HEPATITIS C VIRAL INFECTION LEADS TO SELF DNA RECOGNITION AND AIM2 ACTIVATION IN HEPATOCYTES

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Lay Summary

This thesis focuses on the cross-talk between hepatitis C (HCV) RNA viral infection and cytosolic self-DNA recognition. Cytosolic DNA is a damage associated molecular pattern that, when recognized, triggers a cascade involving DDX41 translocation and degradation, STING translocation and AIM2 inflammasome formation, ultimately leading to death of the cell. During HCV infection, and in the context of inflammation, most researchers focus on the well-studied NLRP3 inflammasome, but we believe HCV infection triggers the release of nuclear or mitochondrial DNA to the cytosol and activates the AIM2 inflammasome. This is reflected through an increase in AIM2 proteins, the visualization of macro-molecular aggregate structures tagged with anti-AIM2 antibodies and the colocalization of AIM2 and caspase-1 after HCV RNA infection.

Abstract

HCV is the leading cause of liver disease, cirrhosis, and hepatocellular carcinoma (HCC) in many countries. HCV, an RNA virus, may engage elements of cytosolic DNA response. Life or death of individual cells can determine disease progression in multicellular organisms, thus pyroptosis, a pro-inflammatory form of cell death, is initiated through the recognition of danger signals associated with pathological stimuli such as HCV. AIM2 inflammasome formation, triggered through the presence of cytosolic DNA, is not well characterized in the context of HCV, especially as it relates to hepatocytes *in vitro*. This is because there is dogma that RNA viral infection leads to NLRP3 inflammasome activation; whereas, DNA viral infection leads to AIM2 inflammasome activation, although there are some well studied exceptions. During HCV infection, AIM2 inflammasomes could potentially be activated through the loss of nuclear envelope or mitochondrial membrane integrity leading to the release of DNA to the cytosol. The

proteins observed in these experiments were specifically chosen in the context of gaining a greater understanding of how JFH-1_T infection in Huh-7.5 cells involves “cross talk” between RNA viral infection and self cytosolic DNA recognition. Here I demonstrated that after a three-day infection, there is a drastic decrease in the amount of DDX41 protein in the nucleus, suggesting DDX41 degradation. I also show that after infection, STING proteins were far less wide spread, and condensed into smaller pockets located just outside the nucleus, pointing to the possibility that STING proteins are being activated by DDX41 proteins. Since HCV is an RNA virus, and DDX41 is triggered by cytosolic DNA, this lays the foundation for understanding the potential for the formation of AIM2 inflammasomes within the same parameters. Next, I found that AIM2 protein colocalizes with caspase-1 after JFH-1_T infection. I believe that the discovery of a new inflammasome activated by HCV may provide insight on programmed cell death and inflammation, which can have an impact on HCV pathogenesis and treatment, especially since AIM2 expression in down regulated in HCV-related cancers.

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COVID-19 Statement

Do to COVID-19, I was prohibited from working in the lab for 5 months, and spent an additional 4 months working at 50% capacity throughout both the experimental and writing stages of this thesis. Unfortunately, because of time restraints, I could not verify my results through additional experimentation and was unable to find a suitable positive control. What could not be completed has been stated in the discussion session.

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List of abbreviations

aa	Amino acids
AIF	Apoptosis-inducing factor
AIM2	Absent in melanoma 2
ALR	(AIM2)-like receptors
apaf-1	Apoptosis protease-activating factor 1
ATP	Adenosine 5'-triphosphate
BAK	BCL-2 killer
BAX	BCL-2-associated X protein
Bcl-2	B-cell lymphoma 2
BID	BH3-interacting domain death agonist
BIM	Bcl-2-like protein 11
BMDC	Bone marrow-derived dendritic cell
BOC	Boceprevir
CAD	Caspase-activated deoxyribonuclease
CARD	Caspase activation and recruitment domain
CD81	Cluster of Differentiation 81
cGAMP	Cyclic GMP-AMP

cGAS	Cyclic GMP-AMP synthase
CHIKV	Chikungunya virus
CLDN1	Claudin-1
COVID-19	Coronavirus infectious disease 19
DAMP	Damage-associated molecular pattern
DAPI	4’,6-diamidino-2-phenylindole
DDX41	Asp-Glu-Ala-Asp box polypeptide 41
DGAT1	Diacylglycerol acyltransferase-1
DISC	Death-inducing signaling complex
DMEM	Dulbecco’s Modified Eagle’s Medium
DMVs	Double-membrane vesicles
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
FADD	FAS-associated with a death domain
FBS	Fetal bovine serum
FFU/ml	Focus forming units per millilitre

FIIND	Function-to-find domain
GSDMD	Gasdermin D
HAV	Hepatitis A virus
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HIN	Hematopoietic interferon-inducible
HIV-1	Human immunodeficiency virus-1
HSV-1	Herpes simplex virus-1
IAV	Influenza A virus
IKK-α	IκB kinase-α
INF-I	Type I interferon
IRES	Internal ribosomal entry site
IRF3	Interferon regulatory factor 3
JFH-1	Japanese fulminant hepatitis 1
KSHV	Kaposi's sarcoma associated herpesvirus
LeTx	lethal toxin

LNA	Locked nucleic acid
LPS	Lipopolysaccharide
MAM	Mitochondria-associated membranes
MDP	Muramyl dipeptide
miRNA	MicroRNA
MMP	Mitochondrial membrane permeabilization
MOI	Multiplicity of infection
MOMP	Mitochondrial outer membrane permeabilization
MPT	Mitochondrial permeability transition
mtDNA	Mitochondrial DNA
MTP	Microsomal triglyceride transfer protein
NANB	Non-A, non-B hepatitis
NEK7	NIMA-related kinase 7
Neo	Neomycin phosphotransferase gene
NFκB	Nuclear Factor-κB
NLR	Nucleotide-binding oligomerization domain, leucine rich repeat receptor

NLRC4	Nucleotide-binding oligomerization domain, leucine rich repeat and CARD domain containing 4/NOD-like receptor family CARD domain containing 4
NLRP1	Nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 1/NOD-like receptor family pyrin domain containing 1
NLRP3	Nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 3/NOD-like receptor family pyrin domain containing 3
NOD	Nucleotide-binding oligomerization domain
NS	Non structural
NTR	Non-translated regions
OCLN	Occludin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCD	Programmed cell death

PHH	Primary human hepatocytes
PI	Protease inhibitor
p.i.	Post infection
PLA2G4	MAPK-regulated cytosolic phospholipase A2
PS	Phosphatidylserine
PTM	Post-translational modification
PYD	Pyrin domain
PYHIN	Pyrin and hematopoietic interferon-inducible (HIN) domain
RBV	Ribavirin
RdRp	RNA-dependent RNA polymerase
RIG- I	RNA helicase retinoic acid-inducible gene I
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SARS-CoV-2	Sever acute respiratory syndrome coronavirus 19
SeV	Sendai virus

STING	Stimulator of interferon genes
SR-BI	Scavenger Receptor class B type I
SVR	Sustained virological response
TBK1	TANK-binding kinase 1
TLR	Toll-like receptor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR-associated protein with death domain
TRIM21	Tripartite motif-containing protein 21, also/E3 ubiquitin-protein ligase
TVR	Telaprevir
T3SS	Type III secretion systems
VACV/ VV	Vaccinia virus
VLDL	Very low-density lipoproteins
WNV	West Nile virus
ZIKV	Zika virus

Chapter 1: Introduction

1.1 Overview

1.1.1 The discovery of HCV

The discovery of hepatitis C (HCV) relied on the ability to distinguish the virus from hepatitis A and B. From the 1960s to the 1970s, viral hepatitis represented two diseases: infectious hepatitis and serum hepatitis. Infectious (now known as hepatitis A or HAV) and serum (now known as hepatitis B or HBV) hepatitis differed via: incubation period, transmission, degree of infectiousness, and severity (Krugman et al., 1967). HAV could have an incubation period of 1-3 weeks with fecal-oral transmission, a low degree of infectiousness and could become severe and fatal but did not lead to chronic hepatitis or liver cirrhosis. HBV was found to have an incubation period of 1-3 months, to be transmitted from mother to child, sexually or through injection drugs with a low degree of contagiousness that often resulted in chronic hepatitis and liver corrosion (Krugman et al., 1967). Blood from individuals with HAV, used for transfusion, did not transmit hepatitis to the recipient (Feinstone et al., 1975) The discovery of the Australia antigen in 1968, now named the hepatitis B surface antigen or HBsAg (Prince, 1968), led to the screening of all donated blood and only a 25%-50% reduction in hepatitis infection via blood transfusion (Alter et al., 1972), suggesting a presence of a "non-a, non-b" (NANB) hepatitis, not detected through HBV screening. The visualization of HAV via immune electron microscopy in 1973 (Feinstone et al., 1973) supported this.

NANB hepatitis was soon to be classified as HCV. Those infected often experienced two distinct stages. The acute stage had mild to no symptoms but could lead to a chronic stage in around 75% of individuals. Chronic infection can result in liver cirrhosis and hepatocellular

carcinoma (HCC) (Dienstag, 1983). After 6 years of study by scientists at a California biotechnology company called Chiron, a single HCV clone was isolated and discovered to be a new flavi-like virus which, in 1989, they named the hepatitis C virus (Choo et al., 1989) (Houghton, 2009).

1.1.2 Epidemiology

Today, HCV is the third leading cause of chronic liver disease in the world (Cheemerla & Balakrishnan, 2021). Although there are new direct-acting antiviral agents which allow for eradication of HCV in over 90% of patients (Stasi et al., 2020), it is estimated that more than 71 million people are affected by HCV globally (WHO, 2020) in 2020. Thirty percent (15%-45%) of individuals can clear the infection without treatment, meaning 70% (55%-85%) of individuals progress to chronic infection. In 2017, 19% of those chronically infected were aware of their illness, leading only 2 million persons (less than 3% of all chronically infected) to receive treatment in the same year (WHO, 2019). Chronic HCV infection is often partnered with lymphoproliferative disorders, type II diabetes mellitus, cardiovascular disease and kidney disease (Zignego et al., 2007). HCV infection is also linked to a decreased quality of life (Dirks et al., 2019). In 2016, 399 000 deaths worldwide were directly related to HCV (WHO, 2020). In Canada, HCV causes more years of life lost than any other infectious disease (Schanzer et al., 2014), (Kwong et al., 2012) and affects nearly 1% of the population (Myers et al., 2015) with an estimated care cost of $160 million per year as of 2013 (Myers et al., 2014). Individuals born between 1945–1975 have the highest prevalence of chronic HCV and because this population is aging, Canada is seeing an increase in complications, even though prevalence is declining (Schanzer et al., 2014). The World Health Organization (WHO) has set targets for 2030 which includes a 90% reduction in HCV infections, 65% reduction in HCV-related mortality, and

treatment of 90% of individuals living with HCV (WHO, 2020). Canada is one of 11 high income countries on track to reach this goal (Pedrana et al., 2021). This means that significant and strategic action is required among 3 out of 4 high income countries while also battling coronavirus disease 2019 (COVID-19).

1.1.3 HCV and SARS-CoV-2 coinfection

COVID-19 increases the mortality of individuals with chronic HCV infection. Within 20 years of acquiring chronic HCV, 15%-30% will experience liver cirrhosis (WHO, 2020), which is especially harmful to those infected with COVID-19. The world is currently experiencing a viral pandemic caused by severe, acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which leads to COVID-19 (H. Li et al., 2020). Of individuals with liver cirrhosis infected with COVID-19, a preliminary study found an overall mortality rate of 40% (Moon et al., 2020). The study found a death rate of 43%-63% in patients with decompensated cirrhosis, compared to only 12% of patients with other liver diseases (Moon et al., 2020). HCV and SARS-CoV-2 coinfection is one factor that may increase mortality, but a number of other systemic factors may be related to risk of death and may also increase the likelihood of acquiring the virus.

1.1.4 At risk populations

Numerous populations are disproportionately affected by HCV. Factors such as age, a history of incarceration, sexuality, gender and immigration should be integrated into strategic actions to eliminate the virus. In children who are exposed to HCV via blood transfusion, only 55%-60% remain HCV RNA positive in adulthood (Vogt et al., 1999). In 2014, it was estimated that 15.1% of incarcerated individuals have HCV (Dolan et al., 2016) and recent incarceration increased the risk of acquiring HCV by 62% (Stone et al., 2018). Men who have sex with men

are at a higher risk, especially if they are living with HIV, with a prevalence of 9% and 37.3%, respectively (Terrault, 2002). Women may be at a decreased risk of developing chronic HCV (Kenny-Walsh, 1999), (Wiese et al., 2000) and in Canada, immigrants are at greater risk than Canadian-born citizens (Cooper et al., 2017). Other factors such as the use of injection drugs, alcohol consumption, being insulin resistant, obesity, and HBV coinfection may contribute to disease progression and one's ability to spontaneously clear the virus, as these factors may indicate decreased viral immune response (Moosavy et al., 2017), (Chen & Morgan, 2006). Symptoms of acute HCV infection may include fever, fatigue, nausea, vomiting, abdominal pain, joint pain, and jaundice with many attributing acute HCV symptoms with a more robust immune response (Chen & Morgan, 2006). One study found that 48% of those who developed symptoms spontaneously cleared the virus, while 100% of those who did not develop symptoms progressed to chronic HCV (Gerlach et al., 2003).

1.1.5 The need for continuing research

Although there are new direct-acting antiviral agents which allow for eradication of HCV in over 90% of patients (Stasi et al., 2020) (see section 1.2.7 "Treatment"), liver function may not normalize even after receiving the cure and those with liver cirrhosis remain at an increased risk of developing hepatocellular carcinoma (Soriano et al., 2017), (Lybeck et al., 2019). Individuals who spontaneously eliminate HCV or who have received direct-acting antiviral treatment and successfully cleared HCV are not immune to HCV reinfection (Newsum et al., 2020). Systemic barriers to access medications are experienced by many at risk populations (McGowan & Fried, 2012) and challenges emerge due to treatment cost (Myers et al., 2014). Research in HCV is a continued need especially as it relates to viral induced inflammation,

which can lead to liver disease, cirrhosis, and liver cancer even after the use of direct-acting antiviral agents.

1.2 The Hepatitis C Virus

1.2.1 Classification

HCV is a member of the *Flaviviridae* virus family which includes four genera with 89 species (Simmonds et al., 2017). The family is described as having 9,000-13,000 bases, with positive-strand, non-segmented RNA, and are typically spherical in shape with a lipid envelope (Simmonds et al., 2017). All genera in the *Flaviviridae* virus family can infect mammals. The four genera in this family include: *Flavivirus, Pestivirus*, *Pegivirus,* and *Hepacivirus* (Simmonds et al., 2017), (Dubuisson & Cosset, 2014). The *Flavivirus* genus include 53 arthropod-borne species; including human pathogens such as Zika virus, yellow fever virus, dengue virus, and West Nile virus (Simmonds et al., 2017). Members of the *Pestivirus* genus are transmitted through infected secretions and take on pig and ruminant hosts (Simmonds et al., 2017). The *Pegivirus* genus is not clearly involved in disease development but infects a number of mammalian species (Simmonds et al., 2017). The hepatitis C virus is a member of the *Hepacivirus* genus.

Hepaciviruses are closely related to *pegiviruses*, and before 2011, HCV was considered to be the only member of this genus, infecting only humans (and possibly other primates) (Hartlage et al., 2016). Through recent discovery, the *Hepacivirus* genus has since expanded to include a number of species with a wide range of hosts, making the origins of HCV difficult to determine (Hartlage et al., 2016). Some of these species include: *Hepacivirus A,* which infects horses and likely dogs (and is the closest relative of HCV) (Hartlage et al., 2016)*; Hepacivirus*

B (GBV-B), described in tamarins in 1995 and likely infects other new world primates*;* *Hepacivirus D* which takes on colobus monkeys as hosts; *Hepacivirus E-J* which can infect rodents; *Hepacivirus K-M* infecting bats; and *Hepacivirus N* which infects cows (ICTV, 2019). HCV is the most studied and well-known species of the *Hepacivirus* genus (ICTV, 2019).

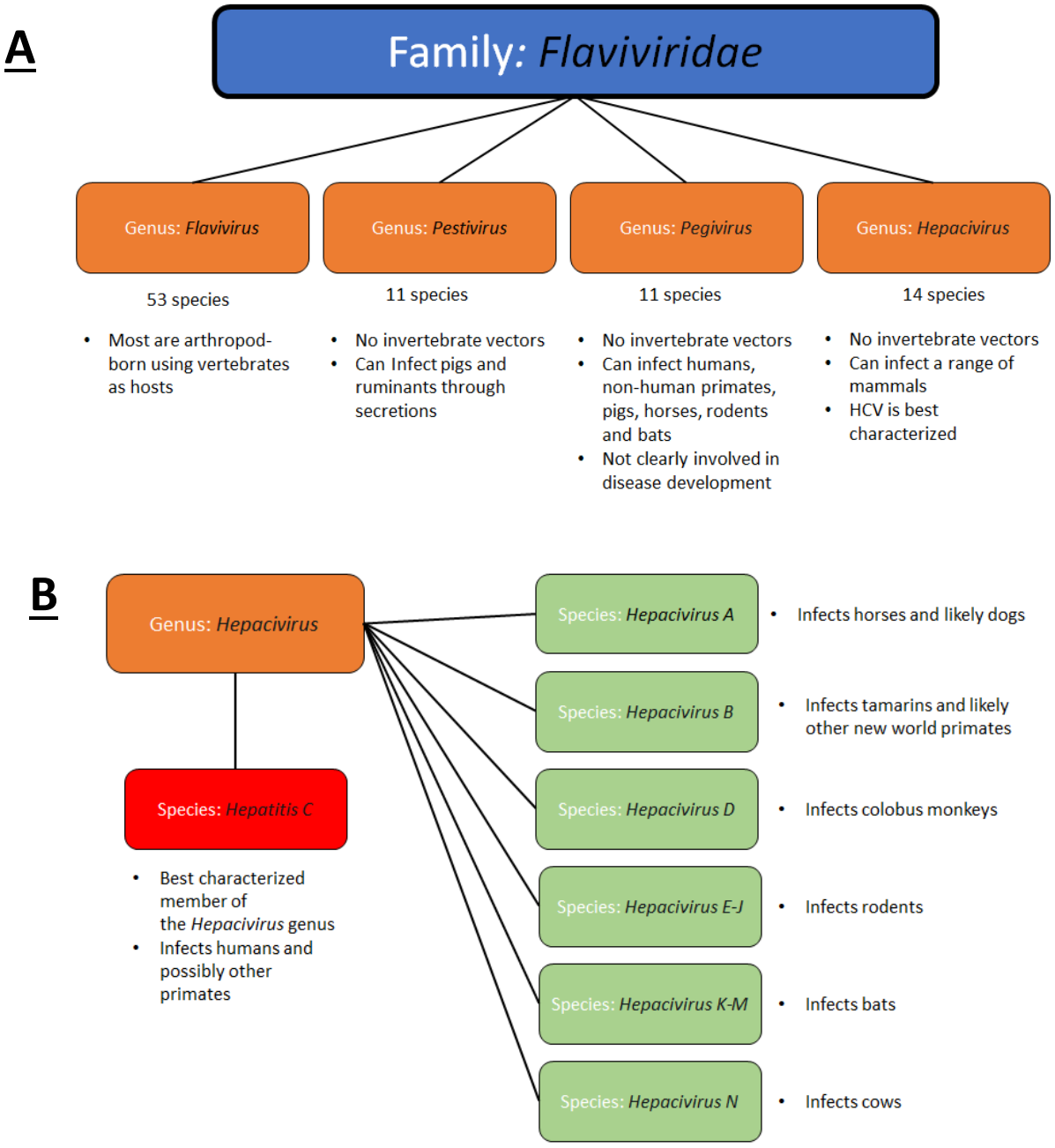


Figure 1.1 HCV classification.

Schematic representation of **(A)** *Flaviviridae* virus family, including number of species and brief description and **(B)** *Hepacivirus* genus, including viral species and know host species (Simmonds et al., 2017).

1.2.2 Genotypes

The hepatitis C virus contains a positive-sense single-stranded RNA genome with considerable variability among genotypes (Irvine et al., 1993) (>30% at the nucleotide level (Smith et al., 2014)). In 1993, HCV was classified into six genotypes (Irvine et al., 1993), and in 2005, rules were established to the assignment and naming of future genotypes and subtypes (Simmonds et al., 2005). These rules required a complete coding region sequence, three or more epidemiologically unrelated isolates, a distinct phylogenetic group, and exclusion of intergenotypic or intersubtypic recombination (Simmonds et al., 2005), leading to the discovery of 18 subtypes (Hedskog et al., 2019) (subtypes have a sequence divergence of >15%). In 2013, the list of genotypes was updated to 7 and 67 subtypes (D. B. Smith et al., 2014). With technological advances the number of genotypes identified expanded to 8 in 2018 (Borgia et al., 2018), with a total of 86 subtypes based on phylogenetic and sequence analyses (Hedskog et al., 2019). Genotypes 1-3 are common around the world with genotype 1 accounting for 46% of infections (Smith et al., 2014), while genotypes 4 and 5 are usually seen in low-income areas (Tagnouokam-Ngoupo et al., 2019) such as Africa and the middle east, and southern Africa respectively (Smith et al., 2014). Genotype 6 is almost restricted to east and south east Asia (Thong et al., 2014), while genotype 7 is thought to have originated from central Africa (Murphy et al., 2015). Genotype 8 has been seen in only four cases in the state of Punjab in India (Hedskog et al., 2019). Genotype 5 and 8 have only 1 subtype each (Smith et al., 2014), (Hedskog et al., 2019). Errors made by the viral RNA-dependent RNA polymerase increases genetic diversity in individuals living with HCV, leading to quasispecies (Pellerin et al., 2004).

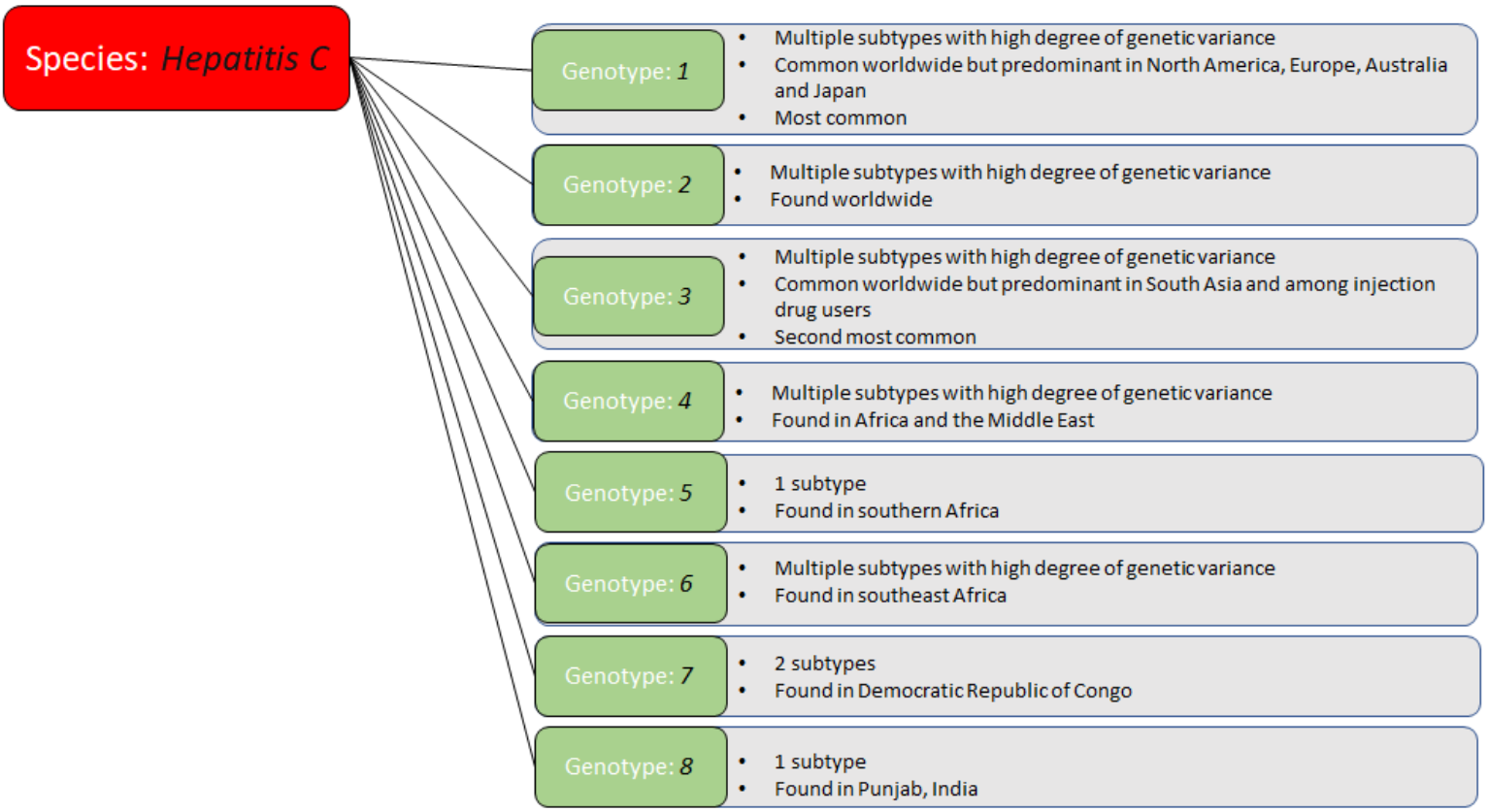


Figure 1.2 HCV genotypes.

