HEPATITIS C VIRUS INFECTION CAUSES APOPTOSIS AND PYROPTOSIS IN BOTH INFECTED AND BYSTANDER CELLS

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Abstract

Hepatitis C virus (HCV) infection is a global health challenge affecting 3% of the world's population. Chronically infected individuals are at high risk of developing progressive liver diseases including cirrhosis and hepatocellular carcinoma (HCC). Apoptosis and pyroptosis are two forms of programmed cell death (PCD) that can cause different pathological outcomes. Studying the induction of these forms of PCD by HCV will help in understanding the development of liver complications and might be useful in designing new treatment. We used a tissue culture adapted strain of HCV ($JFH1_T$) to study the effect of infection on the induction of these two forms of PCD in infected and bystander cells. We found that HCV infection reduces the proliferation rate and induces PCD in the infected cell population. Further analysis revealed that two forms of PCD are induced: apoptosis and pyroptosis, as we were able to detect the activation of both caspase-3 and caspase-1 and we confirmed their role in the induction of PCD. NLRP3 inflammasome activation was found to play a role in the induction of pyroptosis. By performing a co-culture assay containing Huh-7.5 cells and HCV-non-permissive cell lines, we were able to detect the induction of both bystander apoptosis and bystander pyroptosis in the non-permissive cells. Bystander apoptosis, but not bystander pyroptosis, was found to require cell-cell contact between the infected and the bystander cell. In summary, we demonstrated that HCV infection can cause apoptosis and pyroptosis, and both of these forms of PCD can be induced in uninfected bystander cells.

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To My Father

Mohammed Kofahi

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AP-1	Activator protein-1
APAF-1	Apoptotic protease activating factor-1
ASC	Apoptosis-associated speck-like protein containing a CARD
BAD	Bcl-2-associated death promoter
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2-associated protein X
Bcl-2	B-cell lymphoma 2
Bcl-2X_L	Bcl-2 like protein X
BID	BH3 interacting-domain death agonist
BIM	bcl-2 interacting mediator of cell death

BIN-1	Bridging integrator 1
bp	base pair
CAD	Caspase-activated DNase
CARD	Caspase activation and recruitment domain
Caspase	Cysteinyl aspartate specific protease
c-FLIP	Cellular FLICE inhibitory protein
CFSE	Carboxyfluorescein succinimidyl ester
CHOP	CCAAT-enhancer-binding protein homologous protein
cLD	Cytosolic lipid droplet
CLDN-1	Claudin-1
CMV	Cytomegalovirus
Cyp A	Cyclophilin A
Cyt C	Cytochrome C
DAMP	Danger associated molecular pattern
DAA	Direct acting antiviral
DD	Death domain

DED	Death effector domain
DENV	Dengue Virus
DFF	DNA fragmentation factor
DMV	Double membranous vesicle
DNA	Deoxyribonucleic acid
DR	Death receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
FADD	Fas-Associated protein with death domain
FLICE	Caspase-8/FADD-like IL-1β-converting enzyme
FFU	Focus forming unit
GFP	Green fluorescent protein
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HCVcc	HCV cell culture

HCVpp	HCV pseudo particles
HIN200	Hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats
HLA	Human leukocyte antigen
HMGB1	High-mobility group box 1
HPgV	Human Pigivirus
HSC	Hepatic stellate cells
hTAF	human TBP–associated factor
HtrA2	High-temperature requirement A2
ICAD	Inhibitor of caspase-activated DNase
IFN	Interferon
IL	Interleukin
IRES	Internal ribosomal entry site
ISG	IFN-stimulated gene
JAK	Janus kinase
KC	Kupffer cell
kDa	Kilodalton

LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LPS	Lipopolysaccharides
LRR	Leucine rich repeat
LVP	Lipo-viro-particle
MAM	Mitochondria-associated membrane
MAVS	Mitochondrial antiviral-signaling protein
MCL-1	Myeloid cell leukemia-1
MEK-1	Mitogen-activated protein kinase (MAPK)/ extracellular signal–regulated kinase (ERK) kinase 1
MLV	Murine leukemia virus
MOI	Multiplicity of infection
MOMP	Mitochondrial outer membrane permeabilization
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

