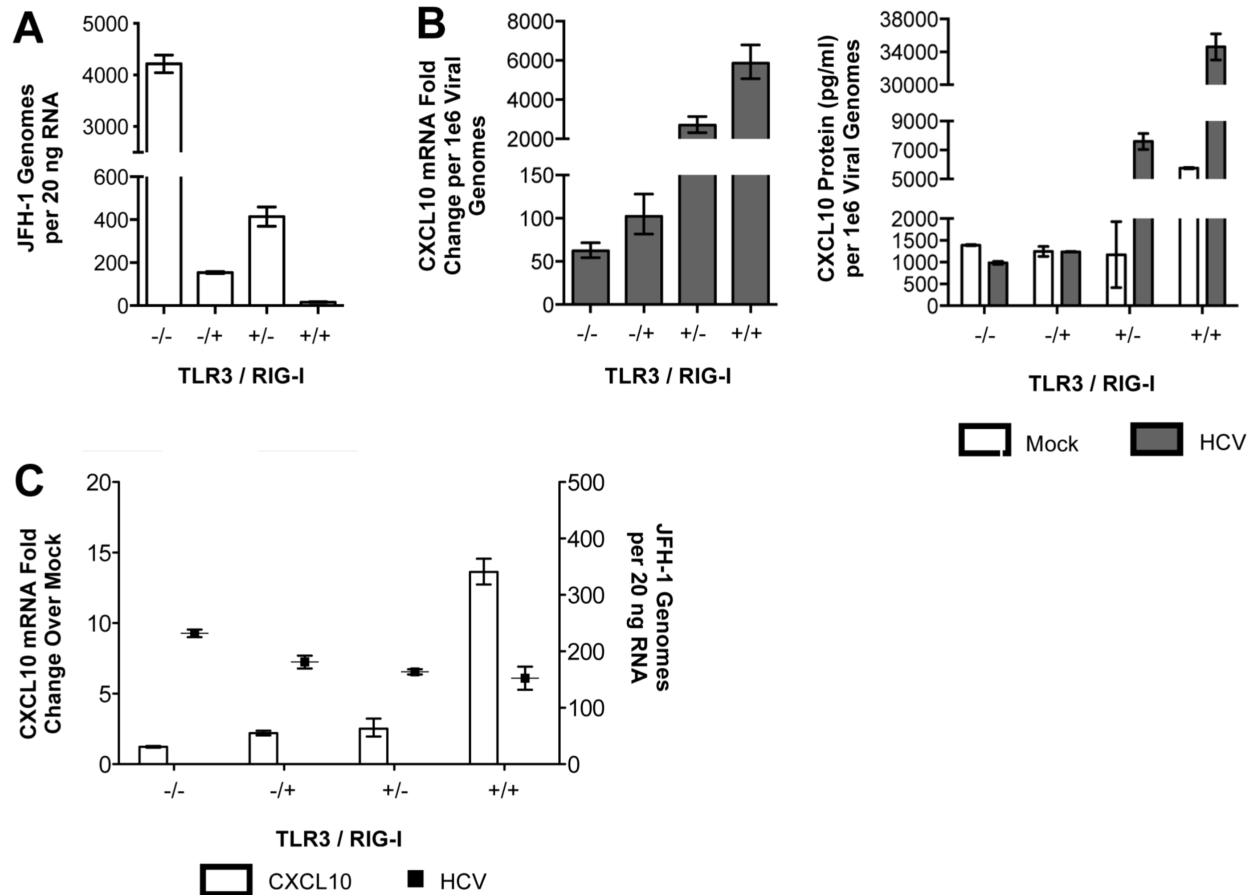
PHH were combined with biotin-conjugated antibodies against CD45 (R&D Systems; BAM1430), CD68 (i.e. SR-D1; R&D Systems; BAF2040), and CD31 (i.e. PECAM-1; R&D Systems; BAM3567) and MACS anti-biotin-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA) before being applied to a magnetic MACS

Cell Separation column (Miltenyi Biotec). Non-adhered cells were collected and plated following the standard culture protocol for PHH (see Chapter 2, pg 30). Adhered cells were then isolated from the column and reserved for analysis. Fluidigm real-time RT-PCR (see above) was run on pre-depletion, post-depletion, and adhered cell mRNA samples to assess efficiency of NPC removal.

RESULTS

Maximal CXCL10 induction during early HCV infection occurs in TLR3+/RIG-I+ cells

To evaluate the roles of TLR3 and RIG-I signaling in CXCL10 induction during *in vitro* HCV infection, we utilized four Huh7-derived hepatoma cell lines that differentially expressed each PRR (see Chapter 3, Figure 3.1B). Receptor functionality was verified experimentally (Chapter 3, Figure 3.2A and [16]). 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 4.1A).



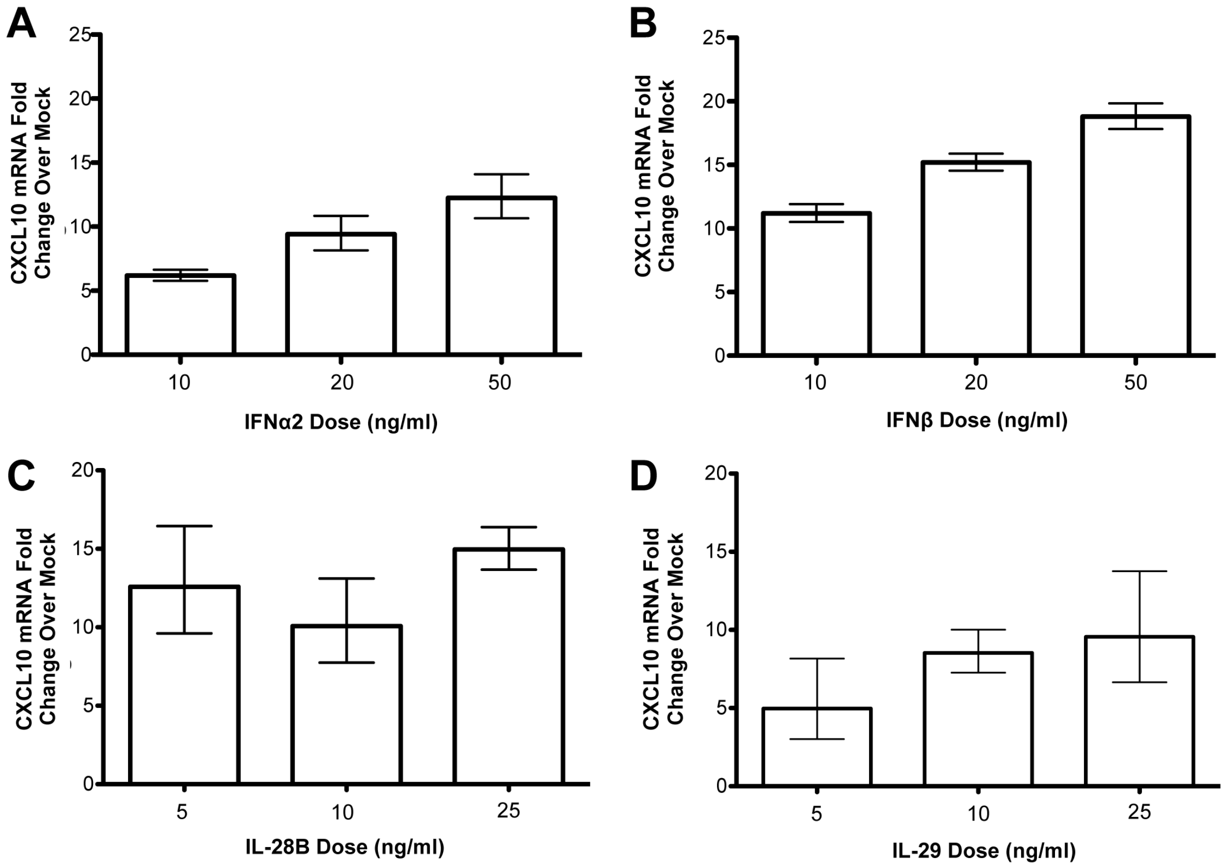
<u>Figure 4.1:</u> Maximal CXCL10 Induction During Early HCV Infection Requires Both TLR3 and RIG-I. (A) Levels of HCV RNA vary with PRR expression in Huh7-derived cells at 72 hours post-infection (MOI .05). **(B)** Huh7 cells expressing both TLR3 and RIG-I produce maximal CXCL10 mRNA (left) and protein (right) following HCV infection (72 hours, MOI .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).

During asynchronous, low MOI infection, TLR3+/RIG-I+ cells had the largest induction of CXCL10 mRNA and protein at 72 hours after normalization to HCV RNA copy number (Figure 1B). Data were normalized to account for variability in cell permissivity to viral replication and thus PAMP exposure. To validate our findings in the absence of normalization, we performed synchronous, high MOI infections in these cell

lines. CXCL10 induction was then 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). These data indicate that both TLR3 and RIG-I signaling pathways are required for maximal CXCL10 induction during early HCV infection in hepatocytes.

CXCL10, Type I IFNs, and type III IFNs are induced simultaneously in TLR3+/RIG-I+ Huh7 Cells during early HCV infection

Type I IFN induction has been reported in Huh7 cells following RIG-I-specific activation and in TLR3+/RIG-I- Huh7 cells in response to polyI:C [14,15]. Since CXCL10 induction was observed in TLR3+/RIG-I+ Huh7 cells after 24 hour treatment with IFN-α2, IFN-β, IL-28B, or IL-29 (Figure 4.2), we hypothesized that early paracrine IFN signaling amplifies the CXCL10 response. Therefore, we examined the kinetics of type I and type III IFNs and CXCL10 during early HCV infection of TLR3+/RIG-I+ Huh7 cells.



<u>Figure 4.2:</u> Type I and Type III IFNs Induce CXCL10 in TLR3+/RIG-I+ Huh7 Cells. An increase in CXCL10 mRNA was observed following treatment with **(A)** IFNα2, **(B)** IFN-β, **(C)** IL-28B, or **(D)** IL-29 for 24 hours.

While CXCL10 was again robustly induced in these cells, only low-level IFNα2 and IFNβ induction was detected (Figure 4.3A [MOI 0.05] and Figure 4.3B [MOI 0.5]). Peak production of IFNα2 and IFNβ mRNAs occurred after induction of CXCL10 mRNA (at 12 and 72 hours post-infection for MOIs 0.5 and 0.05 respectively). IL-28B or IL-29 mRNAs were not detected (data not shown). These kinetic data suggest that while CXCL10 is inducible by type I and III IFN treatment, hepatocyte-derived IFNs do not contribute to CXCL10 induction in Huh7-derived cells during early HCV infection.

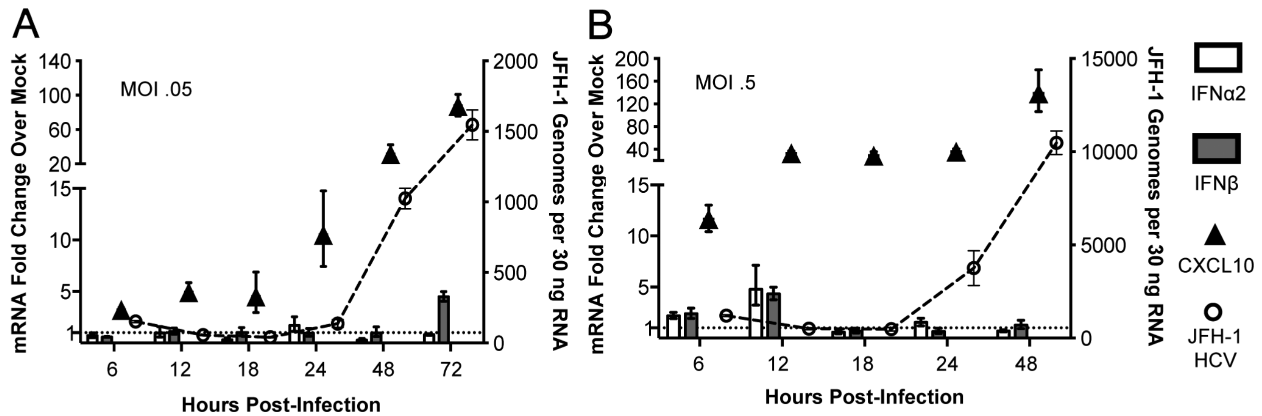
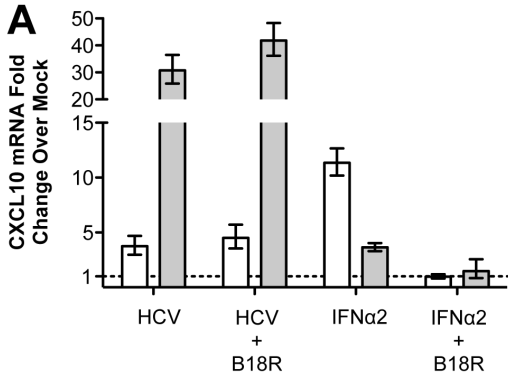
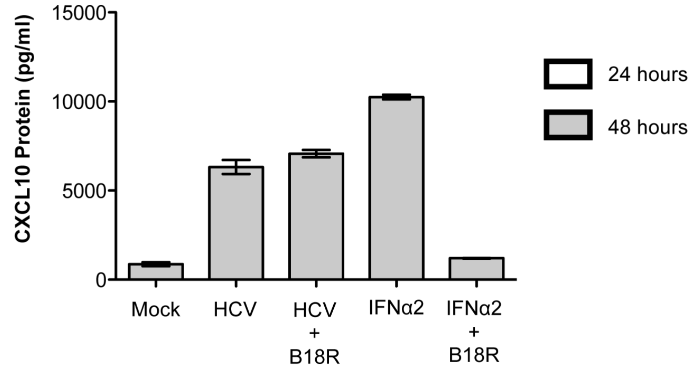


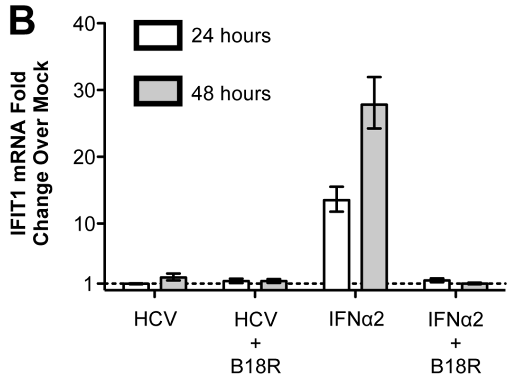
Figure 4.3: Kinetics of CXCL10 and Interferon Induction in TLR3+/RIG-I+ Huh7 Cells. IFNα2 and IFNβ mRNAs were minimally upregulated in TLR3+/RIG-I+ Huh7 cells following HCV infection at **(A)** MOI 0.05 and **(B)** MOI 0.5. IL-28B and IL-29 were not detected (data not shown).

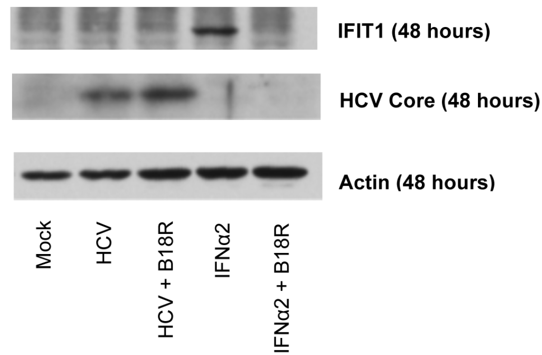
Neutralization of type I or III IFNs does not affect CXCL10 induction during early HCV infection of TLR3+/RIG-I+ Huh7 cells

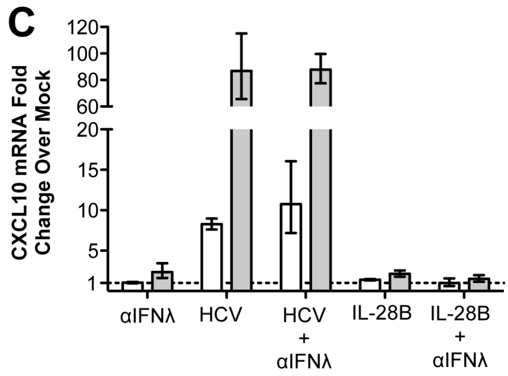
To confirm our observations, we neutralized residual IFNs produced during HCV infection of TLR3+/RIG-I+ Huh7 cells and evaluated the effect on CXCL10 induction. For neutralization of IFNα2 and IFNβ, 2 μg/ml of the *Vaccinia* virus B18R protein (a soluble type I IFN receptor [32]) was added to the culture medium following virus adsorption. This treatment had no impact on CXCL10 mRNA or protein production at 24 or 48 hours post-infection, but completely abrogated CXCL10 induction by recombinant IFNα (Figure 4.4A). Expression of IFN-induced protein with tetratricopeptide repeats 1 (IFIT1), a known highly-induced ISG, was not induced following HCV infection, but was induced with IFNα treatment (Figure 4.4B), This effect was abrogated by B18R co-treatment.

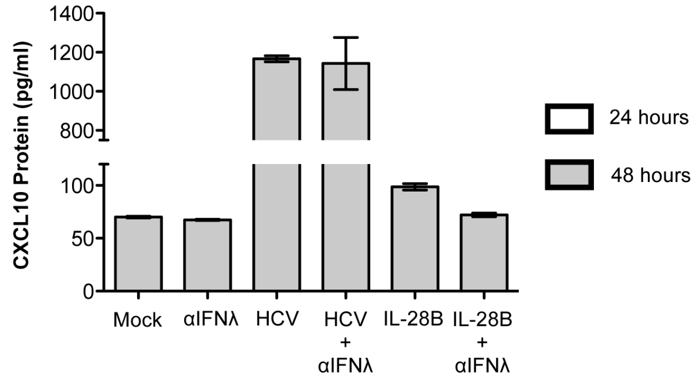

A
CXCL10 mRNA Fold Change Over Mock
50
40
30
20
15
10
5
1
HCV
HCV + B18R
IFNα2
IFNα2 + B18R

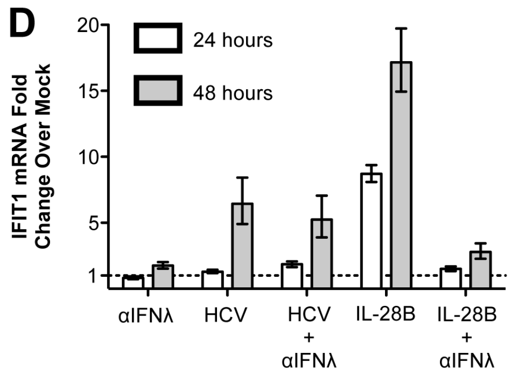

CXCL10 Protein (pg/ml)
15000
10000
5000
0
Mock
HCV
HCV + B18R
IFNα2
IFNα2 + B18R
24 hours
48 hours

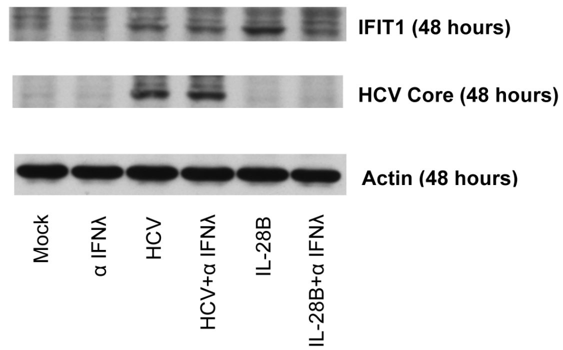

B
IFIT1 mRNA Fold Change Over Mock
24 hours
48 hours
40
30
20
10
1
HCV
HCV + B18R
IFNα2
IFNα2 + B18R


IFIT1 (48 hours)
HCV Core (48 hours)
Actin (48 hours)
Mock
HCV
HCV + B18R
IFNα2
IFNα2 + B18R


C
CXCL10 mRNA Fold Change Over Mock
120
100
80
60
20
15
10
5
1
αIFNλ
HCV
HCV + αIFNλ
IL-28B
IL-28B + αIFNλ


CXCL10 Protein (pg/ml)
1400
1200
1000
800
100
50
0
Mock
αIFNλ
HCV
HCV + αIFNλ
IL-28B
IL-28B + αIFNλ
24 hours
48 hours


D
IFIT1 mRNA Fold Change Over Mock
24 hours
48 hours
20
15
10
5
1
αIFNλ
HCV
HCV + αIFNλ
IL-28B
IL-28B + αIFNλ


IFIT1 (48 hours)
HCV Core (48 hours)
Actin (48 hours)
Mock
α IFNλ
HCV
HCV+α IFNλ
IL-28B
IL-28B+α IFNλ

<u>Figure 4.4:</u> Induction of CXCL10 by HCV in TLR3+/RIG-I+ Huh7 cells is Interferon Independent. **(A)** Neutralization of type I IFNs with B18R does not impact CXCL10 mRNA (left) or protein (right) at 24 or 48 hours after HCV infection (MOI 0.5), but **(B)** negates IFNα2 induction of IFIT1 mRNA (left) and protein (right). **(C)** Addition of a pan-type III IFN neutralizing antibody (αIFNλ) does not impact CXCL10 mRNA (left) or protein (right) at 24 or 48 hours after HCV infection (MOI 0.5), but **(D)** reduces IL-28B induction of IFIT1 mRNA (left) and protein (right). HCV Core expression is reflective of that of other viral proteins (i.e. NS5A).