Schematic representation of HCV genotypes, including geographic location and subtype genetic variance (Smith et al., 2014), (Irvine et al., 1993), (Simmonds et al., 2005), (Tagnouokam-Ngoupo et al., 2019), (Thong et al., 2014).

1.2.3 Quasispecies

An HCV infected individual will have a number of closely related, but distinct, genetic variants of the virus. This is called a quasispecies (Pellerin et al., 2004). The RNA-dependent RNA polymerase lacks proof-reading activity (NS5B discussed in section 1.1.4). This results in random mutations in nucleotides and continuous production of new viral variants during RNA replication (Pellerin et al., 2004). Similar to HIV, HCV has a mutation rate of 10^{-4} to 10^{-5} mutations per site per replication cycle (Tisthammer et al., 2022), (Borzooee et al., 2019). Although the majority of these new variants are defective in some capacity, some variants succeed and propagate throughout the liver (Pellerin et al., 2004). Due to selection pressures, such as immune evasion, some of these new variants are consistently selected, resulting in variants with enhanced survival capacity (Pellerin et al., 2004). Mutations allow for viral evolution, but for evolution to occur, the virus must conserve essential functions.

1.2.4 Structure and Proteins

The HCV virion is circular in shape and ranges from 40 to >100 nm in diameter, suggesting heterogeneity across virion populations and quasispecies (Lussignol et al., 2016). The virion is enveloped and by interacting with host lipoproteins has the ability to hide viral epitopes (Lussignol et al., 2016). The hybrid lipoviral particles create a low buoyant density of around 1.08 to 1.11 gm/mL (Kanto et al., 1994) involving the association with LDL and very-LDL components, which form a chimeric lipoviral particle (LVP) (Lussignol et al., 2016). The virion contains the HCV genome and the HCV core protein, making up the nucleocapsid; and the envelope glycoproteins E1 and E2.

The HCV genome is positive-stranded, non-segmented RNA with 9.6 kb (Simmonds et al., 2017) and due to the single open reading frame, will encode a polyprotein precursor of 3010 amino acids (Ashfaq et al., 2011) (figure 1.1). Ten mature structural and non-structural (NS) proteins will be the result of co- and posttranslational possessing by host and viral proteases, as seen in figure 1.1. (Ashfaq et al., 2011). Translation will be further discussed in section 1.2.5. Of the 10 mature proteins, the three located at the N-terminal region are structural: HCV core, E1 and E2 (Ashfaq et al., 2011).

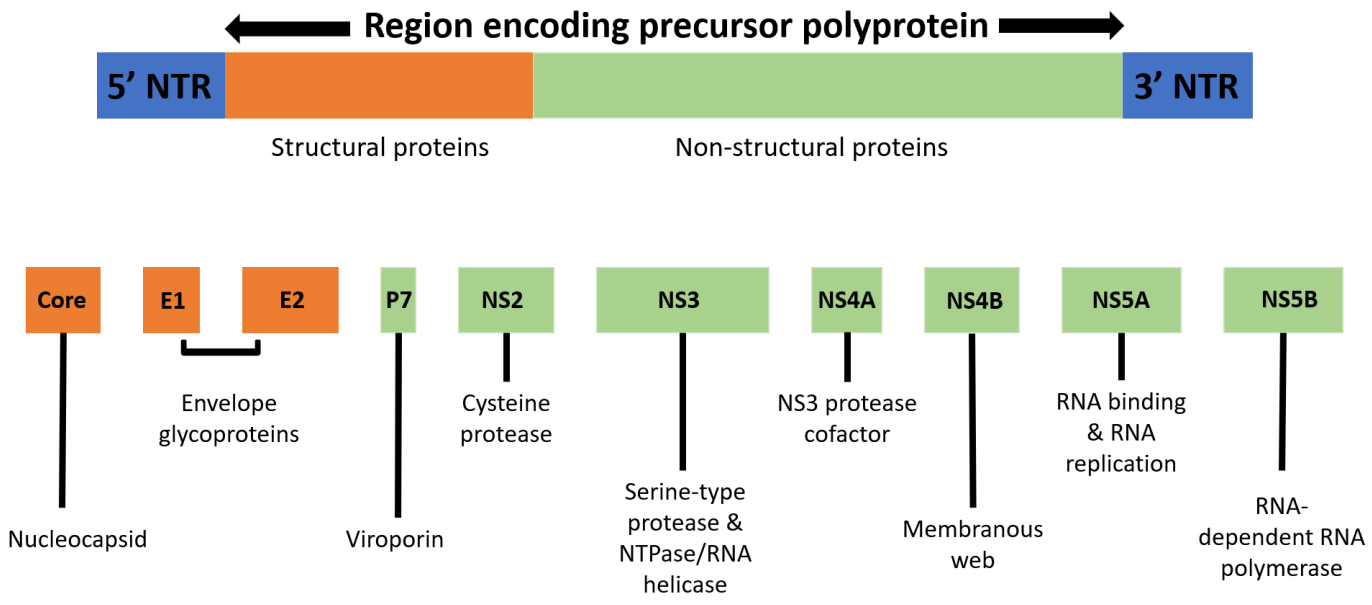


Figure 1.3 HCV proteins.

Schematic representation of the HCV polyprotein and the 10 HCV protein products with descriptions of protein function including the structural proteins: core, E1, E2, and the non-structural proteins: P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Reproduced under Creative Commons CC BY 2.0. Ashfaq, U. A., Javed, T., Rehman, S., Nawaz, Z., & Riazuddin, S. (2011). An overview of HCV molecular biology, replication and immune responses. Figure 1: Proteins encoded by the HCV genome. *Virology Journal*, *8*(1), 161. https://doi.org/10.1186/1743-422X-8-161

HCV core, 1–191 aa, is a 21 kDa protein with two domains housing the nuclear genome and, with HCV RNA, create the nucleocapsid (Gawlik & Gallay, 2014). The fist domain, D1, is located at the N-terminus and is subdivided into three subdomains. D1 is hydrophilic and involved in RNA and lipid binding (Santolini et al., 1994). The D2 domain is more hydrophobic and responsible for lipid droplet localization, folding, and stability of the protein (Boulant et al., 2006). HCV core is well conserved and known for interaction with cellular proteins resulting in the modulation of diverse cellular functions (Klein et al., 2005).

HCV is an enveloped virus (Yost et al., 2018). The envelope is derived from the host membrane and is embedded with viral, highly glycosylated envelope glycoproteins, which are: E1 and E2, aa192-383 and aa384-746, respectively (Ashfaq et al., 2011), (Y. Wang et al., 2017). E1 and E2 form a heterodimer and contain an N-terminal domain which extends into extracellular space (ectodomain), allowing for a receptor-binding domain and a stem region (Helle et al., 2007), and a C-terminal transmembrane domain which allows for anchoring and retention signal to the endoplasmic reticulum (Cocquerel et al., 1999). E1 and E2 play an important role in viral entry. E2 directly binds host cell receptors resulting in receptor-mediated endocytosis and entry into the host cell (Tong et al., 2018). The functions of E1 during viral entry may be limited to its interactions with E2 (Tong et al., 2018) or E1 may be an important component of fusion machinery (Tong et al., 2018). Viral entry will be further discussed in section 1.2.5. Non-structural proteins include: p7 (a viroporin), NS2, NS3, NS4A, NS4B, NS5A, and NS5B.

The p7 monomer is a small 63-amino acid hydrophobic protein, with two assumed transmembrane domains connected by a conserved basic loop region (Oestringer et al., 2019). Both the N- and C- termini are exposed to the ER lumen (Dubuisson, 2007). p7 self-oligomerizes

to predominantly hexameric and heptameric viroporins to form ion channels. These channels are required for the assembly and secretion of the infectious virus but not required for RNA replication (Dubuisson, 2007) (Oestringer et al., 2019). The complete context of p7 involvement in HCV infection is still being studied.

NS2 protein is a 21-23 kDa integral membrane protein (Ashfaq et al., 2011) which is 217 amino acids long (Boukadida et al., 2018). Before cleavage from the polyprotein, NS2 participates in cysteine protease activity responsible for the cleavage between non-structural proteins NS2 and NS3 (Boukadida et al., 2018). The first 180 residues of NS3 are also required (Dubuisson, 2007). NS2 contains 2 domains. The N-terminal domain contains three highly hydrophobic transmembrane helices and serves as a molecular platform for the coordination of HCV proteins in viral assembly (Lange et al., 2014). The crystal structure of the C-terminal domain reveals a dimeric cysteine protease containing two composite active sites. Though NS2 is not vital to the formation of the replication complex, the protein is involved in virus assembly and release (Dubuisson, 2007).

NS3 is 67 kDa, multifunctional, and contains two domains: the N-terminal serine-type protease domain, which is enhanced by the NS4A cofactor (54 amino acids), and the C-terminal NTPase/RNA helicase domain (Dubuisson, 2007). NS3 is dispersed throughout the cytoplasm when expressed alone but when co-expressed with NS4A, is found in association with the ER (Wölk et al., 2000). The heterodimer, NS3-4A, is responsible for the cleavage of downstream NS proteins essential for components of the RNA replication cycle (Dubuisson, 2007). The heterodimer also weakens the cellular immune response through disruption of the RNA helicase retinoic acid-inducible gene I (RIG- I) pathway (Meylan et al., 2005). The RNA helicase domain

is indispensable for RNA replication through RNA unwinding, which requires a NS3 dimer and can be enhanced by the full-length NS3-4A complex (Serebrov & Pyle, 2004).

NS4B is a small (27 kDa) very hydrophobic, transmembrane protein, with both the C and N-termini located in the cytosol, with a fraction of the N-terminus located in the ER lumen (Dubuisson, 2007). The protein contains 4 transmembrane domains and induces intracellular membrane alterations including a membranous web, which may indicate a site for the HCV replication complex formation (Egger et al., 2002). Some studies suggest that other viral components must contribute to this web, as the membranes of cells expressing only NS4B were different than those expressing all HCV proteins (Dubuisson, 2007).

NS5A is a phosphoprotein of 58 kDa in size with a unique amphipathic alpha-helix at its N-terminus that acts as an anchor to the membrane. NS5A has three structural domains (Fridell et al., 2011). Domain I has a motif of 4 fully conserved cysteine residues coordinating a zinc ion which is essential for RNA replication (Tellinghuisen et al., 2004). The other two domains are not well defined (Dubuisson, 2007), though domain III can tolerate insertions and partial deletions meaning less conservation (Appel et al., 2005). NS5A has been shown to bind to the 3' ends of HCV positive and negative strand RNA (Gale et al., 1998) and mutations leading to enhanced viral replication are often found in the NS5A coding region (Dubuisson, 2007). The membranous replication organelles are essential for viral replication. These organelles have double-membrane vesicles (DMVs) which require NS5A for their formation (Lee et al., 2019). Viral replication will be further discussed in section 1.2.5.

NS5B is a 65 kDa protein and contains a C-terminal transmembrane domain which anchors the protein and is essential for RNA replication (Ashfaq et al., 2011). NS5B is an RNA-dependent RNA polymerase which synthesizes a complimentary negative RNA strand from the

positive-sense RNA genome, and in turn, synthesizes a positive strand using the negative strand as a template (Dubuisson, 2007). As mentioned, NS5B lacks proof-reading activity, leading to random mutations in nucleotides and continuous production of new viral variants during RNA replication, resulting in quasispecies (Pellerin et al., 2004). The NS5B catalytic domain contains 3 subdomains: the palm domain containing the active site, and the finger and thumb domains which control interactions with RNA (Dubuisson, 2007). Viral replication will be further discussed in section 1.2.5.

1.2.5 HCV life cycle

The HCV viral lifecycle can be broken down into 6 steps: attachment, where E2 glycoproteins interact with host cell receptors to gain entrance into the cell (Tong et al., 2018); fusion, through clathrin-mediated endocytosis (Blanchard et al., 2006); translation of the viral genome's open reading frame into a large polyprotein, and polyprotein processing (Dubuisson & Cosset, 2014); replication of viral RNA utilizing NS5B (an RNA-dependent RNA polymerase (RdRp)) (Dubuisson, 2007), particle assembly; where lipid droplets play an important role (Dubuisson & Cosset, 2014); and viral particle release through the Golgi secretory pathway (Coller et al., 2012).

The HCV virion circulates in blood both as a free particle, or due to the low buoyant density of HCV, is surrounded by host low-density lipoproteins (André et al., 2002). As mentioned, the envelope proteins play a major role in viral entry. E2 is responsible for receptor binding (Tong et al., 2018) while E1 may be an important component of fusion machinery (Tong et al., 2018). Four cellular proteins have been described in the interaction between the virus and host: scavenger receptor class B type I (SR-BI) (Scarselli et al., 2002), the human cluster of differentiation 81 (CD81) (Pileri et al., 1998), the tight junction proteins claudin-1 (CLDN1)

(Evans et al., 2007), and occludin (OCLN) (Ploss et al., 2009). SR-BI, CD81, and CLDN1 act as initial virus receptors by directly binding to envelope glycoproteins and interact with each other to form co-receptor complexes. Receptor binding starts with the interaction between E2 and the extracellular loop of SR-BI, facilitating exposure of binding sites on E2 allowing viral interactions with CD81 and triggering signals that initiate virion internalization. CD81 associates with CLDN1 and drives HCV entry. Post-binding, OCLN is essential in mediating HCV entry (Colpitts et al., 2020). Hepatocyte to hepatocyte transmission is critical for viral persistence in the liver (Timpe et al., 2008) (Fan et al., 2017) (Ramakrishnaiah et al., 2013) and *in vivo* experiments report that the VLDL receptor mediating lipoprotein uptake into hepatocytes might enable HCV entry (Ujino et al., 2016).

Upon attachment, HCV virions enter the hepatocyte via clathrin-mediated endocytosis, where fusion is triggered by the low pH of the vesicle (Blanchard et al., 2006). Clathrin polymerization leads to changes in membrane curvature and invagination, eventually internalizing the CD81-CLDN1 complexes (Dubuisson & Cosset, 2014) and the HCV viral particle (Egger et al., 2002). Acidification of the early endosomes leads to fusion of the viral envelope to the endosomal membrane, uncoating of the viral particle, and release of the genome into the cytosol where translation takes place (see figure 1.2) (Egger et al., 2002).

The viral RNA genome is approximately 9.6 kb in length and contains an open reading frame flanked by 3' and 5' non-translated regions (NTRs). The NTRs contain elements necessary for RNA replication such as the internal ribosomal entry site (IRES) located on the 5' NTR (Dubuisson & Cosset, 2014). The IRES allows for the host cell ribosomes to begin translation of the open reading frame, creating a single polyprotein which will be processed into the 10 proteins mentioned previously: E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Host

and viral proteases are responsible for polyprotein cleavage. The structural proteins are processed by signal peptidase and host peptides, while the viral proteins: NS3, the NS3/4A (protease complex), and the NS2 (cysteine protease) target NS protein cleavage from the polyprotein (Dubuisson & Cosset, 2014). MiR-122, a liver specific microRNA, recruits Argonaute 2 to the 5′ end of the viral genome which is necessary for translation and genome replication via genome stabilization and protection from degradation by 5′ exonucleases (Dubuisson & Cosset, 2014).

Replication takes place in the membranous web (Egger et al., 2002). To create this, rearrangements of intracellular membranes, mainly originating from the endoplasmic reticulum (ER), are constructed via NS3/4A, NS4B, NS5A or NS5B, with NS4B required as a scaffold (Dubuisson & Cosset, 2014), and NS5A is needed to induce DMVs (Lee et al., 2019). Although each protein alone can induce some membrane remodelling, most or all proteins are required to created the membranous web (Dubuisson & Cosset, 2014) and thus, the membranous web is a membrane-associated multiprotein complex that contains all of the non-structural HCV proteins (Egger et al., 2002).

HCV RNA genome replication is performed by NS5B, which is an RNA-dependent RNA polymerase (RdRp) that synthesizes a complementary negative-strand intermediate of replication from the positive-sense RNA genome, and in turn, synthesizes numerous strands of positive polarity using the negative strand as a template (see figure 1.4) (Dubuisson, 2007). This positive strand will be used for polyprotein translation, synthesis of new negative strands, or packaging into new virions (Bartenschlager et al., 2004). The number of positive strands transcribed is 5 to 10 times greater than the number of negative strands (Bartenschlager et al., 2004). As mentioned,

NS5B lacks proof-reading activity, leading to random mutations in nucleotides and continuous production of new viral variants (Pellerin et al., 2004).

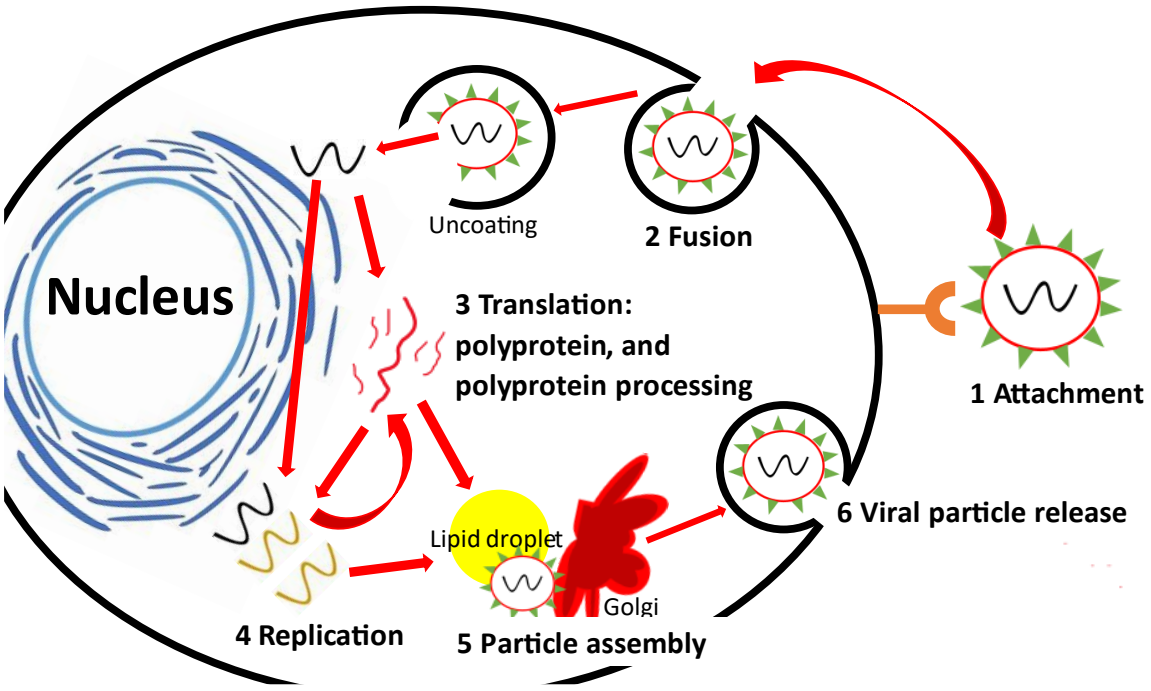


Figure 1.4 HCV life cycle

Schematic representation of the HCV viral life cycle. The HCV viral lifecycle can be broken down into 6 steps: **1** attachment, **2** fusion, **3** translation of the viral genome's open reading frame into a large polyprotein, and polyprotein processing, **4** replication of viral RNA, **5** particle assembly, and **6** viral particle release through the Golgi secretory pathway. Reproduced and adapted to depict HCV replication in a hepatocyte under Creative Commons CC BY 4.0. Ito, M., Kusunoki, H., & Mizuochi, T. (2011). Peripheral B Cells as Reservoirs for Persistent HCV Infection. Figure 1: Proposed lifecycle of HCV in B cell. *Frontiers in Microbiology*, *2*. https://www.frontiersin.org/articles/10.3389/fmicb.2011.00177

An HCV particle contains the HCV genomic RNA and the HCV core protein, which make up the nucleocapsid, and the envelope glycoproteins, which are: E1 and E2. Viral assembly involves bringing these together and takes place near the site of viral replication amongst ER-derived membranes and close to lipid droplets (LDs) (Dubuisson & Cosset, 2014). LDs house core protein heterodimers and accumulation of core protein in LDs which lead to changes in intracellular distribution, causing LDs to collect in the perinuclear region, close to the membranous web (Moradpour et al., 1996). NS2 and p7 coordinate the movement of structural proteins, as well as NS3 and NS5A to the assembly sites (Boson et al., 2011) (Stapleford & Lindenbach, 2011). NS5A is involved in the delivery of the viral RNA to the core proteins, creating the nucleocapsid, through NS5A's domain III which interacts with the LD-bound core protein (Masaki et al., 2008). NS4B may be required for genome encapsulation (Q. Han et al., 2013). One study argues that nucleocapsid assembly utilizes weak-affinity short motifs termed: "packaging signals", located across the HCV genome (Stewart et al., 2016) are the key players in nucleocapsid construction. Interactions between the NS3 helicase and residues 64 to 66 of core D1, after nucleocapsid formation, are necessary for viral assembly to continue, although why this is required is unknown.

Host cell proteins interact with core-LD association. Diacylglycerol acyltransferase-1 (DGAT1) can stop viral production through preventing core-LD association. MAPK-regulated cytosolic phospholipase A2 (PLA2G4) is needed for core trafficking. IκB kinase-α (IKK-α) is a host factor for viral assembly by initiating a pathway that induces lipogenic genes, enhancing core-associated LD formation. Ras-related protein Rab-18, promotes the interaction of the site of viral replication and LDs though binding NS5A (Salloum et al., 2013) and, Diacylglycerol acyltransferase-1 (DGAT1) enhances the interaction between NS5A and core (Camus et al.,

2013). Finally, the μ subunit of the clathrin adaptor protein complex 2 (AP2M1) may interact with core in the retrieval of core from LDs (Dubuisson & Cosset, 2014).

HCV glycoproteins E1 and E2 are necessary in forming the viral particle. Together E1 and E2 form a non-covalent heterodimer, found in the ER (Dubuisson et al., 1994). As mentioned, NS2 and p7 coordinate the movement of structural proteins to the assembly sites (Boson et al., 2011) (Stapleford & Lindenbach, 2011). It has been suggested that the heterodimer, NS2 and p7 form a functional unit, traveling close to the LDs to arrive at the site of assembly (Popescu et al., 2011).

Lipid composition of HCV viral particles resemble that of VLDLs suggesting viral utilization of the VLDL assembly pathway and utilization of microsomal triglyceride transfer protein (MTP), involved in VLDL production. VLDLs biogenesis occurs in the ER and is exported with cholesterol and triglycerides through the Golgi secretory pathway. HCV particles gain their envelope through budding at the ER, where they become infectious (Coller et al., 2012), and likely fuse with newly produced VLDLs at the ER or the Golgi compartment (see figure 1.4), creating lipoviroparticles (LVPs) which can be found circulating in the blood (Syed et al., 2017). HCV matures during the egress process as LVPs display a buoyant density of <1.03 to - 1.25 g/ml, whereas intracellular infectious particles have much higher densities (Jones & McLauchlan, 2010). Other pathways such as the endosomal sorting complex required for transport (ESCRT) may also be involved in viral release (Barouch-Bentov et al., 2016).

