UNMASKING A SILENT KILLER: UNCOVERING THE MECHANISM OF HEPATITIS C VIRUS PROTEIN NS5A-MEDIATED INHIBITION OF THE INNATE IMMUNE RESPONSE

by

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Introduction

Hepatitis C disease

Hepatitis C (Hep C) is an infectious disease caused by the Hepatitis C virus (HCV). The Centers for Disease and Control estimates 170-200 million people world-wide suffer from chronic HCV infection [1,2]. In the US alone there are about 45 000 new cases of HCV infections reported each year with about 20% of the cases ending in death [3]. Pathogenesis of Hepatitis C begins with persistent liver inflammation which leads to liver fibrosis and given time, develops into cirrhosis, massive liver scarring, and finally to end stage liver disease. All through the disease progression the patient is at risk for developing liver cancer. In recent years, instead of AIDs, infection by the hepatitis C virus has emerged as a major public health problem as there is no vaccine and the success rate of the standard and new therapies are far from ideal [4].

A master at concealing from the innate immune defenses of the host, HCV is able to hide out in the human body for more than 50 years [5]. Often the initial HCV infection is asymptomatic or if present, the symptoms are mild, flu-like and nonspecific. As a result, most of the people who have chronic hepatitis C are unaware of the infection and lead normal lives. It is only when the liver becomes cirrhotic beyond repair that symptoms of liver disease become apparent [6]. Generally in a population of newly infected individuals only about 20% of them will spontaneously recover from the infection. Most often HCV infection will develop into chronic hepatitis. Once a patient proceeds to end stage liver disease death becomes inevitable without a transplant [7].

Treatment and Therapy

Persistent infection and resistance to IFN based therapy are the two hall marks of HCV pathogenesis. Treatment of HCV infection is difficult as the 6 virus genotypes each display marked differences in disease progression and response to therapy [17]. Of the different strains infecting humans, genotype 1 is the most wide spread and hard to treat. Further, as the virus replicates in a person the error prone RNA dependent RNA polymerase generates various subtypes within each genotype. The great sequence variability between each HCV particle becomes a natural defense against the host immune system and a great challenge for preventive medicine [16, 17].

The liver is an indispensable organ that plays an integral part in many metabolic processes in the human body. While long term HCV infection methodically destroys the function of this essential organ it is only until recently that the demand for a more effective anti-HCV treatment was met [8]. For the past 20 years, the standard therapy for HCV patients, long acting interferon-alpha (IFN-α)-an innate immune booster- and ribavirin-a viral replication inhibitor, offered low success rates, a menagerie of adverse side effects, and are ineffective against the most prevalent strain of HCV in the US, genotype 1.

The year 2012 ushered a new era of treatment for HCV patients. The Food and Drug Administration (FDA) approved two new medications, synthetic molecules that are designed to inhibit viral protease function. This brought forth a new era of direct acting antivirals (DAA) that specifically targeted the enzymatic proteins of HCV [9]. The new drugs greatly improved the cure efficacy and target range of the existing HCV therapies. However, the drugs when used alone are unable to maintain a sustained virological response (SVR). As most RNA viruses have fast mutation rates and can quickly change the drug targets so can HCV quickly become resistant to its DAAs.

Thus, to maintain a SVR in Hep C patients, the new drugs must be used in conjunction with the older treatments. The current standard of care for Hep C patients are a combination of pegylated IFNα, the nucleoside analog ribavirin (RBV), and a direct acting antiviral (DAA) that specifically targets the viral protease, Boceprevir/ telaprevir , NS3/4A protease inhibitors [10]. Unfortunately, when combined with the latest FDA approved direct-acting antiviral drugs, the already debilitating side effects of HCV therapy compounded. Indeed, in December of 2012 the FDA reported 2 fatalities out of 112 patients that developed serious skin reactions from the combination HCV treatment given to them [36]. Furthermore, many patients who are unable to tolerate the side effects of IFN are deemed ineligible for the IFN based therapies.

The discovery and development of Sofosbuvir, a DAA that inhibits NS5B HCV polymerase, by Gilead Sciences in late 2013 herald the new age of an interferon-ribavirin free therapy. In fact two independent clinical trials published in the New England Journal of Medicine that year showed that Sofosbuvir when either combined with ribavirin or two other DAA's resulted in a 90% cure rate in patients with chronic HCV, in contrast with the standard therapies of today which have a 70-75% cure rate with the addition of high dropout rate due to side effects to IFN. The clinical trials went so well that FDA had granted priority review status to Sofosbuvir because for the first time in hep C history the possibility of a cure may be at hand [37].

HCV life cycle, genome, genes, protein function

HCV is a small enveloped 9600b RNA virus 55nm in diameter. It is classified under the family *flaviviridae* in the genus of *hepacivirus* [11]. To date humans and chimpanzees are the only known hosts that are naturally permissive to HCV infection [12]. Entering through the blood, HCV is carried to the liver. In the liver, hepatocytes are the primary locations for HCV replication [13]. To summarize, the HCV life cycle begins as the virion envelope glycoprotein attaches to its target receptors on the host cells, followed by the virus entering the cell through receptor mediated endocytosis and the fusion of viral envelope to the endosome. Once the viral genome is released into the cytoplasm it is translated and replicated. At the end of HCV life cycle the viral progeny are packaged and released from the host cell [11, 12, 13].

Even though the mechanisms of host and viral factor interactions are not completely understood, the growing consensus are that multiple cellular factors and viral components work together to allow HCV entry. Over the years many host cell receptors were implicated in facilitating HCV entry. Receptors such as Tetraspanin (CD81), Scavenger receptor class B type I (SR-BI), and tight junction proteins such as Claudin-1 (CLDN1) and Occludin are postulated to be essential host receptors for HCV entry [39]. A recent study published by Sainz B et al. in Nature Medicine 2012 revealed that silencing the expression of a cholesterol uptake receptor Niemann-Pick C1–like 1 (NPC1L1) on host cells directly impacted HCV infectivity [38].

In the cell the entire RNA genome of HCV is translated into one polyprotein of approximately 3000 amino acids. The coding sequence on the viral RNA genome is flanked by two untranslated regions (UTR) at its 5' and 3' termini. Both UTRs are critical in the propagation of the virus [14]. The internal ribosomal entry site located at the 5' UTR is required for the cap-independent assembly of the host translational machinery for protein synthesis and the secondary structures assumed by the 3' UTR is required in the initiation of replication. After translation the polyprotein is processed by the host and viral proteases to generate HCV structural and enzymatic polypeptides [15].

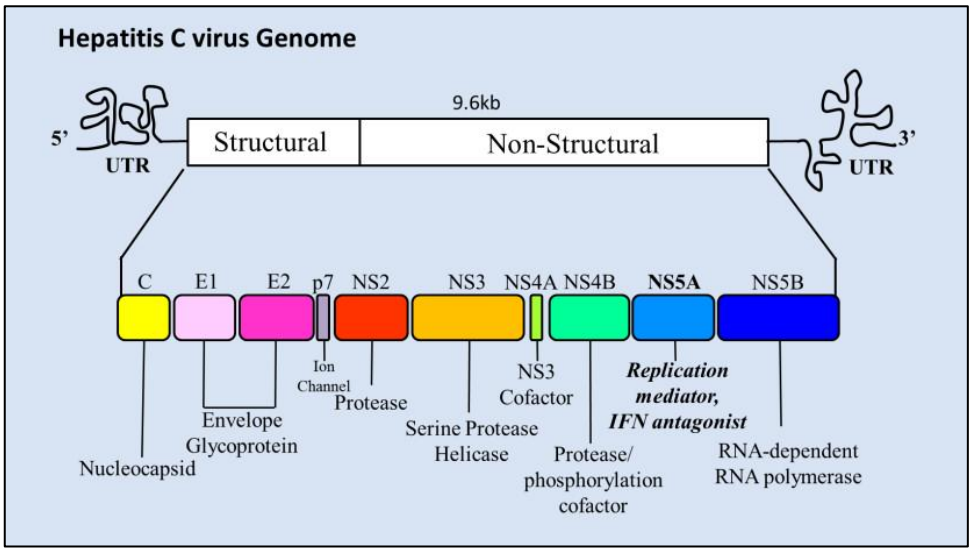


Fig. 1. **Organization of HCV genome.** (Top) organization of HCV positive sense RNA genome structure. The 5' and 3' are essential for viral protein expression and replication. An internal ribosome entry site (IRES) is found within the 5' UTR that mediates cap-independent translation of viral polyprotein. (Bottom) The polyprotein is separated into 10 individual viral proteins with C, E1,and E2 being structural proteins and the rest being viral enzymes that is required for viral replication assembly and production of progenies.

The polyprotein gives rise to 10 individual proteins. Structural proteins are found at the N-terminal part of the polyprotein while the non-structural proteins make up the rest. The core protein and the E1, E2 glycoproteins are the structural proteins that form the viral particle. A P7 viroporin protein which forms an ion channel is found at the junction between the structural and non-structural proteins, and remainder of the polypeptide codes for the non-structural (NS) proteins [14].

NS2, NS3, and NS3/4A are viral proteases (NS4A is a co-factor of NS3). NS3 is also a helicase. NS4B is a membrane protein essential for the production of a replication platform. NS5A is a phosphoprotein found to facilitate the formation of the viral replication complex, and NS5B is the viral RNA-dependent RNA polymerase (fig 1).

Replication of the viral genome is a two-step process. First, the positive strand RNA must be translated and processed into viral proteins that make up the replicative platform for viral replication. Next the replicase complex formed by a conglomeration of proteins including NS5A, NS5B, NS3 and various host cellular components along with the NS5B RNA dependent RNA polymerase make a complimentary negative sensed RNA genome from the positive RNA genome. The negative sense strand then becomes the template for all future positive sense HCV RNA genomes [16].

Antiviral response by the innate immune system

Interferons (IFN) are a family of cytokines utilized by the innate immune system to mediate an antiviral and anti-proliferative response in infected cells and the surrounding[18]. IFNs are grouped according to the type of receptors they signal through. To date three types of IFNs are known: Type I, II and III. IFNα, IFNβ, and IFNω, belong to the type I IFNs, while IFNγ is the only IFN in type II IFN, and IFNλ in type III [18, 19]. By binding to specific transmembrane receptors, type I and II IFNs activate the JAK/STAT signaling pathways that trigger the expression of a large number of genes, such as Protein Kinase R (PKR), 2'-5' oligoadenylate synthase (OAS1) , Chemokine ligand 5 (CCL5), that encode proteins that interfere with pathogen propagation and the activation of immune cells [20].