Treatment with a pan type III IFN neutralizing antibody (αIFNλ) did not affect CXCL10 production during HCV infection, but did reduce induction following treatment with recombinant IL-28B (Figure 4.4C). Neutralization also severely reduced the upregulation of IFIT1 mRNA and protein in response to recombinant IL-28B and IL-29 (Figure 4.4D and Chapter 2, Figure 2.1). Furthermore, simultaneous neutralization of type I and type III IFNs had no effect on CXCL10 production during virus infection while completely abrogating IFIT1 induction by combined treatment with IFNα and IL-28B (Figure 4.5). Taken together, these results indicate that neither type I nor type III IFNs produced by hepatocytes are necessary for CXCL10 induction during early HCV infection in TLR3+/RIG-I+ Huh7 cells.

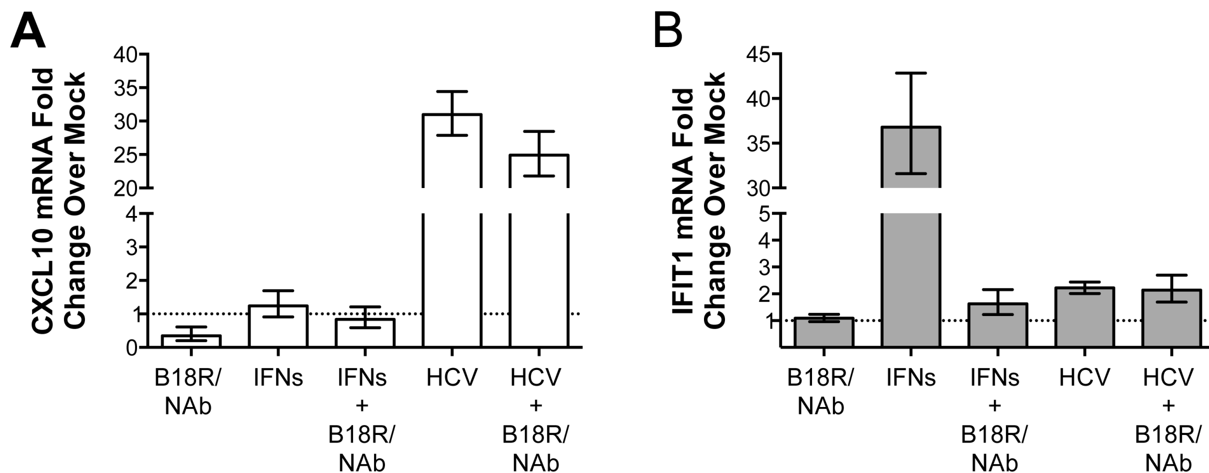
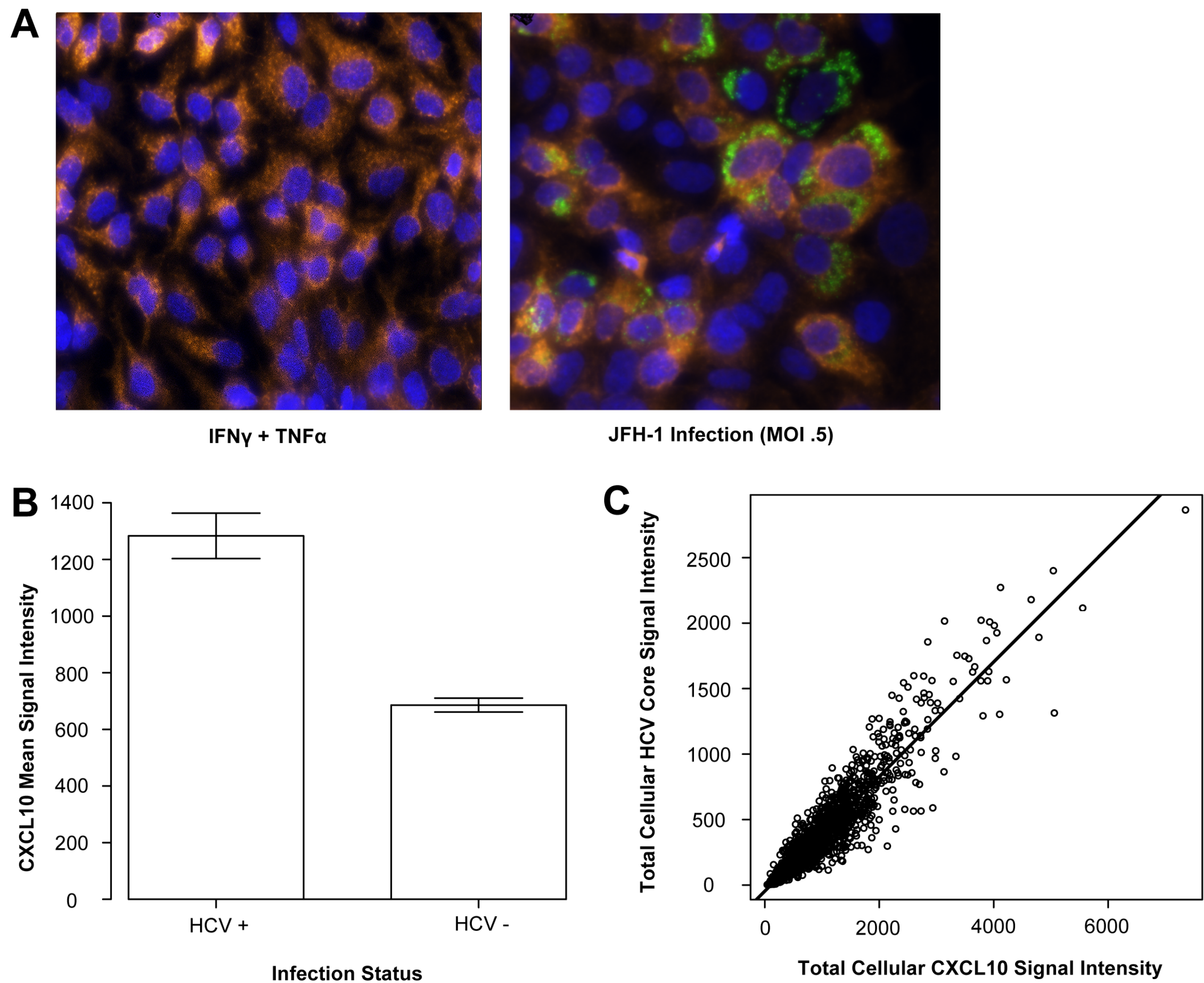


Figure 4.5: No Effect of Simultaneous Type I and Type III Interferon Neutralization. **(A)** Simultaneous neutralization of type I and type III IFNs through co-administration of B18R and αIFNλ had no impact on CXCL10 mRNA induction 48 hours after HCV infection (MOI 0.5) of TLR3+/RIG-+ Huh7 cells, but **(B)** completely neutralized IFIT1 mRNA induction during 24 hour co-treatment with IFNα2 and IL-28B.

HCV infection and paracrine IFNγ/TNFα treatment produce differing 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 IFNγ and TNFα [1] (Figure 4.6). TLR3+/RIG-I+ Huh7 cells were infected with HCV (72 hours, MOI 0.5) or treated with 100 ng/ml IFN-γ and 40 ng/ml TNFα for 24 hours. Brefeldin A was added during the final 5 hours of treatment to prevent export of secreted proteins. Cells were then fixed and stained for CXCL10 (orange) and HCV Core (green) proteins. As expected for a paracrine stimulus, all cells exposed to IFNγ/TNFα were positive for CXCL10 protein (Figure 4.6A, left). In contrast, infected cells (HCV Core positive) showed much stronger CXCL10 staining than non-infected cells (HCV Core negative; Figure 4.6A, right). Quantitative analysis of CXCL10

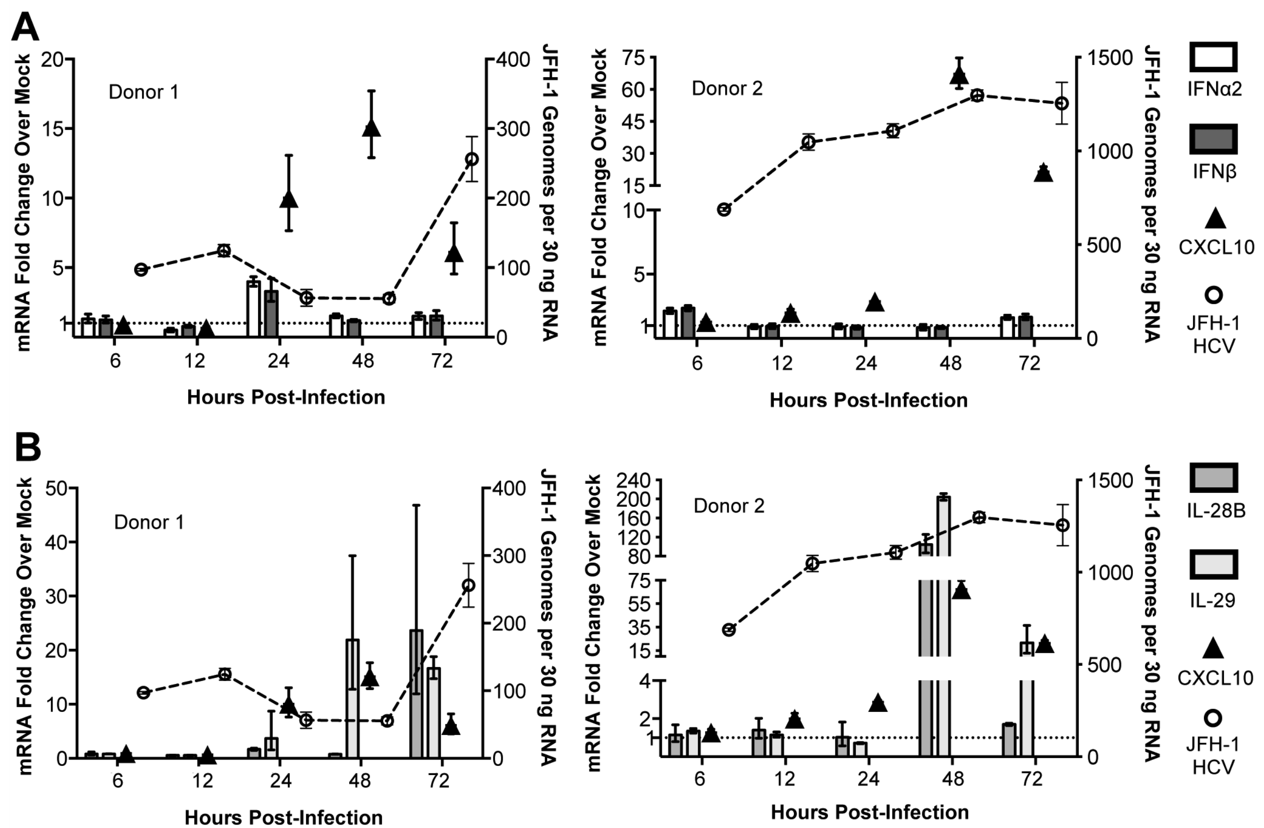
and HCV Core staining was also conducted on a per-cell basis within the HCV-exposed population (n=2145, see Materials and Methods and Chapter 2). HCV Core-positive cells had a significantly higher mean CXCL10 signal than Core-negative cells ($p<0.001$, Figure 4.6B). We also observed a direct, positive correlation between HCV Core signal intensity and CXCL10 signal intensity ($r^2 = 0.88$, p=< 0.001, Figure 4.6C), confirming that the intracellular CXCL10 expression pattern in hepatocytes during early HCV infection is directly virus-dependent and is distinct from a paracrine IFN stimulus.



<u>Figure 4.6:</u> HCV Infection and Paracrine IFNγ/TNFα Treatment Yield Distinct Intracellular CXCL10 Staining Patterns. (A) CXCL10 signal clusters with viral protein in TLR3+/RIG-I+ cells exposed to HCV (right), whereas IFNγ/TNFα 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).

CXCL10, Type I IFNs, and type III IFNs are induced simultaneously in primary hepatocytes during early HCV infection

Although Huh7-derived cells are an optimized cell culture system for studying HCV virology, they differ substantially from primary human hepatocytes (PHH) in terms of inflammatory responses (see Chapter 3 and refs. [24,33]). Thus, we sought to examine whether CXCL10 induction was also independent of type I and type III IFNs during early HCV infection of PHH.



<u>Figure 4.7:</u> Kinetics of CXCL10 and Interferon Induction in Primary Hepatocytes. (A) Peak IFNα2 and IFNβ mRNA induction following HCV infection of primary hepatocytes (MOI 0.2) varied between two donors, while CXCL10 mRNA consistently peaked at 48 hours post-infection. **(B)** IL-28, IL-29, and CXCL10 mRNAs were induced simultaneously 48 hours after HCV infection of primary hepatocytes from two donors (MOI 0.2)

We and others have previously observed that PHH produce both type I and type III IFNs following RIG-I and TLR3-specific stimulation (see Chapter 3, Figure 3.3B and [23]). However, we detected only low levels of type I IFNs following HCV infection of PHH from two donors (Figure 4.7A). The greatest induction of type I IFN (Donor 1; 3.99 [range: 4.35-3.65] and 3.30 [range: 4.22-20.58] fold change over mock infection for IFNα2 and IFNβ, respectively) coincided with CXCL10 expression at 24 hours post-infection. Maximal CXCL10 mRNA expression occurred at 48 hours post-infection in both donors. Similarly, maximal CXCL10, IL-28B, and IL-29 mRNAs were also detected concurrently at 48-72 hours post-infection in both donors (Figure 4.7B). These data indicate that induction of type I and type III IFNs coincides with rather than precedes CXCL10 induction in PHH during *in vitro* HCV infection, suggesting that these IFNs also do not contribute to the initial CXCL10 response in these cells.

Neutralization of type I but not type III IFN reduces CXCL10 induction in primary hepatocyte cultures

To confirm this observation, we neutralized IFNs in PHH during HCV infection. Cells were infected with JFH-1 HCV (MOI 0.2) for 24 and 48 hours and then neutralized for type I and type III IFNs, as described above. In contrast to the Huh7 system (Figure 4.4), neutralization of type I IFNs in PHH culture resulted in 92% and 89% reduction in CXCL10 mRNA and protein production by PHH, respectively, at 24 hours post-infection (Figure 4.8A). Only the mRNA response remained diminished during type I IFN neutralization at 48 hours post-infection in these cells, although this rebound was not observed in other PHH preparations (see Figure 4.12 below). This reduction mirrored

the neutralization of IFIT1 production both during co-treatment with IFNα and during HCV infection (Figure 4.8C, left), indicating that type I IFNs do contribute to both IFIT1 and CXCL10 induction in PHH cultures.

In contrast, neutralization of type III IFNs in PHHs had no effect on HCV induction of CXCL10 at either 24 or 48 hours while IFIT1 induction by recombinant IL-28B was again suppressed by type III IFN neutralization (Figure 4.8B). These results are in accordance with our observations following neutralization of type III IFNs in TLR3+/RIG-I+ Huh7 cells (Figure 4.4C). However, type III IFNs contributed to CXCL10 induction in other PHH preparations (see Figure 4.12 below). Together, our data suggest that both hepatocyte-derived type I and type III IFNs participate in the induction of CXCL10 in PHH cultures in response to early HCV infection. The lack effect of type III neutralization on IFIT1 induction during HCV infection (Figure 4.8C, right) further supports the influence of type I IFNs on cytokine and chemokine induction in this culture system.

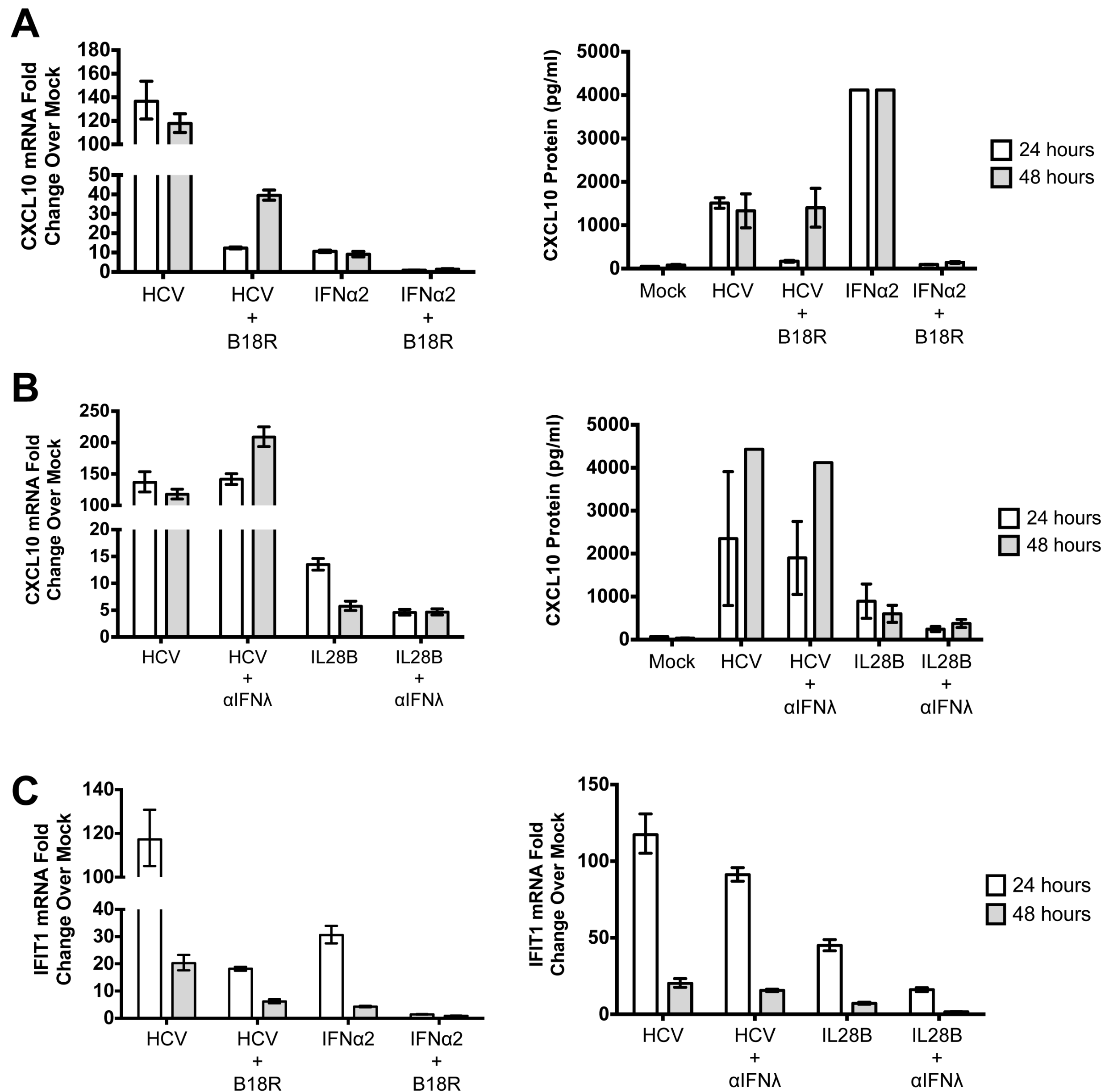


Figure 4.8: Induction of CXCL10 by HCV in Primary Hepatocytes is Type I but not Type III Interferon Dependent. Neutralization of **(A)** type I IFNs but not **(B)** type III IFNs reduces CXCL10 mRNA (left) and protein (right) at 24 and 48 hours after HCV infection (MOI 0.2) in primary human hepatocytes. Reduction of CXCL10 induction was also observed following 24-hour treatment with IFNα2 or IL-28B. **(C)** Neutralization of type I IFNs (left) but not type III IFNs (right) reduces IFIT1 mRNA at 24 and 48 hours HCV infection (MOI 0.2) in primary human hepatocytes. Reduction of IFIT1 mRNA response was also observed following 24-hour treatment with IFNα2 or IL-28B. Data are representative of results from 2 independent donors.

