TOLL-LIKE RECEPTORS: TARGET OF HEPATITIS C VIRUS MANIPULATION

A Dissertation Presented

By

SERENA SOYOUNG YUNMEE CHANG

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The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

Gyongyi Szabo, Thesis Advisor

Eva Tsuda-Szomolanyi, Member of Committee

Stuart Levitz, Member of Committee

Reid Gilmore, Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Alan Rothman, Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school.

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

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DEDICATION

I would like to dedicate this thesis to my family especially those closest to me. They have always been here supporting me for this chapter of my life and without their support and love the road would be much more difficult to travel.

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ABSTRACT

Hepatitis C Virus (HCV) is the primary cause of liver transplantation due to its chronic nature in up to eighty percent of infected cases. Around 3 percent of the world's population is infected with HCV. Treatment for HCV is a combined Ribavirin and interferon-α (IFN-α) therapy effective in only fifty to eighty percent of patients depending on HCV genotype. The growing health concern with this disease is the lack of a cure despite liver transplantation. HCV targets hepatocytes, liver cells, but is not cytolytic. HCV has been shown to induce end stage liver disease through sustained inflammation from the host's immune system in the liver. One of the key dilemmas in HCV research and the search for fully effective treatments or vaccines is the lack of animal models. HCV infectivity and disease is limited to primates, most specifically to humans, which cannot be fully replicated in any other living being. The mechanisms for HCV evasion or activation of the immune system are complex, many and discoveries within this field are crucial to overcoming this destructive hepatic infection.

Toll-like receptors (TLR) are cellular activators of the innate immune system that have been a target of HCV. Activated TLRs trigger both the inflammatory and anti-viral pathways to produce inflammatory cytokines and interferons. HCV proteins have been reported to activate a number of TLRs in a variety of cell types. In order to identify possible targets of HCV within the TLR family, we first characterized TLR presence and function in both human hepatic carcinoma cell lines and purified primary human hepatocytes. RNA from TLRs 1-10 was observed to varying degrees in both the hepatoma cell lines and the

primary hepatocytes. We show the extracellular and/or intracellular presence of TLR2, TLR1, TLR3 and TLR7 proteins in hepatoma cell lines. TLR3 and TLR7 are located within the endosome and recognize viral RNA products. We recently reported that TLR2-mediated innate immune signaling pathways are activated by HCV core and NS3 proteins. TLR2 activation requires homo- or hetero-dimerization with either TLR1 or TLR6. We show NF-κB activation in hepatoma cells by TLR2/1, TLR2/6 ligand and HCV protein stimulation. In primary hepatocytes, HCV proteins induced both IL-8 and IL-6 production. We also show that primary hepatocytes initiate a Type 1 IFN response in addition to IL-8 and IL-6 production upon stimulation with a TLR7/8 ligand. Human hepatoma and primary hepatocytes are responsive to TLR2, TLR1, TLR6, TLR7/8 ligands and HCV proteins. Activation of these TLRs may contribute to the inflammatory mediated destruction caused by HCV or could be targets of HCV contributing to its immune evasion.

We found previously that hepatoma cells and primary hepatocytes are responsive to TLR2 ligands and HCV proteins. We also reported that TLR2 is activated by HCV proteins. Here we aimed to determine whether TLR2 co-receptors participated in cellular activation by HCV core or NS3 proteins. By designing siRNAs targeted to TLR2, TLR1 and TLR6, we showed that knockdown of each of these receptors impairs pro- and anti-inflammatory cytokine activation by TLR-specific ligands as well as by HCV core and NS3 proteins in Human Embryonic Kidney cells (HEK/TLR2) and in primary human macrophages. We found that HCV core and NS3 proteins induced TNF-α and IL-

10 production in human monocyte-derived macrophages, which was impaired by TLR2, TLR1 and TLR6 knockdown. Contrary to human data, results from TLR2, TLR1 or TLR6 knockout mice indicated that the absence of TLR2 and its co-receptor TLR6, but not TLR1, prevented the HCV core and NS3 protein-induced peritoneal macrophage activation. TLR2 may utilize both TLR1 and TLR6 co-receptors for HCV core- and NS3-mediated activation of macrophages and innate immunity in humans. These results imply that multiple pattern recognition receptors could participate in cellular activation by HCV proteins contributing to inflammatory disease.

Two critical factors in chronic HCV infection are inflammatory disease and immune evasion. We have demonstrated that TLR2 and its co-receptors play a role in inflammatory-mediated induction via HCV NS3 and core administration. It has recently been shown that HCV targets the TLR3 pathway to aid in immune evasion. TLR3 is only one of four viral recognition receptors located within the endosome and it is plausible that HCV may target others. We hypothesized that HCV infection may interfere with the expression and function of TLR7, a sensor of single stranded RNA. Investigating any effect on TLR7 by HCV may reveal a new mechanism for HCV immune evasion. Low levels of both TLR7 mRNA and protein were measured in HCV replicating cells compared to control cells while reducing HCV infection with either IFNα or restrictive culture conditions restored the decreased TLR7 expression. Downstream of the TLR7 pathway, an increased baseline IRF7 nuclear translocation was observed in HCV replicating cells compared to controls. Stimulation with a TLR7 ligand, R837, resulted in

significant IRF7 nuclear translocation in control cells. In contrast, HCV replicating cells showed impaired IRF7 activation. Use of RNA polymerase inhibitors on hepatoma cells, control and HCV replicating, revealed a shorter TLR7 half life in HCV replicating cells compared to control cells which was not seen in TLR5 mRNA. These data suggest that reduced TLR7 expression, due to RNA instability, directly correlates with HCV replication and results in impaired TLR7-induced IRF7-mediated cell activation.

In conclusion, Hepatitis C Virus manipulates specific Toll-like receptors' expression and their signaling pathways to induce cytokine production. HCV utilizes surface receptors TLR2 and its co-receptors which once activated could contribute to inflammatory disease by production of inflammatory cytokines and possibly immune evasion. HCV down-regulates TLR7, a viral recognition receptor, by decreasing mRNA stability which could facilitate evasion of host immune surveillance.

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ABBREVIATIONS

ALT-	alanine aminotransferase
BB7-	Blazing Bright 7
DNA-	deoxyribonucleic acid
E1-	envelope protein 1
ER-	endoplasmic reticulum
FL-	Full Length
GAPDH-	glyceraldehyde 3-phosphate dehydrogenase
HCV-	Hepatitis C virus
HEK-	human embryonic kidney cells
HIV-	human immunodeficiency virus
hMDM-	human monocytes derived macrophage
IFN-	interferon
IL-6/IL-8-	interleukin-6/8
IRAK-	interleukin-1 receptor-associated kinase
IRES-	internal ribosome entry site
IRF7-	interferon regulatory factor 7
LAL-	limulus amebocyte assay
LDLR-	low density lipoprotein receptor
MDA-5-	melanoma differentiation associated 5
mRNA-	messenger ribonucleic acid
MyD88-	myeloid differentiation primary response gene 88
NF-κB-	nuclear factor-kappa B

NS3-	non-structural protein 3
NTPase-	nucleoside triphosphate hydrolase
NTR-	non-translated region
PBMC-	peripheral blood mononuclear cells
PGN-	peptidoglycan
pLPS-	purified lipopolysaccharide
RIG-I-	retinoic acid inducible gene
RNAi-	ribonucleic acid interference
RNA-	ribonucleic acid
RT-PCR-	reverse transcriptase polymerase chain reaction
siRNA-	small interfering ribonucleic acid
SRB1-	scavenger receptor class B-1
TLR-	Toll-like receptor
TNF-α-	tumor necrosis factor-alpha
TRAF-	tumor necrosis factor receptor-associated factor

CHAPTER I

INTRODUCTION

Hepatitis C Virus – The deadly hepatitis virus in America

Hepatitis C Virus is a growing clinical health concern afflicting close to three percent of the world's population (1), with the largest percentage occurring in Egypt, 20% of the nation's population (2). Two percent, around five million, of the United States' population (3) is infected with HCV, more than twice as high as HIV infections (4). Unlike other hepatitis viruses, HCV leads to a high rate of chronic infection in about eighty percent of the 170 million people infected worldwide (1, 5). It was soon discovered that HCV-induced liver inflammation and cirrhosis is one of the leading causes for liver transplantations (6, 7).

Since the advent of blood transfusion screening (8, 9) and public education regarding transmission, the incidence of new infections has dramatically declined (10, 11). Yet, chronic and existing infections may not develop for several years to several decades and therefore in America, the incidence of existing infections will continue to rise in the near future. With no vaccine and limited treatment efficiencies, HCV will continue to be a world-wide health concern (12). To control for the increasing cases of infection and liver-related complications due to infection it is essential to continue to make novel discoveries in HCV research.

Discovery of a new hepatitis virus

There are five major characterized hepatitis viruses, hepatitis A-E. (13-16). Of the five in America, HCV is the deadliest and most prominent (1, 3, 5, 7). Unlike HCV, effective vaccines are available for HAV and HBV (17, 18). In the

mid 1970s a new hepatitis virus was recognized in blood transfusion recipients, identified as non-A, non-B hepatitis (19). A few distinctive characteristics aided in separating this new virus from HAV and HBV. Infection in chimpanzees produced chronic disease portrayed by inflammation and hepatocyte structural changes (20, 21). Complete identification of the new virus was hindered by difficulties in producing a cellular model permissive to the viral replication (22). In 1987, Michael Houghton and Daniel Bradley collaborated to create a molecular cloning technique to sequence the viral genome (23). Once HCV was identified, screening for it in blood transfusions became mandatory starting in 1990 which subsequently decreased the rate of infection (24). Transmission of the virus in some areas is still growing despite the advanced techniques to identify HCV in blood (25). The highest transmission rates were initially from unsanitary injection drug use or unsterile needle use as in unregulated body tattoo art and blood transfusions (26). Since the screening of blood there has been a decrease in developed countries but due to the expensive nature of testing for HCV, underdeveloped countries are still struggling to control the spread of the virus (27). Occupational exposure to HCV is and has been the most difficult type of transmission to control as most infections occur accidentally even with proper precautions observed (28). Continued HCV research has consequently led to treatment against the virus.

HCV infection and treatment: acute vs chronic

HCV infection results in two forms, either an acute infection followed by clearance or acute infection followed by persistence (29, 30). Diagnosis of the

acute phase followed by clearance is rarely made as the symptoms are so slight that most HCV acutely infected patients do not require medical assistance (31, 32). In almost all infected individuals antibodies against HCV can be detected within 3 to 6 months after initial infection, while symptoms from chronic infection may not materialize for decades after the initial infection (33). PCR is used to confirm infection in all patients and it is critical for those that have low antibody titers (34). Around twenty to fifty percent of HCV infected patients are spontaneously cured while the chronic disease is seen in eighty to fifty percent of infected individuals (1, 33, and 35). Roughly half of the chronically infected individuals can eliminate HCV after a pegylated IFN-α and ribavirin combined therapy for up to 48 weeks depending on HCV genotype, while non-responders develop potentially fatal chronic disease (5, 35, 36, and 37). A major characteristic and possibly a vital participant in persistent HCV infection is chronic inflammation in and outside the liver mediated by cytokines such as tumor necrosis factor-alpha (TNF-α) (38), interleukin 12 (IL-12) (39), and interleukin 6 (IL-6) (40, 41). Chronic infection can cause liver cirrhosis and/or hepatocellular carcinoma, ultimately leading to liver failure due to inflammatory cell-mediated liver damage (42). Chronic liver inflammation from HCV infection is thought to be either inactivated or maintained by immune-mediated mechanisms.

HCV histology

In HCV infected individuals, chronic infection can lead to elevated alanine aminotransferase (ALT) levels. Even though testing for ALT is the most

inexpensive measure for liver injury, it is not the most accurate and in chronic infection may not be elevated at the time of testing (43, 44). The most accurate procedure to investigate histological changes in chronic HCV is from a liver biopsy of HCV chronically infected individuals. Liver histology shows persistent mononuclear cell invasion into the parenchyma and portal vein areas, hepatocyte necrosis and differing grades of fibrosis. Fibrosis is an accumulation of extracellular matrix proteins such as collagen. Fibrosis can eventually lead to cirrhosis of the liver, a condition where the liver tissue is replaced with fibrotic scar tissue inhibiting liver function, and/or liver failure (45). Recent analysis of liver histology of chronic HCV patients showed that fibrosis as opposed to steatosis, accumulation of lipids within the cell, was associated with HCV infection (46).

The distribution of viral HCV RNA is mainly in the liver, with the majority of RNA within the hepatocytes (47). Viral RNA and HCV core antigen have also been isolated from the serum (47) and HCV core protein was found in the cytoplasm of hepatocytes (48).

HCV virology

HCV is a single-stranded positive sense RNA virus from the *Flaviviridae* family (14, 49). The genome is approximately 9.6 kilo-bases and has six different major genotypes, genotypes 1-6 (14, 36). HCV has a high propensity for genetic variations due to the error prone nature of the RNA replicase (50, 51). Therefore, HCV infected individuals with certain genotypes and subtypes respond atypically to treatment and the identity of HCV genotypes in infected individuals is

important to the specific treatment regimen for the individual. Throughout the history of HCV therapy, treatment has been conventionally less effective, up to 30-50%, in patients harboring HCV genotype 1 compared to up to 80% effective clearance in genotypes 2 and 3 (32, 36, and 52). Thus, genotype 1 is associated with the development of chronic infection and is also the most prevalent genotype in America (36).

1. HCV proteins

HCV is a relatively small 50 nm enveloped RNA virus (53, 54). The genome flanked by small 5' and 3' non-translated regions (NTRs) have one open reading frame that translates to a number of proteins of roughly 3000 residues (29, 55, and 56). The 5'NTR region contains an internal ribosomal entry site (IRES) and is highly conserved among the various genotypes and subtypes. It is essential for RNA translation by binding the 40S ribosome subunit positioning it near the initiation site (57-59). The 3'NTR region with its three domains, variable region, polyU region, and a conserved terminal domain, is highly inconsistent among the genotypes (60, 61). Areas in the latter two regions are required for RNA replication (61-63). The virus has ten proteins with three structural proteins: core, envelope 1 and 2 (64-66). In between the structural and nonstructural proteins there is a small integral membrane protein: p7 (67). The non-structural proteins are: NS2 (68, 69), NS3, NS4A, NS4B, NS5A, and NS5B. NS3 through NS5B are required for replication (68, 69). Extensive research into each of these proteins has revealed specific roles for viral replication and survival.

1a: Structural proteins

The three structural proteins make up the HCV viral particle which consists of the nucleocapsid or core protein covered by two envelope glycoproteins, E1 and E2 (70). The viral particle, shown in Figure 1.1, constitutes these three proteins and possibly the p7 protein which has yet to be confirmed in the virion. The envelope proteins, E1 and E2, are glycosylated and are secured to the lipid membrane by their C-terminal transmembrane domain (71). E1 and E2 form the HCV glycoprotein complex that binds to cellular receptors required for attachment and fusion into the cell (72-74). E1 has been shown to aid in E2 protein folding (75). E1 and E2 can either form heterodimers with noncovalent bonds or misfolded aggregates held together by disulfide bonds (76-78).

HCV core protein is a highly basic RNA binding protein that can also stimulate the innate immune inflammatory pathways via TLR2 and plays an important role in the formation of viral nucleocapsids and interactions with the viral genome (65, 79, and 80). The core protein is located intracellularly in infected hepatocytes; however, there are relatively high levels of core protein detected in patients' blood (81). During infection, HCV core protein is circulating in the blood stream and can stimulate cell surface receptors throughout the host. The core protein consisting of the first 191 amino acids in the polyprotein is a relatively small protein, ~21kDa, also contains DNA binding motifs, nuclear localization signals and phosphorylation sites (82-85). The protein localizes in the cytoplasm but can also localize in the nucleus (86, 87).

The structural proteins compose the viral particle shown in Figure 1.1. The structural proteins, E1, E2 and core are associated with the endoplasmic

reticulum and cleaved from the HCV polyprotein complex and endoplasmic reticulum membrane by host and viral enzymatic reactions at multiple signal peptidase cleavage sites shown in Figure 1.2 (69, 88).

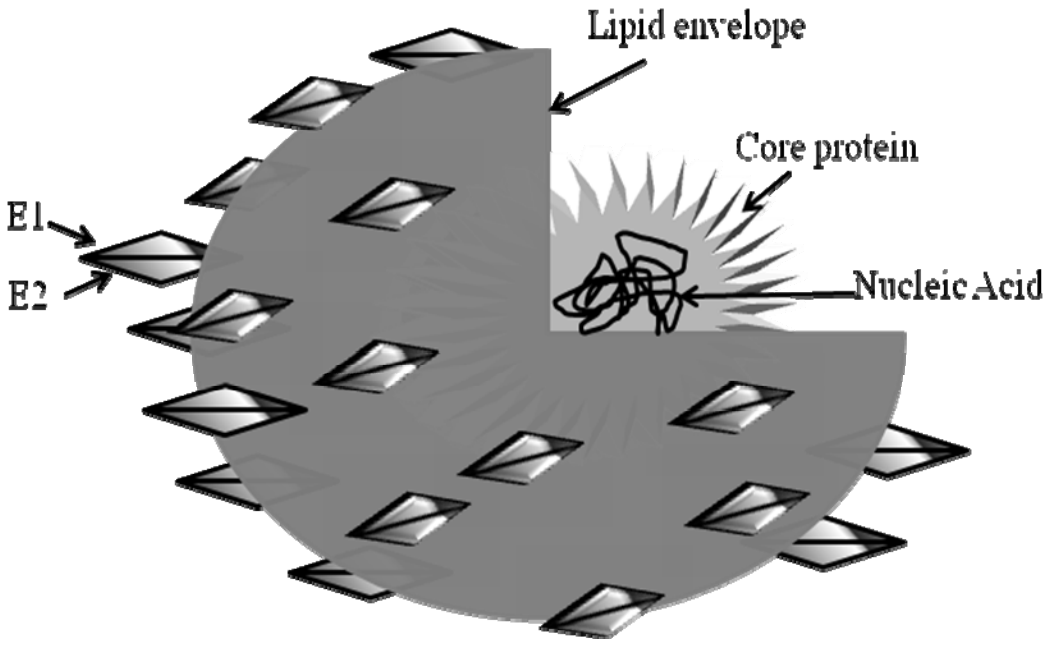


Figure 1.1: Schematic diagram of HCV virus particle. Envelope glycoproteins E1 and E2 are cellular attachment receptors. The core protein, nucleocapsid, encompasses the single stranded RNA.

1b: ion channel protein

The p7 protein is a very small polypeptide, 7kDa, located between the structural and non-structural HCV proteins (Figure 1.2, 67). It has two transmembrane domains positioned so both amino and carboxy termini face the endoplasmic reticulum lumen with peptidase cleavage sites at both ends (89). The protein is observed to have ion channel function in artificial lipid membranes and is necessary for HCV infection in chimpanzees (67, 90). Recent evidence on p7 has supported its vital role in infectious viral production (91), assembly and release of the virus (92, 93).

1c: non-structural proteins

The non-structural proteins are not associated with the viral particle but are essential for viral replication and immune evasion. NS2 is not required for HCV replication and very little is known about its specific function (94). Expressed alone, NS2 localizes to the ER membrane. Dimerization of NS2 yields a cysteine protease with two active sites which cleaves between NS2 and NS3 (95). Due to this function, researchers believe it is involved in viral assembly and release. NS2 can also bind host pro-apoptotic protein, CIDE-B, which subsequently inhibits apoptosis (96). NS2 is marked by the host cell for proteasome degradation soon after it is translated (97).

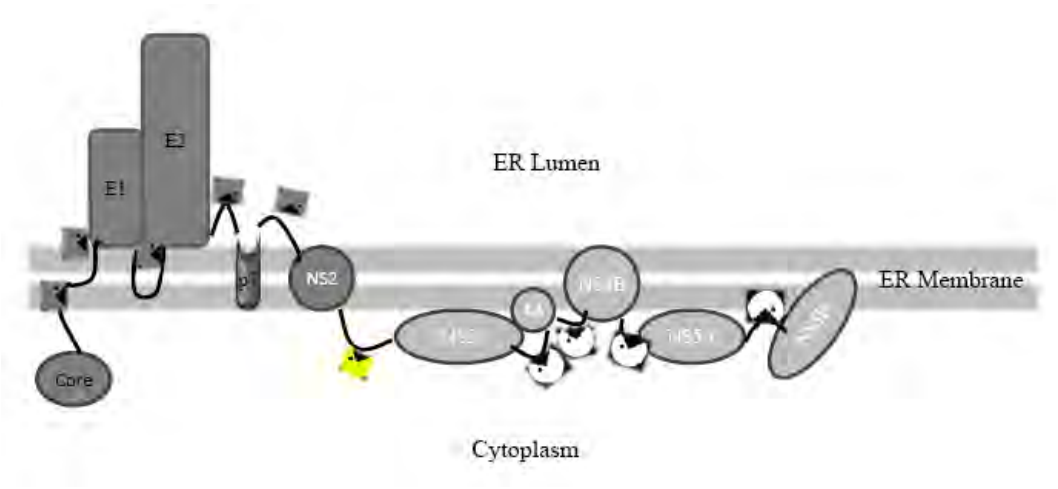


Figure 1.2: HCV protein association with host endoplasmic reticulum. Structural proteins, core, E1, and E2, have signal peptidase sites targeted for ER peptidase cleavage indicated by grey pacmen between core and NS2. Non-structural proteins located either within the membrane or on the cytoplasmic side of the ER have a cysteine protease cleavage site from NS2 to NS3 indicated by the light grey pacman and viral serine protease sites from NS3 to NS5B that are cleaved by viral proteases indicated by white pacmen.

HCV NS3-NS5B proteins have been shown to be required for replication and thus one of the initial HCV cell culture models, Blazing Blight 7 or BB7, contains only these proteins (98). NS3 has a variety of functions. At its amino terminal it contains a serine protease domain yet at its carboxyl terminus there is an RNA helicase/NTPase domain (99, 100). NS3 on its own functions inadequately, diffusing to the cytoplasm and nucleus, and is marked for proteolytic degradation. Upon formation of the NS3-NS4A complex, NS3 localizes to the ER membrane and both the protease and helicase activity are greatly enhanced (101, 102). Monomeric NS3 binds RNA but homodimerization is required for unwinding (103). The serine protease domain in conjunction with NS4A cleaves processing sites located between the rest of the non-structural proteins (101, 102). These functions are required for HCV to form the replication complex (104). NS4A is a membrane protein that covalently binds to NS3 and this complex, NS3/4A cuts processing sites. NS4A also targets NS3 to the cytoplasmic side of the ER membrane and protects NS3 from degradation (101, 102). NS3/4A is also very important for HCV immune evasion and manipulation of host cellular factors (105-107). NS4B is a transmembrane protein that is thought to contribute to the formation of HCV membranous replication complexes by altering intracellular membranes. Mutations in this protein affect replication efficiency (108, 109). Like p7, the exact function is unknown leaving further research to be desired.

NS5A is a phosphoprotein associated with the ER membrane (110, 111). It exists in either a hypo- or hyper-phosphorylated state which regulates RNA

replication (112-114). Hypo-phosphorylation is required for efficient HCV replication in cell culture and therefore modifications such as point mutation, (S2240I), to NS5A are necessary for permissibility (98, 115). NS5A can also bind a number of viral and host proteins to promote RNA replication and immune evasion (116-118). NS5B is the RNA-dependent RNA polymerase absolutely required for HCV replication (119, 120). It is secured to the ER membrane by a carboxy terminal domain and interacts closely with NS5A and NS3 (90, 121). The protein has a distinct polymerase feature of a fully encircled active site which serves to regulate RNA synthesis (122). NS5B has also been implicated in manipulating host survival and defense mechanisms by binding to specific host proteins (123-125).

2. Viral life cycle

HCV invades and replicates in human hepatocytes. Chimpanzees are the only other animal model that will sustain replication (126). The HCV life cycle consists of five distinct steps: entry, protein translation, RNA replication, virion assembly, virion release. The viral infection starts by binding of the envelope proteins to several host cellular receptors: CD81 (127); scavenger receptor class B-1 (SRB1) (128); glycosoaminoglycans (129); low density lipoprotein receptor (LDLR) (129, 130); claudin-1 (131). Endocytosis of the virion occurs by clathrin mediation and acidification of the endosomal compartment. After entry the viral and endosomal membrane fuse allowing for the release of HCV RNA into the cytoplasm (132-134). The positive single stranded HCV RNA is dependent on the 5'NTR IRES to begin translation of the polyprotein (64, 65, 133). The

proteins, core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B, are manufactured in the cytoplasm and localize to the endoplasmic reticulum membrane where HCV negative sense strand RNA production occurs (90, 102, 109, 111, 116, 135-139). In the cytoplasm while HCV proteins are translated from RNA, the proteins may interact with host factors to ensure viral survival. HCV proteins in particular NS4B, mediate the formation of membranous webs postulated to be partial ER membranes, where further RNA replication from negative strand to positive strand occurs (109). Close to the membranous webs, the RNA and structural proteins assemble into viral particles which bud into the ER or similar membrane compartments to exocytose out of the cell leaving it intact (77, 136, 140).

HCV cell culture: advances and setbacks

Despite the great increase in HCV research since its identification in 1989, there are still a number of areas that are unknown. The primary host for HCV is the human hepatocyte (141). HCV tropism is not limited to hepatocytes, but has been found in peripheral blood mononuclear cells and the brain, yet HCV replication in these tissues has not been confirmed (142, 143). Others have reported HCV in the bone marrow, serum, spleen, thyroid, pancreas, lymph nodes and adrenal glands, but some groups have shown in bone marrow and sera that due to the nature of the PCR testing the presence of HCV was most likely an artifact or experimental error (144-147). Since HCV positive patients with liver transplants have a 100% re-infection rate, HCV must at least seek refuge in other non-hepatic tissues (148). The largest challenge for researchers is the lack of an

animal model. The chimpanzee is the only other species that can permit HCV infection with similar pathogenesis as the human (149). Even though the chimpanzee has been used in HCV research, it is not common mainly due to the expense and time required to utilize the animal model for infection (150).

The chronic infection exhibited by HCV infected patients, pressured scientists to develop a cell culture system permissive for HCV replication. More than ten years after the discovery of the HCV genome, the first efficient HCV cell culture system was reported by Dr. Charlie Rice (98, 115). This cell culture system required mutations in the HCV NS5A protein and adaptive mutations in the hepatoma cell line, Huh7 (98, 115). Two distinctive cell culture lines were produced: BB7, an HCV subgenomic (NS3-NS5B) line and the full length (FL) which contains the sequence of all HCV proteins (98, 115). We utilized both cell lines for further investigations with HCV and the innate immune system presented in this thesis. Once these cell cultures were reported and openly shared by Dr. Rice, tremendous advances in HCV research were possible. Even though the HCV field could now move forward, it was still held back by the lack of a complete cell culture system (115). All cell culture systems created up to that point only allowed for viral replication within the cell but did not allow for active viral particle assembly, leaving half the viral life cycle unaccounted for and also a lack of an infectious HCV cell culture model. A few years later in 2003, scientists in Japan discovered the first and to date the only infectious HCV clone able to replicate in hepatoma cell culture (151). Dr. Takaji Wakita isolated an HCV genotype 2a species, JFH-1, from a patient with fulminant hepatitis. JFH-1

replicates, assembles, releases and infects hepatoma cells, Huh7 and Huh7.5, a variant of Huh7 with further adaptive mutations to create a more permissive infectious environment. With the newly discovered infectious clone, the full HCV life cycle is being explored, but the lack of an appropriate animal model is still inhibiting further discoveries for effective vaccines and treatment for non-responders.

Immune reaction to HCV infection

Despite the lack of an appropriate animal model and before the development of the cell culture system, scientists and medical researchers have been investigating the effects of HCV on the host immune system in HCV infected patients and chimpanzees. Researchers have found both an innate and adaptive immune reaction against HCV which the virus has developed ways to circumvent (9, 29, 41, 152, and 153). Initial infection causes activation of the innate immune system before the adaptive system can either clear infection or allow chronic infection to set in.

1. Adaptive immunity against HCV

The adaptive immune response plays a very important role in acute infection and clearance. Seroconversion can take up to three months after initial infection is established (154, 155). With acute HCV infection that leads to clearance of the infection, antibodies in the serum diminish to undetectable levels (156). One mechanism of clearance in acute infections can be attributed to HCV specific antibody neutralization. This was demonstrated in chimpanzees with anti-E2 against the hypervariable region (157). Chronic infection can cause

increasing levels of antibodies to develop throughout the infection (33). Those who do not develop a T-cell response or a weak response are more likely to develop chronic infection (159). The mechanisms for non-functional T-cell response are not well known, yet recently high expression of programmed death-1, PD-1, on CD8+ T-cells was demonstrated in HCV infected patient livers. This inhibitory co-stimulatory receptor inhibits T-cell function when highly expressed and it was shown that PD-1 expression reduced upon low viral titers or clearance of HCV infection (160). Blockade of PD-1 could not fully restore T-cell function in HCV infected patients which proves the complex nature HCV infection can have on adaptive immunity. A major defense mechanism against HCV specific B- and T-cell responses is the generation of escape mutations by the virus that are potentially caused by a variety of B-, T-cell epitopes (161-163). T-cell responses are directed towards these mutations which are ineffective in aiding with clearance.

2. Innate immunity against HCV

We have chosen to focus this research on the innate immune response to HCV infection in HCV replicating cell lines. HCV targets the innate immune response by a number of mechanisms which if discovered can help aid in innovative treatments for chronically infected patients. In addition to hepatocytes, the liver contains a number of different cell types such as stellate cells that store vitamin A (164), Kupffer cells that serve as resident liver macrophages (165), circulating natural killer cells (NK) that are involved in innate immune surveillance (166), circulating T-cells and dendritic cells (DC). Hepatocytes and

other innate immune cells within the liver, such as Kupffer cells (167, 168), have many viral defense mechanisms to clear the infection, including the production of inflammatory cytokines and Type 1 interferons, which are involved in the clinical treatment of HCV (38, 79). Inflammatory cytokines and IFNs are produced within the cell by triggering viral recognition receptors which include the following receptors: cytoplasmic localized receptors, protein kinase R (PKR), RNA helicase receptors (RIG-I and MDA-5), NOD-like receptors, 2'5' oligoadenylate synthetase (OAS) as well as the following membrane bound receptors: TLRs and mannose receptors.

3. Cytoplasmic innate immune receptor response to HCV

PKR binds double stranded RNA and activates apoptosis, preventing further spread of the virus. PKR, the double stranded RNA dependent protein kinase induced by IFNs (169), was one of the first receptors identified to be inhibited by the HCV proteins E2 (170, 171) and NS5A (172, 173). 2'5'OAS is activated by dsRNA, mostly viral (174). Once activated it produces 2'5' adenylate that binds RNaseL and induces RNaseL dimerization. Activated RNaseL cleaves single stranded regions of RNA, with a preference towards viral RNA (175). 2'5'OAS is enhanced by Type 1 IFNs but it is still controversial whether HCV NS5A inhibits or HCV core protein stimulates 2'5'OAS (176, 177). Most recently, HCV NS3/4A protease was shown to inhibit the RNA helicase family (178). RIG-I and MDA-5 are cytoplasmic receptors that sense single and double stranded RNA triggering a cascade of events including phosphorylation, dimerization and nuclear translocation of IRFs which leads to activation of Type 1

IFNs (179-181). Since HCV RNA is found in the cytoplasm, RIG-I and MDA-5, cytoplasmic viral recognition receptors, are extremely important in inducing an initial antiviral response to inhibit further replication and infection. RIG-I and MDA-5 share a very similar IFN induction pathway with TLR3, where all three receptors induce IFNβ production via IRF3/IRF7 activation. With overlapping pathways for IFN induction, these receptors can survey for viral activity in the cytoplasm and in endosomes. HCV NS3/4A cleaves the adaptor protein, IPS-1/MAVS/VISA/Cardif, to MDA-5 and RIG-I at the transmembrane region associated with the mitochondrial membrane which is necessary for receptor activation (178). By inhibiting RIG-I and MDA-5, HCV replication is not hindered by their ability to activate IFN induction.

4. Toll-like receptors

One of the larger families of innate immune surveillance proteins is the Toll-like receptors, TLRs (182). Toll was first discovered to participate in dorsoventral patterning during embryogenesis in *Drosophila melanogaster*, household fruit fly (183). These receptors were then seen to play a role in *Drosophila* immunity (184). It was soon discovered that there were analogous receptors in mammals, 13 discovered to date, 10 of which exist in humans (182, 185). TLRs are cell surface or endosomal transmembrane receptors that initiate signaling pathways that produce pro- and anti-inflammatory cytokines when stimulated by pathogen-derived ligands (182, 186-188). These receptors have extracellular leucine rich repeat domain and an intracellular TIR (Toll/Interleukin-1 receptor) domain (187). The extracellular domain recognizes pathogen

associated molecular patterns while the intracellular domain binds adaptor proteins, such as myeloid differentiation factor 88 (MyD88/Mal) (189) and/or TIR-domain containing adaptor inducing IFNβ (TRIF). Activation of TLRs initiates a cascade of events leading to NF-κB, nuclear factor kappa B, or IRF, interferon regulatory factor, nuclear translocation (190). NF-κB is a transcription factor that binds to promoter regions of a considerable number of genes, producing pro- or anti-inflammatory cytokines and chemokines (191, 192). Another activation pathway utilizes IRFs that are transcription factors that bind specifically to promoter regions of IFNs (193).

Ascertaining TLR expression and function in hepatocytes that respond to HCV proteins

Hepatocytes were thought to contribute very little to innate immune surveillance, yet recently hepatocytes were shown to express a number of TLRs, expanding their function during viral infection (194, 195). We set out to examine the expression and function of TLRs in hepatocytes. We concentrated on two TLRs, TLR2 heterodimers and TLR7. HCV proteins activate TLR2 (79) while TLR7 agonists were seen to reduce HCV infection (196, 197). Both TLRs are highly expressed in innate immune cells including macrophages (198) and dendritic cells, (199) yet it was unknown whether TLRs are expressed and functional in hepatocytes. Therefore we examined the extent of expression and function by looking at RNA, protein and cytokine production. We determined that hepatocytes substantially express TLR2, TLR1, and TLR7. These TLRs activate either the NF-κB pathway and/or the IRF pathway upon specific ligand

stimulation. We also saw a large induction of inflammatory cytokines in hepatocytes when we stimulated with HCV proteins core and NS3 but not NS5A.