To recognize foreign pathogens the innate immune system utilizes evolutionarily conserved pattern recognition receptors (PRRs) found both in and on the cell. These receptors are sentinels that recognize parts of pathogens that are shared across many species, termed pathogen-associated molecular patterns (PAMPs). During virus

replication their replication intermediaries are recognized by the PRRs in the cytoplasm, and through a series of adapter proteins, the PRRs activate transcription factors that lead to the production of IFN-β [18].

In short there are two parts to the antiviral response. First the host recognizes viral components by Toll-like-receptors (TLRs) and (retinoic acid-inducible gene 1) RIG-I PPRs that identifies the PAMPS. When a part of the pathogen is recognized a signal is sent through a series of downstream effector proteins such as TRIF, MyD88, and IPS-1/MAVS. The subsequent activation of the transcription factors IRF3, NF-kB, and ATF2/c-JUN in turn leads to the initial production and secretion of IFNβ.

The second part of the antiviral response begins when the secreted IFNβ binds to its respective trans-membrane receptors on the cell surface. The activated receptors signal through the adaptor protein (Janus kinase) JAK. JAK in turn phosphorylate the transcription factor signal transducer and activator of transcription (STAT) and along with other cellular factors they initiate the expression of genes with inflammatory, antiproliferative, and antiviral properties (eg. TLR3, TLR7, RIG-I, RANTES, p53, OAS1, IFNβ, PKR etc.) Upon completion the innate immune antiviral response establishes an antiviral state in the infected cell and cells surrounding it.

Interactions between cell and virus

Normally viral infection induces host cells to mount an antiviral response to the pathogen resulting in the production of IFN-β in the cells. The infected cell then secretes the IFNβ allowing the cytokine to bind to their receptors and cause the cells

to express antiviral proteins such as PKR, OAS, CCL5/RANTES, and IFNα [18,19]. However, this immune response is thwarted in HCV infections. To persistently infect the cell HCV must disrupt the immune system, and research has shown many HCV proteins interfere with the effector proteins that mediate the antiviral signaling cascade [19].

In the cell, Toll-like receptor (TLR) family and RIG-I are PRRs that activate antiviral pathways. TLR family detects viruses that enter the cell through endocytosis and those that escape into the cytoplasm are recognized by the RIG-I helicases. RIG-I is a receptor that recognizes intracellular dsRNA. The TLR family signals through adaptor proteins such as TIR domain-containing adapter inducing IFN protein, (TRIF) and Myeloid differentiation primary response gene 88 (MyD88) that leads to the activation of transcription factors, ATF-2/c-Jun, NF-κB and IRFs.

RIG-I signals through the adaptor protein that resides in the mitochondrial membrane, mitochondrial anti-viral signaling protein (MAVS). MAVs in turn, through a series of unidentified downstream adaptor proteins, lead to the activation of transcription factors，NF-κB and IRF3. Signaling through both pathways lead to the induction of IFN-β [17].

Studies have shown various HCV proteins to interfere with components of the antiviral pathways. For example, HCV NS3/4A is found to cleave PRR's down-stream effector proteins such as MAVs, the T-cell protein tyrosine phosphatase (TC-PTP), and the TRIF to ablate ds-RNA mediated IFNβ production [21, 22]. HCV NS4B has shown to inhibit PRR-mediated signaling through association with the stimulator of interferon genes protein (STING). [23] Evidence has shown HCV core protein to interfere with IFN

signaling through IFN receptor by degrading STAT1 protein, a type I IFN activated transcription factor that turns on the expression of antiviral proteins [24].

Non-Structural Protein NS5A

Amongst all the HCV proteins, in our lab, NS5A has been an object of interest. Despite its lack of enzymatic activity NS5A is known to play a paramount role in regulating viral replication, infection, and HCV countermeasures against the innate immune response [28,30, 35]. The importance of NS5A in promoting HCV pathogenesis is revealed by the subpicomolar activity its small molecule inhibitor Daclatasvir (BMS-790052) has on HCV infection in cell based assays. In fact Nature reviews commented that the new NS5A inhibitor seem to be the most potent antiviral ever discovered [33].

HCV NS5A protein is classified as a phosphoprotein because in nature it exists in multiple phosphorylated forms. It is postulated that the phosphorylation state of NS5A is a major determinant of what biological functions it is to perform [31]. Crystal structures of the NS5A suggested that it has 3 domains. The N terminus, in addition to a transmembrane region, has a domain that is capable of Zn^{+2} and RNA binding. The transmembrane domain is essential for the formation of a membranous web from host membranes to house the HCV replicase and the zinc and RNA binding motifs are essential for viral RNA transcription [34]. The other two domains, due to their lack of structural motif, are postulated to facilitate protein-protein interactions between NS5A and host and viral proteins [31].

At its C terminus NS5A contains a putative nuclear localization signal (NLS) and a

region enriched with proline residues and this positively charged structural feature is found within many eukaryotic transcriptional factors [32]. Studies by Tanimoto. et al., not only revealed that the C-terminus half of NS5A functions as a potent transcriptional activator they also identified the essential amino acid sequences that permit maximum transcription activity. Their study suggests that NS5A may directly influence cellular gene transcription.

NS5A and the antiviral response

Interestingly in a clinical setting an observation was made regarding HCV NS5A. Variations in the NS5A coding sequences have a deterministic effect on the outcome of Hep C therapy and experimentally certain mutations within the carboxylic end of the NS5A have been shown to increase the efficacy of IFNα treatment[25]. The region where these mutations occur has been termed IFN sensitivity-determining region (ISDR). Many studies have focused on the mechanisms where NS5A mediates the shutdown of IFN signaling. Studies by Gale, M. et al. have shown that NS5A interacts with PKR at its ISDR to inactivate PKR activity [26,28]. Another group, Taguchi, T. et al., showed that NS5A also interacts with 2'-5'OAS to ablate its antiviral function[27,28].

In 2004 Sumpter R. et al. investigated the relative replication rates of different HCV RNA replicon variants in mammalian cell cultures while simultaneously treated with IFNα. Their studies showed that, while the cells were in an antiviral state, when compared to the parental strain, some variants replicated with high efficacy, while some replicons at a very low. Replicons are sequences of virus genome, removed of the structural genes that can replicate autonomously.

The group sequenced the variants and found that both replicons differed from the wild type strain only by one amino acid within the NS5A-coding region. The variant that replicated robustly had a deletion of a lysine residue at the 2040 position in the NS5A amino acid sequence, and was given the name NS5A-10A and the variant that replicated poorly had a substitution of its leucine residue by a serine at 2198 position of the NS5A sequence, and was given the name NS5A-H27. Subsequently the NS5A protein was constructed from both replicons. [29]

Correlating how NS5A mutations contribute to the replicative success of the HCV replicon systems and affect the outcome of IFN-based HCV therapy in the clinic and how IFNβ production is required to inhibit replicon replication, our lab questioned whether NS5A variants can affect a host's ability to mount an antiviral response, characterized by the production of IFNβ. Utilizing cell IFNβ levels as an indicator of the degree of the antiviral response we looked at the IFNβ levels produced by infected cells co-expressing NS5A-10A. Our study showed that the level of IFNβ in cells with NS5A-10A protein is significantly lower than in the control cells.

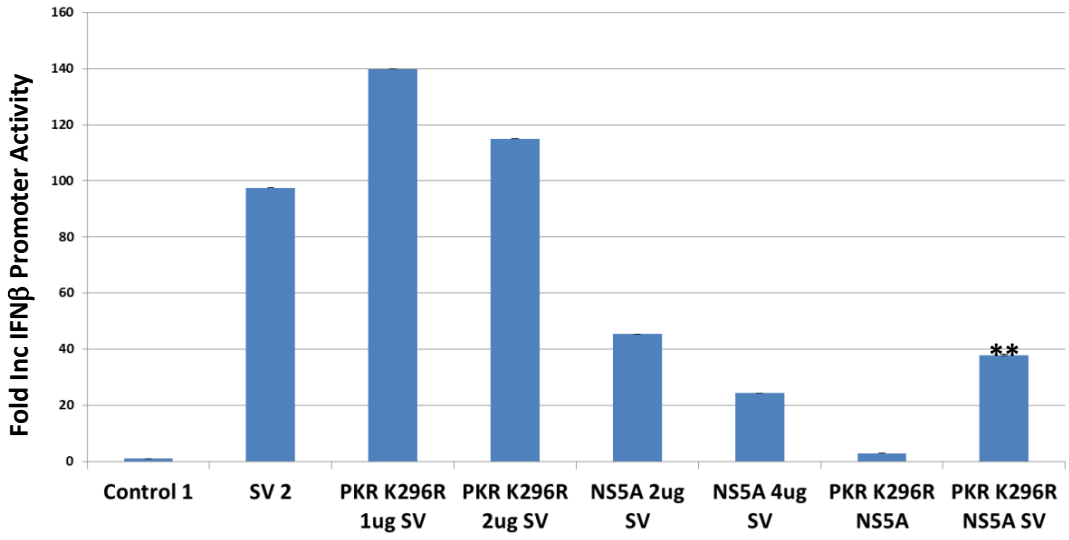


Fig. 2. **PKR is not involved in NS5A-mediated inhibition of the IFNβ promoter**. NS5A was originally identified as an inhibitor of PKR- a key player in the antiviral response. Here, we transfected 293HEK cells with a dominant negative mutant of PKR and subsequently infected with SV. If PKR is required for IFNβ promoter activation, dominant negative PKR should inhibit the promoter. $P<0.0001$ by one-way ANOVA followed by Tukey's post.hoc for Control.

It is possible that the decrease in IFNβ promoter activity may be caused by NS5A and PKR association during viral infection. However, previous studies in our lab with a dominant negative form of PKR showed no decrease in SV induced IFNβ promoter activity in 293 HEK cells revealed that NS5A inhibition of viral induced IFNβ promoter activity is not mediated through PKR inhibition (Fig. 2; G. Akkaraju, Unpublished data).

In this study we were interested in deciphering the mechanism whereby NS5A exerts its inhibitory effect on IFNβ production during viral infection.