1.2.6 Chronic infection

As mentioned, HCV is the third leading cause of chronic liver disease in the world (Cheemerla & Balakrishnan, 2021). Although many individuals are unaware of their acute HCV status, chronic infection can come with symptoms: 80% of people report fatigue, 50% report adnominal pain, 40% report jaundice, and 40% report dyspepsia (Moosavy et al., 2017). In most individuals infected through blood transfusion HCV-RNA can be detected at 7-21 days and the chronic stage can begin 6 months after. This can lead to liver cirrhosis in 15%–35% of these individuals if left untreated for 20 years. 1%–3% of said individuals will develop HCC (Moosavy et al., 2017). Persons with chronic hepatitis can produce up to 10^{12} virus particles daily (Moosavy et al., 2017) but thankfully direct-acting antivirals are now available that can cure over 90% of people who can access treatment.

1.2.7 Treatment

Treatment has come advanced considerably since the 90's**.** The first treatments for chronic HCV were made available in the early 90s. Depending on genotype, patients received a 24- or 48-week course of interferon-alfa 2a or 2b, but less than 10% of individuals could clear the virus. When ribavirin (RBV) was introduced, the number of individuals clearing the virus increased to 30-40%. Sustained virological response (SVR) indicates that this therapy has had successful clearance of the virus and refers to undetectable HCV RNA levels 3–6 months after completing antiviral therapy. SVR further increased in persons following the release of pegylated interferon (PEG-IFN)-alfa 2a and 2b (to be used with RBV) in the late 1990s. This marked the standard treatment for the next 10 years. Although this therapy worked well for genotypes 2, 3, 5 and 6, individuals with HCV genotype 1 (by far the most common genotype) achieved rates of

SVR of only around 40%. This pushed the need to discover direct-acting antivirals (DAAs) (reviewed in Burstow et al., 2017).

The first DAAs were protease inhibitors (PIs), specifically NS3/4A inhibitors and in 2011 included boceprevir (BOC) and telaprevir (TVR) which prevented the cleavage of the HCV polyprotein into non-structural proteins. BOC or TVR given alongside PEG-IFN and RBV increased SVR rates in individuals with chronic HCV genotype 1 to between 64% and 75% in phase III trials. Unfortunately, this treatment came at a cost, including: many side effects, drug interactions, and complex dosing regimens. Real-life outcomes were not comparable with clinical trials, seeing viral clearance in only 40–53% and 53–56% of BOC and TVR patients (reviewed in Burstow et al., 2017).

Soon new DAAs would become available including new NS3/4A inhibitors: Simeprevir, Grazoprevir, Paritaprevir, Asunaprevir, voxileprevir, and glecaprevir. NS5A inhibitors are now produced and they include: Daclatasvir, Ledipasvir, elbasvir, ombitasvir, velpatasvir, and odalasvir. Finally, NS5B inhibitors include: Sofosbuvir and Dasabuvir. Although most inhibitors were designed to be taken alongside PEG-IFN and RBV, interferon therapy still required weekly injection (and possible side effects). Now, depending on genotype, a combination of different inhibitors can be taken without interferon therapy leading to less side effects and SVR rates of over 90% (reviewed in Burstow et al., 2017).

The use of an anti-microRNA (miR) may become available for HCV treatment in the near future. MiRNAs are single stranded, non-coding RNA, which in animals, participate in RNA-silencing and post transcriptional gene regulation by base-pairing with target mRNA, resulting in translational inhibition and mRNA destabilization. When miRNAs bind to sites in the 3’ untranslated region (UTR), gene expression is typically repressed, although miRNAs, like

in the context of HCV replication, have been reported to repress or activate gene expression by binding to 5'-UTR sites (Roberts et al., 2011). MiR-122 is a liver-specific miRNA which regulates liver metabolism, and is conserved among vertebrates (Gebert et al., 2014). MiR-122 has been shown to positively regulate the HCV RNA life cycle by binding directly to a site in the 5'-UTR of the viral RNA, stimulating HCV translocation (Jopling, 2008), (Roberts et al., 2011). Miravirsen (SPC3649) is an anti-miR-122 drug currently in clinical trials for treatment of HCV infections (Gebert et al., 2014), (E. S. Smith et al., 2022), (Janssen et al., 2013). Miravirsen is composed of locked nucleic acid (LNA) ribonucleotides spaced within a DNA phosphorothioate sequence complementary to mature miR-122 that hybridize to mature miR-122 and block its interaction with HCV RNA (Gebert et al., 2014). Treatment with miravirsen has shown to support a potent and dose-dependent suppression of viral load in chronic HCV patients during clinical trials (Janssen et al., 2013).

1.3 HCV in cell culture

1.3.1 Cell culture HCV

The first infectious clones to be used in cell culture required patient sera as a source for HCV. Early cell culture systems leading to perpetual HCV infection required the use of primary hepatocytes from chronically infected HCV patients and secondary *in vitro* infection (Shimizu et al., 1992). The use of primary hepatocytes from chronically infected HCV patients or primary hepatocytes infected with patient sera *in vitro* lead to limited time models resulting in time dependent cell viability. Huh-7 and Hep-G2 cell lines could support HCV replication for up to 130 days (Theilmann et al., 1997) (N. Kato et al., 1995) but these methods resulted in low replication levels, which were difficult to detect (reviewed in Bartenschlager & Lohmann, 2000).

The use of patient sera in primary human hepatocytes proved unlikely to be very useful as a means to study HCV (Bartenschlager & Lohmann, 2000).

Researchers looked to using cloned full-length genomes in the development of a reverse genetics model for HCV, which could put transcription under the control of a phage promoter (reviewed in Lohmann, 2019). This involved viral replication via RNA polymerase within a plasmid amplified by bacteria. The new genomes could then be transfected into permissive cells or into hepatocytes, thus starting an HCV replication cycle. In 1997 two cloned genomes from the same isolate (H77, genotype 1a) successfully infected chimpanzees intrahepatically (Yanagi et al., 1997) but were unable to be replicated in cell culture (Blight et al., 2000).

In 1999, HCV subgenomic replicons that efficiently replicated in the human hepatoma cell line, Huh-7, were developed from the consensus Con1 cDNA genotype 1b (Lohmann et al., 1999). This was achieved using bicistronic vectors which allowed for the simultaneous translation of two genes of interest: the HCV internal ribosomal entry site (IRES), for translation of the selector gene: neomycin phosphotransferase gene (neo); and the encephalomyocarditis virus (EMCV) IRES, for translation of the non-structural genes (NS3 to NS5B). This was flanked by the HCV 5' and 3' non-translated regions, resulting in a replicon encoding the HCV polyprotein NS3-5B, which could be used to transfect Huh-7 cells, producing autonomous viral replication. The selector gene, neo, expression brought resistance to the cytotoxic drug G418, which can be used to select upon, and construct cell lines from, parental HCC cells, creating such cells as Huh-7.5, showing persistent replication. Selection with G418 resulted in a low number of surviving cell colonies (~1 colony per 10^6 transfected cells) but produced adaptive viral and cellular mutations and provided selective pressures which cannot act upon structural proteins absent from the replicon. Subgenomic replicons may be effective in studying specific

subgenomes, but only through their non-structural proteins and replication complexes (Lohmann et al., 1999). Later, full-length genomic replicons were used, but could only produce low numbers of viral particles (Ikeda et al., 2002).

In 2003, researchers sought out to study HCV entry and neutralization but with no available model, the HCV pseudo-particle (HCVpp) system was created (Bartosch et al., 2003). HCVpp provides a system closely related to human infection by using functionally conserved envelope glycoproteins via the incorporation of the full-length E1 and E2 onto lenti- or retroviral core particles. This system has been used to isolate cellular receptors involved in viral entry (Evans et al., 2007).

The use of full-length genomic replicons saw advancement when researchers looked at successful *in vitro* mutations in both the HCV genome and the host Huh-7 cells. The use of the full-length Con1 wildtype had a very low transfection efficiency and only a small amount of Huh-7 cells supported persistent high-level HCV replication (Lohmann et al., 2003). Of such, HCV colonies were found to have mutations across most non-structural proteins, not present in the original replicon sequence (Lohmann, 2019), that which, when transferred back into the wild type, Con1 replicon, could increase colony formation intensely (Lohmann et al., 2003). Many host factors and mutations are also involved. As much as 100-fold differences in permissiveness to support RNA replication can be dependent on naïve vs late passages (Lohmann et al., 2003).

Using an isolate of Japanese fulminant hepatitis 1 (JFH-1), (genotype 2a), a successful replicon was produced in 2003, allowing for the production of infectious virus in cell culture in 2005 (T. Kato et al., 2003). This is now known as HCVcc. Compared to Con1 subgenomic RNA with highly adaptive mutations, the G418-resistant colony-forming ability of JFH-1 replicons, absence of adaptive mutations, is 60-fold higher in Huh-7 cells (T. Kato et al., 2003). Clones of

different genotypes were not universally infectious using this model so chimeric viruses were constructed and ultimately, increased viral titer. One example is J6CF- and JFH-1-derived sequences fused via a site located right after the first transmembrane domain of NS2, creating a GT2a/2a chimera, named JC-1, which can yield infectious titers 100– to 1,000-fold higher than the parental isolate and is infectious in chimpanzees. Many chimeras can be made utilizing different genomes, and of those produced, all but the GT3a/JFH-1 chimera can result in efficient virus production (Pietschmann et al., 2006). This is the most efficient system tested so far. Some subclones of Huh-7, such as Huh-7.5 are known to be more permissive to HCV infection vs their parental strain (Feigelstock et al., 2010).

Finally, coding mutations found in E2, p7, and NS2 of JFH-1 produced titers up to 1,000-fold more infectious virus than the wild-type (N417S [E2], N765D [p7], and Q1012R [NS2]) (Russell et al., 2008). The JFH-1 strain with said mutations increased viral titers closest to resembling HCV-infected individuals, and later called JFH-1_T, is the virus used in all experiments within this report.

1.3.2 Huh-7/Huh-7.5 cells

In 1982, the Huh-7 cell line was established as a convenient experimental substitute for primary hepatocytes. This well differentiated and epithelial-like, hepatocyte derived cellular carcinoma cell line was originally taken from a liver tumor in a 57-year-old Japanese male (Nakabayashi et al., 1982). This cell line was found to represent host cells suitable for propagating HCV *in vitro*, used for an HCV replicon system (T. Kato et al., 2003). Huh-7 cells grow as adherent monolayers with a p53 gene (tumor suppressor) mutation producing immortality (Toshiyuki & Reed, 1995). The Huh-7 cell line is characterised by a high degree of heterogeneity with chromosome abnormalities involving all 60 (average number) chromosomes

and heterozygosity, accounting for 55.3% of the genome (Kasai et al., 2018). Further mutations are presented in subclones, such as the Huh-7.5 cell line, used in this report.

Huh-7.5 cells, developed from the Huh-7 cell line are highly permissive for subgenomic and genomic hepatitis C virus RNA replication and can produce infective progenitor virions when infected with HCV. (Blight et al., 2002). A single point missense mutation (T55I) found in the dsRNA sensor retinoic acid-inducible gene-I (RIG-I or DDX58), which is an important function for innate antiviral response, leads to a defect in pattern recognition of RNA in Huh-7.5 cells (Yoneyama et al., 2004). This, with a low expression of toll-like receptor (TLR) 3 (K. Li et al., 2005), a lack of translocation of interferon regulatory factor 3 (IRF3) from the cytosol to the nucleus, and low levels of ISG56 gene expression (a well-defined interferon stimulating gene), may prevent a lack of IFN activation and support high permissiveness of HCV RNA replication.

1.4. Programmed Cell Death

Programmed cell death (PCD) has been observed since the 19th century. In the 1960s, researchers began to understand cell death as biologically controlled programs, originally focused on apoptosis and the common morphology of cells participating (Zakeri & Lockshin, 2008). PCD has since evolved to include many different forms and the stimulation of specific signalling pathways in multicellular organisms, which leads to cellular suicide. PCD can be involved in homeostasis, the elimination of cells no longer needed, the removal of superfluous and damaged cells, cellular differentiation of specific cell types, innate immune defence against bacterial and viral infection, etc. (Jorgensen et al., 2017). Although there are many types of PCD including, but not limited to, autophagy, necroptosis, entosis, ferroptosis, apoptosis and pyroptosis, this report focuses specifically on apoptosis and pyroptosis as it relates to HCV and other viral or bacterial infections.

1.4.1 Apoptosis

Apoptosis refers to an orderly process where intrinsic and extrinsic signals lead to cellular contents being packaged and engulfed by immune cells via phagocytosis. This is an immunologically silent reaction where inflammation is not evoked (V. Fadok et al., 1998). Non-inflammatory, initiator caspases (caspase 2, 8, 9, and 10) and executioner caspases (caspase-3, 6, and 7) are involved, or apoptosis can be caspase independent (Elmore, 2007). Pathological induced apoptosis can occur as a defence mechanism in immune reactions or when cells are damaged via disease, or infection. Apoptosis in response to viral infection can lead to a reduction or an increase in viral progeny (Thomson, 2001).

Cells undergoing apoptosis present a particular morphology which can be visualized through light microscopy. First the cell shrinks, where the cytoplasm becomes dense, tightly packaging the organelles. Condensation of chromatin in the nucleus can be visualized in pyknosis. Next the plasma membrane begins blebbing with fragmentation of the nucleus followed by the budding of apoptotic bodies (Elmore, 2007).

The intrinsic pathway can be activated by a diverse array of non-receptor-mediated stimuli including: cytokine deprivation, DNA damage, oxidative stress, cytosolic $Ca2^{+}$ increase, endoplasmic reticulum stress and viral proteins (reviewed in Zhou et al., 2017). These signals converge to open the mitochondrial permeability transition (MPT) pore resulting in mitochondrial outer membrane permeabilization (MOMP), leading to the loss of mitochondrial transmembrane potential and the release of cytochrome c and other key molecules into the cytosol, thus securing death through the generation of initiator and effector caspases. This may also cause the release of mitochondrial DNA into the cytosol (Rogers et al., 2019), (McArthur et al., 2018). Specifically, mitochondrial membrane permeabilization (MMP) is controlled by

members of the B-cell lymphoma 2 (Bcl-2) family, which can determine if the cell commits to apoptosis or aborts the process. Members of the BCL-2 protein family can have up to four different BCL-2 homology domains (BH1–4). Many pro-apoptotic members of the BCL-2 family, such as BCL-2-associated X protein (BAX) will translocate to the mitochondrial membrane in response to apoptotic signals. BH3-only proteins, including: BH3-interacting domain death agonist (BID), Bcl-2-like protein 11 (BIM) and p53 upregulated the modulator of apoptosis (PUMA) which can activate BAX and the mitochondrial protein BCL-2 killer (BAK) causing activation through oligomerization and insertion into the outer mitochondrial membrane. This can lead to MOMP (Chipuk et al., 2006). Once cytochrome c is released, apoptosis protease-activating factor 1 (apaf-1) and procaspase-9 are recruited to form a multicomponent holoenzyme, known as the apoptosome (Elmore, 2007). Apoptosome formation will cleave procaspase-3, activating caspase-3 and initiate the executioner pathway. Oligomerization of the caspase-9 is mediated by a caspase activation and recruitment domain (CARD) where apaf-1 is required for procaspase-9 cleavage to caspase-9 (Jiang & Wang, 2000). MOMP may also result in the release of apoptosis-inducing factor (AIF), leading to DNA fragmentation and endonuclease G, causing cleavage of nuclear chromatin to catalyze caspase-independent apoptosis (Chipuk & Green, 2005). The execution pathway and the activation of executioner caspases will be discussed below.

HCV is well known to induce apoptosis (Deng et al., 2008) (Fischer et al., 2007) and in HCV infected Huh-7.5 cells, apoptosis in one cell can trigger apoptosis in another through cell-cell contact (Kofahi et al., 2016). HCV viral gene products may interfere with the apoptotic intrinsic pathway via encoding anti-apoptotic proteins that allow for HCV to complete viral replication before destruction of the host cell; however, HCV may also take advantage of

apoptosis to disseminate progeny viruses. The role of HCV proteins in the apoptotic intrinsic pathway is still controversial. As reviewed in (Deng et al., 2008) and (Fischer et al., 2007) core, E2, NS2, NS3, and NS5A have all been shown to function as both anti-apoptotic and pro-apoptotic proteins within the intrinsic pathway.

The extrinsic pathway is activated by extracellular stress stimulation and involves transmembrane receptor-mediated interactions triggered through death receptors. These are a family of type I transmembrane proteins belonging to the tumour necrosis factor (TNF) receptor gene superfamily which have 1-5 cysteine-rich extracellular domains and homologous, cytoplasmic death domains of 60-80 amino acids long (reviewed in Zhou et al., 2017). Death ligands and their receptors include: FasL/FasR (also called CD95 or Apo-1 or TNFRSF6), TNF-α/TNFR1, Apo3L/DR3 (also called Apo3, WSL-1 or TRAMP), Apo2L/DR4 (also called TRAIL-R1) and Apo2L/DR5 (also called Apo2 or TRAIL-R2) (reviewed in Elmore, 2007). Death cell receptor binding is best described with the FasL/FasR, and TNF-α/TNFR1 models. Ligand binding to the death receptor on the cell surface results in the recruitment of cytoplasmic adapter proteins. FAS-associated with a death domain (FADD) is recruited through Fas D4 and D5 receptor binding where FADD then associates with procaspase-8 via dimerization of the death effector domain, resulting in the assembly of the death-inducing signaling complex (DISC), activating caspase-8. The binding of TNF to TNFR1 recruits TNFR-associated protein with death domain (TRADD) and with recruitment of FADD and receptor-interacting protein (RIP), FADD can then assemble DISC and activate caspase-8, which can directly activate executioner caspases (reviewed in Thomson, 2001). The execution pathway will be discussed below.

In regard to HCV and the extrinsic pathway: individuals with chronic HCV experience increased hepatic apoptosis, leading to disease progression, which may be linked to the upregulation of death receptors and their ligands (Mundt et al., 2003). Examples include: upregulated Fas expression in peripheral blood mononuclear cells (PBMC) collected from HCV-infected patients (Taya et al., 2000) and increased expression of DR4 and DR5 in Huh-7.5 cells infected with HCV (Lan et al., 2008). Although the roles of HCV proteins are not well understood, core may bind the cytoplasmic domain of TNFR1 thus increasing the outcome of extrinsic apoptosis (Zhu et al., 1998).

The execution pathway is considered "the final common pathway" as it brings both extrinsic and intrinsic pathways together. The activation of executioner caspases-3, -6 and -7 can be through apoptosome formation or the direct action of caspase-8. Executioner caspases activate cytoplasmic endonuclease and proteases which effectively break down the cell and are responsible for the common apoptotic morphology (Slee et al., 2001). Caspase-3 is the most significant of the executioner caspases as it will destroy the cytoskeleton, intracellular transport, cell division, and signal transduction through the cleavage of gelsolin, which will then cleave actin filaments and can be activated by caspase-8, caspase-9, or caspase-10 (Kothakota et al., 1997). Caspase-3 can activate caspase-activated deoxyribonuclease (CAD), degrading chromosomal DNA and caspase-3 will break the cell down into apoptotic bodies to be removed by immune cells (Sakahira et al., 1998).

The clearance of apoptotic cells is a rapid and silent innate immune response (Arandjelovic & Ravichandran, 2015). Cells are broken down into apoptotic bodies where no release of cellular constituents can leak out and damage surrounding tissues, thereby limiting inflammation (Lauber et al., 2004); however, when there are too many dead cells or when the

activity of phagocytes is inhibited, the DNA fragments of apoptotic cells will enter the bloodstream (Hu et al., 2021). During this process, the cells release proteins, lipids, nucleotides, and their receptors to act as "find-me" signals. Phagocytes begin engulfment when they bind to ligands ("eat-me" signals) on the surface of the cell undergoing apoptosis (Ravichandran, 2011). Phosphatidylserine (PS) is a common "eat-me" signal and PS translocation to the outer leaflet allows early uptake and clearance (V. A. Fadok et al., 2001).

1.4.2 Pyroptosis

Life or death of individual cells can determine disease progression in multicellular organisms, thus pyroptosis, a pro-inflammatory form of cell death, is initiated through the recognition of danger signals associated with pathological stimuli such as stroke, heart attack, cancer, and microbial invasion. Recognition of these signals lead to pyroptotic cell death which involves the formation of an inflammasome complex (reviewed in Man & Kanneganti, 2015). This report focuses specifically on pyroptosis as it relates to HCV and other viral or bacterial infections.

Danger signals can be comprised of molecules regularly associated with pathogens called pathogen-associated molecular patterns (PAMPs) (Janeway & Medzhitov, 2002). These are conserved compounds of infectious agents such as viral and bacterial proteins. Signals of host cellular distress are called damage-associated molecular patterns (DAMPs), which can include the presence of reactive oxygen species (ROS), calcium influx, mitochondrial DNA (mtDNA) in the cytosol, etc. (Janeway & Medzhitov, 2002). PAMPs and DAMPs are recognized by pattern recognition receptors (PRRs). When activated by a PAMP or DAMP, many cytoplasmic PRRs can assemble into an inflammasome complex such as members of the nucleotide-binding oligomerization domain (NOD), leucine rich repeat receptor (NLR) family (NLRP1, NLRP3 and

NLRC4 are the best characterised), as well as proteins belonging to the pyrin and hematopoietic interferon-inducible (HIN) domain (PYHIN) family (AIM2 is the best characterized) (Zheng et al., 2020). Some inflammasomes require more than one PRR signal to assemble into a complex.

The inflammasome complex regulates the activation of proinflammatory caspases (not involved in apoptosis) (reviewed in Man & Kanneganti, 2015). Active caspases, in turn, activate gasdermins (Wang et al., 2019) and are responsible for proteolytic maturation of the inflammatory cytokines: interleukin-1β (IL-1β) and IL-18. Gasdermins form membrane pores, which lead to ion fluxes resulting in rapid plasma-membrane rupture and the release of proinflammatory intracellular contents. Pyroptosis has been widely studied in inflammatory disease models especially as it relates to infectious agents either as a consequence of infecting host cells or by producing toxic products. The proinflammatory caspases in humans include: 1, 4, 5 and 12 (reviewed in Man & Kanneganti, 2016). Pyroptosis takes place through the "canonical inflammasome" and the "non-canonical inflammasome" pathways.

The canonical inflammasome pathways involve the formation of, and are named for, an inflammasome such as NLRP1, NLRP3, NLRC4, and AIM2 to activate caspase-1. These inflammasomes include a sensor protein that can recruit the caspase-1 activating machinery called caspase recruitment domain (CARD), which may be achieved through the employment of an apoptosis-associated speck-like protein (ASC) (Elizagaray et al., 2020), discussed in detail below. The non-canonical inflammasome pathway involves the direct sensing and binding of caspase 4 or 5 (caspase-11 in mice) to cytosolic lipopolysaccharide (LPS) produced from gram-negative bacteria. This activates a noncanonical inflammasome, and like the canonical inflammasome pathway, leads to the cleavage of gasdermin D (GSDMD), producing membrane pores causing pyroptosis. The non-canonical inflammasome also activates the canonical NLRP3

inflammasome leading to caspase-1 activation and the release of IL-1β and IL-18 (Downs et al., 2020). The NLRP3 inflammasome will be discussed below. Caspase-12 does not participate in the activation of inflammasomes (reviewed in Man & Kanneganti, 2016). This report will outline the canonical pathway inflammasomes: NLRP1, NLRP3 and NLRC4, with a focus on AIM2.

The apoptosis-associated speck-like protein containing a CARD (ASC) is an adaptor protein found in inflammasomes where the scaffold protein utilises a pyrin domain (PYD) (such as NLRP1, NLRP3 and AIM2). ASC comprises both a PYD and a CARD. ASC is recruited via PYD-PYD interaction. The exposed CARD domain will then engage with the CARD domain of procaspase- 1, resulting in procaspase-1 cleavage and activated caspase-1. If the scaffold protein contains a CARD, the inflammasome may engage with procaspase-1 without the use of ASC (such as NLRP1 and NLRC4) (reviewed in Schroder & Tschopp, 2010).