TLR2 signaling pathway

Activation of TLRs results in homo-dimerization (TLR4, 200) or hetero-dimerization (TLR2/1 or TLR2/6; 201). TLR2 can recognize a diverse number of ligands from gram^{+} and gram^{-} bacterial products such as peptidoglycan (PGN), or mycobacterial lipoproteins, to zymosan from yeast (202). Formation of TLR2 heterodimers activates MyD88 which recruits a number of other kinases, mitogen activated protein kinase 37 (TAK1), and inhibitor of kappa B kinases (IKK). IKKs phosphorylate the inhibitor of kappa B (IκBα) which prevents the NF-κB complex from translocating into the nucleus (203). Phophorylated IκBα is marked for proteosomal degradation while the released NF-κB complex is free to move into the nucleus activating a number of genes. Current research revealed that HCV core and NS3 proteins could stimulate the innate immune system in monocytes and macrophages by activating the TLR2-mediated inflammatory cytokine pathway (79).

Investigating the identity of TLR2 heterodimer contributing to HCV protein recognition

Some TLRs, such as TLR2, 4, 3, 7, and 9, are involved in detection of viral proteins (106, 179, 204, and 205). Kupffer cells in the liver have the ability to produce a significant amount of cytokines when stimulated (206). Kupffer cells may contribute to inflammatory cell recruitment to the liver and be responsible for further liver damage such as fibrosis or cirrhosis (167). In a

previous study, TLR2-specific activation by HCV NS3 and core proteins but not HCV E2 protein was shown in monocytes and macrophages (79). HCV core and NS3 proteins interact with many host proteins (79, 207); however, all the mechanisms by which HCV proteins modulate the innate immune system that contribute to disease are not fully answered. Existing research illustrates that TLR2 homo- or hetero-dimerizes with TLR1 or TLR6 (186, 201). Therefore, to further identify the receptor recognition of TLR2-specific HCV protein activation, we created a knockdown system using RNAi in TLR-expressing human cells, human embryonic kidney cells (HEK/TLR2), and primary macrophages, human monocyte derived macrophages (hMDMs). We also utilized TLR-specific knockout mice to confirm our results. Our results revealed that in HCV protein stimulated HEK/TLR2 or hMDM cells, TLR1 or TLR6 siRNA knockdown caused a significant loss of cytokine production. These results suggest that in both HEK/TLR2 and hMDM cells HCV protein activation of TLR2 utilizes either TLR1 or TLR6. We show that HCV core and NS3 proteins stimulate the host's innate immune system (TLRs) to produce inflammatory cytokines. Identifying receptors activated by HCV proteins is important in discovering novel therapeutic targets to reduce inflammation in the liver (79, 208).

HCV immune escape via interference with IFN-mediated pathways

Some HCV-derived products, including HCV RNA and several HCV proteins trigger host defense (79, 173). Immune responses, including cell-mediated immunity and Type 1 IFNs are vital in controlling and clearing HCV infection (209, 210). A number of host receptors, including TLR3, TLR7, TLR8,

TLR9, RIG-I and MDA-5 lead to a Type 1 IFN-mediated response upon stimulation with viral RNA (179, 181, 211, and 212). However the virus has developed mechanisms to bypass the immune defense and facilitate viral persistence. HCV proteins not only stimulate TLRs but can inhibit TLR-mediated pathways by disrupting TLR3-mediated type 1 interferon induction (106). HCV NS3/4A cleaves TRIF, the adaptor protein for the IFN-induction pathway of TLR3. Viral recognition receptors can compensate for the loss of one another although during HCV infection, disruption of TLR3, MDA-5, and RIG-I pathways greatly diminishes IFNβ production and allows for continued viral replication and infection. Immune escape from HCV proteins may manifest not only in the disruption of TLR3 or RIG-I/MDA-5-mediated signaling machinery but also affect other IFN-inducing pathways such as activation of IRF7 which contributes to IFNα production (178).

IFN and its detrimental effects against HCV

Over fifty years ago, a new family of cytokines, IFNs, was discovered to interfere with influenza virus replication (213, 214). IFNs are an important component involved in innate immune activation against viruses and have been manufactured as effective therapy against a number of viral infections. There are three subfamilies of IFNs, type 1, type 2, and type 3. Type 1 IFNs include: 13 IFNα subtypes, IFNβ, IFNκ, IFNε, IFNo, IFNτ and IFNδ, that all bind the IFNα receptors 1 and 2. Type 2 is IFNγ signaling through the IFNγ receptor. Type 3 consists of the IFNλ1, 2 and 3 genes and signal through the IL-10 receptor 2 and the IFNλ receptor 1 (215). All IFNs stimulate the JAK/STAT pathway to activate

a number of anti-viral or anti-microbial interferon stimulated genes (ISGs) (216). ISGs activate a number of immune cells such as macrophages (217), NK cells (218), T-cells (219) which up-regulate major histocompatibility class 1 and 2 (220). They also induce activation of anti-viral and anti-microbial enzymes such as PKR and 2'5'OAS/RNaseL which cleaves viral RNA (221, 222). TLRs are one of many types of receptors that stimulate the production of IFNs.

Like IRF3, mediated by TLR3 activation, IRF7 is a transcription factor responsible for Type 1 IFN production (223). It is mainly activated by viral recognition receptors, TLR7, TLR8, or TLR9, through a series of phosphorylation events starting with MyD88, the main adaptor protein for most TLRs (223, 224). Once the receptor is stimulated, a complex is formed between MyD88, TRAF6, IRAK4, and IRAK1 that allows activation of IRF7 (225). Both TRAF6 and IRAK1 have been reported to be responsible for phosphorylating IRF7 leading to its homodimerization and subsequent nuclear translocation. Unlike TLR3, TLR7 pathway mainly utilizes IRF7 for activation of Type 1 IFNs, mainly IFNα.

TLR7: another target for HCV immune evasion

Recent reports indicated that robust TLR7 agonists decreased HCV RNA in HCV infected patients (196) and HCV RNA and NS5A protein in HCV replicating hepatoma cells (197). TLR7 has also been reported to recognize other single stranded RNA viruses such as influenza (226, 227) and dengue (228). TLR7 also recognizes polyU sequences in viral RNA which are found in the HCV 3'NTR (229, 230). Based on these reports, we postulated that TLR7 may play a role in HCV infection and analyzed the hypothesis that HCV, in an effort to

bypass immune surveillance, interferes with TLR7 expression and function. Our results demonstrate that HCV infection causes instability of TLR7 RNA, thus leading to low TLR7 RNA and protein expression and impaired TLR7-mediated activation of the IRF7 signaling pathway.

General Thesis Objectives

The general objective for this thesis was to determine the effects of HCV infection on Toll-like receptors. I concentrated my research on investigating these effects in two cell types: hepatocytes, the main cell type in the liver and host to HCV replication; human monocyte derived macrophages, similar to Kupffer cells (liver macrophages) that participate in inflammatory cytokine production upon viral infection. It is very important to understand the mechanisms behind HCV induced cellular activation and HCV immune escape as further research will pave the way for effective treatment in those chronically infected patients who are not responsive to current treatment.

We pursued the discovery of TLR expression in hepatocytes to see whether these cells might participate or be a target of HCV cell activation. We also wanted to learn whether hepatocytes could be activated by HCV proteins to produce inflammatory cytokines. These results might suggest another role for hepatocytes in inflammatory liver disease. Previously it was shown that HCV proteins, core and NS3, stimulated human monocytes (innate immune cells actively participating in inflammatory cytokine production) through TLR2. TLR2 heterodimerizes with TLR1 or TLR6 and we aimed to further identify which receptor complex is required for HCV protein mediated TLR activation in human

macrophages. Findings for these objectives will help distinguish which co-receptor could be involved in the activation by HCV proteins and show that HCV NS3 and core could be broadly recognized by multiple cellular receptors. Others have shown in hepatocytes that HCV NS3/4A protease activation inhibits the TLR3 and RIG-I/MDA-5 IFN-mediated pathway. There are a number of receptors that can trigger IFN production in hepatocytes and HCV has developed ways to inhibit a majority of them. Single stranded RNA viruses trigger TLR7 to produce IFNα. Alterations of TLR7 in HCV infected hepatocytes have not been investigated and we aimed to explore the effects on TLR7 and mechanisms by which the effects may occur. We intended to determine the expression of TLR7 in HCV replicating cells and any mechanisms for effects on TLR7. We aim to demonstrate the possibility for another target of HCV and a novel mechanism by the virus to continue infection.

CHAPTER II

MATERIALS AND METHODS

PART 1: Characterization of TLRs in hepatocytes.

Reagents:

Dulbecco's Modified Eagle Medium, (D-MEM) and RPMI 1640 were from Gibco (Grand Island, NY), fetal calf serum (FCS) was from HyClone (Logan, UT). Blasticidin was from Invivogen (San Diego, CA). Phenol-purified lipopolysaccharide (pLPS) was from List Biological Laboratories (Campbell, CA); PAM2CSK4 (Pm2) and PAM3CSK4 (Pm3) from EMC Microcollections GmbH (Germany); R848 was from 3M Pharmaceuticals (St. Paul, MN); TNF-α was from BD Biosciences (San Jose, CA). Recombinant HCV core, NS3, and NS5a proteins were purchased from Biodesign (Saco, ME). HCV NS3 and core proteins were fused with *E coli* derived β-galactosidase at the N-terminus and had >95% purity as evaluated by SDS-PAGE. Further purification of recombinant proteins was performed by S-Sepharose-Ceramic Hydroxyapatite-Affinity purification by Biodesign. β-galactosidase, was expressed and purified identically to HCV core and NS3 proteins (kindly provided by Biodesign), and was used as a control where indicated. To confirm there were no immunogenic affects from β-galactosidase we stimulated cells with β-gal alone. Lipopolysaccharide contamination of recombinant proteins (at a concentration of 10 μg/ml) was lower than 0.01 EU/mL, as determined by *Limulus amebocyte* assay (Kit QCL-1000; BioWittaker Inc., Walkersville, MD). The minimum limit of detection by the LAL assay is 12 picograms of endotoxin.

Cells:

All cells were grown in 5% CO_2 at 37°C and passed at 75% confluency. Human (*Homo sapiens*) hepatoma cell lines, Huh7 and Huh7.5 were maintained in DMEM with 10% heat inactivated FCS, 1% non-essential amino acids and 500 μg/ml Ciprofloxacin. Human hepatocytes were obtained from Cambrex (North Brunswick, NJ) adhered to 24 well plates coated with matrigel. Human hepatocytes were 97% pure and purified by high speed centrifugation and several other purification techniques undisclosed by Cambrex. After purification, a sample of hepatocytes was tested for enzymatic activity and functions for albumin production, phase 2 activities, and cytochrome p450 activity. Hepatocytes tested negative for Hepatitis C virus, Hepatitis B virus, human immunodeficiency virus, human T-cell lymphotrophic virus 1 and 2, and Syphilis by RPR (rapid plasma reagin). Hepatocytes with abnormal, low or high functional or enzymatic activities were not released by Cambrex. They were maintained in hepatocyte media consisting of hepatocyte basal medium with ultraglutamin-1 (Cambrex) with a 1:50 dilution of bovine serum albumin, rhEGF (epidermal growth factor), insulin, ascorbic acid, gentamicin sulfate amphotericin B, transferrin, and hydrocortisone 21 hemisuccinate.

Peripheral blood mononuclear cells (PBMCs) were isolated from HCV negative, healthy human blood donors using Ficoll-Paque density gradient centrifugation.

Human monocyte-derived macrophages were differentiated from monocytes isolated from peripheral blood mononuclear cells of HCV negative,

healthy human blood donors using Ficoll-Paque density gradient centrifugation. Monocytes were separated from PBMCs by adherence to flasks coated for two hours with 2% sterile, endotoxin-free gelatin and dried overnight in a 37°C, 5% CO_2 incubator. Before addition of peripheral blood mononuclear cells, the flasks were additionally coated for one hour with platelet-free autologous human serum and rinsed with Hanks' Balanced Salt Solution (Gibco). PBMCs were seeded into the gelatin and serum coated flasks for one-hour at 37°C, 5% CO_2. Adherent monocytes were washed three times with RPMI and removed from the flasks using a mixture of RPMI with 10% FCS and 10 mM EDTA in a 1:1 dilution. Isolated monocytes were washed three times with RPMI and plated at $5x10^5$/ml in RPMI supplemented with 18% autologous serum for eight days.

Human embryonic kidney (HEK 293T) cells stably transfected with TLR7 (HEK/TLR7) or TLR8 (HEK/TLR8) were a generous gift from Dr. Jennifer Wang at the University of Massachusetts Medical School in Worcester and were maintained in D-MEM with 10% heat inactivated FCS. HEK/TLR7 was cultured in blasticidin at 10 μg/ml and was originally from Invivogen. Both HEK cell lines were subcultured 3 times/week.

Reverse Transcription Polymerase Chain Reaction (RT-PCR):

RNA was extracted from cells collected from PBMCs, hepatoma cell lines and primary hepatocytes using the RNeasy kits (Qiagen, Valencia, CA), as manufacturer instructed. RNA was DNase (Qiagen) treated on column according to manufacturer's instructions. Reverse transcription was performed using the Reverse Transcription System® from Promega (Madison, WI) according to the

manufacturer's instructions. PCR products were run on a 1% agarose gel with DNA ladders (Roche, #1336045). The sequences for the primers used are shown in Table 2.1.

Table 2.1: Primer Sequences of TLR 1-10 and Innate Immune genes for RT-PCR.

Primer	Forward sequence	Reverse sequence	Product Size
TLR1	5'CTGGTATCTCAGGATGGTGTGC3'	5'TTGGAGTTCTTCTAAGGGTATGTTCC3'	76
TLR2	5'GGCCAGCAAATTACCTGTGTG3'	5'AGGCGGACATCCTGAACCT3'	67
TLR3	5'TCCCAAGCCTTCAACGACTG3'	5'TGGTGAAGGAGAGCTATCCACA3'	68
TLR4	5'CTGCAATGGATCAAGGACCA3'	5'TTATCTGAAGGTGTTGCACATTCC3'	74
TLR5	5'TCGAGCCCCTACAAGGGAA3'	5'CACTGAGACTCTGCTATACAAGCTA3'	74
TLR6	5'CTATTGTTAAAAGCTTCCATTTTGT3'	5'ACCTGAAGCTCAGCGATGTAGTTC3'	187
TLR7	5'TTACCTGGATGGAAACCAGCTAC3'	5'TCAAGGCTGAGAAGCTGTAAGCTA3'	72
TLR8	5'GAGAGCCGAGACAAAAACGTTC3'	5'TGTCGATGATGGCCAATCC3'	73
TLR9	5'TGGTGTTGAAGGACAGTTCTCTC3'	5'CACTCGGAGGTTTCCCAGC3'	115
TLR10	5'GAAAGGTTCCCGCAGACTTG3'	5'TGGAGTTGAAAAAGGAGGTTATAG3'	73
18S	5' GGAACTGAGGCCATGATTAA 3'	5' TCGGAACTACGACGGTATCT 3'	177
MyD88	5'GAGCGTTTCGATGCCTTCAT3'	5'CGGATCATCTCCTGCACAAA3'	65
ISG56	5'TAGCCAACATGTCCTCACAGAC3'	5'TCTTCTACCACTGGTTTCATGC3'	394
IP-10	5'AGTGGCATTCAAGGAGTACCT3'	5'ATCCTTGGAAGCACTGCATCG3'	289
ITAC	5'GCTATAGCCTTGGCTGTGATAT3'	5'GCCTTGCTTGCTTCGATTTGGG3'	232

MIG	5’TTCAGCAGATGTGAAGGAACTG3’	5’AGCATGATGAAATTCAACTGGT3’	389
TRIF	5’CCAGATGCAACCTCCACTGG3’	5’CTGTTCCGATGATGATTCC3’	339
RIG-I	5’CAGTATATTCAGGCTGAG3’	5’GGCCAGTTTTCCTTGTC3’	389
MDA-5	5’AGTTTGGCAGAAGGAAGTGTC3’	5’GGAGTTTTCAAGGATTTGAGC3’	480

All primers were supplied by IDT (Coralville, IA). The PCR protocol consisted of 95°C for 5 min, 30 cycles of 95°C – 30 seconds, 55°C – 30 seconds, 72°C – 1 minute, and 10 min at 72°C. PCR was conducted using the BioRad I-cycler. The PCR products were separated in agarose gel, stained with 0.01% ethidium bromide, detected with UV light.

Flow Cytometry:

Hepatoma cells were detached from plates using trypsin and washed with cell culture medium, stained with antibody or fixed and permeabilized with BD Cytofix/Cytoperm Kit (BD Biosciences). Cells were stained with antibodies against human TLR1 (Abcam, Cambridge, MA) at 20 μg/ml, TLR2 (Imgenex, San Diego, CA) at 5 μl/10^6 cells, TLR3 (Imgenex) at 20 μg/ml, and TLR7 antibody (Imgenex) at 5 μg/ml for 1 hour on ice in Cytoperm buffer, washed with Cytoperm buffer and then stained with the corresponding secondary antibodies either FITC (Santa Cruz Biotechnology, Santa Cruz, CA) at 10 μg/ml or PE conjugated antibody 1 μg/ml (Invitrogen, Carlsbad, CA) for 1 hour on ice; secondary PE-conjugated or FITC-conjugated antibody alone was used as a control. Cell fluorescence was analyzed using an LSRII (BD Biosciences) flow cytometer and FlowJo FACS analysis program (TreeStar, Ashland, OR).

Nuclear and Cytoplasmic Extraction:

Hepatoma cells were stimulated for 1 hour with either TNF-α, Pm2, Pm3, HCV core, HCV NS3, or HCV NS5a ligands. Nuclear and cytoplasmic samples from stimulated and unstimulated hepatoma cells were extracted by the method of Schatzle et al. (232). Briefly, after stimulation, cells were scraped and washed in

ice-cold PBS. Cells were then resuspended in cold hypotonic buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 10 µg/ml protease inhibitors, aprotinin, antipain, and leupeptin (Sigma-Aldrich)) and incubated on ice for 30 min. Cells were then lysed in 0.6% Nonidet P-40 by vortexing for 20 sec. The lysate was then centrifuged at 12,000 x *g* for 30 sec to pellet the nuclei, and the supernatant was stored at –80°C as the cytoplasmic extract. The nuclear pellet was then resuspended in ice-cold buffer B (20 mM HEPES (pH 7.9), 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 20% glycerol). All tubes were kept on a shaker at 4°C for 30 min. The lysate was then centrifuged at 12,000 x *g* for 15 min and the supernatant was stored at –80°C as the nuclear extract. Protein content was determined in both the cytoplasmic and nuclear extract by the Bio-Rad Dye Reagent assay.

Electrophoretic Mobility Shift Assay (EMSA):

Nuclear extracts were used for EMSA analysis. A consensus dsNF-κB oligonucleotide (5'-AGTTGAGGGGACTTTCGC-3') was used to bind NF-κB. End-labeling was accomplished by treatment with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (DuPont-NEN). Labeled oligonucleotide was purified on a polyacrylamide copolymer column (Bio-Rad). Nuclear protein (5 µg) was added to a binding reaction mixture containing 20 mM HEPES (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 5% glycerol, 200 µg/ml BSA, 2 µg of polydeoxyinosinic-polydeoxycytidylic acid, and 50,000 cpm of γ-^{32}P-labeled NF-κB oligonucleotide. Samples were incubated at room temperature for 30 min. All reactions were run on a 6% polyacrylamide gel, dried overnight, and the gel was

exposed to x-ray film at –80°C overnight. For the cold competition reaction, a 20-fold excess of specific unlabeled double-stranded probe was added to the reaction mixture before adding the labeled oligonucleotide.

Enzyme-Linked Immunosorbent Assay (ELISA):

Hepatoma, primary hepatocytes, HEK/TLR7, HEK/TLR8, and hMDM cells were stimulated for 10 hrs or for a time course up to 144 hours with TLR-specific ligands or HCV core or NS3 recombinant proteins. Cell culture supernatants were collected and analyzed using cytokine specific ELISAs (IL-6 and IL-8 ELISAs from BD Bioscience (San Jose, CA), IFN-α (limit of detection =10 pg/ml) and IFN-β (limit of detection=25 pg/ml) ELISA from PBL Interferon Source (Piscataway, NJ)), all performed according to the manufacturer's instructions.

Statistical analysis:

Statistics were calculated by student's t-test using EXCEL with a p-value less than 0.05 as statistical significance. Human hepatocytes were from one individual performed in three or one independent setting as stated in figure legends.

PART 2: Toll-like receptors 1 and 6 are both involved in TLR2-mediated macrophage activation by Hepatitis C Virus core and NS3 proteins

Reagents:

S. aureus peptidoglycan (PGN) was from Fluka (Milwaukee, WI). HCV NS3 and core proteins were fused with *E coli* derived β-galactosidase at the N-terminus and had >95% purity as evaluated by SDS-PAGE. Further purification of recombinant proteins was performed by S-Sepharose-Ceramic Hydroxyapatite-

Affinity purification by Biodesign. β-galactosidase was expressed and purified identically to HCV core and NS3 proteins (kindly provided by Biodesign), and was used as a control where indicated. To confirm there were no immunogenic affects from β-galactosidase we stimulated cells with β-gal alone.

Cells:

HEK 293T cells stably transfected with TLR2/YFP (HEK/TLR2) were maintained in D-MEM with 10% heat inactivated FCS and selective antibiotics and subcultured 3 times/week.

C57BL/6J (wild type), TLR2, TLR1, TLR4 and TLR6 knockout mice and TLR4 mutant mice were a gift from Dr. Robert Finberg at the University of Massachusetts Medical School, Worcester, MA. TLR4 mutant mice were C3H/HeJ mice that have a spontaneous point mutation in the intracellular domain of TLR4 that blocks LPS signaling. Mice were injected with a 4% thioglycollate medium intraperitoneal and peritoneal macrophages were extracted from peritoneal exudates five-days later. Mouse macrophages were plated in 96 well plates (BD Biosciences, Franklin Lakes, NJ) at 10^6/ml.

Small Interfering RNA:

TLR2, TLR1, TLR6, and control siRNA oligonucleotides (Cat. 1027280) were purchased from Xeragon (Valencia, CA). The full mRNA sequence was located from GeneBank and siRNA sequences were identified as 21-nucleotides starting with AA and ended with TT and were chosen after the first 150 base pairs of the full mRNA sequence. Sequences with G/C content between 30 to 70%

were used. Sequence alignment tests confirmed specificity to corresponding TLR.

HEK/TLR2 cells were plated in a 24 well plate at 10^6/ml and grown to yield 70% confluency. SiRNAs (final concentration 300 nM/well) were combined with Mirus TransIT transfection reagent and Opti-MEM medium for 15 minutes. The culture medium was changed prior to transfection and the siRNA mix was added to each well and incubated for eight hrs at 37°C. After transfection, 1 ml of fresh culture medium was added to each well and the cells were grown for an additional time up to 72-96 hours.

Human monocyte-derived macrophages (hMDM) were transfected with siRNAs using Lipofectamine 2000. Briefly, 3 µls of Lipofectamine 2000, 350 µl D-MEM, and 60 pmol of siRNA/well were incubated for 15 min at room temperature. The macrophages were washed with serum-free D-MEM and incubated at 37°C, 5% CO_2 for three hours with siRNA transfection mixture as described above. After incubation, the cells were washed with serum-free D-MEM and maintained in RPMI with 18% autologous serum for up to 72-96 hours. Knockdown of a specified gene was confirmed by Real Time PCR analysis for the mRNA expression and by Western Blot analysis for protein expression.

Protein quantification by Western Blot:

Twenty-four and seventy-two hours after siRNA transfection the cells were counted and lysed for 10 min on ice with lysis solution: [1% NP40, 0.5% docetaxel, 1.5 mM NaCl, 0.5 M EDTA, Complete Mini® Protease Inhibitor Cocktail (Roche)]. The lysate was spun at 4°C for 10 min at 14,000 rpm,

collected and mixed with SDS sample buffer. Protein samples were heated at 95°C for 2 minutes, loaded onto a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Precision Plus Protein standard Bio-Rad (Hercules, CA) was used for the molecular weight ladder. Membranes were blocked overnight with 5% non-fat milk in PBS, and then probed with anti-TLR6 (Zymed, San Francisco, CA) or anti-β-actin (Abcam, Cambridge, MA) antibodies at 1:10,000 dilution in PBS for 1hr at room temperature. Secondary HRP-labeled goat anti-mouse antibody (Santa Cruz, Santa Cruz, CA) and enhanced chemiluminescence (LumiGlo kit Cell Signaling Technology (Danvers, MA) were employed to identify the immunoreactive bands. The densitometry analysis of the immunoreactive bands was performed using the Fuji UVP system and LabWorks program.

Reverse transcription and polymerase chain reaction:

RNA was extracted from cells collected 48 and 72 hrs after siRNA transfection using the RNeasy kits (Qiagen, Valencia, CA), as manufacturer instructed. Reverse transcription was performed using the Reverse Transcription System® from Promega (Madison, WI) according to the manufacturer's instructions. The sequences for the primers used are shown in Table 2.2. Primers for TLR2, TLR1, and TLR6, were all supplied by IDT (Coralville, IA). 18S primers were from Ambion (Austin, TX). The PCR protocol consisted of 95°C for 5 min, 30 cycles of 95°C – 30 seconds, 55°C – 30 seconds, 72°C – 1 minute, and 10 min at 72°C. PCR was conducted using the BioRad I-cycler. The PCR products were separated in an agarose gel, stained with 0.01% ethidium bromide,

detected with UV light and quantified using the Fuji UVP system and LabWorks program.

Table 2.2: Primer Sequences for TLRs recognizing HCV proteins using RT-PCR

Primer	Forward Sequence	Reverse Sequence	Product Size
TLR1	5'-GGGTCAGCTGGACTTCAGAG-3'	5'-AAAATCCAAATGCAGGAACG-3'	214
TLR2	5'-ACTCAGGAGCAGCAAGCAC-3'	5'-ATCAGCAGGAACAGAGCACA-3'	173
TLR6	5'-GAACATGATTCTGCCTGGGT-3'	5'-GCTGTTCTGTGGAATGGGTT-3'	282
18S	5'-GGAACTGAGGCCATGATTAA-3'	5'-TCGGAACTACGACGGTATCT-3'	177

Flow cytometry analysis:

HEK/TLR2 cells were detached from plates using trypsin and washed with cell culture medium. Control and siRNA-treated cells were incubated with anti-human TLR2 antibody (a kind gift from Dr. Robert Finberg, University of Massachusetts Medical School, Worcester, MA) and secondary APC-conjugated antibody (Caltag Laboratories, Burlingame, CA). APC-conjugated antibody alone was used as a background control. Cell fluorescence was analyzed using an LSR II (BD Biosciences) flow cytometer and FlowJo FACS analysis program (TreeStar, Ashland, OR).

Cytokine quantification by ELISA:

SiRNA-transfected cells and mouse peritoneal macrophages were stimulated for 10 hrs with TLR-specific ligands or HCV core or NS3 recombinant proteins. Cell culture supernatants were collected and analyzed using cytokine specific ELISAs (IL-6, IL-8, and TNF-α ELISAs from BD Bioscience (San Jose, CA), IL-10 ELISA from eBioscience (San Diego, CA)), all performed according to the manufacturer's instructions.

Statistical analysis:

All data derived from cell lines were analyzed with t-test, those from mouse macrophages employed ANOVA and those from human monocyte-derived macrophages with non-parametric Wilcoxon statistical analysis methods. A $p<0.05$ was considered statistically significant. Statistics were calculated by using EXCEL.

PART 3: Impaired expression and function of Toll-like receptor 7 in Hepatitis C virus infection in human hepatoma cells

Reagents:

DMEM, Dulbecco's Modified Eagle Medium and NEAA, nonessential amino acids, Geneticin (G418) and TrypLE Express were from Gibco (Grand Island, NY) and FCS, fetal calf serum, was from Hyclone (Logan, UT). Ciprofloxacin was from CellGro (Herndon, VA). IFNα, Actinomycin D and Alpha amanitin were from Sigma Aldrich (St. Louis, MO) and R837 was from Invivogen (San Diego, CA).

Cells:

All cells were grown in 5% CO_2 at 37°C and passed at 75% confluency. Human hepatoma cell lines, Huh7 and Huh7.5 were maintained in DMEM with 10% heat inactivated FCS, 1% NEAA and 500 μg/ml Ciprofloxacin. HCV stable cell lines FL, generated on the Huh 7.5 background, and BB7, generated on the Huh7 background, were maintained in DMEM with 10% FBS, 1% NEAA, 500 μg/ml Ciprofloxacin and either 750 μg/ml G418 or 500 μg/ml G418. All four cell lines were gifted from Dr. Charlie Rice at the Rockefeller University (New York, NY). JFH-1 plasmid was a generous gift from Dr. Takaji Wakita at the National Institute of Infectious Disease (Tokyo, Japan). The JFH-1 cell line, generated on the Huh7.5 background, was propagated by following the methods outlined previously (233) and briefly stated here. JFH-1 plasmid was transformed into JM109 cells (Promega) and grown overnight in LB broth with ampicillin. Plasmids were extracted by a Maxiprep kit from Qiagen and cut with Xba1 from

New England Biolabs, (Ipswich, MA). RNA transcription was performed using MEGAscript T7 kit by Ambion, Austin, TX. Purified RNA was electroporated into Huh7.5 cells using Gene Pulser II™ apparatus by Bio-Rad (Hercules, CA). All cells were grown in 5% CO_2 at 37°C and passed at 75% confluency.

Quantitative Real Time Polymerase Chain Reaction:

RNA isolated from HCV patients' liver tissue samples were obtained from Dr. Gyongyi Szabo (University of Massachusetts Medical School). RNA was isolated using the RNeasy Kit (Qiagen) for tissue samples according to manufacturer's protocols and subjected to DNase treatment (Qiagen) during isolation. For analysis by quantitative PCR, cellular RNA was extracted with RNeasy Kit (Qiagen, Valencia, CA) and on column DNase (Qiagen) treatment was performed according to manufacturer's instructions. Complementary cDNA was transcribed with the Reverse Transcription System (Promega, Madison, WI) according to manufacturer's instructions. TLR7, TLR5 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) quantification was performed by real-time PCR (QRT-PCR) with Sybr Green Master Mix (Eurogentec, San Diego, CA) according to manufacturer's instructions; HCV and GAPDH was assessed with Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA), all on the Eppendorf Realplex4 Mastercycler (Eppendorf, Westbury, NY). β-actin was used for RNA stability experiments as it was more stable throughout the 24 hour time course than GAPDH. The sequences of the specific primers are listed in Table 2.3. TLR7 and TLR5 primer sequences are listed in Table 2.1. The delta delta ct method was used to calculate fold change in all quantitative PCR graphs.

Table 2.3: Primer Sequences for HCV Quantitative Real Time-PCR

Primer	GAPDH	HCV	β-actin (232bp)
Sense	5’-TGCCTTCTTGCCTCTTGTCT-3’	5' NTR: 5'-CCTCTAGAGCCATAGTGGTCT-3’	5’-GCGGGAAATCGTGCGTGACAT-3’
Anti-sense	5’-GGCTCACCATGTAGCACTCA-3’	5'-CCAAATCTCCAGGCATTGAGC-3'	5’-GATGGAGTTGAAGGTAGTTTC-3’
Taqman probe	FAM-TTTGGTCGTATTGGGCGCCTGG-BHQ1	FAM-CACCGGAATTGCCAGGACGACCGG-BHQ1	Not Applicable

Western Blot:

For assessment by Western Blot, cells were lysed for 10 min on ice with 1% Nonidet P-40, 0.5% docetaxel, 1.5 mM NaCl, 0.5 M EDTA, Complete Mini protease inhibitor cocktail (Roche, Indianapolis, IN) and the lysate was spun at 4°C for 10 min at 14,000 rpm. Equal amounts of total cellular proteins from each experimental group were mixed with 4x SDS-containing loading buffer, heated at 95°C for 5 min, separated on 10% SDS-PAGE, and transferred to a nitrocellulose membrane. Precision Plus protein standard (Bio-Rad, Hercules, CA) was used as a molecular weight ladder. Membranes were blocked overnight with 5% nonfat milk in PBS and probed with anti-NS5A antibody 1:500 (a generous gift from Dr. Charlie Rice) or anti-β-actin (Abcam, Cambridge, MA) 1:10,000. Secondary HRP-labeled goat anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL [LumiGlo kit, Cell Signaling Technology (Danvers, MA)] were used to identify the immunoreactive bands.

Flow Cytometry:

For flow cytometry analysis, cells were detached from plates using cell stable TrypLE Express, washed, fixed and permeabilized with BD Cytofix/Cytoperm Kit (BD Biosciences). Cells were stained with anti-human TLR7 antibody (Imgenex, San Diego, CA) 1:100 for 1 hour on ice in Cytoperm buffer, washed with Cytoperm buffer and then stained with secondary anti-mouse PE conjugated antibody 1:1000 (Invitrogen, Carlsbad, CA) for 1 hour on ice; secondary PE-conjugated antibody alone was used as a control. Cell fluorescence

was analyzed using an LSRII (BD Biosciences) flow cytometer and FlowJo FACS analysis program (TreeStar, Ashland, OR).

Immunofluorescence:

For immunofluorescence, cells were plated at 10^5/ml on 4 well BD Falcon CultureSlides (BD Biosciences, San Jose, CA) and grown overnight in 37°C, 5% CO_2. Cells were washed with PBS and fixed on slide with methanol:acetone 1:1 mix for 10 minutes. Slides were dried, rinsed with PBS, blocked for 1 hour with 10% human serum in PBS and probed with anti-HCV core antibody 1:250 (Affinity BioReagents, Golden, CO) overnight at 4°C. Slides were washed with 0.5% Tween 20-PBS for 30 min followed by AlexaFluor 568 goat anti-mouse IgG antibody 1:1000 (Invitrogen) for 1 hour. During the final wash step DAPI (2 μg/ml) was added for 10 minutes. Slides covered with a glass coverslip using ProLong Antifade kit (Invitrogen) and analyzed for fluorescence using a Leica DM IRE2 confocal microscope.

