

HEPATITIS C VIRAL PROTEIN NS3/4A AND ITS EFFECT
ON THE ANTI-VIRAL RESPONSE

By

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ON THE ANTI-VIRAL RESPONSE

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ABSTRACT

Hepatitis C Virus (HCV) is estimated to infect 3% of the world's population and is transmitted by contaminated blood. HCV can be asymptomatic or lead to cirrhosis of the liver, liver cancer or hepatocellular carcinoma. Understanding the virus life cycle and the viral proteins generated by HCV will help expand the knowledge of mechanisms that the virus uses to inhibit the immune system. HCV produces 10 viral proteins when it infects hepatocytes that increase the reproduction ability of the virus. The anti-viral response of the body uses transcription factors such as ATF-2, NFKB, and IRF-3 that translocate into the nucleus and bind to the interferon gene that produces interferon to alert the body of a viral infection. HCV viral protein NS3/4A acts as protease to cleave the polycistronic strand of viral proteins made by HCV and is known to inhibit IRF-3 movement into the nucleus to inhibit the production of interferon. HCV viral protein NS5A is known to inhibit the movement of transcription factor NFKB into the nucleus, thus inhibiting the anti-viral response. We have shown that HCV viral protein NS3/4A inhibits the movement of both transcription factors NFKB and IRF-3 supporting the hypothesis that NS3/4A is able to block the anti-viral response inhibiting both the movement of NFKB and IRF-3 following infection. These findings suggest the importance of the interferon gene in alerting the immune system of an infection, as HCV produces two viral proteins that inhibit the production of interferon so it can establish a chronic infection within the host.

ACKNOWLEDGEMENTS

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INTRODUCTION

Natural History

Hepatitis C Virus (HCV) is estimated to chronically infect 3% of the world's population (130-170 million people).⁽¹⁾ In many cases, an acute HCV infection progresses to a chronic infection and can lead to liver cirrhosis or hepatocellular carcinoma. At its peak in the 1980s, the incidence of acute HCV was estimated to be around 180,000 cases per year. HCV is transmitted from host-to-host via blood contamination which includes, but is not limited to, blood transfusion, sharing of needles, and sexual encounters. Those at the highest risk for HCV infection are those who have received a blood transfusion, use injectable drugs, and/or are infants born to infected mothers. A sharp decline in the prevalence of HCV was correlated with needle exchange programs and increasing awareness of behavior that increases the risk of HCV infection.⁽²⁾

HCV has been grouped into seven genotypes (each has about a 30% sequence divergence). Distinct geographical distributions have been observed with the different genotypes. Genotype 1 is mainly observed in the Americas, Japan, and Europe; along with genotypes 2 and 3. Genotypes 3 and 6 are observed mainly in South and Southeast Asia. Genotypes 4 and 5 are observed in Africa.⁽¹⁾

Virology

Hepatitis C Virus is an enveloped, positive-strand RNA virus that belongs to the *Flaviviridae* family which requires mammals as hosts. The viral genome is 9.6 kilobases long. Following infection, HCV produces 10 viral proteins, three of which are structural proteins and seven of which are nonstructural proteins. The structural proteins include core, E1, and E2 and

produce the capsid and envelope. The nonstructural proteins produce viral proteins and are required for replication of the virus within its host. HCV protein p7 encodes for an ion channel and assembly factor. NS2 is an auto-protease and assembly factor. NS3 is a serine protease and helicase that binds to its co-factor NS4A. NS4B organizes the replication complex and membranous web. NS5A is a regulator of replication and viral assembly. NS5B codes for an RNA dependent RNA polymerase for HCV transcription.⁽¹⁾

Life Cycle

Hepatitis C Virus uses blood circulation of the host to travel to its target cells, hepatocytes. HCV binds to receptors on the liver cells via glycoproteins on the cell envelope of the virus. Following attachment, the enveloped virus is endocytosed by the cell into an endosome. The endosome acidifies which results in the uncoating of the viral nucleocapsid releasing the viral RNA of HCV into the cytoplasm of the cell. Once in the cytoplasm, HCV RNA hijacks the machinery of the host to begin translation and replication of its genome in the endoplasmic reticulum of the cell. HCV RNA is positive sense, which means it can be immediately translated once the RNA is translocated into the endoplasmic reticulum. HCV RNA is translated into a polycistronic strand of proteins that must be cleaved by the NS2 and NS3/4A proteases to become active proteins.⁽³⁾ Three of these viral proteins are structural proteins (Core protein, E1, E2) and are important components to the structure of the nucleocapsid and envelope of the viral particle. The remaining seven are non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) which are essential for the replication of the viral genome.⁽¹⁾ Following assembly of the nucleocapsid, the nucleocapsid buds off the endoplasmic reticulum taking some of the membrane of the ER with it as it moves across the cytosol of the cell to the membrane.⁽³⁾ Once the

nucleocapsid reaches the cell membrane, the ER membrane containing the nucleocapsid fuses with the cell membrane to release the nucleocapsid into the extracellular environment where it goes on to infect other host cells.

NS5A

NS5A is a nonstructural protein produced by Hepatitis C Virus that helps down-regulate the immune response. NS5A localizes in the cytoplasm of cells in specialized cytoplasmic structures that could be derived from the Golgi body or endoplasmic reticulum and near the HCV core proteins. NS5A possibly regulates viral assembly through the direct interaction with HCV capsid proteins. However, not much is known specifically about the interaction of NS5A with other proteins, but research shows that NS5A plays an important role in the inactivation of the pathways that induce the anti-viral response. NS5A inhibits virus-induced activation of transcription factor NF κ B, and by inhibiting the activation, NF κ B is unable to move into the nucleus of the cell to bind to the genome and induce transcription of interferons.⁽⁴⁾ This inhibition results in the decrease of the anti-viral response which is beneficial to the virus.

NS3/4A

NS3/4A is a serine protease complex produced by HCV. The NS3/4A complex is responsible for the proteolytic cleavage of the polycistronic strand of proteins made by HCV RNA. This proteolytic cleavage is essential to make these proteins functionally active. It has been shown that NS3/4A could be one of the mechanisms that HCV uses to breakdown the immune response of the host. HCV NS3/4A protease inhibits both the activation of TLR-3-dependent and TLR-3-independent signaling transduction cascades.⁽⁵⁾ These signaling into the

nucleus to produce interferons as part of the anti-viral response of the host. NS3/4A allows HCV to replicate without being detected by the immune response of the host and is essential for the lifecycle of HCV.

Anti-Viral Response

The immune response in our bodies is responsible for killing virally infected cells to stop the spread of the virus to other cells. The innate immune response is the first defense of the body that responds nonspecifically to kill virally infected cells. The innate immune response uses Natural Killer (NK) cells to kill virally infected cells. MHC (major histocompatibility complex) Class I receptors are normally expressed on the surface of cells and provide an inhibitory signal for NK cells. Once it has infected the cells, the virus reduces the number of MHC Class I receptors and therefore decreases the inhibition signal to the NK cells. NK cells kill the virally infected host cell via perforin and granzyme which create holes in the membrane of the cell and induce apoptosis.⁽⁶⁾

If the innate immune system is unable to clear the virus, the adaptive immune system is the second line of defense. The adaptive immune system is slower to respond but highly specific for recognizing specific viral antigens. The adaptive immune system does this by recognizing PRRs (pattern recognition receptors) like toll-like-receptors (TLR) that present specific antigens on the surface of cells.⁽⁷⁾ Cytotoxic T cells arrive at the site of infection and kill the virally infected cells by apoptosis.