NLRP1 (nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 1/NOD-like receptor family pyrin domain containing 1) was the first NLR family member shown to form an inflammasome complex and like all NLRs contains a NOD, leucine rich domain (reviewed in Zheng et al., 2020). NLRP1 is unique in that it encompasses two effector-recruitment domains: PYD, located at the N-terminus and CARD, located at the C-terminus (see figure 1.3). It is also the only NLR to contain a function-to-find domain (FIIND) which is responsible for autoproteolysis and activation of NLRP1 (Finger et al., 2012). NLRP1 is well studied in mice, where NLRP1b serves the function of human NLRP1, though NLRP1b does not have a PYD. Most notably: *Bacillus anthracis* produces anthrax lethal toxin (LeTx), leading to anthrax disease and rapid caspase-1-dependent cellular lysis. In mice, NLRP1b proteins are proteolytically cleaved by anthrax LeTx creating activated NLRP1b, leading to the degradation of the N-terminal domains and presenting the bioactive CARD (Levinsohn et al.,

2012). In humans, NLRP1 has been described as a "tripwire sensor for pathogen-encoded proteases" as a wide range of *picornavirus* proteases cleave NLRP1 leading to the bioactive CARD (Tsu et al., 2021). Activated NLRP1 will oligomerize to form the NLRP1 inflammasome which may or may not include ASC. CARD of NLRP1 may recruit procaspase-1 via CARD-CARD directly or may engage ASC by way of CARD-CARD interactions followed by a second ASC via PYD-PYD resulting in CARD-CARD engagement between the second ASC and procaspase-1 (see figure 1.5) (Finger et al., 2012). A decrease in adenosine 5'-triphosphate (ATP) (Liao & Mogridge, 2013) as well as muramyl dipeptide (MDP), a component of bacterial peptidoglycan, will also result in NLRP1 activation (reviewed in Chavarría-Smith & Vance, 2015). The interaction of ASC with the N-terminal PYD of NLRP1 can result in inflammasome activation (Martinon et al., 2002) or may enhance NLRP1 CARD engagement with the CARD of procaspase-1 (Faustin et al., 2007).

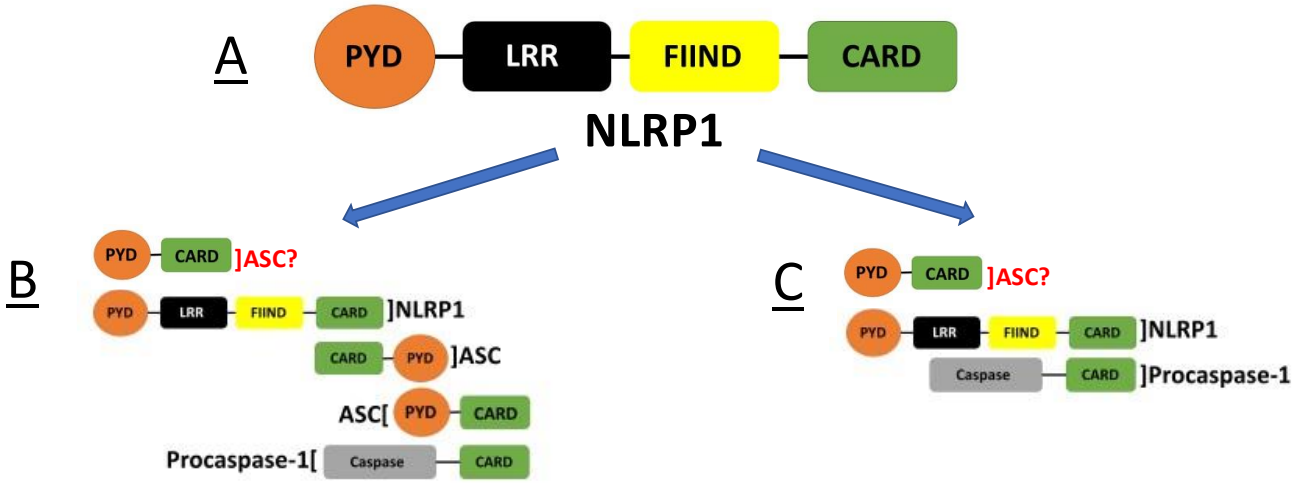


Figure 1.5 NLRP1 inflammasome formation

(A) The NLRP1 protein represented by an N-terminal PYD, a leucine rich repeat domain, a function to find domain, and a C-terminal CARD domains **(B)** A possible NLRP1 inflammasome complex. The C-terminal CARD associates with the CARD of ASC. The PYD associates with a second ASC through PYD-PYD interactions. The CARD of the second ASC associates with the CARD of procaspase-1. ASC may or may not bind to the PYD of the NLRP1 protein. **(C)** A possible NLRP1 inflammasome complex. The C-terminal CARD associates with the CARD of procaspase-1. ASC may or may not bind to the PYD of the NLRP1 protein. Reproduced and adapted to depict inflammasome arrangements under Creative Commons CC BY 4.0. Mi, L., Min, X., Chai, Y., Zhang, J., & Chen, X. (2022). NLRP1 Inflammasomes: A Potential Target for the Treatment of Several Types of Brain Injury. Figure 1: Structures of NLRP1 inflammasome proteins. *Frontiers in Immunology*, 13. https://www.frontiersin.org/articles/10.3389/fimmu.2022.863774

NLRP3 contains a C-terminal leucine-rich domain, a central NOD domain and an N-terminal PYD. Without the presence of a CARD within the NLRP3 scaffold protein, ASC must be recruited to the inflammasome complex through PYD-PYD interaction (Martinon et al., 2002). The NLRP3 inflammasome responds to a wide range of stimuli that are structurally and chemically dissimilar suggesting that the formation involves two signals: priming and activation (reviewed in Zheng et al., 2020). The first signal, priming, involves the engagement of toll-like receptors (TLRs) or tumor necrosis factor receptors (TNFRs) by pro-inflammatory stimuli such as microbial or endogenous molecules (for example the engagement of TLR4 with the agonist LPS). This induces NLRP3, pro-caspase-1 and pro-IL-1β expression through nuclear factor-κB (NFκB) (Bauernfeind et al., 2009). The second signal, activation, is triggered by PAMPs and/or DAMPs which can be provided by a variety of sources such as extracellular ATP, pore-forming toxins and RNA viruses which can result in multiple molecular and cellular events such as: ion fluxes (including K+ efflux, Ca2+ signaling, Na+ influx, and chloride efflux), mitochondrial dysfunction and reactive oxygen species (ROS) production, and lysosomal destabilization (reviewed in Yang et al., 2019). Once both signals have been generated, post-translational modifications (PTMs) regulate NLRP3 inflammasome activation where NEK7 (NIMA-related kinase 7) binds the leucine rich repeat domains and mediates NLRP3 oligomerization (Sharif et al., 2019). This leads to the recruitment of ASC, completing the inflammasome complex. The NLRP3 inflammasome is triggered through a wide variety of RNA viral infections (da Costa et al., 2019).

Chronic inflammation is the basis of HCV-mediated liver damage which points to NLRP3-mediated pyroptosis as a mechanism for cell death in infected individuals (Kofahi et al.,

2016). HCV infection contains all the features required to trigger both priming and activation of the NLRP3 inflammasomes in hepatocytes and macrophages (Negash et al., 2019). Upon pyroptotic cell lysis, DAMPs released can recruit immune cells creating a cascade of secondary inflammation (reviewed in Kubes & Mehal, 2012) and one study found that HCV-infected Huh-7.5 cells underwent NLRP3-mediated pyroptosis resulting in both the death of the cell as well as the death of nearby uninfected "bystander" cells without cell contact (Kofahi et al., 2016). The exact mechanisms involved in linking HCV infection to NLRP3-mediated pyroptosis are unclear. Activation signals could include or be induced by: potassium efflux (Negash et al., 2013), ROS production (W. Chen et al., 2014), HCV RNA (Bergsbaken et al., 2009), and HCV core protein (Negash et al., 2019). It has been shown that NLRP3 expression is upregulated in HCV-infected hepatocytes and macrophages (Chattergoon et al., 2014) and IL-1β production correlates with increased inflammation in individuals with HCV (Negash et al., 2019).

NLRC4 (nucleotide-binding oligomerization domain, leucine rich repeat and CARD domain containing 4/NOD-like receptor family CARD domain containing 4) contains a C-terminal leucine rich domain, a central NOD and an N-terminal CARD. The CARD can directly interact with the CARD of procaspase-1, bypassing the need for ASC or may engage ASC by way of CARD-CARD interactions followed by a second ASC via PYD-PYD resulting in CARD-CARD engagement between the second ASC and procaspase-1 (Y. Li et al., 2018). NLRC4 differs from NLRPs in that it requires a unique binding partner: neuronal apoptosis inhibitory protein (NAIP) which will first bind the agonist and then associate with NLRC4, releasing autoinhibition, for inflammasome activation to occur (Kofoed & Vance, 2011). NAIPs recognize PAMPs mainly derived from gram-negative bacteria such as *Salmonella, Legionella, Shigella*, and *Pseudomonas* species (Zhao et al., 2011). NAIP5 and NAIP6 can detect cytoplasmic

flagellin which is a monomeric subunit from flagella. NAIP1 and NAIP2 identify needle and rod components of type III secretion systems (T3SS) of bacteria (Lage et al., 2014). Completion of the NAIP-NLRC4 inflammasome complex, like all inflammasomes, leads to procaspase-1 cleavage, the release of proinflammatory cytokines and pyroptotic cell death.

AIM2 (absent in melanoma 2) is the best characterized non-NLR protein belonging to the AIM2-like receptors (ALR) family, which are a class of PRRs capable of detecting cytosolic double-stranded DNA (dsDNA) (reviewed in Zheng et al., 2020). AIM2 is composed of a C-terminal hematopoietic interferon-inducible nuclear domain (HIN) that is 200 amino acids long (HIN200) and an N-terminal PYD (see figure 4.2 A). The recognition of DNA happens through the electrostatic binding of double-stranded DNA to the positively charged HIN (reviewed in Zheng et al., 2020). AIM2 detects dsDNA in a non-sequence-specific manner as long as the sequence is at least 80 base pairs long. This leads to AIM2 activation where the PYD recruits ASC, creating the AIM2 inflammasome, mediating pyroptosis in a caspase-1-dependent manner (see figure 4.2 A)(reviewed in Sharma & Kanneganti, 2016).

Eukaryotic organisms partake in cellular compartmentalization where DNA is found in the nucleolus or within the mitochondria and is absent from the cytosol. DNA in the cytosol can be considered a DAMP and a PAMP. This DNA can be a pattern recognition receptor agonist when the recognition of cytosolic DNA belongs to a pathogen and a DAMP when said DNA belongs to the host (reviewed in Rathinam et al., 2010). DNA is a damage associated molecular pattern as a result of the loss of nuclear envelope or mitochondrial membrane integrity where the subsequent release of DNA into the cytosol results in the sensing of self-DNA (reviewed in Rathinam et al., 2010). Bacterial organisms are comprised of DNA genomes and many, such as *Francisella tularensis, L. monocytogenes, S. pneumoniae, Mycobacterium species, Legionella*

pneumophila, S. aureus, etc. (reviewed in Rathinam et al., 2010), can enter the cytosol of eukaryotic organisms and trigger activation of the AIM2 inflammasome. Genetic material from DNA viruses such as HBV (Y. Han et al., 2015), cytomegalovirus (CMV) (Huang et al., 2017), vaccinia virus (Rathinam et al., 2010) human papilloma viruses (HPV) (Reinholz et al., 2013), etc. can trigger AIM2 inflammasome activation in infected cells as well.

Contrary to the popular belief that RNA viral infection will trigger NLRP3 inflammasome formation (da Costa et al., 2019) and AIM2 inflammasome construction is the result of DNA viral infection (Fernandes-Alnemri et al., 2009), many studies are now focusing on the "cross talk" between both inflammasomes. Particularly, many report that AIM2 can drive anti-viral inflammasome responses against some RNA viruses. This includes: Chikungunya virus (CHIKV) (Ekchariyawat et al., 2015), West Nile virus (WNV) (Ekchariyawat et al., 2015), Influenza A virus (IAV) (Zhang et al., 2017), Zika virus (ZIKV) (He et al., 2020), SARS-CoV-2 (Junqueira et al., 2021), etc.

AIM2 inflammasome activation is not well characterized in the context of HCV, especially as it relates to hepatocytes. Although it is clear that the expression of AIM2 in the liver is inducible (Y. Han et al., 2015), one study found that, through biopsy, only 2/23 (8.7%) of individuals living with chronic HCV were expressing AIM2 in their liver tissue (Y. Han et al., 2015). Although NLRP3 inflammasome activation occurs through all stages of HCV infection (Negash et al., 2013), AIM2 inflammasomes could potentially be activated through the loss of nuclear envelope or mitochondrial membrane integrity, or TLR binding and type I IFN signaling in the presence of HCV. This report focuses on *in vitro*, early AIM2 activation, through the use of HCVcc: JFH-1_T infection of Huh-7.5 cells (which are derived from Huh-7 cells, a hepatocellular carcinoma cell line that continues to express AIM2 (X. Ma et al., 2016).

1.5 Non-programed cell death related DNA sensing

IFI16 (IFN-inducible protein 16) is a non-NLR protein belonging to the AIM2-like receptors (ALR) family and is closely related to AIM2 (Zheng et al., 2020). It contains two C-terminal HIN domains and an N-terminal PYD (see figure 3.1 A). IFI16 can function both as an inflammasome in the nucleus and as a DNA sensor in the cytosol that does not result in cell death and instead results in the production of type I interferons (IFN-I) (Zheng et al., 2020). In the context of an IFI16 inflammasome, the protein behaves just like AIM2, where the HIN domains directly bind dsDNA resulting in ASC association and downstream release of IL-18 and IL-1B, and pyroptosis; however, unlike AIM 2, IFI16 is described as a "nucleus-associated inflammasome sensor component" (Xiao, 2015). Inactive IFI16 binds DNA components of viruses replicating within the nucleus such as in the case of HSV-1 (Herpes simplex virus-1) and KSHV (Kaposi's sarcoma associated herpesvirus). Active IFI16 then recruits ASC and procaspase-1 and is redistributed into the cytosol (see figure 3.1 B). The mechanisms used for the transportation of the IFI16 inflammasome complex from the nucleus to the cytosol are not yet known (Xiao, 2015). IFI16 keeps from binding self DNA by having a low affinity to DNA in general. The length of exposed linker-dsDNA between nucleosomes and DNA transcription bubbles are only 10-20 base pairs long. IFI16 proteins increase affinity to DNA as the number of exposed base pairs increase such as just after viral invasion when the entire naked DNA genome of the virus is exposed (Morrone et al., 2014). When dsDNA is found in the cytosol, such as during Human Immunodeficiency Virus-1 (HIV-1) and Vaccinia virus (VV) infection, IFI16 recognise dsDNA and performs its second function: a DNA sensor that does not result in cell death and instead results in the production of IFN-1 (Zheng et al., 2020). After activation by DNA, IFI16 triggers the downstream stimulator of interferon genes-TANK-binding kinase 1-

interferon regulatory factor 3 (STING-TBK1-IRF3) pathway which is described below (Zheng et al., 2020).

DDX41 (Asp-Glu-Ala-Asp box polypeptide 4) is a member of the DExD/H-box helicases superfamily, found widely across organisms, and is believed to be conserved from bacteria (J. Ma et al., 2018). This protein is both an RNA helicase involved in the alteration of RNA secondary structure (Y. Jiang et al., 2017) and a critical cytosolic DNA sensor functioning through the STING (stimulator of interferon genes)-TBK1 (TANK-binding kinase 1)-IRF3 pathway (J. Ma et al., 2018). This report focuses on the latter of its two functions. DDX41 is located in the nucleus of most cells, including hepatocytes, until these cells are stimulated with cytosolic DNA molecules where DDX41 becomes a cytosolic DNA sensor (J. Ma et al., 2018) (see figure 3.3 A). The exact mechanism of transport from the nucleus to the cytosol is unknown. Once in the cytosol DDX41 is phosphorylated by BTK kinase and activated. Activated DDX41 can bind DNA PAMPs and then STING, both through the DEAD domain, ultimately leading to expression of type I interferons (see figure 3.2A) (reviewed in Zahid et al., 2020). STING is the only downstream platform for DDX41 (J. Ma et al., 2018) in regard to immune response. DDX41 is quickly degraded by the E3 ligase TRIM21 (Tripartite motif-containing protein 21/E3 ubiquitin-protein ligase TRIM21), using Lys9 and Lys115 of DDX41 as the ubiquitination sites (see figure 3.2 A) (Y. Jiang et al., 2017).

Once STING has been bound and activated, STING is translocated from the ER to the Golgi apparatus where it interacts with TBK1 and forms a STING-TBK1 complex (see figure 3.3 A) (Y. Jiang et al., 2017). This complex will result in the phosphorylation and nuclear translocation of IRF3, an interferon transcription factor (Dobbs et al., 2015).

Type one interferon responses can also be triggered by cytosolic DNA through RIG-I (Ablasser et al., 2009) via STING. RNA polymerase III can transcribe dsDNA into dsRNA with a 5'-triphosphate moiety which is recognized by RIG-I. This study does not look at this pathway because as mentioned, Huh-7.5 cells are deficient in RIG-I (Ablasser et al., 2009) which also excludes STING activation via cytosolic RNA (the HCV genome).

1.6 Project design and research questions

As noted, there are new direct-acting antiviral agents which allow for eradication of HCV in over 90% of patients but liver function may not normalize even after receiving the cure and those with liver cirrhosis remain at an increased risk of developing hepatocellular carcinoma. Research in HCV is a continued need especially as it relates to viral induced inflammation. There are very few studies that focus on AIM2 associated inflammation through the lens of HCV infection and next to no studies which reflect the complete life cycle of the virus. Where the literature is found, studies direct their effort to the comprehension of AIM2 inflammasome activation in immune cells without any attempt to gauge the hepatocyte response. In this study, we believe that the use of the tissue culture-adapted JFH-1_T strain for the infection of the Huh-7.5 cell line, in the absence of immune cells, allows for the best way to investigate AIM2 inflammasome activation in hepatocytes. Although Huh-7.5 cells are an HCC cell line, leading to a relative decrease in AIM2 expression, the lower levels of endogenous AIM2 allow us to differentiate between the little to no AIM2 expression in uninfected cells vs. increased expression after HCV infection. Most importantly, we used the Huh-7.5 cell line because the use of these cells together with the HCVcc system remains unmatched as the best representation of viral infection and replication *in vitro* as compared to physiological conditions.

The first part of this study focuses on the ability of HCV to engage with proteins traditionally thought to represent elements of the viral/bacterial DNA response. This involved the preparation of viral (JFH-1_T) stocks and the culturing of Huh-7.5 cells. JFH-1_T was then used to infect Huh-7.5 cells where the expression and translocation of proteins was documented via immunofluorescence. Any indication of the following may reveal a cellular response to foreign and/or cytosolic DNA during HCV infection. First, we used anti-IFI16 antibodies to detect the possible formation of IFI16 inflammasomes and perhaps the redistribution of IFI16 in the cytosol as this relates to both inflammasome formation and/or STING association. Next, we used anti-DDX41 antibodies as an attempt to illustrate the movement of DDX41 from the nucleus to the cytosol or potential DDX41 protein degradation as this would mean an immune response to DNA has taken place. Finally, we used anti-STING antibodies as a means to visualize translocation of STING from the ER to the Golgi apparatus.

The second part of this study, HCV infection activates the AIM2 inflammasome complex, focuses on the relationship between HCV and the AIM2 inflammasome. We were determined to elucidate the "cross talk" of HCV, an RNA virus, and its potential to activate an inflammasome that recognises cytosolic DNA. As this concept has not been studied before *in vitro*, using JFH-1_T and Huh-7.5 cells, we believe that this report may provide insight on a secondary inflammasome (not NLRP3) which can have an impact on HCV pathogenesis. To do this we exploited our adapted HCVcc system to study the induction of pyroptosis via AIM2 inflammasome complex formation. First, after JFH-1_T infection, we utilized anti-AIM2 antibodies and immunofluorescent microscopy to look for macro-molecular aggregate structures of 1–2 μM in size (ASC specks/puncta) (Kesavardhana & Kanneganti, 2017) which would indicate the formation of an AIM2 inflammasome complex. Once inflammasomes were

identified, we attempted to utilize positive controls to verify our findings. As discussed, the formation of an AIM2 inflammasome would be associated with caspase-1 production, so we looked for active caspase-1 and colocalization of caspase-1 and AIM2. Next, to determine whether we were seeing AIM2 inflammasome caspase-1 activation and not NLRP3 inflammasome caspase-1 activation, we utilized an NLRP3 knock-out Huh-7.5 cell line generated via CRISPR-Cas9. Finally, because Huh-7.5 cells originated from HCC and likely contain decreased endogenous levels of AIM2 (X. Ma et al., 2016), we used Western blotting to determine an increase in AIM2 protein levels after JFH-1_T infection.

1.7 Objectives

The objectives of this project are:

1- To determine if HCV infection engages elements of cytosolic DNA response
2- To determine if HCV infection leads to the formation of the AIM2 inflammasome complex

Chapter 2: Materials and methods

2.1 Cell culture

Huh-7 is a well differentiated hepatocyte-derived cellular carcinoma cell line, which was isolated from the liver tumor of a 57-year-old Japanese male (Kawamoto et al., 2020). A number of studies indicate that in vitro cellular response may differ according to the sex of the cells used, regardless of whether or not these cells were exposed to sex hormones (Pollitzer, 2013). Specifically, it should be noted that male and female cells may differ in their response to stress. One study found that female primary human hepatocytes were more susceptible to mitochondrial injury, nuclear condensation, ER status, and plasma membrane permeability when exposed to Diclofenac, Chlorpromazine, Acetaminophen, Verapamil, and Omeprazole (Mennecozzi et al., 2015). Furthermore, neurons and splenocytes of female mice have been found to be more susceptible to programmed cell death in comparison to their male counterparts (Penaloza et al., 2009). This means that the male cells used in this study may be less vulnerable to pyroptosis, though viral-induced programmed cell death, and its relevance to the use of Huh-7.5 cells, have yet to be determined.

Human hepatoma cells, Huh-7.5 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 1% penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (FBS) (Invitrogen), referred to as complete medium, in 15 cm plates (Thermofisher), in a humidified incubator at 37 °C in 5% CO_2. All experiments described in this study were performed using these cells. Cells were split every 3-4 days up to 30 passages. The splitting process involved removing the culture medium and washing the cells with 3 mL of Trypsin (Thermofisher). Then 7 mL of trypsin was added and the cells were incubated for 6 mins at 37 °C in 5% CO_2. Next 10 mL of complete medium was added and transferred to a 50 ml tube

(FroggaBio), bringing the trypsinized cells to the same 50 mL tube. This step was repeated with another 10 mL medium. Next the cells were centrifuged and re-suspended in 10 mL medium. The cells were counted and 2 million were added back to a new 15 cm plate (in 30 ml medium).

2.2 Generation of viral stock

To generate JFH-1_T viral stock, 1 million Huh-7.5 cells were cultured overnight in 10 cm culture dishes (FisherScientific) within a humidified incubator at 37 °C in 5% CO_2. The following day the medium was removed and 1 mL of JFH-1_T viral stock provided was added to each plate in addition to 1.5 mL complete medium and incubated for 3 hours. This was followed by the removal of medium containing virus and was replaced with 8 mL of complete medium and incubated for 3 days. Finally, the supernatant was centrifuged, where the pellet is removed. The supernatant was separated into 1 mL eppendorf tubes (ThermoFisher) and stored at -80 °C. Titre was determined using a limiting dilution focus-forming assay described below.