NANB	Non-A, Non-B hepatitis
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLR	Nod-like receptor
NPHV	Non-primate hepacivirus
OCLN	Occludin
ORF	Open reading frame
PAMP	Pathogen associated molecular pattern
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCD	Programmed cell death
PHH	Primary human hepatocytes
PI	Propidiuim iodide
p.i.	Post infection
PKR	Protein kinase R
PRR	Pathogen recognition receptors

PS	Phosphatidyl serine
PUMA	P53 upregulated modulator of apoptosis
PYD	Pyrin domain
RIG-I	Retinoic acid-inducible gene I
RIPK	Receptor interacting protein kinases
RLR	RIG-I-like receptor
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RT/PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SCID	Severe combined immunodeficiency
SR-BI	Scavenger receptor class B type I
ssRNA	Single stranded RNA
STAT	Signal transducers and activators of transcription
SVR	Sustained virological response

TLR	Toll-like receptor
TMD	Trasmembrane domain
TNF	Tumor necrosis factor
TRADD	Tumor necrosis factor receptor type 1-associated death domain
TRIF	TIR-domain-containing adapter-inducing IFN-β
TRAIL	TNF-related apoptosis inducing ligand
TUDCA	Tauroursodeoxycholic acid
uPA	Urokinase plasminogen activator
UPR	Unfolded protein response
UTR	Untranslated regions
VLDL	Very low density lipoprotein
WNV	West Nile Virus

Chapter 1: Introduction

1.1 Overview.

Hepatitis C is a blood-borne disease that is estimated to have originated and started to spread globally more than a 100 years ago [1]. The first use of the term "hepatitis C" goes back to 1974 when it was suggested to describe a post-transfusion hepatitis that could not be classified as either hepatitis B or hepatitis A, both of which were well-known at the time [2]. In later studies, the form of hepatitis that was non-reactive for hepatitis B and hepatitis A was referred to as "Non-A, Non-B hepatitis" (NANB) [3]. Subsequent studies confirmed that the agent responsible for the NANB hepatitis could be transmitted to chimpanzees by transfusion [4,5]. Despite these early findings, the actual virus that causes NANB hepatitis remained a mystery for more than a decade until it was discovered in 1989 and a specific assay for detecting viral antibodies was developed [6,7]. The newly discovered virus was referred to as Hepatitis C virus (HCV).

Hepatitis C is one of the major health challenges in the modern world. It is estimated that 185 million are infected worldwide, which constitutes about 3% of the world's population [8]. High prevalence of HCV infection is found in central and East Asia and North Africa/Middle East with more than 3.5% of that population infected. Lower prevalence is found in the industrialized world including North America [8]. The highest prevalence of HCV infection in the world is found in Egypt with 14.7% of population testing positive for HCV antibodies [9]. Here in Canada, it is estimated that 252,000 are infected with HCV as of 2013 (approximately 1% of the population) with a significant

variation among provinces from as low as 0.13% in Newfoundland and Labrador to as high as 3.9% in the Yukon [10,11]. More than half of these cases were found among former or current drug users, 20% of the cases are infected immigrants and 11% got the infection by contaminated blood products [12].

Acute HCV infection progresses to chronicity in 75%-85% of cases, and the remaining cases spontaneously clear the infection [13]. It is estimated that 5-20% of chronically infected individuals develop cirrhosis in a period of 20-30 years and 1-5% of the chronically infected develop hepatocellular carcinoma (HCC) [14].

Historically, HCV infection was treated with a combination of pegylated interferon-α (IFN-α) and ribavarin. However, the rates of the sustained virological response (SVR) associated with this treatment were low (50% or less) [15]. Furthermore, treatment with IFN results in many side effects that range from relatively mild, such as fever and fatigue, to more severe or even life threatening complications (reviewed in [16]). Fortunately, enormous progress was achieved in the past few years in developing new HCV treatments. Nowadays, a group of direct-acting antiviral agents (DAA) are available. These drugs show very high efficacy (SVR rate of 90-99%) and high tolerability. The currently available drugs are divided into three major categories: protease inhibitors (e.g. Simeprevir), NS5A inhibitors (e.g. Daclatasvir), and polymerase inhibitors (e.g. Sofosbuvir). The use of different regimens including single or combinations of these DAAs is replacing IFN-based treatments.