Before the cell dies, the infected cell will release interferons to alert the host of an infection. In order for interferons to be produced and released by the infected cell, the infected cell must recognize the pathogen within the cell via pattern recognition receptors (PRRs).⁽⁷⁾

RIG-I recognizes RNA viral genome via the tri-phosphorylated characteristic, and once RIG-I is activated, it induces MAVS which is located on the mitochondria (Figure 1). MAVS is an antiviral signaling protein that leads to the activation of proteins TBK, IKK, and JNK. I κ B complex is composed of three isoforms of I κ B: I κ B- α , I κ B- β , I κ B- γ . The I κ B complex regulates NF κ B by binding to it and masking the nuclear localization signal of NF κ B and therefore does not result in the localization of NF κ B in the nucleus of the cell. Phosphorylation of I κ B by I κ K leads to the ubiquitination of I κ B by ubiquitin ligases. Following ubiquitination of I κ B, I κ B is degraded by the 26S proteasome resulting in the release of NF κ B from I κ B unmasking the nuclear localization signal of NF κ B, and NF κ B translocates to the nucleus where it can bind to DNA and induce gene transcription.⁽⁸⁾ IRF-3 is another transcription factor of the cell that is activated by phosphorylation via I κ K. Inactive IRF-3 can constitutively move in and out of the nucleus. However, following phosphorylation, IRF-3 translocates to the nucleus and is retained in the nucleus as it binds DNA.⁽⁹⁾ The translocation of NF κ B and IRF-3 are essential for the transcription of interferon genes such as IFN- β . To prepare neighboring cells for the possibility of infection, IFN- β is released by the cell which will induce the neighboring uninfected cell to begin the production of anti-viral proteins by activating the interferon stimulating genes.

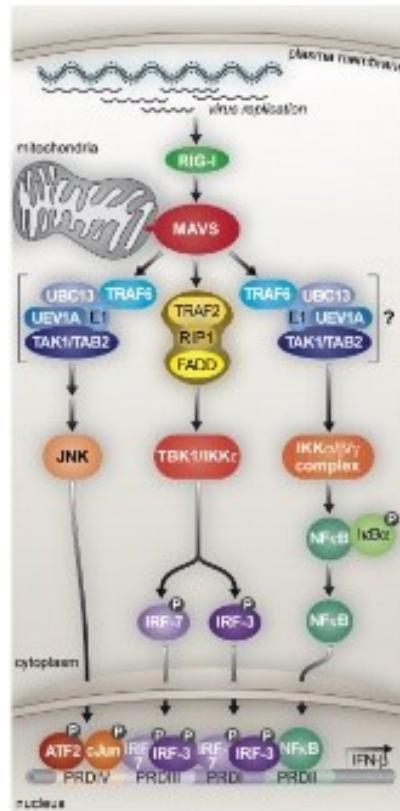


Figure 1: Virus induced activation of the RIG-I pathway leading to the activation of IFN- β .⁽¹⁰⁾

Prevention and Treatment

Because of increased awareness of how HCV is transmitted, hepatitis C incidence has decreased over the past few decades. HCV is transmitted by exposure to infected blood through IV drug use, blood transfusions, the use of inadequately sterilized needles or other medical equipment, and through sexual contact. Because acute HCV can often be asymptomatic, blood tests are often used to diagnose and prevent the spread of HCV via blood and organ donations.

Because there are no vaccines for hepatitis C, prevention involves reducing the risk of exposure to the virus through hand hygiene, gloves, proper handling and disposal of needles, and educating health professionals and the public about the modes of HCV transmission.⁽¹¹⁾ Immunizations for hepatitis A and B are recommended to protect against a co-infection of the liver once infected with hepatitis C. Regular monitoring of liver enzymes of patients at risk of hepatitis C infection is recommended so that an early diagnosis can be made and proper medical treatment can be started to manage the infection.

For about 15-50% of those with an acute infection of HCV, the infection will spontaneously resolve without treatment. Because of this, for a minimum of six months following the diagnosis of hepatitis C, patients will be monitored for viral spontaneous clearance. In the event that the infection is not resolved, patients will begin anti-viral medication which has a 95% cure rate. Current treatments of HCV have two objectives: to sustain the absence of HCV RNA in serum for 12 weeks after completion of the anti-viral therapy (which deems the patient cured) and to prevent or decrease the progression of damage to the liver.⁽¹²⁾

Historically, interferon was used as the treatment for hepatitis C. Almost every cell in the body releases interferons in response to viral infection. Interferons act by directly increasing the immune response by activating the JAK-STAT pathway that leads to transcription factors

binding to regulating sequences known as IFN- γ -activated sites. By treating infected patients with interferon, the antiviral response within the body of the patient is boosted by this increased amount of interferon. Because many patients are treated for extended periods of times with interferon, polyethylene glycol (PEG) was added to increase the effectiveness of the treatment. PEG slows the rate of clearance of interferons and allows for sustained absorption so that the patients only need to receive doses of interferons once a week.⁽¹³⁾

Currently, treatments for hepatitis C use a combination of antiviral drugs and PEG-INF therapy. Ribavirin is often combined with PEG-INF because Ribavirin inhibits viral RNA synthesis. Patients with HCV genotypes 2 and 3 are more responsive to therapy with ribavirin than other genotypes. The addition of Ribavirin to PEG-INF therapy for HCV showed greater improvement in suppressing serum HCV RNA levels during long term treatments.⁽¹⁴⁾ This suppression of HCV RNA levels is important because “curing” HCV involves sustained absence of the viral RNA in the blood of the patient.

Direct-acting antiviral drugs (DAAs) were most recently approved to treat hepatitis C. DAAs target different proteins made by HCV to interrupt the lifecycle of HCV. For example, Telaprevir targets the NS3/4A protease and Ledipasvir inhibits the HCV viral protein NS5A. Combinations of the DAAs are used to inhibit the life cycle of the virus at multiple points. For example, Harvoni is a combination of Ledipasvir and Sofosbuvir (an inhibitor of NS3/4A like Telaprevir) to inhibit viral proteins of HCV.

Treatment with DAAs are associated with a 71% reduction in the risk of developing liver cancer.⁽¹⁵⁾ However, many of these treatments have severe side effects. Adverse effects of IFN include, and are not limited to, memory and concentration problems, pulmonary complications, and neutropenia. Some adverse effects of ribavirin include birth defects, anemia, and gout.⁽¹²⁾

Sendai Virus

Sendai Virus (SV) is a negative stranded RNA virus. SV is part of the *Paramyxoviridae* family and has a viral envelope. It typically infects the respiratory systems of rodents and swine through aerosols or contact with respiratory secretions. SV is capable of infecting human cells in tissue cultures. Infection with SV leads to an immune response through the production of type I interferons and the virus is highly contagious. However, infection only persists in immunocompromised animals. SV is safer to work with in the lab than HCV, so SV is used in place of HCV because both viruses are RNA viruses and induce the production of interferons in the cell by activating the RIG-I pathway.⁽¹⁶⁾

TNF Signaling Pathway

Tumor Necrosis Factor (TNF) is a cytokine that is produced when the immune system is activated and is used to regulate the immune response. When TNF- α binds to its receptor on the cell surface, the TNF- α receptor is activated and recruits TRADD which recruits more proteins like RIP, TRAF2, and FADD to bind and interact with the TNF- α receptor.⁽¹⁷⁾ The interaction of these proteins is responsible for the phosphorylation of the I κ B complex that masks the nuclear localization signal of NF κ B. The phosphorylation of I κ B results in the ubiquitination of I κ B which marks I κ B for degradation by a proteasome.⁽⁸⁾ The degradation of I κ B unmasks the nuclear localization signal of NF κ B and allows NF κ B to translocate into the nucleus where it acts as a transcription factor and binds to the DNA. TNF is involved with the regulation of many biological processes that include lipid metabolism, apoptosis, cell proliferation, and differentiation.⁽¹⁷⁾

Hypothesis

This project investigated the effects of HCV viral protein NS3/4A on the anti-viral response. NS3/4A is known to inhibit the translocation of transcription factor IRF-3 by cleaving MAVS off the mitochondria and rendering it inactive. We hypothesized that because NS3/4A cleaves MAVS and inhibits the signaling cascade that also activates both IRF-3 and NF κ B, the presence of NS3/4A should also inhibit the translocation of NF κ B into the nucleus following infection. Immunofluorescence was used to determine the location of transcription factors within the cell.