Residual non-parenchymal cells produce type I IFNs that contribute to virus-induced CXCL10 induction in PHH cultures

The involvement of type I and type III IFNs in CXCL10 induction during early HCV infection of PHH cultures directly contrasts our results in Huh7 cells, where IFNs were dispensable for CXCL10 induction (Figure 4.4A). Non-parenchymal cells (NPCs) such as macrophage-like Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and hepatic stellate cells are a known source of type I IFNs and other cytokines in the liver [34], we hypothesized that contaminating NPCs in our PHH cultures 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 developed for use with the microfluidic real-time RT-PCR Fluidigm platform (see Chapter 2, Table 2.2). Nine sets of mRNA samples from PHH cultures obtained from 8 independent donors were run in duplicate against the panel, and results were displayed as heat maps of Ct threshold values (Figure 4.9). Samples from TLR3+/RIG+ Huh7 cells were included for comparison of baseline expression. Additionally, seven sets of mRNA samples from JFH-1 HCV-infected PHH cultures (MOI 0.2, 48 hours) were run and evaluated for fold change in expression over untreated samples (Figure 4.10).

TLR3+/RIG+ Huh7 cells showed low or non-detectable expression of most immune cell lineage markers (Figure 4.9). In contrast, all PHH sample sets showed strong baseline and HCV induced expression of cytokines, chemokines (including CXCL10), and immune cell lineage markers such as EMR1, CD86 (i.e. SR-D1), and

MARCO. The intensity of induction varied between sample sets, suggesting that the level of NPC contamination is different for each primary hepatocyte preparation.

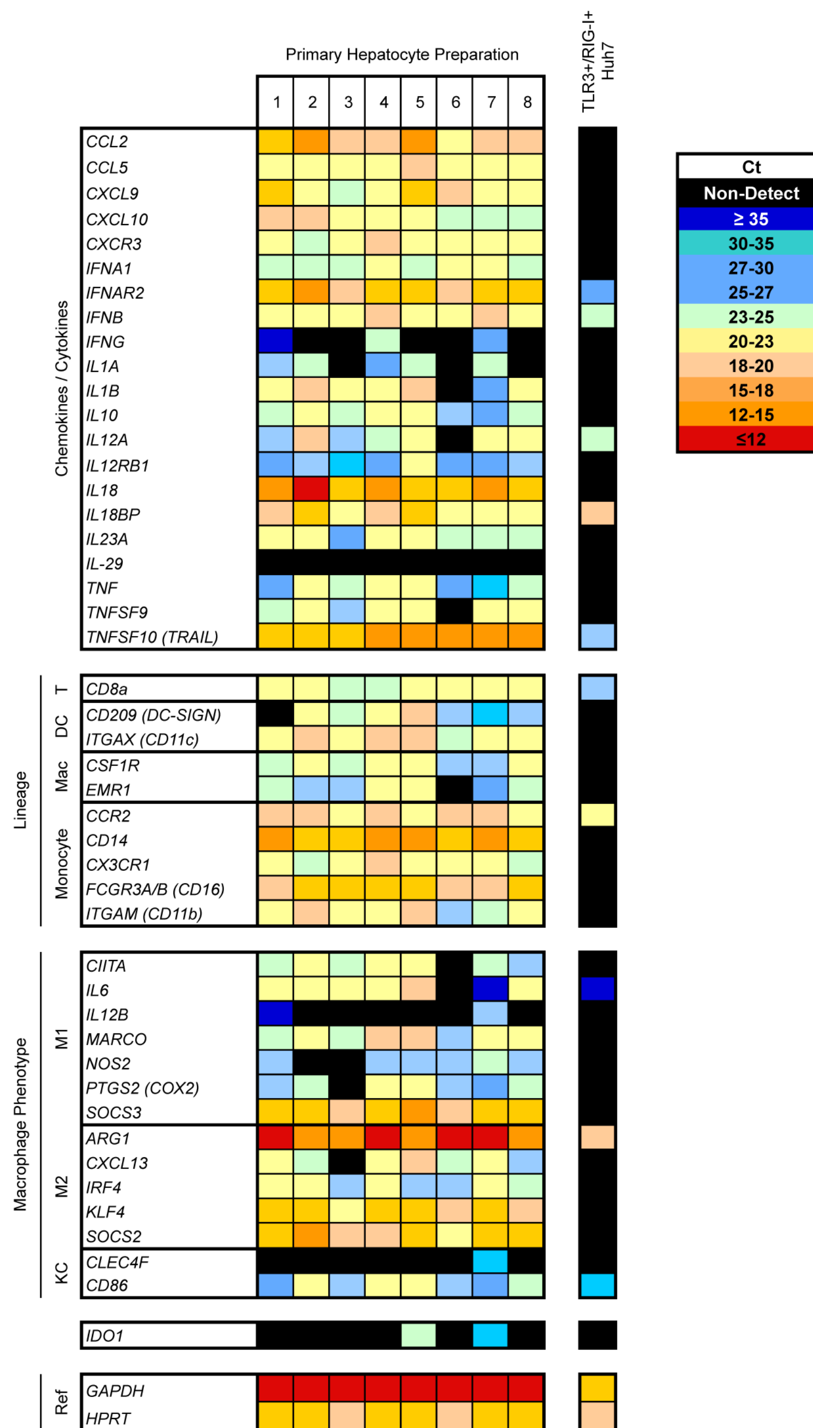


Figure 4.9: **Immune Cell Markers are Highly Expressed in Primary Hepatocyte Cultures.** cDNA from nine untreated preparations of primary human hepatocytes isolated from eight different donors was run in duplicate against a panel of immune cell markers using the Fluidigm real-time RT-PCR platform (see Table 2.2, Chapter 2, pg 34). cDNA from untreated TLR3+/RIG-I+ Huh7 cells was included for comparison. Heat map generated from raw Ct values.

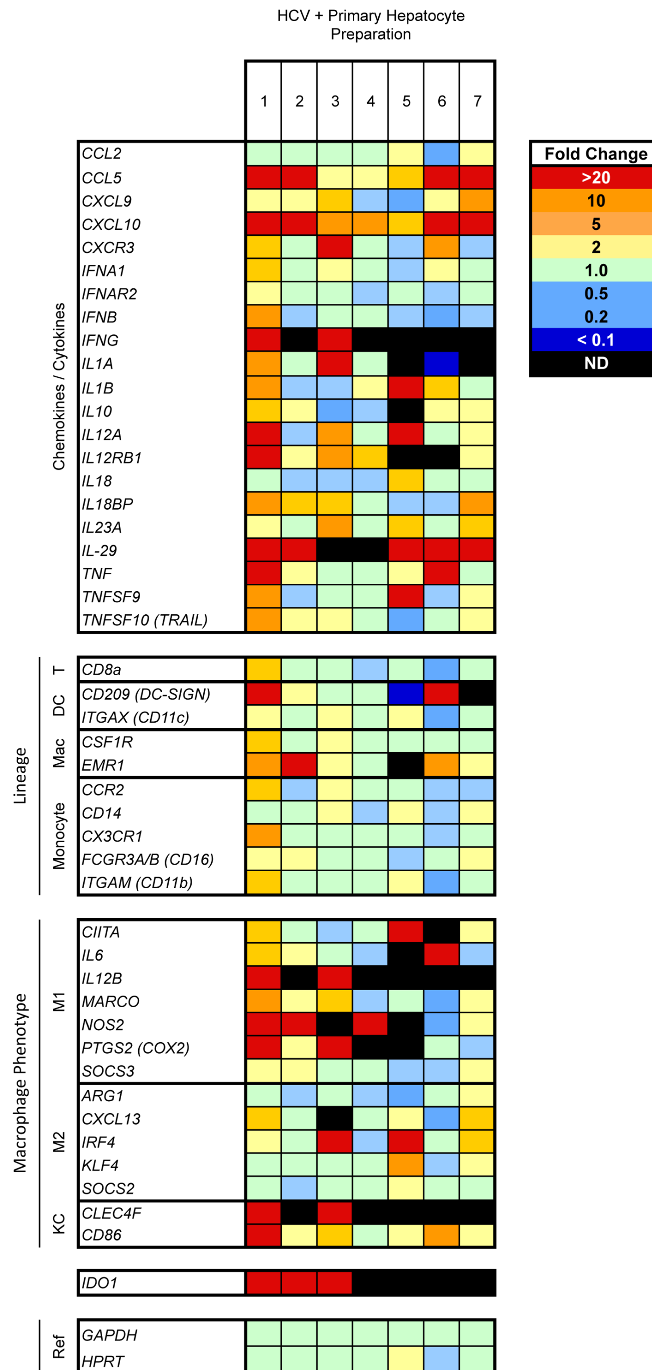
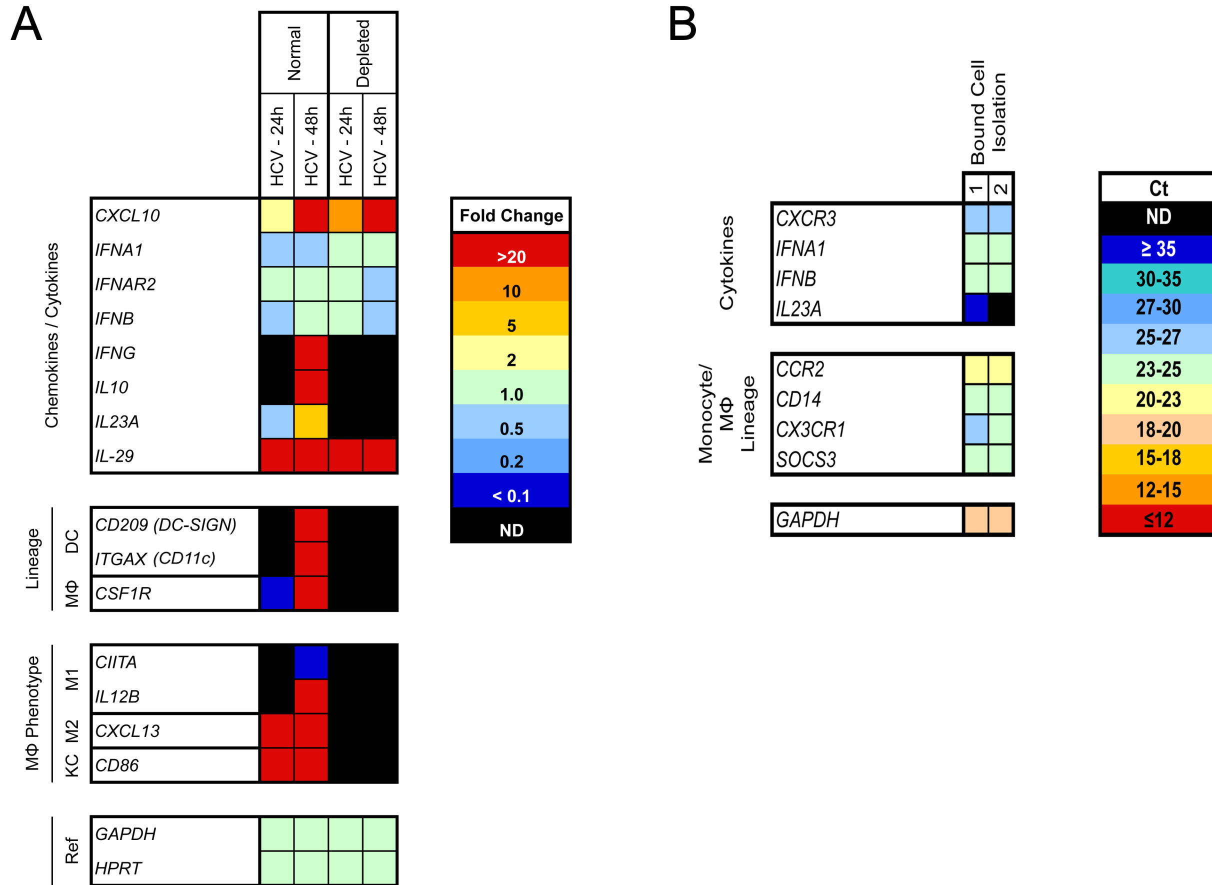


Figure 4.10: Induction of Immune Cell Markers in Primary Hepatocyte Cultures During HCV Infection. cDNA from seven HCV-infected (MOI 0.2, 48 hours) preparations of primary human hepatocytes isolated from six different donors was run in duplicate against a panel of immune cell markers using the Fluidigm real-time RT-PCR platform (see Table 2.2, Chapter 2, pg 34). Heat map values represent the fold change of gene induction over mock-infected cells.

We next sought to remove the contaminating NPCs from our PHH cultures using immunodepletion. PHH were incubated with using a mixture of streptavadin-conjugated magnetic beads and biotin-conjugated antibodies against pan-CD45 (leukocytes), CD68 (monocytes/macrophages [including KCs]), and CD31 (LSECs) [35-39]. The cell-antibody slurry was applied to a magnetic separation column and the non-binding PHH collected for experimentation (see below). cDNA from these cells as well as from non-depleted PHH from the same donor were run against the Fluidigm panel described above to assess the efficacy of depletion.

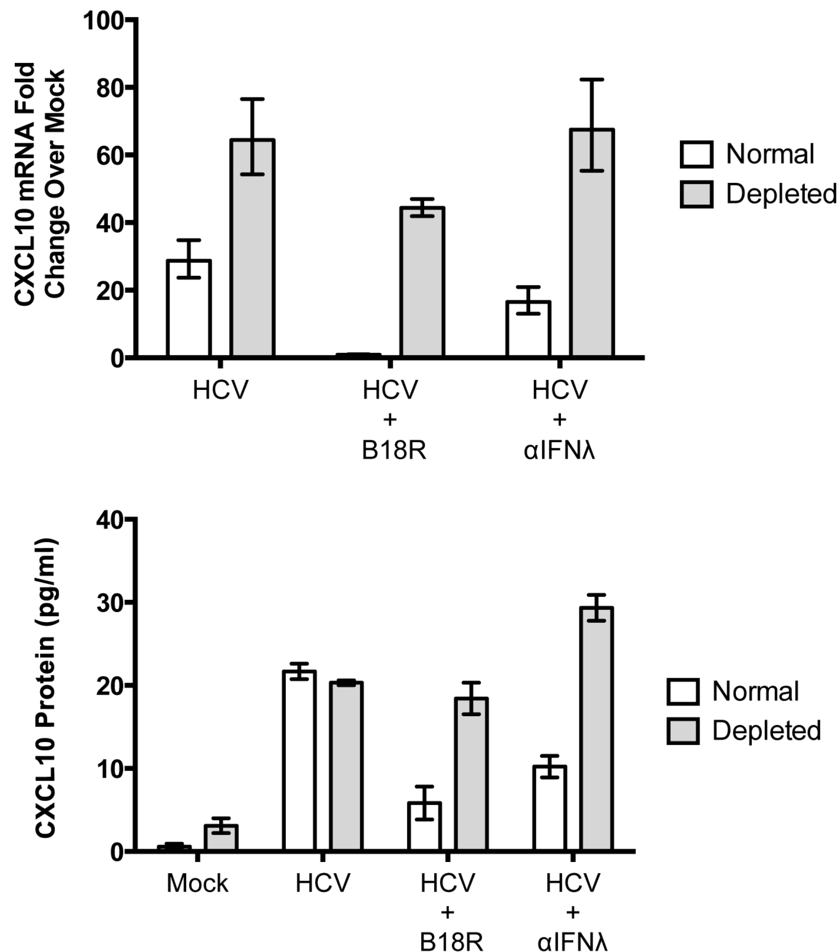
As expected, NPC-depleted PHH cultures had much lower induction of immune cell markers following HCV infection at 24 and 48 hours (MOI 0.2) (Figure 4.11A). Genes that were strongly induced in standard cultures ("Normal") that were absent from NPC-depleted cultures ("Depleted") included markers of dendritic cells (CD209) and macrophages (CXCL13, CD86). Additionally, cells that bound to the column ("Bound Cells") expressed several markers characteristic of the monocyte/macrophage lineages (Figure 4.11B). Bound Cells also showed expression of type I IFNs. This subset of genes represents the only genes detected by the Fluidigm real-time RT-PCR for Bound Cells.



<u>Figure 4.11:</u> Immune Cell Marker Induction is Lower in NPC-Depleted Primary Hepatocyte Cultures. (A) A selection of genes from the Fluidigm real-time RT-PCR immune cell marker panel (see Table 2.2, Chapter 2, pg 34) illustrating changes in HCV-induced gene expression between standard primary hepatocyte cultures (Normal) and primary hepatocyte cultures depleted of contaminating non-parenchymal cells and lymphocytes (Depleted) at 24 and 48 hours post-infection (MOI 0.2). Heat map values represent the fold change of gene induction over mock-infected cells. **(B)** A selection of genes from the immune cell marker panel that were detectably expressed by unstimulated cells depleted from primary hepatocytes cultures in two separate experiments (Bound Cell). Heat map generated from raw Ct values.

Donor-matched, NPC-depleted and non-depleted PHH cultures were used to repeat the type I and type III IFN neutralization experiments depicted in Figures 4.4 and 4.8. As expected, neutralization of type I IFN reduced CXCL10 mRNA to undetectable levels (Figure 4.12, top) and reduced CXCL10 protein by 73% (Figure 4.12, bottom) in non-depleted ("Normal") PHH cultures during early HCV infection (MOI 0.2, 48 hours).

Neutralization of type III IFN modestly reduced induction of CXCL10 mRNA and protein by 42% and 53% respectively in these same cells. In contrast, HCV-induction of CXCL10 mRNA and protein in Depleted PHH were comparatively unaffected by neutralization of either IFN. These data indicate that residual NPCs in PHH preparations produce type I 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 IFN independent.



<u>**Figure 4.12:**</u> **Reduced Impact of Type I IFN Neutralization on CXCL10 Induction in NPC-Depleted Primary Hepatocyte Cultures. (A)** Reduction in HCV-induced CXCL10 mRNA (top) and protein (bottom) observed in Normal primary hepatocyte cultures at 48 hours post-infection (MOI 0.2) following neutralization of type I IFNs is recovered in Depleted cultures to 69% of the non-neutralized response. Neutralization of type III IFNs had no impact on CXCL10 induction in Normal or Depleted cultures. **(B)** Neutralization of type I IFNs has no impact on IFIT1 mRNA induction in standard primary hepatocyte cultures at 48 hours post-HCV infection (MOI 0.2) but reduces IFIT1 response in Depleted cultures. Type III IFN neutralization did not impact IFIT1 induction in either culture.