MATERIALS AND METHODS

Cell Culture

HEK293 (human embryonic kidney fibroblast cells) and Hela (human cervical cancer cells), expressing the hyperactivated mutant form of NS5A-10A, and transiently transfected cells were maintained in Dulbecco's essential modified eagles' medium (DMEM) supplemented with 10% fetal bovine serum, 1% 2nM L-glutamine, and 1% antibiotics (100ug/ml penicillin and streptomycin). All cell lines were cultured in 5% CO^2 and 95% air incubator at 37^oC. NS5A plasmids (wild type, 10A, H27) were a gift from Michael Gale (University of Washington). NS3/4A plasmids were a gift from James Chen.

Transfections

Transient expression of HCV NS5A-WT, NS5A-10A, NS5A-H27, and NS3/4A genes. HEK 293 cells were transfected with expression vectors containing the above mentioned genes. $5x10^5$ cells were seeded on 60mm plates and then transfected with plasmids that expressed NS3/4A, or individual NS5A proteins. For a standard 60mm plate containing 4ml medium 1ug of plasmid was mixed in 100ul of LyoVec (Invivogen), a lyophilized cationic lipid-based transfection reagent. The mixture was allowed to equilibrate in room temperature for 20 min and then added drop-wise to the cells. Cells were incubated and harvested after 48h post transfection.

Sendai virus and TNF-α challenge

Sendai virus (SV) single stranded positive sense RNA virus (Cantell Strain) purchased from Charles River Labs was used to stimulate cells to mount antiviral response mimicking HCV infection with a final concentration at 4000 HAU/ml. TNF-α is an inflammatory cytokine that activates NF-κB pathway it was used to trigger pathway activation in cells. Final concentration was 10ng/ml.

Antibodies

Primary

Anti-IRF3 rabbit polyclonal IgG Santa Cruz sc-9082

Anti-tubulin mouse monoclonal IgM Santa Cruz sc-8035

Anti-Flag M2 mouse monoclonal IgG Sigma F3165

Anti- HCV NS5A mouse monoclonal IgG Santa Cruz sc-52417

Secondary Antibodies-Western Blot

Goat anti-mouse IgM alkaline phosphatase AP conjugated Santa Cruz sc-2070

Goat anti-mouse IgG AP conjugated Santa Cruz sc-2008

Goat anti-rabbit IgG AP conjugated Sigma A-3687

Secondary Antibodies-Immunofluorescence

Goat anti-mouse IgG Alexa Fluor 546 Invitrogen 45705A

Goat anti-rabbit IgG Alexa Fluor 488 Invitrogen 34639A

Protein Analysis by native polyacryliamide gel electrophoresis (PAGE) and Western Blot

Cytosolic cell extracts were prepared by adding 100ul of lysis buffer to collected cells (20mM Tris-Cl, [pH7.5], 150mM NaCL, 10% Glycerol, 0.5% NP40, 1mM Na_3VO_4, Roche EDTA free protease inhibitor cocktail). Cell buffer mixture was left on ice in room temperature for 5min, and centrifuged in 4^oC at 14,000 rpm 5min. Supernatant was isolated as cytosolic fraction. Lysis buffer from above was supplemented with Sodium deoxycholate (DOC-Na2) to a final concentration of 0.5% DOC-Na2 and added to the remaining cell pellet to extract nuclear proteins. Cytosolic and nuclear protein fractions were evaluated using the Bradford assay (Bio Rad). Equal amounts of proteins were electrophoresed on a 9% native PAGE gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was subsequently blocked in Tris buffered saline (TBS) containing 0.1% Tween 20 and 5% milk proteins. Proteins were probed with antibodies against IRF3, tubulin, Flag, NS3/4A, and NS5A, followed by Alkaline Phosphatase (AP) tagged secondary antibodies. Protein bands were visualized by AP staining with substrate Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in dimethylforamide (DMFA). Beta-actin was probed as a loading control, and tubulin was probed as a cytoplasmic marker.

Immunofluorescence

$4x10^4$ cells were seeded on glass coverslips in six well plates. Cells were transfected with NS3/4A expression vector and infected as described. 48 hours later

cells were removed of medium rinsed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature. Removed PFA and added 0.2% Triton X-100 in PBS for 15 min to permeabilize cells. Removed solution and incubated cells in 10% goat serum PBS 1hr room temperature. Rinsed with PBS and added 1:500 primary antibody in PBS to cells incubated in room temperature for 1 hour. Subsequently washed the cells 3 times with 0.5% Tween in PBS, added Alexa Fluor conjugated secondary antibody to cells, and cells were incubated in the dark for 1 hour room temperature. Cells were again washed 3 times, air dry, and mounted on to glass slides with fluoromount-G (Southern Biotech). Coverslips sides were sealed with clear nail polish to prevent drying up. Fluorescence pictures were taken using a Carl Zeiss Axio Observer.Z1 LSM 710 confocal microscope under 63x objective oil immersion.

RNA extraction and Reverse Transcription to cDNA

Total RNA was isolated from SV challenged cells. RNA isolation was performed using TRI reagent (SIGMA) according to manufactures protocol. Total RNA was treated with DNAse using the Turbo DNA-free kit (Ambion Life Technologies) and quantified using Nano Drop measuring absorbance at 260nm. RNA integrity was determined by running the samples on 1% agarose gel. RNA purity was determined using absorbance ratio at 260:280nm. Only RNA with a purity ratio of $\geqslant$1.80 was used to synthesize cDNA. cDNA was synthesized using high capacity cDNA reverse transcription kit from Applied biosystems. Reverse transcription (RT) was performed by incubating 5 ug of total RNA in a 10ul volume, 2uM random primer, 2uM RT buffer,

0.8uM dNTP, 4.2ul H20, and 250 units of Superscript III reverse transcriptase (Invitrogen) in a 20ul reaction volume for 10min at 25°C followed by 40min at 50°C.

Real time polymerase chain reaction (PCR) assay

Quantification of IFN-β, CCL5, OAS1, and P53 gene expression profile in SV challenged 293 cells expressing different NS5A proteins. PCR amplification is carried out using human gene specific primers for genes of interest with Power SYBR Green PCR Master Mix (Applied Biosystems). Primers to Glyceraldehyde phosphate dehydrogenase (GAPDH) used as an internal control. cDNA is amplified with IFNβ (5' CAG CAA TTT TCA GTG TCA GAA GC; 3' TCA TCC TGT CCT TGA GGC AGT), CCL5 (5' TTT CTT CTC TGG GTT GGC AC; 3' CTG CTG CTT TGC CTA CAT), OAS1 (5' TGT CCA AGG TGG TAA AGG GTG; 3' CCG GCG ATT TAA CTG ATC CTG), and P53 (5' CAG CAC ATG ACG GAG GTT GT; 3' TCA TCC AAA TAC TCC ACA CGC) specific primers. All reactions including no-template controls were run in triplicate and quantitated on an ABI 7500 Real Time PCR System. Expression level of gene of interest mRNA will be evaluated relative to that of GAPDH in ABI Prism 7500 sequence detection software V.1.4 (Applied Biosystems). The average threshold (Ct) was determined for each gene and normalized to GADPH mRNA level as an internal normalization control. Relative expression was calculated using the comparative threshold cycle (Ct) method. The real time PCR was done using 25ul of total reaction mixture- 12.5ul SYBR Green Mix, 0.2ul cDNA, 1ul primer pair mix (5 pmol/ul each primer), and 11.3ul H2O.

Dual Luciferase assay

Luciferase reporters driven by 3 copies of an NF-κB responsive element (3xNF-κB-luc), IFNβ promoter sequences (IFNβ-Luc), and CMV promoter driving renilla luciferase (RL-CMV) were used in this study. 293 HEK, 293 NS5A, 293 NS3/4A cells (5 x 10^4cells/well) growing in 12 well plates were transfected with 100ng of the indicated reporter plasmid using Lyovec reagent (Invivogen). Normalization of transfection efficiency was done by co-transfection with 50ng/ml of RL-CMV. 24 hrs later cells were treated with SV or TNF alpha. Day after cells were harvested in passive lysis buffer 100ul/ml (Promega). Luciferase activity was measured with a dual luciferase assay kit (Promega) and a luminometer.

20ul aliquot of cell mixture was used for luminescence measurements in a Berthold luminometer (Sirius Detection System). 50ul of firefly luciferase reagent (LARII) was first added to sample with measurement of luminescence with a 10 sec time lapse, following by addition of 50ul of Renilla luciferase reagent (Stop & Glo) luminescence measured as above. The promoter activity was assayed by sequentially measuring the firefly and Renilla luciferase activity from the same sample. The results were expressed as the ratio of firefly to Renilla luciferase activity (F-Luc/RL-Luc). Data were collected from at least three experiments and standard deviation was calculated.

Results

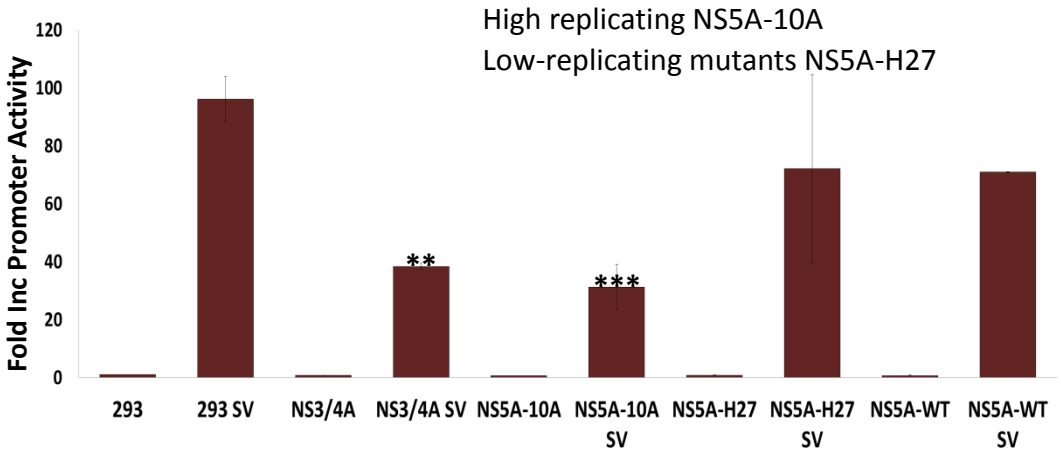
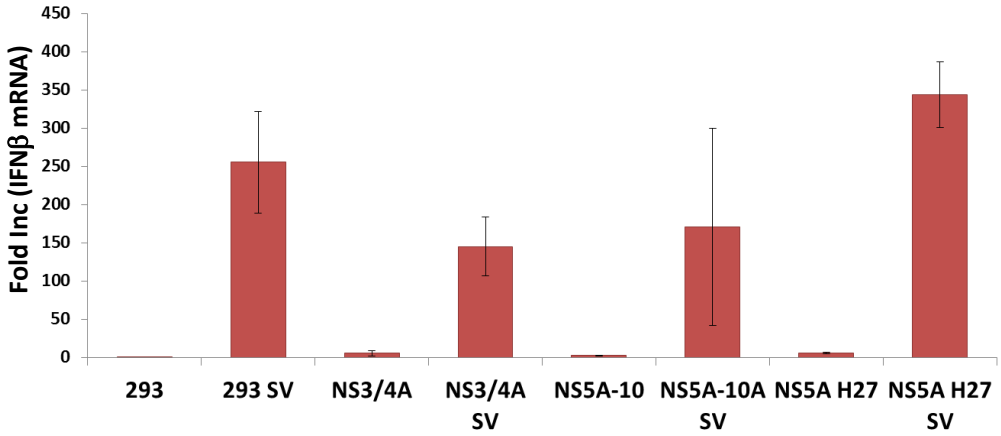