METHODS AND MATERIALS

Cell Culture

HeLa (human cervical cancer cells) and HeLaNS5A (HeLa cells that express the HCV NS5A-10A mutant) cells were used for these experiments. The cells were grown in 6mL of complete DMEM in 25cm² flasks. DMEM is composed of 500 mL of Dulbecco's Modified Eagle Medium, 50 mL of 10% fetal bovine serum, 5 mL of 200 mM L-Glutamine, 5 mL of 100% Penicillin-Streptomycin and 5 mL of 100% Non-essential Amino Acid Solution. The cells were maintained in an incubator with an atmosphere of 5% CO₂ and 95% air. When the cells reached 80% confluence, 10% of the cells were transferred into a new 25cm² flask. To split a new flask, under the hood, medium was aspirated and the cells were washed with 1mL of 1X Phosphate Buffered Saline (PBS). The PBS was aspirated and 1mL of trypsin was added to degrade the proteins that attach the cells to the bottom of the flasks, freeing the cells. The Trypsin was quenched with 3mL of complete DMEM and triturated. A new 25cm² flask with 6mL of complete DMEM was prepared and 0.4mL of the cell solution was added. HeLaNS5A

were grown in the presence of puromycin (1 mg/mL of DMEM) to select for the cells that contained NS5A since the plasmid used to transfect the cells with NS5A contains the puromycin resistance.

Cell Plating

Glass coverslips were prepared by soaking in 1M HCl for 6 hours at 50-60°C. The coverslips were then washed twice with distilled water, and once in 100% ethanol. The coverslips were set out and allowed to air-dry. The coverslips were placed in a 6-well dish. After splitting the cells, the cells were counted to determine the density after which 25,000 cells per well in a volume of 0.5mL of the cell solution were added to each glass coverslip in the 6-well dish, and the cells allowed to settle on the coverslip for 30 minutes. Afterwards, 3.5mL of complete medium were added to each well, and the plated cells were placed in the incubator for 24 hours before starting the experiment.

Transfection

In order to have HeLa cells that expressed the HCV NS5A-10A or HCV NS3/4A protein, the cells were transfected with a plasmid containing the specific gene. Under the hood, 0.5µg of DNA was added to 100µL of Lyovect. The solution was mixed and left to incubate at room temperature for 20 minutes. Then 100µL of the solution was added drop-wise to the cells. The cells were left for 48 hours in the incubator before continuing the experiment.

Infection with Sendai Virus

The cells were plated 24 hours before infecting the cells with Sendai Virus. For each experiment, 10 μ L of Sendai Virus (4000 HAU/mL, Charles River Labs) was added to each well containing medium and coverslips. The dish was rocked carefully back and forth to evenly distribute the viral particles throughout the medium. The cells were placed in the incubator for the desired amount of time before the next step of the experiment.

Immunofluorescence

After cells were plated, transfected and/or infected, the cells were stained for a specific protein of interest. Under the hood, the medium was aspirated from the wells containing the coverslips and the wells were washed with 0.5mL of PBS per well. The PBS was removed and 0.5mL of methanol at 4°C was added to each well for 30 minutes. Methanol was removed and 0.5mL of 10% Triton X-100 diluted in PBS was added to each well. After 15 minutes, 10% Triton X-100 was removed, and 0.5mL 10% Bovine Calf Serum diluted in PBS was added to each well for 1 hour. 10% Bovine Calf Serum was removed and 0.5mL of primary antibody (1/250 dilution in 10% Calf Serum) was added to each well. Depending on the experiment, anti-NF κ B (Santa Cruz Biotechnology), anti-M2 (Sigma Aldrich), anti-NS5A (Santa Cruz Biotechnology), and anti-IRF-3 (Santa Cruz Biotechnology) primary antibodies were used. After one hour, the primary antibody solution was removed and each well was washed with 0.5mL 0.5% Tween20 (diluted in PBS) for 5 minutes on the rocker. This was repeated for a total of 3 washes. After the washes, the lights were turned off to prevent photobleaching. Then 0.5mL of secondary antibody (1/500 dilution in 10% Calf Serum) was added to each well for one hour. Depending on the experiment, fluorescent antibodies anti-mouse (Thermo Fischer Scientific)

and/or anti-rabbit (Thermo Fischer Scientific) were used. The coverslips were then washed 3 times with 0.5mL of 0.5% Tween20 on the rocker for 5 minutes per wash. The 0.5% Tween20 was removed and 0.5mL of PBS was added per well.

The coverslips (cells facing down) were mounted onto glass slides using Fluoromount-G (Southern BioTech). The coverslips were slowly lowered at an angle onto the glass slides to prevent the formation of bubbles under the slide. The edges of the coverslips were sealed with nail polish to fix the coverslip on the glass slide. The glass slides were viewed under a fluorescent microscope at either 488nm or 546nm wavelength depending on the secondary antibody used. The location of proteins in the cell were determined by the location of fluorescence in the cell.

HeLaNS5A 10A Cell Line

HeLaNS5A 10A cells are HeLa cells that overexpress the HCV protein mutant NS5A 10A. HeLaNS5A cells were used as a control in the experiments. 100,000 HeLa cells were plated into two 60mm dishes. After 48 hours, each dish was transfected with 1 μ g of NS5A 10A and 200 μ L of Lyovect following the protocol from the manufacturer. The transfected HeLa cells were grown in the presence of puromycin because the plasmid containing the NS5A gene also had a gene for puromycin resistance so that only the cells that were successfully transfected would continue to grow.

Once the 60mm plates were confluent, the HeLa cells were plated on 10cm dishes. One dish had 1/10 of the HeLa cells while the other plate had 9/10. Puromycin was added to each dish to select for the HeLa cells that took up the NS5A 10A DNA. If the HeLa cells were successfully transfected and took up the NS5A 10A DNA, then those HeLa cells would be

puromycin resistant while the HeLa cells that were unsuccessfully transformed would be killed by the puromycin. Every 3 days the medium and puromycin was replaced in each dish to continue to select only for the HeLaNS5A 10A cells.

Once colonies of about 30 cells were formed on the plates, the medium in the 10cm dishes was aspirated. The 10cm dishes were then washed with PBS and an additional 5mL of PBS were added to each dish. Each cell colony was isolated using a cloning chamber and 100 μ L of trypsin was added to detach the cells from the bottom of the dish. The colonies were then triturated and each colony was transferred into one well of a 12-well dish containing 2mL of complete medium supplemented with 1mg/mL of puromycin.

Once the colonies were more the 50% confluent, they were transferred to a 6-well plate with 4mL of complete medium supplemented with puromycin. The remainder of the cells was placed into a 1.5mL centrifuge tube and centrifuged for 5 minutes at 8,000 rpm to pellet the cells. The medium was removed and the pellet was washed with 0.5mL PBS. The tube was centrifuged again for 5 minutes at 8,000rpm. The remaining supernatant was carefully removed. To break up the pellet of HeLaNS5A cells, 50 μ L of SDS PAGE Lysis buffer (sodium dodecyl sulfate and bromophenol blue) was added and left to sit for 15 minutes on ice. Then the solution was centrifuged in 4°C for 15 minutes at 13rpm. The HeLaNS5A cell supernatant was resuspended n 1xSDS Loading Buffer and then boiled for 5 minutes before being and frozen.

Western Blot

A Western Blot was performed to confirm the presence of NS5A in the isolated cell lines. First a Bradford Assay was performed to determine the concentration of HeLaNS5A cells in each of the cell line tubes that were frozen. A standard curve was constructed to compare the A₅₉₅

values obtained from each of the cell lysates in order to determine the concentration of HeLaNS5A cells. Each HeLaNS5A cell line was loaded into a 96-well plate in triplicates and the A₅₉₅ absorption values of each cell line were averaged to calculate the concentration of NS5A 10A protein in each HeLaNS5A 10A cell line.

Cell lysates were separated on a 9% acrylamide gel using SDS PAGE following which the proteins were transferred to Immobilon membrane at 100V for 1 hour. The membrane was blocked with BLOTTO Blocking Buffer to reduce random antibody binding to the membrane. The membrane was placed in a solution of primary antibody and BLOTTO Blocking Buffer (1:1000 dilution) was added to the membrane and allowed to sit overnight at 4°C on a rocker. After sitting overnight in the primary antibody solution, the membrane was washed with BLOTTO Blocking Buffer three times at room temperature. Then the membrane was added to a solution containing secondary antibody and BLOTTO Blocking Buffer (1:1000 dilution) and the membrane left again to sit overnight at room temperature.