DISCUSSION

Hepatocytes express both TLR3 and RIG-I and produce both type I and type III IFNs *in vivo* [21,23,27]. 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 showed that both TLR3 and RIG-I signaling are required for maximal induction of CXCL10 during *in vitro* HCV infection of hepatocytes. A direct, positive correlation between intracellular CXCL10 and viral protein expression was also observed via immunofluorescence, while CXCL10, type I IFN, and type III IFN induction occurred simultaneously in TLR3+/RIG-I+ Huh7 cells and PHH. Furthermore, neutralization of hepatocyte-derived IFNs did not affect CXCL10 production during HCV infection in TLR3+/RIG-I+ Huh7 cells. However, neutralization of type I and, to a lesser extent, type III IFN did have an effect on CXCL10 production during early HCV infection of standard PHH cultures. The IFN requirement was significantly reduced following depletion of NPCs from the culture, similar to the previous finding that IFNs are not required for CXCL10 induction in response to HCV in Huh7 monoculture. Thus, our study reveals that CXCL10 induction in hepatocytes during early 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 [40]. Indeed, patients with robust ISG

expression in the liver before treatment are less likely to respond to standard IFN-based anti-HCV therapy [41]. We and others have also observed that PHH generate weak type I and robust type III IFN responses following PRR stimulation (see Chapter 3, Figure 3.3) and during HCV infection *in vitro* (See Figure 4.7 and [23,24,42]). Robust induction of IL-29 mRNA was also observed in serial liver biopsies from chimpanzees with acute HCV infection[42]. While these IFNs may induce expression of other documented ISGs, the kinetics of type I and type III IFN responses did not reflect a causal role in the induction of CXCL10 in PHH (Figure 4.7). Maximal production of type III IFNs coincided with the peak of CXCL10 expression at 48 hours in PHH, while peak type I IFN production varied by donor and did not coincide with, nor precede, CXCL10. The low-level induction of type I IFNs in TLR3+/RIG-I+ Huh7 cells also coincided with the rise in CXCL10 production. Furthermore, neutralization of these residual responses failed to impact CXCL10 production during HCV infection of these immortalized cells (Figure 4.4). This lack of effect was replicated in PHH cultures that had been depleted of contaminating NPCs (Figure 4.12). Together, these data suggest that hepatocyte-derived IFNs do not play a significant role in CXCL10 production during the initial hepatocyte response to HCV infection, although they may induce other ISGs such as IFIT1.

Our data instead suggest that CXCL10 induction in hepatocytes during the early phase of infection occurs through direct activation via transcription factors following TLR3 and RIG-I engagement. Previous studies have shown that the CXCL10 promoter can be directly activated by IRFs in non-hepatic cell types following polyI:C exposure or virus infection [43,44]. IRF3 has been implicated in the direct induction of several other

ISGs during viral infections, including IFIT1 (ISG56), ISG54, and ISG15 [44,45]. This binding can occur independently of type I IFN [44,46], supporting the novel observations reported here regarding HCV induction of CXCL10 in hepatocytes. Production of CXCL10 and other pro-inflammatory factors is also induced by direct NF-κB activation during HCV infection in Huh7-derived cells [17,30]. Binding sites for the transcription factors AP-1 and C/EBPβ are also annotated in the CXCL10 promoter [25,47,48]. These transcription factors regulate the expression of other chemokines including CXCL8 and CCL5 [49-51]. Since we observed a linear correlation between HCV Core and intracellular CXCL10 expression via immunofluorescence (Figure 4.6), 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. For example, C/EBPβ activation has been reported following signaling from multiple TLRs but not RIG-I [48,52]. Therefore, cells expressing only one of the two required PRRs would exhibit a smaller CXCL10 induction during HCV infection, as was observed for Huh7-derived cell lines expressing either TLR3 or RIG-I alone (Figure 4.1B). In these two cell lines, greater CXCL10 induction was observed in cells expressing only TLR3. This suggests that TLR3 preferentially activates more potent transcription factors for CXCL10 induction. Indeed, induction of the NF-κB-dependent inflammatory cytokines TNF-α 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) [17]. 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 [8,15,16]. 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-κB) transcription factors activated by these two PRRs [47]. We explore which transcription factors drive CXCL10 transcription in hepatocytes during HCV infection in Chapter 5 (pg 115).

While IFNs appear to be dispensable for the initial wave of CXCL10 induction during early *in vitro* virus 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 of the whole liver *in vivo*. Indeed, neutralization of type I IFNs in non-depleted PHH cultures during 24- and 48-hour HCV infection substantially reduced CXCL10 production (Figures 4.8 and 4.12). Exogenous administration of type I or type III IFNs also led to CXCL10 induction in TLR3+/RIG-I+ Huh7 cells (Figure 4.2), and pegylated-IFNα induces robust intrahepatic ISG expression in patients who respond to anti-HCV therapy [41].

However, the minimal effect of IFN neutralization during HCV infection in Depleted PHH (Figure 4.12) 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. De-repression of this pathway by the removal of anti-inflammatory cytokines through NPC depletion (ex. IL-10; Figure 4.11) may have contributed to the increased CXCL10 mRNA induction observed in all Depleted PHH cultures (Figure 4.12). Since hepatocytes are the predominant cell type infected by HCV [53], this mechanistic shift early in infection would be crucial for maintaining the chemokine gradient responsible for recruiting NK cells, CD8+ T_c cells,

and CD4+ T_H1 to the liver during acute HCV infection *in vivo* [2,4]. Type II IFN, a potent inducer of CXCL10 in many cells types, is produced primarily by these infiltrating cells and would trigger a secondary wave of CXCL10 induction both intrahepatically and in the periphery [1,54,55]. This may explain why CXCL10 is only first detectable 3-11 weeks after HCV RNA in the plasma of acutely infected HCV patients [13].

Thus, our results 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 production results in the recruitment of the first immune effector cells to the site of infection. Secretion of type I, II, and III IFNs by these cells and NPCs then amplifies the existing 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. The IFN-independent (i.e. direct) induction of CXCL10 therefore initiates the cycle of inflammation that can lead to the development of liver fibrosis, cirrhosis, and carcinoma. Indeed, higher levels of intrahepatic CXCL10 have been found in chronic hepatitis C patients with necroinflammation and fibrosis [10]. The development of host-oriented treatments to blockade this pathway may therefore help prevent HCV-induced inflammation and reduce the incidence of liver disease among patients with chronic hepatitis C.

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CHAPTER 5:

TRANSCRIPTIONAL REGULATION OF THE CXCL10 PROMOTER DURING HCV INFECTION OF HEPATOCYTES

“IF YOU CANNOT CONTROL YOURSELF,

YOU CANNOT COMMAND OTHERS."

- KLINGON PROVERB, *STAR TREK*

OVERVIEW

This chapter surveys the transcription factors involved in the induction of CXCL10 in hepatocytes during HCV infection and following exposure to TLR3 and RIG-I specific PAMPs. We identified both positive and negative regulators of induction using wild-type and mutant CXCL10 promoter-luciferase reporter constructs. Follow-up experiments focused on endogenous IRF3-mediated induction of CXCL10. These data expand upon the current knowledge regarding the mechanism behind CXCL10 induction in hepatocytes and lays the foundation for further mechanistic studies that help further elucidate the combinatorial and synergistic nature of the PRR signaling pathways.

A manuscript is currently being prepared from this chapter. As in Chapter 1, portions of the Introduction were reprinted in our review "Molecular Pathways: Hepatitis C Virus, CXCL10, and the Inflammatory Road to Liver Cancer." (*Clinical Cancer Research*, 2013, 19(6): 1347-1352; see Appendix A).

INTRODUCTION

Recognition of viral components by pattern recognition receptors (PRRs) during hepatitis C virus (HCV) infection leads to the induction of various pro-inflammatory and anti-viral genes including interferons (IFNs), cytokines, and chemokines (See Chapters 3 and 4 and ref. [1-5]). The specific set of induced genes depends upon the transcription factors that are activated and translocate into the nucleus during this signaling cascade [6,7]. However, considerable redundancy also exists within the PRR signaling network [7,8]. For example, signaling from either TLR3 or RIG-I following exposure to double-stranded viral RNAs activates an overlapping set of transcription factors that includes nuclear factor (NF)-κB and interferon-regulatory factors (IRFs) [9]. Both of these PRRs also activate mitogen-activated protein (MAP) kinase signaling pathways, which in turn regulate activator protein (AP)-1 and CCAAT/enhancer-binding protein-β (C/EBP-β) activity [10-14]. Putative binding sites for all of these transcription factors have been annotated in the promoter for the pro-inflammatory chemokine CXCL10, the serum and intrahepatic levels of which are predictive of a patient's response to anti-HCV therapy [15-18].

NF-kB is considered a central positive regulator of the pro-inflammatory response, and its role in the induction of genes such as TNFα, IL-8, and IL-1β has been well-characterized [19]. Prior to activation, NF-κB heterodimers are held in a dormant state within the cytoplasm by the IκB family of repressor proteins [19,20]. Virus-induced PRR signaling leads to phosphoryation and ubiquitin-mediated proteasomal degradation of these repressor proteins, allowing activated NF-κB to translocate into the nucleus and bind to the promoters of pro-inflammatory genes such as CXCL10 [21]. For example,

NF-κB drives CXCL10 transcription during rhinovirus infection *in vitro* and *in vivo* [15].

Recently, Li *et al* showed that HCV infection of TLR3-expressing hepatoma cells can also induce NF-κB binding to the CXCL10 promoter [4]. While this study indicates that NF-κB is a critical pro-inflammatory regulator of CXCL10 induction during early HCV infection, other pro-inflammatory transcription factors may still modulate this response. One likely contributor is the dimeric transcription factor AP-1, which can consist of members of the Fos, Jun, ATF, and JDP families of proteins [22]. AP-1 is involved in the regulation of a wide variety of genes, and its regulatory function can vary depending on which subunits comprise the dimer and the surrounding cellular environment. For example, a JunB homodimer induces expression of IL-4 in T helper (T_H) cells under T_H2 polarizing conditions, while JunD homodimers inhibit IL-4 induction in these cells and prevent differentiation [23,24]. Alternatively, phosphorylation and activation of Jun homodimers by the MAP kinase JNK under cell-stress conditions triggers apoptosis via induction of TNFα and FasL [22]. Such dynamic functionality is also observed with the transcription factor C/EBP-β, which can form a homodimer or a heterodimer with other C/EBP proteins to induce a wide variety of gene products including pro-inflammatory cytokines [25-28]. As described in Chapter 1, both AP-1 and C/EBP-β have also been shown to activate transcription of CXCL8 (i.e., interleukin [IL]-8) [29,30]. This chemokine possesses many structural and functional similarities to CXCL10, and is also elevated in patients with chronic hepatitis C [31,32]. Thus, AP-1 and C/EBP-β may contribute to the pro-inflammatory induction of CXCL10 during HCV infection in a manner similar to NF-κB.

The promoters for both CXCL8 and CXCL10 can also be bound by IRF1 and

IRF3 during viral infection, with additional binding by IRF2 and IRF7 previously demonstrated for CXCL10 [33-36]. Activation of IRF3 and IRF7 can also lead to the induction of antiviral type I IFNs (IFNα and IFNβ) and type III IFNs (IL-28A, IL-28B, IL-29) in hepatocytes [7,37]. These secreted cytokines can act in a paracrine manner to amplify chemokine and cytokine responses in adjacent liver cells through activation of Janus kinases (JAK) and various STAT proteins [37,38]. Activation of JAK-STAT signaling induces IFN-stimulated genes (ISG) through the binding of STAT dimers to IFN-stimulated response elements (ISRE) or IFNγ activation site elements in their promoters [37,38]. Type II IFN, a related cytokine produced by NK cells, CD8+ T_c cells, and CD4+ T_H1 cells, can also induce STAT1 signaling through these elements [38,39]. As the CXCL10 promoter contains both putative ISREs and putative STAT-binding sites, it can potentially respond to all 3 types of IFN [15]. Despite these observations in other systems, we observed that neutralization of type I and type III IFNs had no effect on CXCL10 production by primary human hepatocytes and hepatoma cells expressing functional TLR3 and RIG-I during early HCV infection in (see Chapter 4, pg 73). It has yet to be determined if IRFs play a role in CXCL10 induction independently of the action of type I and type III IFNs.

Our previous data suggest that CXCL10 induction in hepatocytes during the initial steps of HCV infection occurs predominantly through direct activation of transcription factors following PRR signaling rather than through secondary paracrine signaling of IFNs. Therefore, we sought to identify and characterize the pro-inflammatory and anti-viral transcription factors responsible for this induction. Our data show that NF-κB and IRF3 are crucial positive regulators of CXCL10 induction both

during early HCV infection and following treatment with TLR3- and RIG-I-specific PAMPs. In contrast, AP-1 and C/EBP-β appear to be minor negative regulators of CXCL10 induction during PAMP treatment but have limited impact during early HCV infection.

MATERIALS AND METHODS

Detailed protocols, reagents, and statistics are included in Chapter 2 (pg 29).

Cells

PH5CH8 immortalized hepatocytes [40] and Huh7 hepatoma cells stably transduced with a FLAG-TLR3-encoding lentiviral vector (TLR3+/RIG-I+ Huh7 cells) [41] were maintained in culture as described in Chapter 2 (see pg 30). Cells were previously confirmed to express functional TLR3 and RIG-I (see Chapter 3, pg 45).

Viruses

JFH-1 HCV (genotype 2a) viral stock preparation and titration were performed as described previously [42,43]. Cell cultures were infected at MOIs (multiplicities of infection) of 0.6 or 1.0 using the protocol described in Chapter 2 (pg 32). Infected hepatocyte cultures were incubated for 12, 18, or 24 hours.

Sendai Virus (SeV; Cantrell Stain; Charles River Laboratories [Wilmington, MA]) was diluted in serum-free DMEM and 100 hemagglutination units (HAU) added to cells as described in Chapter 2 (pg 32). Infected hepatocyte cultures were incubated for 24 hours.

Pathogen Associated Molecular Patterns (PAMPs)

For specific activation of RIG-I, cells were transfected with 0.5 μg of 5' pU HCV PAMP RNA (provided by Michael Gale Jr. [University of Washington]). Specific activation of TLR3 was achieved by adding 5 μg/ml polyI:C (Amersham, now GE Healthcare Life Sciences [Pittsburgh, PA]) to cell culture medium. Cells were incubated with all polyI:C and 5' pU HCV PAMP treatments for 24 hours.

Plasmids

Firefly luciferase reporter pGL4 plasmids expressing the wild-type, ΔκB1, ΔκB2, ΔAP-1, ΔC/EPB-1, or ΔISRE CXCL10 promoter were provided by David Proud (University of Calgary) [15]. ΔISRE and wild-type CXCL10 constructs were sequenced through SimpleSeq DNA Sequencing (Eurofins MWG Operon [Huntsville, AL] in combination with ThermoFischer Scientific [Rockford, IL]) to verify the ΔISRE mutation. A CMV-BL plasmid encoding the constitutively active mutant IRF3 (IRF3-5D) [44] was provided by John Hiscott (McGill University). The empty vector pcDNA3.1 plasmid was obtained from Invitrogen (Carlsbad, CA).

Luciferase Assays

For PRR-specific activation, 50 ng of each CXCL10 Firefly luciferase reporter plasmid (see above) was transfected into cells using MATra-A. After 24 hours, exogenous polyI:C or transfected 5' pU HCV PAMP was added to designated wells as described above. PBS and 100 ng/ml IFNγ in combination with 40 ng/ml TNFα were

also added as negative and positive controls respectively. Luciferase activity was read after an additional 24 hours using BriteLite reagent (PerkinElmer, Waltham, MA).

For JFH-1 HCV-based activation, CXCL10 plasmids were transfected into cells using X-treme Gene 9 (Roche, Indianapolis, IN). Cells were then infected 48 hours post-transfection with JFH-1 HCV (MOI 1) or treated with IFNγ/TNFα or PBS as above. Luciferase activity was read after an additional 24 hours using BriteLite reagent. Cell viability in each well during infection was assessed via Cell Titer Fluor (Promega) and used to normalize luciferase readings.

To evaluate direct activation via IRF3, 50 ng of IRF3-5D plasmid or pcDNA3.1 was co-transfected with 50 ng of the wild-type or ΔISRE CXCL10 reporter plasmid into cells using MATra-A. Two μg/ml B18R protein (eBioscience, San Diego, CA) or 4 μg/ml IL-28B/IL-29 neutralizing antibody (R&D Systems, Minneapolis, MN; MAB15981) was then immediately added to the culture media to neutralize type I IFN or type III IFN respectively (PBS was added to control samples). Luciferase activity was read after 24 hours using BriteLite reagent and were normalized for cell viability as above.

Chromatin Immunoprecipitation (ChIP)

Cells were mock infected, infected with SeV for 6 hours, or infected with JFH-1 HCV for 12 or 18 hours (MOI 0.6; see above for protocol). Chromatin was harvested following formaldehyde-fixation and sheared by sonication using a probe-Ultrasonic Homogenizer (Cole-Parmer Instrument Co; 4710 series). ChIP was performed on 15 μg of each chromatin preparation using the ChIP Express Chromatin Immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer’s protocol. ChIP reactions

were incubated overnight using polyclonal rabbit anti-IRF3 sera (Active Motif) or normal rabbit sera (Jackson ImmunoResearch) at equivalent concentrations. Chromatin fragments were PCR amplified using Phusion Hot Start II High-Fidelity DNA Polymerase (ThermoFischer Scientific) and primers directed against the ISRE region of the CXCL10 promoter (Forward: 5'-TGGATTGCAACCTTTGTTTTT-3'; Reverse: 5'-GTCCCATGTTGCAGACTCG-3'; Tm: 64°C). Input DNA samples and a no template reaction were included as positive and negative controls respectively. PCR products were resolved on a 1% TBE agarose gel and visualized using a GelDoc (BioRad, Hercules, CA). Bands were quantified using ImageJ (Version 1.46; http://rsb.info.nih.gov/ij/).

RESULTS

Positive and Negative Regulation of the CXCL10 Promoter

The CXCL10 promoter contains binding sites for multiple different transcription factors involved in pro-inflammatory and anti-viral innate immune response [15]. In order to evaluate the importance of these factors for TLR3 and RIG-I signaling to CXCL10 induction, luciferase reporter gene constructs driven by either wild-type or mutated CXCL10 promoters were transfected into PH5CH8 immortalized hepatocytes, which express both PRRs (Figure 5.1) [40]. The mutated CXCL10 promoters contain point mutations in the proximal ISRE (ΔISRE) as well as in the proximal binding sites for NF-κB (ΔkB1, ΔκB2), AP-1 (ΔAP-1), C/EPB-β (ΔC/EPB-β1, ΔC/EPB-β2) [15,45]. After 24 hours, 1 μg/ml polyI:C was added to the culture medium or .2 ug 5' pU HCV PAMP was transfected into cells to activate TLR3 and RIG-I respectively. Combination treatment

with 100 ng/ml IFNγ and 40 ng/ml TNFα served as a positive control. Luciferase signal was then measured after an additional 24 hours.