RNA stability:

RNase inhibitors, alpha-amanitin and actinomycin D, were added to cell culture media at a concentration of 10 μg/ml and 2 μg/ml for up to 24 hours. Cell RNA samples were extracted at 0, 2, 4, 8 and 24 hours. QRT-PCR was run on RNA samples using TLR7, HCV, and β-actin primers.

ImageStream: IRF7 Nuclear Translocation analysis

Hepatoma control and HCV replicating cells were plated at $8x10^5$/ml and grown overnight in 5% CO_2 37°C and stimulated with R837 (10 μg/ml) for a time course up to 4 hours. Cells were fixed and permeabilized as described for the

flow cytometric analysis and stained for IRF7 (Santa Cruz Biotechnology, Santa Cruz, CA) 1:50 for 1 hour on ice. Cells were stained with secondary anti-mouse IgG conjugated with Alexa Fluor 488 (Invitrogen) 1:1000 for 1 hour on ice, then with 5 μM DRAQ5 (Biostatus Limited, Leicestershire, UK) for nuclear staining. Image files of 10,000 events were collected for each sample using the ImageStream system (Amnis, Seattle, WA) and analyzed using IDEAS software (Amnis). Single cells were identified by in-focus gating on IRF7 or DRAQ5 fluorescent events with high nuclear aspect ratios (minor to major axis ratio, a measure of circularity) and high nuclear contrast (as measured by the Gradient Max feature). Nuclear localization of IRF7 was measured using the similarity score, which quantifies the correlation of pixel values of the nuclear and IRF7 images on a per-cell basis (234). If IRF7 is nuclear, the two images will be similar and have large positive values. If it is cytoplasmic, the two images will be anti-similar and have large negative values. Events with positive values had visually apparent nuclear distributions of IRF7 and were gated to quantify the percentage of cells within the hepatoma population with nuclear-localized IRF7 (234)

Statistical analysis:

Statistics were calculated by student's t-test using EXCEL with a p-value less than 0.05 as statistical significance.

CHAPTER III

Characterization of TLRs in hepatocytes.

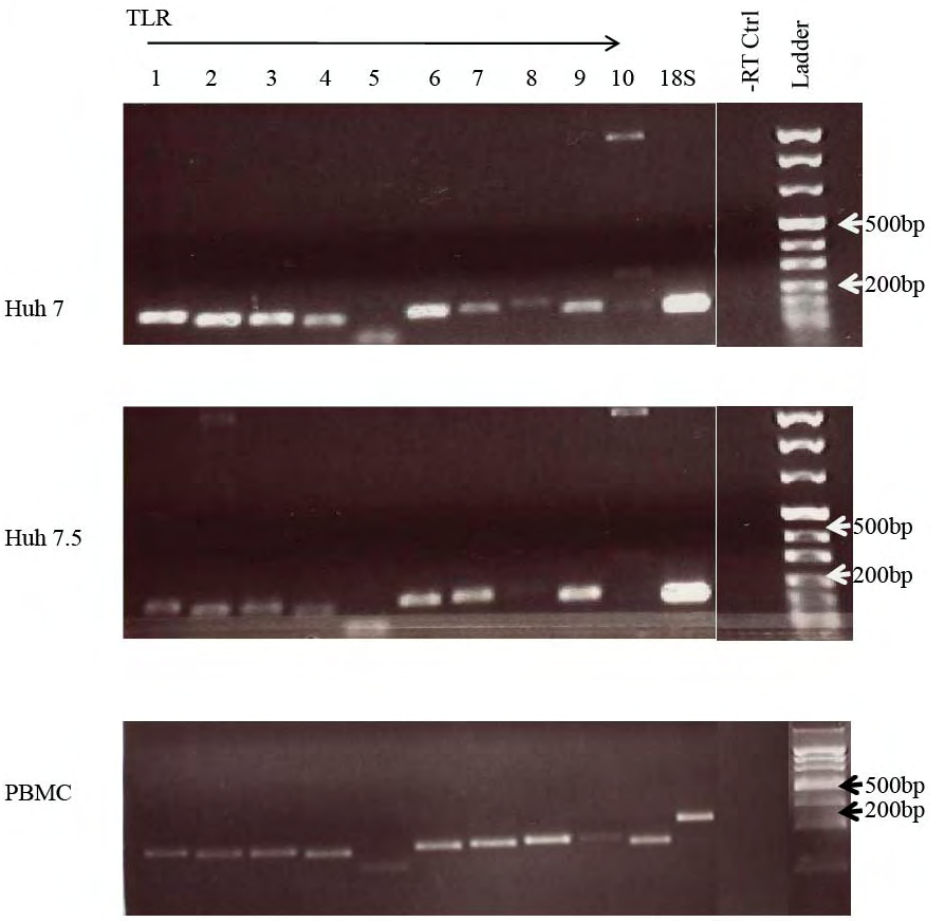
There are a number of host factors that are affected by HCV either directly or indirectly. Since the discovery of a cell culture model that supports HCV infection, intense research is ongoing. Previous groups have uncovered a number of host innate immune factors that are manipulated by HCV proteins (235, 236). HCV core and NS3 proteins were shown to activate TLR2 in human monocytes, HEK/TLR2, and murine peritoneal macrophages (79). HCV E2 binds to CD81 causing inhibition of NK cell activation (153). To further investigate innate immune mechanisms activated by HCV in hepatocytes, we hypothesized that both hepatoma and primary hepatocytes express functional TLR1, TLR6, and TLR7 that may be involved in the recognition of HCV.

Identification of TLR RNA in Hepatocytes

Previously, a number of innate immune receptors including some TLRs were thought to be exclusive to specific cell types (237). In particular, TLR7, TLR8, and TLR9 were thought to be restricted to dendritic cells (238, 239). To investigate our hypothesis and establish TLR expression, we used hepatoma cell lines Huh7 and Huh7.5 and purified primary hepatocytes to determine the TLR mRNA expression levels by RT-PCR (Figure 3.1). Using primers shown in Table 1, directed towards TLRs 1-10 and other various innate immune factors, we determined that hepatoma cells express almost all TLRs except for TLR10 in both cell lines and TLR8 in Huh 7.5. PBMCs express all ten TLRs and were thus run alongside the hepatoma cells as a control for TLR expression. No RT controls

were run alongside all cell types to ensure no contaminating products contributed to expression. Because hepatoma cells are transformed hepatocytes and could have changes in cellular RNA expression, we used primary hepatocytes to confirm that TLR expression is similar in both transformed and untransformed cell types (Figure 3.1B). Primary hepatocytes express very similar levels of TLRs as the hepatoma cells. The major difference is that TLR10 and TLR8 are expressed in primary hepatocytes compared to hepatoma cells. Primary hepatocytes also express a variety of innate immune factors associated with TLRs. The differences in TLR8 and TLR10 expression between hepatoma cells and hepatocytes may indicate possible functional variations when responding to HCV. Band sizes vary due to the time agarose gels were run for. Figure 3.1A and Figure 3.1B upper half, were run for a short time period shown by their condensed DNA ladders and thus the sizes of the bands are more condensed, while Figure 3.1B lower half was run for a longer period of time resulting in much more space aliquoted between the band sizes. No RT controls were run for every PCR in this figure and all subsequent figures with PCR to ensure no contaminating products.

A.


TLR
1
2
3
4
5
6
7
8
9
10
18S
-RT Ctrl
Ladder
Huh 7
500bp
200bp
Huh 7.5
500bp
200bp
PBMC
500bp
200bp

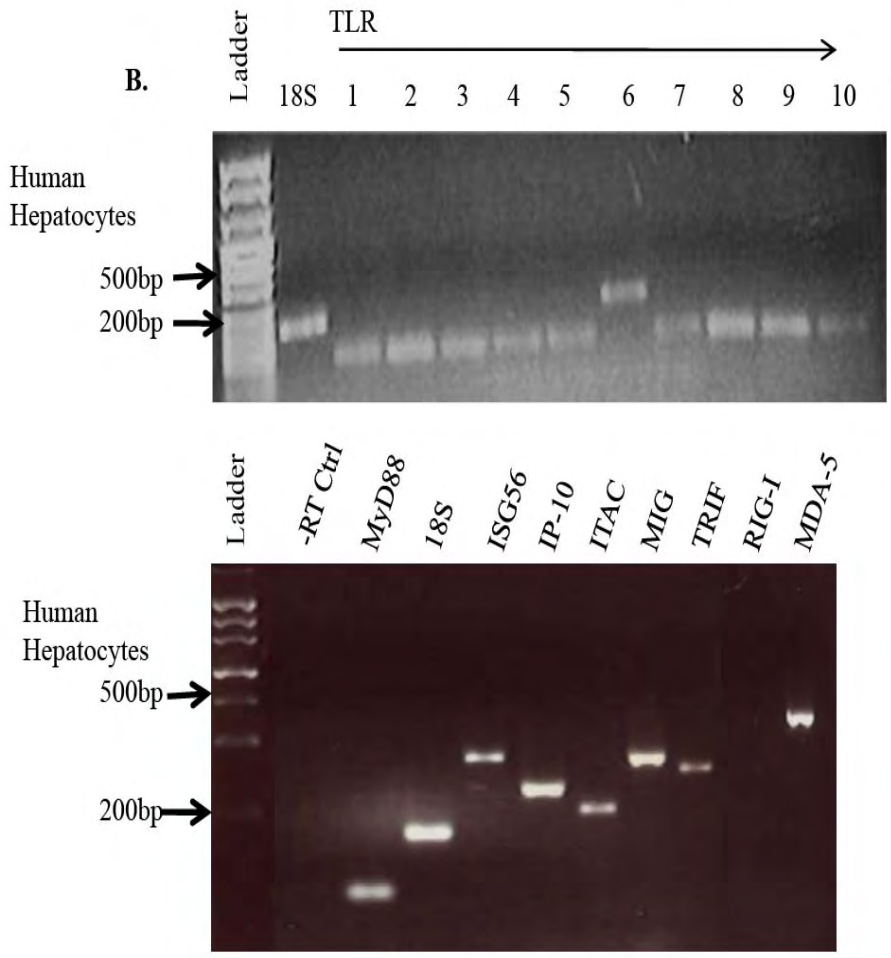


Figure 3.1: Messenger RNA expression of Toll-Like Receptors in human hepatoma cell lines and primary hepatocytes. (A.) Reverse Transcription PCR was performed showing TLR 1-10 expressions from hepatoma cell lines, Huh7 and Huh7.5, and PBMCs used as a control for TLR expression. 18S was used as an experimental control to ensure proper PCR technique. DNA ladder is also shown with basepair markers indicated with arrows. (B.) TLR expression on pure primary human hepatocytes is representative of two independent experiments. Targets of both TLR-mediated pathways and other innate immune receptors were also performed shown in the bottom half of (B). No RT controls are shown next to the DNA ladder for each cell type. Figures shown are representative of three independent experiments.

Possible HCV Targets within the TLR Family: TLR2/1, TLR2/6, and TLR7

HCV targets specific TLRs to induce either an immune response or immune escape (235, 236). Others have reported TLR2 immune activation (79) and TLR3-mediated pathway inhibition by HCV proteins (106). TLR2 immune activation was not fully characterized as TLR2 heterodimerizes with TLR1 or TLR6, thus we examined the activation of TLR2 and its heterodimers. TLR3 is a viral recognition receptor in a small family of TLRs contained in the endosome (240). TLR3-mediated pathway inhibition was thoroughly described and so we decided to investigate other viral recognition receptors (106). Since HCV is a single stranded RNA virus, we decided to investigate TLR7, a single stranded RNA viral recognition receptor. TLR2, TLR1, TLR6 and TLR7 mRNA was expressed in both hepatoma and primary hepatocytes (Figure 3.1).

Protein Receptor Expression of TLR2 Heterodimers and TLR7

We next looked into the protein expression levels either on the cell surface or within the cell using flow cytometry. Previous groups have reported protein expression of TLR3 within hepatoma cells and we confirmed these results in Figure 3.2 (240). There are moderate levels of TLR1 on the cell surface compared to high protein expression of TLR2 both on the cell surface and within the cell. There is a slight tail of increased fluorescent intensity to the right of the histogram of TLR1 expression which could be from variations in staining. There was no expression of TLR7 on the cell surface but moderate expression levels within the cell. Flow cytometry antibodies specific for TLR6 were not optimized at that time and so we do not show TLR6 protein expression. These results

confirm the presence, both RNA and protein, of TLR2, TLR1 and TLR7 in human hepatoma cell lines.

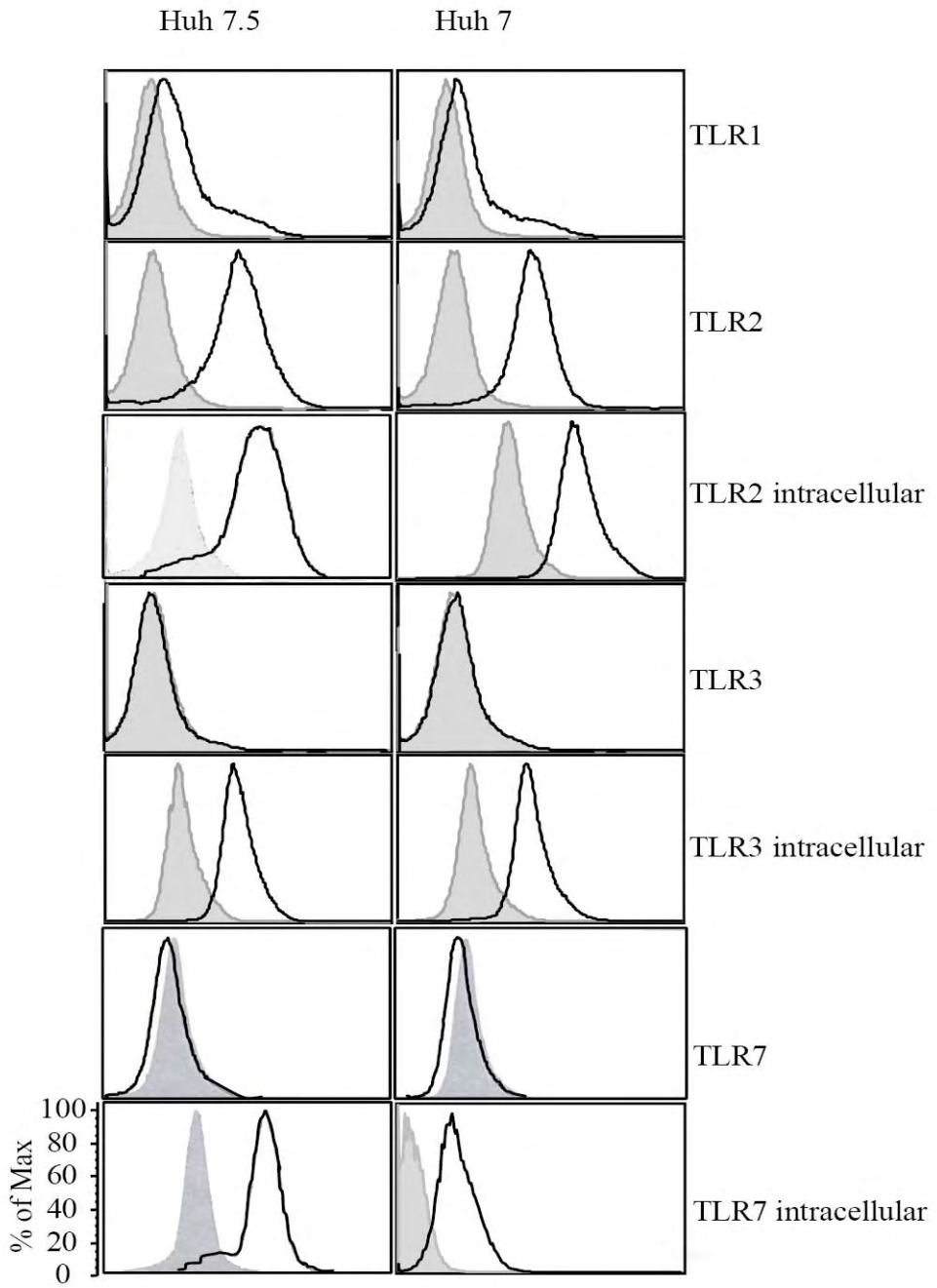


Figure 3.2: TLR protein expression levels in human hepatoma cell lines. Human hepatoma cells were either fixed or permeabilized and fixed for flow cytometry analysis. TLR specific antibodies stained cell surface and intracellularly, where indicated, on Huh7 and Huh 7.5 cells. Grey shaded histogram represents the corresponding secondary antibody control. Data shown are representative of three separate experiments.

TLR Functional Confirmation in Hepatoma Cells: Activation of NF-κB

In mice, TLR8 RNA and protein is expressed yet reported non-functional (241). Non-functional receptors may not be an active target of HCV; therefore to confirm functionality in the TLR2 heterodimer complexes and TLR7 we stimulated hepatocytes with TLR2 heterodimer and TLR7 specific ligands.

On unstimulated hepatoma cells, we observed a high background of IL-8 and IL-6 cytokines which upon treatment with TLR2 or TLR7 ligands did not induce a significant increase (data not shown). Therefore, we tested the function of these receptors by investigating the NF-κB nuclear activation after ligand stimulation using electrophoretic mobility shift assays. NF-κB is a major transcription factor that is activated upon numerous stimulations such as TLR ligand stimulations (79, 191). Activation of this factor elicits a variety of pro- and anti-inflammatory cytokines and chemokines such as IL-6 and IL-8. EMSAs were performed using nuclear extracts from Huh7 (Figure 3.3B) or Huh7.5 (Figure 3.3A) cells stimulated for one hour with TLR specific ligands. Using TLR2/1, Pm3, or TLR2/6, Pm2, specific ligands (242), we found more NF-κB activation with ligands specific to TLR2/6 compared to TLR2/1, although both ligands activated NF-κB. After one hour I did not see a measurable increase from R848 stimulated cells in NF-κB activation compared to the control (data not shown) using EMSA. These results show functional TLR2, TLR1 and TLR6 receptors in Huh7 and Huh7.5 cells.

Recombinant HCV proteins, core and NS3, can elicit a cellular response in epithelial and immune cells (79, 108). It is not known whether hepatocytes react

to HCV protein administration, therefore we investigated whether hepatoma cells activate NF-κB after a one hour stimulation using recombinant HCV core, NS3 and NS5A proteins (Figure 3.3). We found that both Huh7 and Huh7.5 cells respond to HCV core and NS3 stimulation. The NS5A response in Huh7.5 cells showed a small amount of NF-κB activation while in the Huh7 cells it was relatively weak compared to the media control. These results show that despite the lack of cytokine production from hepatoma cell lines that would be released into the media, these cells are activated by TLR2 heterodimer ligands and HCV proteins.

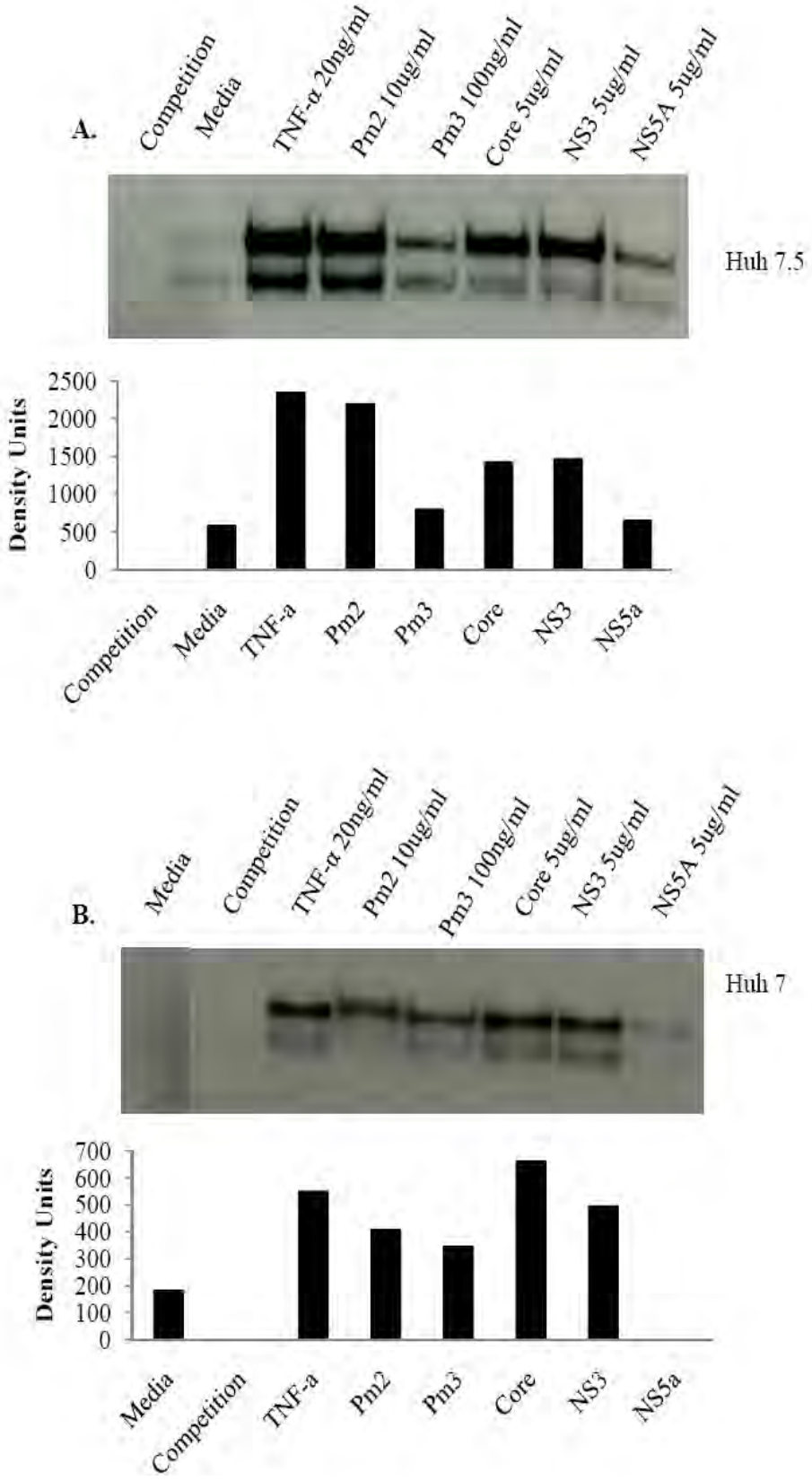

A.
Competition
Media
TNF-α 20ng/ml
Pm2 10ug/ml
Pm3 100ng/ml
Core 5ug/ml
NS3 5ug/ml
NS5A 5ug/ml
Huh 7.5
Density Units
2500
2000
1500
1000
500
0
Competition
Media
TNF-a
Pm2
Pm3
Core
NS3
NS5a
B.
Media
Competition
TNF-α 20ng/ml
Pm2 10ug/ml
Pm3 100ng/ml
Core 5ug/ml
NS3 5ug/ml
NS5A 5ug/ml
Huh 7
Density Units
700
600
500
400
300
200
100
0
Media
Competition
TNF-a
Pm2
Pm3
Core
NS3
NS5a

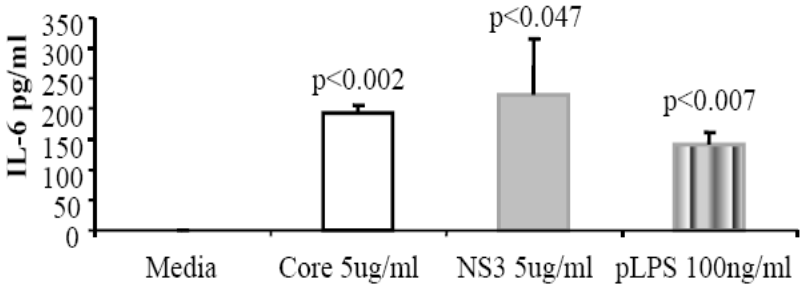
Figure 3.3: NF-κB activation in hepatoma cell lines by electrophoretic mobility shift assay. (A.) Huh7.5 and (B.) Huh7 cells were stimulated with the indicated concentrations for one hour by TNF-α (stimulation control), Pm2 (TLR2/6 ligand), Pm3 (TLR2/1 ligand), HCV core, HCV NS3, HCV NS5A and plain media as a negative control. EMSA was used to study the protein-DNA interaction for the NF-κB complexes in nuclear extracts from hepatoma cell lines. TNF-α is a positive stimulation control for NF-κB activation. The media alone lane were cells that did not have any stimulants and controlled for excess baseline activation of NF-κB. There was no excess baseline seen in the media control lanes. Competition lanes were used to identify NF-κB complexes. Competition lanes contained cold (unlabeled) oligonucleotide sequence that competitively bound to NF-kB oligonucleotide sequence in the TNF-α stimulated sample and was administered 20 minutes before labeled oligonucleotide was added to the extracts. Densitometry included both upper and lower bands. Density graphs are shown below and correspond to the bands directly above the bars. Data shown is representative of four independent experiments.

Primary Hepatocytes Show Cytokine Production when stimulated with HCV Proteins

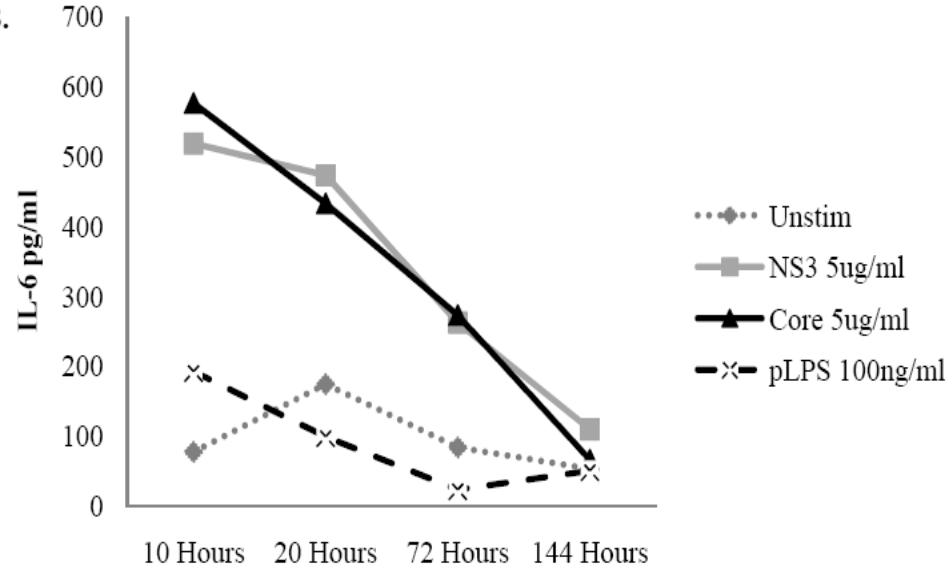
Since hepatoma cells are not an exact representation of how TLRs function in primary hepatocytes, we investigated cellular activation using primary human hepatocytes obtained from Cambrex. Unlike hepatoma cell lines, primary hepatocytes do produce measurable amounts of cytokines in media upon stimulation. The caveat to using human hepatocytes is the expense in obtaining them, therefore where noted in the figures, most human hepatocyte experiments were completed only using one individual either in triplicates or singlets. We stimulated the cells for ten hours with HCV proteins, core and NS3, and used purified lipopolysaccharide (pLPS), a TLR4 specific ligand, as a positive control. We ran enzyme-linked immunosorbent assays (ELISAs) for IL-6 and IL-8. Both HCV core ($p<0.002$) and NS3 ($p<0.047$) produced a moderate IL-6 response, 200-300 pg/ml, after ten hours (Figure 3.4A) which was significant compared to the media control. This response was acute showing the peak levels, around 550 pg/ml, at ten hours and diminished in half after three days and back to control levels after 6 days (Figure 3.4B). We observed a more substantial and significant IL-8 response, around 3000 pg/ml, when stimulated with the same proteins after ten hours (Figure 3.4C). Unlike the rapid IL-6 response, the IL-8 response was much slower, peaking, between 25,000 and 30,000 pg/ml, after 3 days and sustaining the response up to 6 days (Figure 3.4D). These results show that primary hepatocytes do respond when stimulated with HCV core and NS3 proteins and the response is both a rapid and slow production of cytokines. Beta-

galactosidase was run as a negative control in Figure 4.2 to assure that β-gal fusion to HCV core or HCV NS3 proteins was not contributing or interfering with cellular cytokine production. There was no contribution by β-gal to cytokine production in either primary macrophages or HEK/TLR specific cells.

A.


IL-6 pg/ml
350
300
250
200
150
100
50
0
p<0.002
p<0.047
p<0.007
Media
Core 5ug/ml
NS3 5ug/ml
pLPS 100ng/ml

B.


IL-6 pg/ml
700
600
500
400
300
200
100
0
10 Hours
20 Hours
72 Hours
144 Hours
Unstim
NS3 5ug/ml
Core 5ug/ml
pLPS 100ng/ml

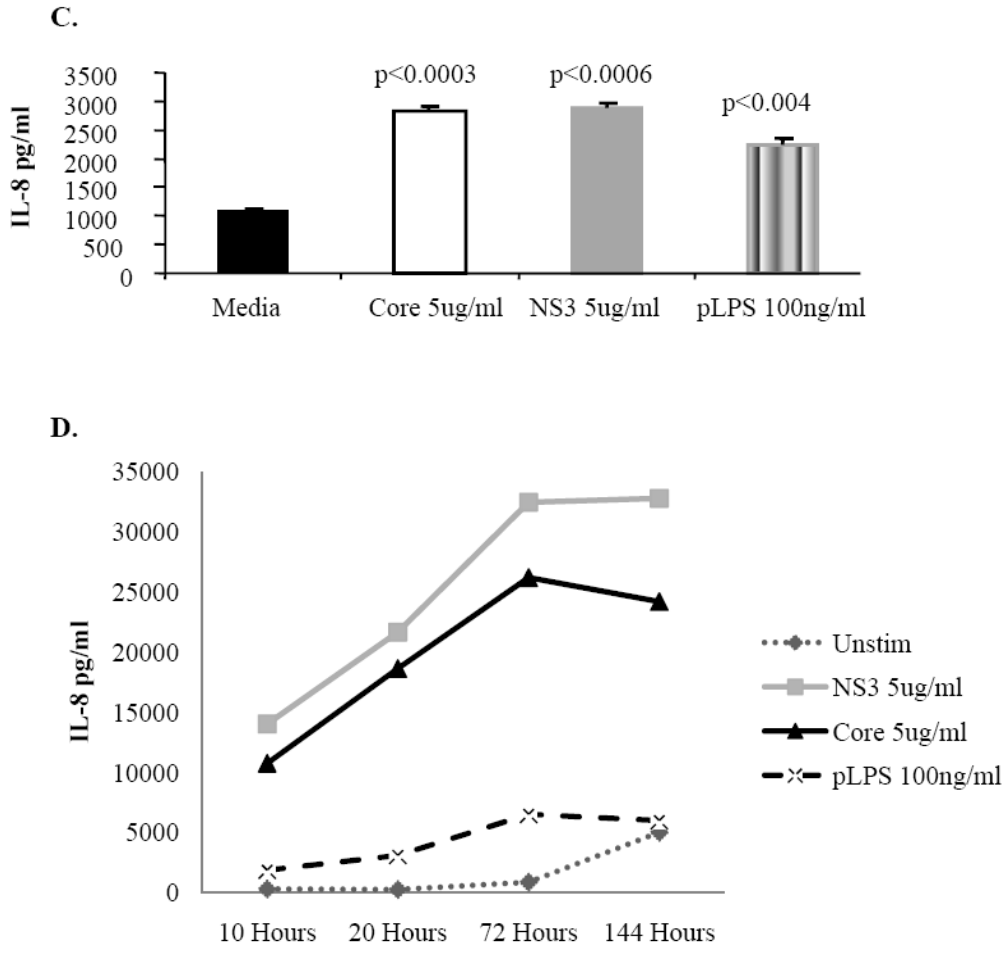


Figure 3.4: IL-6 and IL-8 response in primary human hepatocytes to HCV proteins. (A.) IL-6 and (C.) IL-8 were measured with ELISA after a ten hour stimulation with HCV proteins, core or NS3, or pLPS with the indicated concentrations. Data are shown as an average ± SD pg/ml of n=3 replicate wells experiments from one non-HCV infected control. P-values compared with the media control were calculated using the student's T-test. Time courses of (B.) IL-6 and (D.) IL-8 were measured with ELISA using the same stimulations as in (A.) and (C.) but were performed with one non-HCV infected individual of n=1 experiment. Data are shown with different non-HCV control hepatocytes from (A.) and (C.).

Examining Type 1 IFN Response in Hepatocytes

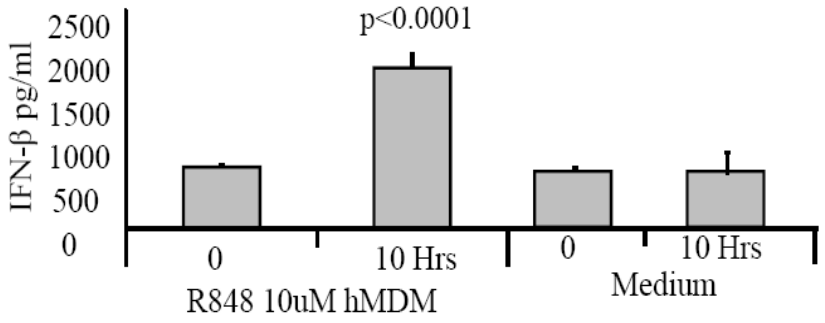
HCV infection is treated with a combined therapy of pegylated IFN-α and ribavirin (35, 37). Specific cell types in the liver produce Type 1 IFNs upon activation from cellular factors, such as TLR7 or TLR3 (179, 181, and 195). Unlike TLR3 where IFN-β is the primary product after stimulation, IFN-α is the main product of the activated TLR7 IFN-mediated pathway (199, 243). Earlier reports have already identified inhibition of Type 1 IFN production by HCV proteins, thus showing the viral defense mechanisms against anti-viral products (106, 171, 172, and 178). Since IFN-α is involved in combating HCV infection, we investigated whether primary hepatocytes and representative cell types in the liver could produce Type 1 IFNs using a TLR7/TLR8 specific ligand, R848.