RESULTS

HeLaNS5A-10A Cell Line

NS5A is a nonstructural protein produced by HCV that is known to inhibit the translocation of transcription factor NFκB into the nucleus following viral infection. The HeLaNS5A-10A cell line was used as a positive control for the inhibition of NFκB translocation. HeLa cells were grown in 60mm dishes and 48 hours later were transfected with a plasmid encoding for NS5A-10A. The cells were grown in puromycin to select for only the cells that had successfully incorporated the introduced plasmid into its genome because the plasmid, in

addition to coding for NS5A, also coded for a puromycin resistant gene. The colonies were isolated and a western blot was performed to confirm that the cell lines were expressing the HCV viral protein NS5A (Figure 2). Beta-actin was utilized as a loading control for the different cell line. Only two of the cell lines designed HeLaNS5A (3A) and HeLaNS5A (3B) showed the presence of NS5A protein (Figure 2B).

To further confirm that the NS5A protein was expressed in HeLaNS5A 10A cell lines 3A and 3B immunofluorescence was performed. HeLa cells were plated in one well on a pre-treated coverslip in a 6-well plate as a negative control as they do not express the HCV viral protein NS5A. Cell lines HeLaNS5A (3A) and (3B) were each plated in one well on a pre-treated coverslip. All cells were stained with an anti-NS5A antibody (mouse) and then with a secondary anti-mouse fluorescent antibody. The NS5A band appears at 56kDa. HeLa cells did not show the presence of NS5A and both NS5A cell lines, HeLaNS5A (3A) and HeLaNS5A (3B) showed cytoplasmic NS5A present (Figure 3B, 3C).

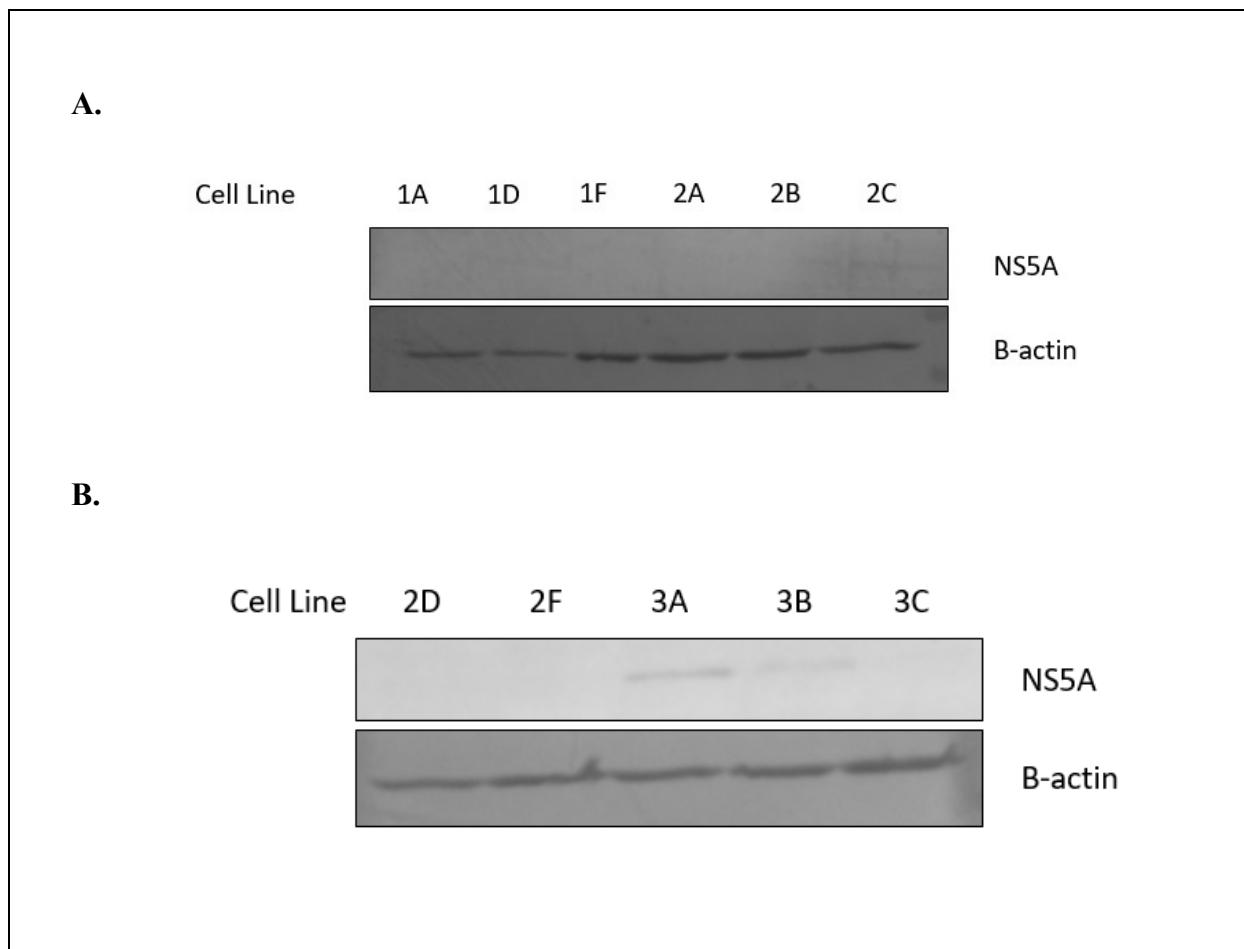


Figure 2: (A) Western Blot for beta-actin and NS5A in cell lines 1A, 1D, 1F, 2A, 2B, and 2C. (B) Western Blot for beta-actin and NS5A in cell lines 2D, 2F, 3A, 3B, and 3C.