Despite these advancements in the field, many challenges remain to be overcome in order to effectively cure or control HCV infection and its complications globally. These challenges include but are not limited to, the high cost of the treatment (approximately $60,000-$100,000 in North America), the emergence of DAA resistant mutations, and the fact that curing HCV infection reduces but does not eliminate the risk of development of HCC in these patients [17,18]. As such, research on the many aspects of HCV biology are still required, particularly in terms of pathogenesis and virus-host interactions.

1.2 Hepatitis C Virus.

1.2.1 Classification and diversity.

HCV is a member of the *hepacivirus* genus that is part of the *Flaviviridae* family. The *Flaviridae* family contains four genera: *pestivirus*, *flavivirus*, *pegivirus*, and *Hepacivirus*. Pestiviruses are particularly important for the livestock agricultural industry, because they can cause serious diseases in cattle and pigs [19]. In contrast, flaviviruses are known to cause many human diseases. Some of the well-known human pathogens within this group are West Nile Virus (WNV), Dengue Virus (DENV) and yellow fever virus. Most of the flaviviruses transmit between arthropods and vertebrates. Human pathogens within this family transmit from either a mosquito (e.g. WNV) or a tick (e.g. tick-borne encephalitis virus) to humans [19]. The genus *pegivirus* was recently described [20,21]. This new genus contains Human Pegivirus (HPgV) (formerly known as hepatitis G virus/GB virus-C). This is the most closely related human virus to HCV and it causes a persistent infection in human lymphocytes and NK cells [22-24]. Unlike HCV, HPgV seems to cause relatively mild pathogenicity with clinically important effect only observed in

patients who are co-infected with HIV. In these individuals HPgV infection affects the HIV prognosis significantly [25].

Until recently, HCV was the only known member of the genus *hepacivirus*. However, development of deep sequencing technologies enabled the identification of a large number of hepaciviruses capable of infecting various species of animals [26]. The first non-primate hepacivirus (NPHV) was discovered in dogs [27]. However, in subsequent studies this virus was found to infect mainly horses [28]. More hepaciviruses were reported in later studies and the host range of hepaciviruses has expanded to include: dogs, horses, bats, cows, rodents, humans and non-human primates [29]. Cross-species transmission of hepaciviruses between horses and dogs has been reported before, which has raised the question as to whether HCV has a zoonotic origin [29].

HCV strains are classified into seven genotypes (1-7) and 67 subtypes based on phylogenetic analysis of genome sequences from available HCV sequences [30,31]. Strains that belong to the same genotype have 65-70% sequence identity at the nucleotide level, whereas subtypes have at least 85% sequence identity at the nucleotide level [30]. Genotype 1 is the most common worldwide (46% of all infections) and it is the most prevalent genotype in many regions of the world including North America. The second most prevalent genotype is genotype 3 (22%) and it dominates in South Asia and some parts of Scandinavia. Followed by Genotype 2 (13%), which dominates in West Africa, and Genotype 4 (13%), which dominates in Central and North Africa, including Egypt. Genotypes 5 and 6 have relatively low prevalence (2% and 1%, respectively). Genotype 5 dominates in South Africa and most of the cases of Genotype 6 are found in South East

Asia [32,33]. Only one case of genotype 7 has been reported globally. This case was identified here in Canada where a virus was isolated from an immigrant who came from Central Africa [34,35].

The final level of diversity for HCV occurs within the body of a single infected individual during the course of chronic infection and is termed the quasispecies. The quasispecies is a population of related but genetically distinct variants of virus within the host [36,37]. These variants are generated during genome replication because the viral RNA-dependent RNA polymerase lacks proofreading ability. These variants are continuously selected on the bases of their fitness and by the pressure exerted on them by the immune system [38]. These variants are clustered around a few sequences of highest fitness that are known as master sequences [39,40].