2.3 Titration of infectious HCV

The titre of infectious HCV was determined by a limiting dilution focus-forming assay using immunofluorescence, where the number of HCV core-positive cell foci was determined. Focus (or the plural foci) cells stand out from their surrounding cells via their appearance and in this case, positive stain absorbance, when utilizing microscopy. Cell supernatants were serially diluted 10-fold in DMEM. The supernatants were used to infect 50 000 naïve Huh-7.5 cells in 8-well chamber slides (FisherScientific). Cells were incubated in a humidified incubator at 37 °C in 5% CO_2, washed and incubated with complete DMEM for 2 days. Two days post infection (p.i.), cells were fixed and stained for HCV core protein. The slides were viewed with a Zeiss Axio Imager.M2 immunofluorescence microscope and the number of positive stained regions

were counted and multiplied by the dilution factor to determine focus forming units per millilitre (FFU/mL).

2.4 Antibodies

The primary antibodies used for immunofluorescence include: mouse monoclonal anti-HCV core antibody (MO-I40015B, Anogen), rabbit polyclonal anti-HCV (AI20006P, Anogen), mouse monoclonal anti-AIM2 (ab180665, Abcam), rabbit polyclonal anti-AIM2 (ab93015, Abcam), mouse monoclonal anti-DDX41 (ab182007, Abcam), mouse monoclonal anti-IFI16 (ab55328, Abcam), and mouse monoclonal anti-STING (MAB7169-SP, Novus). The secondary antibodies used for immunofluorescence include: goat anti-mouse AlexaFluor® 488 (A11029, Thermofisher), goat anti-mouse AlexaFluor® 594 (A11020, Thermofisher), goat anti-rabbit AlexaFluor® 488 (A11008, Thermofisher), and goat anti-rabbit AlexaFluor® 594 (A11037, Thermofisher). VectaShield with DAPI Vibrance (VECTH180010, BioLynx) was used for curing, mounting, and as a nuclear stain. The primary antibodies used for Western blotting include: mouse monoclonal anti-HCV core antibody (MA1-080, Thermofisher), mouse monoclonal anti-AIM2 (ab233756, Abcam), and mouse monoclonal anti-GAPDH (sc-32233, Santa Cruz). The secondary antibodies used for Western blotting include: HRP-conjugated anti-mouse (sc-516102, Santa Cruz). All antibodies were obtained commercially. All antibody dilutions were titrated them to ensure optimal concentrations.

2.5 Immunofluorescence microscopy

Medium was removed from the wells of the 2-chamber slides (FisherScientific) and cells were washed by immersing the slide in 1X phosphate-buffered saline (PBS) (pH 7.4) (Thermofisher) for 1 minute. Cells were fixed and permeabilized by immersion in 100% acetone

(MPD) for 1 minute. The use of paraformaldehyde as a fixative was also tested but acetone offered better immunofluorescent visualization. The cells were then labeled with: mouse monoclonal anti-HCV core antibody (MO-I40015B, Anogen) at a 1: 200 dilution for 20 mins, or rabbit polyclonal anti-HCV (AI20006P, Anogen) at a dilution of 1:200 for 2 hours, and/or mouse monoclonal anti-AIM2 (ab233756, Abcam) at a 1:200 dilution overnight, or rabbit polyclonal anti-AIM2 (ab180665, Abcam) at a dilution of 1:600 for 2 hours, or mouse monoclonal anti-DDX41 (ab182007, Abcam) at a dilution of 1:200 for 2 hours, or mouse monoclonal anti-IFI16 (ab55328, Abcam) at a dilution of 1:100 overnight, or a mouse monoclonal anti-STING (MAB7169-SP, Novus) at a dilution of 1:200 for 2 hours. Dilutions were done with 5% BSA in PBS. The cells were then washed by immersion in PBS for 5 mins followed by staining with goat anti-mouse AlexaFluor® 488 (A11029, Thermofisher), goat anti-mouse AlexaFluor® 594 (A11020, Thermofisher), goat anti-rabbit AlexaFluor® 488 (A11008, Thermofisher), goat anti-rabbit AlexaFluor® 594 (A11037, Thermofisher) at a 1:100 dilution in PBS for 20 minutes. Finally, cells were washed in PBS for 5 mins after which VectaShield with DAPI Vibrance (VECTH180010, BioLynx) was used for mounting to be viewed with a Zeiss Axio Imager.M2 immunofluorescence microscope.

2.6 Measurement of active caspase-1

To measure the levels of active caspase-1, FAM-FLICATM Caspase-1 Assay Kits were used (ImmunoChemistry Technologies). Cells were seeded into 2-well slides at 200 000 cells/well and incubated overnight. The next day JFH-1_T was added at a multiplicity of infection (MOI) of 1 for 3 hours. Medium was removed and replaced. Then the cells were left to incubate for 3 days after which the medium was removed and a 1:20 dilution (in medium) of 30x FLICA was added. The slides were then incubated for 45 mins and washed then incubated again for 60

mins. The medium was removed and replaced then the slides incubated for 5 mins. The medium was removed again and 300 µL of apoptosis wash buffer was added. The slides were left at room temperature for 15 mins. The buffer was removed and the slides were fixed in acetone for 45 seconds. Next antibodies were added for co-staining or DAPI Vibrance (VECTH180010, BioLynx) was used for curing and mounting to be viewed with a Zeiss Axio Imager.M2 immunofluorescence microscope.

2.7 Western blot AIM2 analysis

2.7.1 Infection

10 cm plates were seeded with 1 million Huh-7.5 cells and incubated overnight. The next day, one plate was left as a control while the other was infected with JFH-1_T at an MOI of 1. After 3 days, the cells were harvested

2.7.2 Preparation of cell lysates

The medium was removed and the plates were rinsed with 4 mL of 1x PBS. The plates were rinsed a second time. 200 µL of RIPA++ (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40) plus protease (1X complete Mini protease inhibitor cocktail tablets) and phosphatase (10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 25 mM NaF, 2 mM NaVO3) inhibitors) buffer is added to the plates and using a cell scraper, the cells are removed and centrifuged.

The cells were vortexed and placed on ice on a rocker for 30 mins. The cells were then centrifuged at 4°C at 6800g for 10 mins. The supernatant was then removed and the pellet was discarded.

2.7.3 Protein concentration assay

The sample buffer used was Laemelli buffer (4% SDS 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl pH approx. 6.8), (Millipore Sigma).

The samples were diluted at 1:10 in distilled water and vortexed. Two cuvettes were prepared for each sample. The first was 10 µL of the 1:10 dilution in 790 uL distilled water. The second was 20 µL of the 1:10 dilution in 780 µL distilled water. Next increasing concentrations of BSA were added to cuvettes to prepare a standard curve. An amount of 200 µL of Bio-Rad protein dye reagent was then added to each cuvette and each cuvette was vortexed. Using the spectrophotometer, first the known standards were added to create the curve. Next the samples were added and the spectrophotometer read the µg/mL. The 20 µL sample was divided by two and averaged with the 10 µL sample. The Western blot samples were then prepared, each containing the same protein concentration. 5 µL of loading buffer (3x SSB) (Millipore Sigma), along with the correct volume of the sample calculated are added to a 1 mL eppendorf tube. Finally, RIPA++ buffer was added to each sample brining to total volume to 20 µL. The prepared samples were held on ice.

2.7.4 Electrophoresis and electrophoretic transfer

The polyacrylamide gels for transfer and Western blotting were prepared with a 15% separating gel. The samples were heated to 100°C for 8 mins and loaded into the gel. The power was set to 20 mA and ran for about 1 hour before the gels were transferred to the membrane at 300 mA in the cold room for 45 mins.

2.7.5 Immunodetection

The membranes were placed in blocking solution (5% milk powder in 1x TBST) for 30 mins. Next the blocking solution was removed and mouse monoclonal anti-AIM2 (ab93015, Abcam) at a dilution of 1:100 in TBST (in 4 mL) was added. The membranes were rocked overnight in the cold room. The next day the membranes were washed three times in 1x TBST for 5 mins each before HRP-conjugated anti-mouse (sc-516102, Santa Cruz) was added at a 1:100 dilution. The membranes were rocked for 45 mins at room temperature. Next the membranes were washed. Finally, 1 mL of Western blot sensitive substrate (BioRad) was added on top of the membranes and the membranes were viewed using ImageQuant LAS 4000 to detect AIM2. These steps were repeated using mouse monoclonal anti-HCV core antibody (MA1-080, Thermofisher) to detect the presence of an HCV infection and/or mouse monoclonal anti-GAPDH (sc-32233, Santa Cruz) as a control.

2.8 Using as poly(dA:dT)/LyoVec™ a positive control

We transfected Huh-7.5 cells with poly(dA:dT)/LyoVec™ at 1 μL/mL- 10 μL/mL for 24 hours before the cells were fixed by submersion in acetone for 1 minute and then washed in PBS for 1 minute. The cells were then stained with anti-AIM2, anti-DDX41, and anti-STING antibodies.

2.9 Using vaccinia virus as a positive control

We infected Huh-7.5 cells with VACV, gifted to us by and performed by Dr. Michael Grant's lab, at varying MOIs from 0.001 to 15 for 3 days before the cells were fixed via submersion in acetone for 1 minute and washed in PBS for 1 minute. The cells were stained with anti-AIM2 antibodies.

Chapter 3: Results – HCV engages elements of cytosolic DNA response

In this chapter we focus on the ability of HCV to engage with proteins traditionally thought to represent elements of the cytosolic DNA response. This may involve the expression/overexpression and/or translocation of proteins which we observe via immunofluorescence. The proteins we look at in these experiments were specifically chosen in the context of gaining a greater understanding of how JFH-1_T infection in Huh-7.5 cells involves "cross talk" between RNA viral infection and foreign and/or cytosolic DNA recognition.

3.1 HCV infection does not trigger a response from IFI16

We looked for macro-molecular aggregate structures of 1–2 μM in size (ASC specks/puncta) (Kesavardhana & Kanneganti, 2017) which could indicate the formation of an IFI16 inflammasome complex, or we looked for the translocation of IFI16 from the nucleus to the cytosol, which could indicate engagement with the STING-TBK1-IRF3 pathway. To do this, we infected Huh-7.5 cells with JFH-1_T at an MOI of 1 for 3 days before the cells were fixed and stained with anti-HCV core and anti-IFI16 antibodies. Immunofluorescence analysis indicated that although HCV infection did take place, IFI16 proteins could not be observed anywhere in the cell indicating that HCV infection does not trigger a response from IFI16 (see figure 3.1 B). Our results also show a lack of IFI16 proteins in uninfected cells indicating low levels of endogenous protein which may be required for an accurate result. In the absence of a positive control cell for IF116 antibody, we cannot conclude with certainty that HCV infection did not trigger a response from IFI16.

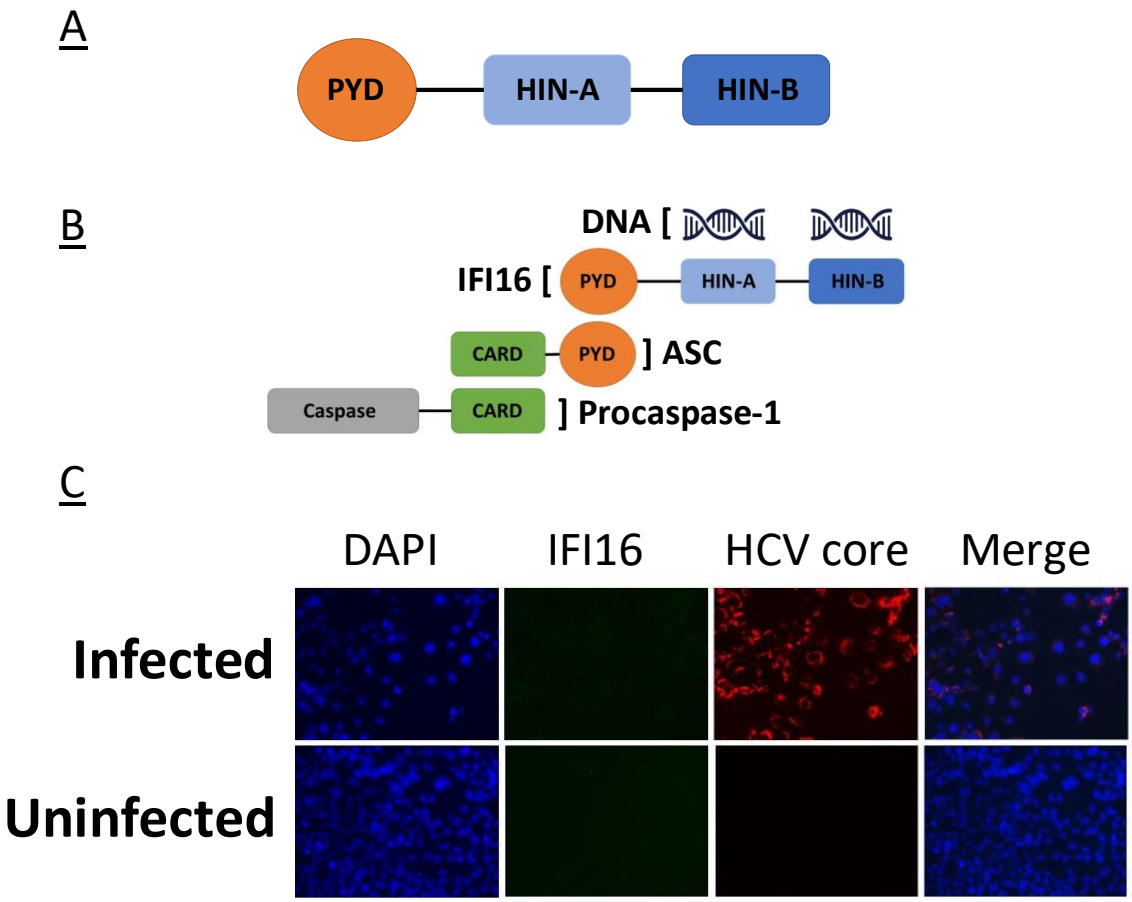


Figure 3.1 HCV infection does not trigger a response from IFI16.

(A) The IFI16 protein represented by an N-terminal PYD and two C-terminal HIN domains. **(B)** The IFI16 inflammasome complex. Both HIN domains are capable of binding DNA. The N-terminal PYD associates with the PYD of ASC. The CARD associates with the CARD of procaspase-1. **(C)** Huh-7.5 cells were infected at an MOI of 1. The cells were stained 3 days p.i. for DAPI (blue), IFI16 (green) and HCV core protein (red). This experiment was completed in duplicate 5 times at 200x magnification. Figure A has been reproduced and adapted to depict inflammasome arrangements under Creative Commons CC BY 4.0. Zheng, D., Liwinski, T., & Elinav, E. (2020). Inflammasome activation and regulation: Toward a better understanding of complex mechanisms. Figure 1: Pattern diagram for inflammasome assembly and activation *Cell Discovery*, *6*(1), 1–22. https://doi.org/10.1038/s41421-020-0167-x

3.2 HCV infection prompts DDX41 to leave the nucleus resulting in degradation

We wanted to test whether HCV infection could cause the translocation from within the nucleus to the cytosol and/or the degradation of DDX41 proteins. To determine this, we infected Huh-7.5 cells with JFH-1_T at an MOI of 1 for 3 days before the cells were fixed and stained with anti-HCV core and anti-DDX41 antibodies. Immunofluorescence analysis indicated that HCV prompts DDX41 to leave the nucleus resulting in degradation. As expected, DDX41 protein can be seen in the nucleus of the uninfected cells, as noted by the overlap of the DAPI (blue) and the anti-DDX41. (green) staining (see 3.2B). After a three-day infection has taken place, we see a drastic decrease in the amount of DDX41 protein in the nucleus and no DDX41 protein in the cytosol. This suggests that HCV infection triggers a release of DDX41 into the cytosol but that after three days p.i., DDX41 has already been degraded by the E3 ligase TRIM21. Since cytosolic DNA is what ultimately drives DDX41 degradation, these finding support the "cross talk" theory between RNA viral infection and foreign and/or cytosolic DNA recognition.

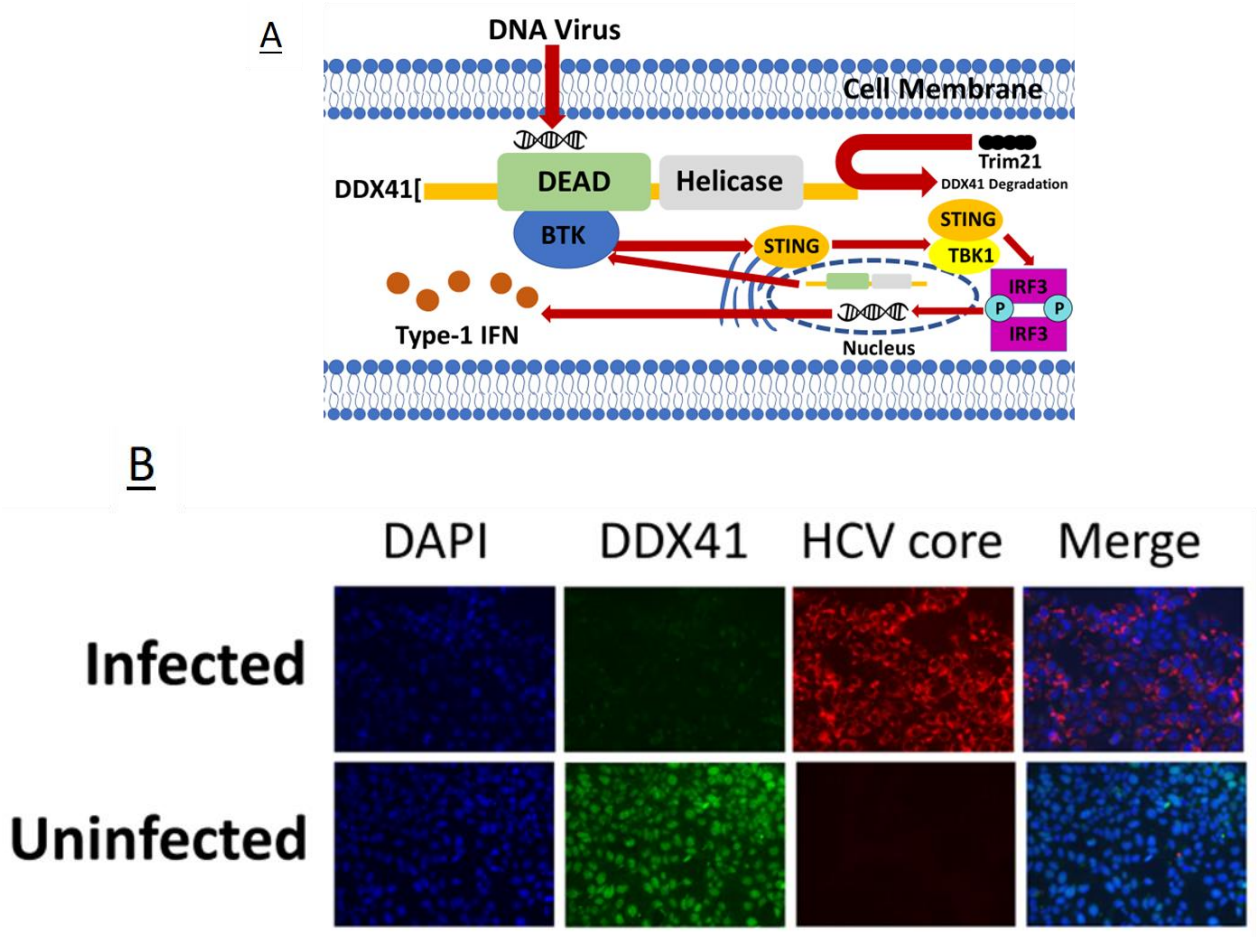


Figure 3.2 HCV prompts DDX41 to leave the nucleus resulting in degradation.

(A) Schematic representation of DDX41 acting as a cytosolic DNA sensor and functioning through the STING-TBK1-IRF3 pathway. DDX41 is located in the nucleus of hepatocytes until stimulated with cytosolic DNA where DDX41 enters the cytosol. Once in the cytosol DDX41 is phosphorylated by BTK kinase and activated. Activated DDX41 can bind DNA PAMPs and then STING, both through the DEAD domain. Once STING is bound, it is translocated from the ER to the Golgi apparatus where it interacts with TBK1 and forms a STING-TBK1 complex. This complex will result in the phosphorylation and nuclear translocation of IRF3, an interferon transcription factor, promoting the release of type 1 IFNs. After immune response, DDX41 is quickly degraded by the E3 ligase TRIM21. **(B)** Huh-7.5 cells, in triad, were infected at an MOI

of 1. The cells were stained 3 days p.i. for DAPI (blue), DDX41 (green) and HCV core protein (red). This is a depiction of the average result. This experiment was completed in duplicate 3 times at 200x magnification. Figure A has been reproduced and adapted for simplification under Creative Commons CC BY 4.0. Jiang, Y., Zhu, Y., Liu, Z.-J., & Ouyang, S. (2017). The emerging roles of the DDX41 protein in immunity and diseases. Figure 1: The signaling pathway of DDX41 in innate immunity. *Protein & Cell*, 8(2), 83–89. https://doi.org/10.1007/s13238-016-0303-4

3.3 HCV entices STING to move from the ER and gather at the Golgi apparatus

Activated STING is translocated from the ER to the Golgi apparatus where it interacts with TBK1 and forms a STING-TBK1 complex (see figure 3.3 A) (Y. Jiang et al., 2017). This complex will result in the phosphorylation and nuclear translocation of IRF3 (Dobbs et al., 2015) and the release of type 1 IFNs (see figure 3.3 A). Since we know we will not see the release of IFN-I in our experiment (Bender et al., 2015), we chose to visualize the movement of STING from the ER into clusters within the Golgi apparatus. We did this by infecting Huh-7.5 cells with JFH-1_T at an MOI of 1 for 3 days before the cells were fixed and stained with anti-HCV core and anti-STING antibodies. Using immunofluorescence, we were able to detect, what looks like, a large endogenous population of STING proteins surrounding the ER of uninfected cells (see figure 3.3 B). After a three-day infection, STING proteins were far less wide spread, and condensed into smaller pockets located just outside the nucleus, as indicated by the green stain (DDX41) proximity to the blue stain (DAPI) (see figure 3.3 B). Since we know that we are using an RNA virus for infection, in a sterile, bacteria free environment, and that Huh-7.5 cells are deficient in RIG-I, this supports that STING proteins are being activated by DDX41 proteins leading to the translocation of STING from the ER to the Golgi apparatus.