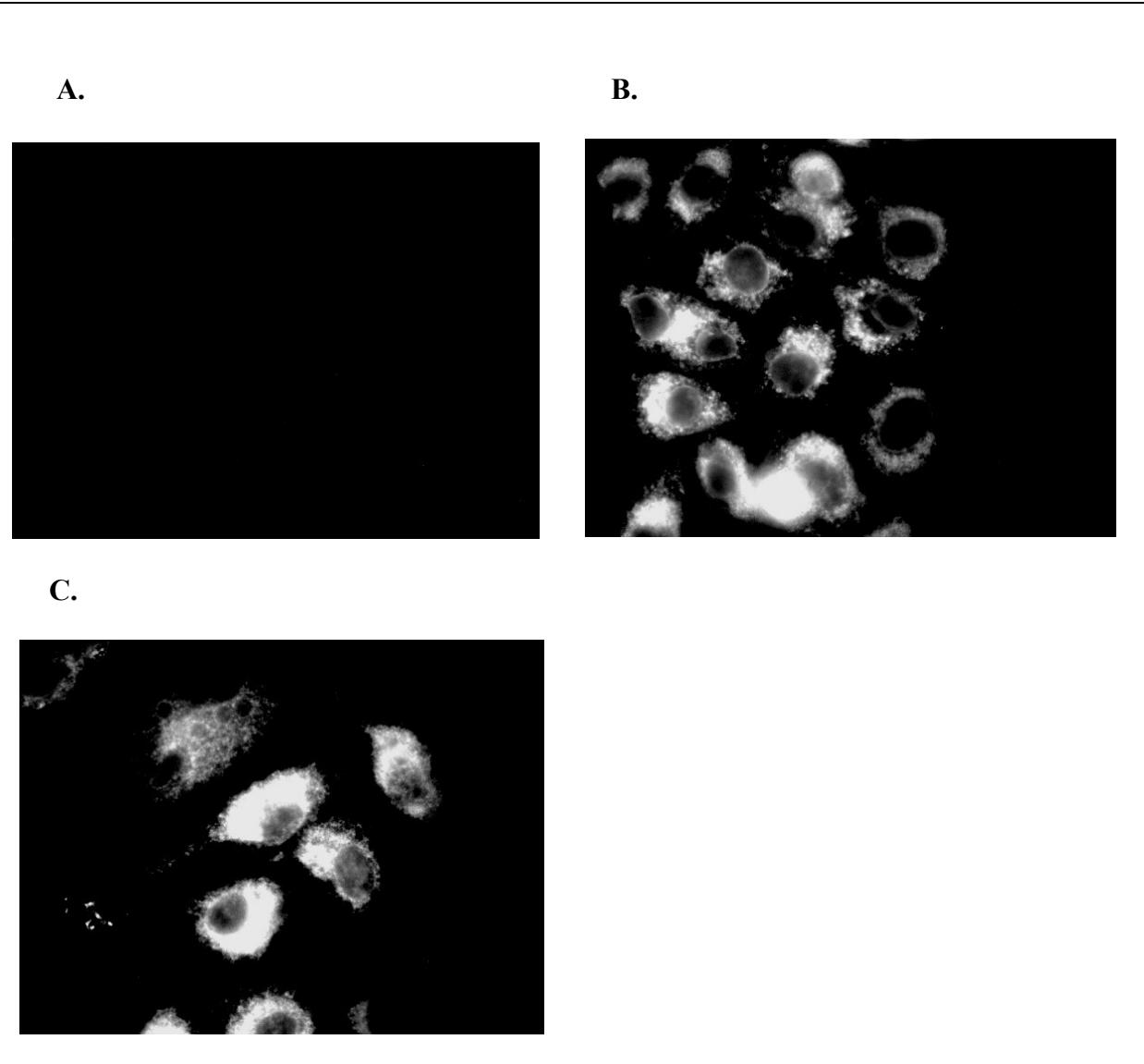


Figure 3: All slides were viewed under a 100x fluorescent microscope with a wavelength of 488nm. (A) HeLa cells stained with anti-NF κ B antibody. (B) HeLaNS5A 3A cells stained with anti-NF κ B antibody. (C) HeLaNS5A 3B cells stained with anti-NF κ B antibody.

Translocation of NF κ B at Different Times Post Infection with Sendai Virus

NF κ B is a transcription factor that translocates into the nucleus of a cell following viral infection. The purpose of this experiment was to determine at what time post-infection NF κ B had the highest nuclear localization. HeLa cells were plated on coverslips and treated with 10 μ L of Sendai Virus (SV) per well for 8 hours, 16 hours, and 20 hours. One well of uninfected HeLa cells was also plated on coverslips as a negative control. All wells were stained with an anti-NF κ B (rabbit) antibody and a secondary anti-rabbit fluorescent antibody. Uninfected HeLa cells showed cytoplasmic localization of NF κ B (Figure 4A). HeLa treated with SV for 16 hours showed the highest nuclear localization of NF κ B with about 85% of the cells with NF κ B in the nucleus (Figure 4C). HeLa cells treated with SV for 8 hours and 20 hours showed low nuclear localization of NF κ B (about 5% and 25% respectively) (Figure 4B, 4D). As a result, 16 hours was determined to be the optimal time of infection with SV as the highest nuclear localization of NF κ B occurred at 16 hours. This was consistent with previous findings in our lab and 16 hours of infection with SV was used for future experiments.

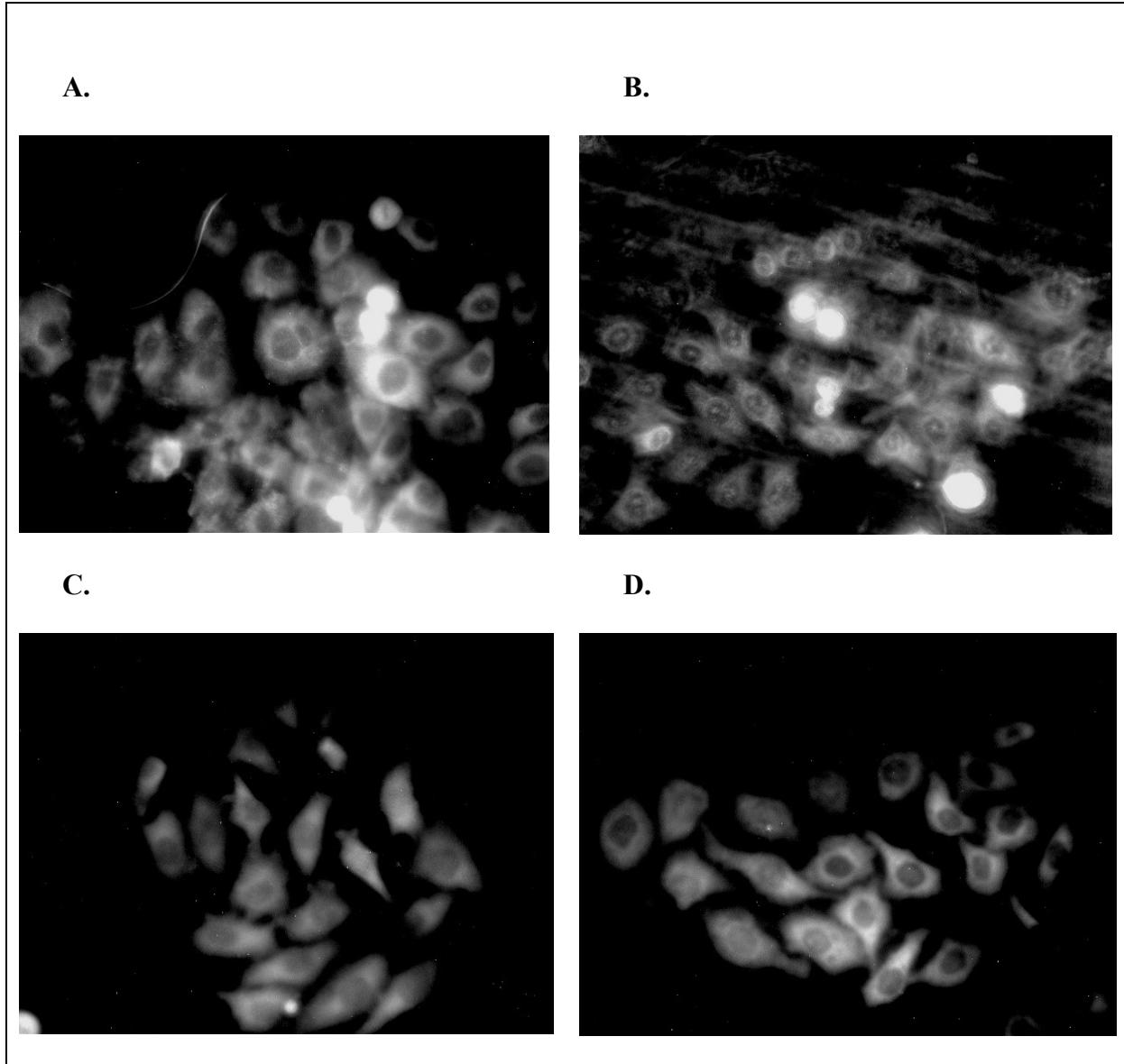


Figure 4: All cells were viewed under 40x fluorescent microscope at a wavelength of 488nm. (A) HeLa cells were stained with anti-NF κ B antibody. (B) HeLa cells infected with Sendai Virus for 8 hours and stained with anti-NF κ B antibody. (C) HeLa cells infected with Sendai Virus for 16 hours and stained with anti-NF κ B antibody. (D) HeLa cells infected with Sendai Virus for 20 hours and stained with anti-NF κ B antibody.

Translocation of NFκB at different times post treatment with TNF-alpha

NFκB translocation can result from the stimulation of the TNF- α receptor. TNF- α induces NFκB translocation through a separate pathway from viral infection. This experiment was conducted to determine the time post treatment that would have the highest NFκB nuclear localization. Four wells of HeLa cells were plated on coverslips and infected with 10ng/mL of TNF- α for 1 hour, 2 hours, and 4 hours. One well of untreated HeLa cells was also plated as a negative control. All cells were then fixed and stained with an anti-NFκB antibody (rabbit) and with a secondary anti-rabbit fluorescent antibody. Untreated HeLa cells showed cytoplasmic NFκB while all the cells treated with TNF- α showed nuclear localization of NFκB (Figure 5A). HeLa cells treated for 1 hour with TNF- α showed 100% nuclear localization of NFκB while 2 hours and 4 hours showed 90% and 75% respectively (Figure 5B, 5C, 5D). NFκB moves from the nucleus to the cytoplasm following exposure to TNF- α which is consistent with previous experiments.