Fig. 3. **Spontaneously generated mutants of NS5A have varying effects on Sendai Virus (SV)-mediated IFNβ promoter activation.** High replicating and low-replicating mutants of NS5A were isolated from replicon cell lines. The mutants were over-expressed in 293 cells transfected with an IFNβ-luciferase reporter gene. NS3/4A, a known inhibitor of the RIG-I mediated signaling pathway was used as a positive control. P=0.0006 by One way ANOVA followed by Tukey's post hoc for SV control.

NS5A-10A inhibits IFNβ promoter activation in Sendai virus (SV) challenged 293HEK Cells. Since the entire early antiviral response largely centers on the ability of the cell to produce IFNβ and the sequence of NS5A variants are correlated to a patient's response towards IFN-based therapy, we investigated whether different NS5A variants may affect the ability of the cell to activate the IFNβ promoter. To investigate whether the expression of NS5A-10A in HEK 293 cells could interfere with innate immune response, characterized by the production of IFNβ, we transfected plasmids that encode the Luciferase reporter under the control of IFNβ promoter (IFNβ-Luc) into a 293 HEK cell line over-expressing the NS5A-10A gene, and infected the cells with SV, a known activator of the antiviral pathway.

As a control HEK293 cells transiently expressing the HCV NS3/4A gene are transfected with IFNβ-Luc. Both NS3/4A is a known inhibitor of the IFNβ mediated antiviral response. NS3/4A terminates the antiviral response by cleaving the mitochondrial antiviral signaling protein (MAVS) downstream of RIG-I. The level of IFNβ promoter activity was measured by the relative amounts of light produced by the Luc protein. Compared to SV infected HEK 293 cells a 40% reduction in IFNβ promoter activity was observed in the infected NS5A-10A expressing 293 cells. As expected, expression of NS3/4A strongly inhibited promoter activity (Fig. 3).

4a



4b

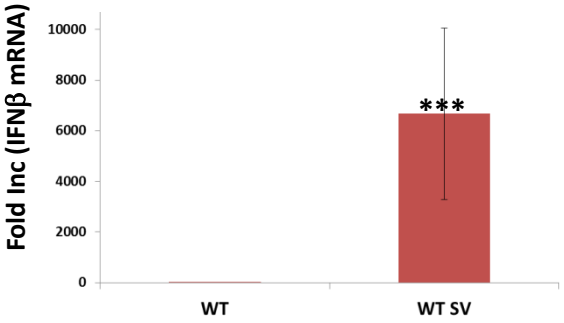


Fig. 4. **HCV NS5A K2040 inhibits expression of IFNβ mRNA.** 293HEK cells were transfected with different mutants of NS5A and infected with SV following which RNA was isolated and subject to qPCR. The results confirm those obtained using the promoter-reporter assay. 4b: WT. P=0.0001 by One way ANOVA followed by Tukey's post hoc for SV control.

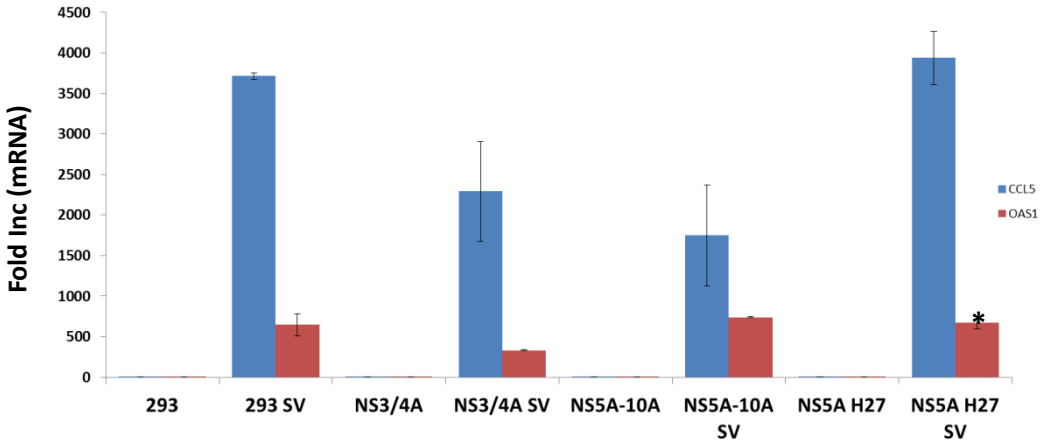
NS5A variants alter the expression of endogenous IFNβ levels in infected cells.

To take our observations a step closer towards the actual transcription activity in infected cells and to authenticate our initial findings we investigated whether the NS5A variants could perturb the endogenous expression levels of IFNβ. To determine the effect the NS5A variants have on endogenous IFNβ production we performed Q-PCR analyses on IFNβ mRNA levels in SV infected 293 cells expressing either the wild-type (WT) NS5A, NS5A-10A, or the NS5A-H27 variants.

Cells were plated in 60mm dishes and transfected with the expression vectors of the aforementioned genes and infected with SV. 16 hours post infection cells were harvested and total mRNA was isolated and reverse transcribed into cDNA. cDNA was then subjected to quantitative polymerase chain reaction to determine the relative expression levels of IFNβ under each condition, as a control we calibrated our mRNA levels of the gene of interest to the endogenous constitutively expressed glyceralaldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA levels.

As expected endogenous IFNβ mRNA levels in NS5A-10A expressing cells showed a 30% reduction when compared to the IFNβ levels in control infected HEK293 cells. The IFNβ levels in NS5A-H27 and NS5A-WT expressing cells were two fold higher than the level seen in infected HEK293 cells. The result suggests that certain variants of the NS5A may enhance the initial effector phase of the innate immune response. Our results showed that certain NS5A variants repressed the expression of IFNβ while others enhance it (Fig. 4).

5a



5b

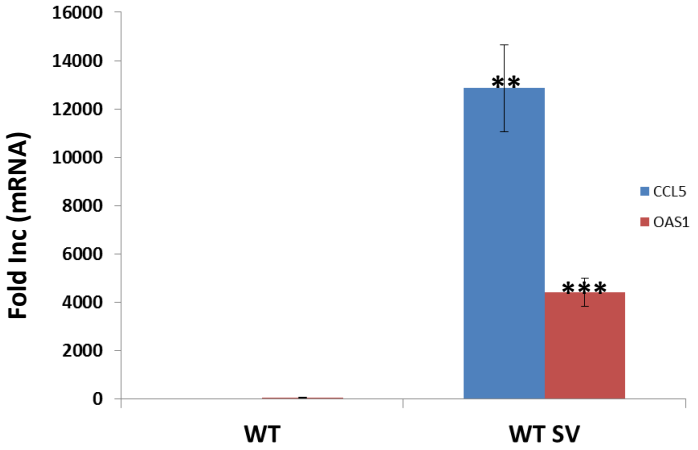


Fig. 5. **Inhibition of IFNβ expression results in a concomitant decrease in the expression of genes downstream of the IFNβ signaling pathway.** The experiment was carried out as described in the previous figure. qRT-PCR was carried out using primers for 3'-5' oligo-A synthetase (OAS) and RANTES/CCL5, both known to be induced following IFNβ binding to its receptor on the cell surface. 5b: WT. CCL5: $P<0.0011$, OAS1: $P<0.0001$ by One way ANOVA followed by Tukey's post hoc for SV control.

The IFNβ expressed by infected cells triggers an autocrine and paracrine response in the surrounding cells to induce the production of a large number of downstream antiviral proteins which are collectively called Interferon Stimulated Genes (ISGs). To better characterize the effect of NS5A variants on the entire antiviral pathway we looked at the expression profiles of two ISGs that are specifically up-regulated downstream of the IFNβ signaling pathway, 2'-5'-Oligoadenylate Synthetase 1 (OAS1), and Regulated on Activation, Normal T cell Expressed and Secreted protein (RANTES). OAS1 when activated synthesizes 2’5’-Oligoadenylates (2-5As) which in turn, activates the latent RNase L which degrades all RNAs in the cell. RANTES is a chemotactic cytokine that recruits leukocytes to the site of inflammation and it also activates Natural Killer (NK) cells.

In addition, if a virus infected cell is unable to eradicate the pathogen through the initial establishment of an antiviral state, programmed cell death, apoptosis is another way a cell could inhibit virus spread. p53 is a tumor suppressor protein that initiates apoptosis in stressed cells, thus we looked at the cellular expression levels of the p53 gene in infected cells over-expressing the NS5A variants.

NS5A deregulate the activation of genes controlled by the IFNβ signaling pathway. Q-PCR was used to analyze the mRNA levels of the interferon stimulated genes (ISGs) in SV infected 293 cells expressing either the wild-type (WT) NS5A, NS5A-10A, or the NS5A-H27 variants. In the infected NS5A-10A expressing cells which showed decreased SV-induced IFNβ mRNA levels, we observed a twofold decrease in the expression of RANTES, while no correlation was observed between

the IFNβ and OAS1 or p53 levels (results not shown). The OAS1 and RANTES mRNA expression levels in both the WT NS5A and the NS5A-H27 cell lines were unperturbed, corresponding to their unperturbed upstream IFNβ mRNA levels (Fig. 5). Since our results suggested that the presence of NS5A in a cell negatively affects its IFNβ production we went on to try and decipher the mechanism by which NS5A is exerting this inhibitory effect.