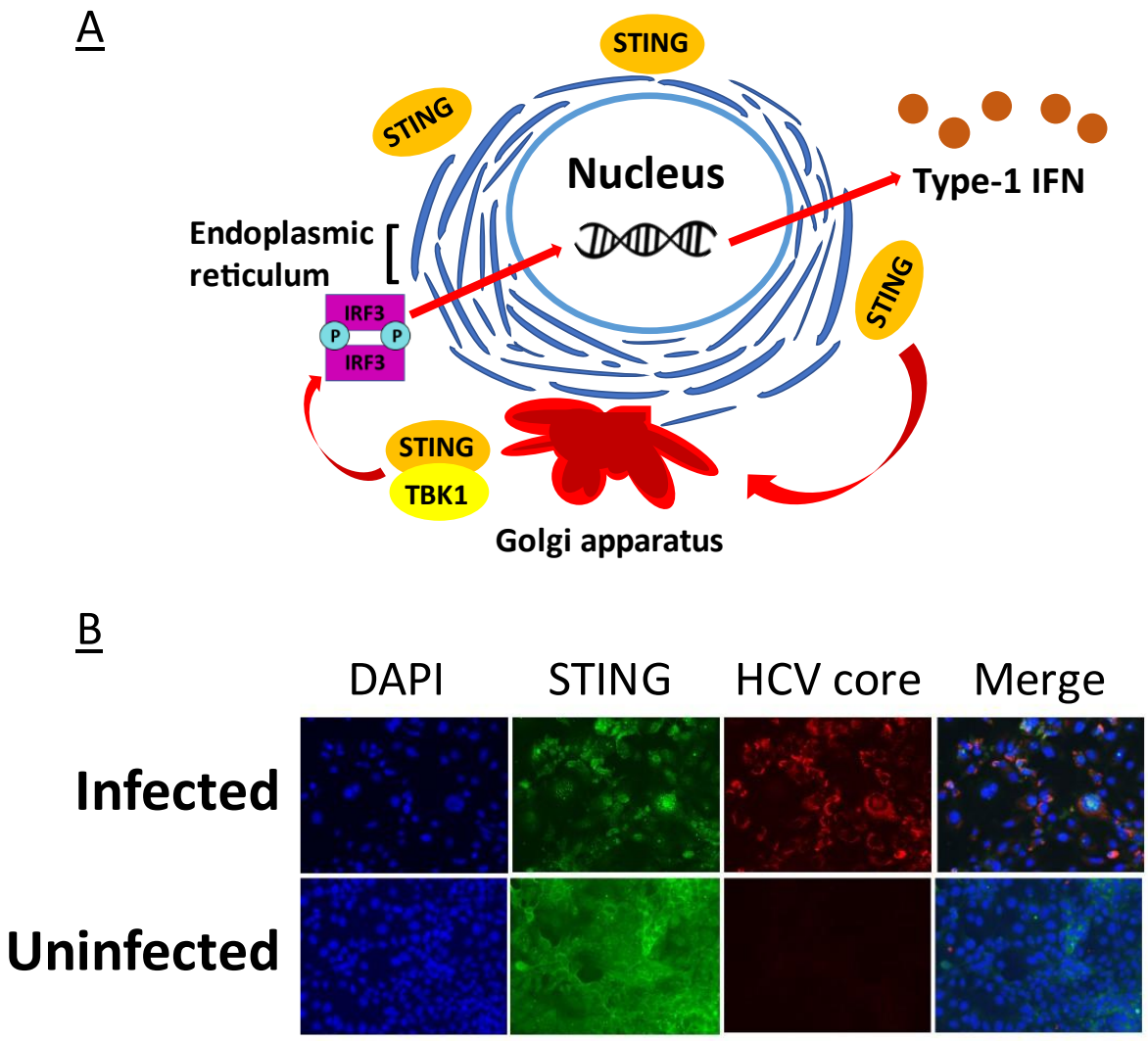


Figure 3.3 HCV entices STING to move from the ER and gather at the Golgi apparatus

(A) Schematic representation of the translocation of STING from the ER to the Golgi apparatus where it interacts with TBK1 and forms a STING-TBK1 complex. This complex will result in the phosphorylation and nuclear translocation of IRF3, an interferon transcription factor, promoting the release of type 1 IFNs. **(B)** Huh-7.5 cells, in triad, were infected at an MOI of 1. The cells were stained 3 days p.i. for DAPI (blue), STING (green) and HCV core protein (red). This experiment was completed in duplicate 3 times at 200x magnification. Figure A was reproduced and adapted to depict the role of STING in the cGAS-STING Pathway under

 Xu, D., Tian, Y., Xia, Q., & Ke, B. (2021). The cGAS-STING Pathway: Novel Perspectives in Liver Diseases. FIGURE 1 The cytosolic DNA-sensing cGAS-STING pathway in innate immunity. *Frontiers in Immunology*, *0*. https://doi.org/10.3389/fimmu.2021.682736

Chapter 4: Results - HIV infection activates the AIM2 inflammasome complex

In the previous chapter we demonstrated that JFH-1_T infection in Huh-7.5 cells could result in the activation and/or utilization of protein elements of the cytosolic DNA response. Specifically, after a three-day infection had taken place, we saw a drastic decrease in the amount of DDX41 protein in the nucleus suggesting that HCV infection prompts DDX41 to leave the nucleus leading to DDX41 degradation. We also saw that after infection, STING proteins were far less wide spread, and condensed into smaller pockets located just outside the nucleus, suggesting that STING proteins are being activated by DDX41 proteins. Since we know that HCV is an RNA virus, and that DDX41 is triggered by cytosolic DNA, this lays the foundation for understanding the potential for the formation of AIM2 inflammasomes within the same parameters. In this chapter, we utilize immunofluorescence microscopy and Western blotting to look exclusively at JFH-1_T and AIM2 engagement. We believe that the discovery of the AIM2 inflammasome activated by HCV may provide insight on a PCD which can have an impact on HCV pathogenesis, especially since AIM2 expression is down-regulated in HCV-related cancers (Chen et al., 2017).

4.1 AIM2 expression increases after HCV infection

Since Huh-7.5 cells are an HCC cell line, leading to a relative decrease in AIM2 expression (DeYoung et al., 1997) (X. Ma et al., 2016), we wanted to evaluate the level of AIM2 protein in Huh-7.5 cells before and after JFH-1_T infection. To do this, we infected Huh-7.5 cells with JFH-1_T at an MOI of 1 for 3 days before the cells were harvested, and lysed. Anti-AIM2 antibodies were used to detect AIM2 protein in cell lysates after the use of a Western blot. Our data indicated a very low endogenous expression of AIM2 that increased after HCV infection

(see figure 4.1). Note that AIM2 should be detected at about 39kDa and we were detecting it at around 63kDa.

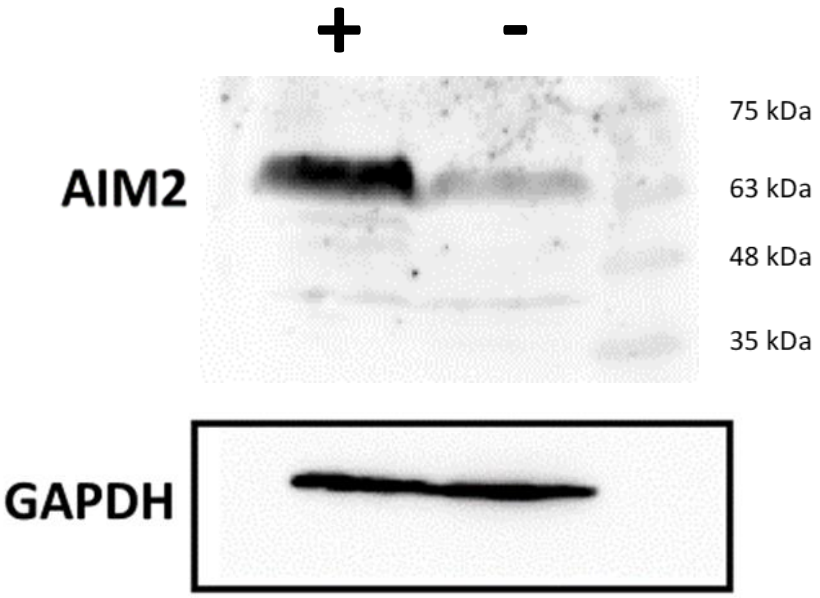


Figure 4.1 AIM2 expression increases after HCV infection.

Western blot to detect AIM2 protein in infected and uninfected Huh-7.5 cells. Huh-7.5 cells were infected with JFH-1_T at an MOI of 1 for 3 days before the cells were harvested and lysed. Anti-AIM2 antibodies were used to detect AIM2 protein after the use of a Western blot. (Left lane infected, right lane uninfected, GAPDH control). This experiment was completed 7 times, though this result was only achieved twice. Note that AIM2 should be detected at about 39kDa and we were detecting it at around 63kDa.

4.2 HCV infection triggers the formation of AIM2 inflammasomes

To detect the presence of AIM2 inflammasome formation, we infected Huh-7.5 cells with JFH-1_T at an MOI of 1 for 3 days before the cells were fixed and stained with anti-HCV core and anti-AIM2 antibodies. Then we looked for macro-molecular aggregate structures of 1–2 μM in size (ASC specks/puncta) (Kesavardhana & Kanneganti, 2017).

Immunofluorescent microscopy showcased a very noticeable difference between the location of AIM2 (green) found in the infected vs. the uninfected cells. In the uninfected cells, AIM2 is spread out through the cytosol, which we believe may be background stain or diffuse localization (see figure 4.2 B). In the infected cells, AIM2 has formed the traditional ASC specks/puncta shape which confirms the presence of the AIM2 inflammasome complex in Huh-7.5 cells after JFH-1_T infection.

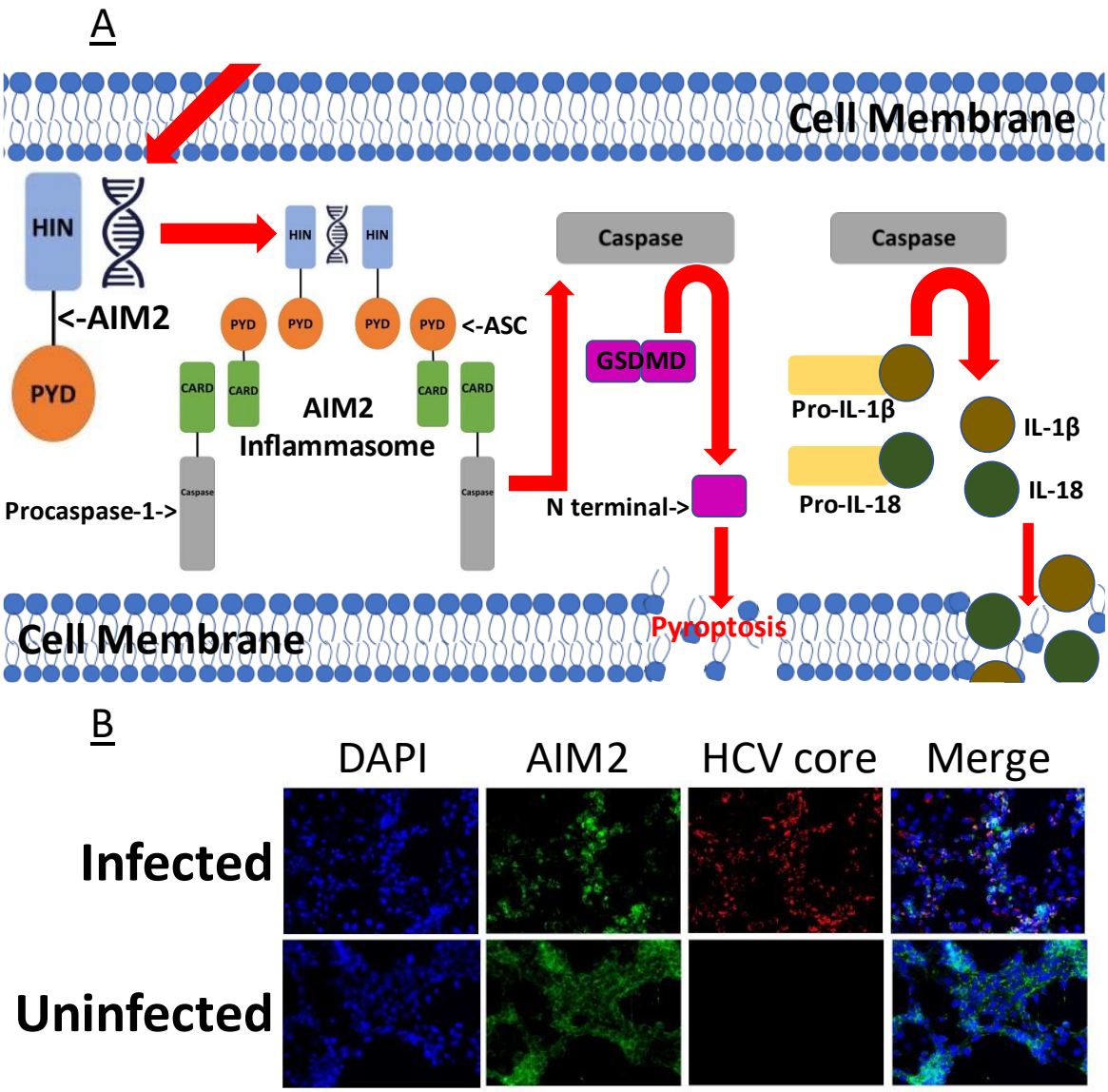


Figure 4.2 HCV infection triggers the formation of AIM2 inflammasomes.

(A) Schematic representation of pyroptosis through the formation of the AIM2 inflammasome complex. AIM2 is composed of a C-terminal HIN and an N-terminal PYD. AIM2 binds dsDNA at the HIN leading to AIM2 activation, where the PYD recruits ASC through PYD-PYD interactions. ASC then associates with procaspase-1 through CARD-CARD binding. Procaspase-1 is then cleaved by the complex. As a result, caspase-1 cleaves GSDMD, pro-IL-1β and pro-IL-18, resulting in pyroptosis and the release of proinflammatory cytokines (IL-1β and IL-18). **(B)** Huh-7.5 cells were infected at an MOI of 1. The cells were stained 3 days p.i. for DAPI (blue), AIM2 (green) and HCV core protein (red). This experiment was completed in duplicate 4 times

at 200x magnification. Figure A was reproduced and adapted to specifically depict the role of AIM2 in DNA viral infection under Creative Commons CC BY 4.0. Lozano-Ruiz, B., & González-Navajas, J. M. (2020). The Emerging Relevance of AIM2 in Liver Disease. Figure 1: Mechanism of AIM2 inflammasome assembly. *International Journal of Molecular Sciences*, *21*(18), 6535. https://doi.org/10.3390/ijms21186535

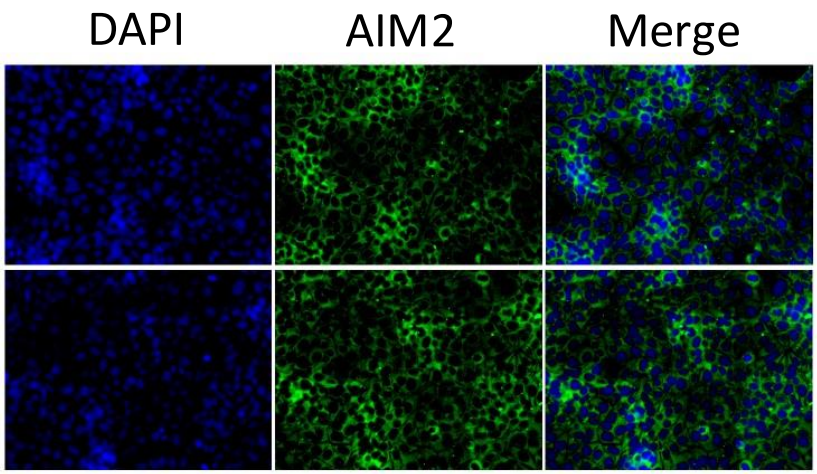
4.3 Evaluation of poly(dA:dT)/LyoVec™ and vaccinia virus as potential positive controls for AIM2 activation

Cytosolic poly(dA:dT) should activate AIM2 resulting in the formation of an inflammasome of 1–2 μM in size (Kesavardhana & Kanneganti, 2017) detectible by immunofluorescence. To determine the presence of AIM2 inflammasome formation, we transfected Huh-7.5 cells with poly(dA:dT)/LyoVec™ at 1 μl/ml for 24 hours before the cells were fixed and stained with anti-AIM2 antibodies. This experiment was repeated with up to 10 μL/mL poly(dA:dT)/LyoVec™ for 24 hours (data not shown). To test the similarities between JFH-1_T infection and poly(dA:dT)/LyoVec™ transfection on other elements of the cytosolic DNA response, we used anti-DDX41, anti-STING and anti-IFI16 antibodies. Our results indicated that it is unlikely that the poly(dA:dT)/LyoVec™ transfection worked. We did not see the translocation from within the nucleus to the cytosol and/or the degradation of DDX41 proteins (see figure 4.3 B), the translocation of STING from the ER to the Golgi apparatus (see figure 4.3 C) or the traditional ASC specks/puncta shape confirming the presence of the AIM2 inflammasome complex (see figure 4.3 A); all of which was seen during a JFH-1_T infection.

VACV replicates in the cytosol (Lorenzo et al., 2000) where the virus should be recognized by AIM2. VACV is also known to infect Huh-7 cells (Gentschev et al., 2011), so we attempted to use a VACV infection as a positive control as a means to demonstrate AIM2 inflammasomes in Huh-7.5 cells. To detect the presence of acute AIM2 inflammasome formation we infected Huh-7.5 cells with VACV, gifted to us by and preformed by Dr. Michael Grant's lab, at varying MOIs from 0.001 to 15 for 3 days before the cells were fixed and stained with anti-AIM2 antibodies. Then we looked for macro-molecular aggregate structures of 1–2 μM in size (ASC specks/puncta) (Kesavardhana & Kanneganti, 2017). Immunofluorescent microscopy

showed no difference between infected or uninfected cells regardless of MOI (see figure 4.3 D), and according to Dr. Michael Grant, significant death of cells should have taken place at higher MOIs. This indicates that a VACV infection did not take place.

A


DAPI
AIM2
Merge
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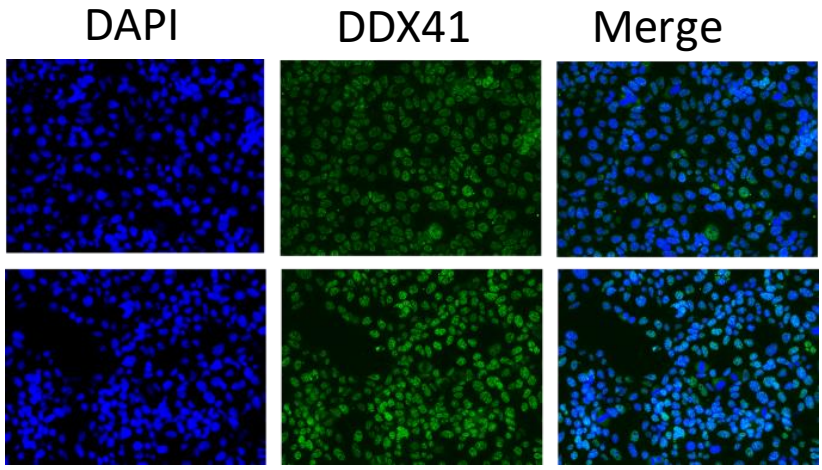
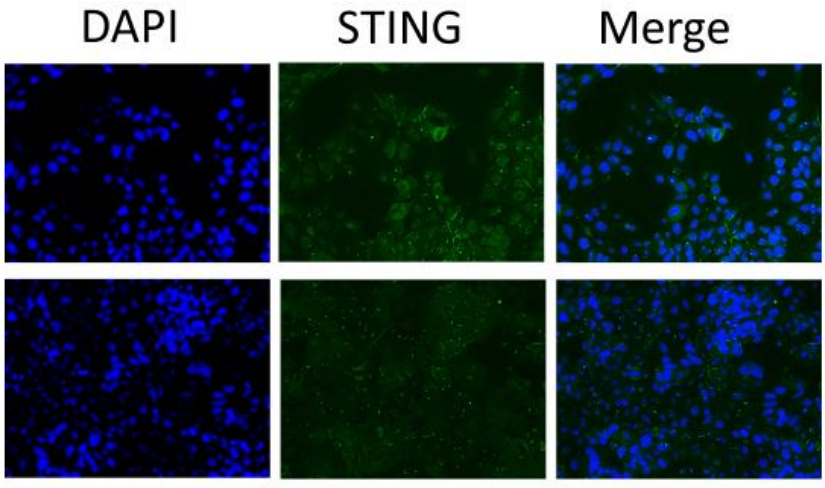
Transfected with poly(dA:dT)/LyoVec™

B


DAPI
DDX41
Merge
Not transfected
Transfected with poly(dA:dT)/LyoVec™

C


DAPI
STING
Merge
Not transfected
Transfected with poly(dA:dT)/LyoVec™

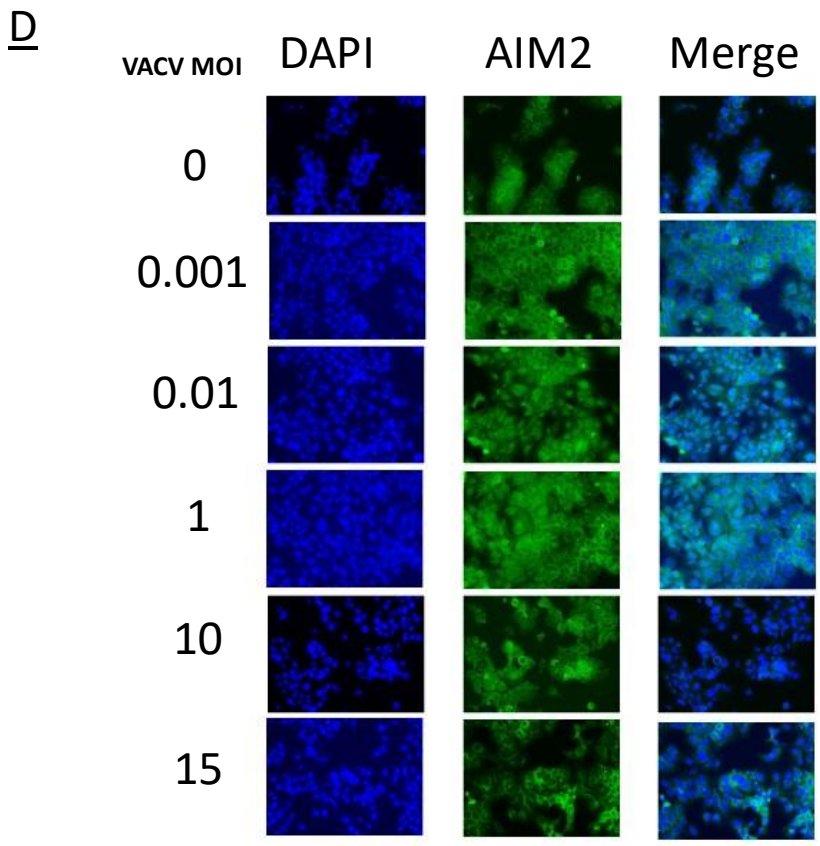


Figure 4.3 Evaluation of poly(dA:dT)/LyoVec™ and vaccinia virus as potential positive controls for AIM2 activation.

(A) Huh-7.5 cells were transfected with poly(dA:dT)/LyoVec using 1ug/ml in 2 ml medium for 24 hours. The cells were stained for DAPI (blue) and AIM2 (green). **(B)** Huh-7.5 cells were transfected with poly(dA:dT)/LyoVec using 1ug/ml in 2 ml medium for 24 hours. The cells were stained for DAPI (blue) and DDX41 (green). **(C)** Huh-7.5 cells were transfected with poly(dA:dT)/LyoVec using 1ug/ml in 2 ml medium for 24 hours. The cells were stained for DAPI (blue) and STING (green). **(D)** Huh-7.5 cells were infected with vaccinia virus at an MOI from 0.001 to 15. The cells were stained 3 days p.i. for DAPI (blue) and AIM2 (green). Experiments A and B were completed 3 times. Experiment C was completed twice at a magnification of 200x.

4.4 AIM2 colocalizes with caspase-1 in HCV infected cells.

AIM2 contains a N-terminal PYD which recruits ASC through PYD-PYD interactions. ASC then recruits procaspase-1 through CARD-CARD interactions, creating the AIM2 inflammasome complex (see figure 4.2 A) (reviewed in Sharma & Kanneganti, 2016). This means that if AIM2 inflammasome complex formation is taking place, active caspase-1 should be found in close proximity to the AIM2. To test whether AIM2 colocalizes with caspase-1 we infected Huh-7.5 cells with JFH-1_T at an MOI of 1 for 3 days before the cells were probed for caspase-1 activity using the FAM FLICA Caspase-1 Assay Kit. The kit requires the use of live cells where the FLICA reagent FAM-YVAD-FMK irreversibly binds to activated caspase-1, which is then detected by Immunofluorescence microscopy (FAM-FLICA® Caspase-1 (YVAD) ImmunoChemistry Technologies, Assay Kit data sheet). The cells were then fixed and stained with anti-AIM2 antibodies. To ensure the presence of active caspase-1 was related to HCV infection, we repeated the experiment substituting the anti-AIM2 antibody with an anti-HCV core antibody. Using immunofluorescence, we could see a clear overlap in AIM2 and caspase-1 indicating colocalization (see figure 4.4 A). This is especially apparent in the merge image where the red and green stain create an orange colour. We can also determine that HCV infection is the cause of caspase-1 production, as there were very little caspase-1 producing cells in those uninfected compared to those infected (see figure 4.4 B).