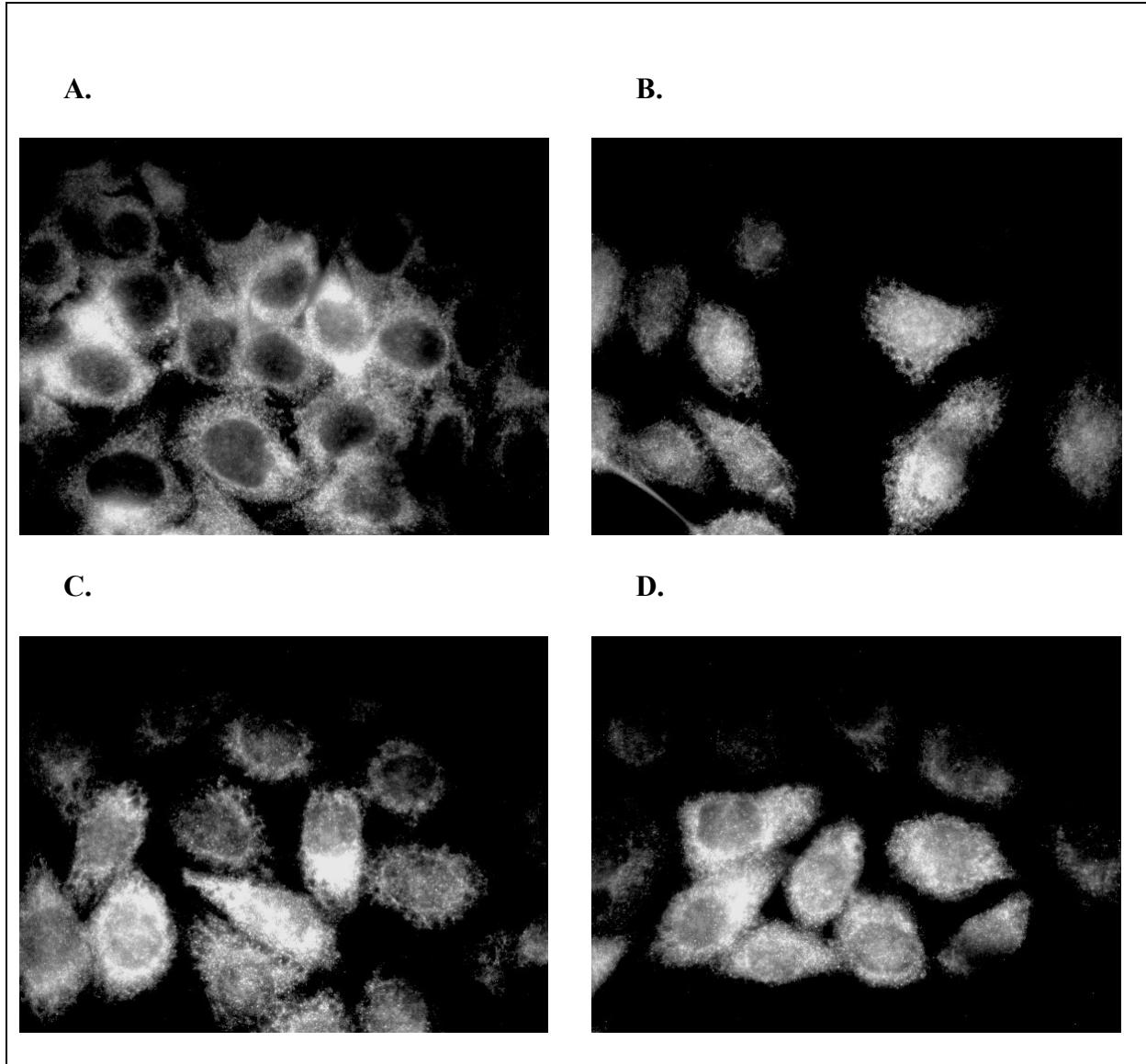


Figure 5: All cells were viewed under 100x fluorescent microscope. (A) HeLa cells were stained with an anti-NF κ B antibody. (B) HeLa cells were treated with TNF- α for 1 hour and stained with an anti-NF κ B antibody. (C) HeLa cells were treated with TNF- α for 2 hours and stained with an anti-NF κ B antibody. (D) HeLa cells were treated with TNF- α for four hours and stained with an anti-NF κ B antibody.

NS3/4A inhibits nuclear localization of IRF-3 post infection with Sendai Virus

IRF-3 is a transcription factor that translocates from the cytoplasm to the nucleus following viral infection and binds to DNA which leads to the production of interferon-beta.

NS3/4A is a HCV viral protease that is known to inhibit the virus infection-induced movement of IRF-3 into the nucleus. This experiment was conducted to demonstrate a positive control of inhibiting IRF-3 movement into the nucleus. HeLa cells were plated on coverslips and two wells were transfected with HCV viral protein NS3/4A and Lyovec. All wells were stained with anti-IRF-3 (rabbit) antibody and anti-rabbit fluorescent antibody. The wells transfected with NS3/4A were double stained with anti-M2 (mouse) antibody and anti-mouse fluorescent (M2 recognizes a peptide sequence that is found on NS3/4A). Uninfected HeLa cells approximately 3% nuclear localization of IRF-3 while infected HeLa cells showed approximately 95% nuclear localization of IRF-3 (Figure 6A, 6B). When HeLa cells were transfected with NS3/4A and infected with Sendai virus, there was no nuclear localization of IRF-3 showing that NS3/4A effectively inhibited the movement of IRF-3 into the nucleus (Figure 6D). This finding was consistent with previous experiments.