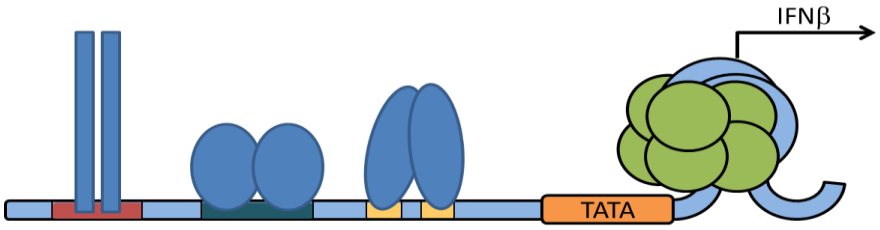


Fig. 6. **IFNβ gene regulation schematics**. The IFNβ promoter contains binding domains for three transcription factor families, IRF-3, NF-κB and ATF-2.

In eukaryotic cells the activation of specific genes by specific stimuli is regulated by transcription factors. The transcription of the human IFNβ gene requires the simultaneous activation of distinct transcription factors, IRF3, ATF2/c-Jun, and NF-κB. This is a highly specific control mechanism that governs the activation of antiviral pathway (Fig. 6). We suspected that NS5A may be interfering with the activation of one or more of the transcription factors that lead to IFNβ expression because the inactivation of any one the three transcription factors would led to the observed decrease in IFNβ levels.

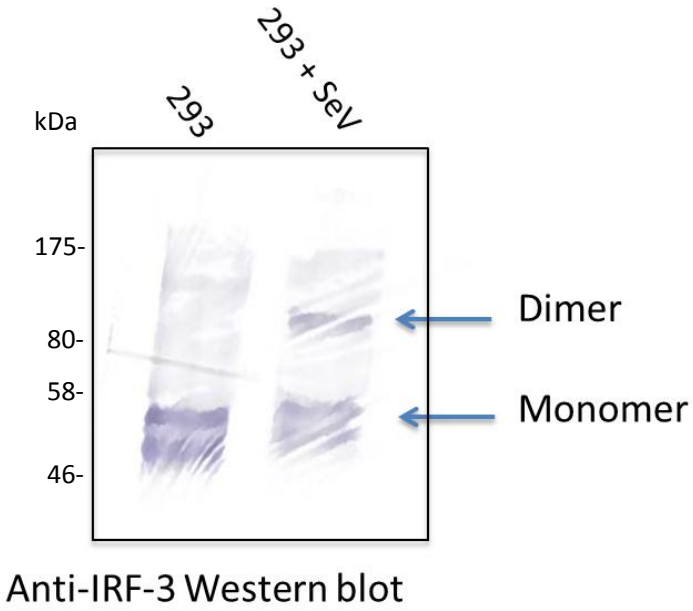


Fig. 7. **Activation of IRF3 by Sendai virus Infection.** 293 HEK cells were infected and IRF3 were targeted by immunoblot. When compared to uninfected cells the presence of IRF3 dimers were clearly observed in infected cells.

IRF3 dimerizes upon virus activation of the antiviral pathway. When activated, IRF3 forms dimers before going into the nucleus. We confirmed its activation by western blot. HEK 293 Cells were plated and infected with SV. 16hr post infection cells were harvested and total protein were extracted. Uninfected cells were used as a negative control. The endogenous IRF3 protein was analyzed by immunoblotting with an IRF3 polyclonal antibody. When compared to uninfected cells, there were 70% more IRF3 dimers in infected cells (Fig. 7).

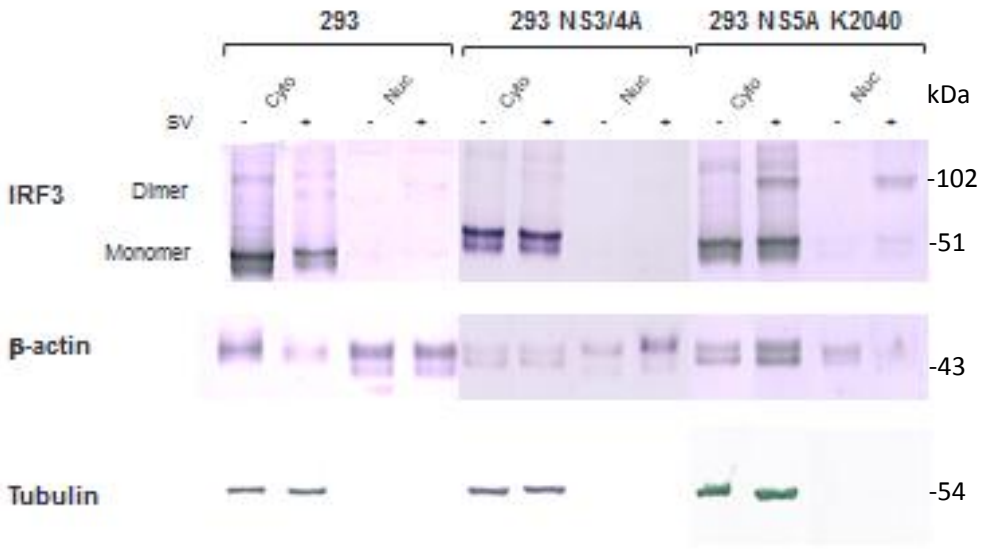


Fig. 8. **NS5A has no inhibitory effect on IRF3 dimerization**. 293HEK cells over expressing NS5A clearly have more IRF3 dimers in the nucleus 293HEK or 293HEK NS3/4A cells. NS3/4A is used as a positive control.

NS5A-10A does not inhibit transcription factor IRF3 activation. To determine whether NS5A-10A mediates its inhibitory effect on IFNβ production through inhibition of IRF3 activation we looked at the presence of IRF3 dimers in infected cells either expressing the NS5A-10A or NS3/4A protein. Uninfected cells were used as a control. The above-mentioned cells were plated, transfected, and infected as described in the previous experiments. 16hr post infection cells were harvested and cytosolic or nuclear proteins were individually extracted. Endogenous IRF3 protein in each of the cytosolic and nuclear fractions was analyzed by immunoblotting with an IRF3 polyclonal antibody (Ab). β-actin, a ubiquitously expressed protein, was used as a loading control. Tubulin was used as a cytoplasmic marker. When compared to the IRF3 dimer levels in infected and non-infected 293 HEK cells, a reduction of IRF3 dimerization level was observed in infected and non-infected cells expressing NS3/4A, while IRF3 dimerization level remains constant in the mock infected and infected NS5A-10A expressing cells (Fig. 8).

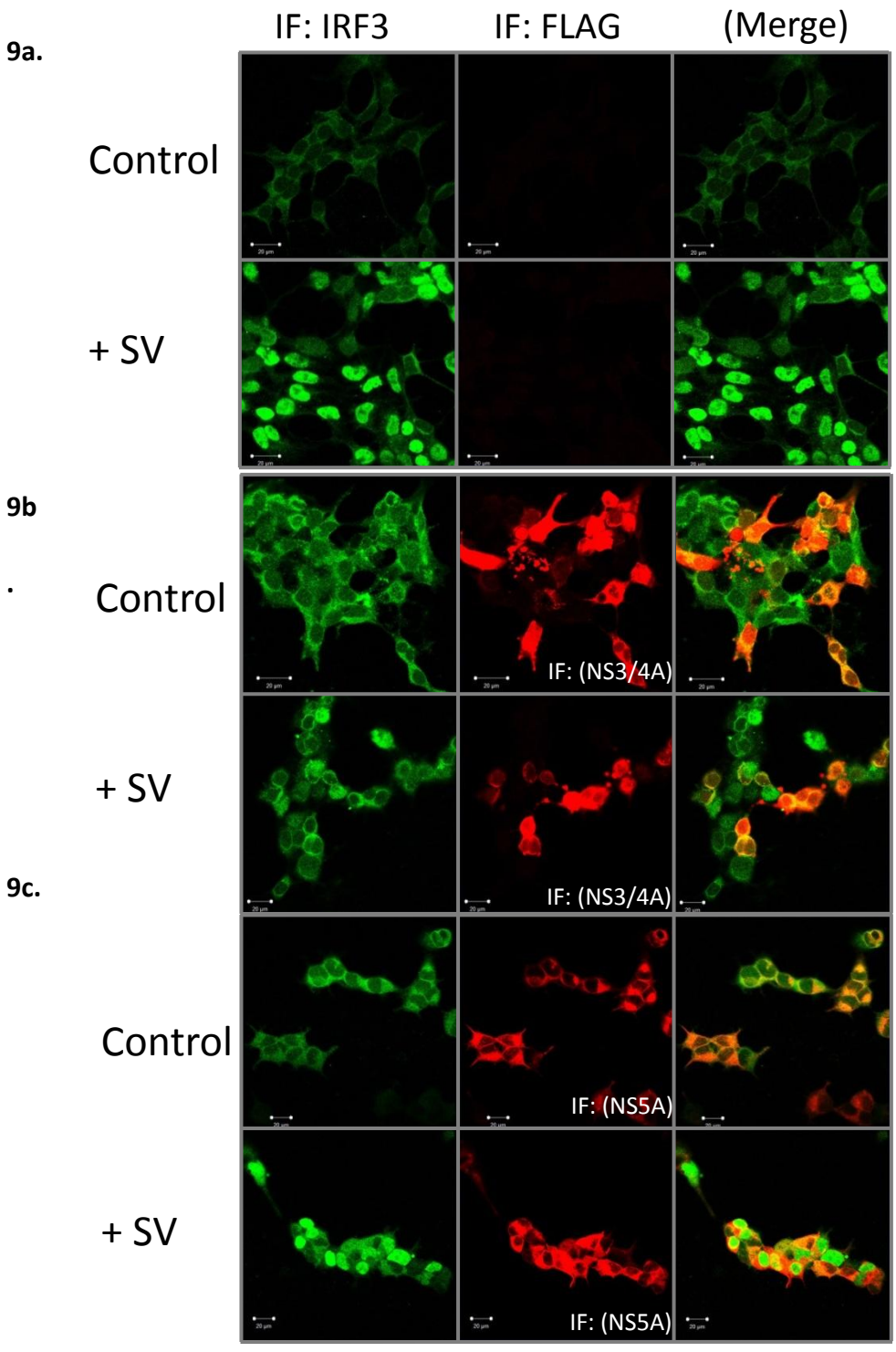
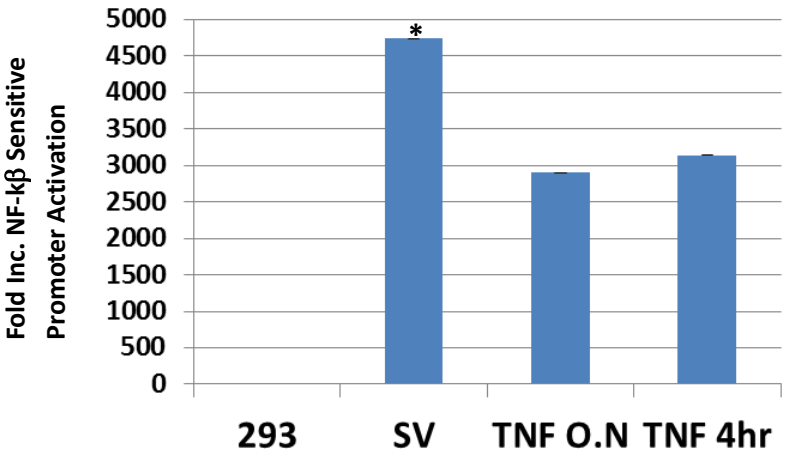


Fig. 9. **HCV NS5A K2040 does not inhibit nuclear localization of the transcription factor IRF-3.** 293HEK cells were transfected with NS5A K2040 and then infected with SV. (a). 293HEK cells. (b). 293 NS3/4A cells. (c). 293 NS5A cells. FLAG-tagged NS3/4A and NS5A (red). IRF3 (Green).