1.2.2 Virus Structure, Genome and proteins.

HCV virions are spherical and 30-65nm in diameter [41-44]. The density of circulating HCV particles varies widely and ranges between 1.06 and 1.25g/ml. The density was found to inversely correlate with the infectivity, with higher infectivity observed for lower density particles [45]. This can be attributed to the association of HCV particles with the lipoproteins in the plasma of infected individual. The virion is composed of a capsid, which is an icosahedral shell made of multiple copies of the same protein, which coat and protect the genome [46]. The capsid and the genome together comprise what is termed the nucleocapsid. HCV is an enveloped virus, i.e. it covers its nucleocapsid with a host cell-derived lipid bilayer, in which the viral envelope glycoproteins are anchored and exposed on the surface.

HCV is a positive-sense single-stranded RNA (ssRNA) virus. The genome of HCV is 9.6kb in length and contains a 3000 amino acid open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs). The 5′ UTR folds and forms a secondary structure that serves as an internal ribosomal entry site (IRES) and allows the binding of ribosomes and the initiation of translation of the ORF in the absence of a 5′ cap [47]. The 3′ UTR does not have a poly-A tail. Instead, it is composed of a variable region, a poly (U/UC) tract and a conserved region (also termed 3′X region) that contains 3 stem-loop structure [48-50]. The conserved region and the poly (U/UC) tract of the 3′ UTR was found to play a crucial role in RNA replication [51-53]. Poly-A tails are known to stimulate the translation of the capped messenger RNAs [54]. It is not clear whether the 3′ UTR plays a similar role in IRES-dependent translation as similar and apposing roles were reported by different groups [55-57]. Finally, the poly (U/UC) tract acts as a pathogen-associated molecular pattern (PAMP) that is recognized by the innate immunity [58,59].

During and after translation, the polyprotein is cleaved by viral and host proteases into 10 proteins: three structural proteins (core, E1, and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The structural proteins and p7 are cleaved by host signal peptidases [60]. Following the cleavage of the signal peptides between the core and E1 proteins, the C-terminus of core is further processed by signal peptide peptidase to cleave the hydrophobic region and release the core protein from the endoplasmic reticulum (ER) allowing it to traffic to cytosolic lipid droplets (cLDs) [61]. The non-structural proteins are cleaved by the activity of two viral proteases. The NS2/NS3 junction is cleaved by the cysteine protease activity of NS2, which requires the

N-terminus of NS3 as a cofactor [62]. The remaining cleavages downstream of NS3 (NS3-4A, NS4A-4B, NS4B-5A and NS5A-5B junctions) are all carried out by NS3 and its cofactor NS4A, which has chemotrypsin-like serine protease activity [63].

Core protein is a highly basic protein and the main component of the nucleocapsid. Core is a dimeric protein that is composed of two domains. The N-terminal domain (D1) contains a large number of positively charged amino acids and is responsible for RNA binding [64]. The C-terminal domain is formed by two amphipathic α-helices and it is important for the association of the core protein to cLDs, an important step in the assembly of the nucleocapsid [65,66]. Besides its main function in nucleocapsid formation, the core protein was reported to have a variety of other roles in the HCV life cycle. In HCV core gene transgenic mouse models, core protein was reported to induce oxidative stress and liver steatosis, which can contribute to the development of HCC [67,68]. Core was also reported to cause insulin resistance that might lead to the development of type 2 diabetes [69,70]. Finally, core protein has been reported to have both pro-apoptotic and/or anti-apoptotic effects. The details of these contradicting results will be discussed in more detail in section 1.6.

E1 and E2 are transmembrane glycoproteins that form the viral envelope. Both of these glycoproteins are composed of two domains: an N-terminal ectodomain and a conserved C-terminal transmembrane domain (TMD) that anchors these glycoproteins into the ER membrane [71,72]. During the post-translational modification of E1 and E2, they are heavily glycosylated. This glycosylation is important for many functions, i.e, it helps in properly folding the protein, and facilitates escape from the adaptive immune

responses [73]. E1 and E2 form a stable noncovalent heterodimeric complex that lies on the surface of the virion [74]. The main function of the E1 and E2 glycoproteins is to form a complex that is able to interact with the cellular receptors on the surface of target cells and mediate fusion with the cell membrane, which results in the entry of the virion into its target cell [75-77]. The cellular receptors for HCV will be discussed in section 1.2.3. E2 has been better characterized than E1, and it was found to be responsible for cell receptor binding, and it is the target of neutralizing antibodies [78]. The exact functions of E1 are poorly understood; however, E1 has been reported to be important for the fusion of viral and cellular membranes [79,80].