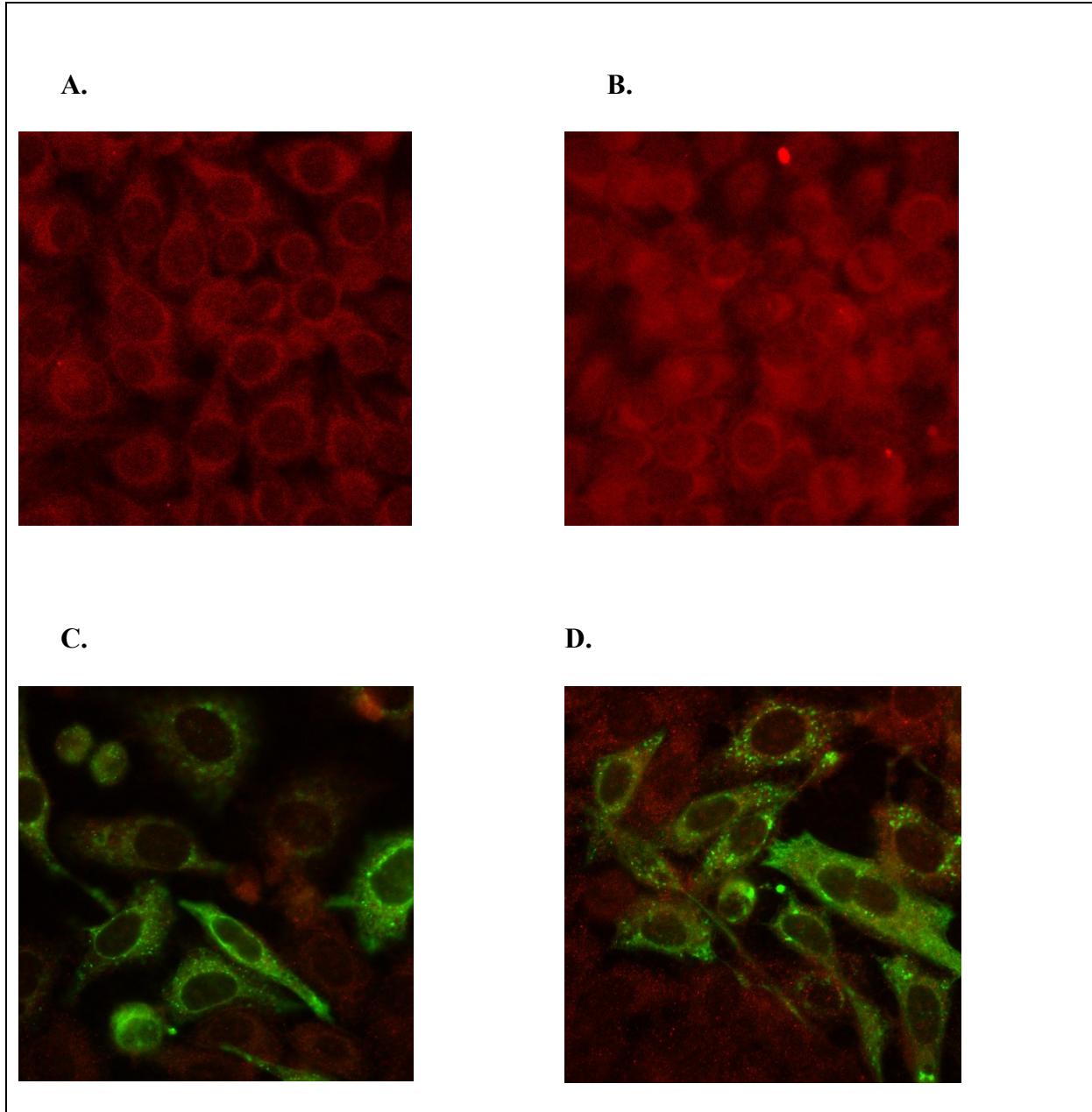
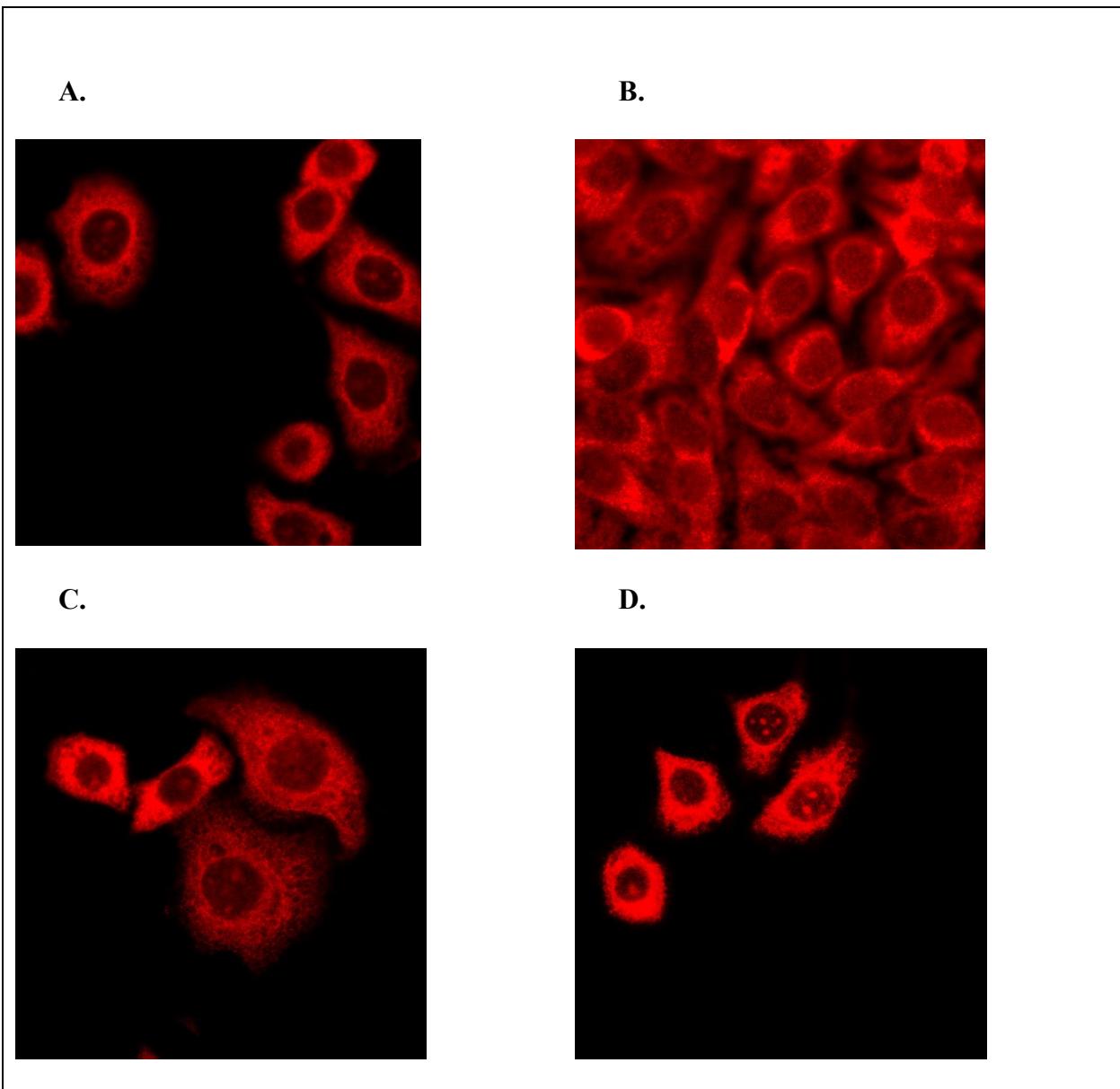


Figure 6: All cells were viewed under 63x confocal microscope at a wavelength of 544nm.

(A) HeLa cells were stained with an anti-IRF-3 antibody. (B) HeLa cells were infected with Sendai Virus for 16 hours and stained with an anti-IRF-3 antibody. (C) HeLa cells were transfected with NS3/4A and stained with an anti-IRF-3 (red) and anti-M2 (green) antibody. (D) HeLa cells were transfected with NS3/4A and infected with Sendai Virus for 16 hours. The cells were stained with an anti-IRF-3 (red) and anti-M2 (green) antibody.

NS3/4A Inhibits Nuclear Localization of NFκB Post Infection with Sendai Virus

NFκB is a transcription factor that is known to translocate into the nucleus following viral infection to produce IFN-β and alert the immune system of an infection. Because viral protein NS3/4A inhibits the production of IFN-β by cleaving MAVS, we hypothesized that NS3/4A inhibits the movement of both transcription factors IRF-3 and NFκB into the nucleus of a cell. As a control, HeLa cells were infected with SV to observe the movement of NFκB into the nucleus of the cell following viral infection (Figure 7A and 7B). Viral protein NS5A is known to inhibit the movement of NFκB into the nucleus of the cell. Therefore, NS5A was used as a positive control for inhibiting the movement of NFκB into the nucleus of the cell (Figure 7C and 7D). HeLa cells were transfected with NS3/4A and the location of NFκB following viral infection was observed. NS3/4A was able to inhibit the movement of NFκB into the nucleus of the cell (Figure 7E and 7F). This supports our hypothesis that NS3/4A is able to inhibit the movement of both IRF-3 and NFκB into the nucleus of the cell. HeLaNS5A and HeLa cells transfected with NS3/4A were also stained for the presence of NS5A and NS3/4A respectively in order to show that these cells were indeed expressing these viral proteins (Figure 7G and 7H).