NS5A-10A does not inhibit transcription factor IRF3 nuclear translocation. After activation and dimerization IRF3 translocates to the nucleus. We investigated whether NS5A's inhibitory effect of IFNβ could be mediated a step down stream of IRF3 activation, IRF3 nuclear translocation, using immunofluorescence. 293 HEK Cells, 293 HEK over-expression NS5A cell line, and NS3/4A transfected 293 HEK cells were seeded on glass cover slips then infected with SV. 16 hours post infection cells were fixed in 4% paraformaldehyde (PFA) and incubated in primary Abs targeting IRF3, NS5A and NS3/4A following with incubation with Alexa488 and Alexa 647 fluorophore-linked secondary Abs.

Cells were visualized by confocal microscopy. We studied IRF3 subcellular localization in mock infected and infected cells over-expressing NS5A or NS3/4A. We observed similar degree of IRF3 nuclear localization in infected cells that either expresses NS5A compared to control cells. However, in cells that are over-expressing NS3/4A IRF3 nuclear translocation was clearly stifled (Fig. 9). Since NS3/4A terminates IFNβ expression by cleaving MAVs the signal transducer that is part of the IRF3 activation pathway we used it as a positive control.

10a



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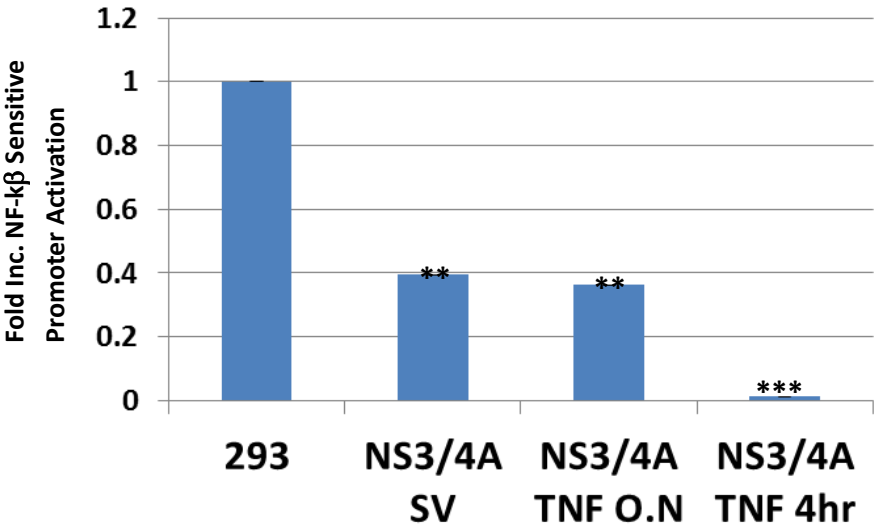

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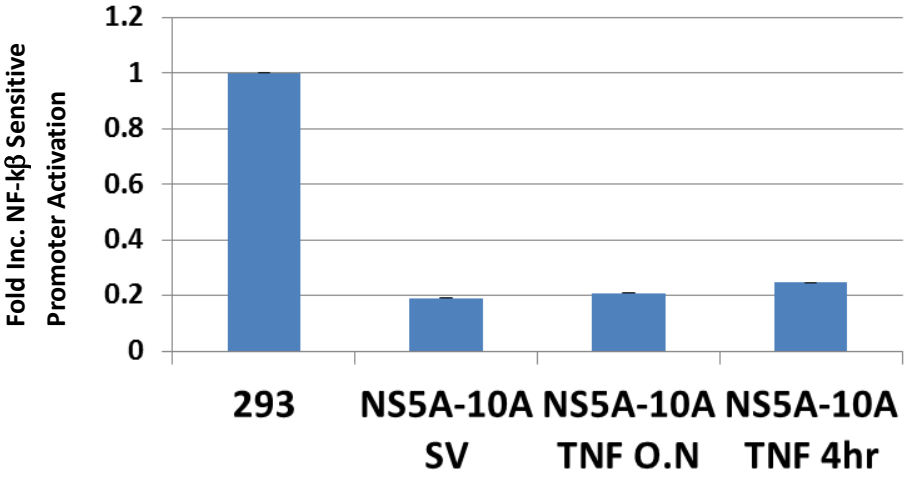

Fig. 10. **NS5A and NS3/4A inhibits activation of the NF-κB sensitive promoter.** Luciferase assay where NF-κB sensitive promoter activation was measured following stimulation with SV and TNFα. 10a. 293 cells, 10b. 293 NS3/4A, 10c. 293 NS5A-10A. P=0.0383 (10a) P=0.0002 (10b) P=0.0708 (10c) by one-way ANOVA followed by Tukey's post.hoc for 293 or 293 SV.

NS5A represses NF-κB sensitive promoter activation. Another transcription factor that governs the expression of IFNβ is NF-κB. During viral infection it is activated when TLR3, an endosomal PRR, recognizes dsRNA and signals through its adaptor protein, TIR-domain-containing adapter-inducing interferon-β (TRIF). To determine whether NS5A mediates its inhibitory effect on IFNβ production through deregulation of NF-κB activation we performed dual luciferase assay with a luciferase reporter plasmid driven by a promoter containing three copies of NF-κB binding site and a cytomegalovirus (CMV) promoter driving Renilla luciferase reporter gene.

Cells were seeded and co-transfected with the reporter genes mentioned. 24hr after transfection cells were treated with SV or Tumor Necrosis Factor Alpha (TNFα at four hour and overnight time points. TNFα is a cytokine that induces inflammation in cells by activation of NF-κB. After treatment with either stimulus cells were harvested 48hr post transfection in passive lysis buffer and fold increase of luciferase activity was analyzed using the dual luciferase assay system (Promega Corp., enMadison, WI) in a luminometer and Renilla luciferase activity was used to normalize transfection efficiency.

Data analysis from the two treatments at either time points showed a 2 fold inhibition of the NF-κB promoter activity in cells that are over expressing NS5A or NS3/4A (Fig. 10).

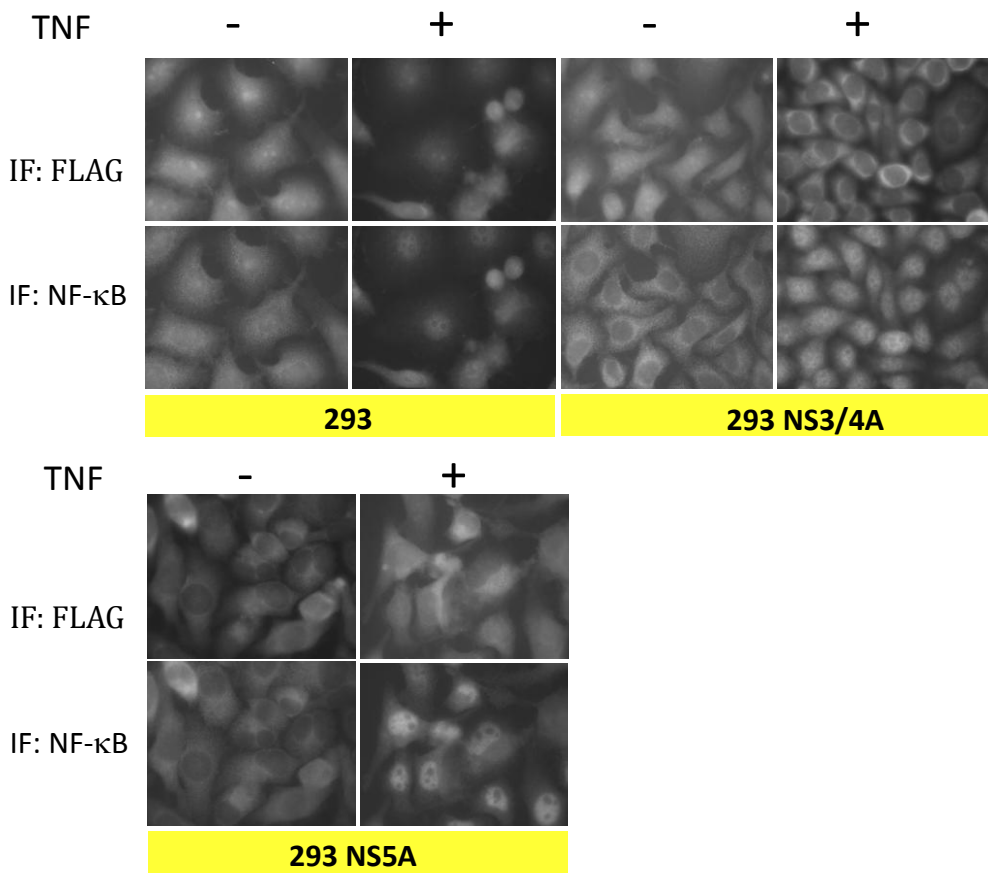

Fig. 11. **NS5A does not prevent TNF mediated nuclear localization of NF-κB** nor does NS3/4A (used as a positive control).