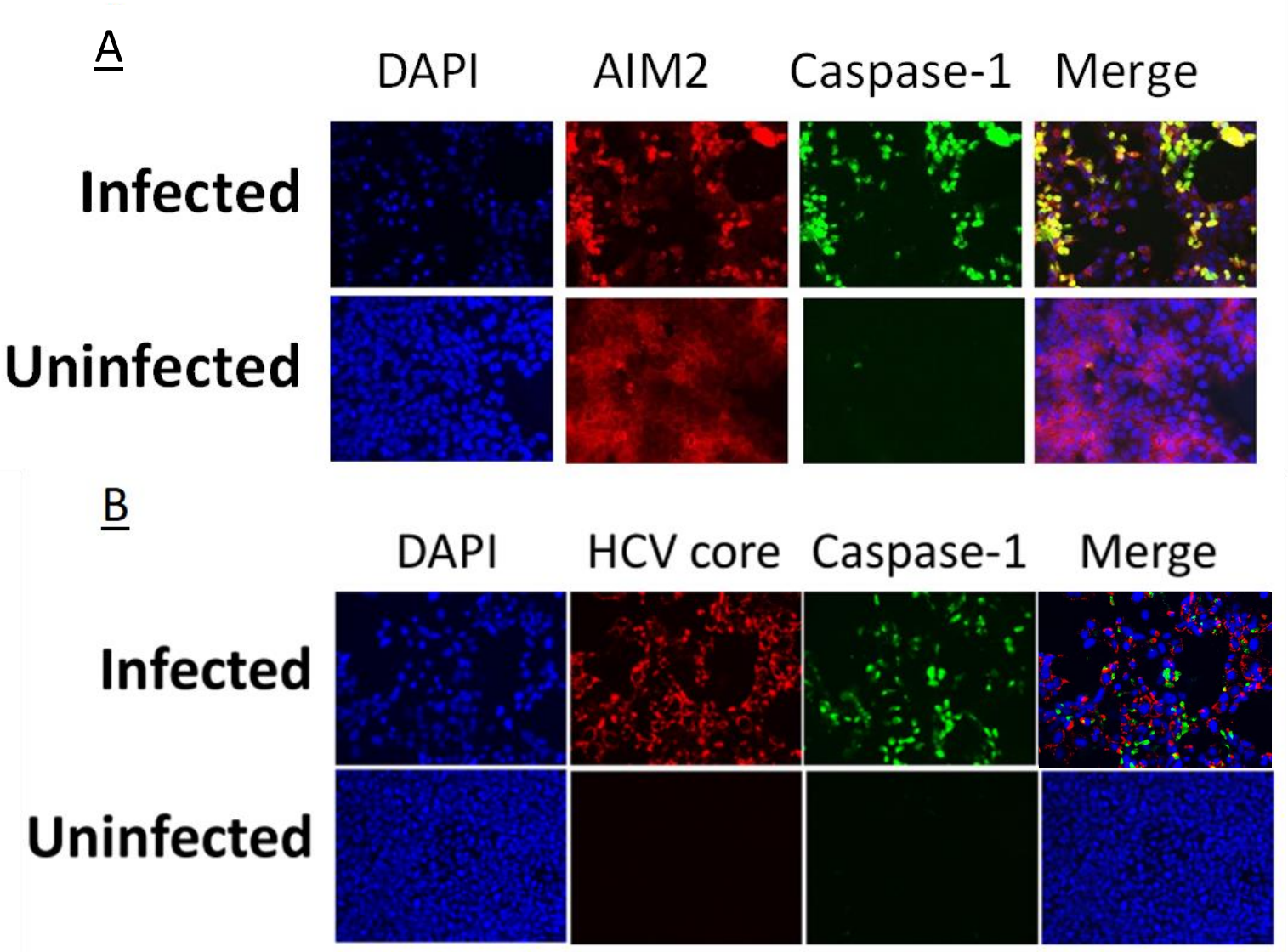


Figure 4.4. AIM2 colocalizes with caspase-1 in HCV infected cells.

(A) Huh-7.5 cells were infected at an MOI of 1. The cells were stained or probed using the FAM FLICA Caspase-1 Assay Kit 3 days p.i. for DAPI (blue), AIM2 (red) and caspase-1 (green). **(B)** Huh-7.5 cells were infected at an MOI of 1. The cells were stained or probed using the FAM FLICA Caspase-1 Assay Kit 3 days p.i. for DAPI (blue), HCV core protein (red) and caspase-1 (green). This experiment was completed in duplicate 3 times at a magnification of 200x.

4.5 AIM2 colocalizes with caspase-1 in HCV infected Huh-7.5 NLRP3 K/O cells

NLRP3 inflammasome complex formation is known to take place during HCV infection. HCV infection contains all the features required to trigger both priming and activation of the NLRP3 inflammasomes in hepatocytes and macrophages (Negash et al., 2019) and JFH-1_T infection has been shown to activate NLRP3 inflammasomes in Huh-7.5 cells (Kofahi et al., 2016). To ensure that we are not detecting activated caspase-1 cleaved via the NLRP3 inflammasome complex, we sought to detect caspase-1 in Huh-7.5 NLRP3 knock-out (K/O) cells (created by Lingyan Wang, PhD candidate in Dr. Russell's lab). First, we wanted to visualize and compare caspase-1 activation in Huh-7.5 and Huh-7.5 K/O cells. We infected both cell lines with JFH-1_T at an MOI of 1 for 3 days before the cells were probed for caspase-1 activity using the FAM FLICA Caspase-1 Assay Kit and visualized with immunofluorescence. The resulting data indicated that although caspase-1 activation was taking place in Huh-7.5 K/O cells, it was doing so to a far lesser degree than in Huh-7.5 cells (see figure 4.5 A). To test if AIM2 colocalizes with caspase-1, we infected Huh-7.5 K/O cells with JFH-1_T at an MOI of 1 for 3 days before the cells were probed for caspase-1 activity with the FAM FLICA Caspase-1 Assay Kit. The cells were then fixed and stained with anti-AIM2 antibodies. To ensure the presence of active caspase-1 was related to HCV infection, we repeated the experiment substituting the anti-AIM2 antibody with an anti-HCV core antibody (see figure 4.5 C). We found that AIM2 colocalizes with caspase-1 in Huh-7.5 K/O though to a lesser degree than AIM2 colocalizes with caspase-1 in Huh-7.5 cells (see figure 4.5 B).

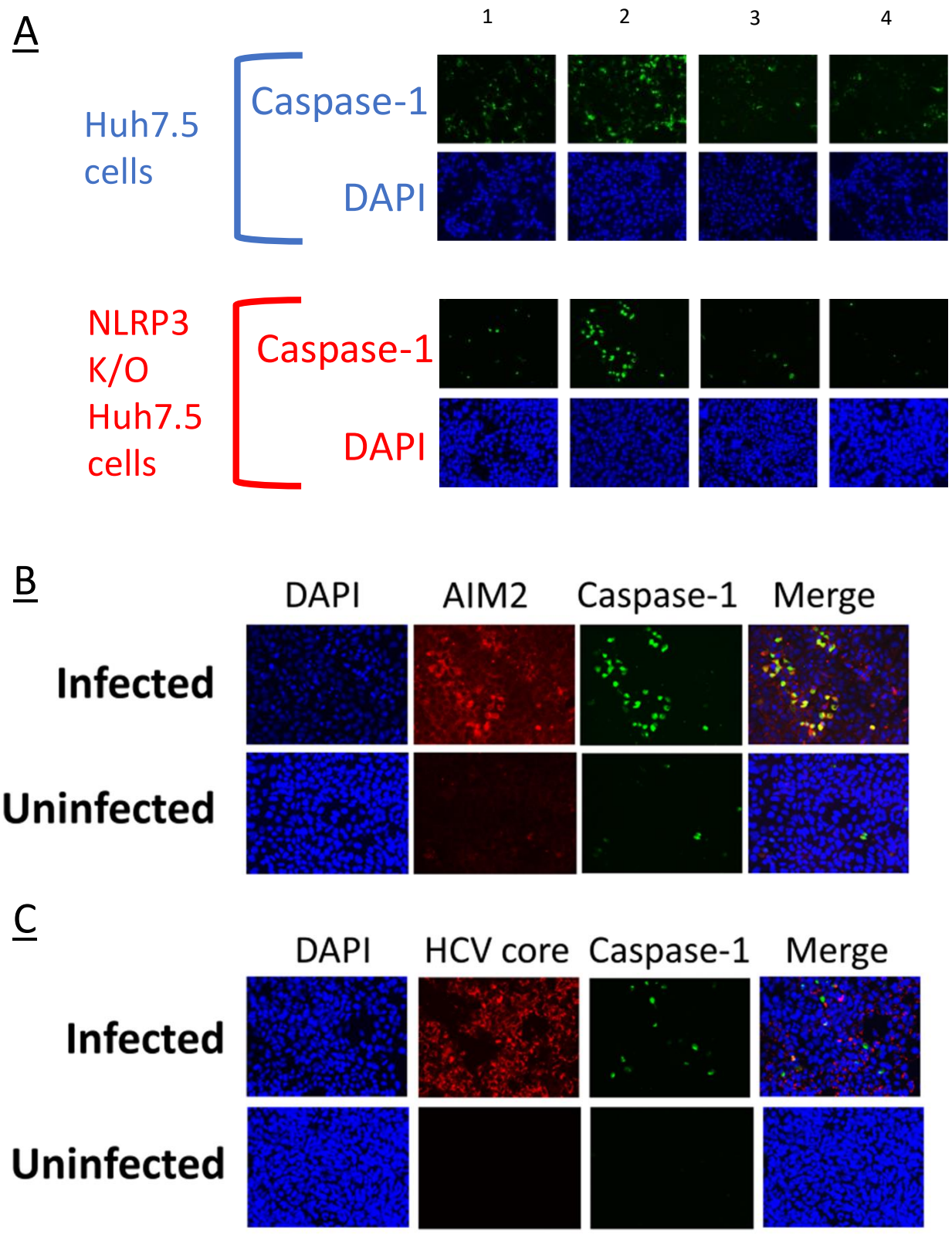

A
1
2
3
4
Huh7.5 cells
Caspase-1
DAPI
NLRP3 K/O Huh7.5 cells
Caspase-1
DAPI
B
DAPI
AIM2
Caspase-1
Merge
Infected
Uninfected
C
DAPI
HCV core
Caspase-1
Merge
Infected
Uninfected

Figure 4.5 AIM2 colocalizes with caspase-1 in HCV infected Huh-7.5 NLRP3 K/O cells.

(A) A comparison between activated caspase-1 in infected Huh-7.5 cells and Huh-7.5 NLRP3 K/O cells. Each of the 4 images are from the 4 corners of the same slide where 1 is the top left, 2 is the top right, 3 is the bottom left, and 4 is the bottom right. **(B)** Huh-7.5 NLRP3 K/O cells were infected at an MOI of 1. The cells were stained or probed using the FAM FLICA Caspase-1 Assay Kit 3 days p.i. for DAPI (blue), AIM2 (red) and caspase-1 (green). **(C)** Huh-7.5 NLRP3 K/O cells were infected at an MOI of 1. The cells were stained or probed using the FAM FLICA Caspase-1 Assay Kit 3 days p.i. for DAPI (blue), HCV core protein (red) and caspase-1 (green). Experiments B and C were completed in duplicate twice at 200x magnification.

Chapter 5: Discussion

Although there are new direct-acting antiviral agents which allow for eradication of HCV in over 90% of patients (Stasi et al., 2020), it is estimated that more than 71 million people are affected by HCV globally, (WHO, 2020) in 2020, and in Canada HCV causes more years of life lost than any other infectious disease (Schanzer et al., 2014), (Kwong et al., 2012) and of individuals with liver cirrhosis infected with COVID-19, a preliminary study found an overall mortality rate of 40% (Moon et al., 2020). Many report that AIM2 can drive anti-viral inflammasome responses against some RNA viruses (Ekchariyawat et al., 2015), (Zhang et al., 2017), (He et al., 2020), (Junqueira et al., 2021) (discussed in section 1.4.2), but this has yet to be studied using HCVcc, although in this context, the mechanisms for AIM2 inflammasome activation would be unknown. In this report we investigated how JFH-1_T infection in Huh-7.5 cells could involve "cross talk" between RNA viral infection and foreign and/or self cytosolic DNA recognition. I started by examining the ability of HCV to engage with proteins traditionally thought to represent elements of cytosolic DNA response pathways and found that although IFI16 remained unprovoked by the virus, DDX41 and STING translocate in a manner seemingly similar to that which would occur in the presence of cytosolic dsDNA. Next, I found that not only does AIM2, a cytosolic dsDNA sensor, expression increase after JFH-1_T infection, the protein forms an AIM2 inflammasome complex and colocalizes with caspase-1.

AIM2 inflammasome activation is not well characterized in the context of HCV, especially as it relates to hepatocytes, although it is clear that the expression of AIM2 in the liver is inducible (Y. Han et al., 2015). Research in HCV is a continued need especially as it relates to viral induced inflammation. Unfortunately, there are very few studies which focus on AIM2 associated inflammation through the lens of HCV infection and next to no studies which reflect

the complete life cycle of the virus. Where the literature is found, studies direct their effort to the comprehension of AIM2 inflammasome activation in immune cells without any attempt to gauge the hepatocyte response.

5.1 IFN signalling

Although NLRP3 inflammasome activation occurs through all stages of HCV infection (Negash et al., 2013), it is interesting to speculate whether or not AIM2 expression exists in hepatocytes at the acute stage and decreases as symptoms progress, as loss of AIM2 expression promotes hepatocellular carcinoma progression (HCC) (S.-L. Chen et al., 2017). As stated, HCV is the third leading cause of hepatocellular carcinoma (Cheemerla & Balakrishnan, 2021). AIM2 is a tumor suppressor gene (DeYoung et al., 1997) and lower AIM2 expression correlates with more advanced HCC (X. Ma et al., 2016). AIM2 inflammasomes could potentially be activated through the loss of nuclear envelope or mitochondrial membrane integrity, or TLR binding and type I IFN signaling in the presence of HCV. Here I focused on *in vitro*, early AIM2 activation, through the use of HCVcc: JFH-1_T infection of Huh-7.5 cells (which are derived from Huh-7 cells), which is a hepatocellular carcinoma cell line that continues to express AIM2 (X. Ma et al., 2016), though levels are low.

Huh-7.5 cells, developed from the Huh-7 cell line, are highly permissive for subgenomic and genomic hepatitis C virus RNA replication and can produce infectious progenitor virions when infected (Blight et al., 2002). Unfortunately, these cells carry a number of mutations making the study of foreign and/or self cytosolic DNA recognition difficult (Sumpter et al., 2005). Huh-7.5 cells do not produce IFNs (Tsugawa et al., 2014). I could therefore not use IFN production (a common indicator used) as a tool to gauge the activation of proteins associated with elements of cytosolic DNA responses. As mentioned, Huh-7.5 cells carry a single point

missense mutation (T55I) found in the dsRNA sensor retinoic acid-inducible gene-I (RIG-I or DDX58) (Yoneyama et al., 2004), and have low expression of TLR 3 (K. Li et al., 2005), which senses double-stranded RNA and is found on plasma membranes. This may help to explain the lack of IFNs produced after RNA viral infection in Huh-7.5 cells; however, nuclear, and especially mitochondrial, dsDNA may be released by pyroptotic cells regardless of viral genome (Man et al., 2017), which should then be recognized by dsDNA sensors (such as DDX41 and IFI16 viewed in this report). Nuclear and mitochondrial dsDNA present in the serum after pyroptotic cell death should be recognized by TLR 9 (Wagner & Bauer, 2006). Both cytosolic and cell membrane recognition of self dsDNA should result in an IFN response but in the context of Huh-7.5 cells infected with JFH-1 virus, no IFNs are produced (Tsugawa et al., 2014). This may indicate a mutation in the dsDNA IFN production pathway. In Huh-7 cells, IRF3 is located in the cytosol and redistributes to the nucleus after the creation of activated IRF3 dimers, in response to Sendai virus (SeV) (a negative strand RNA virus) infection or transfection of HCV 5′NTR (non-translating region) (Blight et al., 2002). In Huh-7.5 cells, IRF3 fails to leave the cytosol (Blight et al., 2002). Since IRF3 translocation is a requirement for IFN activation in a number of DNA recognition pathways (including downstream of IFI16, DDX41 and STING activation) (J. Ma et al., 2018), this may help illustrate why IFNs are not formed in response to self dsDNA in Huh-7.5 cells. Interestingly, the same study found that ectopic expression of RIG-I led to accumulation of activated IRF3 dimers and IRF3 translocation (Blight et al., 2002). Unfortunately, ectopic restoration of RIG-I would complicate our study, as it would be difficult to determine if IFN production was a result of self DNA recognition (what we would be looking for) or a response from RIG-I after identifying the RNA HCV viral genome. In the future, I would like to utilize anti-IRF3 antibodies via immunofluorescence as a means to visualize IRF3

translocation (or lack there of) in our Huh-7.5 cell line after JFH-1_T infection. Regardless of mutations in Huh-7.5 cells, I believe that the use of the tissue culture adapted JFH-1_T strain for the infection of the Huh-7.5 cell line, in the absence of immune cells, allowed for the best way to investigate cytosolic DNA response and AIM2 inflammasome activation in hepatocytes as this system best represents the large-scale production of infective virions found in the human model of disease.

5.2 IFI16 response

I present here evidence that HCV infection does not trigger a response from IFI16. Our results also show evidence of a lack of IFI16 proteins in uninfected cells, indicating little to no endogenous protein production. It is not surprising that IFI16 inflammasome activation did not occur after JFH-1_T infection. IFI16 is described as a "nucleus-associated inflammasome sensor component" meaning inactive IFI16 binds DNA components of viruses replicating within the nucleus, such as in the case of HSV-1 and KSHV (Xiao, 2015). HCV replication takes place in a membrane-associated multiprotein complex (membranous web) (Egger et al., 2002), outside of the nucleus and HCV contains an RNA genome without DNA intermediates. IFI116 is unlikely to bind self DNA inside the nuclease during pyroptosis because IFI16 has a low affinity to DNA in general. IFI16 proteins increase affinity to DNA as the number of exposed base pairs increase, such as just after DNA viral invasion when the entire naked DNA genome of the virus is exposed (Morrone et al., 2014). IFI16 could potentially bind cytosolic self DNA during pyroptosis and engage the STING-TBK1-IRF3 pathway resulting in the production and release of the of type I interferons (Zheng et al., 2020), but as noted Huh-7.5 cells do not release interferons because the IRF3 in the STING-TBK1-IRF3 pathway does not participate in nuclear translocation (Blight et al., 2002). In both infected and uninfected cells, we may not have seen IFI16 due to the nature of

the parental, Huh-7, cell line. Of the HCC cell lines: HePG-2, Huh-7, Bel-7402, HL-7702, and SMMC-7721 (which carry decreased IFI16 mRNA); Huh-7 cells produce the least amount of endogenous IFI16 (very low levels which may not be detectable via immunofluorescence) (Lin et al., 2017). Notably, IFI16 is a tumor suppressor gene and ectopic expression in Huh-7 cells increases apoptotic and pyroptotic cell death, and IL-1β and 1L-18 production (Lin et al., 2017), which may explain the low levels of IFI16 discovered in Huh-7.5 cells. Finally, these results may simply indicate a lack of binding between IFI16 proteins and the mouse monoclonal anti-IFI16 (ab55328, Abcam) antibody used for immunofluorescence in this report.

5.3 DDX41 response

I showed in section 3.2 that HCV infection prompts DDX41 to leave the nucleus resulting in DDX41 degradation. DDX41 is located in the nucleus of hepatocytes until stimulated with cytosolic DNA, where DDX41 enters the cytosol. After which it is quickly degraded by the E3 ligase TRIM21 (Y. Jiang et al., 2017). Using immunofluorescence, the prominent decrease in nuclear visualization of DDX41 in JFH-1_T infected cells vs. uninfected cells indicates the presence of cytosolic DNA. Furthermore, the lack of DDX41 proteins in the cytosol in infected cells suggests that a 3-day infection is enough time for degradation to take place. In the future, I would like to repeat this experiment every 12 hours in a time course as an attempt to capture DDX41 translocation before degradation. I would also like to perform a Western blot to verify this result. DDX41 mRNA expression should increase after the presence of cytosolic DNA and therefore I would like to perform a quantitative RT-PCR (QRT-PCR) for the expression analysis. DDX41 may play a role in HCV viral replication and viral replication might be responsible for DDX41 leaving the nuclease. It has been shown that DDX41 can bind the 3′ (+) untranslated region (UTR), as well as its reverse complementary 5′ (−) UTR (Tingting et al., 2006). After I

discover the time point at which DDX41 is found in the cytosol but has not yet been degraded, I would like to utilize confocal microscopy to determine if colocalization of DDX41 and HCV core is occurring in Huh-7.5 cells infected with the HCV tissue culture adapted JFH-1_T virus or if the presence of cytosolic DNA is indeed responsible for DDX41 redistribution.

5.4 cGAS-STING pathway

Our team did not investigate the cyclic GMP-AMP synthase (cGAS)-STING pathway, which is considered to be responsible for the most robust response to cytosolic DNA stimulation (reviewed in Xu et al., 2021). Cytosolic DNA from damaged mitochondria, dying cells, DNA damage, genomic instability, bacteria, DNA viruses, and retroviruses can all bind cGAS regardless of DNA sequence, which in turn will prompt cGAS to synthesize cyclic GMP-AMP (cGAMP) from ATP and GTP (see figure 5.1) (reviewed in Xu et al., 2021). cGAMP will bind and activate STING. In liver cells, cGAS can be found at relatively high endogenous levels and is located close to the nucleus (reviewed in Xu et al., 2021). There are a number of anti-cGAS antibodies available and in the future, I would like to utilize immunofluorescence to detect recolonization after HCV infection. Recently, a 2',3'-cyclic GAMP enzyme immunoassay kit has been created (Miyakawa et al., 2020) and is now available for purchase through Arbor Assays (Catalog Number: K067-H1 or K067-H5). Since cGAMP in created by cGAS, which is activated through cytosolic DNA binding, in the future, I would like to utilize the 2',3'-cyclic GAMP enzyme immunoassay kit to detect the presence of cGAMP. This may further support our belief that JFH-1_T infection in Huh-7.5 cells could involve "cross talk" between RNA viral infection and foreign and/or self cytosolic DNA recognition.

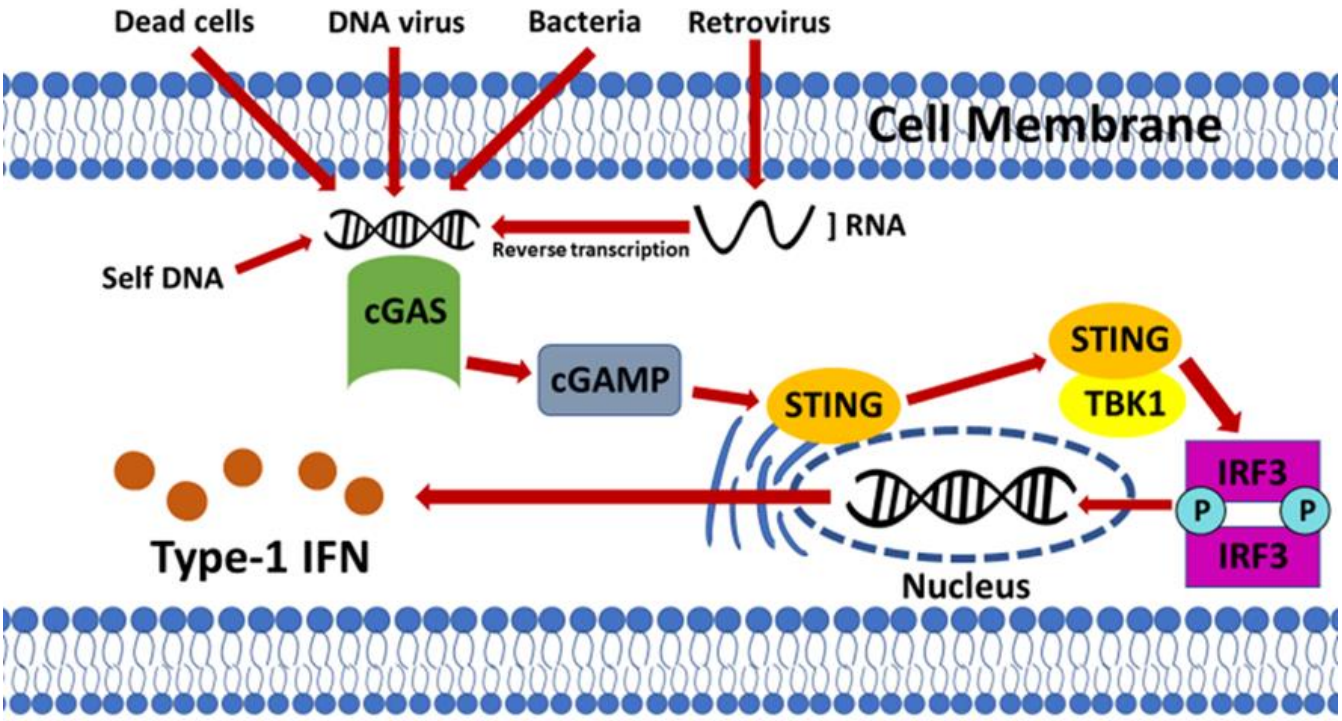


Figure 5.1 Schematic representation of cGAS acting as a cytosolic DNA sensor and functioning through the STING-TBK1-IRF3 pathway.