We used pure human hepatocytes and human monocytes derived macrophages (hMDMs) which are similar to Kupffer cells, hepatic macrophages. We measured IFN-α and IFN-β by ELISA in all cell types (Figure 3.5A, B and data not shown). Human hepatocytes barely produced the detectable limit of IFN-β after 20 hours of stimulation (data not shown); hMDMs elicited a much larger increase of IFN-β compared to hepatocytes when stimulated with R848 (Figure 3.5A). This suggests that the primary cell type to produce IFN-β in the liver could be Kupffer cells.. Unlike IFN-β, human hepatocytes did not produce any IFN-α above the limit of detection of 12.5 pg/ml (data not shown). The hMDMs showed a much reduced amount of IFN-α production compared to its IFN-β production but was measurable above the limit of detection (Figure 3.5B). These results show that human hepatocytes do not produce measurable amounts of extracellular IFN-α

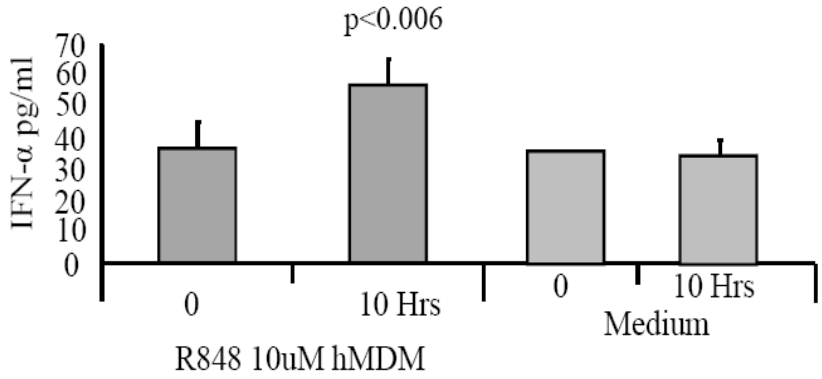
and very little IFN-β when stimulated with R848 up to 144 hours (data not shown) while human macrophages do produce both IFNs.

TLR7 stimulation results in activation of two separate pathways: production of inflammatory cytokines through NF-κB transcription factor or Type 1 IFN production through interferon regulatory factor (IRF) 7 (179, 243, 228, and 229). In Figure 3.5A and 3.5B we show the limited Type 1 IFN response from hepatocytes. To investigate the other pathway in hepatocytes, we stimulated cells with R848 during a time course and measured IL-6 (Figure 3.5C) and IL-8 (Figure 3.5D) levels using ELISA. Primary hepatocytes showed a similar pattern of cytokine induction when stimulated with HCV proteins or R848. IL-6 levels after R848 treatment were rapid in response after ten hours quickly diminishing after 3 days and showed similar quantities compared with stimulation using HCV proteins. IL-8 levels, on the other hand, were induced after ten hours yet slowly peaked by 3 days, showing the same pattern of induction as when stimulated with HCV proteins. The only difference between the two stimulants (HCV proteins vs R848) was the order of magnitude; HCV proteins induced a larger cytokine production compared to R848. These results show that the TLR7 receptor in human hepatocytes previously thought to be rare in expression and dysfunctional, is fully functional and does elicit a response to ligand stimulation.

A.


p<0.0001
IFN-β pg/ml
2500
2000
1500
1000
500
0
0
10 Hrs
0
10 Hrs
R848 10uM hMDM
Medium

B.


p<0.006
IFN-α pg/ml
70
60
50
40
30
20
10
0
0
10 Hrs
0
10 Hrs
R848 10uM hMDM
Medium

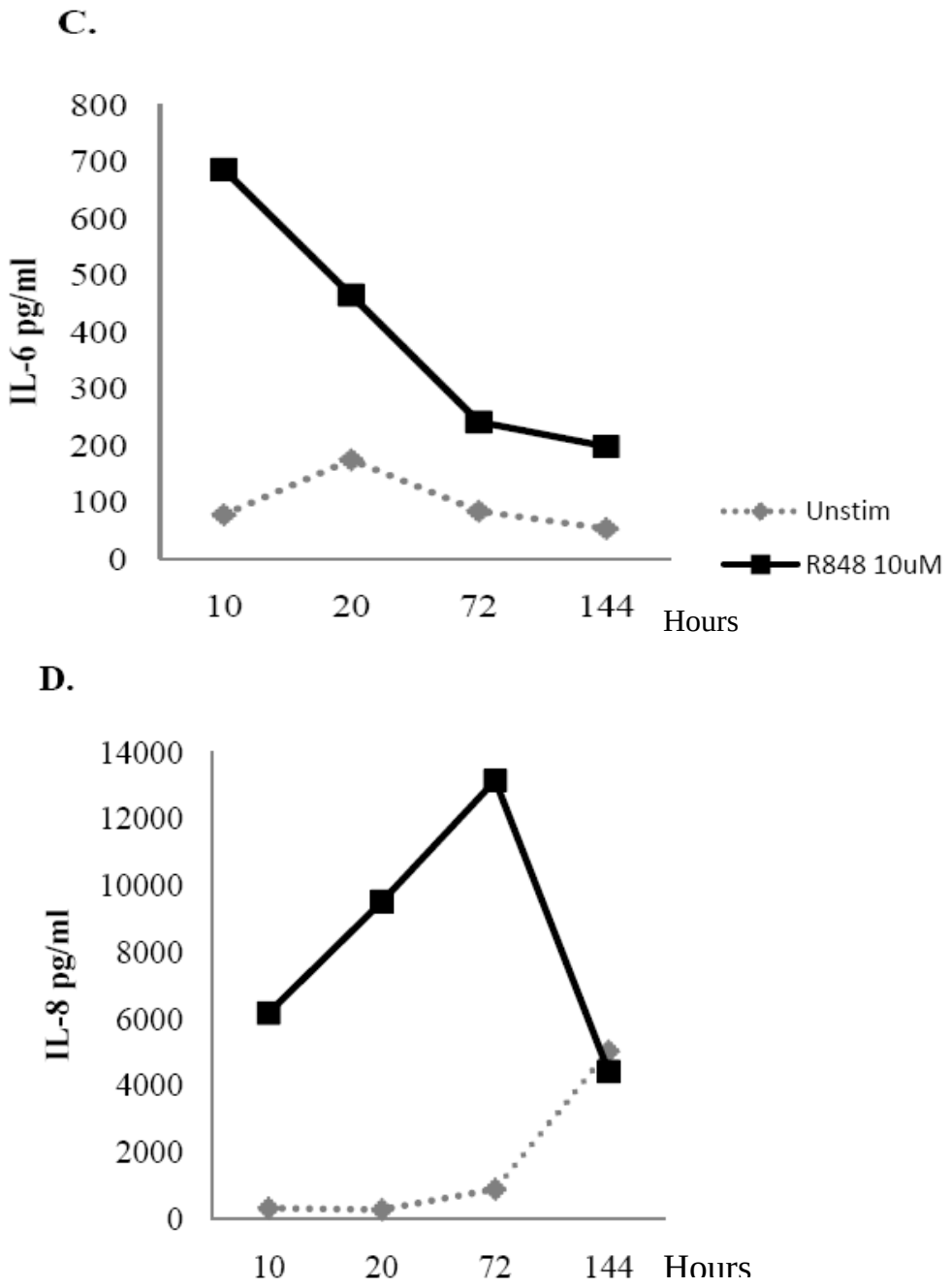


Figure 3.5: Cytokine response from TLR7/8 specific ligand in primary hepatocytes and macrophages. (A.) IFN-β or (B.) IFN-α levels were measured by ELISA after stimulation with R848, TLR7/8 specific ligand, for the indicated time periods from human monocytes derived macrophages, hMDMs. (C.) IL-6 and (D.) Il-8 was also measured by ELISA after stimulation with R848 from primary hepatocytes. Data is shown as an average ± SD of n=3 independent experiments or for primary hepatocytes n=1 experiment. Primary hepatocytes originated from one non-HCV infected individual performed in one experiment. P-values compared with the media control were calculated using the student's T-test.

Chapter Summary

Human hepatocytes are the primary host for HCV infection which can lead to liver failure. Although many advances have been made in HCV research, there are still a number of unknown areas of interest. The diverse innate immune reaction occurring in HCV infected patients has prompted many to identify factors responsible for chronic HCV. Though there are many immune cell types in the liver responsible for yielding inflammatory and IFN cytokines, the majority of cells are hepatocytes, host to HCV. We have studied the identity and function of specific TLRs in hepatocytes which until now was unknown. Others have previously shown that HCV proteins stimulated peripheral blood mononuclear cells in healthy human controls, for the first time we have shown that these proteins can also stimulate hepatocytes.

TLR RNA was expressed in hepatoma cell lines, Huh7 and Huh7.5 with absence of expression in TLR8 and TLR10 in Huh7.5 cells compared to both Huh7 and PBMC controls. Primary human hepatocytes also showed TLR expression without absence of TLR8 or TLR10. Primary hepatocytes additionally showed expression of various innate immune factors except for RIG-I. TLR2 was previously reported to become activated by HCV proteins, yet this activation was not identified in hepatocytes, the main cell type in the liver. TLR7 was identified as a viral recognition receptor of single stranded RNA viruses and agonists were involved in reduction of HCV replication. Therefore, we investigated receptor surface and intracellular levels of the TLR2 and its heterodimers, and TLR7. We found measurable amounts of TLR2, TLR1, and TLR7 within the cell.

Next, we tested the function of these cells in hepatoma cell lines and primary hepatocytes. We found NF-κB activation in hepatoma cell lines upon TLR2/1 or TLR2/6 specific ligand stimulation, yet did not find measurable amounts using R848. We were able to measure IL-8, IL-6, and Type 1 IFN cytokine production from activated primary hepatocytes and found that the TLR7 and TLR4 receptor is functional. We also found HCV proteins, core and NS3, activated both hepatoma cell lines and hepatocytes, producing a large amount of inflammatory cytokines. These results show that hepatocytes do respond to TLR ligands and HCV proteins suggesting that they are not only a host to HCV but could contribute demonstrably to innate immune functions against HCV.

CHAPTER IV

Toll-like receptors 1 and 6 are both involved in TLR2-mediated macrophage activation by Hepatitis C Virus core and NS3 proteins

Previous work showed that HCV proteins, core and NS3, stimulated human monocytes, TLR2 expressing HEK cells, and mouse peritoneal macrophages in a TLR2-specific manner and induced production of TNF-α and IL-10 (79). HCV infection targets the liver where Kupffer cells, resident liver macrophages, are the major cell type involved in innate immune pathogen surveillance and pro-inflammatory cytokine production. Similar to human monocytes and monocyte-derived macrophages (hMDMs), Kupffer cells express TLR2, TLR1, TLR6, TLR4, and TLR9 (207, 244). Purified human or mouse Kupffer cells are very difficult to acquire, therefore to investigate the effects of HCV proteins on these cells we used hMDMs and mouse peritoneal macrophages which are within the same family as Kupffer cells.

HCV proteins cause dose dependent inflammatory cytokine production in hMDMs

We found that normal human monocytes and hMDMs express TLR2, TLR1 and TLR6 (Figure 4.1A). To determine the effect of HCV proteins on hMDMs, we stimulated hMDMs with recombinant HCV core or NS3 protein. We found that as little as 10 ng/ml of HCV protein induced a dose dependent induction of TNF-α in hMDMs (Figure 4.1B). Despite the high concentrations of HCV proteins used in Figure 4.1 the lowest concentrations of protein we used are similar to concentrations found in HCV patient serum (209, 245). It is difficult to

confirm the highest levels of HCV proteins circulating or concentrating in the human body as most acute infections are not detected or misdiagnosed by physicians. Human MDMs contribute to not only the generation of inflammatory responses triggered by pathogen-derived ligands but also to the generation of anti-inflammatory cytokines, such as IL-10, which can inhibit pathogen-specific T cell activation (246). It is unknown whether TLR2 alone, and/or in combination with TLR1 or TLR6, participate in HCV-specific anti-inflammatory production during stimulation with HCV core and NS3 protein. Here we established that hMDMs produced IL-10 when stimulated with HCV core or NS3 proteins in a dose dependent manner (Figure 4.1C). Importantly the levels of IL-10 induced with as little as 76 ng/ml of HCV proteins were closely comparable with cytokine and HCV core protein levels reported in the serum of HCV-infected patients (209, 245). Beta-galactosidase as stated in the methods was fused with HCV proteins as recombinant proteins and was run as a negative control in Figure 9 to assure that β-gal fusion to HCV core or HCV NS3 proteins was not contributing or interfering with cellular cytokine production. There was negligible contribution by β-gal at a concentration of 5μg/ml to cytokine production in primary macrophages therefore we did not run this control in the subsequent figures. Since there was a high induction of cytokine from 5ug/ml of HCV protein stimulation and there was no induction of cytokine from β-gal at the same concentration, we presumed that the activation of macrophages was indeed due to HCV protein stimulation as opposed to a general response of adding a high concentration of protein onto the cells.

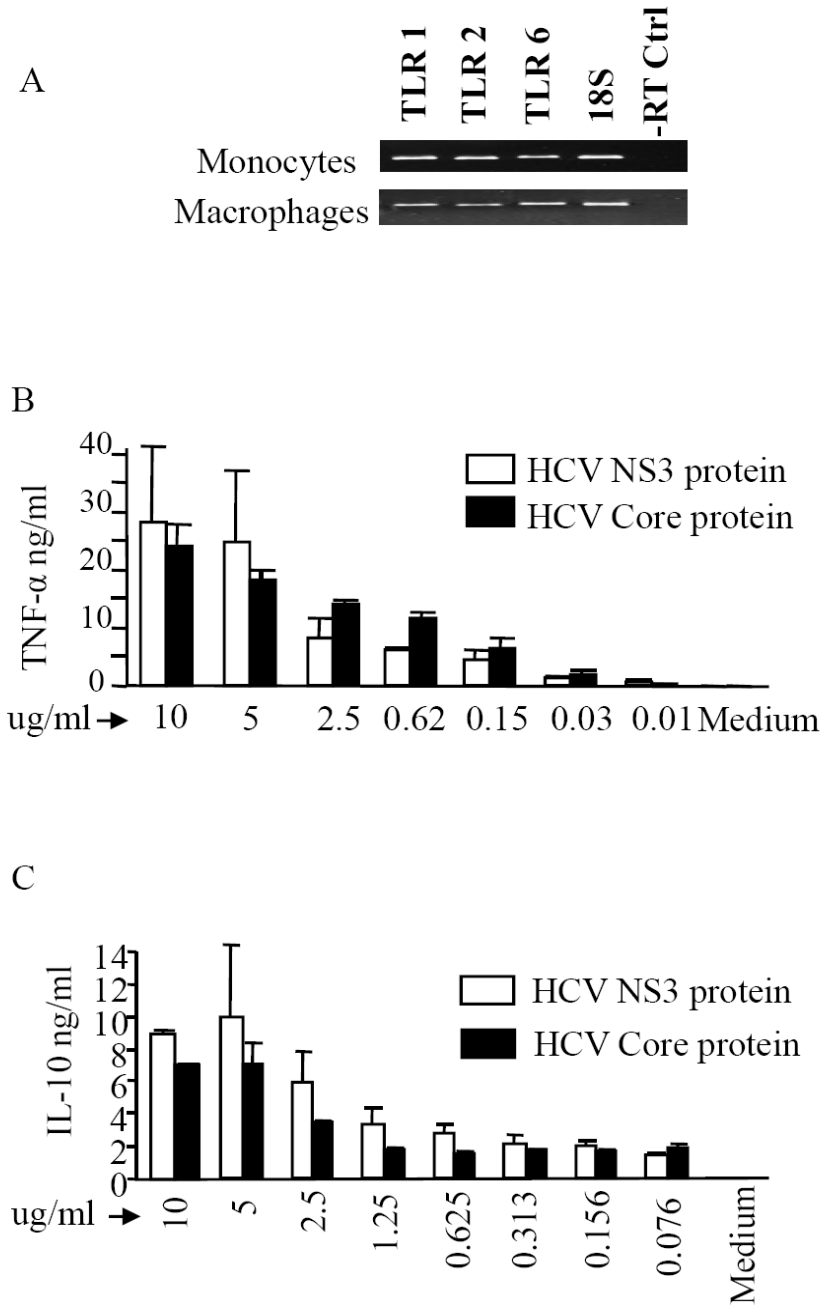


Figure 4.1: HCV core and NS3 proteins induce TNF-α and IL-10 production in a dose dependent manner in human monocytes and macrophages. (A) Equal amounts of mRNA extracted from normal human monocytes (top gel) and macrophages (bottom gel) were transcribed into cDNA and amplified using TLR1-, TLR2-, TLR6- and 18S-specific primers in reverse transcription (RT)-PCR. No RT control for contaminating products is also shown for each cell type. One individual out of n=3 independent experiments from three subjects with similar results is shown. Normal human monocyte-derived macrophages were stimulated with recombinant HCV core (open bars) or NS3 (black bars) β-gal fusion proteins for ten hours. Culture supernatants were analyzed for TNF-α (B) and IL-10 (C) using specific ELISA. Data are shown as an average±SD ng/ml from three or more experiments.

Uncontaminated HCV proteins specifically activate TLR2 in knockout mice

Before performing siRNA knockdown, we determined that the HCV core and NS3 recombinant proteins were TLR2 specific and were void of endotoxin. Murine peritoneal macrophages were extracted from wild-type C57BL/6 (Figure 4.2), TLR2-/- (Figure 4.2B), TLR4 mutant (Figure 4.2C) and TLR4-/- mice (Figure 4.2D) and stimulated with TLR2 and TLR4 ligands, HCV core and NS3 proteins. IL-6 production from peritoneal macrophages showed that stimulation with HCV core and NS3 proteins rendered no response in the TLR2-/- mice as opposed to the wild-type, TLR4-/-, or TLR4 mutant mice. The purified LPS was used as a TLR4 specific ligand and the PGN was used as a TLR2 specific ligand. Recombinant proteins HCV core and NS3 were fused with β-galactosidase. β-galactosidase is commonly used for molecular biology techniques to screen for recombinant clones. To test whether β-gal causes an immune reaction in macrophages, we added β-gal provided by Biodesign to macrophages from either wild-type, TLR2-/-, TLR4 mutant, or TLR4-/- mice. There was a negligible cytokine response when stimulated with β-gal in any set of macrophages tested.

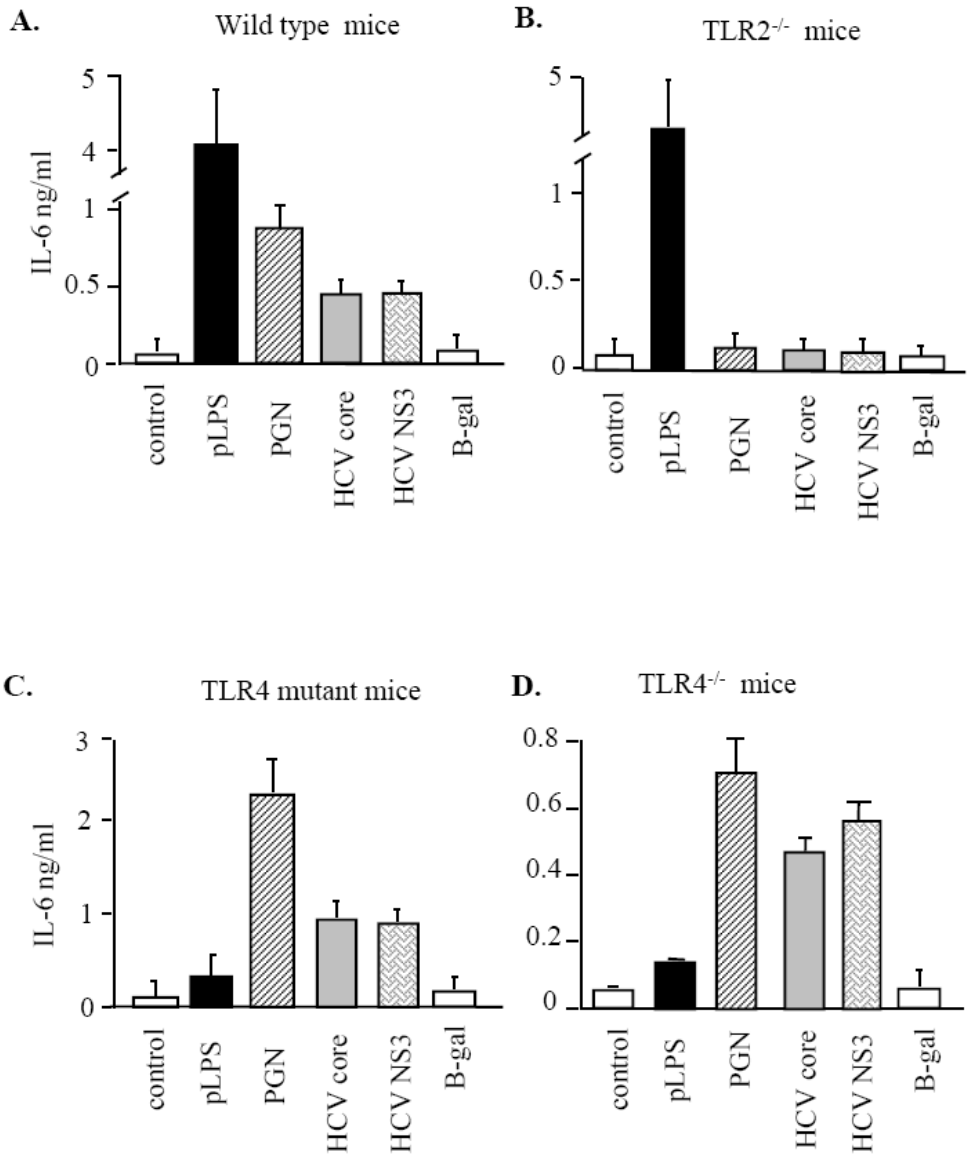


Figure 4.2A-D: Ligand specificity to TLR2 by HCV core and NS3 proteins. Peritoneal Macrophages from (A) wild-type C57BL/6, (B) TLR2 -/-, (C) TLR4 mutant, and (D) TLR4 -/- were stimulated with plain media (no stimulant) as the control, pLPS (100 ng/ml), PGN (10 µg/ml), HCV core and NS3 proteins (5 µg/ml), and Beta-galactosidase (5 µg/ml). β-gal was used as a control for non-specific cytokine production from the fusion proteins HCV core and NS3. After twelve hours, the culture supernatants were analyzed for IL-6 content using ELISA. Data are shown as an average±SD ng/ml from n=3 independent experiments.

TLR2 Specificity in Human Cells

In human cells over-expressing TLR2 or TLR4 (Figure 4.2E), stimulation with TLR2 or TLR4 ligands, HCV core or NS3 proteins showed the same pattern of TLR2 specificity. In HEK/TLR4 cells, PGN, HCV core and NS3 proteins did not elicit NF-kB activation as opposed to HEK/TLR2 cells, which showed significant activation of NF-kB. To further investigate the specificity to TLR2 we utilized TLR2 blocking antibodies in human monocytes stimulated with the same ligands and proteins as in Figure 4.2E, and measured TNF-α production (Figure 4.2F). The blocking antibodies resulted in a significant reduction of cytokine production compared to the isotype antibody control but did not completely inhibit cytokine output. The remaining cytokine production was most likely due to the incomplete blocking by the antibody and not to TLR2 contaminants in the HCV core and NS3 proteins as the anti-TLR2 antibody also failed to achieve full inhibition of the classical TLR2 ligands (Figure 4.2F). IL-1β was used as a positive cytokine stimulation control in Figure 4.2E to confirm functionality of the cells.

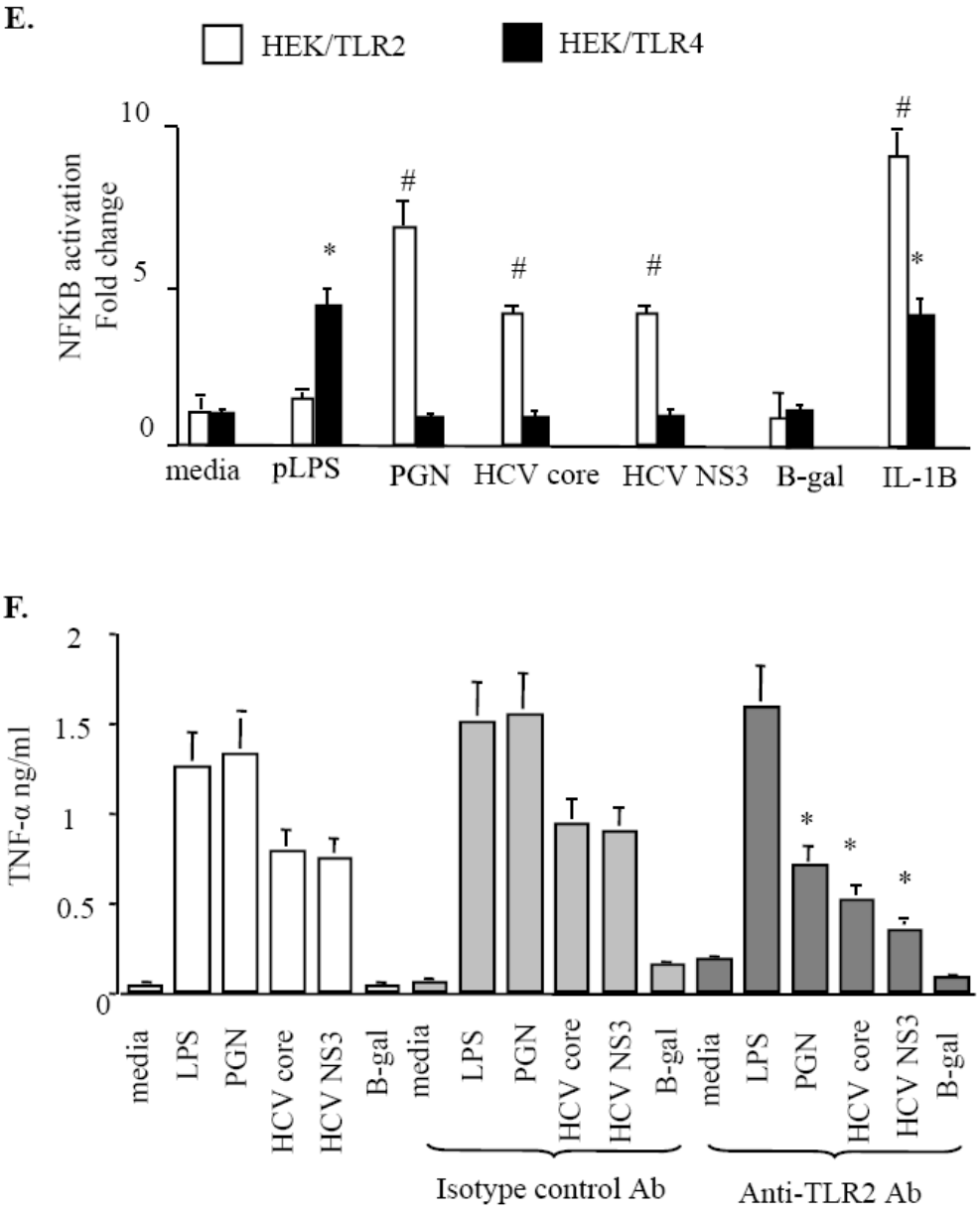


Figure 4.2E-F: Ligand specificity to TLR2 by HCV core and NS3 proteins. (E) HEK293/TLR2 or HEK293/TLR4 cells were stimulated with pLPS (100 ng/ml), PGN (10 µg/ml), HCV core and NS3 proteins (5 µg/ml), Beta-galactosidase (5 µg/ml) and IL-1β (20 µg/ml). After twelve hours, the cells were lysed and luciferase NF-kB levels were measured. Data are shown as an average±SD ng/ml from n=3 independent experiments. Asterisks (TLR4) and pound (TLR2) symbols represent p-values < 0.05 compared to the controls calculated by student's t-test. (F) Mouse peritoneal macrophages were stimulated with LPS, PGN, HCV core and NS3 proteins, and Beta-galactosidase in the same concentrations as in (E). Culture supernatants were analyzed for TNF-α by ELISA. Blocking antibodies, isotype and TLR2, were added 2 hours before the 16-hour stimulation. Asterisks indicate p-values< 0.05 compared to the controls calculated by student's t-test.

Reducing TLR Expression by siRNA

Based on these findings, we hypothesized that the TLR2 co-receptors, TLR1 and/or TLR6, are involved in TLR2-mediated recognition and activation by HCV core and NS3 proteins. HEK 293 cells have endogenous levels of TLR1 and TLR6 shown in Figure 4.3. We specifically knocked down TLR2, TLR1 and TLR6 receptor-specific mRNA in HEK/TLR2 cells and primary human macrophages using siRNA technology to evaluate which TLR2 co-receptor participates in TLR2-mediated cell activation by HCV core and NS3 proteins. First, to determine the efficiency in the knockdown of the siRNAs, we examined the expression of target mRNA over a three day time course after siRNA transfection. For each target TLR, there were two separate sequence siRNAs, named sequence (seq)-A and seq-B. We found a significant reduction (>50%) of mRNA in at least one or both of the two separate siRNA sequences for TLR2, TLR1 and TLR6 on day 2 after transfection (Figure 4.3A). To determine whether mRNA reduction correlated with loss of protein expression, we examined surface protein levels by FACS (Figure 4.3B) and cellular protein levels by Western Blot analysis (Figure 4.3C). By day 2, TLR2 surface expression levels dramatically decreased with TLR2 seq-a siRNA but not in control siRNA transfected HEK/TLR2 cells (Figure 4.3B). By day 3, there was a >75% decrease in TLR6 protein expression using TLR6 seq-b siRNA knockdown in HEK/TLR2 cells (Figure 4.3C). Due to the lack of efficient Western Blot- or FACS-suitable antibody, TLR1 protein levels could not be determined. These results suggest that the designed siRNAs were specific and functional for knockdown, portrayed by

the significant decrease in mRNA and/or protein expression levels of TLR 2, TLR1 and TLR6 in HEK/TLR2 cells.

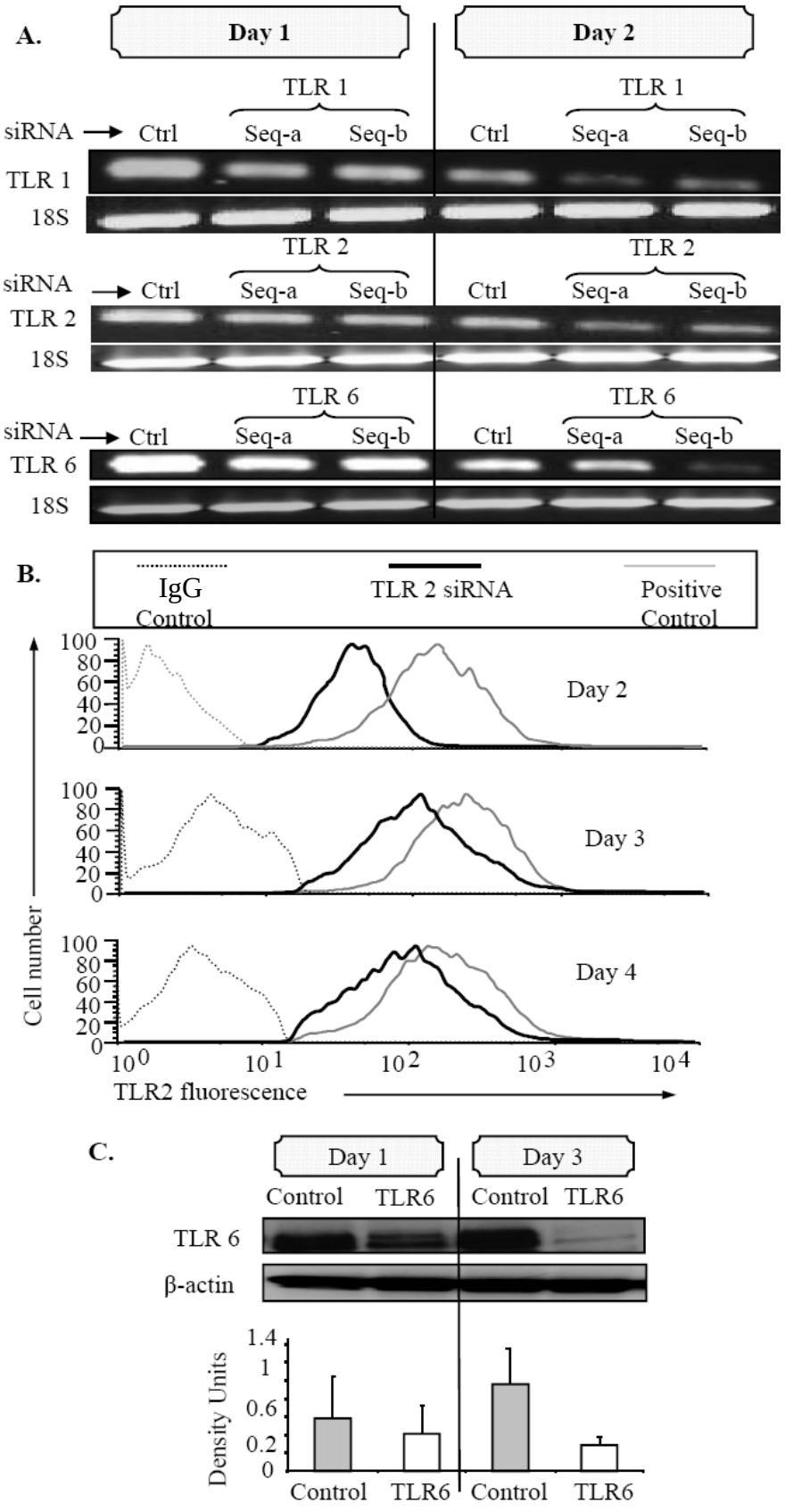

A.
Day 1
Day 2
TLR 1
siRNA
Ctrl
Seq-a
Seq-b
TLR 1
18S
TLR 2
TLR 2
TLR 6
TLR 6
B.
IgG Control
TLR 2 siRNA
Positive Control
Day 2
Day 3
Day 4
Cell number
TLR2 fluorescence
C.
Day 1
Day 3
Control
TLR6
TLR 6
β-actin
Density Units

Figure 4.3: TLR-specific siRNAs knockdown TLR mRNA and protein expression in HEK293/TLR2 cells. (A) Two different RNAi sequences, Seq-a, Seq-b, were constructed to target TLR2, TLR1 and TLR6 and transfected in HEK/TLR2 cells, as described in Methods. The control (Ctrl) represents the baseline expression of the corresponding TLR in a negative control siRNA-transfected cells. A negative control siRNA refers to the non-specific scrambled siRNA that does not target any gene in the cell for knockdown. At 24 hrs (day 1) and 48 hrs (day 2) after siRNA transfection, the mRNA from HEK/TLR2 cells was extracted and equal amounts of mRNA were transcribed into cDNA. The levels of TLR2 (top gel), TLR1 (middle gel), and TLR6 (bottom gel) were determined using specific primers and PCR. 18S was included as an internal control for all experiments. PCR products were separated on a 1% agarose gel, stained with ethidium bromide and detected upon exposure to UV light. Shown are representative gels from one individual donor out of n=3 independent experiments with similar results. (B) HEK/TLR2 cells were transfected with siRNA specific to TLR2 seq-a. The cells were stained with secondary antibody alone (IgG control) or anti-TLR2 antibody followed by secondary APC-labeled antibody. The fluorescence was analyzed by flow cytometry. The TLR2 expression in cells transfected with the negative control siRNA, as described in Methods, was considered as positive control for full TLR2 expression. TLR2 expression was determined over a five-day time course; shown are representative fluorescence histograms from one experiment analyzed on day 2 (top), day 3 (middle panel) and day 4 (bottom pane) out of n=4 independent experiments with similar results. (C) HEK/TLR2 cells were transfected with siRNA specific to TLR6 seq-b. Control represents cells transfected with the negative control siRNA, as described in Methods. On day 1 and day 3 after siRNA transfection, the cells were lysed, equal amounts of proteins from each experimental group were separated in SDS-PAGE, transferred to a membrane and probed with specific anti-TLR6 or anti-β-actin antibodies followed by secondary-HRP labeled antibodies. The expression of TLR6 (top blot) or β-actin (bottom blot) and the densitometric analysis of TLR6 expression adjusted to β-actin from one experiment out of n=3 independent experiments with error bars representing $\pm$ SD density units with similar results is shown.