NS5A-10A does not impede NF-κB nuclear translocation. To determine whether NS5A mediates its inhibitory effect on NF-κB promoter through restricting it in the cytosol we performed immunofluorescence assay to investigate the sub-cellular localization of NF-κB in Hela cells that are treated with TNFα while over expressing NS5A-10A or NS3/4A. In both cases NF-κB nuclear translocation was unimpeded (Fig. 11).

DISCUSSION

HCV NS5A is involved in many aspects of the HCV life cycle and its presence greatly influences the ability of the virus to replicate and maintain an active infection in its host as shown by the correlation between certain NS5A variants and the host response to therapy. However, to date aside from knowing that the protein seems to interact with various host proteins and is able to transduce to the nucleus via a nuclear localization signal (NLS) in its N terminus, the exact role of NS5A in promoting virus survival remains elusive because it does not participate in any of the replication events.

NS5A seems to be more of a by-stander that facilitates all the events but is not directly involved in any. In the arena where NS5A's ability inhibits innate immune response, studies have shown that it is capable of attenuating the expression of interferon sensitive genes and inhibiting the antiviral activity of PKR and 2'5'OAS. This results in the suppression of initial antiviral response post infection, an elevated expression of IFNβ, and downstream events brought on by IFNβ signaling.

Since the entire early antiviral response largely centers on the ability of the cell to produce IFNβ and there is an growing awareness of how the variability of the NS5A sequence determines the outcome of the IFN based therapy, we were interested in investigating how the different NS5A variants may affect the ability of the cell to express IFNβ. Previously, using a luciferase reporter gene driven by the IFNβ promoter, we have identified a variant of NS5A, NS5A-10A, which when over

expressed in infected cells, inhibited IFNβ promoter activation (unpublished results SJG and GA). Our research focused on deciphering the mechanisms behind this observed attenuation in IFNβ production by infected cells.

To verify the discovery and to further identify the inhibitory effect of NS5A variants on IFNβ endogenous expression levels of IFNβ in HEK293 cells over-expressing the wild type (WT) NS5A, or the NS5A mutants, NS5A-10A and NS5A-H27 were assayed. Comparing their IFNβ expression profile to controls (HEK 293±SV, HEK 293 NS3/4A±SV) We observed a down regulation of IFNβ expression by 2 fold in infected cells over expressing NS5A-10A, but not in the cells over expressing NS5A-H27 or WT. In comparison an up-regulated expression of IFNβ in infected cells over expressing NS5A-H27 or WT. This perhaps suggests that certain variants of the NS5A protein in an in vivo infection are more immunogenic thus supporting the laboratorial evidence of NS5A sequence dependent sensitivity to IFN therapy.

Furthermore, our data showed that certain mutants of the NS5A protein are more beneficial to the virus as they are able to interfere with the signaling pathways that lead to the antiviral response. NS3/4A is used in our experiment as a positive control. NS3/4A is a known inhibitor of the IFNβ pathway the down regulation of the IFNβ mRNA levels by this protein validates the functionality of the pathway in our cell system.

To better characterize the entire antiviral pathway we subsequently looked at the downstream effect of IFNβ attenuation. Similar to the experiment described above, we assayed for the expression profile of two ISGS which are known to be

positively regulated by IFNβ, RANTES and OAS1. RANTES is a chemotactic cytokine that lures inflammatory cells to the site of infection and OAS1 activates RNase L, an intracellular RNA degrading enzyme. As expected IFNβ, OAS1 and RANTES mRNA expression in infected HEK 293 cells expressing variants of NS5A showed a similar trend to the levels of IFNβ expression from the initial infection. Cells that expressed less IFNβ also showed a reduction in their RANTES and OAS1 expression. The results suggest that the level of IFNβ directly affects the establishment of the antiviral state and by just shutting down IFNβ production the virus is also curtailing many downstream antiviral responses as well.

In addition we were interested in the mRNA levels of p53. p53 is a known tumor suppressor that has the ability to arrest the cell cycle and induce apoptosis . Furthermore in a paper by Takaoka, et al. it was reported that the sequences within the promoter regions of many ISGs was similar to those found in the p53 promoter [40]. Since a major arm of innate immune defense against virus infection is programed cell death we investigated whether p53 has a role in the IFNβ-mediated antiviral response. Preliminary data showed that IFNβ had no influence on the expression levels of endogenous p53. One explanation could be that RNA extractions were performed at 12-16 hour post infection while perhaps p53 mRNA levels fluctuated at an earlier time point to prepare the cell for program apoptosis. Indeed previous research has shown upon infection p53 mRNA induction in murine embryonic fibroblasts (MEF) lasted for 9 hours [40].

In eukaryotic cells the activation of specific genes by specific stimuli is regulated by transcription factors. Transcription factors are effector proteins that relay the cytoplasmic signal into the nucleus and turn on expression of specific genes in response to specific stimuli. However, sequences that are recognized by the transcription factors are found upstream of a multitude of genes though only a subset of them may be expressed in response to a particular stimulus. This secret of this specificity lies in the organization of transcriptional enhancers, which are regulatory sequences, located upstream of the promoter. There can be multiple binding sites within the enhancer that are recognized by distinct transcription factors. Thus, the gene that is regulated by the enhancer only will be activated by a combinatorial mechanism where transcription factors act synergistically to allow its expression.

It is long known that IFNβ is produced only during viral infection and part of this specificity lies in the enhancer sequence found upstream of the IFNβ promoter and in the transcription factors binding to this enhancer. The transcription of the human IFNβ gene requires three distinct transcription factors that are activated simultaneously by virus components to bind to their respective recognition sequences with in the enhancer. Along with architectural proteins that reside in the nucleus the transcription factors assemble into a multi-component complex on the enhancer forming an enhanceosome. The enhanceosome then permits the basic transcriptional machinery to access the IFNβ promoter more efficiently. The three transcription factors, interferon regulatory factor 3 (IRF3), nuclear factor

kappa-light-chain-enhancer of activated B cells (NF-κB), and activating transcription factor 2 (ATF2)/c-JUN heterodimer exert a combinatorial synergistic effect on the IFNβ transcription rate.

We hypothesized that NS5A-10A mediates its inhibitory effect on the antiviral response, the attenuation of the IFNβ promoter activation and its gene transcription, by interfering with the activation of one or more of the IFNβ transcription factors. We first looked at the effect of NS5A-10A on IRF3 transcription factor activation. Forming dimers upon activation is a characteristic behavior of IRF3. Upon dimerization a nuclear localization sequence is exposed on the IRF3 dimers thus facilitating their import into the nucleus. Taking those two factors into account we looked at whether NS5A-10A could disrupt these behaviors.

As a proof of theory we first looked at IRF3 dimer levels in Sendai Virus (SV) challenged HEK293 cells. In comparison to uninfected cells our western blot results showed a 70% induction of IRF3 dimers in infected cells. Subsequently we followed on to compare IRF3 dimerization levels in SV challenged cells over-expressing the NS5A-10A or NS3/4A gene. Both our western blot and densitometry results showed that aside from the expected inhibition of IRF3 dimers in cells over-expressing NS3/4A, NS5A-10A did not inhibit IRF3 dimerization in infected cells.

To determine whether the inhibitory effect on IRF3 exerted by NS5A-10A happens downstream of IRF3 activation we looked at subcellular localization of IRF3 in infected cells expressing NS5A-10A and NS3/4A. After plating cells and transfecting and infecting them, we fixed the cells and fluorescently tagged IRF3 with an antibody.

We visualized IRF3 localization patterns in cells co-expressing the viral protein using confocal microscopy. We observed that IRF3 translocated to the nucleus with equal efficiency in both the SV challenged 293 HEK cells and cells over expressing NS5A-10A. But as expected IRF3 nuclear translocation was inhibited in cells expressing NS3/4A.

To confirm our results we proceeded to determine the subcellular localization of both IRF3 and NS5A-10A during virus infection. The results showed that in during SV infection NS5A-10A stayed in the cytoplasm while IRF3 transduced to the nucleus with similar efficiency as seen in infected 293 cells, while IRF3 remained in the cytoplasm in infected cells expressing the NS3/4A. The results suggested the inhibitory effect of NS5A-10 on the antiviral response may not be working through IRF3 dimerization or localization.

Next we tried to determine whether the anti-antiviral response by NS5A-10A is mediated through NF-κB inhibition. When a cell is infected by virus an assortment of PRRs recognizes replication intermediates or structural motifs of the virus and sends signals by various pathways to induce the antiviral response. RIG-I in the cytoplasm recognizes dsRNA and signals through the mitochondria antiviral protein MAVs which activates IRF3. On the other hand TLR3 is an endosomal PRR which when it recognizes dsRNA sends its signal through an adaptor protein, TIR-domain-containing adapter-inducing interferon-β (TRIF). TRIF then activates a series of kinases that leads to the activation of NF-κB. We looked at the impact of NS5A-10A on NF-κB promoter activation via a dual luciferase assay.

Comparing the amount of promoter activity in infected cells to the amount of promoter activity in infected cells either expressing NS5A-10A or NS3/4A we could observe a down regulation of the NF-κB promoter activity in cells expressing both viral proteins. There was a 3-fold decrease of NF-κB promoter activity in SV challenged NS5A-10A expressing cells and this fold decrease was of a larger extent then the inhibitory effects mediated on promoter activation by NS3/4A. To verify the results we repeated the experiment this time with an extracellular stimulus, tumor necrosis factor alpha (TNFα), known to trigger the activation of NF-κB and observed a similar inhibitory effect on its promoter activation in SV challenged cells over expressing either NS5A-10A or NS3/4A. When TNFα binds to its receptor intracellular pathways are activated to promote inflammatory responses and one of the pathways involves in the activation of NF-κB. Even though initially SV and TNFα signal by different pathways, eventually the pathways converge at the phosphorylation of IκB kinase (IKK) which leads to the activation of NF-κB.

To decipher the mechanism whereby NS5A-10A may be inhibiting NF-κB promoter activation we performed immunofluorescence on NF-κB looking at its subcellular localization in SV challenged cells co-expressing NS5A or NS3/4A. Using TNFα as a stimulus to trigger NF-κB nuclear translocation we hoped to identify whether NS5A could inhibit this translocation behavior. My results showed no inhibition of NF-κB nucleus translocation in NS5A-10A and NS3/4A expressing cells. One explanation could be that TNFα is an extrinsic factor and it may stimulate NF-κB through a completely different pathway than the pathway activated by viral

components. Another explanation could be that the inhibitory effect of NS5A-10A on the antiviral pathway may work a step down stream of the nuclear translocation of transcription factors. As described previously the IFNβ enhanceosome assembly also requires intrinsic structural proteins. Thus perhaps NS5A-10A could disrupt the assembly of these architectural proteins indirectly. NS5A nuclear translocation and transcription activation could also directly interfere with the transcription silencing of IFNβ. All the above are avenues of further research our lab is currently exploring.