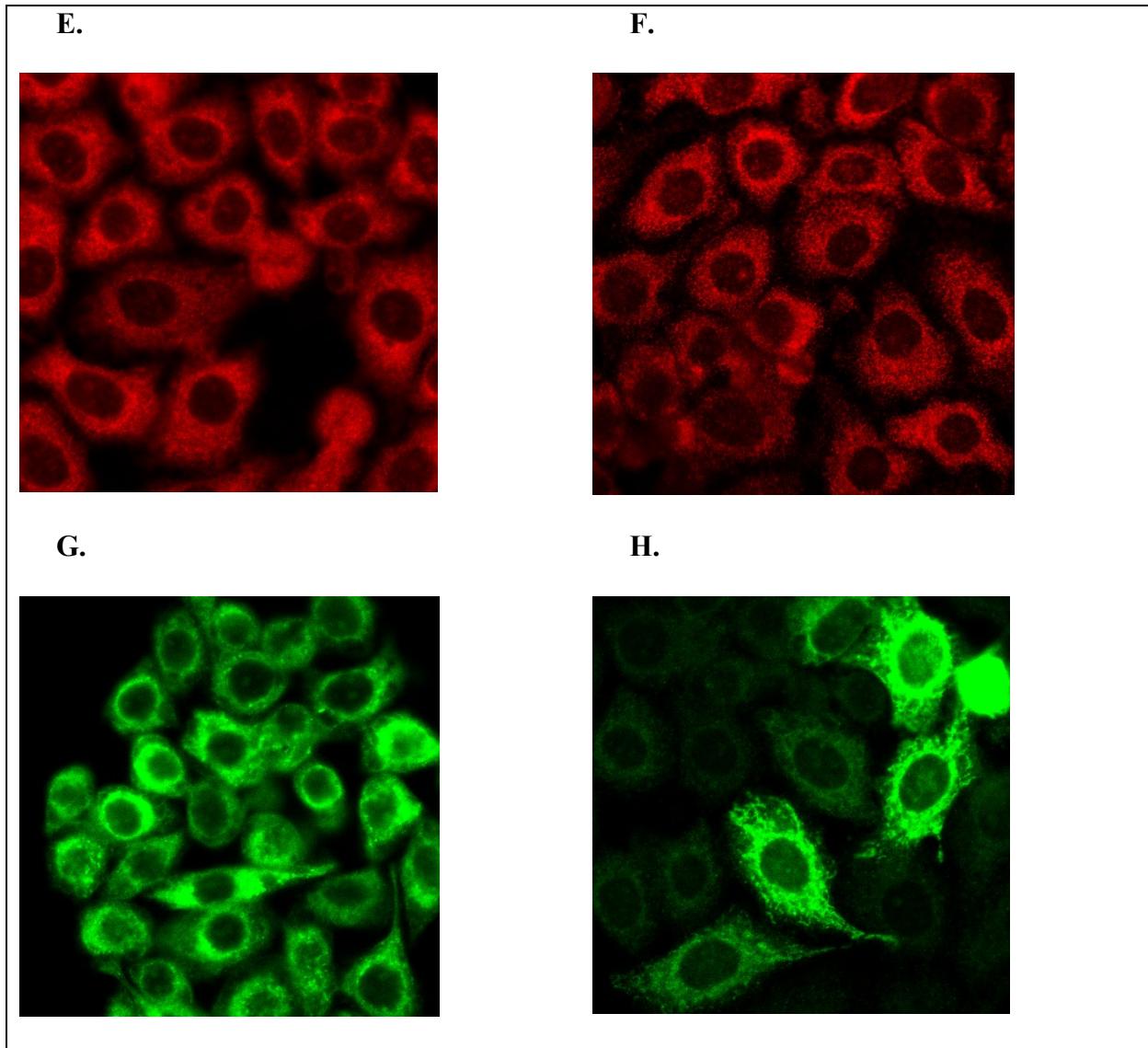


Figure 7: All cells were viewed under 63x confocal microscope. A) HeLa cells stained with anti-NF κ B antibody. B) HeLa cells infected with Sendai Virus for 16 hours stained with anti-NF κ B antibody. C) HeLaNS5A cells stained with anti-NF κ B antibody. D) HeLaNS5A cells stained infected with Sendai Virus for 16 hours stained with anti-NF κ B antibody. E) HeLa cells transfected with NS3/4A stained with anti-NF κ B antibody. F) HeLa cells transfected with NS3/4A and infected with Sendai Virus for 16 hours stained with anti-NF κ B antibody. G) HeLaNS5A cells stained with anti-NS5A antibody. H) HeLa cells transfected with NS3/4A stained with anti-M2 antibody.

DISCUSSION

Hepatitis C Virus produces viral proteins that aid in the replication of its genome and help the virus evade the innate immune response of the host. HCV viral protein NS3/4A is known to cleave MAVS and by doing so it inhibits the movement of IRF-3 into the nucleus and inhibits the production of interferon. NS5A is known to inhibit the movement of NF κ B into the nucleus of the cell thereby inhibiting the production of interferon. Activation of MAVS by RIG-I leads to the assembly of the inflammasome which leads to the activation of both NF κ B and IRF-3. We hypothesized because NS3/4A cleaves MAVS and makes the inflammasome unable to assemble, NS3/4A should be able to inhibit the translocation of both IRF-3 and NF κ B into the nucleus.

To establish a positive control for inhibition of NF κ B translocation into the nucleus, a cell line overexpressing the HCV protein NS5A (HeLa NS5A-10A) was made since NS5A-10A is known to inhibit the movement of NF κ B into the nucleus. A Western blot was used to identify the transfected cell lines that had successfully incorporated the plasmid with NS5A into their genome and expressed the NS5A-10A protein. HeLa NS5A-10A cell lines 3A and 3B were the only cell lines out of the twelve cell lines that expressed NS5A. One of these lines (HeLa NS5A 10A-3A) was used in subsequent experiments. Immunofluorescence with anti-NS5A antibody also confirmed the presence of the viral protein NS5A in the cytoplasm of HeLa NS5A-10A 3A and 3B cell lines as expected. These cell lines will be useful in future experiments because the plasmid expressing NS5A-10A also contains a gene for puromycin resistance that enables allows for only the selection of cells expressing the NS5A-10A protein. The HeLaNS5A-10A cell lines can be used in experiments to ensure that every cell expresses the NS5A protein and to examine

the properties of NS5A, which is also known to inhibit the virus infection-induced movement of NFκB into the nucleus.

To optimize the localization of NFκB into the nucleus for future experiments and as a positive control for the movement of NFκB, HeLa cells were infected with Sendai Virus for various lengths of times following which they were stained with anti-NFκB antibodies. Infection with Sendai Virus for 16 hours showed the highest nuclear localization of NFκB in HeLa cells (approximately 85% of the cells had NFκB in the nucleus) as determined by the increased intensity of fluorescence in the nucleus of these cells. For future experiments, cells should be exposed to Sendai Virus for 16 hours because this time length optimized NFκB localization into the nucleus.

HeLa cells were also exposed to TNF- α as another positive control for NFκB movement into the nucleus and to optimize the localization of NFκB into the nucleus. Exposure to TNF- α for 1 hour, 2, hours, and 4 hours all showed nuclear localization of NFκB; however, exposure to TNF- α for one hour appeared to have 100% nuclear localization as determined by increased fluorescence in the nucleus of the cell. Treatment with TNF- α for one hour should be used for future experiments.

HCV viral protein NS3/4A is known to inhibit the movement of IRF-3 into the nucleus following infection. To establish a negative control demonstrating the inhibition of IRF-3 movement into the nucleus, HeLa cells were transfected with a plasmid containing the NS3/4A gene and infected with Sendai Virus. HeLa cells exposed to Sendai Virus for 16 hours showed 100% nuclear localization of IRF-3 while HeLa cells transfected with NS3/4A and subsequently exposed to Sendai Virus inhibited the movement of IRF-3. These results demonstrated the NS3/4A inhibited the movement of IRF-3 into the nucleus.

NS3/4A was also shown to inhibit the nuclear localization of transcription factor NFκB following viral infection. HeLaNS5A cells were used as a positive control for inhibit the movement of NFκB into the nucleus of the cell as NS5A has already been shown to inhibit the nuclear localization of NFκB. HeLa cells transfected with NS3/4A showed that NFκB remained in the nucleus of the cell following viral infection. We were able to conclude that NS3/4A is able to block the movement of both transcription factors IRF-3 and NFκB with the likely mechanism involving cleaving MAVS.

HCV uses two viral proteins (NS3/4A and NS5A) to inhibit the production of IFN. Because the virus has evolved multiple mechanisms to inhibit IFN, this suggests the importance of IFN production in alerting the immune system of an infection. Inhibition of IFN is essential to the viral life cycle so that viruses like Hepatitis C Virus can hide from the immune system and establish a chronic infection within cells.

The more we know about the life cycle of the virus, the more we can direct treatment toward inhibiting essential parts of the viral life cycle. As shown in our results, NS3/4A and NS5A both have crucial roles in inhibiting the anti-viral response. This can inform physicians on the importance of using certain drugs like Telaprevir and Daclatasvir that specifically inhibit the viral proteins NS3/4A and NS5A respectively. By understanding the life cycle of Hepatitis C Virus, we also able to gain more insight about other viruses that are closely related to Hepatitis C Virus. The more we know about a virus and its life cycle the greater likelihood we have to develop better treatment options for patients.

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