Specific siRNA knockdown significantly reduces function in HEK/TLR2 cells

To evaluate if the receptor knockdown correlated with reduced receptor function, we stimulated siRNA-transfected cells with TLR specific ligands, including the TLR2 ligand, PGN, TLR2/1 ligand, Pam3CSK4, and TLR2/6 ligand, Pam2CSK4, and measured cell activation by means of cytokine production. TLR-specific siRNAs knocked down IL-8 cytokine production in HEK/TLR2 cells when compared to control siRNAs (Figure 4.4). TLR2 RNAi transfection resulted in a significant loss of function indicated by a decrease in IL-8 production (50-75%) compared to control siRNA after stimulation with all three TLR2 ligands: PGN (TLR2/6), Pam2CSK4 (TLR2/6), and Pam3CSK4 (TLR2/1) (Figure 4.4A). TLR1 silencing resulted in significant IL-8 reduction (~57%) after stimulation with TLR2/1 ligand, Pam3CSK4, and not with the TLR2/6 ligand, Pam2CSK4, indicating specific TLR1 silencing (Figure 4.4B). TLR6 silencing also showed specificity in functional knockdown as significant reduction occurred with Pam2CSK4 (TLR2/6) and not with Pam3CSK4 (TLR2/1) ligand (Figure 4.4C). These results implied that knockdown of mRNA transcripts and protein expression by RNA interference method correlates with a significant reduction in TLR receptor function and inhibition of ligand-stimulated cytokine production. We used IL-1β as a stimulation control, which was unaffected by transfection of any of the siRNAs (Figure 4.4D) since IL-1β induces cell activation via IL-1β receptor and not via TLRs. We also tried a variety of ways to increase siRNA knockdown, from increasing siRNA concentration to increasing and varying transfection reagents (data not shown). No other techniques yielded a higher

knockdown and in some cases such as increasing transfection reagent or high siRNA concentrations, caused cell death. We also attempted to produce a double knockdown (TLR1/TLR6) siRNA to confirm that only these co-receptors were involved in TLR2 activation by HCV proteins. Double knockdowns also yielded cell death or little knockdown of one but not the other.

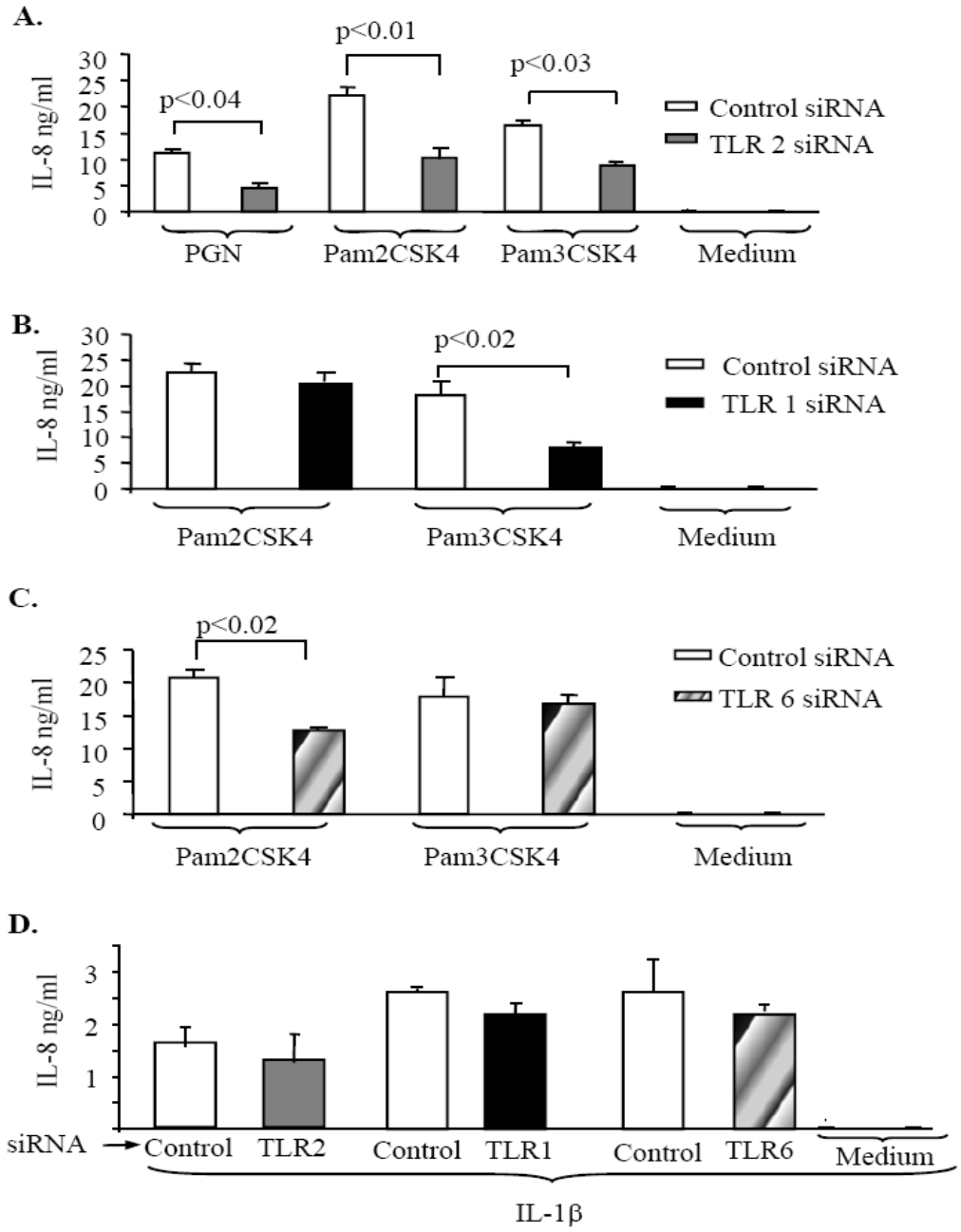


Figure 4.4: Specific siRNAs for TLR2, TLR1 and TLR6 affect functional outcome of these receptors in HEK/TLR2 cells. HEK/TLR2 cells were transfected with siRNAs specific for TLR2 (A), TLR1 (B) and TLR6 (C) receptors. Control represents cells transfected with a negative control siRNA, as described in Methods. On day 3 after siRNA transfection the cells were stimulated for ten hours with PGN (TLR2/TLR6 ligand) at 5 µg/ml, Pam_3CSK_4 (TLR2/TLR1 ligand) at 50 ng/ml, Pam_2CSK_4 (TLR2/TLR6 ligand) at 5 µg/ml or recombinant IL-1β at 50 ng/ml (D) and the IL-8 production in culture supernatants was ascertained using a specific ELISA. Data are shown as an average±SD ng/ml from n=3 independent experiments. P-values were compared to the controls and calculated by student's t-test.

Identifying the TLR2 Co-receptor Participating in HCV Protein Recognition

After we confirmed the specificity of siRNAs to TLR2, TLR1 and TLR6, we intended to establish which TLR2 co-receptor participates in recognition of HCV proteins, core and NS3. TLR2 ligand controls were tested on siRNA knockdowns along with the HCV proteins (data not shown). In HEK/TLR2 cells, knockdown of all three targeted siRNAs, TLR2 (Figure 4.5A), TLR1 (Figure 4.5B) and TLR6 (Figure 4.5C) individually, resulted in significant inhibition of HCV core- ($p<.009$, $p<.02$, $p<.005$, respectively) and NS3- ($p<.003$, $p<.03$ $p<.002$, respectively) induced IL-8 production compared to a control siRNA (Figure 4.5A, B, C). Figure 12C tested a higher concentration of HCV proteins to confirm that knockdown was not affected by concentration of stimulant. Both TLR2 and TLR1 were also tested at 10 μg/ml with HCV proteins but yielded the same results as in Figure 4.5 (data not shown). Knockdown of at least 50% was sufficient enough to cause a loss of function of the receptor when stimulated with HCV proteins. The absence of TLR2 or either of its co-receptors, TLR1 or TLR6, greatly diminished cellular activation by HCV NS3 and core proteins. These results suggest that both TLR1 and TLR6 participate in TLR2 activation by HCV NS3 and core.

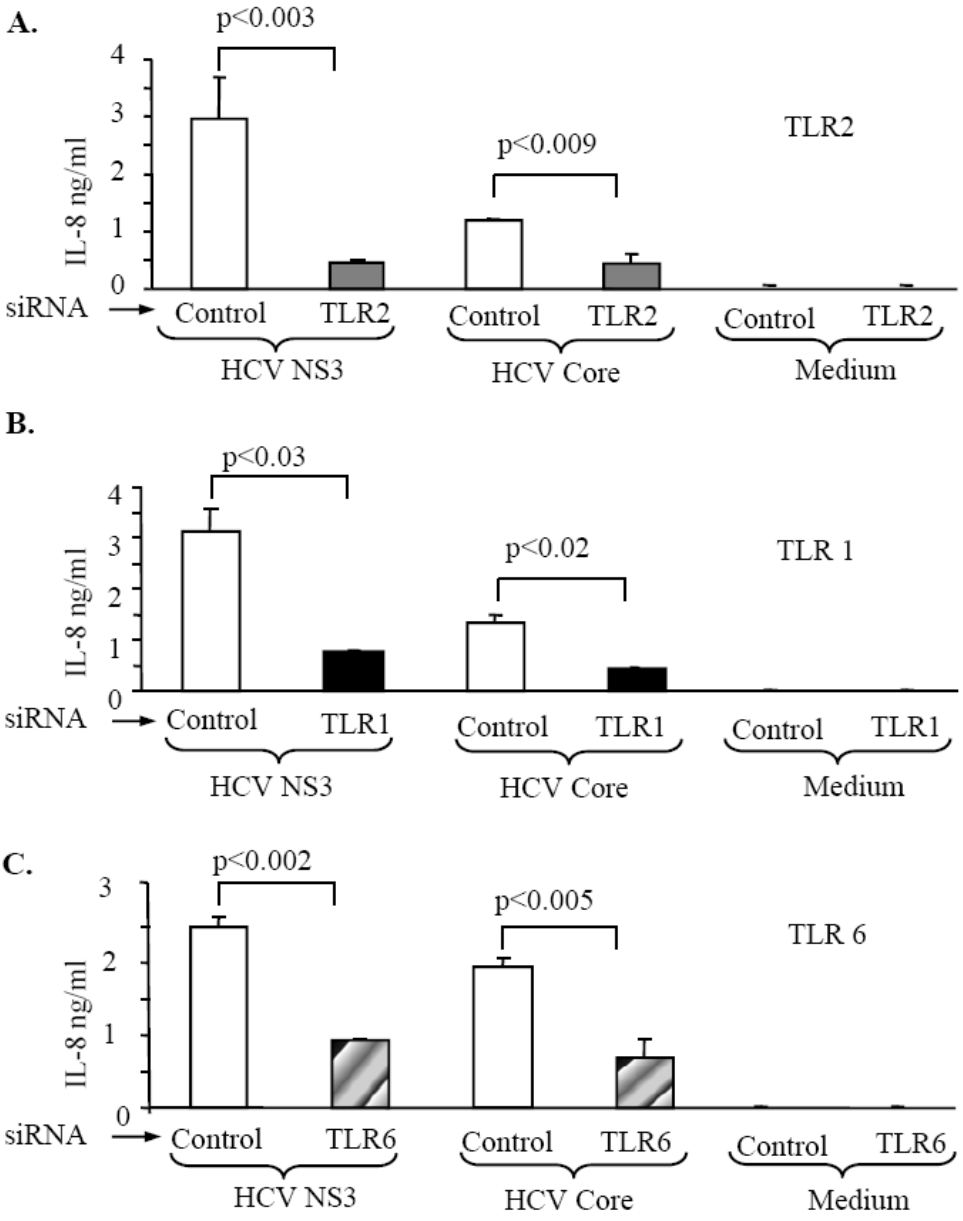


Figure 4.5: TLR2, TLR1 and TLR6 participate in recognition of HCV core and NS3 proteins in HEK/TLR2 cells. HEK/TLR2 cells were transfected with siRNA specific for TLR2 (top panel), TLR1 (middle panel) and TLR6 (bottom panel). Control represents cells transfected with a negative control siRNA, as described in Methods. Three days after siRNA transfection, the cells were stimulated for ten hours with HCV core and NS3 proteins at 5 µg/ml for (A and B) 10µg/ml for (C) and the IL-8 production in culture supernatants was ascertained using IL-8 specific ELISA. Data are shown as an average±SD ng/ml from n=3 independent experiments. P-values were compared to the controls and calculated by student's t-test.

Both TLR2 co-receptors participate in HCV protein activation in hMDMs

To assess the involvement of TLR1 or TLR6 in HCV protein stimulation in macrophages, we stimulated TLR-specific siRNA-transfected hMDMs with HCV proteins. There was a significant reduction in TNF-α (Figure 4.6A) and IL-6 (Figure 4.6B) production in all three TLR-specific siRNA-transfected macrophages stimulated with Pam2SCK4 (TLR2/TLR6), Pam3CSK4 (TLR2/TLR1) or PGN (TLR2/TLR6), suggesting a loss of function. Upon sole transfection of siRNA (medium control) there were no cytokines detected (Figure 4.6A, B). Silencing of TLR2, TLR1 or TLR6 did not affect TLR4-induced (pLPS) IL-6 or TNF-α production in hMDMs, confirming the lack of cross reactivity. More important, both TLR1 and TLR6 silencing caused reduced levels of inflammatory cytokines, TNF-α (Figure 4.6A) and IL-6 (Figure 4.6B), when stimulated with HCV core or NS3 proteins. TLR2, TLR1 and TLR6 knockdowns in hMDM cells followed the same pattern as in the HEK/TLR2 cells (Figure 4.4), supporting the hypothesis that both TLR1 and TLR6 are involved in conjunction with TLR2, in cellular activation by HCV core and NS3. In Figure 4.6, p-values were insignificant where there were no symbols.

To evaluate whether the anti-inflammatory response from HCV protein stimulation in macrophages is dependent on TLR2, TLR1 or TLR6, we measured IL-10 levels after TLR-specific knockdown in hMDMs stimulated with HCV core or NS3 proteins. There was a significant attenuation in IL-10 induction in TLR2 siRNA-transfected hMDMs after stimulation with core or NS3 proteins, suggesting that TLR2 was important for IL-10 production (Figure 4.6C). TLR1

and TLR6 siRNA attenuated IL-10 induction by HCV core and NS3 proteins in macrophages, but only TLR1 knockdown resulted in significant inhibition of NS3-induced IL-10 (Figure 4.6C). These results suggest that TLR2 is involved in IL-10 induction by HCV core and NS3 stimulation.

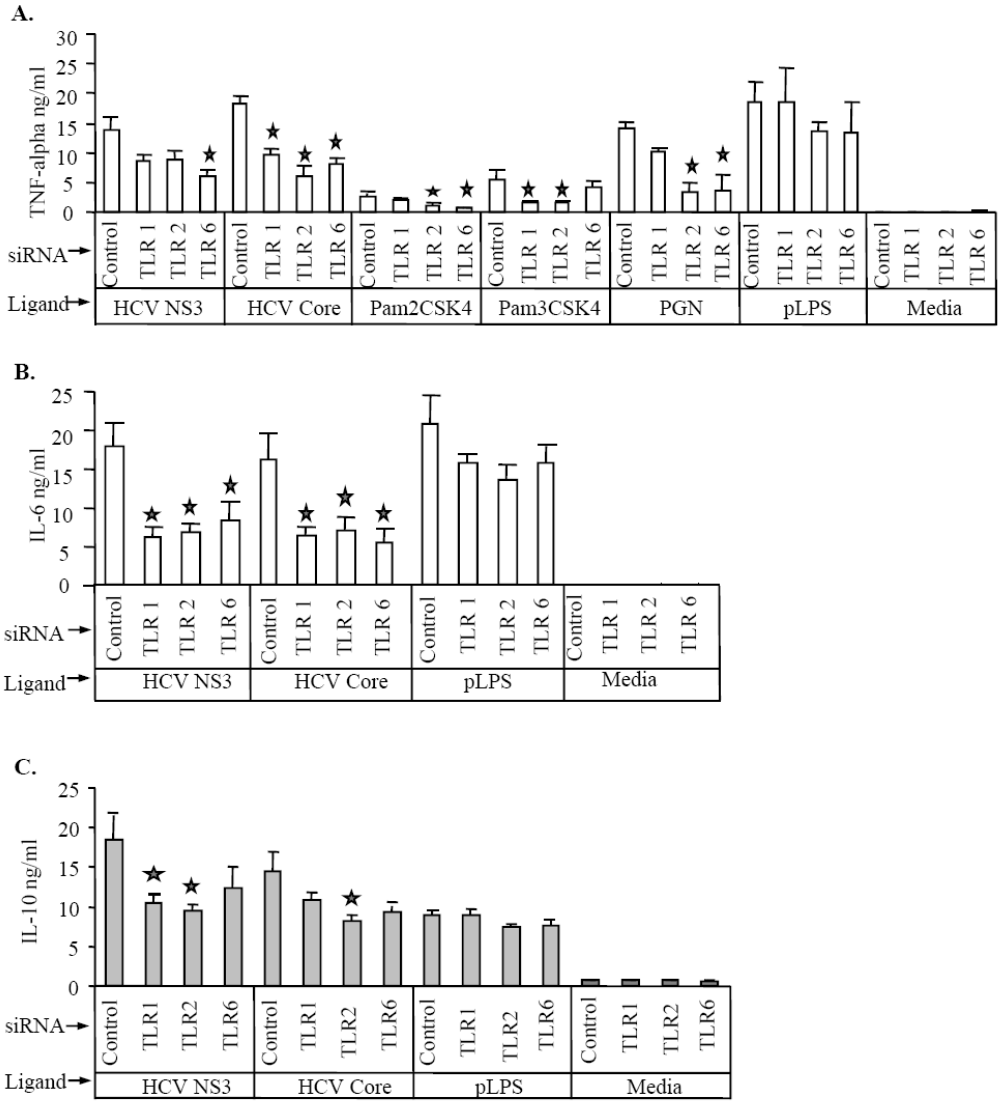


Figure 4.6: Targeting of TLR2, TLR1 and TLR6 with specific siRNA conditions reduced cytokine production in human monocyte-derived macrophages. Human monocyte-derived macrophages were transfected with siRNA specific for TLR2, TLR1, TLR6 or non-specific scrambled negative control (Control). Columns marked Media were unstimulated cells transfected with one of the four siRNAs. On day 3 after siRNA transfection, the cells were stimulated with Pam_3CSK_4 (TLR2/TLR1 ligand) at 50 ng/ml, Pam_2CSK_4 (TLR2/TLR6 ligand) at 5 µg/ml, PGN (TLR2/TLR6 ligand) at 10 µg/ml, phenol extraction-purified LPS (TLR4 ligand) at 100ng/ml or HCV core and HCV NS3 proteins at 5 µg/ml, as indicated. TNF-α (A), IL-6 (B) and IL-10 (C) were measured in culture supernatants using specific ELISA. Data are shown as an average±SD ng/ml from n=5 independent experiments. The stars indicated the $p<0.05$ calculated using student's T-test in siRNA-transfected samples compared to corresponding scrambled siRNA controls.

Investigating effects of HCV in complete TLR2, TLR1, or TLR6 knockout mice shows a significant TLR2/6 dependence for HCV activation

Our method of knockdown by siRNA transfection was neither complete nor stable and left residual cytokine production during stimulation. Thus, we employed peritoneal macrophages isolated from TLR-specific knockout mice as an alternate approach to determine the roles of TLR2, TLR1, or TLR6 receptors in HCV protein-induced stimulation. In accordance with previous findings (79), the absence of TLR2 prevented TNF-α induction by HCV core, NS3 or TLR2 ligand stimulation (Figure 4.7A). Macrophages from $TLR1^{-/-}$ mice showed a slight decrease yet not significant in TNF-α production when stimulated by recombinant HCV core or NS3 proteins (Figure 4.7B). Contrary to the $TLR1^{-/-}$, macrophages from $TLR6^{-/-}$ mice had a significantly attenuated TNF-α production upon HCV core or NS3 protein stimulation (Figure 4.7C), compared to wild type animals. These results imply that in mouse peritoneal macrophages TLR2 and TLR6, but possibly not TLR1, are substantially involved in HCV core or NS3 protein-induced activation.

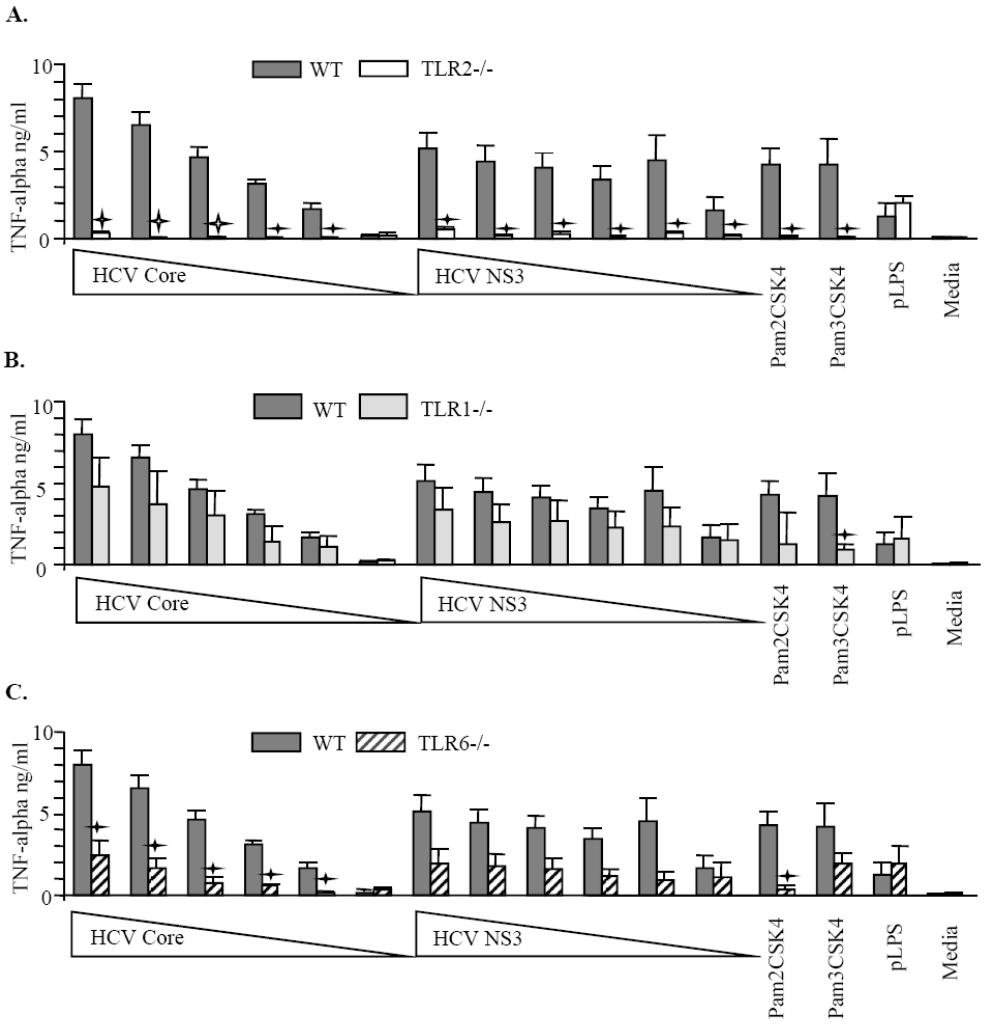


Figure 4.7: HCV core and NS3 protein employ TLR2 and TLR6 to activate mouse peritoneal macrophages. The peritoneal macrophages of TLR2 (A), TLR1 (B), TLR6 (C) knockout mice or wild-type mice were stimulated with Pam_3CSK_4 (Pm3, TLR2/TLR1 ligand) at 200 ng/ml, Pam_2CSK_4 (Pm2, TLR2/TLR6 ligand) at 10 µg/ml, or phenol extraction-purified LPS (TLR4 ligand) at 200 ng/ml. HCV core and HCV NS3 proteins were employed at serial dilution concentrations between 10-0.1 µg/ml, as indicated. After ten hours, the culture supernatants were analyzed for TNF-α content using ELISA. Data are shown as an average±SD ng/ml from n=3 independent experiments. The stars indicated the $p<0.05$ calculated using student's T-test in cells with siRNA-transfected samples compared to corresponding stimulation in wild-type cells.

Chapter Summary

TLR2 is a diverse innate immune receptor involved in HCV protein recognition and activation. Effects of innate immune activation by HCV proteins, core and NS3 identified the specific involvement of TLR2 in mononuclear cells. TLR2 activation occurs by heterodimerization with either TLR1 or TLR6 (202). We wanted to further identify which heterodimer was responsible for mononuclear cell activation by HCV proteins. We established comparable expression of TLR 1, TLR2, and TLR6 in both monocytes and macrophages and showed that both HCV core and NS3 stimulate human monocytes derived macrophage production of TNF-α or IL-10 in a dose dependent manner. We also confirmed HCV protein specificity to TLR2 using TLR knockout mice and HCV protein purity, devoid of contaminants that might stimulate TLR2.

To determine which TLR2 heterodimer was responsible cellular activation from HCV proteins, we utilized siRNA knockdown technology in HEK/TLR2 cells and primary human macrophages. TLR2, TLR1 and TLR6 knockdowns were observed from both RNA and protein levels and a significant yet not complete reduction of function was perceived in HEK/TLR2 cells when stimulated with various TLR2 ligands. Knockdowns were specific to the TLR gene and did not affect functionality of other TLR2 heterodimers or the IL-1β receptor. Administration of HCV proteins showed participation of both TLR2/1 and TLR2/6 heterodimers as there was a significant loss of function in all three separate knockdowns upon stimulation.

RNAi knockdown experiments in primary macrophages showed a TLR1, TLR2, and TLR6 requirement for complete production of IL-6 when stimulated with HCV proteins. Yet for production of either TNF-α or IL-10 there was variable use of TLR2 and its heterodimers in primary macrophages. Since siRNA technology was not a complete knockout of TLRs, we wanted to confirm our findings in TLR specific knockout mice. TLR6 showed significant but not full reduction of function with both HCV proteins compared to TLR1 which was not significant with either protein. Therefore, in mice, TLR2/6 seems to be required for full HCV protein-mediated cellular activation.

CHAPTER V

Impaired expression and function of Toll-like receptor 7 from Hepatitis C Virus infection in human hepatoma cells

Expression of Toll-like receptors is not restricted to immune cells and recent studies suggested that human hepatocytes express mRNA for all TLRs (247). TLR7 agonist, Isatoribine, reduced HCV infection in patients after one week of treatment. Another group generated a more potent specific TLR7 agonist which reduced HCV replication and protein in HCV replicating cell lines. Based on these previous reports connecting TLR7 with the reduction of HCV infection (197, 198), we postulated that TLR7 may play a role in anti-HCV immunity. Specifically, we analyzed whether HCV interferes with TLR7 expression and/or functions in a disruptive manner.

Active HCV replication in HCV cell lines

To address our hypothesis, we used Huh7 and Huh7.5 hepatoma control cell lines and HCV-replicating stable cell lines FL, containing the full length HCV genome transfected into Huh7.5, BB7, containing the subgenomic HCV genome transfected into Huh7, and JFH-1, containing the infectious full length HCV genome transfected into Huh7.5 (Figure 5.1A). HCV FL and BB7 have an antibiotic selectable marker (Neomycin) before the core gene for FL and before EMCV for BB7. JFH-1 does not have a selectable marker as it is the only infectious HCV genome readily electroporated into cells and contains only the HCV RNA sequence. The genome for JFH-1 was not contained within a plasmid that would contain the Neomycin gene. HCV NS5A protein expression was

confirmed by Western analysis for all HCV-expressing cell lines (Figure 5.1B) and by immunofluorescence detection of HCV core protein, expressed in FL and JFH-1 but not in BB7 (Figure 5.1C).

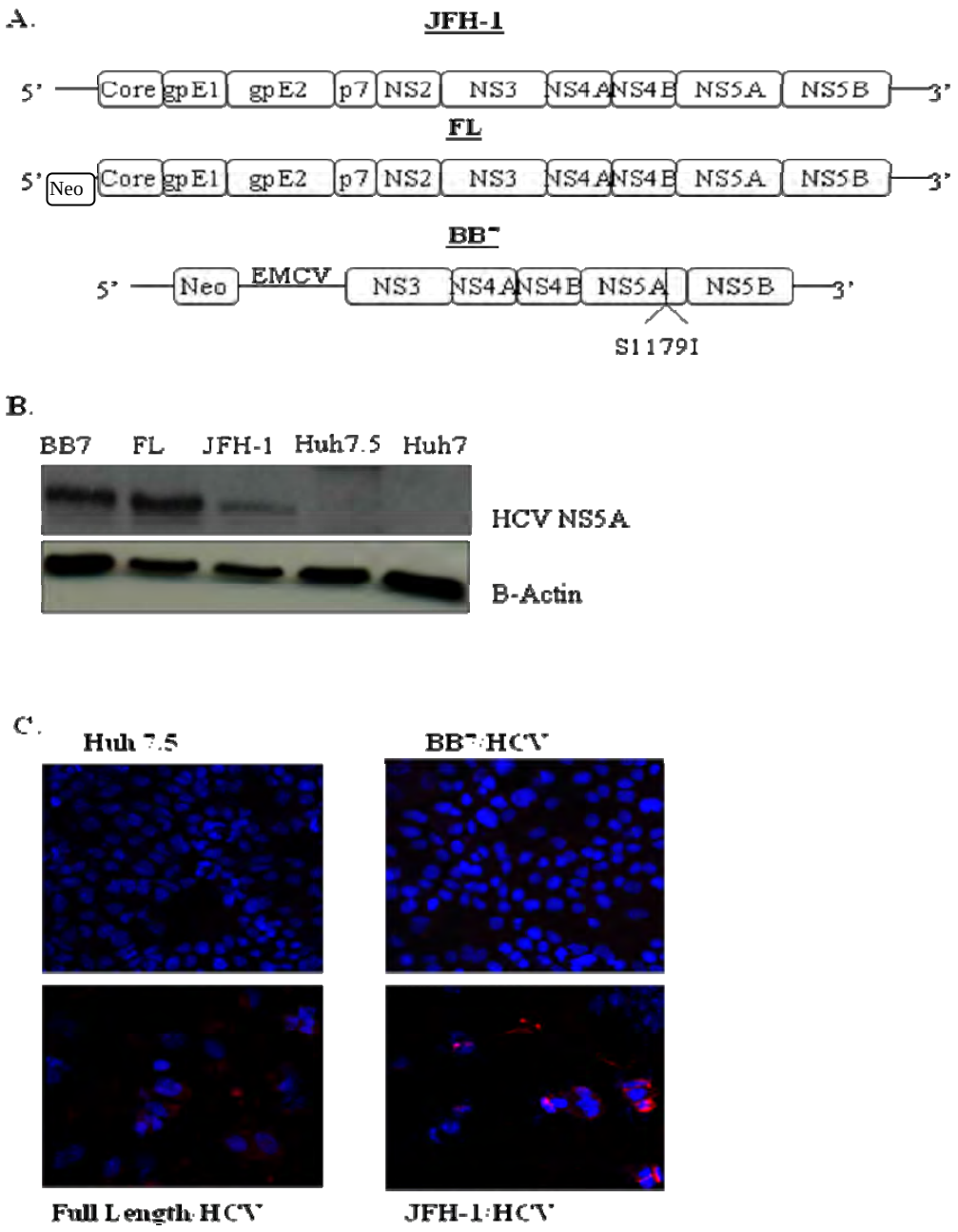


Figure 5.1: Hepatoma cells transfected with various HCV genomes show actively replicating HCV. (A) Protein map encoded by HCV genome in HCV replicating cell lines JFH-1, full length HCV genotype 2a, FL, full length HCV genotype 1a, and BB7, subgenomic HCV genotype 1b. Neo refers to neomycin selectable marker. (B) HCV replicating cells and hepatoma control whole cell extracts (5 µg) immunoblotted against antibodies for HCV NS5A or B-Actin. B-Actin was used as a loading control. Data shown is one representative experiment of n=3 independent experiments with similar results. (C) Immunofluorescence of fixed hepatoma and HCV replicating cells on slide. Nucleus stained in blue (DAPI) HCV core protein stained in red. Data shown is one representative experiment of n=4 independent experiments with similar results.