The p7 protein is a small protein that is composed of two trans-membrane domains connected by a short conserved loop [81,82]. p7 is not necessary for RNA replication but is essential for the infectivity of HCV and the production of infectious virus particles [83,84]. p7 homo-oligomerizes in artificial membranes to form hexa- or hepata-oligomers that can act as ion channels, which led to the classification of p7 as a member of the viroporin family of proteins [85-87]. In a recent study conducted in the Russell lab, p7 was shown to protect E2 from premature degradation during virus production [88].

NS2 is a 24kDa protein that is composed of two domains: the N-terminal membrane-binding domain, which contains three transmembrane segments, and a C-terminal globular cytosolic domain, which dimerizes to form the active site of the cysteine protease [89,90]. Besides its protease activity, NS2 is believed to play an important role in orchestrating the assembly of the new virions. This is achieved by interacting with and bringing together E1-E2 and p7, as well as NS3, which is part of the replicase complex

[91-94]. It was also shown that an interaction between NS2 and NS3-4A is essential for recruiting the core protein from cLDs to the site of assembly [95].

The NS3 protein contains two functional domains, a protease and a helicase domain [96]. It forms a heterodimer with NS4A that catalyzes the cleavage of most of the non-structural proteins, as described above. The protease activity of NS3-4A is also important for suppressing the innate immune response to HCV. NS3-4A blocks the retinoic acid-inducible gene I (RIG-I) signaling pathway by cleaving the Mitochondrial antiviral-signaling protein (MAVS), which abolishes IFN-β expression [97-99]. TIR-domain-containing adapter-inducing IFN-β (TRIF) is a second adaptor protein that is also a victim of cleavage by NS3-4A resulting in blocking toll-like receptor (TLR)-3 signaling [100]. The role of NS3-4A in evading innate immune responses will be discussed further in section 1.4.1. The helicase domain of NS3 is important for hydrolyzing ATP and the resultant energy is used to unwind the viral RNA [101-103].

NS4B is a membrane integral protein that is composed of two N-terminal amphipathic helices, a highly hydrophobic central core domain that contains four putative transmembrane segments, and a highly conserved C-terminal domain [104,105]. Expression of NS4B and the oligomerization of the NS4B molecules were found to cause rearrangement of ER membranes [106,107]. These results led to the belief that NS4B is solely responsible for the formation of the membranous web, the membranous structure in which replication takes place. However, more recent studies have shown that the expression of another non-structural protein (NS5A) is necessary for the formation of the double membrane vesicles (DMV), which are a constituent of the membranous web,

while only single membrane vesicles result from the expression of NS4B alone [108]. As such, it is now widely accepted that the formation of the membranous web requires the expression of multiple non-structural proteins.

NS5A is a proline-rich phosphoprotein that is essential for RNA replication and virus assembly [109,110]. It was found to be capable of binding the 3' end of the positive and negative strands of HCV RNA [111,112]. NS5A is comprised of an amphipathic helix at its N-terminus that is responsible for its interaction with the membranes, as well as three functional domains (DI, DII, and DIII) [113,114]. The structure of the first domain (DI) is well characterized, and it contains a motif that is capable of coordinating a zinc atom, which is essential for NS5A function within the replicase complex [114]. The structure of the other two domains is still not fully elucidated [115]. Nevertheless, there is strong evidence that the assembly function of NS5A is carried out mainly by DIII [116-118]. As discussed above, NS5A is the main viral protein that is able to induce the formation of DMVs [108]. NS5A was reported to achieve this by interacting with Phosphatidyl-inositol-4-kinase IIIα (PI4KIIIα), which activates a pathway that ultimately results in cholesterol enrichment of the membranous web [119]. Furthermore, NS5A was found to interact with cyclophilin A (Cyp A) and this interaction is believed to be essential for HCV replication [120]. Inhibiting the interaction between NS5A and Cyp A pharmacologically was found to block *de novo* formation of DMVs [121].