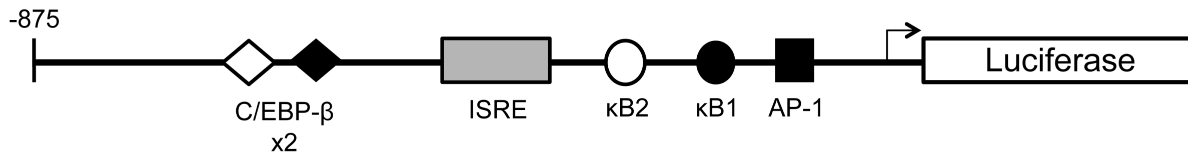
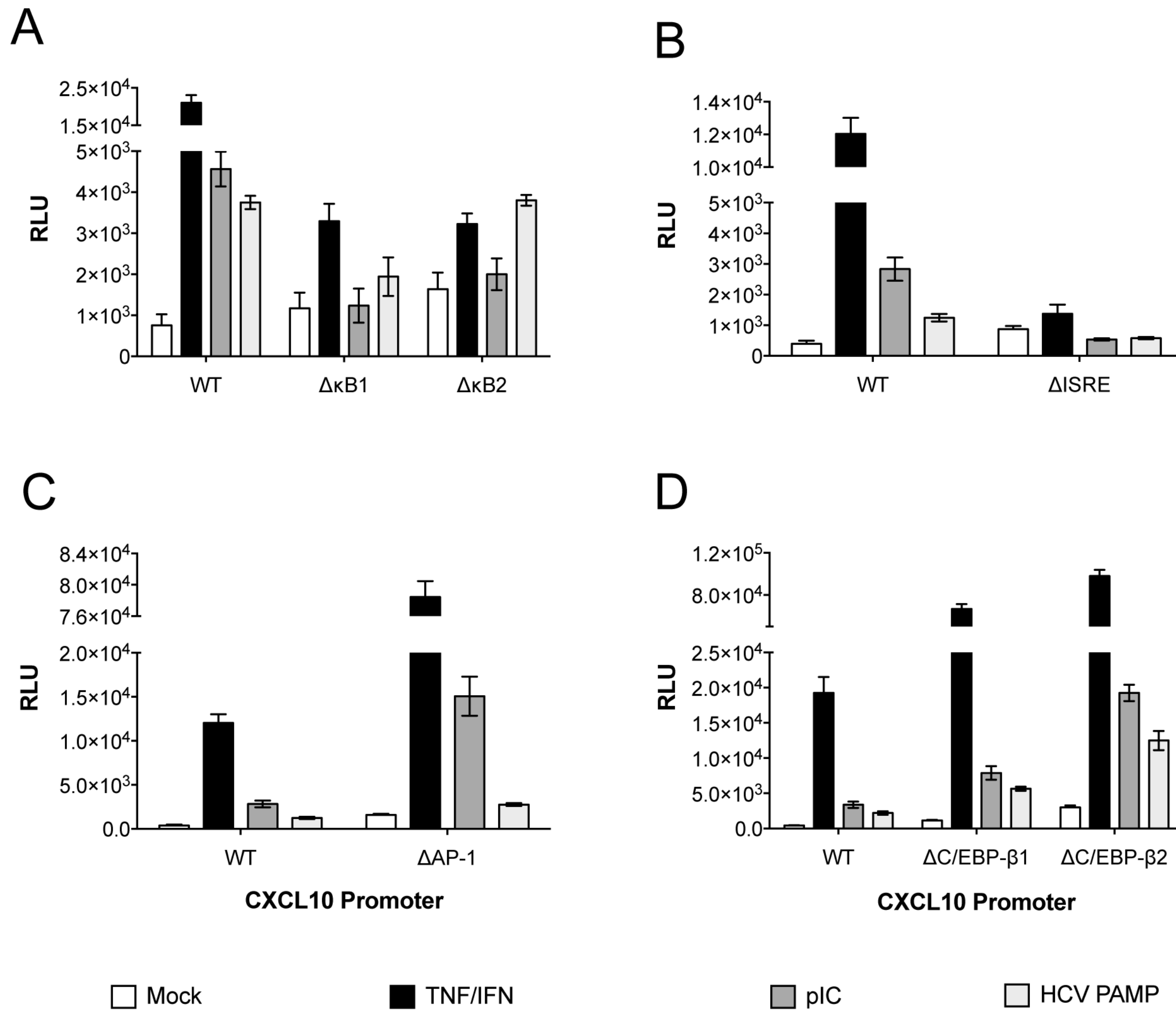


Figure 5.1: Schematic of the CXCL10 Promoter Constructs. Putative transcription factor binding sites are labeled. Schematic adapted from ref. [15].

As expected, the wild-type promoter strongly responded to the IFNγ/TNFα treatment as well as both PRR stimuli (Figure 5.2). Mutating the more proximal NF-κB site (ΔkB1) significantly reduced these responses ($p<0.05$), with the induction from the HCV PAMP and polyi:C returning to baseline levels (Figure 5.2A). The ΔkB2 mutation also resulted in a significant decrease in the CXCL10 response to IFNγ/TNFα and polyI:C ($p<0.01$). However, no decrease in signal was observed in response to the HCV PAMP, suggesting that NF-κB binding to these sites is stimulus-specific.

CXCL10 transcription was also significantly decreased in response to all three treatments after mutation of the proximal ISRE (ΔISRE; $p<0.01$; Figure 5.2B). In contrast, treatment with either PAMP lead to an increase in luciferease signal over wild-type for the ΔAP-1, ΔC/EPB-β1, and ΔC/EPB-β2 constructs ($p<0.01$; Figure 5.2C-D). These data indicate that AP-1 and C/EBP-β act as negative regulations of CXCL10 induction following TLR3 and RIG-I activation. Furthermore, as all constructs responded with similar intensity to either stimulus, TLR3 and RIG-I likely independently and equally activate all of these transcription factors in response to non-viral PRR-specific PAMPs.



<u>Figure 5.2:</u> Wild-Type and Mutant CXCL10 Promoters Respond to TLR3 and RIG-I PAMPs. Deletion of the **(A)** NF-κB binding sites (κB1, κB2) or **(B)** proximal ISRE eliminates activation of the CXCL10 promoter following 24 hour treatment with either exogenously added polyI:C (5 μg/ml) or 0.5 μg transfected 5' pU HCV PAMP ($p<0.05$). Deletion of the **(C)** AP-1 binding site or **(D)** C/EBP-β binding sites (C/EBP-β1, C/EBP-β2) results in increased activity of the CXCL10 promoter in response to these stimuli ($p<0.01$). Combination treatment with 100 ng/ml IFN-γ and 40 ng/ml TNFα for 24 hours served as a positive control for induction.

We also sought to evaluate the responsiveness of these constructs during HCV infection. TLR3+/RIG-I+ Huh7 cells were transfected as above and infected with JFH-1 HCV (MOI 1.0) after 48 hours. Luciferase values were read after an additional 24 hours and normalized according to cell viability, which was comparable between mock and

HCV infected cells (data not shown). Similar to the PAMP treatments, the wild-type CXCL10 promoter responded strongly to HCV infection (Figure 5.3). This is consistent with our previous observations of CXCL10 mRNA and protein induction during virus infection (see Chapter 4). The ΔkB1, ΔkB2, and ΔISRE promoter responses were also similar to those observed with the PAMPs, showing significant decreases in signal in comparison to the wild-type promoter ($p<0.05$; Figure 5.3A,B). However, the ΔAP-1 response was not significantly different from the wild-type response ($p>0.1$; Figure 5.3C), contrary to the results from treatments with PAMPs. The increase in CXCL10 induction observed with the ΔC/EPB-β1 construct was also less than that observed during PAMP stimulation, although it remained significantly higher than wild-type ($p<0.05$; Figure 5.3D). Increased CXCL10 induction above wild-type from the ΔC/EPB-β2 construct remained at the same level of significance ($p<0.01$; Figure 5.3D).

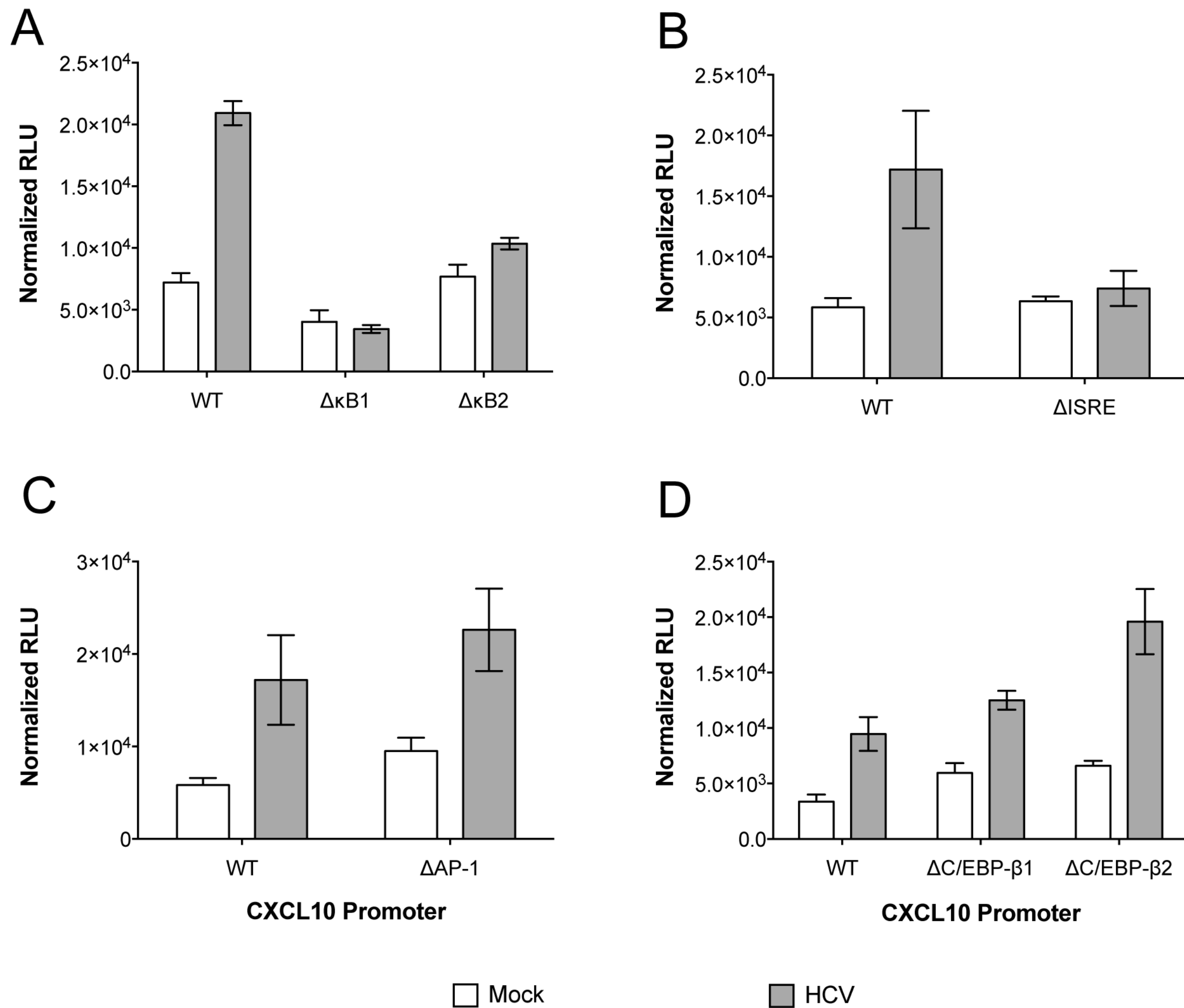
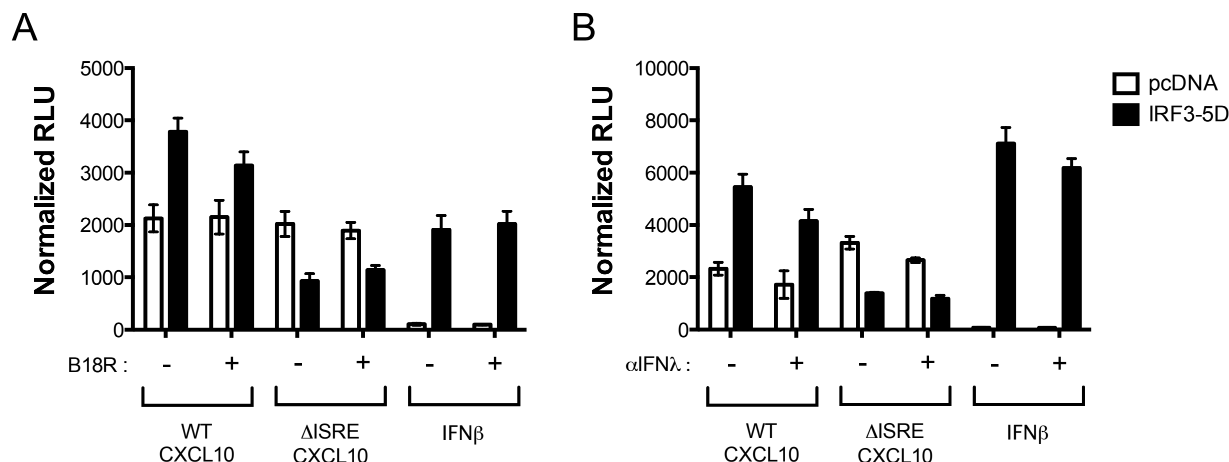


Figure 5.3: Wild-Type and Mutant CXCL10 Promoters Respond to HCV Infection. Deletion of the **(A)** NF-κB binding sites (κB1, κB2) or **(B)** proximal ISRE significantly decreases activation of the CXCL10 promoter following 24 hour HCV infection ($p<0.05$; MOI 1.0). **(C)** Deletion of the AP-1 binding site had no significant effect on the CXCL10 promoter response during HCV infection ($p>0.1$). **(D)** Deletion of the C/EBP-β binding sites (C/EBP-β1, C/EBP-β2) increased the CXCL10 promoter response during HCV infection ($p<0.05$).

IRF3-5D Activates CXCL10 Transcription During Interferon Neutralization

We previously observed no contribution of type I or type III IFNs, which induces the ISGF3G complex that bind ISREs [46], to CXCL10 induction in immortalized cell lines (see Chapter 4, pg 73). This contradicts the data presented above. Therefore, we

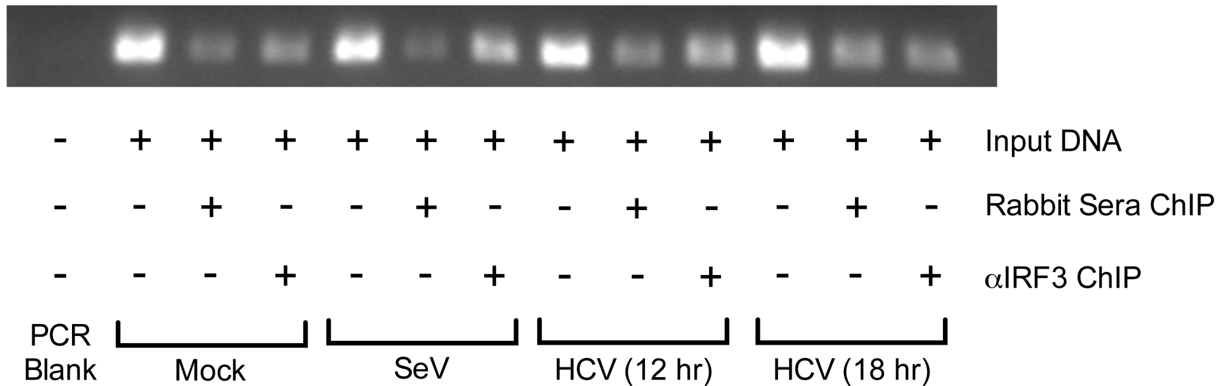
hypothesized that one or more IRFs may be directly binding to the CXCL10 promoter. IRF3, which binds to the CXCL8 promoter following HCV infection and RIG-I activation, was chosen as a likely candidate [35]. A constitutively active mutant form of IRF3 (IRF3-5D) or an empty vector (pcDNA 3.1) was co-transfected with the wild-type CXCL10 promoter construct into PH5CH8 immortalized hepatocytes. IRF3-5D was also co-transfected with an IFNβ luciferase reporter construct as a positive control. Luciferase activity was then read in the presence and absence of B18R (Figure 5.4A) or a pan-type III IFN-neutralizing antibody (αIFNλ; Figure 5.4B) to observe the effects of blocking interferon signaling and normalized for cell viability as above. In both systems, IRF3-5D induced transcription of the wild-type CXCL10 and the IFNβ promoter constructs. While the addition of either neutralizing agent significantly reduced CXCL10 induction compared to non-neutralizing conditions ($p<0.05$), expression during neutralization was still significantly higher than baseline ($p<0.05$). Induction was also completely absent for the ΔISRE CXCL10 construct, indicating the specificity of IRF3 binding for this site.

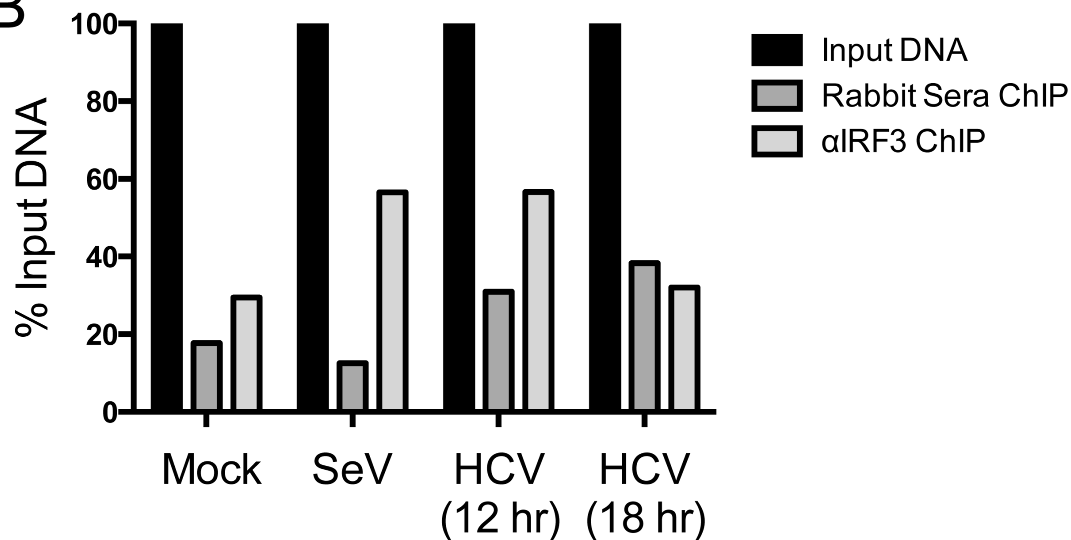


<u>Figure 5.4:</u> Constitutively Active IRF3 Drives CXCL10 Transcription During Interferon Neutralization. Neutralization of **(A)** type I IFNs via B18R or **(B)** type III IFNs via αIFNλ did not impact IRF3-5D-induced WT CXCL10 promoter activity 24 hours after co-transfection in PH5CH8 immortalized hepatocytes. IRF3-5D was not able to induce expression of the ΔISRE CXCL10 mutant promoter above background levels induced by a control vector (pcDNA3.1). IFNβ promoter responses to IRF3-5D were included as a positive control.

IRF3 Binds to the CXCL10 Promoter During HCV Infection

To confirm that IRF3 binding to the CXCL10 promoter also occurs during HCV infection, we performed a chromatin immunoprecipitation (ChIP) for IRF3 on TLR3+/RIG-I+ Huh7 cells that were infected with JFH-1 HCV for 12 or 18 hours (MOI 0.6). Uninfected and cells infected with SeV (100 HAU) for 6 hours were included as negative and positive experimental controls, respectively (Figure 5.5, “Mock” and “SeV”). Bound IRF3 bound was detected in cells infected with HCV for 12 hours (Figure 5.5, “HCV (12 hr)”). This signal was reduced to baseline by 18 hours (Figure 5.5, “HCV (18 hr)”).