Low TLR7 expression measured in HCV replicating cells.

We identified detectable TLR7 RNA (Figure 5.2A) and protein levels (Figure 5.2B) in control Huh7 and Huh7.5 cells. There was a significant decrease of TLR7 RNA (Figure 5.2A) and protein (Figure 5.2B) in all HCV cell lines. The TLR7 high population in the Huh7 cells could be artifact staining as this population was not consistent with other staining of TLR7 in Huh7 cells. It most likely results from improper wash techniques after temporary permeabilization of the cells. These data suggest that TLR7 levels are impaired in the presence of HCV in vitro. To determine if low levels of TLR7 correlate with the presence of HCV infection in patients, we analyzed the levels of TLR7 RNA in the HCV infected and control livers (Figure 5.2C). Liver tissue samples were from HCV chronically infected individuals with high viral titers measured by PCR (data not shown). Expression of TLR7 RNA was significantly reduced in livers of patients with chronic HCV infection compared to healthy, HCV-naive controls. These results suggest that the presence of HCV correlated with reduced levels of TLR7.

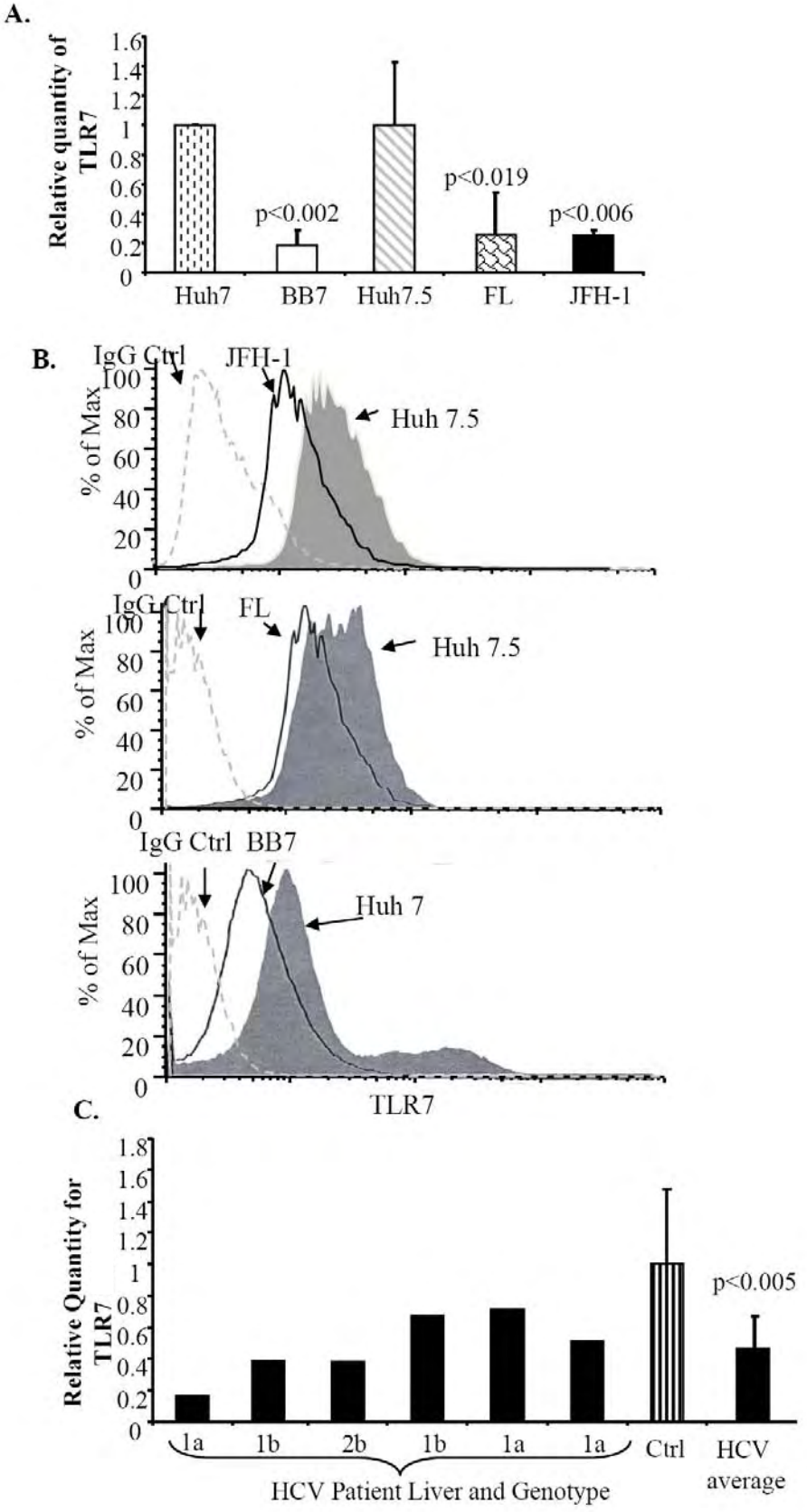

A.
Relative quantity of TLR7
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
p<0.002
p<0.019
p<0.006
Huh7
BB7
Huh7.5
FL
JFH-1
B.
IgG Ctrl
JFH-1
Huh 7.5
% of Max
IgG Ctrl
FL
Huh 7.5
IgG Ctrl
BB7
Huh 7
TLR7
C.
Relative Quantity for TLR7
p<0.005
1a
1b
2b
1b
1a
1a
Ctrl
HCV average
HCV Patient Liver and Genotype

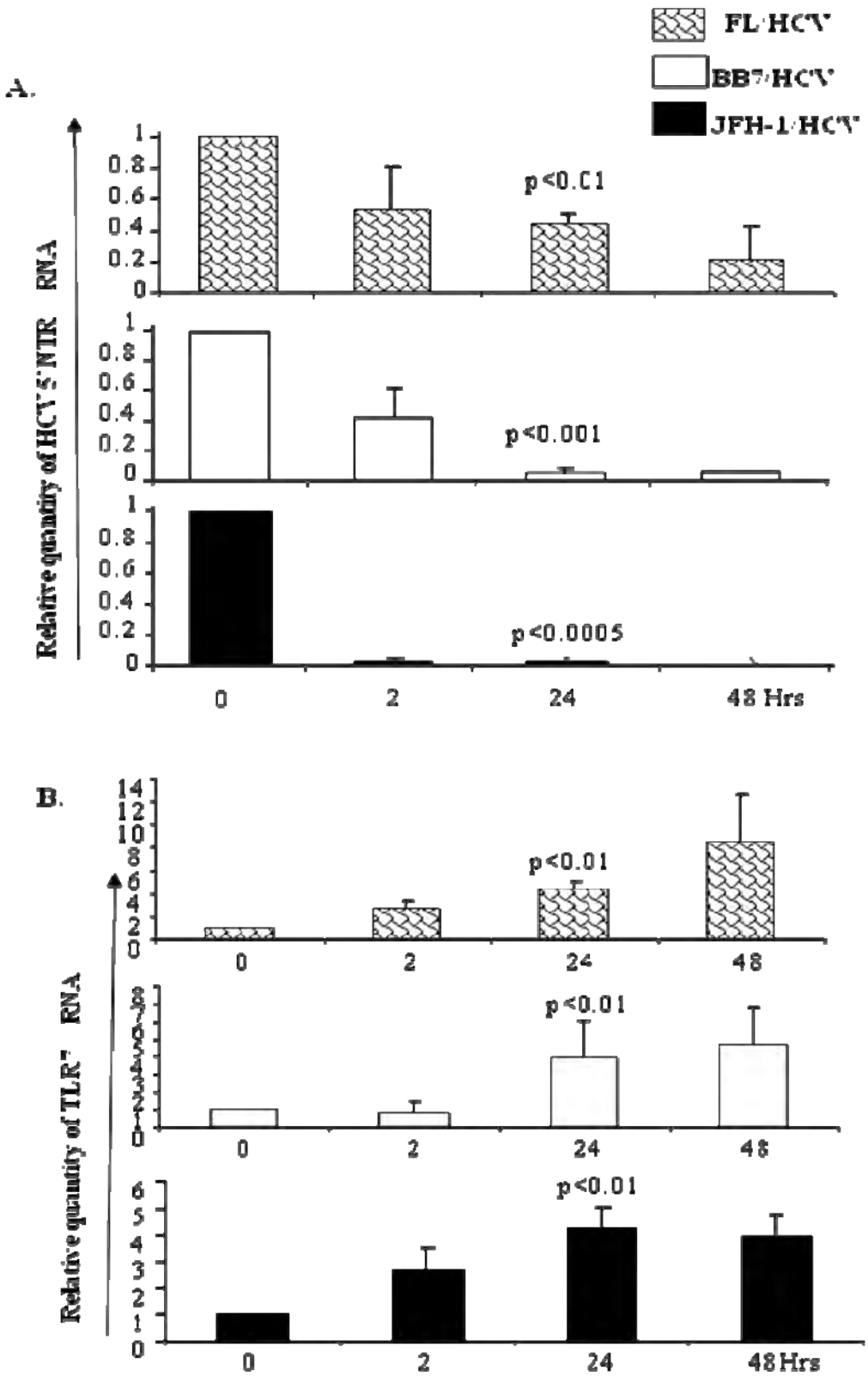
Figure 5.2: Hepatoma cells actively replicating HCV show reduced TLR7 mRNA and protein. (A) RNA extracts of HCV replicating and hepatoma control cells used to amplify TLR7 mRNA by quantitative real time PCR normalized to GAPDH mRNA. Data are shown as n=3 independent experiments Data was quantitated using the $2^{-\Delta\Delta Ct}$ method. Data are shown as an average ± SD with p-values calculated using student's t-test. (B) Hepatoma and HCV replicating cells fixed, permeabilized and stained for TLR7 receptor using flow cytometry. Negative controls included unstained samples (not shown) and secondary antibody only stained samples (shown as dotted grey line) Data is representative of one of n=3 independent experiments with similar results (C) RNA extracts of HCV patient liver samples and control liver samples obtained as stated in methods. HCV average indicates the average TLR7 mRNA of the 6 HCV patient samples. Three healthy control liver samples were tested and averaged. Data are shown as an average ± SD with p-values calculated using student's t-test.

HCV clearance by IFNα re-establishes TLR7 expression

Reduced TLR7 expression in HCV replicating hepatoma cells and in the livers of HCV infected patient indicated a novel phenotype possibly caused by HCV infection. Thus, we hypothesized that the reduced TLR7 expression was due to HCV replication. To test whether there is a direct effect of HCV infection on TLR7 levels, we reduced HCV replication by either administration of IFNα treatment, cultured stable HCV-expressing cell lines in selection antibiotic-depleted media, or self-restriction of viral replication through high passage number of JFH-1 cells, followed by the analysis of TLR7 levels. We analyzed mRNA content through quantitative real-time PCR calculated using a relative standard curve method. HCV mRNA was performed with the more precise and expensive Taqman probes while TLR7 mRNA was performed with the less precise but still quantitative Sybr green protocol. Sybr green QRT-PCR was used for economical reasons. Both HCV and TLR7 were normalized against GAPDH.

Treatment of HCV replicating cells with IFNα over a forty-eight hour time course led to time-dependent reduction of HCV replication in all three HCV cell models including FL, BB7 and JFH-1 (Figure 5.3A). In contrast to significant decreasing HCV levels (Figure 5.3A), TLR7 RNA significantly increased upon IFNα treatment in all three HCV replicating cells (Figure 5.3B). TLR7 levels doubled in control cells treated with IFN-α after 2 hours (Figure 5.3B). TLR7 levels in control cells increased slightly or decreased after 2 hours. Even though TLR7 RNA levels in control cells did increase upon IFN-α administration, there was a significant ($p<0.05$) TLR7 RNA increase in HCV replicating cells

compared to the control cells after the 2 hour time point. The only significant TLR7 RNA increase in control cells was at the 2 hour time point when compared with the subgenomic cell line, BB7, which did not increase TLR7 levels above baseline. These results suggest that IFN-α does have some control over TLR7 RNA levels. Curing HCV replicating cells with IFNα directly correlated with conventional levels of TLR7.


A.
FL-HCV
BB7-HCV
JFH-1-HCV
Relative quantity of HCV 5'NTR RNA
p<0.01
p<0.001
p<0.0005
0
2
24
48 Hrs
B.
Relative quantity of TLR7 RNA
p<0.01
p<0.01
p<0.01
0
2
24
48
48Hrs

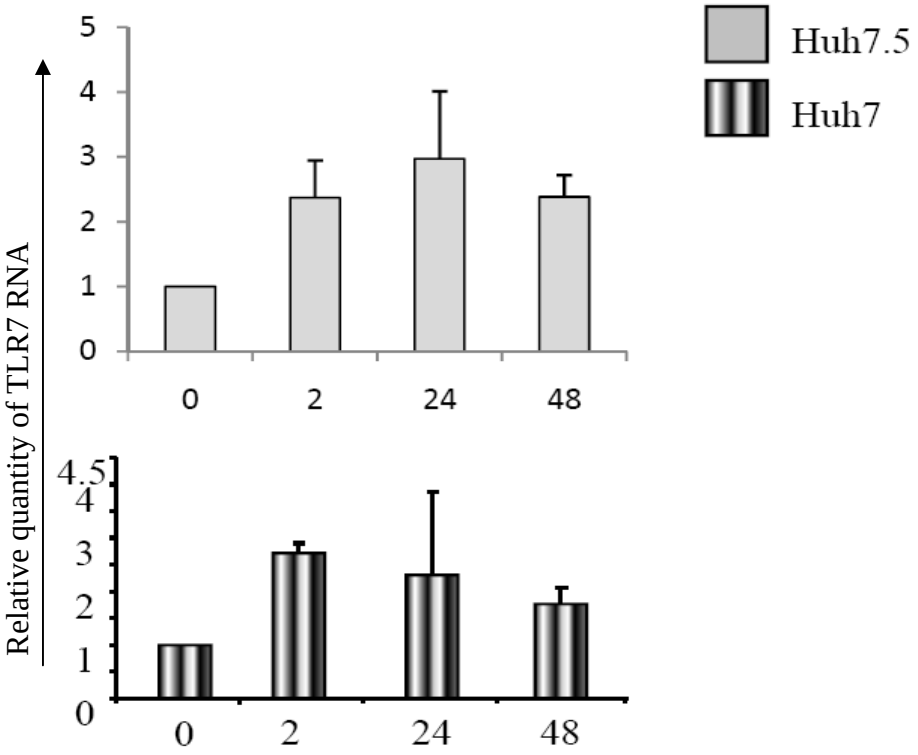
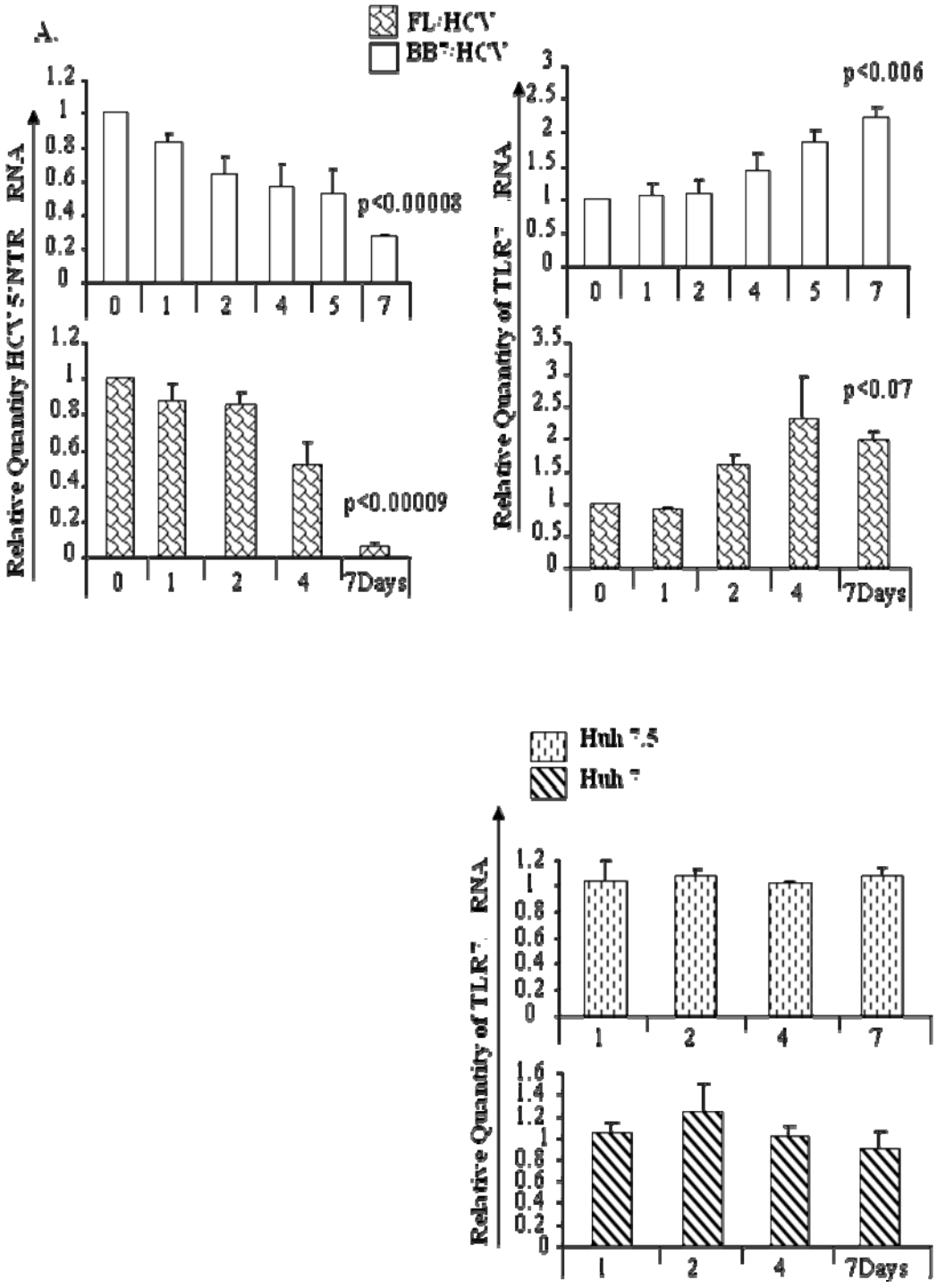


Figure 5.3: IFNα curing of HCV replication increases TLR7 mRNA expression. (A) HCV mRNA was amplified by Taqman QRT-PCR normalized to GAPDH mRNA levels and (B) TLR7 mRNA amplified by Sybr Green QRT-PCR in HCV replicating cells, FL, JFH-1, and BB7 and in control cells, Huh7 and Huh7.5. 1ng/ml of IFNα was administered to cells for a 48 hour time course and samples were taken at indicated intervals. Data was quantitated using the $2^{-\Delta\Delta Ct}$ method. Data are shown as an average ± SD of n=3 independent experiments. P-values were calculated by student's t-test and compared to the controls.

Natural 'curing' due to depletion of selective media in stable HCV replicating cell lines confirms TLR7 protein restoration

To verify these findings we followed up these results by culturing FL and BB7 HCV replicating cells without selection media, G418. After seven days, we observed similar restoration of TLR7 RNA (Figure 5.4A) upon diminishing HCV replication. TLR7 expression was not affected by cell growth phases over the seven day time course in control cells. Effects of G418 on TLR7 expression was not properly tested and would need to be performed to confirm that these effects are not from addition of G418. G418 was added to control hepatoma cells for a seven day period (data not shown). 48 hours with G418 was enough to cause cell death in the control cells. The proper experiment would be to transfect hepatoma control cells with an empty vector containing the G418 resistance, add G418 and measure TLR7 expression levels over time. Experiments with the IFNα curing did not show any difference in TLR7 protein restoration after two days (data not shown). Therefore we investigated protein levels during a longer time course in HCV replicating cells without selection media. Protein levels returned to non-HCV control cells after four days (Figure 5.4B) upon diminished HCV expression. These results confirm the direct effect HCV replication has on diminished TLR7 expression in both FL and BB7.


A.
FL-HCV
BB7-HCV
Relative Quantity HCV 5'NTR RNA
p<0.00008
p<0.00009
0
1
2
4
5
7
7Days
Relative Quantity of TLR7 RNA
p<0.006
p<0.07
Huh 7.5
Huh 7

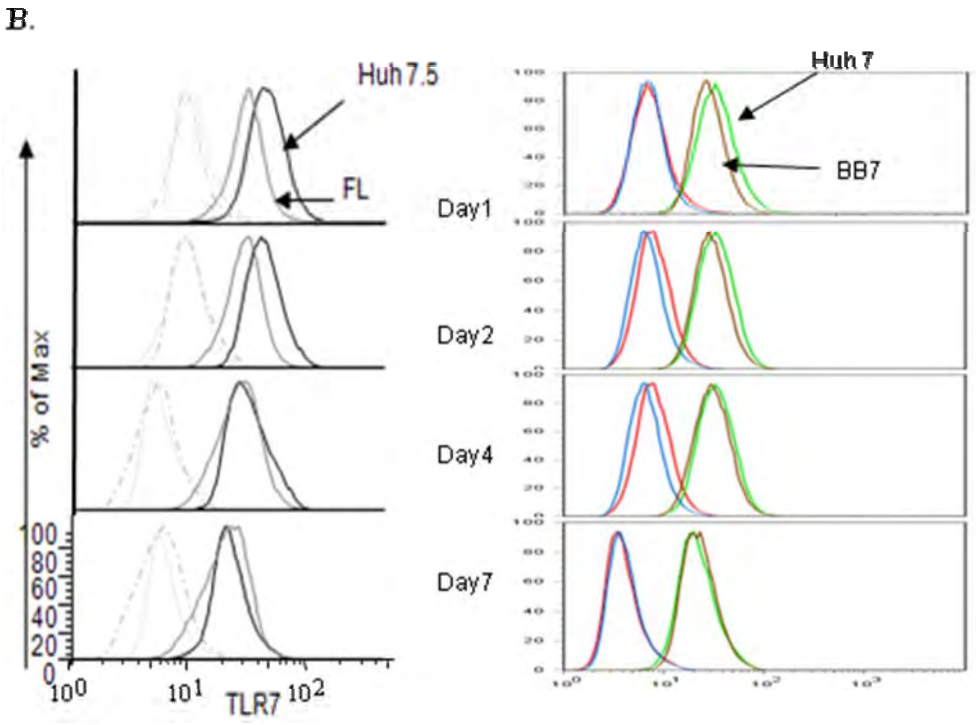


Figure 5.4: Reduction of HCV replication by exclusion of selective media, G418, in HCV stable cell lines increases TLR7 mRNA and protein expression. (A) RNA was extracted from HCV stable cell lines FL and BB7 and amplified by Taqman QRT-PCR normalized to GAPDH mRNA levels. TLR7 mRNA was amplified by Sybr Green QRT-PCR in HCV stable cell lines FL and BB7. Stable TLR7 expression in hepatoma control cells. RNA was extracted from hepatoma control cells and used to amplify TLR7 mRNA by Sybr Green QRT-PCR normalized with GAPDH. Cells were maintained in G418 deficient media and were used as a TLR7 mRNA time course control. Data was quantitated using the $2^{-\Delta\Delta Ct}$ method. Data are shown as an average $\pm$ SD of n=4 independent experiments. P-values were calculated by student's t-test and compared to the controls. (B) Cell samples stained with TLR7 antibody representing TLR7 protein levels using flow cytometry. Cells stained with IgG controls are shown by (—•—) in the left panel and red in the right panel. Unstained controls are shown in blue in the right panel and solid grey in the left panel. HCV replicating cells were passed and maintained in G418 deficient media on day 0 and RNA and protein samples were taken on the indicated days over a period of one week. Data is representative of one of n=4 independent experiments with similar results.

To corroborate these results in JFH-1 cells, we examined TLR7 protein levels in parallel with HCV expression over a series of passages. JFH-1 replication was depleted after fourteen serial passages while TLR7 protein levels were restored (Figure 5.5). These data show a direct correlation between active HCV replication and decreased TLR7 expression.

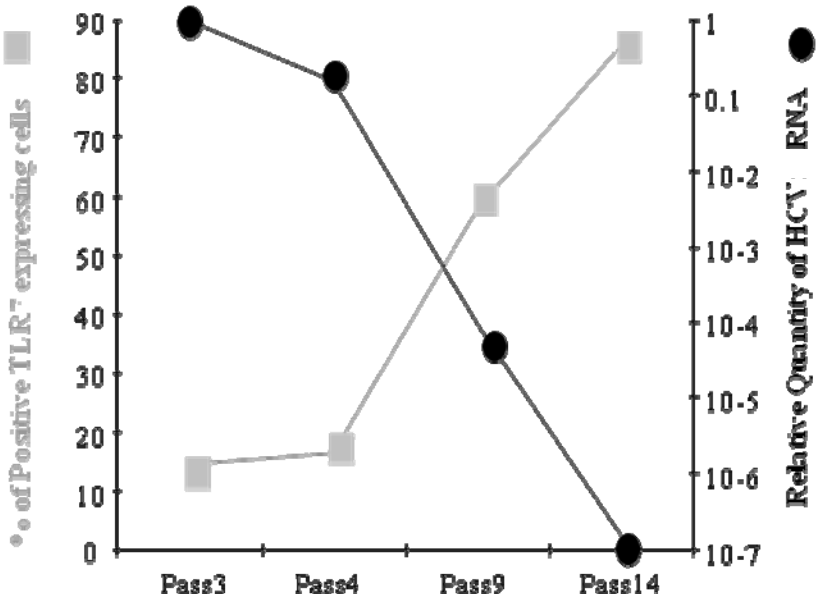
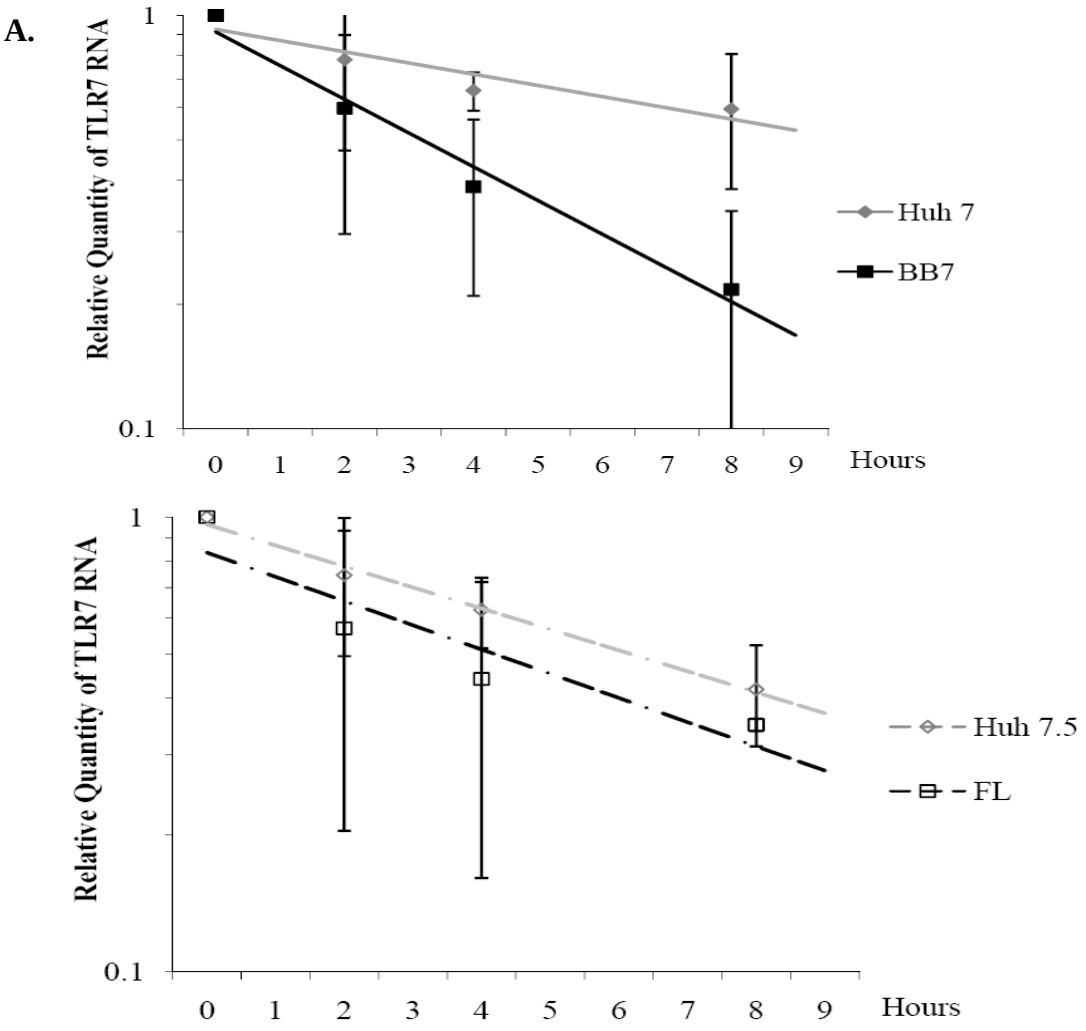


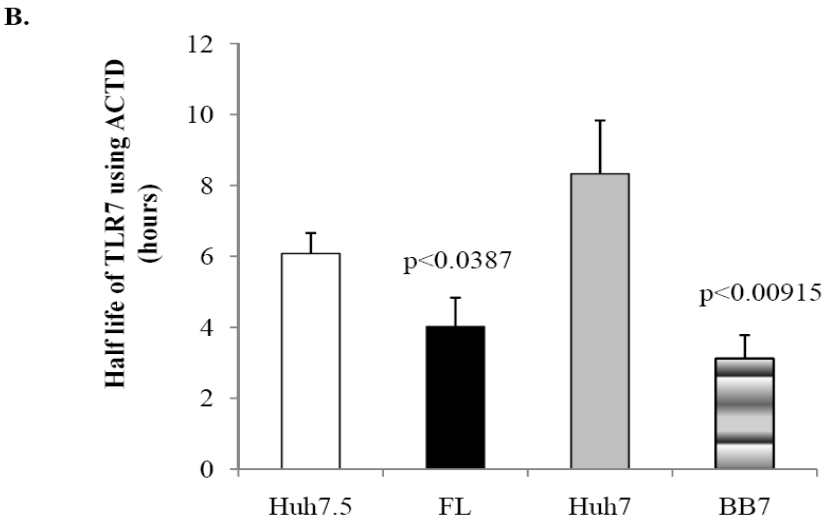
Figure 5.5: Diminished HCV infection from serial passages of JFH-1 cells increases TLR7 protein expression. RNA extracts were taken from JFH-1 cells from the indicated passage number and HCV mRNA was amplified using Taqman QRT-PCR normalized with GAPDH and displayed on the right side Y-axis, black line. TLR7 protein levels were quantified by flow cytometry from cells fixed, permeabilized and stained against TLR7 from the indicated passage number displayed on the left side Y-axis, gray line. JFH-1 cells were passed every three to four days with the first passage performed after electroporated cell recovery described in the Materials and Methods. Data was quantitated using the $2^{-\Delta\Delta Ct}$ method. Data is representative of one independent experiment with n=3 replicate wells.