Cytosolic DNA from damaged mitochondria, dying cells, DNA damage, genomic instability, bacteria, DNA viruses, or retroviruses bind cGAS, which in turn will prompt cGAS to synthesize cyclic GMP-AMP (cGAMP) from ATP and GTP. cGAMP will bind and activate STING. Once STING is bound it is translocated from the ER to the Golgi apparatus where it interacts with TBK1 and forms a STING-TBK1 complex. This complex will result in the phosphorylation and nuclear translocation of IRF3, an interferon transcription factor, promoting the release of type 1 IFNs. This Figure was reproduced and adapted for simplicity under Creative Commons CC BY 4.0. Lozano-Ruiz, B., & González-Navajas, J. M. (2020). The Emerging Relevance of AIM2 in Liver Disease. Figure 1: Mechanism of AIM2 inflammasome assembly. *International Journal of Molecular Sciences*, *21*(18), 6535. https://doi.org/10.3390/ijms21186535

5.5 STING translocation

STING translocation was tested using immunofluorescence where our team discovered STING movement from the ER to the Golgi apparatus in JFH-1_T infected Huh-7.5 cells. STING activation occurs down stream of many cytosolic DNA sensors, suggesting the presence of cytosolic DNA. Activated STING is translocated from the ER to the Golgi apparatus where it interacts with TBK1 and forms a STING-TBK1 complex (Y. Jiang et al., 2017). This complex will result in the phosphorylation and nuclear translocation of IRF3 (Dobbs et al., 2015) and the release of type 1 IFNs. As mentioned, in Huh-7.5 cells, IRF3 fails to leave the cytosol (Blight et al., 2002) so our team did not test for IFN production. It should be noted that the HCV NS4B protein may also block the interaction of STING and TBK1, a viral mechanism used to sense IFN production and evade host innate immune responses (Ding et al., 2013). My findings replicate what would be seen during STING activation: after a three-day infection, STING proteins were far less dispensed, and condensed into small pockets located just outside the nucleus. In the future, I would first like to investigate STING and Golgi colocalization by employing immunofluorescence and confocal microscopy. We will make use of our anti-STING antibodies (MAB7169-SP, Novus) and an anti-58K Golgi protein antibody (used as a Golgi apparatus marker), for visualization. I would also like to test HCV NS4B inhibition of STING-TBK1 complex formation through the use of anti-STING, anti-TBK1 and anti- NS4B antibodies by using immunofluorescence and confocal microscopy to view NS4B and STING and/or TBK1 colocalization.

5.6 AIM2 expression

We present evidence that AIM2 expression increases after JFH-1_T infection. Since Huh-7.5 cells are an HCC cell line, leading to a relative decrease in AIM2 expression, we tested the

amount of AIM2 proteins in both JFH-1_T infected and uninfected Huh-7.5 cells. Using Western blot, I discovered very low endogenous expression of AIM2 that increased during JFH-1_T infection. AIM2 expression can be increased in response to type 1 IFNs, though without the presence of cytosolic DNA, an inflammasome will not form (Duan et al., 2011). AIM2 expression can also increase in response to injury, such as after a stroke (Kim et al., 2020) or heart attack (Ruppert et al., 2020), though such injuries may lead to the loss of nuclear envelope or mitochondrial membrane integrity, causing the movement of self DNA to the cytosol and lead to activated AIM2 inflammasomes. It is unclear if, in the context of HCV infection in hepatocytes, an increase in AIM2 expression is caused by cytosolic DNA recognition, which would mean AIM2 inflammasome activation is occurring, or if AIM2 expression is increasing as a response to cellular stress, in the absence of cytosolic DNA. To this effect, from these results alone, it is impossible to determine if AIM2 inflammasome formation is taking place in Huh-7.5 cells infected with JFH-1_T. It should be noted that this Western blot experiment proved difficult for me. Although I replicated the results a few times in a row when I first attempted it, later results were less reproducible. Also, AIM2 should be detected at about 39kDa and I was detecting it at around 63kDa. Although unlikely, this may indicate lack of separation between AIM2 and ASC. In the future I would like to transfect Huh-7.5 cells with an AIM2 producing plasmid and repeat the Western to verify that the anti-AIM2 (ab233756, Abcam) antibody is binding to the correct protein. I would also like to repeat this experiment but through a time course assay to determine the length of time p.i. it takes for AIM2 levels to increase. Finally, I want to verify this experiment with QRT-PCR. We purchased an inflammasome array to detect elements of human inflammasomes called "RT² Profiler™ PCR Array Human Inflammasomes" via Qiagen (and recommended to us by Jordan Osmond in Dr. Moore's lab) which identifies 84

inflammasome pathway or disease focused genes, including AIM2, but due to time restrains caused by pandemic-related disruptions, I was unable to use it.

With an increase in AIM2 expression, we witnessed AIM2 inflammasome activation. Eukaryotic organisms partake in cellular compartmentalization where DNA is found in the nucleus or within the mitochondria and is absent from the cytosol. DNA in the cytosol via the breakdown of the nuclear envelope or the mitochondrial membrane can be considered a DAMP and can be detected by AIM2 (reviewed in Rathinam et al., 2010), thus forming the AIM2 inflammasome complex. Our team visualized this in infected cells, where AIM2 had formed the traditional ASC specks/puncta shape. HCV contains an RNA genome without a DNA replication intermediate so AIM2 visualized in the ASC specks/puncta formation was most likely in response to self-DNA. HCV replication or viral protein production may have led to the loss of nuclear or mitochondrial membrane integrity, as described. It should be noted that our DAPI stain, used to visualize the nucleus, is actually binding to DNA. If there was a loss of nuclear or mitochondrial membrane integrity, we should therefore see DAPI stain in the cytosol, but we did not. Perhaps, there was not enough DNA in the cytosol to visualize through the use of the DAPI stain. AIM2 activation leads to cell death via pyroptosis so we believe that this finding may provide insight on PCD which can have an impact on HCV pathogenesis, especially since AIM2 expression is down-regulated in HCV-related cancers (Chen et al., 2017). To achieve this result, we used both a mouse monoclonal anti-AIM2 (ab180665, Abcam) antibody, that produced a lot of background staining in uninfected cells and a rabbit polyclonal anti-AIM2 (ab93015, Abcam) antibody, that produced very little background staining. Although both gave great results, it should be recognized that the rabbit polyclonal anti-AIM2 (ab93015, Abcam) antibody has since been taken off the market. In the future we would like to repeat the immunofluorescence

experiment using Huh-7.5 cells transfected with AIM2 proteins to ensure the correct binding by antibodies. We would also like to repeat this experiment in a time course and measure speck size during imaging to make sure the ASC specks/puncta formations are the correct size (1–2 μM (Kesavardhana & Kanneganti, 2017)).

5.7 AIM2 and caspase-1 colocalization

As expected, our results showcased AIM2 and caspase-1 colocalization in both Huh-7.5 cells and NLRP3 K/O Huh-7.5 cells. Once dsDNA is detected by AIM2, the PYD recruits ASC through PYD-PYD interactions. ASC then recruits procaspase-1 through CARD-CARD interactions, creating the AIM2 inflammasome complex. This complex will then cleave procaspase-1 to the active enzyme caspase-1 (reviewed in Sharma & Kanneganti, 2016). This means that if AIM2 inflammasome complex formation is taking place, active caspase-1 should be found in close proximity to the AIM2, which was visualized in both Huh-7.5 cells and NLRP3 K/O Huh-7.5 cell models. Immunofluorescence may not be enough to prove colocalization, so in the future I want to utilize confocal microscopy to provide greater evidence. In regards to only the NLRP3 K/O Huh-7.5 cells, I want to apply the use of a Western blot to determine AIM2 protein expression (I have done this but have yet to acquire concrete results), and use flow to determine caspase-1 production as a means to compare with Huh-7.5 cells.

The "AIM2 colocalization with caspase-1 in HCV-infected Huh-7.5 NLRP3 K/O cells" experiment provided our team with some concerns. In regards to the Huh-7.5 cells, as mentioned: NLRP3 inflammasome complex formation is known to take place during HCV infection. HCV infection contains all the features required to trigger both priming and activation of the NLRP3 inflammasomes in hepatocytes and macrophages (Negash et al., 2019) and JFH-1_T infection has been shown to activate NLRP3 inflammasomes in Huh-7.5 cells (Kofahi et al.,

2016). This means that regardless of AIM2 inflammasome formation, NLRP3 inflammasomes should exist in Huh-7.5 cells after JFH-1_T infection. Although we are not looking for NLRP3 inflammasomes, the caspase-1 probe should detect caspase-1 created via the NLRP3 inflammasome complex and thus not all caspase-1 visualized in this experiment should colocalize with AIM2, but it did. Initially I wondered if the rabbit polyclonal anti-AIM2 (ab93015, Abcam) antibody was binding to NLRP3 (or both NLRP3 and AIM2) but found that to be impossible since the antibody corresponds to amino acids 93-341 of human AIM2, meaning the epitope is located just outside of the PYD (which both AIM2 and NLRP3 have) and mostly within the HIN200 domain (which NLRP3 does not have) (B. Wang & Yin, 2017). Due to the redundancy of AIM2 and NLRP3 (both lead to pyroptosis), it may be possible that, as with *A. fumigatus* infection of bone marrow-derived dendritic cells (BMDCs), AIM2 and NLRP3 were binding together via PYD-PYD interactions and creating a NLRP3-AIM2 heterodimer inflammasome complex (see figure 5.2) (Briard et al., 2021). This would explain why AIM2 was found in close proximity of all caspase-1 formation. Due to lack of NLRP3-AIM2 inflammasome discovery outside of *A. fumigatus* infection, this is unlikely. In regards-to the NLRP3 K/O Huh-7.5 cells, as expected, our team witnessed less caspase-1 production in JFH-1_T infected cells, which likely suggests that all caspase-1 was produced via AIM2 inflammasome formation (though due to the large number of NLRs containing a PYD, and the multiple binding sites located within the PYD (Vajjhala et al., 2014), the existence of other AIM2-NLR inflammasome complexes, ex. AIM2-NLRP1, is open to speculation).

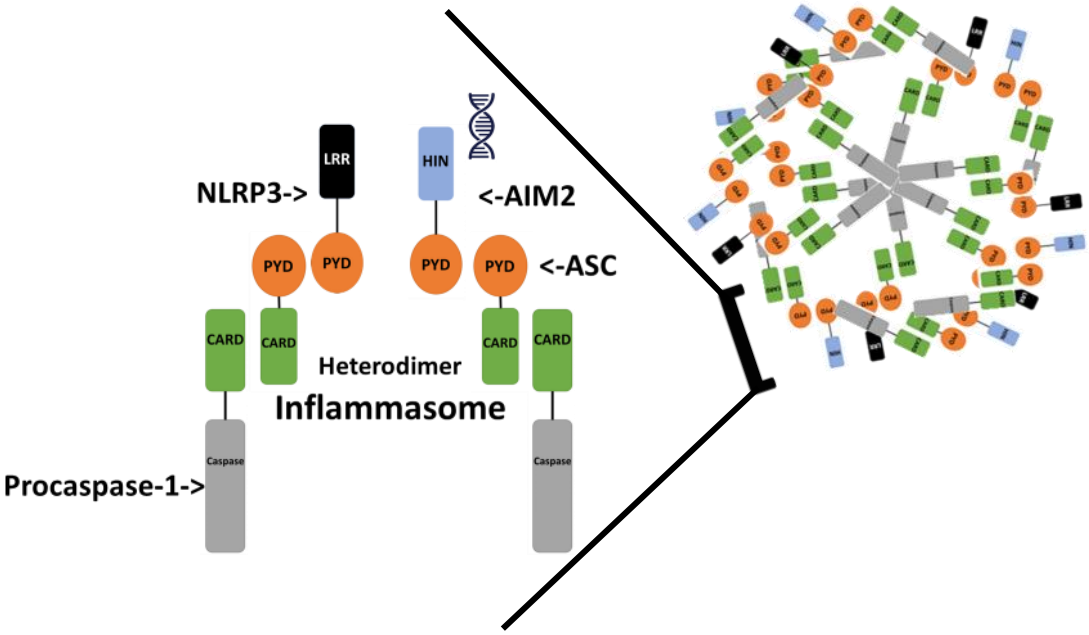


Figure 5.2 Schematic representation of a heterodimer inflammasome

AIM2 is composed of a C-terminal HIN and an N-terminal PYD. AIM2 binds dsDNA at the HIN leading to AIM2 activation. AIM2 binds NLRP3 via PYD-PYD where both PYDs recruit ASC through PYD-PYD interactions. ASC then associates with procaspase-1 through CARD-CARD binding. NLRP3 may be activated first, thus recruiting AIM2 through PYD-PYD binding. This figure was reproduced and adapted to fit HCV viral infection under Creative Commons CC BY 4.0. Briard, B., Malireddi, R. K. S., & Kanneganti, T.-D. (2021). Role of inflammasomes/pyroptosis and PANoptosis during fungal infection. Figure 3: Mechanisms of *A. fumigatus*-induced inflammasome activation. *PLOS Pathogens*, 17(3), e1009358. https://doi.org/10.1371/journal.ppat.1009358

5.8 Positive controls

All experiments in this report require the use of a positive control. Unfortunately, our team attempted to utilize both poly(dA:dT)/LyoVec™ and vaccinia virus for this purpose but neither worked well enough. Poly(dA:dT) is a synthetic analog of B-DNA. LyoVec™ is a transfection reagent capable of delivering poly(dA:dT) to the cytosol. Cytosolic poly(dA:dT) should activate AIM2 and form an inflammasome of 1–2 μM in size (Kesavardhana & Kanneganti, 2017) detectable by immunofluorescence. This did not happen for us. In the future, I would like to try this again but first prime our cells with lipopolysaccharides (LPS), as this has worked for some researchers (Zannetti et al., 2016). Vaccinia virus (VACV) is a member of the family *Poxviridae* with a large double stranded DNA genome of approximately 190 kbp in length (Lorenzo et al., 2000). VACV replicates in the cytosol (Lorenzo et al., 2000) where, since AIM2 detects dsDNA in a non-sequence-specific manner as long as the sequence is at least 80 base pairs long (reviewed in Zheng et al., 2020), the virus should be recognized by AIM2. This did not happen for us either. In the future we may attempt to try cytomegalovirus (Benz et al., 2001), HBV (Le et al., 2021), or creating our own plasmid containing dsDNA. Finally, I would like to try the positive control in HCC cell lines: HePG-2, Huh-7, Bel-7402, HL-7702, and SMMC-7721 which have higher endogenous AIM2 and IFI16 expression, or may help indicate a deficit in the transfection efficiency of Huh-7.5 cells.

5.9 Theories to explain findings

I have developed several theories explaining why and how AIM2 activation is initiated in JFH-1_T infected Huh-7.5 cells. Since HCV has an RNA genome without a DNA intermediate, it is plausible that the AIM2 in our experiment is responding to self-DNA. The self-DNA may be

released to the cytosol: in response to viral proteins, during NLRP3-initiated pyroptosis, or after the initiation of apoptosis.

The production of HCV proteins may lead to release of mtDNA into the cytosol and in turn activate AIM2 inflammasomes. A number of HCV proteins, including core (T. Wang et al., 2010) and NS3/4a (Machida et al., 2006), are thought to bind to, or interact with, the mitochondria leading to ultrastructural abnormalities and oxidative stress. Although HCV core is most notably found amongst the ER or lipid droplets (Boulant et al., 2006), this protein can interact with many different mitochondrial elements such as: the outer membrane (Korenaga et al., 2005), the inner membrane (Chu et al., 2011) and the mitochondria-associated membranes (MAM) fraction of the ER (McLauchlan, 2009). NS3/4a binds the mitochondrial outer membrane (Nomura-Takigawa et al., 2006). These protein interactions can lead to increased mitochondrial ROS production and ER stress causing the transfer of Ca^{2+} from ER to the mitochondria leading to a disruption in electron transport. This makes the mitochondria susceptible to mitochondrial permeability transition (MPT) pore formation resulting in mitochondrial outer membrane permeabilization (MOMP) and the release of mtDNA to the cytosol where the mtDNA can be recognized by AIM2 (reviewed in Wang & Weinman, 2013), (Patrushev et al., 2004). It should be noted that oxidative stress is very common in individuals with HCV and that the severity of mitochondrial dysfunction in the liver correlates with disease severity (reviewed in Wang & Weinman, 2013). Finally damaged mitochondria are removed via mitophagy which may also result in the release of mtDNA (Jing et al., 2020). Both ROS production and Ca^{2+} transport are activation signals for NLRP3 inflammasome production.

NLRP3 inflammasome production may activate AIM2 inflammasomes down stream. As noted, the final step of pyroptosis involves the activation of gasdermin-D (GSDMD), which

produces membrane pores leading to the death of the cell (Downs et al., 2020). Though these membrane pores are often associated with the cell membrane, GSDMD can permeabilize the mitochondria causing the release of mitochondrial contents, including mtDNA, into the cytosol (Rogers et al., 2019). The mtDNA can then bind AIM2 leading to the formation of the AIM2 inflammasome (see figure 5.3). This theory suggests that HCV causes the activation of the NLRP3 inflammasome which indirectly leads to the activation of the AIM2 inflammasome.

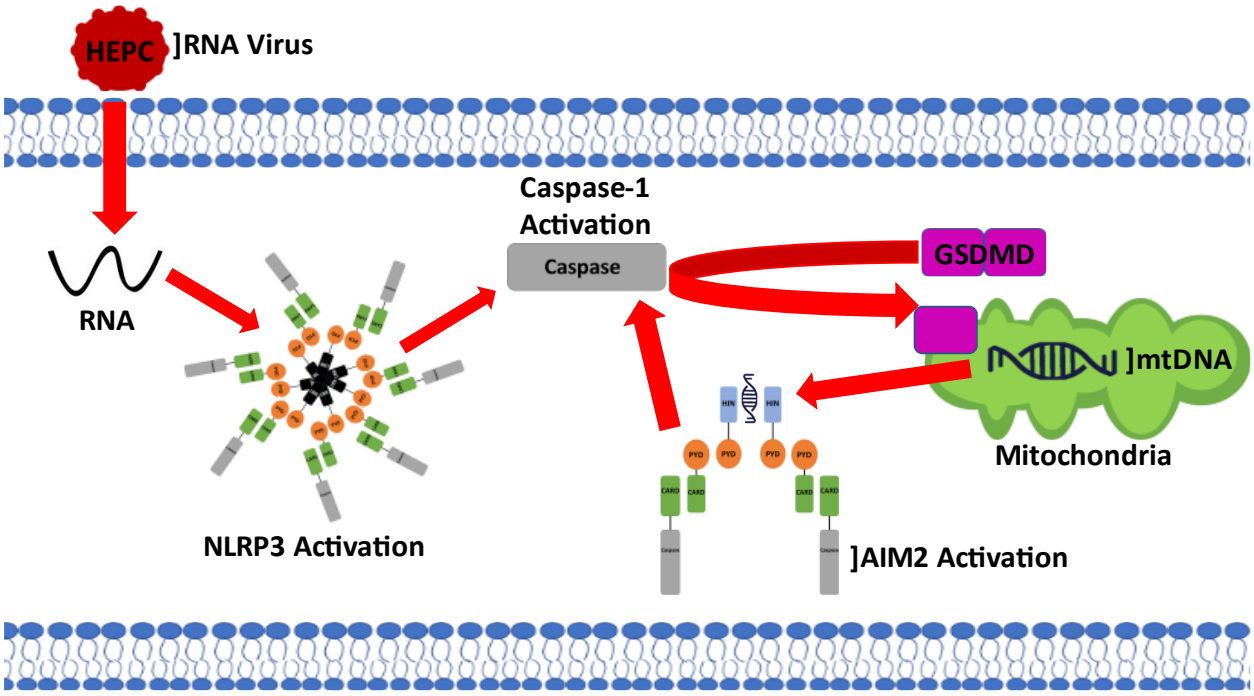


Figure 5.3 Schematic representation of mitochondrial DNA activating the AIM2 inflammasome after NLRP3 activation via HCV RNA

HCV enters a cell and is uncoated to reveal RNA. The RNA activates the NLRP3 inflammasome, which in turn activates caspase-1. Caspase-1 cleaves gasdermin-D which creates a hole in the mitochondrial membrane allowing mitochondrial DNA to enter the cytosol and activate AIM2.

Apoptosis may lead to the activation of the AIM2 inflammasome. As mentioned, the AIM2 inflammasome is activated by cytosolic DNA and I continued to see AIM2 activation and caspase-1 colocalization in NLRP3 K/O JFH-1T-infected Huh-7.5 cells (see figure 4.5). This may mean that NLRP3 inflammasome activation is not a prerequisite for AIM2 activation in JFH-1_T-infected Huh-7.5 cells, as theorized above. Instead JFH-1T-infected Huh-7.5 cells undergoing apoptosis, may indirectly activate the AIM2 inflammasome as this process does not require NLRP3 inflammasome formation.

The initiation of apoptosis may result in nuclear or mitochondrial DNA release, thus triggering AIM2 inflammasome formation. As mentioned, the mitochondrial permeability transition (MPT) pore is opened within the intrinsic pathway, resulting in mitochondrial outer membrane permeabilization (MOMP), leading to the loss of mitochondrial transmembrane potential and may also cause the release of mitochondrial DNA into the cytosol (Rogers et al., 2019), (McArthur et al., 2018). This can be recognized by AIM2. Furthermore, when there are too many dead cells or when the activity of phagocytes is inhibited, the DNA fragments of apoptotic cells will enter the bloodstream (Hu et al., 2021). Since our experiment is *in vitro*, existing without the presence of phagocytes, we may assume that apoptosis results in the presence of DNA in the supernatant. Cell free DNA can be sensed by T cells (Roth et al., 2021), macrophages (Dholakia et al., 2020) and human embryonic cortical neurons (Man et al., 2016) resulting in AIM2 activation. It is unclear if our team's Huh-7.5 cells are sensing DNA in the supernatant, leading to AIM2 inflammasome formation. If the hepatocytes are sensing AIM2, it is unclear by what mechanism. In the future I would like to use PCR to measure DNA in the supernatant after JFH-1_T infection. This theory suggests that self-DNA is recognized by AIM2 in cells undergoing apoptosis.

5.10 Conclusion

In these experiments, I focused on the ability of HCV to engage with proteins traditionally thought to represent elements of the cytosolic DNA response. This involved the expression/ overexpression and/or translocation of proteins which we observed via immunofluorescence and Western blotting. Although there are new direct-acting antiviral agents which allow for eradication of HCV in over 90% of patients (Stasi et al., 2020), liver function may not normalize even after receiving the cure and those with liver cirrhosis remain at an increased risk of developing hepatocellular carcinoma (Soriano et al., 2017), (Lybeck et al., 2019). The proteins I studied in these experiments were specifically chosen in the context of gaining a greater understanding of how JFH-1_T infection in Huh-7.5 cells involves "cross talk" between RNA viral infection and cytosolic DNA recognition. I believe that the discovery of a new inflammasome activated by HCV may provide insight on a PCD which can have an impact on HCV pathogenesis, especially since AIM2 expression in down-regulated in HCV-related cancers (Chen et al., 2017). As explained, I recognize that a large amount of further research is required.

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