<u>Figure 5.5:</u> IRF3 Binds the CXCL10 Promoter During HCV Infection. (A) TLR3+/RIG-I+ Huh7 cells were infected with HCV (12 and 18 hours; MOI 0.6) or SeV (6 hours; 100 HAU) and formaldehyde-fixed for chromatin immunoprecipitation (ChIP) using polyclonal anti-IRF3 serum. Normal rabbit sera was used as a negative control for ChIP. Isolated chromatin fragments were PCR amplified using primers specific for the proximal ISRE of the CXCL10 promoter and resolved on an agarose gel. Input DNA was used as a positive control during PCR. Under these conditions, increased IRF3 binding to the CXCL10 promoter was detected during SeV infection and after 12 hours of HCV infection. IRF3 binding returned to baseline levels 18 hours after HCV infection. (**B**) Quantification of PCR band intensities from (A) expressed as percentage of Input DNA.

DISCUSSION

Binding sites for a wide variety of transcription factors have been annotated within the CXCL10 promoter, including those for NF-κB, AP-1, C/EBP-β, and IRFs [15]. In the current study, we not only confirmed previous reports that NF-κB is a critical positive regulator of this promoter during HCV infection and PRR activation, but we have also demonstrated for the first time that AP-1 and C/EBP-β are negative or neutral regulators of CXCL10 induction under these conditions. The observed negative or neutral regulation occurred in a PAMP-dependent manner, which is summarized in Table 5.1. The ISRE most proximal to the CXCL10 transcriptional start site was also found to be a site of strong positive regulation, and we determined that IRF3 binds directly to this site during early HCV infection. Together, these data indicate that CXCL10 induction during both early HCV infection and PRR-specific stimulation is predominantly mediated by NF-kB and IRF3, with minor negative modulation by AP-1 and C/EBP-β.

	Effect on CXCL10 Promoter Activation		
Transcription Factor	**HCV PAMP**	**polyI:C**	**HCV Infection**
NF-κB	+ + +	+ + +	+ + +
IRF3	+ +	+ +	+ +
AP-1	- -	- -	NE
C/EBP-β	- -	- -	-

<u>Table 5.1:</u> PAMP-Dependent Effects of Transcription Factor Binding to the CXCL10 Promoter. Proposed effect of each transcription factor on CXCL10 promoter activation in response to transfected HCV PAMP ("HCV PAMP"), exogenously-added polyI:C ("polyI:C), or HCV infection (MOI 1.0, 24 hours). Listed effects are extrapolated based on the results presented for mutated CXCL10 promoters in Figures 5.1 and 5.2. NE: No Effect.

Although AP-1 and C/EBP-β are typically considered positive regulators of gene expression, instances of negative regulation have also been documented. C/EBP-β has

been shown to negatively regulate transcription of the tumor suppressor microRNA miR-145 in breast cancer cells [47]. There is also evidence that C/EBP-β inhibits collagen synthesis in fibroblasts in response to type II IFN and ERK1/2 MAP kinase signaling [48]. Similarly, Fos/Jun AP-1 heterodimers have been shown to negatively regulate transcription of the steroidgenic enzyme CYP17 following ERK1/2 activation [49], and we previously described the negative regulation of IL-4 by the JunD AP-1 homodimer [24]. Given the vast array of subunits that can comprise AP-1, other less well-studied heterodimers may also negatively regulate the induction of target genes. Different heterodimers could also be activated in response to different stimuli. This could potentially explain the differential effect of AP-1 on CXCL10 induction during treatment with PRR-specific PAMPs (negative regulation) in comparison to early HCV infection (no significant regulatory effect). Future studies may seek to identify the specific subunit combinations responsible for modulating CXCL10 induction in response to each stimulus.

Induction of CXCL10 in hepatocytes may also be influenced by transcription factors not surveyed in our experiments. For example, type II IFN (i.e. IFNγ) signaling leads to the formation of STAT1 homodimers that bind to Gamma Interferon Activation Site (GAS) elements in ISGs [38]. Type II IFN is the canonical inducer of CXCL10 in immune cells, and GAS elements have recently been identified within the CXCL10 promoter [50,51]. Additionally, other IRFs besides IRF3 may bind the proximal ISRE in the CXCL10 promoter. IRF1 and IRF7 have been shown to directly bind this site in lung epithelial cells following influenza-A infection [33]. Secretion of type I and type III IFNs and activation of JAK/STAT signaling during viral infection also leads to formation of the

canonical ISRE-binding ISGF3 transcription factor complex [46]. This complex may compete with IRF3 for binding to the proximal CXCL10 ISRE during late acute and chronic hepatitis C when non-parenchymal cells and peripheral immune cells have been activated and recruited to the site of infection within the liver. However, ISGF3 is unlikely to play a central role in our experimental system since type I and type III IFNs were neutralized during instances of significant CXCL10 induction by IRF3-5D (Figure 5.4) and HCV infection (see Chapter 4). Other ISREs have also been annotated further upstream within the CXCL10 promoter [15]. ISGF3 and other IRFs may alternatively bind to these sites during later stages of HCV infection and work synergistically with IRF3 to promote CXCL10 induction.

Non-traditional signaling pathways may also be responsible for activation of transcription factors that drive CXCL10 induction. Ho and colleagues reported IFN-independent activation of STAT1 and STAT3 proteins during infection with dengue virus, another member of the *Flaviviridae* [52]. STAT1 can also be activated via p38 mitogen-activated protein (MAP) kinase following TLR7 stimulation in plasmacytoid dendritic cells [53]. As STAT1 can bind to both ISREs and GAS elements it is possible that this alternative pathway also contributes to CXCL10 induction during early HCV infection, although this has not yet been shown experimentally. It also remains to be demonstrated whether IFN-independent STAT activation can be induced following TLR3 and RIG-I signaling in hepatocytes.

In summary, our results indicate that NF-κB and IRF3 are crucial regulators of the CXCL10 response during early HCV infection in hepatocytes, and that this response can be partially downregulated by AP-1 and C/EBP-β. Other transcription factors,

including other IRFs and STAT proteins, may also modulate this response. Antagonism of any of these factors by viral proteins during early HCV infection could interfere with CXCL10 induction and alter the character of the initial innate immune response to one that favors perpetual inflammation and viral persistence. For example, the HCV NS5a protein alone can induce NF-kB-mediated activation of genes that can contribute to the development of interferon resistance, fibrosis, and hepatocellular carcinoma [54]. HCV has also been shown to prevent nuclear translocation of activated IRF7 during later stages of infection [55], and the Core protein specifically is known to inhibit IRF3 dimerization and activation of IFNβ transcription [56]. Further elucidation of the complex and combinational mechanisms behind transcriptional control of CXCL10 may help to identify novel targets for host-oriented therapies for controlling the persistent and damaging liver inflammation that is characteristic of chronic hepatitis C.

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CHAPTER 6:

DISCUSSION AND FUTURE DIRECTIONS

"HAVE RULED OUT ARTIFICIALLY INTELLIGENT VIRUS. UNLESS IT'S *VERY* INTELLIGENT. AND TOYING WITH ME. HMM. TESTS..."

- PROFESSOR MORDIN SOLUS, *MASS EFFECT 2*

OVERVIEW

This chapter summarizes the results presented in the preceding chapters and discusses their scientific implications regarding hepatic inflammation and hepatitis C *in vivo*. The importance of the direct, interferon-independent CXCL10 induction pathway during hepatitis C is discussed in the context of the larger literature on hepatitis C pathogenesis and hepatitis C virus immune evasion strategies. Possible future directions for this research are proposed, including the potential for CXCL10 regulatory pathways as targets for host-oriented therapies for controlling HCV-related inflammation and liver disease.

Portions of this chapter were reprinted in our review "Molecular Pathways: Hepatitis C Virus, CXCL10, and the Inflammatory Road to Liver Cancer" (*Clinical Cancer Research*, 2013, 19(6): 1347-1352; see Appendix A).

SUMMARY OF RESULTS

The preceding chapters presented data on the induction and regulation of CXCL10 during early HCV infection of hepatocytes *in vitro*. Results described in Chapter 3 confirmed that primary human hepatocytes (PHH), PH5CH8 immortalized hepatocytes, and specifically engineered Huh7 hepatoma cell lines express functional versions of the pathogen recognition receptors (PRRs) Toll-like receptor 3 (TLR3) and retinoic acid inducible gene-I (RIG-I). Each PRR could be activated by a specific pathogen associated molecular pattern (PAMP) to induce production of CXCL10, other chemokines (CXCL8 and CCL4), and interferons (IFNs). While the presence of overlapping downstream pathways suggested the possibility of cooperative induction of CXCL10, synergism between TLR3 and RIG-I was only observed in cell lines overexpressing TLR3 (Chapter 3, Figure 3.7).

As presented in Chapter 4, both TLR3 and RIG-I were required for maximal CXCL10 induction during HCV infection using a collection of Huh7-derived cell lines that differentially expressed each receptor. Type I and type III IFNs were dispensable for CXCL10 induction during HCV infection of the TLR3 and RIG-I expressing Huh7 cell line (TLR3+/RIG-I+ Huh7 cells; Chapter 4, Figure 4.4), suggesting that CXCL10 is induced in an IFN-independent manner in these cells. This was confirmed through the observation of a positive correlation between intracellular CXCL10 and HCV Core proteins via immunofluorescence (Chapter 4, Figure 4.6).

Type I and, to a lesser extent, type III IFNs were also required for CXCL10 induction in standard PHH cultures (Chapter 4, Figures 4.8 and 4.12). Since non-parenchymal cells (NPCs) such as Kupffer cells (KCs), liver sinusoidal endothelial cells

(LSECs), and hepatic stellate cells can produce IFNs and other cytokine *in vivo* [1], it was hypothesized that these cell types instead of hepatocytes were responsible for the IFN requirement in PHH cultures. To test this hypothesis, baseline and HCV-induced mRNA expression of a panel of 46 cytokine and immune cell markers were examined in 8 different PHH preparations (Chapter 4, Figures 4.9 and 4.10). TLR3+/RIG-I+ Huh7 cells included for comparison saw little to no expression of these mRNAs at baseline. In contrast, all 8 PHH cultures showed baseline expression of these markers, which included type I IFNs, CXCL13, CD14, MARCO, and CD86. The level of expression varied between samples, suggesting that the level of NPC contamination was different for each PHH preparation.

Contaminating NPCs were removed from PHH cultures by immunodepletion using antibodies against CD45 (leukocytes), CD68 (macrophages [including KCs]), and CD31 (LSECs). While non-depleted PHH cultures (“Normal”) had elevated expression of macrophage markers (CXCL13, ITGAX, CD86) and cytokines (IFNγ and interleukin [IL]-10) in response to HCV infection, these transcripts were undetectable in PHH cultures depleted of NPCs (“Depleted”; Chapter 4, Figure 4.11). Furthermore, the cells removed from Depleted PHH cultures (“Bound Cells”) expressed transcripts characteristic of monocyte/macrophage lineages as well as type I IFNs, suggesting that contaminating NPCs were the source of these cytokines in PHH cultures. This was confirmed by data showing that type I and type III IFNs were necessary for CXCL10 induction during HCV infection of Normal but not Depleted PHH (Chapter 4, Figure 4.12).

Thus, CXCL10 induction in hepatocytes during early HCV infection appears to be independent of the influence of hepatocyte-derived IFNs. Instead, the data indicate that the CXCL10 promoter is under the direct transcriptional control of NF-kB and IRF3, which are activated downstream of TLR3 and RIG-I signaling. Minor negative regulation of the CXCL10 promoter by AP-1 and C/EBP-β was also observed, but only during treatment with artificial PRR-specific PAMPs. Thus, CXCL10 production in hepatocytes appears to be regulated by both immediate, direct transcriptional activation following PRR sensing of HCV infection as well as delayed, secondary induction via IFNs secreted by other resident liver cell types and infiltrating peripheral immune effector cells.

CXCL10 AND THE ROLE OF PERSISTENT INFLAMMATION IN HEPATITIS C-RELATED LIVER DISEASE .

In Vivo *Relevance of CXCL10 Direct Induction*

The dual mechanism model detailed in this work suggests that the second, IFN-controlled wave of induction is likely the source of serum and intrahepatic CXCL10 during the later stages of acute and during chronic HCV infection. Indeed, levels of CXCL10 in the serum of acute hepatitis C patients peak at 3 months post-infection [2], while plasma levels of type I and type III IFNs are detectable at 1 and 9 weeks post-infection respectively [3,4]. Systemic levels of both IFNs and CXCL10 remain elevated over normal in chronically infected patients prior to undergoing pegylated-IFNα/Ribavirin therapy [5-7]. This suggests that the IFN response pathway quickly overshadows the proposed direct induction pathway for CXCL10 during infection *in vivo*.

However, as proposed in Chapter 4 (pg 73), the direct, IFN-independent transcriptional induction of CXCL10 within hepatocytes is likely necessary for the initial recruitment of IFN-producing effector cells and resident NPCs to sites of infection within the liver during the early stages of HCV infection. Since hepatocytes are the primary cell type infected by HCV [8], they are the first cells to generate an immune response to the nascent infection. Evidence presented in Chapter 4 indicates that this response includes weak induction of type I and type III IFNs and strong induction of CXCL10 (Figures 4.3 and 4.7). Observations of strong CXCL10 expression in hepatocytes (but not NPCs) from chronic hepatitis C patients further support a central role for this chemokine in the intrinsic, hepatocyte-initiated innate immune response [9]. IFN-producing effector cells are recruited by this local production of CXCL10 and are activated by exposure to HCV and signals from infected hepatoyctes (see below). A new wave of NPC and leukocyte derived type I, type II, and type III IFNs then leads to paracrine induction of CXCL10 in both infected and non-infected hepatocytes, dramatically increasing the production of CXCL10 in the liver and its diffusion into the bloodstream. Thus, IFN-based induction of CXCL10 is a necessary but derivative aspect of the pro-inflammatory hepatic immune response to HCV infection.

It should be noted that CXCL10 is not the only chemokine capable of inducing pro-inflammatory immune responses. For example, CXCL9 (i.e. Monokine induced by gamma interferon [MIG]) and CXCL11 (i.e. Interferon-inducible T-cell alpha chemoattractant [I-TAC]) are structurally similar to CXCL10, bind to the same receptor (CXCR3), and are induced by HCV infection [10]. However, these chemokines have not been strongly associated with the outcome of anti-HCV therapy despite being

associated with liver inflammation *in vivo* [6,11,12]. While levels of non-CXCR3 binding chemokines such as CCL5 (i.e. Regulated on Activation, Normal T cell Expressed and Secreted [RANTES]) and CXCL8 (i.e. IL-8) are also secreted in response to HCV *in vitro* and *in vivo* [13-16], they do not target the same cells for chemotaxis as CXCL10 The receptors for CCL5 (CCR5, CCR3, and CCR1) are found on many different cell types (ex. CD4+ type I helper T (T_H1) cells, eosinophils, and monocytes), while CXCL8 is primarily responsible for the chemotaxis of neutrophils via the receptors CXCR1 and CXCR2 [17,18]. In contrast, CXCR3 is expressed on CD4+ T_H1, CD8+ cytotoxic T (T_c), and natural killer (NK) cells [18-22]. This suggests that CXCL10 is a unique and central player in the recruitment of type I adaptive immune responses following HCV infection, with other chemokines playing supportive roles by recruiting additional pro-inflammatory innate immune cells. This process is described in more detail below.

Development and Perpetuation of Persistent Liver Inflammation

IFN-producing effector cells recruited to the site of infection in the liver by the initial wave of hepatocyte-produced CXCL10 include peripheral immune cells (notably NK cells, CD8+ T_c cells, and CD4+ T_H1 cells as described above) as well as the liver's own resident immune cells, known as non-parenchymal cells (NPCs). These make up 20% of total liver volume and include Kupffer cells, liver sinuosoidal endothelial cells (LSECs), and hepatic stellate cells (HSCs) [23]. While LSECs comprise the walls of hepatic blood vessels (i.e. sinusoids), Kupffer cells and HSCs are free-floating cells found within sinusoids and the Space of Disse respectively (See Figure 6.1 and ref. [23]). Kupffer cells are resident tissue macrophages involved in lipid metabolism and the

removal of damaged red blood cells from circulation [23,24]. HSCs are quiescent, Vitamin A-storage bodies that can differentiate into proliferative pro-fibrotic myofibroblasts upon activation [25]. Both Kupffer cells and HSCs also express the CXCL10 receptor CXCR3 [26,27]. Therefore, CXCL10 produced by HCV-infected hepatocytes could direct these NPCs as well as incoming CXCR3-expressing CD4+ T_H1, CD8+ T_c, and NK cells [20] to specific sites of infection within the liver.

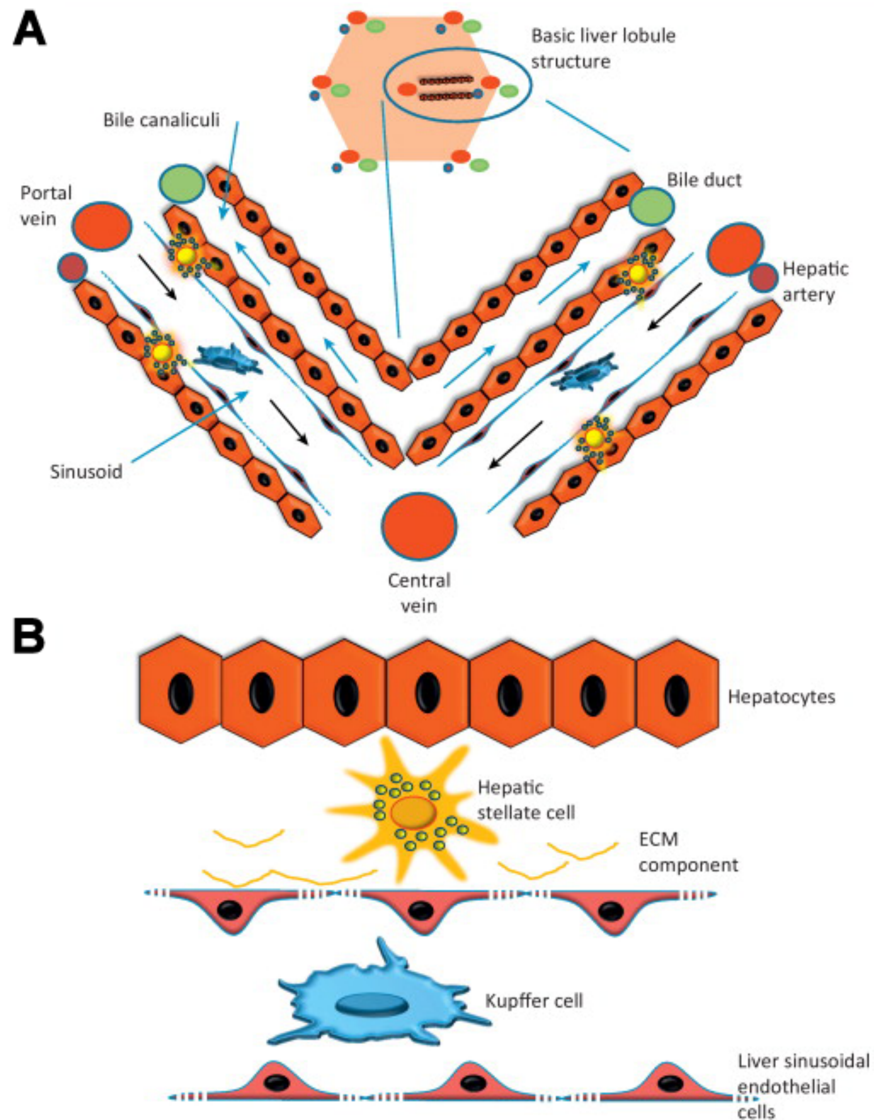

Figure 6.1: **Normal Liver Architecture. (A)** Liver lobules are constructed from sheets of hepatocytes that encapsulate and process materials from herpatic arteries (sinusoids). **(B)** Schematic representation of hepatic sinusoid, which is lined with liver sinusoidal endothelial cells (LSECs). Kupffer cells reside within the sinusoid. Hepatic stellate cells (HSCs) are found in the space between the basal membrane of LSECs and the surface of hepatocytes (i.e. the space of Disse). *Reprinted with minor modification from Trends in Parasitology, 28, Barrie J. Anthony ,Grant A. Ramm, Donald P. McManus, "Role of resident liver cells in the pathogenesis of schistosomiasis", 572-579, Copyright (2012), with permission from Elsevier*

Once recruited to the liver by CXCL10, activated CD8+ T_c and NK cells kill HCV-infected hepatocytes via Fas/TRAIL-mediated apoptosis, the release of granzymes and perforin, and secretion of type II IFN (IFNγ) [28,29]. Apoptotic bodies released from dying hepatocytes are then phagocytosed by Kupffer cells, which further promote Fas-mediated hepatocyte apoptosis and release reactive oxygen and nitrogen species (ROS/NOS) [30]. In addition, Kupffer cells can activate HSCs by releasing TGF-β [30]. This causes HSCs to differentiate into myofibroblasts and secrete type I collagen as part of the general wound healing response to liver injury [25]. Meanwhile, continued secretion of type II IFNs leads to further production of CXCL10 [10] and recruitment of additional immune effector cells to the site of infection.