RNA instability is a mechanism for HCV modulation of TLR7

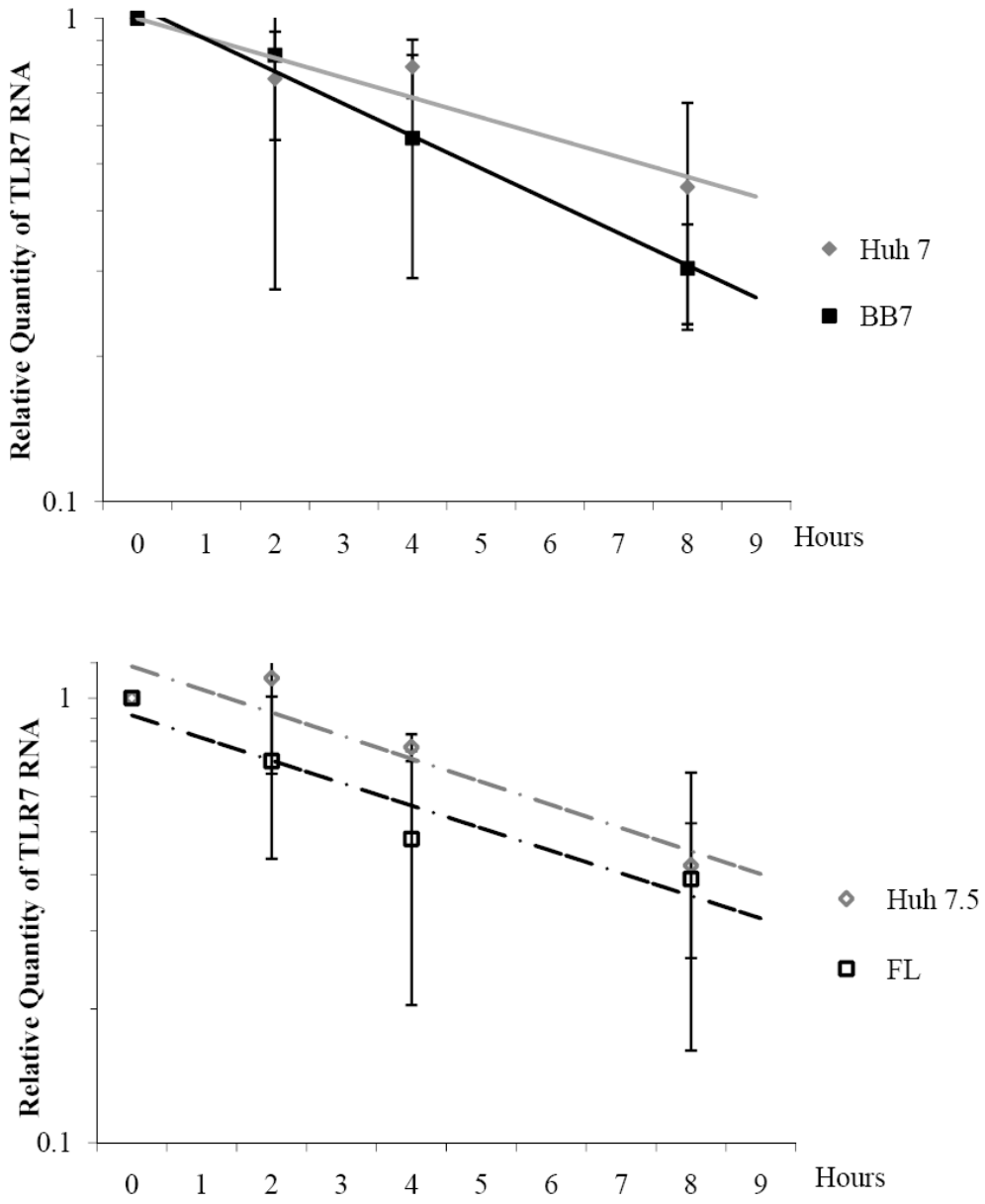
Deficient production of RNA coding for a gene, which can lead to impaired protein expression, is often caused by modifications at the level of gene transcription (248). More importantly, some viruses manipulate host mRNA stability to achieve delayed and/or impaired immune responses, thus leading to viral persistence (249, 250). Considering that our results in HCV replicating cells showed decreased TLR7 RNA due to HCV replication, and recent report of HCV NS5A protein interference with the TLR7-mediated signaling pathway resulting in impaired cytokine production (236), we predicted that HCV may affect the stability of TLR7 mRNA. To investigate this hypothesis, we utilized actinomycin D, an inhibitor of mRNA transcription achieved by binding the DNA at the transcription initiation complex and thus preventing elongation by RNA polymerase (251), and quantified the TLR7 mRNA half life levels using real time PCR over a twenty-four hour time course. TLR7 quantification was normalized to β-actin instead of GAPDH. β-actin mRNA is more stable than GAPDH and TLR7 which makes this gene more appropriate to use for normalization than one that is less stable. The 24 hour time point was not used to calculate the half life as it yielded slightly reduced viability. There were values detected up to 8 hours but at the 24 hour time point there were a few undetectable values which did not affect half life values since I did not include the 24 hour time point in this calculation. If any experiment had one abnormal CT value (no value at all or extremely high) detected in the PCR, I would discard the whole experiment. The half-life of TLR7 mRNA using actinomycin D was significantly shorter in HCV-

replicating cells compared to controls (Figure 5.6A, Figure 5.6B). A similar pattern of shortened half-life of TLR7 mRNA instability in HCV replicating cells was observed using alpha-amanitin, another inhibitor of mRNA transcription which interacts with the bridge helix in RNA polymerase II, allowing it to disrupt the translocation of RNA required to vacate the area for the next round of RNA synthesis (252) (Figure 5.6C, Figure 5.6D). The larger standard deviation in the Huh7 cells using alpha-amanitin caused a p-value greater than 0.05. Even though these results did not fall under the strict p-value limit for significance, the trend of reduced stability of TLR7 mRNA in HCV replicating cells compared to controls was followed. These results suggest mRNA instability as one mechanism that HCV utilizes to circumvent induction of TLR7.


A.
Relative Quantity of TLR7 RNA
1
0.1
0 1 2 3 4 5 6 7 8 9
Hours
Huh 7
BB7
Relative Quantity of TLR7 RNA
1
0.1
0 1 2 3 4 5 6 7 8 9
Hours
Huh 7.5
FL


B.
Half life of TLR7 using ACTD (hours)
12
10
8
6
4
2
0
p<0.0387
p<0.00915
Huh7.5
FL
Huh7
BB7

C.


Relative Quantity of TLR7 RNA
1
0.1
0 1 2 3 4 5 6 7 8 9
Hours
Huh 7
BB7
Relative Quantity of TLR7 RNA
1
0.1
0 1 2 3 4 5 6 7 8 9
Hours
Huh 7.5
FL

D.

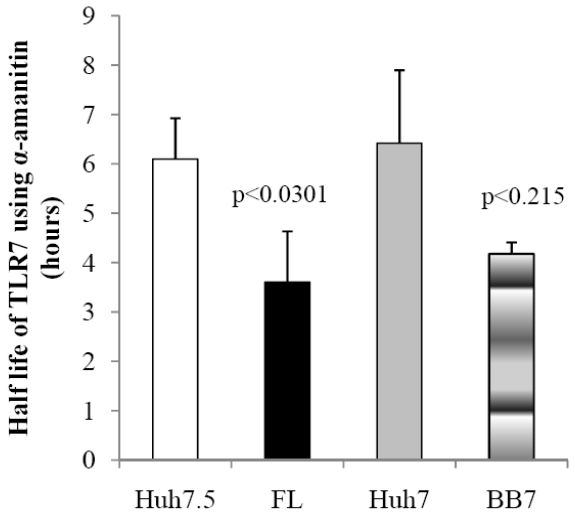
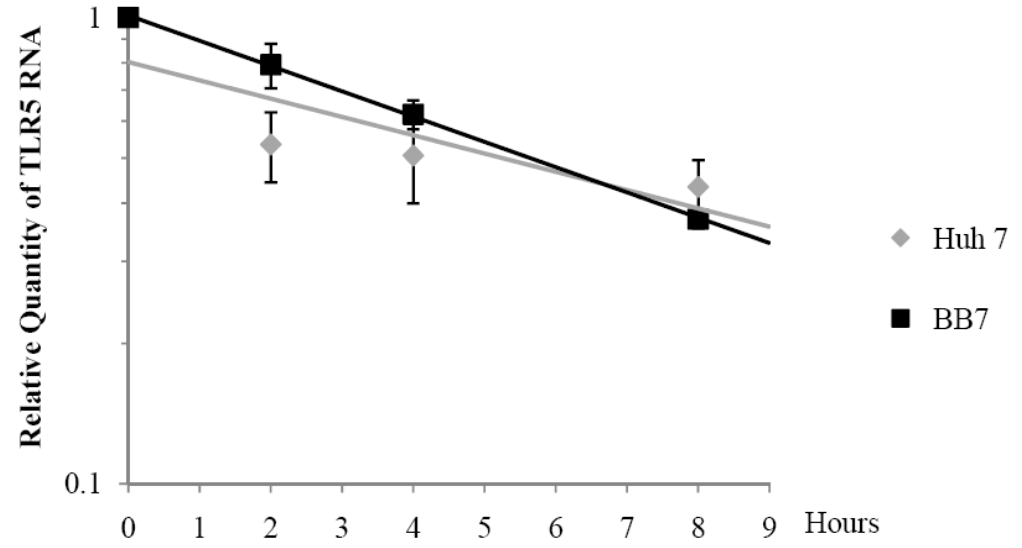


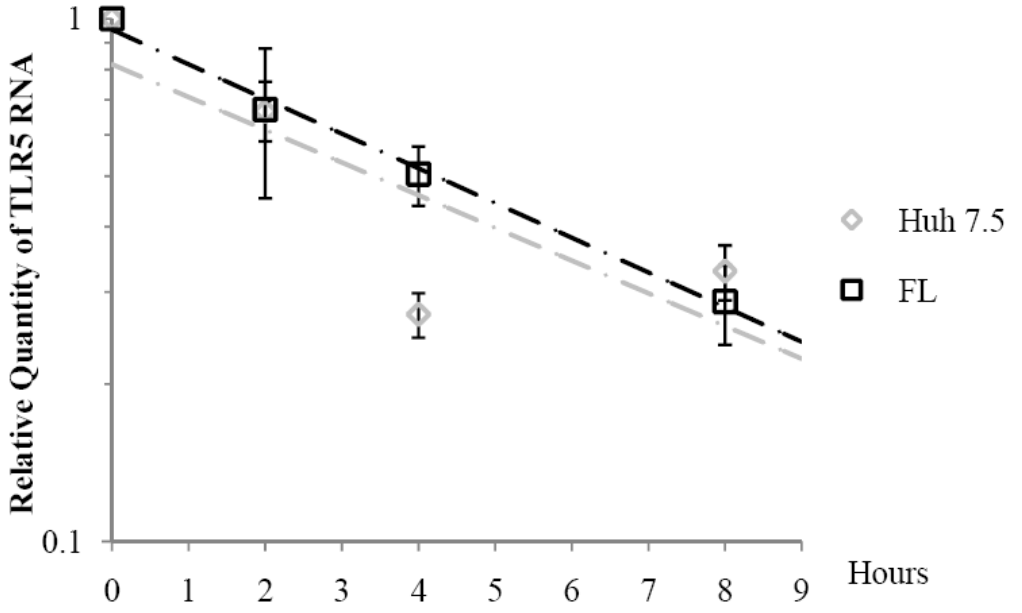
Figure 5.6: RNA polymerase inhibitors reveal TLR7 mRNA shortened half-life in HCV replicating cells compared to control cells. (A and B) Cells were treated with RNA polymerase inhibitor actinomycin D (2 µg/ml) or (C and D) alpha amanitin (10 µg/ml) up to 24 hours. (A. and C.) are shown as semi-log representation up to 9 hours with the respective regression lines for each cell type. RNA extracts were taken at the indicated time points and TLR7 mRNA was quantitated by QRT-PCR normalized with β-actin. Data was quantitated using the $2^{-\Delta\Delta Ct}$ method. Data are shown as an average ± SD of n=3 independent experiments. (B and D) Half life was calculated using the first order rate equation from the slope of the natural log of points shown in (A and C). Data are shown as an average ±SD of n=3 independent experiments. P-values were calculated by student's t-test and compared to the controls.

RNA instability is specific to TLR7 in HCV replicating cells

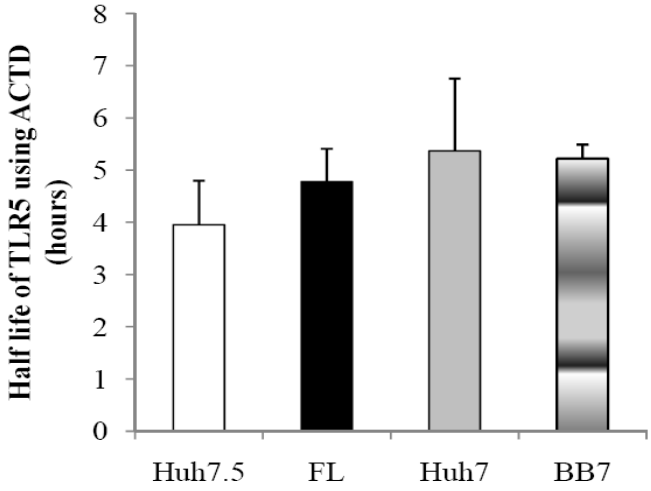
To verify that this mechanism is specific to TLR7 and not a global effect of HCV replication, we investigated the half-life of TLR5 in HCV replicating cells. TLR5 and TLR7 baseline levels in hepatoma cells are very similar unlike TLR4 levels, which we also investigated but half-life of TLR4 mRNA was more erratic (data not shown). Reduction in mRNA stability was specific to TLR7, as the half-life of TLR5 mRNA, a TLR which does not recognize viruses or viral-derived products (253), was similar in control cells and HCV replicating cells (Figure 5.7). There were no significant changes to TLR5 half-life between HCV replicating cells and non-HCV control cells in either actinomycin D (Huh7.5/FL - $p<0.52$, Huh7/BB7 - $p<0.92$; Figure 5.7B), or alpha amanitin (Huh7.5/FL – $p<0.67$, Huh7/BB7 – $p<0.72$; Figure 5.7D). These results establish specificity to decreased TLR7 mRNA stability in HCV replicating cells.

A.

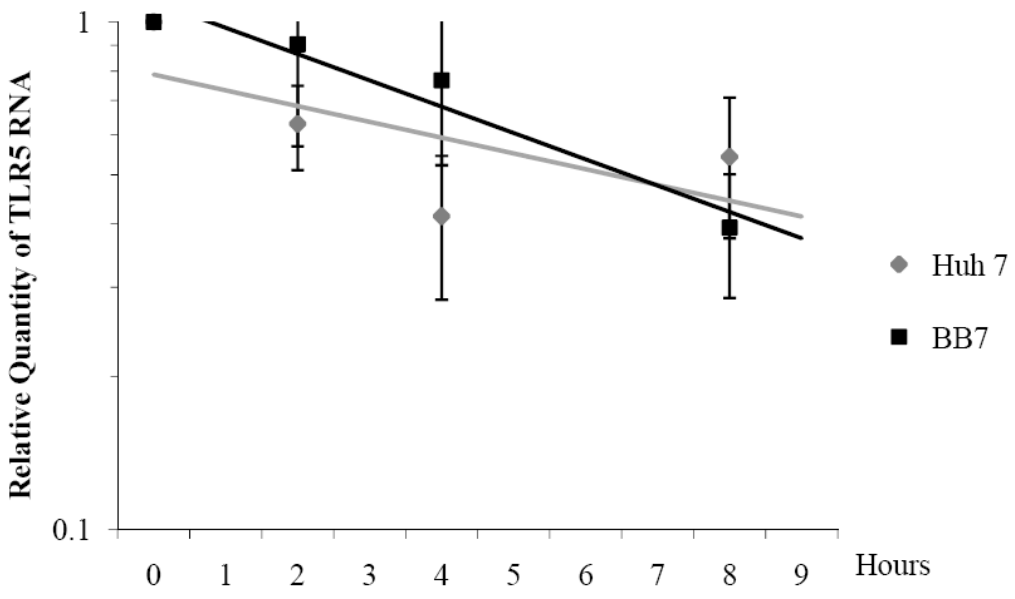

Relative Quantity of TLR5 RNA
1
0.1
0 1 2 3 4 5 6 7 8 9
Hours
Huh 7
BB7

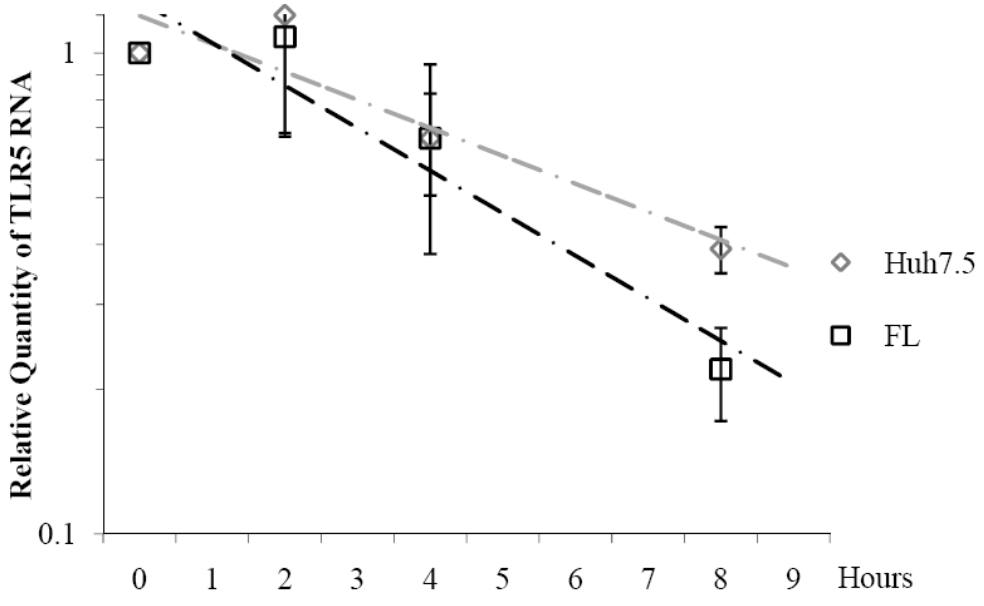

Relative Quantity of TLR5 RNA
1
0.1
0 1 2 3 4 5 6 7 8 9
Hours
Huh 7.5
FL

B.


Half life of TLR5 using ACTD (hours)
8
7
6
5
4
3
2
1
0
Huh7.5
FL
Huh7
BB7

C.


Relative Quantity of TLR5 RNA
1
0.1
0 1 2 3 4 5 6 7 8 9
Hours
Huh 7
BB7


Relative Quantity of TLR5 RNA
1
0.1
0 1 2 3 4 5 6 7 8 9
Hours
Huh7.5
FL

D.

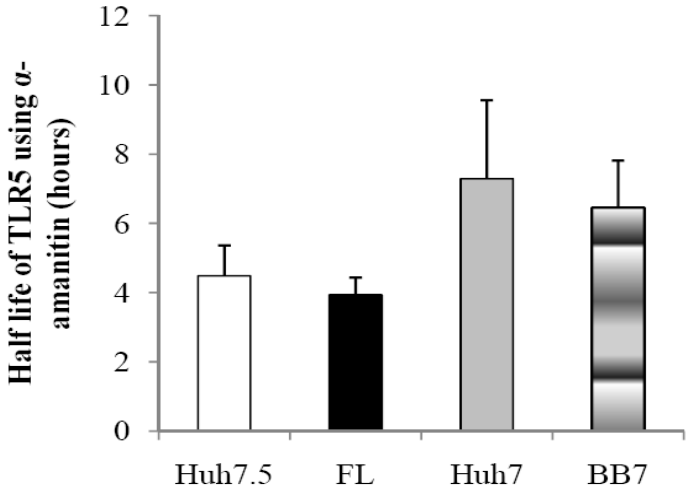


Figure 5.7: HCV replication does not affect TLR5 half-life. Cells were treated with RNA polymerase inhibitors either actinomycin D (A and B) or alpha-amanitin (C and D) for 24 hours. (A. and C.) are shown as semi-log representation up to 9 hours with the respective regression lines for each cell type. RNA extracts were taken at the specified time points and were used to quantify TLR5 mRNA by QRT-PCR normalized with β-actin. Data was quantitated using the $2^{-\Delta\Delta Ct}$ method. Data are shown as an average ± SD of n=3 independent experiments. (B and D) Half life was calculated using the first order rate equation from the slope of the natural log of points shown in (A and C). Data are shown as an average ±SE of n=3 independent experiments.

Furthermore, actinomycin D and alpha-amanitin, inhibitors used in the message stability experiments had little effect on HCV replication (Figure 5.8). β-actin was used for normalization of HCV levels and β-actin did not decrease after 24 hours with inhibitors. HCV replication with inhibitors neither increased nor decreased dramatically after 8 hours. Up to 24 hours the HCV replication increased in FL and BB7 despite treatment with RNA polymerase inhibitors unlike a third inhibitor that did reduce HCV replication (data not shown). These results indicate that HCV replication can decrease TLR7 expression by way of TLR7 mRNA instability.

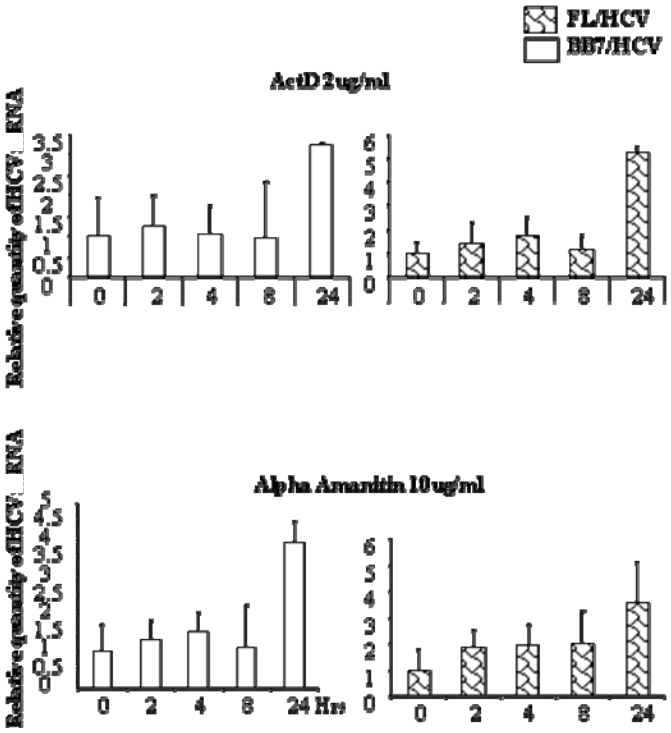
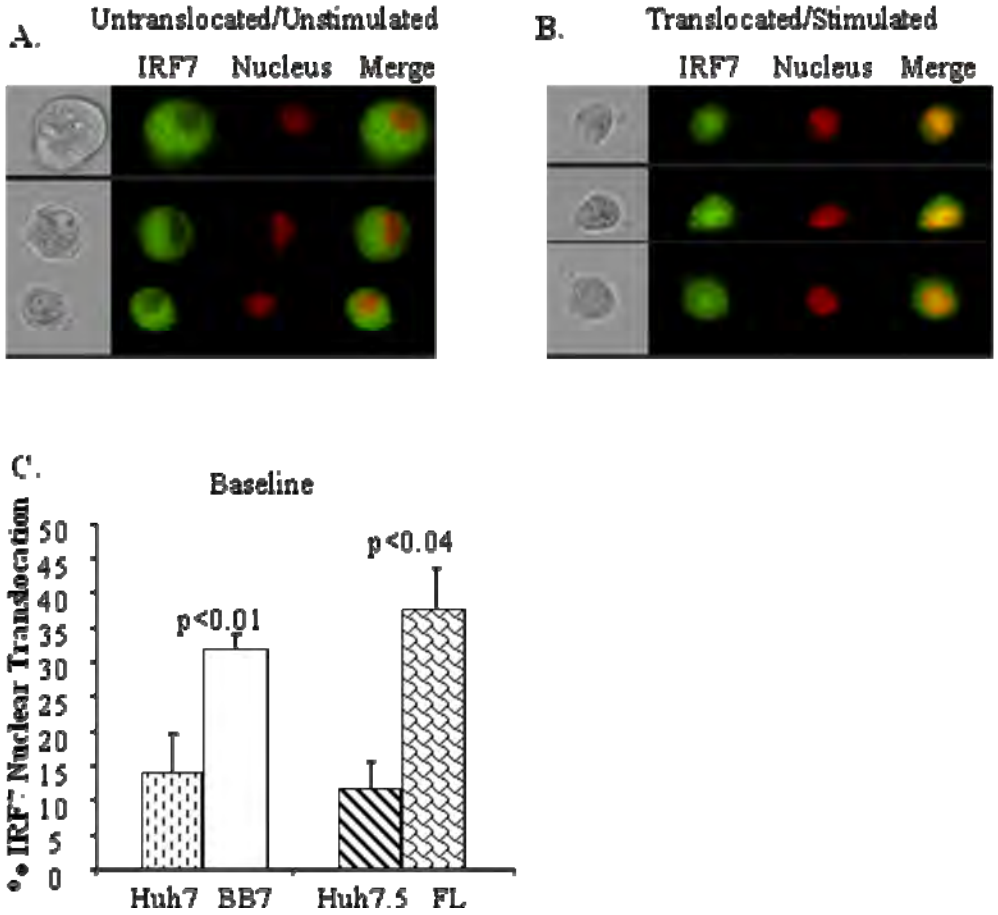


Figure 5.8: HCV replication is not affected by RNA polymerase inhibitors. Cells were treated with RNA polymerase inhibitors either actinomycin D or alpha-amanitin for 24 hours. RNA extracts were taken at the specified time points and were used to quantify HCV replication by QRT-PCR normalized with β-actin. Data was quantitated using the $2^{-\Delta\Delta Ct}$ method. Data are shown as an average ± SD of n=3 independent experiments.

IRF7 activation is stunted in HCV replicating cells when stimulated with TLR7 agonist

Our results so far showed a correlation between HCV replication and impaired TLR7 expression. Others have reported an increased baseline expression of Type 1 IFN-inducible genes in HCV-infected patients (247, 254), but no significant increase in IFN-dependent genes, ISG-15 and OAS, in HCV infected patients when given isatoribine, a TLR7 agonist (197). These data together prompted us to explore the effect of HCV replication on the TLR7-mediated Type 1 IFN signaling pathway. IRF7 is vital to TLR7-induced type I IFN production (255). Using ImageStream technology, we analyzed the activation of IRF7 by investigating IRF7 nuclear translocation in control and HCV replicating hepatoma cells. Unstimulated control cells show IRF7 localized in the cytoplasm separate from the nuclear compartment (Figure 5.9A) while stimulated cells present IRF7 co-localized with the nucleus (Figure 5.9B). The majority of control cells showed minimal IRF7 nuclear translocation and baseline activation of IRF7 (Figure 5.9C). In contrast to control cells, unstimulated HCV replicating cells, FL ($p<0.01$) and BB7 ($p<0.04$), had a significantly higher baseline activation of IRF7 (Figure 5.9C). Upon stimulation with the TLR7 ligand, control cells, Huh7 ($p<0.01$) and Huh7.5 ($p<0.04$), show a significant up regulation of IRF7 activation. HCV replicating cells did show an increase in IRF7 nuclear translocation, yet it was not significant in either FL or BB7 ($p>0.05$) (Figure 5.9D). The observation that stimulation of HCV replicating cells with R837 triggered only minimal activation of IRF7 compared to the existing high baseline

activation was in agreement with low expression of TLR7 (Figure 5.1). These results indicate that the presence of HCV modulates the baseline expression of IRF7; however the TLR7-induced activation of HCV replicating cells is impaired. Due to the existing upregulated IRF7 nuclear translocation at baseline in HCV replicating cells, we can not rule out the possibility that statistically significant increases in IRF7 nuclear translocation may not be possible.


A.
Untranslocated/Unstimulated
IRF7
Nucleus
Merge
B.
Translocated/Stimulated
IRF7
Nucleus
Merge
C.
Baseline
% IRF7 Nuclear Translocation
50
45
40
35
30
25
20
15
10
5
0
p<0.01
p<0.04
Huh7
BB7
Huh7.5
FL

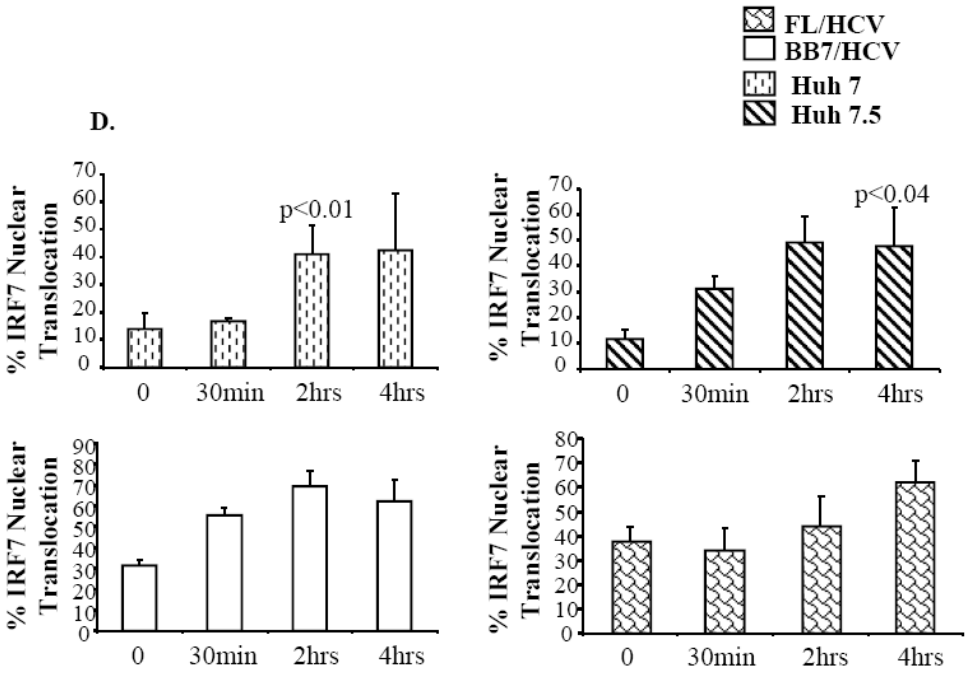


Figure 5.9: Type 1 IFN-mediated pathway via IRF7 nuclear translocation is elevated in HCV replicating cells yet less responsive by TLR7 stimulation compared to control cells. Fluorescent images of unstimulated (A) or stimulated with TLR7 ligand, R837, (B) hepatoma control cells using ImageStream technology. Control cells were fixed, permeabilized and stained with IRF7, green, and nucleus, red. Cells were analyzed by the ImageStream system that individually excites fluorescently stained cells with a 488 nm laser and a brightfield light source while collecting cell images equivalent to 40-60X magnification and fluorescent intensity data through a custom CCD camera. The brightfield is shown to the left and co-localization is shown by a merged image of the IRF7 and nuclear images shown to the right. (C) Unstimulated control and HCV replicating cells were fixed, permeabilized and stained for IRF7 and nucleus then analyzed on the ImageStream system. Percent of IRF7 nuclear translocation was calculated from the IDEAS software using data collected from 10,000 events in each sample. (D) Percent of IRF7 nuclear translocation after TLR7 ligand, R837 10µg/ml, stimulation up to 4 hours. Data are shown as an average ± SD of n=3 independent experiments.

Chapter Summary

HCV proteins disrupt innate immune receptors that generate Type 1 IFNs. TLR7 is a viral recognition receptor that warns the cell of single stranded RNA viruses by releasing Type 1 IFNs, particularly IFNα. Stimulating TLR7 in HCV patients or HCV replicating cell lines has proven detrimental for viral survival. The major caveat to this treatment is the immense quantities of TLR7 agonists necessary for potency. Therefore, we investigated whether TLR7 was a target of HCV infection in hepatocytes. First, we confirmed HCV replication in all three HCV replicating cell lines by NS5A and core protein presence. TLR7 RNA and protein levels in HCV cell lines and HCV infected patient liver samples showed a significant reduction compared to healthy controls. The direct link to HCV with this phenotype was verified by exploring TLR7 levels after HCV was reduced. Curing HCV replicating cells with IFNα restored TLR7 RNA levels after 24 hours, but did not show protein restoration after 48 hours. To confirm these results and show protein restoration, we grew HCV replicating cells requiring selection media for HCV replication, devoid of selection antibiotics. After 7 days, the HCV replication dramatically decreased while the TLR7 levels were re-established. The TLR7 protein levels also returned to normal but only after 4 days, which accounts for the lack of TLR7 protein restoration during IFNα curing. JFH-1 cells do not sustain HCV replication indefinitely and perform a self-curing, therefore corroborated our previous results by showing a reduction in HCV replication over time in JFH-1 cells proved to restore TLR7 protein levels.

One of the mechanisms by which HCV reduces TLR7 expression is reducing TLR7 RNA stability. The half-life of TLR7 in HCV replicating cells was shorter than control cells when utilizing two different RNA polymerase inhibitors. Stability of other TLRs such as TLR5 was not affected in HCV replicating cells and thus proves specificity to TLR7. RNA polymerase inhibitors did not cause much change in HCV replication. We further investigated the downstream IFNα-mediated pathway which in TLR7 is mainly directed by IRF7 nuclear translocation. Using ImageStream technology we quantitated IRF7 nuclear translocation in stimulated and unstimulated HCV and control hepatoma cell lines. HCV replicating cell lines have an already high baseline IRF7 activation that is not significantly amplified with further TLR7 stimulation compared to control cells. These results suggest further downstream consequences to diminishing TLR7 expression.

CHAPTER VI

DISCUSSION

The aim of this dissertation was to establish the influence Hepatitis C virus has on Toll-like receptors in hepatocytes and macrophages. We hypothesized that HCV modulates TLRs to escape the immune system and ultimately establish chronic infection. We explored this aim in three parts: first establishing the TLR profile in hepatocytes to identify two potential targets of HCV, testing their function and determining whether hepatocytes like many other cell types can be activated by HCV; next, we continued to determine the identity and extent of functional efficiency of specific TLR2 heterodimers responsible for macrophage activation by HCV; finally, we sought to investigate how HCV affects TLR7 expression. Mechanisms for HCV chronic infection and immune modulation are unknown and therefore exploring the unique phenotypes caused by infection provides insight and possibilities for novel therapies in the future.

We did not use any correction method such as Bonferroni in our statistical analysis. We used the student's t-test for analysis with the cell culture lines because we always compared two paired data sets from each other. The student's t-test is used for analysis of two data sets that are normally distributed with similar variances. Using a Bonferroni correction would yield strict parameters with smaller p-values that would only result in a few changes to significance on a few figures.

Hepatocytes involved in inflammatory response to HCV

Previous literature surrounding HCV research established immune activation in innate immune cells (79, 208). While there has been much research in hepatocytes regarding HCV immune evasion from activation of IFN-mediated pathways (105-107, 171, 172) hepatocytes were not thought to contribute to the immune mediated inflammation caused by HCV. Despite this notion, hepatocytes were characterized as containing functional TLRs yet it was unknown whether these cells greatly contributed to inflammation due to HCV infection (256). We examined the possibility of innate immune activation in hepatocytes by HCV proteins which could contribute to the inflammatory response. We also confirmed the expression and function of specific TLRs that might be involved in HCV modulation.

1. Expression of TLRs in hepatocytes

A number of groups have investigated effects of TLR4, TLR9, TLR2, or TLR3 in the liver (256-260). Yet the liver is also composed of stellate cells, Kupffer cells, epithelial cells and circulating and resident lymphocytes (261). All of these cells express a number of innate immune receptors including TLRs (262). These cells also produce a significant amount of inflammatory cytokines via NF-kB pathway and IFNs via IRF pathway (262). Therefore, studies of TLRs in the liver do not reflect the expression or function of TLRs in individual cell types and since hepatocytes play host to HCV, we concentrated our work on purified hepatocytes.