To conclude NS5A is a promiscuous protein that interacts with a plethora of host cellular proteins. It is a phosphoprotein which is capable of modulating signaling pathways that lead to the antiviral response and transduce to the nucleus to influence gene transcription. Exactly how it down regulates the IFN antiviral system unfortunately remains obscured. We took a stab at solving parts of the puzzle but much more is required in our search.

REFERENCES

1. Chisari FV. Unscrambling hepatitis C virus-host interactions. Nature. 2005;436: 930-932.

2. Hepatitis Foundation International (800) 891-0707 info@hepatitisfoundation.org

3. Lindenbach BD, Rice CM. Unravelling hepatitis C virus replication from genome to function. Nature. 2005;436:933-938.

4. Bowen DG, Walker CM. Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature. 2005;436:946-952.

5. Sklan EH, Charuworn P, Pang PS, Glenn JS. Mechanisms of HCV survival in the host. Nature. 2009;6:217-227.

6. Tellinghuisen TL, Evans MJ, Hahn TV, You S, Rice CM. Studying hepatitis C virus: making the best of a bad virus. J Virol. 2007;81:8853-8867.

7. Brown Jr RS. Hepatitis C and liver transplantation. Nature. 2005;436:973-978.

8. Francesco RD, Migliaccio G. Challenges and successes in developing new therapies for hepatitis C. Nature. 2005;436:953-960.

9. Hagan LM, Schinazi RF. Best strategies for global HCV eradication. Liver Int. 2013;33(s1):68-79.

10. Asselah T, Marcellin P. Interferon free therapy with direct acting antivirals for HCV. Liver Int. 2013;33(s1):93-104.

11. Houghton M. Discovery of the hepatitis C virus. Liver Int. 2009;29(s1):82-88.

12. Sandmann L, Ploss A. Barriers of hepatitis C virus interspecies transmission. Virology 2013;435:70-80.

13. Joyce MA, Tyrrell DLJ. The cell biology of hepatitis C virus. Microb. Infect. 2010: 1-9.

14. Brar I, Baxa D, Markowitz N. HCV enters the twenty-first century. Curr. Infect. Dis Rep. 2013;15(1):52-60.

15. Piñeiro D, Martinez-Salas E. RNA structural elements of hepatitis c virus controlling viral RNA translation and the implications for viral pathogenesis. Viruses. 2012;4:2233-2250.

16. Yamasahi LHT, Arcuri HA, Jardim ACG, Bittar C, Carvalho-Mello IMVG, Rahal P. New insights regarding HCV-NS5A structure/function and indication of genotypic differences. J Virol. 2012;9(14): 1-10.

17. Imran M, Waheed Y, Manzoor S, Bilal M, Ashraf W, Ali M, Ashraf M. Interaction of hepatitis C virus proteins with pattern recognition receptors. Virology. 2012; 9:1-18.

18. Calland N, Dubuisson J, Rouillé Y, Séron K. Hepatitis C virus and natural compounds: A new antiviral approach? Viruses. 2012;4:2197-2217.

19. Heim MH. Innate immunity and HCV. J. Hepatol. 2012, http://dx.doi.org/10.1016/j.jep.2012.10.005

20. Oshiumi H, Funami K, Aly HH, Matsumoto M, Seya T. Multi-step regulation of interferon induction by hepatitis C virus. Arch. Immunol. Ther. Exp. 2013;8(44):1-12.

21. Darnell Jr JE, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science. 1994;264(5164):1415-1420.

22. Foy E, Li K, Wang C, Sumpter Jr R, Ikeda M, Lemon SM, Gale Jr M. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. Science. 2003;300:1145-1148.

23. Li XD, Sun LJ, Seth RB, Gabriel P, Chen ZJ. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. PNAS. 2005;102(49) 17717-17722.

24. Nitta S, Sakamoto N, Nakagawa M, Kakinuma S, Mishima K, Kusano-kitazume A, Kiyohashi K, Murakawa M, Nishimura-Sakura Y, Azuma S, et al. Hepatitis C virus NS4B protein targets STING and abrogates RIG-I-mediated type I interferon-dependent innate immunity. Hepatology. 2013;57(1):46-58.

25. Inoue K, Tsukiyama-Kohara K, Matsuda C, Yoneyama M, Fujita T, Kuge S, Yoshiba M, Kohara M. Impairment of interferon regulatory factor-3 activation by hepatitis C virus core protein basic amino acid region 1. Biochem. Biophys. Res. Commun. 2012;428:494-499.

26. Gale Jr. M, Blakely CM, Kwieciszewski B, Tan SL, Dossett M, Tang NM, Marcus JK, Polyak SJ, Gretch DR, Katze MG. Control of PKR protein kinase by hepatitis c virus nonstructural 5A protein: Molecular mechanisms of kinase regulation. Mol. Cell. Biol. 1998;18(9):5028-5218.

27. Taguchi T, Nagano-Fujii M, Akutsu M, Kadoya H, Ohgimoto S, Ishido S, Hotta H. Hepatitis C virus NS5A protein interacts with 2',5'-oligoadenylate synthetase and inhibits antiviral activity of IFN in an IFN sensitivity-determining region-independent manner. J Gen Virol. 2004;85(4):959-969.

28. Kriegs M, Bürckstümmer T, Himmelsbach K, Bruns M, Frelin L, Ahlén G, Sällberg M, Hildt E. The hepatitis C virus non-structural NS5A protein impairs both the innate and adaptive hepatic immune response in vivo. J. Biol. Chem. 2009;284(41):28343-28351.

29. Sumpter Jr R, Wang C, Foy E, Loo YM, Gale Jr M. Viral evolution and interferon resistance of hepatitis C virus RNA replication in a cell culture model. J Virol. 2004;78(21):11591-11604.

30. Khaliq S, Latief N, Jahan S. Role of different regions of the hepatitis C virus genome in the therapeutic response to interferon-based treatment. Arch Virol. 2013;10(1007):1-15.

31. Yamasaki HT L, Arcuri A H, Jardim G AC, Bittar C, Carvalho-Mello de VG M, Rahal P. New insights regarding HCV-NS5A structure/function and indication of genotypic differences. J Virol. 2012;9(14):1-10.

32. Maqbool A M, Imache R M, Higgs R M, Carmouse S, Pawlotsky MJ, Lerat H. Regulation of hepatitis C virus replication by nuclear translocation of Nonstructural 5A protein and transcriptional activation of host genes. J Virol. 2013;87(10):5523-5539.

33. Scheel KH T, Rice M C. Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. Nature. 2013;19(7):837-849.

34. Horner M S, Gale Jr M. Regulation of hepatic innate immunity by hepatitis C virus. Nature. 2013;19(7):879-888.

35. Qashqari H, Al-Mars A, Chaudhary A, Abuzenadah A, Damanouri G, Alqahtani M, Mahmoud M, Zaki SE M, Fatima K, Qadri I. Understanding the molecular mechanism(s) of hepatitis C virus (HCV) induced interferon resistance. Infect. Gen. Evo. 2013;19:113-119.

36. WHO Drug Information Vol. 27, No. 1, 2013. http://apps.who.int/medicinedocs/documents/s20154en/s20154en.pdf.

37. Lawitz E, Mangia A, Wyles D, Rodriguez-Torres M, Hassanein T, Gordon S C, Schultz M, Davis N M, Kayali Z, Reddy K R, Jacobson I M, Kowdley K V, Nyberg L, Subramanian G M, Hyland R H, Arterburn S, Jiang D, McNally J, Brainard D, Symonds W T, McHutchison J G, Sheikn A M, Younossi Z, Gane E J. Sofosbuvir for previously untreated chronic hepatitis c infection. N. Engl. J. Med. 2013;368(20):1878-1887.

38. Sainz B, Barretto N, Martin D N, Hiraga N, Imamura M, Hussain S, Marsh K A, Yu X, Chayama K, Alrefai W A, Uprichard S L. Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. Nat. Med. 2012;18(2):281-185.

39. Thomas V H, Charles M R. Hepatitis C virus entry. J. Biol. Chem. 2008;283(7):3689-3693.

40. Takaoka A, Hayakawa S, Yanai H, Stoibe D, Negishi H, Kikuchi H, Sasaki S, Imai K, Shibue T, Honda K, Taniguchi T. Integration of interferon-(alpha)/(beta) signaling to p53 responses in tumor suppression and antiviral defense. Nature. 2003;424(6948):516-523.

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ABSTRACT

UNMASKING A SILENT KILLER: UNCOVERING THE MECHANISM OF HEPATITIS C VIRUS PROTEIN NS5A-MEDIATED INHIBITION OF THE INNATE IMMUNE RESPONSE

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Hepatitis C Virus (HCV) is a human liver pathogen. In the host its infection leads to acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma. This positive stranded RNA virus is extremely efficient in establishing persistent infection by escaping immune detection by hindering the host immune responses. An important component of the host's innate immune response in viral infection is the production of type I interferons (IFNs). Typically, viral infection induces the synthesis and secretion of interferon α/β (IFN α/β) by the infected cell, which in turn activates signaling pathways leading to the establishment of an antiviral state in the cell. This raises the question of how HCV circumvents the antiviral immune responses of host cells. Previous studies have shown that HCV nonstructural protein NS3/4A interferes with the activation of signaling pathways that leads to the activation of the IFNβ promoter. Our lab has identified another HCV protein, NS5A, that also interferes with host antiviral signaling independent of the NS3/4A-mediated inhibition of the host antiviral response. Activation of IFNβ gene expression involves the activation of three transcription factors (ATF-2, IRF3/7 and NF-kB) and the formation of an enhanceosome on the promoter. To investigate the influence of HCV NS5A on innate immunity, we study the effect of NS5A over-expression on Sendai Virus (SV)-mediated IFNβ gene induction via qPCR and reporter gene assay. We have identified NS5A to be a potent inhibitor of the host innate immune system, possibly through inhibition of IRF3 activation. We are currently investigating the effect of NS5A on transcription factor activation. NS5A inhibition of IFNβ induction, may be another factor contributing to the persistence of HCV in the host, and may play a key role in designing therapies for the treatment of HCV infection.