Kupffer cells, HSCs, and LSECs also perpetuate the existing inflammatory state by secreting additional cytokines and chemokines as part of a positive feedback loop (Figure 6.2). As in hepatocytes, this secretion can be triggered by pro-inflammatory cytokines (TNFα, IFNs, etc) or by recognition of viral components through innate PRRs. Recognition of HCV non-structural proteins by TLR4 in Kupffer cells during chronic infection can increase secretion of TNFα [31]. TNFα and IL-1β activated HSCs show increased secretion of CXCL8 when exposed to ligands for TLR2, which recognizes HCV Core and NS3 proteins [32,33]. Additionally, supernatants from LSECs treated with TLR3 and TLR4-specific PAMPs were able to suppress HCV replication in HCV replicon-bearing cells [1]. Production of pro-inflammatory cytokines by these NPCs in response to PRR activation is further augmented by direct hepatocyte-NPC interactions [34,35]. Together, these studies support the proposed dual mechanism model whereby the initial production of CXCL10 by hepatocytes during the early stages of HCV infection

initiates an anti-viral, pro-inflammatory response that involves recruitment of multiple immune cell types to the liver, which further amplify the CXCL10 and inflammatory responses via interactions with viral components and other cytokines. The data reported in Chapters 4 and 5 elaborate upon this model by demonstrating that initial CXCL10 production is IFN-independent and instead controlled by direct binding of the transcription factors NF-κB and IRF3 to the CXCL10 promoter.

Deregulation of CXCL10-Directed Inflammation Leads to Liver Damage and Disease

Despite this robust inflammatory response, virus infection persists and chronic hepatitis C develops in up to 85% of subjects with acute infection [36]. Viral evolution plays a considerable role in establishing this persistence, as immune escape variants of the HCV NS3 epitope recognized by CD4+ T_H1 cells fail to stimulate proliferation while simultaneously causing these cells to shift to a T_H2 response profile [37]. This causes induction of anti-inflammatory cytokines (i.e. IL-10) and reduction of CD8+ T_c and NK cell-stimulating cytokines (i.e. type II IFN and IL-2) [38]. Direct inactivation of infiltrating effector cells can also lead to ineffective viral clearance. HCV-specific CD8+ T_c cells from patients with chronic hepatitis C display an exhausted phenotype, with decreases in both type II IFN production and epitope-specific degranulation [39]. Virus-mediated dendritic cell dysfunction may contribute to the development of anergy through ineffectual co-stimulation or antigen presentation [40,41]. Higher frequencies of both intrahepatic and peripheral CD4+ CD25+ FoxP3+ immunosuppressive regulatory T (T_{reg}) cells have also been reported in HCV-infected patients, further indicating that

suppression of effector immune responses maintains viral persistence in chronic hepatitis C [40,42].

Meanwhile, continued PRR sensing of HCV by hepatocytes leads to chronic production of inflammatory cytokines and chemokines (including CXCL10), which are elevated in the serum of chronically infected patients as described above. This leads to further recruitment of type I immune effector cells to the liver that perpetuate destruction of infected hepatocytes. Chronic production of apoptotic bodies and viral particles leads to continued activation of Kupffer cells, which in turn continue to release ROS/NOS and TGF-β (Figure 6.2). This perpetuates HSC activation and type I collagen deposition. Eventually, chronic activation causes HSCs to secrete tissue inhibitor of metalloproteinases (TIMPs), which inhibit collagen-degrading matrix metalloproteinases (MMPs) and leads to an excessive accumulation of fibrotic scar tissue known as fibrosis [25]. Progressive disruption of the liver architecture and continued hepatocyte turnover can then lead to cirrhosis, a condition where the liver parenchyma is divided into isolated nodules of regenerative tissue with severely reduced functionality (See Figure 6.2 and ref. [30]). Accumulation of genetic aberrations from repeated rounds of cell death and renewal within these nodules then leads to neoplasm and hepatocellular carcinoma (HCC) [43].

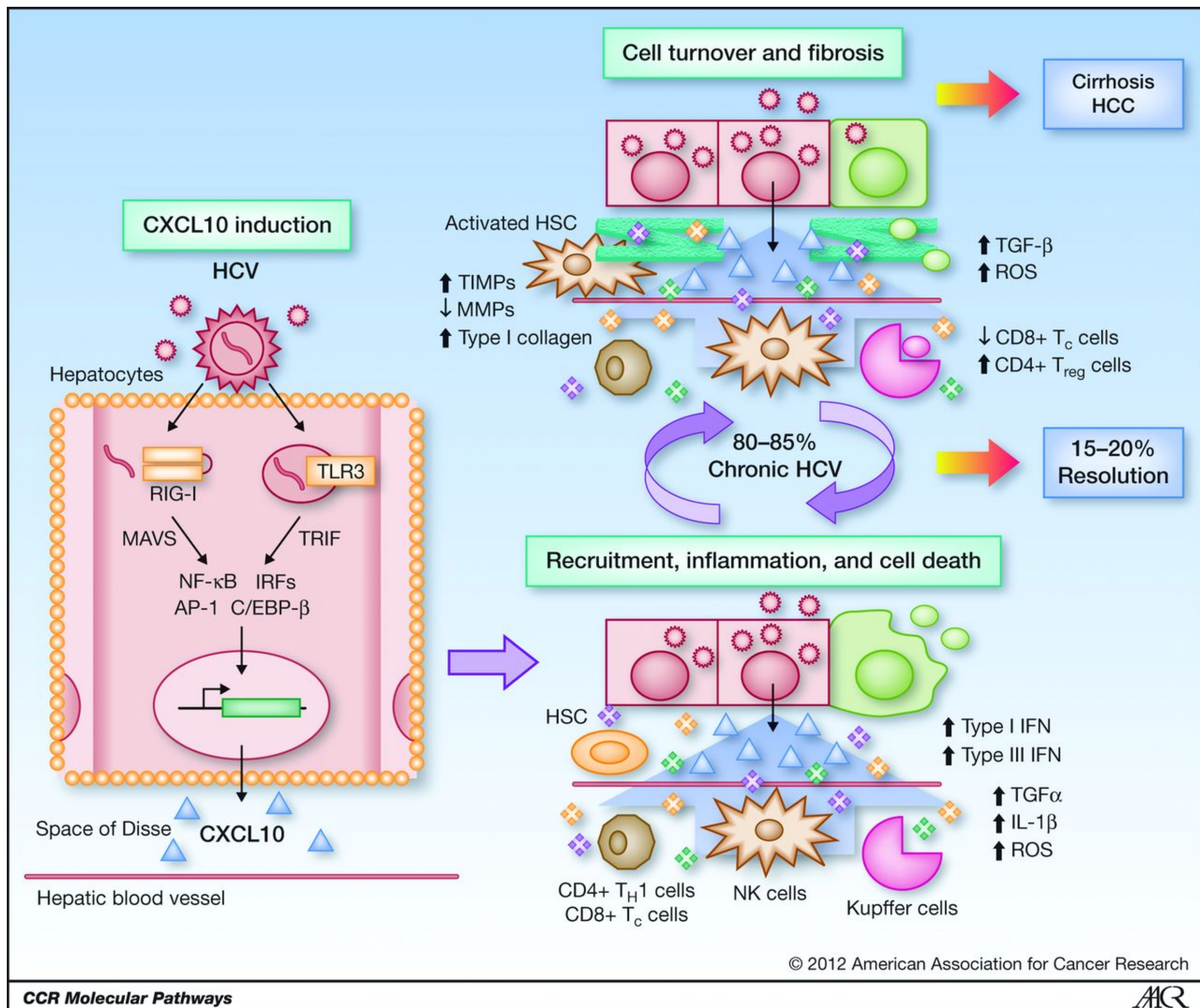

Figure 6.2: Deregulation of the Inflammatory Response Recruited by CXCL10 Following HCV Infection. Sensing of viral RNA by TLR3 and RIG-I following HCV (☼) infection of the hepatocyte leads to signal transduction through TRIF and MAVS, respectively, activation of transcription factors (NF-κB, IRFs, AP-1, C/EBP-β), and transcription of CXCL10 (Δ) ("CXCL10 Induction"). Secreted CXCL10 recruits immune cells (Natural Killer [NK], CD4+ T_H1, and CD8+ T_c cells) and NPCs (Kupffer cells and Hepatic Stellate Cells [HSCs]) to the site of infection ("Recruitment, Inflammation, and Cell Death"). Upon arriving, these cells produce pro-inflammatory, pro-apoptotic mediators (❖) such as type I IFN, type III IFN, TNFα, IL-1β, and reactive oxygen species (ROS). This response fails to clear HCV and instead generates persistent inflammation and hepatocyte turnover. It also leads to liver fibrosis through chronic HSC activation, the overproduction of type I collagen, and the inhibition of collagen-degrading matrix metalloproteinases (MMPs) by tissue inhibitor of metalloproteinases (TIMPs) ("Cell Turnover and Fibrosis"). Over several decades, progressive fibrosis can lead to cirrhosis and hepatocellular carcinoma. *Reprinted Clinical Cancer Research, 19, Brownell J and Polyak SJ, "Molecular Pathways: Hepatitis C Virus, CXCL10, and the Inflammatory Road to Liver Cancer", 1347-52, Copyright (2013).*

The pro-inflammatory and cytotoxic immune responses recruited by CXCL10 and other chemokines can normally eliminate pre-cancerous and cancerous cells through recognition of tumor-specific antigens [43]. However, as these responses are impaired during chronic hepatitis C (see above), it is likely that the ability to identify and eliminate neoplastic cells is also defective. CXCL10 specifically may still inhibit development of HCC through its reported angiostatic activity, but recent literature suggests that CXCL10 may accelerate cancer growth in non-immune cell types [44,45]. Neoplastic cells may also exploit chemokine gradients as "roads" during metastasis. Treatment with CXCL10 increases motility of prostate cancer-derived but not normal prostate epithelial cells via reduced expression of the alternate receptor isoform CXCR3B, which normally inhibits cell growth and migration in non-motile cell types [46,47]. CXCR3B expression was also reduced in two breast cancer cell lines, while induction of normal CXCR3 ("CXCR3A") and repression of CXCR3B have been reported in clear cell ovarian cancers [48,49]. It remains to be determined whether down-regulation of growth inhibitory receptor CXCR3B and/or up-regulation of the growth promoting receptor CXCR3A occurs during hepatocyte transformation to HCC and metastasis. In either case, these studies suggest that CXCL10 may be involved in disease beyond the initiation of persistent inflammation and play a role throughout the development of liver disease as a result of chronic HCV infection.

CXCL10 INDUCTION DURING HCV INTERFERENCE WITH INNATE IMMUNITY

While viral evolution is important for evasion of adaptive immune responses, HCV proteins also interfere with the anti-viral type I and type III IFN innate responses in hepatocytes during chronic infection [50,51]. The NS3/4A protease is able to disrupt RIG-I signaling by cleaving the signaling adaptor MAVS and preventing the formation of signaling complexes on the mitochondrial membrane [50,52]. Cleavage of MAVS has been documented as early as 24 hours post-infection *in vitro* [52]. NS3/4A is also capable of cleaving the adaptor TRIF to inhibit TLR3 signaling [51]. Cleavage of these adaptor prevents PRR-mediated activation and translocation of IRF3 and IRF7 into the nucleus during later stages of infection [52,53]. Other HCV proteins (ex. Core, NS5A) directly inhibit or alter the activity of pro-inflammatory and anti-viral transcription factors [54,55]. NS5A can also interfere with the function of known ISGs including protein kinase R [56].

The reports described above appear to conflict with the results presented in Chapter 4 that CXCL10 induction depends on the TLR3 and RIG-I pathways at 48 and 72 hours following HCV infection. One explanation for this discrepancy may be a delay between the initial and maximal effects of adapter cleavage on the disruption of downstream signaling. Cooperative protein-protein interactions between transcription factors bound to the CXCL10 promoter may help maintain binding and activation during the early stages of this HCV-mediated PRR signaling decline [57,58]. Enhanced mRNA stability could also prolong detection of CXCL10 [59]. Such a delay may have prevented an observable decline in CXCL10 induction during the time window of our kinetic experiments, although CXCL10 production leveled off at later time points of infection

(72-144 hours) in both PHH and TLR3+/RIG-I+ Huh7 cells (Chapter 4, Figures 4.3 and 4.7).

The involvement of TLR3 and RIG-I in CXCL10 induction after detectable NS3/4A cleavage of MAVS and TRIF could also reflect the use of alternate, albeit less favorable, signaling adaptors by these PRRs. Although alternate adaptors have yet to be identified for TLR3, RIG-I has been shown to interact with the inflammasome adaptor ASC to induce caspase-1 activation (See Chapter 1, Figure 1.1 and ref. [60]). ASC has also been linked to MAPK activation during bacterial infection, as well as NF-kB and AP-1-dependent induction of CXCL8 [61,62]. Thus, activated RIG-I in HCV-infected hepatocytes may bind ASC in the absence of functional MAVS in order to perpetuate the pro-inflammatory, anti-viral immune response that includes production of CXCL10.

FUTURE DIRECTIONS

The identification and evaluation of potential alternate signaling adaptors could help clarify the role of TLR3 and RIG-I in the regulation of CXCL10 during early (and/or late) HCV infection, as well as expand the broader scientific understanding of the complex PRR signaling network. Genetic differences in these adaptors that affect expression, binding ability, or signaling capacity may explain the clinical observations of high pre-treatment hepatic expression of ISGs in some, but not all, chronic hepatitis C patients [63]. Similarly, using genome wide association studies to assess the relationship between common regulatory and gene-encoding CXCL10 SNPs and the likelihood of achieving SVR may identify new genetic screening markers. These could be used to improve existing models for predicting patient treatment outcomes that

currently include viral genotype, viral load, IL-28B SNPs, and pre-treatment levels of CXCL10 [64].

Post-transcriptional aspects of CXCL10 regulation also remain to be explored. For example, a N-terminal truncated form of CXCL10 that inhibits migration of CXCR3+ immune cells from plasma into tissue has been detected in the bloodstream of patients with hepatitis C [41]. This antagonist form may contribute to the dysfunction of the type I adaptive immune response described above that leads to liver disease. Conversion of the arginine residue at position 5 to citrulline via deimination can also downregulate the GAG binding and CXCR3-signaling capacity of CXCL10 [65]. In contrast, a C-terminal truncated form of CXCL10 generated by furin has been shown to retain full chemotactic activity [66]. It remains to be determined how these alternate forms of CXCL10 found in the periphery are related to intrahepatic CXCL10 and the persistent hepatic inflammation that is characteristic of chronic hepatitis C.

Further research into these areas may help inform the development of anti-CXCL10 therapies for controlling persistent liver inflammation and damage, which would provide an alternative to the current standard of care. These IFN-containing regimens are poorly tolerated, require 24-48 weeks of administration, and do not address the underlying inflammatory sequelae [67,68]. Current anti-HCV therapy is also less efficacious and associated with increased toxicity in patients experiencing re-infection of a newly transplanted liver [69]. Since transplant is the only treatment option for patients that have already progressed to decompensated cirrhosis [69], and the prevalence of advanced HCV-related liver disease is predicted to rise [70], new treatments that prevent or reverse the onset of inflammatory liver damage must be pursued. As a

master regulator of the infiltrating pro-inflammatory response, the CXCL10/CXCR3 signaling pathway makes an attractive therapeutic target.

Agents that selectively neutralize CXCL10 would theoretically increase patient responsiveness to traditional IFN-based HCV therapy while simultaneously dampening inflammatory immune cell activation. For example, specific inhibitors of the pro-migration CXCR3A isotype could prevent aberrant activation of CD8+ T_c cells and NK cells that lead to excessive hepatocyte death. This would limit Kupffer cell and HSC activation and delay or prevent development of fibrosis. Such drugs would likely mimic Maraviroc, an antagonist of the chemokine receptor CCR5 that is used clinically to block HIV entry [71-74]. However, it is possible that reducing a patient's sensitivity to CXCL10 by blocking its receptor may also interfere with the immune system's ability to respond to other pathogens [75,76]. Indeed, null mutations in CCR5 render patients more susceptible to severe symptoms during West Nile Infection, which would theoretically extend to patients taking Maraviroc [76,77]. More study of the long-term effects of these medications will be necessary before they can be used in the general clinical population.

Overall, the results presented herein provide insight in the regulation of a key chemokine of the inflammatory immune response to HCV infection. Evidence presented herein indicate that CXCL10 is induced via independent, parallel pathways involving both IFN-dependent and IFN-independent mechanisms in hepatocytes following PRR-detection of HCV infection, and that both pathways are required for successful recruitment of the anti-viral immune response to the liver. The immune control exhibited by CXCL10 and its receptor CXCR3 may also be exploited by HCV to allow for viral

persistence and perpetuate hepatic inflammation. Further investigation of the regulatory pathways controlling CXCL10 induction may therefore reveal new targets for host-oriented therapies to treat hepatitis C as well as novel PRR signaling pathways within the complex network of the innate immune system.

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Published OnlineFirst January 15, 2013; DOI: 10.1158/1078-0432.CCR-12-0928

Clinical Cancer Research

Molecular Pathways

Molecular Pathways: Hepatitis C Virus, CXCL10, and the Inflammatory Road to Liver Cancer

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Abstract

An estimated 170 million people worldwide are chronically infected with the hepatitis C virus (HCV), which is characterized histologically by a persistent immune and inflammatory response that fails to clear HCV from hepatocytes. This response is recruited to the liver, in part, by the chemokine CXCL10, the serum and intrahepatic levels of which have been inversely linked to the outcome of interferon-based therapies for hepatitis C. Bystander tissue damage from this ineffective response is thought to lead to increased hepatocyte turnover and the development of fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). However, CXCL10 is traditionally viewed as an orchestrator of the angiostatic and antitumor immune response. In this review, we will explore this duality and the pathways by which CXCL10 is produced by hepatocytes during HCV infection, its effects on resident and infiltrating immune cells, and how deregulation of these cell populations within the liver may lead to chronic liver inflammation. We will also discuss potential host-directed therapies to slow or reverse HCV-induced inflammation that leads to fibrosis, cirrhosis, and HCCs. *Clin Cancer Res; 19(6); 1347–52. ©2012 AACR.*

Background

Chronic hepatitis C virus (HCV) infection affects an estimated 170 million people globally and is the leading cause of liver transplantation in many countries (1, 2). Activation of innate immune pathways in hepatocytes following infection leads to infiltration of proinflammatory, antiviral immune effector cells into the liver (3). Many of these cells are recruited to the liver by the chemokine CXCL10, which binds to and activates the CXCR3 receptor found most commonly on proinflammatory CD8+ cytotoxic T (T_c) cells, CD4+ type I helper T (T_H1) cells, and natural killer (NK) cells (4, 5). However, this response is incapable of eliminating the virus in approximately 85% of patients with acute infection and instead contributes to a chronic immune cell presence in the liver (6). Indeed, CXCR3+ CD8+ T_c cells have been identified among intrahepatic immune cells in patients with chronic hepatitis C (4, 5). Damage to bystander tissue from this persistent yet ineffective inflammatory response has been linked to the development of fibrosis, cirrhosis, and hepatocellular carcinoma (HCC; ref. 7). CXCL10 plasma levels are also negatively correlated with the outcome of interferon (IFN)-based therapy for HCV infection (8). However, as an angiostatic chemokine that recruits CD8+ T_c and NK cells, CXCL10, could orchestrate an antitumor response (9). Herein, we will explore this apparent paradox by defining the innate immune signaling pathways that lead to CXCL10 induction in hepatocytes, examining how deregulation of the recruited immune response during HCV infection may lead to inflammatory liver disease, and discussing possible avenues for controlling inflammation and preventing the development of HCCs.