We determined the expression of TLR2, TLR1, TLR6 and TLR7 in human hepatoma cells and primary hepatocytes. In doing so, we were able to postulate

that hepatoma cells are not only host to HCV but must be targeted for innate immune suppression in order for infection to continue or possibly occur. Hepatocytes compose around 80% of the liver and act in many ways to metabolize substances taken up in the body, either good or bad (263). They produce bile to aid in the process of digestion, cholesterol and lipids for cellular membranes, break down and form carbohydrates in gluconeogenesis and store proteins; they also make clotting factors vital to the body's initial defense mechanisms (263). Of course classically, hepatocytes and the liver are most known for detoxifying the Friday night rendezvous so many of us make, a nice stiff drink of alcohol (264). The liver is also the only known organ that can regenerate due to the restoring nature of hepatocytes (265). It is quite an accomplishment that hepatocytes can also act in an innate immune function. Traditional to hepatocytes' original occupation, we can conclude that in hepatocytes the expression of most TLRs, in particular TLR2, its co-receptors and TLR7, alert other immune cells of potentially harmful pathogens by inducing inflammatory cytokines. R848, TLR7/TLR8 specific ligand has been shown to induce NF-κB activation after 7 hours in HEK293/TLR7 cells (266) and in murine peritoneal macrophages (267). I did not see a measurable increase after one hour but to correctly ascertain NF-κB activation by R848 in hepatoma cells, I would perform a time course using R848 stimulation and obtaining nuclear extracts to run EMSAs using dsNF-κB oligonucleotides. Another route would be to transfect in an NF-κB luciferase reporter construct into hepatoma cells and run a time course stimulating with R848.

A caveat to obtaining primary hepatocytes from outside sources is the lack of 100% purity of cell type. Cambrex guarantees up to if not greater than 97% pure human primary hepatocytes. There were no phenotyping tests for contaminating cells performed. Three percent or fewer cells in the hepatocyte samples could be contaminating cells from the liver, such as Kupffer, stellate, or epithelial cells. All of these cells can also contribute to not only the expression of TLRs but also the functional cytokine production upon stimulation with TLR specific ligands or HCV proteins. We can not rule out the possibility that the TLR expression or the cytokine production could partially be from other cells. Even though the cells were allowed to recover from the stress of transportation, we can not also rule out the possibility that the cells were slightly activated and may express varying degrees of TLRs or innate immune genes from this activation. There was no elevated baseline of cytokine production in the hepatocyte media during any of these experiments which assures us that any cellular stress or contamination was minimal if any and did not contribute to the activity of hepatocytes upon ligand stimulation.

2. HCV proteins core and NS3 are responsible for increased inflammatory cytokine production in primary hepatocytes that have functional TLRs.

Earlier we brought notice to the intriguing finding that TLR8, in particular, is fully expressed in mice yet dysfunctional (241). A few have postulated whether the receptor is redundant in mice and therefore unnecessary. Studies with murine macrophages showed non-responsive TLR8 upon ligand,

R848, stimulation. In humans both TLR7 and TLR8 are expressed and fully functional to R848, yet for reasons unknown TLR8 in mice may not be utilized. A possible caveat to this theory is the small yet disruptive difference in genomic sequences between mice and men (268), which could cause TLR receptors to malfunction when stimulated with functional ligands in mice possibly due to a point mutation that could cause misfolding of the receptor's recognition site (269, 270). Because of this unanswered query, we sought to determine whether TLR expression in hepatocytes is functional or defunct.

The lack of extracellular cytokine production in hepatoma cells forced us to investigate activation of TLRs via NF-kB translocation. Only TLR2 and not TLR7 ligands were able to activate NF-kB in both hepatoma cell types, which we also saw with the little TLR2 mediated IL-6 and IL-8 production made from these cells (data not shown). Stimulation with TLR2/1, TLR2/6 or TLR7 ligands in primary hepatocytes showed a rather large response of inflammatory cytokines and modest production of Type 1 IFNs. Novel to these findings is the activation of purified primary human hepatocytes by HCV core and NS3. HCV core and NS3 were shown to activate monocytes and murine macrophages in a TLR2 specific manner. We can thus postulate the likelihood that HCV core and NS3 activation of hepatocytes is also a TLR2-mediated mechanism.

Because the HCV proteins were recombinant fusion proteins with β-galactosidase and produced in *E. coli* there is a chance that lipopeptides from *E. coli* were contaminating the HCV proteins. The HCV proteins had only been purified by the company using affinity column purification stated in the methods.

We did not perform further purification steps but would consider in the future purifying the proteins further. Since the limulus assay does not detect lipopeptides further purification using detergent extraction and isolation by reverse-phase high performance liquid chromatography may be necessary to assure purification of proteins.

3. Consequences to hepatocyte activation

IL-6 and IL-8 are important aspects of the immune response. IL-6 acts as either a pro- or anti-inflammtory cytokine (271). As an anti-inflammatory cytokine it can inhibit TNF-α, IL-1 and IL-10 (272). As a pro-inflammatory cytokine it can cause a febrile response from release of prostaglandin E2 (273). Important to the acute phase response, IL-6 has also been implicated in modulation of osteoclast cells (274). A consequence to chronic HCV is hepatocellular carcinoma (HCC) for which the mechanism for transformation of hepatocytes is not fully elucidated (275). It could be possible that excess IL-6, which is required for Ras oncogenic behavior (276), from stimulated hepatocytes by the TLR2 or TLR7 receptors, contributes to the mechanism of HCC. Even though these receptors proved functional, there was a preference for production of inflammatory cytokines, particularly IL-8, over Type 1 IFNs. IL-8 is a chemokine that attracts and directs immune cells, such as: neutrophils, macrophages, mast cells (277). There was a much larger IL-8 induction compared to IL-6 in hepatocytes which also occurred with HCV protein stimulation. This huge influx of immune cells in acute infections could help cause spontaneous resolution of the infection, but in chronic infections a steady

influx of IL-8 or IL-6 production from HCV proteins or RNA eventually causes more damage than good. We conclude that hepatocytes have a very important function in mediation of self created inflammatory cytokines, yet don't seem to play as important of a role in Type 1 IFN generation. Functional TLR2/1 or TLR2/6 receptors in hepatocytes provide further knowledge of the identity of cells producing inflammatory cytokines during HCV infection which in chronic infections leads to an over abundance of inflammatory cytokines causing damage to the liver. Blocking TLR2 production of excess inflammatory cytokines during chronic HCV infection could be a novel therapy to aid in the fight against the virus.

The lack of stimulation from TLR7 ligands in hepatoma cells could represent a mutation in the receptor due to carcinogenesis or possibly a methodology error. Since primary hepatocytes administered with R848, resulted in a large IL-8 and moderate IL-6 production, the ligand performed as should. TLR7 is located within the endosome and therefore isolated from free flowing stimulants, whereas TLR2 exists both intracellularly but more importantly on the cell surface where upon the receptor is totally accessible to stimulants. Primary hepatocytes were stimulated for ten hours or greater with R848, whereas hepatoma cells were stimulated for only 1 hour. Due to the nature of the hepatoma cell lines and their inability to produce measurable amounts of extracellular cytokines upon stimulation, we could not test hepatoma cells using the ELISA technique. On the other hand, it would be worth testing NF-κB activation in primary hepatocytes after one hour of stimulation to compare the

results with hepatoma cell lines. Due to restrictions of obtaining enough primary hepatocytes for these experiments, NF-κB activation was not tested. Extracellular cytokines were measured using ELISA after ten hour stimulation with R848 in primary hepatocytes whereas in hepatoma cells NF-κB was measured by EMSA. NF-κB activation occurs much faster than cellular production of cytokines and extracellular release, therefore samples for EMSAs were performed with a one hour ligand incubation compared to the ELISA testing where the cells were incubated with ligand for ten hours. Activation of NF-kB occurs immediately in TLR2 receptors within 1 hour. Since TLR7 is located within the endosome, ligands must fuse with TLR containing endosomes before ligands can bind and activate TLRs. This process may take a longer time in accessing or allowing for endosomal maturation partially required by the receptor, thereby delaying NF-kB activation as opposed to TLRs located on the cell surface where ligands have immediate access to binding and activation of the receptor. Although the NF-kB pathway was not activated after 1 hour, the TLR7/IRF7 pathway was activated upon R848 or R837 stimulation after 2 hours. This phenomenon could be addressed by performing a time course with an NF-kB luciferase reporter assay transfected into hepatoma cells.

On a molecular level, it is quite possible that TLR7 is engaged by HCV RNA. TLR7 recognizes and is activated by single stranded RNA and polyU sequences. HCV not only exists in a single stranded RNA form but also has a significant polyU sequence in the 3'NTR region. Although there is strong evidence that TLR7 would recognize and become activated by HCV RNA, the

limitation to TLR7 recognition of HCV is based on its physical location strictly within the endosome. HCV entry and fusion into the host cell results in release of its genomic material directly into the cytoplasm without contacting TLRs within the endosome (278). Recent evidence suggests that HCV infection induces an incomplete autophagic response in hepatocytes (279). Autophagy involves cellular membrane invagination around cytosolic products which the resulting autophagosome fuses with a lysosome leading to degradation. TLR7 located within the endosome/lysosome recognizes vesicular stomatitis virus in this manner (226). HCV replicating cells showed presence of autophagosomes and HCV infection led to an increase of autophagy despite different genotypes (279, 280). It is quite possible that HCV RNA is also recognized in this manner yet it is still unknown whether HCV RNA is present within these autophagosomes and whether they fuse efficiently with lysosomes containing TLR7. The efficiency for autophagosomes to fuse with lysosomes and cause protein degradation was low in HCV replicating cells, and therefore without further evidence autophagy may not be the major recognition mechanism of HCV RNA by TLR7 (279).

Both TLR2 co-receptors function in response to HCV core and NS3

We established the identity and involvement of TLR2 and its co-receptors in cellular activation by HCV NS3 and core proteins in both human and mouse cells. HCV core protein is found not only in the cell cytoplasm but is present circulating within the host blood stream. Location of core outside of the virus and cell implicates the protein in immunogenicity. HCV NS3 unlike core protein is

not located in the blood stream but has been shown to be immunogenic also with its ability to activate monocytes and dendritic cells (208, 281). HCV does not induce cell lysis, but signaling to other immune cells from infected cells may cause their cell lysis. Viral products such as HCV NS3 protein may be released in the serum upon cell death and it is here where it may be possible for NS3 to activate TLR2/1 or TLR2/6 on the cell surface. We have also been able to locate TLR2 intracellularly by flow cytometry (data not shown). NS3 protein as a protease cleaves TRIF and IPS-1 in the cytoplasm, therefore it is possible that NS3 may be autophagosized by the cell as a host cellular defense mechanism where TLR2/1 or TLR2/6 complexes may be traveling intracellularly to the surface. These mechanisms might explain the activation induced from NS3 on TLR2 and its co-receptors. Without further research in this area the exact mechanism is still unknown yet important to understand as NS3 is immunogenic.

Here we show that HCV core and NS3 proteins activate not only monocytes (79), but also monocyte-derived macrophages to produce both TNF-α and IL-10. We confirmed that TLR2 siRNA knockdown significantly reduced activation by HCV core and NS3 proteins. Innate immune cells in the liver that are rich in TLRs include resident macrophages, Natural Killer (NK) cells, and Natural Killer T (NKT) cells, but also recruited monocytes and macrophages (282). Since Kupffer cells, the resident hepatic macrophages, compose ~20% of the liver, we used macrophages derived from human monocytes to parallel Kupffer cells and to examine the effects of HCV core and NS3. Kupffer cells produce the majority of TNF-α in the liver, which can mediate liver injury (167).

Here we show for the first time that TLR1 and TLR6 are involved in macrophage activation by HCV core and NS3 proteins. Involvement of both TLR1 and TLR6 receptors is another way for HCV infection to cause an inflammatory cytokine response.

1. **Both TLR1 and TLR6 are involved in TLR2 activation by HCV proteins**

Our results indicate that in human cells, the absence of either TLR1 or TLR6 had a dramatic negative effect on HCV core and NS3 stimulation, suggesting the involvement of both of these TLR2 co-receptors. Selective silencing of only one co-receptor did not result in the complete loss of cytokine induction by the HCV ligands. SiRNA knockdown of TLR1 or TLR6 showed very little cytokine production when stimulated with HCV proteins. These results might suggest that HCV proteins utilize not only both TLR1 or TLR6 receptor, along with TLR2, but also that HCV proteins may require a complex of TLR2 and both heterodimers for full activation by the HCV proteins. Knockdown of either TLR1 or TLR6 did not affect the others' function as shown in Figure 4.4. Due to an incomplete knockdown using RNAi technology, we utilized TLR2, TLR1, and TLR6 knockout mice. In the knockout mouse model, our data demonstrate that HCV core and NS3 utilize the TLR2/TLR6 complex. The observation of only minimal inhibition of HCV core or NS3 induced TNF-α in $TLR1^{-/-}$ suggests that in mice, recognition or activation by HCV core or NS3 proteins may not involve TLR1 as much as it may involve TLR6. Since there was some downregulation of cytokine production in TLR1-/- mice upon HCV protein stimulation, it is possible

that TLR1 might contribute to slight recognition of HCV core and NS3. Since HCV does not infect mice and their TLRs have slightly different sequences than humans, it is not surprising that there may be some differences in ligand activation between these two TLR2 co-receptors. The mechanisms for HCV proteins to activate innate immune receptors in mice and in humans might also be different. HCV does not infect mice; therefore the innate immune receptors in mice might not be as sensitive to HCV proteins. HCV proteins which are accustomed to the human host may not have the conformation or sequence to stimulate mice receptors.

2. Other receptors could be involved with TLR2 activation via HCV proteins

We also found that, although there was a significant reduction in TNF-α in the HCV protein stimulated TLR6 knockout macrophages, residual cytokine production remained. The residual cytokine could be due to a functional TLR2 and an unknown co-receptor. These results could imply alternate utilization of TLR1 or the possibility of another TLR2 co-receptor without the use of TLR1 or TLR6, such as CD36 (dectin-1) (201, 283) or CD14 (202). It is unknown whether other possible co-receptors such as β3 integrins (284) or ganglioside GD1a (285) also utilize TLR1 or TLR6 when activating TLR2. Alternatively, our results cannot rule out utilization of a different receptor in HCV core and NS3-induced innate cell activation. Several groups demonstrated that HCV core protein can inactivate T cells via gC1qR (286-288) and the implication of gC1qR-mediated HCV-induced innate immune suppression awaits confirmation. Recent reports

also indicate that HCV core protein may activate pro-inflammatory cell activation in an IFN-γ receptor-dependent manner (289, 290). Others and we have previously reported that HCV core and NS3 proteins inhibits macrophage-derived dendritic cell differentiation and functional capacity (208, 291-293).

3. Non-specificity of TLR2 and its co-receptors could lead to activation by many other viral products

Utilization of either TLR1 or TLR6 as TLR2 co-receptors in macrophage activation by HCV core and NS3 proteins supports the potential for broad-range recognition and cell activation by these proteins. Recent reports show that a number of viruses, such as cytomegalovirus (294, 295) and vaccinia virus (296), activate TLR2 and either TLR1 or TLR6 inflammatory cytokine response. It is unclear as to whether specific TLR2, TLR1 and TLR6 receptor-mediated activation by HCV proteins is helpful or harmful to the host. These proteins elicit both an inflammatory and anti-inflammatory response in human monocyte derived macrophages. Considering that HCV core and NS3 proteins induce inflammatory cytokine production, TLR2-mediated signaling may represent a mechanism for non-specific inflammatory activation seen in chronic HCV. In support of this contention, patients with chronic HCV infection exhibit an activated phenotype of Kupffer cells (297). However, it is not negligible that HCV core and NS3 proteins trigger production of IL-10, a potent anti-inflammatory cytokine. Such dual modulation of innate immunity by triggering both pro- and anti-inflammatory pathways seems to be common for TLR2-signaling pathogens of different origin (298). Further, in HCV infected patients

there are reports of elevated levels of serum IL-10 (299) and increased IL-10 production in immune cells (300). Importantly, the modulation of expression of TLR2 and its co-receptors in both immune and liver compartments is common for multiple liver diseases, including chronic HCV infection (38, 301).

With recent research progress, the possibility of cytokine regulation and the modulation of TLR function for therapeutic purposes become realistic (196). Our data provide novel insight into the mechanisms of HCV protein-induced activation of immune cells and indicate a new potential direction in managing the imbalanced immune functions during chronic infection with Hepatitis C virus.

TLR7: HCV's newest target for immune evasion from IFNs

Immune evasion by HCV has been documented in a number of different host cell types and it has been suggested to play a key role in viral persistence and development of chronic infection (106, 178, 236, 302-304). HCV interferes with host defense at multiple levels: HCV NS3/4A cleaves the adaptor proteins TRIF and MAVS preventing further downstream signaling in the Type 1 IFN pathway (106, 178), core protein interferes with cell cycle progression and apoptosis (303), and NS5A binds MyD88, a central adaptor protein for TLR downstream signaling and inhibits anti-viral cellular activation (235). NS5A could not fully inhibit IFN production by viral stimulation of TLRs in macrophages. NS5A displayed a weaker inhibition using the TLR7 ligand R-837 than viral stimulation and therefore this HCV immune evasion mechanism can not fully explain the lack of TLR7 expression and decreased stability shown in chapter V. It is possible that

NS5A may contribute to the small increase in IRF7 nuclear translocation of HCV replicating cells by inhibiting signaling through TLR7/MyD88. Without further analysis I cannot conclude whether NS5A is involved in the stunted IRF7 nuclear translocation in HCV replicating cells. Here we show that HCV employs a novel mechanism for immune evasion by specifically targeting TLR7 expression, mRNA stability, and function.

1. Diminished TLR7 expression caused by HCV induced TLR7 RNA instability

We identified a significant decrease of TLR7 expression in the presence of HCV infection both in vitro and in vivo. We established the direct effect of HCV replication on TLR7 expression, as indicated by restoration of TLR7 levels upon viral suppression. Among others, the most frequently encountered mechanisms of viral-dependent impaired expression of a cellular receptor include interference with gene transcription or protein translation, posttranscriptional and posttranslational receptor modifications and disruption of the downstream signaling events (249, 305, 306, 106). We identified that HCV infection, interferes at least with two of these events regarding TLR7. First, HCV infection interfered with the TLR7 gene during transcriptional regulation as shown by TLR7 mRNA instability in HCV replicating cells. Induction of host mRNA instability is a common evasion strategy employed by other viruses, including Herpesviruses, Vaccinia, and Influenza to bypass immune surveillance (249, 307, 308). Among the mechanisms of induction of host mRNA instability are association of viral proteins with several members of the mammalian exosome,

activation of the exosomal enzymes and/or recruitment of the exosome to mRNAs, usage of the exosome as a vehicle to access cellular mRNAs if the viral protein possesses RNase activity and enhancement of exosomal activity whether or not viral functions as an RNase; most often viruses employ more than one mechanism to achieve total or partial host shutoff (309, 310).

2. Further HCV-induced mechanisms for reduced TLR7 expression

The detailed mechanisms of HCV-induced TLR7 mRNA instability are yet to be discovered; it is unknown whether HCV produces siRNA's targeting TLR7 mRNA, exploits host regulatory factors that bind to TLR7 mRNA or uses an HCV protein to aid the TLR7 mRNA instability. HCV requires the use of liver specific host microRNA, miR122, for HCV replication (311). Further studies confirmed the utilization of RNAi by HCV for efficient viral replication (312). During HCV viral replication, it is quite possible that a product from viral RNA cleavage could target TLR7 mRNA in the cytoplasm. Although there is no evidence yet that HCV produces an siRNA to target TLR7, there is evidence that other viruses in plants have the ability to target host mRNA for degradation by producing their own viral siRNAs (313). Nevertheless, impaired TLR7 mRNA stability results in low levels of TLR7 protein expression during HCV infection. Since we did not investigate the mechanisms for reduced TLR7 protein, it may be possible that HCV utilizes the ubiquitin machinery to target TLR7 protein for degradation, thus reducing protein expression.

It is not impossible to ponder that HCV-induced TLR7 mRNA instability is not solely responsible for the observed impaired TLR7 protein levels. Several

viruses affect different steps of the host protein expression, including transcriptional, posttranscriptional and posttranslational modifications; such possibility in regards to TLR7 remains to be analyzed in the case of HCV (314, 315). Finally, by analogy with TLR3 and RIG-I/MDA-5, HCV may partially interfere with TLR7-mediated signaling pathways. We identified impaired TLR7-mediated activation of IRF7 in HCV replicating cells compared to controls. Because the baseline activation in HCV replicating cells was higher compared to controls, we concluded that TLR7-independent activation of IRF7 signaling pathways was intact in the presence of HCV infection. Baseline IRF7 activation has been correlated with endogenous IFN levels in some cells while other ligands like LPS have been shown to activate IRF7 also. In addition to TLR7, a number of other receptors can activate IRF7, such as TLR8, TLR9 or TLR3. It is unknown whether these receptors may play a role in the basal activation of both control hepatoma and HCV replicating cells. Further, these data narrowed TLR7 as a specific target of HCV by the fact that only TLR7, but not TLR5 was targeted during HCV infection suggesting the specific influence.

3. IFN-induced exhaustion from HCV infection?

A decrease in TLR7 levels during HCV infection might not affect the production of Type 1 IFNs via TLR7 signaling; therefore we tested whether the Type 1 IFN pathway was functional in the remaining TLR7 receptors. Our results revealed the TLR7-induced IRF7 nuclear translocation was significantly increased in control cells but it failed to appreciably enhance further from elevated background activation of IRF7 in HCV infected cell lines. These results indicated

that the remaining TLR7 receptors cannot fully compensate in function for the diminished TLR7 levels. We can not rule out the possibility that the high IRF7 baseline in HCV infected cells could cause an immune exhaustion of Type 1 IFN production. Further IFN production by viral stimulation could inhibit activation of other anti-viral interferon stimulated genes creating a negative feedback loop (316, 317). Suppressors of cytokine signaling (SOCS) expression is induced by Type 1 IFNs and SOCS are known to inhibit the IFNα receptor pathway (318).

Recent reports suggested a substantial cellular activation in HCV replicating cells and HCV infected patients without external stimuli (196, 247, 254, and 319). In chronically infected HCV patients, several groups reported an increase of interferon-stimulated genes (ISGs) before therapy and regardless of stimulation (247, 319). These reports support our findings of the initially high IRF7 nuclear translocation in the absence of stimulation in HCV replicating cells and suggest pre-activation of IRF7 nuclear translocation due to HCV infection.

In conclusion, we identified that HCV interferes with TLR7 expression and function. Our findings aid the understanding of the HCV-induced immune evasion mechanisms.

Model of Mechanisms

We propose a representative model for TLR2 co-receptor activation by HCV proteins in Figure 6.1. I propose that TLR1 and TLR6 in conjunction with TLR2 are activated by HCV proteins which stimulate the receptors to activate a cascade of events that produces a large quantity of inflammatory cytokines. These inflammatory cytokines can activate other adjacent cells in the liver, cause cell

death by over stimulation, immune cell deactivation by negative regulators stimulated by inflammatory cytokines. All of these effects of TLR1, TLR6, and TLR2 activation by HCV proteins may participate in creating a more permissive environment for HCV infection persistence. This research has left the possibility of other TLR2 co-receptors that are activated by HCV proteins or other receptors beyond TLR2 that are activated by HCV proteins.

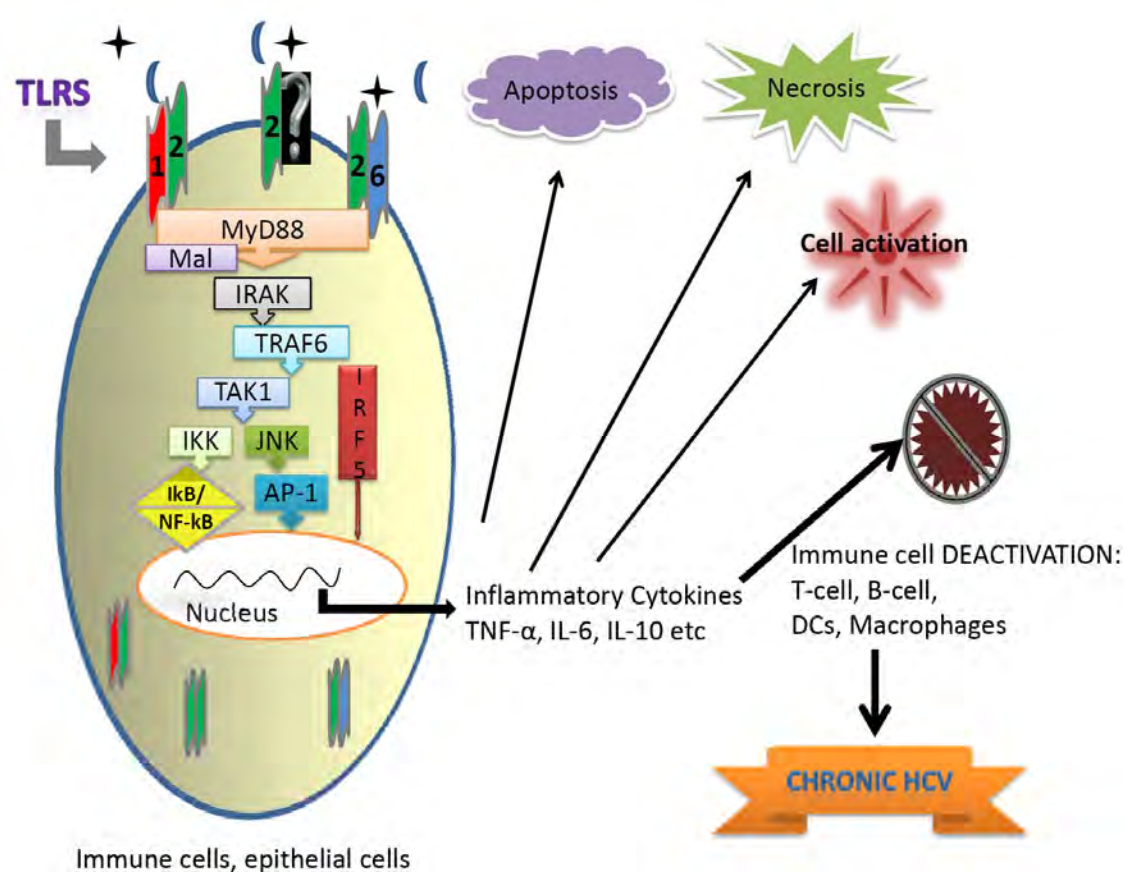


Figure 6.1: TLR2 co-receptor mechanism model for cellular activation by HCV core and NS3 proteins. HCV proteins representated by the black star and blue moon binds TLR2/1 or TLR2/6 complexes with the possibility of TLR2/? receptor that also is activated by HCV proteins. TLR2/1 or TLR2/6 activation leads to initiation of events dependent on the MyD88 pathway that results in translocation of numerous transcription factors, in particular NF-κB, into the nucleus. Inflammatory cytokines produced by these events can cause cell activation, cell death, or immune cell deactivation which can all aid in chronic HCV infection.

HCV does not induce cell lysis, but signaling to other immune cells from infected cells may cause their cell lysis. Viral products such as HCV NS3 protein may be released in the serum upon cell death and it is here where it may be possible for NS3 to activate TLR2/1 or TLR2/6 on the cell surface. We have also been able to locate TLR2 intracellularly by flow cytometry (data not shown). NS3 protein as a protease cleaves TRIF and IPS-1 in the cytoplasm, therefore it is possible that NS3 may be autophagosized by the cell as a host cellular defense mechanism where TLR2/1 or TLR2/6 complexes may be traveling intracellularly to the surface. These mechanisms might explain the activation induced from NS3 on TLR2 and its co-receptors. Without further research in this area the exact mechanism is still unknown yet important to understand as NS3 is immunogenic.

Immune evasion mechanisms are highly prevalent in chronic HCV infected cells. In Figure 6.2, I outline the mechanism of HCV immune evasion involving reduced TLR7 levels and decreased mRNA stability. HCV infection correlates with reduced TLR7 mRNA and protein levels in hepatoma cells. HCV replication causes decreased TLR7 mRNA stability but increased endogenous IRF7 nuclear translocation. Reducing TLR7 RNA expression will ultimately reduce TLR7 protein expression as there is less RNA to translate from. Lowered TLR7 levels will reduce the Type 1 IFN response and inflammatory cytokine response. We did not investigate the mechanism of how TLR7 mRNA expression is destabilized or possibly reduced. HCV core has been found in the host nucleus and it is possible that core may bind to sites, such as IRF1, within the region of the TLR7 promoter to negatively regulate TLR7 transcription. Increased

endogenous IRF7 levels may cause cytokine tolerance or exhaustion as an overabundance of cytokines can cause activation of negative regulators that inhibit anti-viral pathways or also TLR7 transcription pathways. It is unclear whether the stunted IRF7 nuclear translocation response from TLR7 ligand stimulation is due to the already increased endogenous IRF7 translocation levels or the decreased TLR7 levels. We did not explore the possibility that HCV infection may inhibit TLR7 protein stability as a mechanism for the reduced TLR7 protein levels. By decreasing TLR7 expression and yet another Type 1 IFN inducible pathway, hepatocytes are more permissive for chronic HCV infection.

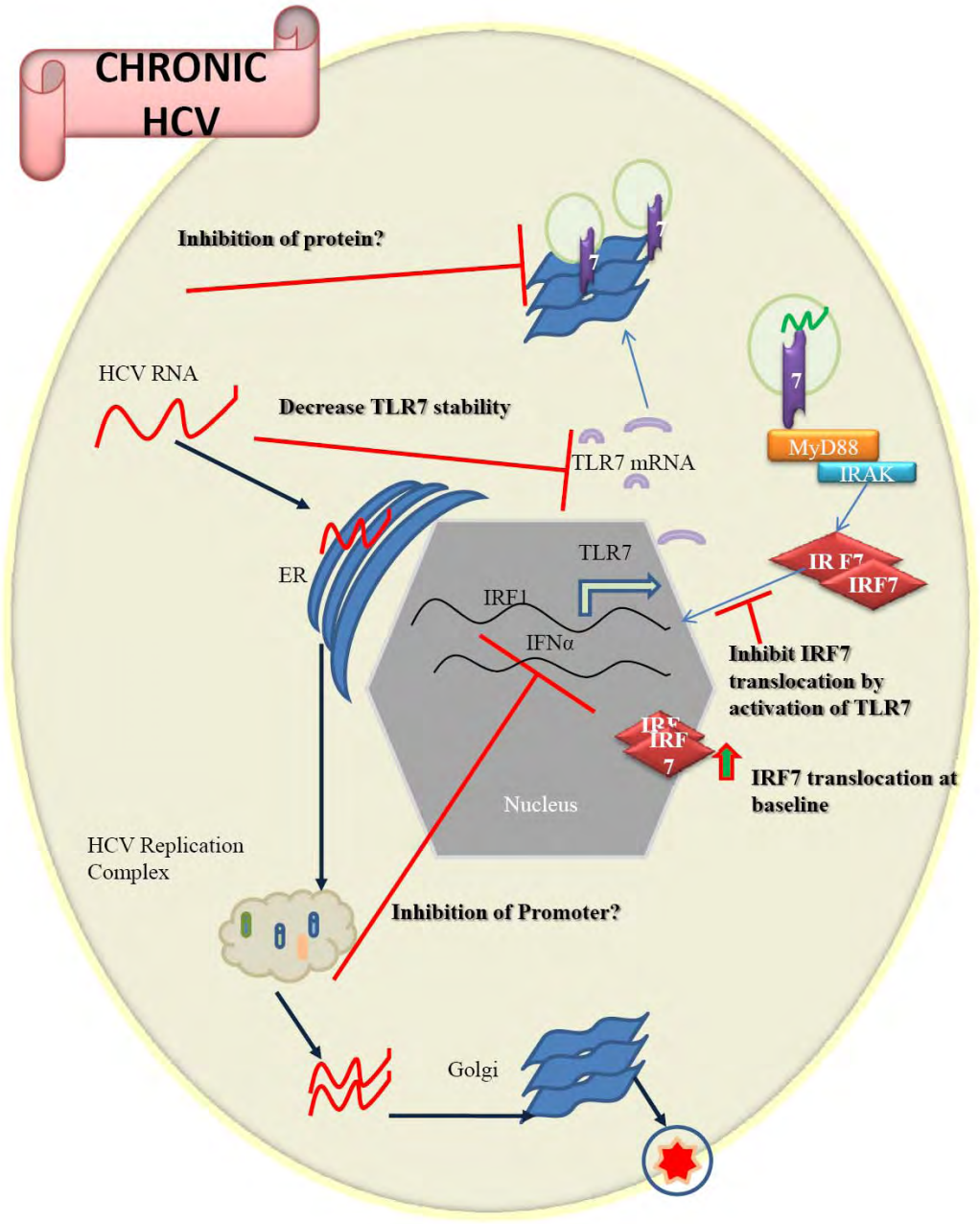


Figure 6.2: Model for contributions to HCV persistence: reduction of TLR7 expression. Model of possible mechanisms to reduced TLR7 expression in HCV replicating hepatoma cells. HCV RNA shown as a red curved line goes through replication in the cytoplasm and within the endoplasmic reticulum (ER) membrane. This replication causes reduced TLR7 mRNA (shown in purple) stability which ultimately reduces TLR7 protein expression. HCV infection also causes increased IRF7 translocation shown with the red and green arrow, yet upon TLR7 activation, there was a stunted increase of IRF7 nuclear translocation. A mechanism for reduced TLR7 expression could be HCV protein negative regulation in the nucleus by inhibiting binding sites at the TLR7 promoter. Another possible mechanism to reduced TLR7 expression could be inhibition of the protein by increasing TLR7 protein degradation. All of these mechanisms could contribute to chronic HCV infection.

Conclusions

The results presented in this composition show various mechanisms for the manipulation of the host innate immune system by Hepatitis C virus. We provide evidence for two separate means that the virus upon infection utilizes for survival in the liver. One mechanism involves an immune exhaustion by Kupffer cells and hepatocytes in which HCV stimulates these cells to manufacture excessive amounts of inflammatory cytokines, IL-8, IL-6, and TNF-α. This mechanism has the possibility to not only confuse the adaptive immune system by over stimulating the cells and masking infection but also to exhaust both arms, innate and adaptive, of the immune system ultimately leading to tolerance or anergy. Treatment for infection will not clear the virus unless the host has working defense mechanisms. The other mechanism is quite the opposite of stimulating the cells non-specifically, but quietly manipulating anti-viral facets of the cell to leave it unprotected. HCV immune evasion is well documented among the innate faction, from HCV NS3/4A cleavage of TRIF and MAVS to HCV E2 or NS5A binding and inhibition of PKR. Increased degradation of TLR7 RNA is a completely novel mechanism exhibited by HCV, yet well used among other viruses. Despite their opposing natures, when exploited together, HCV as an ever developing virus against host immunity is able to assemble these mechanisms for near invincible survival.

CHAPTER VII

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