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doi: 10.1158/1078-0432.CCR-12-0928

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Innate Immune Sensing of HCV in Hepatocytes

Activation of cellular innate immune pathways depends upon recognition of foreign DNA, RNA, or protein motifs known as pathogen-associated molecular patterns (PAMP). Specific PAMPs are recognized by innate pattern recognition receptors (PRR) from 1 of 3 families: Toll-like receptors (TLR), retinoic acid inducible gene 1 (RIG-I)-like receptors (RLR), or Nod-like-receptors (NLR). The interplay of these receptors and their downstream signaling pathways is what determines the resultant innate immune response. For example, the positive sense HCV RNA genome is separately recognized by 2 different PRRs within the hepatocyte: RIG-I and TLR3 (Fig. 1; refs. 10, 11).

RIG-I is a cytoplasmic sensor of double-stranded, 5′ triphosphate RNAs containing poly-U or poly-A motifs (12). Following the binding of this PAMP, RIG-I undergoes a conformational change and binds to the mitochondrial antiviral-signaling protein (MAVS) signaling adaptor (13). In contrast, TLR3 recognizes longer double-stranded RNAs generated during viral replication that have been relocalized to the endosome (11). Activated TLR3 binds the signaling adaptor TIR domain–containing adapter-inducing IFN-β (TRIF) through its cytoplasmic receptor domain (13).

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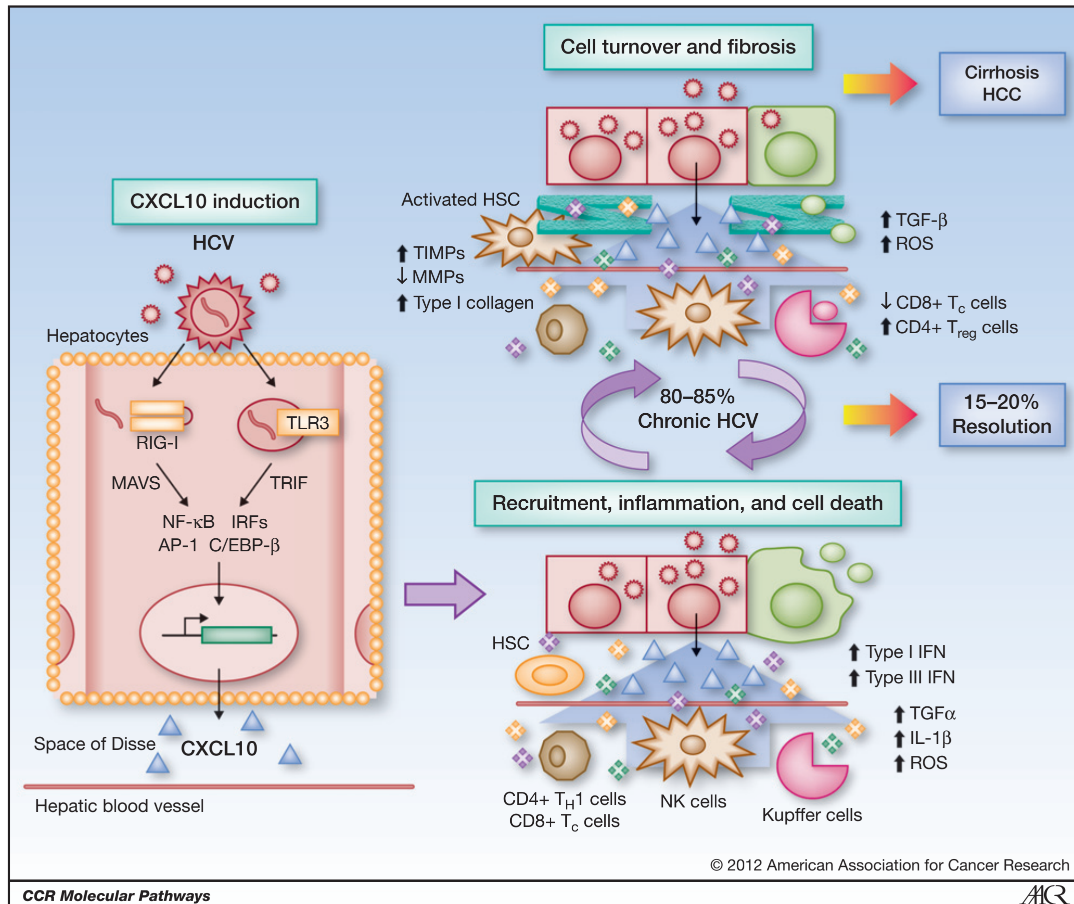

Figure 1. Deregulation of the inflammatory response recruited by CXCL10 following HCV infection. Sensing of viral RNA by the innate immune receptors RIG-I and TLR3 following hepatitis C virus (HCV ☼) infection of the hepatocyte leads to signal transduction through MAVS and TRIF, respectively, activation of transcription factors (NF-κB, IRFs, AP-1, C/EBP-β), and transcription of CXCL10 (△; "CXCL10 Induction"). Secreted CXCL10 forms a chemotactic gradient that recruits immune cells (NK, CD4+ T_H1, and CD8+ T_c cells) and nonparenchymal liver cells (Kupffer cells and HSCs) to the site of infection ("Recruitment, Inflammation, and Cell Death"). Upon arriving, these cells produce proinflammatory, proapoptotic mediators (❖) such as type I IFN, type III IFN, TNFα, IL-1β, and ROS. This response fails to clear HCV in 80% to 85% of patients and instead generates persistent inflammation and hepatocyte turnover. It also leads to liver fibrosis through chronic HSC activation, the overproduction of type I collagen, and the inhibition of collagen-degrading MMPs by TIMPs ("Cell Turnover and Fibrosis"). Over several decades, progressive fibrosis can lead to cirrhosis and HCCs.

Induction of CXCL10 in Hepatocytes

MAVS and TRIF signaling activates various transcription factors including NF-κB, activator protein (AP)-1, C/EBP-β, and IFN-regulatory factors (IRF), which translocate into the nucleus to induce gene transcription. (13, 14). Putative binding sites for these transcription factors have been annotated in the *CXCL10* promoter (15). Indeed, HCV can induce NF-κB binding to this site in TLR3-expressing hepatoma cells (11). NF-κB also drives *CXCL10* transcription during rhinovirus infection, whereas AP-1 and C/EBP-β activate transcription of the structurally similar chemokine CXCL8 [i.e., interleukin (IL)-8; refs. 15–17]. IRF1, IRF2, IRF3, and IRF7 also reportedly bind the *CXCL10* promoter during influenza A infection (18).

Activation of IRF3 and IRF7 can also lead to the induction of antiviral type I IFNs (IFN-α and IFN-β) and type III IFNs (IL-28A, IL-28B, IL-29) in hepatocytes (14, 19). These secreted cytokines can act in a paracrine manner to amplify chemokine and cytokine responses in adjacent liver cells through activation of Janus kinases (JAK) and various STAT proteins (19, 20). Activation of JAK-STAT signaling induces IFN-stimulated genes (ISG) through the binding of STAT dimers to IFN-stimulated response elements (ISRE) or γ-IFN activation site elements in their promoters (19, 20). Type II IFN, a related cytokine produced by infiltrating

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NK cells, CD8+ T_c cells, and CD4+ T_H1 cells, can also induce STAT1 signaling through these elements (20, 21). As the *CXCL10* promoter contains both putative ISREs and putative STAT-binding sites, it can potentially respond to all 3 types of IFN (15).

Despite these observations in other systems, we observed that neutralization of type I and type III IFNs had no effect on CXCL10 production during HCV infection in hepatoma cells expressing functional TLR3 and RIG-I (22). These data suggest that CXCL10 induction in hepatocytes during the initial steps of HCV infection occurs predominantly through direct activation of transcription factors following PRR signaling rather than through secondary paracrine signaling of IFNs. Of course, IFNs secreted from immune cells recruited to the HCV-infected liver as well as from nonparenchymal cells likely contribute to CXCL10 induction *in vivo*. This secondary induction would supplement the initial CXCL10 output by hepatocytes.

Induction of CXCL10 in hepatocytes may also involve nontraditional PRR signaling pathways. Ho and colleagues reported IFN-independent activation of STAT1 and STAT3 proteins during infection with dengue virus, another member of the *Flaviviridae* (23). STAT1 can also be activated via p38 mitogen-activated protein kinase (MAPK) following TLR7 stimulation in plasmacytoid dendritic cells (24). As STAT1 can bind to ISREs, it is possible that this alternative pathway contributes to CXCL10 induction in hepatocytes.

CXCL10 Recruits Proinflammatory Effector Cells for the Anti-HCV Response

Once induced, CXCL10 recruits a proinflammatory, antiviral immune response to sites of infection by binding to the CXCR3 receptor on CD4+ T_H1 and CD8+ T_c cells (Fig. 1; refs. 4, 5). CXCR3 was recently reported to be universally expressed and exists in 2 isoforms: CXCR3A and CXCR3B (25). CXCR3A is the activating isoform highly expressed by leukocytes and is associated with proliferation and chemotactic migration of these cells (25, 26). CXCR3 is also expressed by NK cells as well as by minority cell populations within the liver including resident macrophages (i.e., Kupffer cells) and hepatic stellate cells (HSC; refs. 4, 27–29). Thus, CXCL10 induction from hepatocytes could also localize nonparenchymal cells within the liver to specific sites of infection.

Once recruited to the inflamed liver, activated CD8+ T_c and NK cells kill virus-infected cells via Fas/TRAIL-mediated apoptosis, the release of granzymes and perforin, and secretion of type II IFN (27, 30). Apoptotic bodies released from dying hepatocytes are then phagocytosed by Kupffer cells, which further promote Fas-mediated hepatocyte apoptosis and release reactive oxygen and nitrogen species (ROS/NOS; ref. 31). Kupffer cells also activate HSCs by releasing TGF-β (31). This causes HSCs to differentiate from quiescent vitamin A storage bodies into proliferative myofibroblasts that secrete type I collagen as part of the general wound-healing response to liver injury (32).

Kupffer cells, HSCs, and liver sinusoidal endothelial cells (LSEC) also perpetuate the existing inflammatory state by secreting additional cytokines and chemokines as part of a positive feedback loop. As in hepatocytes, this secretion can be triggered by proinflammatory cytokines produced by infiltrating immune cells (TNFα, IFNs, etc.) or by innate PRRs. Recognition of HCV nonstructural proteins by TLR4 in Kupffer cells during chronic infection can increase secretion of TNFα (33). TNFα- and IL-1β–activated HSCs show increased secretion of CXCL8 when exposed to ligands for TLR2, which recognizes HCV core and NS3 proteins (34, 35). Supernatants from LSECs treated with TLR3- and TLR4-specific PAMPs were also able to suppress HCV replication in HCV replicon–bearing cells (36). Thus, the primary sensing of HCV RNA by hepatocytes initiates an antiviral, proinflammatory response that involves recruitment of multiple immune cell types to the liver that further amplifies the response.

Deregulation of Recruited Cells Leads to Fibrosis, Cirrhosis, and HCC

Despite the robust inflammatory response initiated and recruited by CXCL10, chronic hepatitis C develops in up to 85% of subjects with acute infection (6). Viral evolution plays a considerable role in establishing this persistence, as immune escape variants of the HCV NS3 epitope recognized by CD4+ T_H1 cells fail to stimulate proliferation while simultaneously causing these cells to shift to a T_H2 response profile (37). This causes induction of anti-inflammatory cytokines (i.e., IL-10) and reduction of CD8+ T_c and NK cell–stimulating cytokines (i.e., type II IFN and IL-2; ref. 38). Direct inactivation of infiltrating effector cells can also lead to ineffective viral clearance. HCV-specific CD8+ T_c cells from patients with chronic hepatitis C display an exhausted phenotype, with decreases in both type II IFN production and epitope-specific degranulation (39). Virus-mediated dendritic cell dysfunction may contribute to the development of anergy through ineffectual costimulation or antigen presentation, as could the presence of an antagonistic variant of CXCL10 which may inhibit migration of these CXCR3+ cells from plasma into tissue (40, 41). Higher frequencies of both intrahepatic and peripheral CD4+ CD25+ FoxP3+ immunosuppressive regulatory T (T_{reg}) cells have also been reported in HCV-infected patients, further indicating that suppression of effector immune responses maintains viral persistence in chronic hepatitis C (40, 42).

HCV proteins also interfere with antiviral and IFN responses in hepatocytes during chronic infection (43, 44). Despite this interference, elevated levels of inflammatory cytokines and chemokines are still found in the liver parenchyma of patients with chronic hepatitis C (see above). Kupffer cells also remain activated and continue to release ROS/NOS and TGF-β, perpetuating HSC activation and type I collagen deposition. Eventually, chronic activation causes HSCs to secrete tissue inhibitor of metalloproteinases (TIMP), which inhibit collagen-degrading matrix metalloproteinases (MMP) and lead to an excessive accumulation of fibrotic scar tissue known as fibrosis (32).

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Progressive disruption of the liver architecture and continued hepatocyte turnover can then lead to cirrhosis, a condition in which the liver parenchyma is divided into isolated nodules of regenerative tissue with severely reduced functionality (31). Accumulation of genetic aberrations from repeated rounds of cell death and renewal within these nodules then leads to neoplasm and HCCs (7).

The proinflammatory and cytotoxic immune responses recruited by CXCL10 can normally eliminate precancerous and cancerous cells through recognition of tumor-specific antigens (7). However, as these responses are already impaired during chronic hepatitis C, it is likely that the ability to identify and eliminate neoplastic cells is also defective. CXCL10 may still inhibit development of HCCs through its reported angiostatic activity, but recent literature suggests that CXCL10 may accelerate cancer growth in nonimmune cell types (45, 46). Neoplastic cells may also exploit chemokine gradients as "roads" during metastasis. Treatment with CXCL10 increases motility of prostate cancer–derived but not normal prostate epithelial cells via reduced CXCR3B expression, which normally inhibits cell growth and migration in nonmotile cell types (25, 47). CXCR3B expression was also reduced in 2 breast cancer cell lines, whereas induction of CXCR3A and repression of CXCR3B have been reported in clear cell ovarian cancers (48, 49). It remains to be determined whether downregulation of growth-inhibitory receptor CXCR3B and/or upregulation of the growth-promoting receptor CXCR3A occurs during hepatocyte transformation to HCCs and metastasis.

Clinical–Translational Advances

Current therapies for chronic hepatitis C seek to limit the development of persistent inflammation by reducing systemic viral load using a combination treatment of PEGylated IFN-α and the nonspecific antiviral Ribavirin (peg-IFNα/RBV). Unfortunately, this regimen fails to eliminate the infection in roughly 50% of patients (6). While recently developed HCV-specific protease inhibitors improve the likelihood of success for some patients, IFN-containing regimens are still poorly tolerated, require 24 to 48 weeks of administration, and do not address the underlying inflammatory sequelae that cause liver disease (50, 51). For patients who have already progressed to decompensated cirrhosis, liver transplantation represents the only available treatment option (52). However, reinfection of the new liver occurs in nearly all cases of active infection, and anti-HCV therapy is both less efficacious and associated with increased toxicity after transplantation (52). Thus, new treatments that prevent or reverse the onset of these inflammatory sequelae must be pursued. As a master regulator of the infiltrating proinflammatory response, the CXCL10/CXCR3 signaling pathway makes an attractive therapeutic target.

Potential Anti-CXCL10 Therapies

Agents that selectively neutralize CXCL10 would theoretically increase patient responsiveness to traditional IFN-based HCV therapy while simultaneously dampening inflammatory immune cell activation. For example, specific inhibitors of the CXCR3A isotype could prevent aberrant activation of CD8+ T_c cells and NK cells that lead to excessive hepatocyte death. This, in turn, would limit Kupffer cell and HSC activation and delay or prevent development of fibrosis. Such drugs would likely mimic Maraviroc, an antagonist of the chemokine receptor CCR5 that is used clinically to block HIV entry (53). However, it is possible that reducing a patient's sensitivity to CXCL10 by blocking its receptor may also interfere with the immune system's ability to respond to other pathogens (54).

Broadly Acting Anti-inflammatory Therapies

A safer alternative may be to identify new applications for existing anti-inflammatory drugs. One advantage to this approach is the ability to counteract the excessive immune response recruited by CXCL10 through multiple mechanisms. For example, as oxidative stress causes direct cellular damage in addition to activating HSCs, herbal antioxidant compounds have been suggested as both antifibrotic and anti-inflammatory therapy for liver diseases of multiple etiologies (31). Vitamin E has successfully reduced inflammation and halted fibrosis progression among those with nonalcoholic steatohepatitis (NASH) in clinical trials (55). The routinely consumed herbal medications Sho-saiko-to and Silymarin also appear to have direct antifibrotic activity on HSCs as well as general hepatoprotective properties, although their mechanisms of action remain undefined (56, 57). Traditional antifibrotic drugs have also had demonstrable effects on oxidative stress within the liver: long-term treatment with Losartan reduces NADPH oxidase activity in patients with HCV (58).

Successful anti-inflammatory therapies may also target pathways other than those involved in generating oxidative stress. Broadly acting corticosteroids remain a standard therapy for autoimmune hepatitis (59). Sorafenib, a chemotherapeutic agent already approved to treat HCCs, also inhibits the Raf/ERK proinflammatory and profibrotic signaling pathways (60). Finally, TNFα inhibitors have been used reduce serum levels of liver enzymes, IL-6, and TGF-β in animal models, although limited success has been seen in human clinical trials for alcohol-related liver disease or advanced cirrhosis (31).

Targeting multiple pathways simultaneously may also increase the risk of adverse events occurring during treatment. Severe side effects have been reported among patients taking experimental broadly antiapoptotic drugs such as caspase-3 inhibitors (31). The duration of anti-inflammatory therapy will also likely depend upon the extent of fibrosis or cirrhosis present within the liver, increasing the likelihood of adverse events occurring in patients with severe disease. In addition, administering anti-inflammatory drugs to patients simultaneously undergoing IFN treatment for hepatitis C may interfere with the antiviral efficacy of IFN.

Ultimately, a better understanding of immune and inflammatory signaling within the liver is required before

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the full extent of the efficacy and side effects for these proposed treatments can be known. As HCV-related cirrhosis and HCCs are predicted to increase substantially in the next decade (61), it is imperative that research into this area accelerates. Routine clinical application of hepatoprotective therapies in the near future may help to prevent or reverse the effects of end-stage liver disease in millions of chronically infected patients with HCV worldwide. Furthermore, these types of host-directed therapies may be beneficial to other, nonviral forms of liver diseases that include an inflammatory component.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Brownell, S.J. Polyak
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.J. Polyak
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Brownell, S.J. Polyak
Writing, review, and/or revision of the manuscript: J. Brownell, S.J. Polyak
Study supervision: S.J. Polyak

Grant Support

The study was supported by NIH (U19AI066328 to S.J. Polyak) and University of Washington Pathobiology Training Grant (NIH 2T32AI007509 to J. Brownell).

Received November 15, 2012; revised December 10, 2012; accepted December 12, 2012; published OnlineFirst January 15, 2013.

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