NS5B is an RNA-dependent RNA polymerase (RdRp) that catalyses the replication of the HCV RNA. This replication takes place by first using the HCV genome to synthesize a complementary negative strand, which is subsequently used as a template for the

synthesis of additional positive strands. NS5B lacks proofreading ability and therefore is associated with a very high mutation rate of approximately 10^{-4} substitutions per site [122]. This lack of proofreading activity, in combination with the high rate of virus production, generates the viral quasispecies (discussed above), which contributes to virus persistence and helps establish chronic infection despite the presence of strong adaptive immune responses [123]. The NS5B protein is anchored through its C-terminus to ER-derived membranes [124]. The crystal structure of the NS5B catalytic domain has been reported by several groups and was found to contain several features that are shared by other known RdRps including a GDD motif in the active site, and fingers, palm and thumb sub-domains [125-127].

1.2.3 Life cycle.

The target cells for HCV are the hepatocytes. Nevertheless, the ability of the virus to cause extrahepatic infections in immune cells and central nervous system cells has been reported by some groups [128-132]. The target cells are determined largely by E2 and E1, and their ability to interact with receptors on the surface of susceptible cells. The low-density lipoprotein (LDL) receptor was believed to be a good candidate for the initial attachment of the virus to its target cells due to the fact that HCV circulates in the blood of infected patients as a lipo-viro-particle (LVP), in which the virus is associated with the host LDL or very low density lipoprotein (VLDL) [133,134]. However, it was reported later that this receptor is not essential for infectious particle entry and it leads, at least in some cases, to non-productive entry that results in the degradation of the virus particle [135].

To date, four receptors for HCV have been identified. The most important among them is CD81, which is a member of the tetraspanin family, a family of cell surface proteins with four transmembrane domains and two extracellular loops [136]. CD81 is expressed on the surface of hepatocytes, as well as many other cell types, and can bind to E2 [137]. Scavenger receptor class B type I (SR-BI) is an additional receptor for HCV that can also bind E2 and is necessary for HCV entry [138]. Later, two more receptors were found to be necessary for HCV entry, these are the tight junction components claudin-1 (CLDN-1) and occludin (OCLN) [139,140]. Interestingly, it was recently reported that the VLDL receptor may also serve as an HCV receptor, as it can mediate entry of HCV independent from the canonical CD81 pathway [141].

HCV entry starts with the interaction of the circulating lipoprotein-associated virus particles with surface receptors glycosaminoglycans (GAGs), SR-BI, CD81 and then, upon relocalization to tight junctions, with CLDN-1 (Fig. 1.1) [142]. The exact role of OCLN is not known but it also believed to work at late steps of entry [143]. Next, HCV is internalized by clatherin-dependent endocytosis, which internalizes the virus particle into an endosome [144]. The low pH inside the endosome stimulates the next step of the virus life cycle, which is fusion between the viral and endosomal membranes (Fig. 1.1) [77,145,146]. The exact mechanism for the fusion process is not fully known. However, according to the published data describing the structure of E2, it is unlikely that E2 is involved in the fusion process because it lacks any structural hallmarks of fusion proteins [137,147]. This fact, and the available computational analysis model for E1, has led to the suggestion that E1 alone or in combination with E2 is responsible for the fusion process

[79,80,148]. However, the available structural data for E1 contradicts this suggestion, but it is too limited to exclude the role of E1 in this process [149]. Membrane fusion results in the release of the nucleocapsid into the cytosol followed by uncoating, which releases the viral genome into the cytosol. Uncoating is the least understood step in HCV life cycle.

Upon uncoating, the viral RNA is directly translated into the viral polyprotein that in turn is processed to generate the ten mature viral proteins (Fig. 1.1). Then, the non-structural proteins (mainly NS4B and NS5A) alter the ER membranes to form the membranous web. Inside the membranous web, membrane-associated replication complexes form and the process of RNA replication takes place.

Virus assembly is a very complex and poorly understood process. This process requires three different components: the core protein, envelope glycoproteins (E1 and E2) and the viral RNA. cLDs play a crucial role in assembly as newly synthesised core proteins are recruited to their surface [150,151]. It is assumed that sequesteration of core protein on the surface of cLDs prevents core from competing with the replicase proteins for the viral RNA [152]. NS5A is also recruited to the cLD and interacts with the core protein by the function of its DIII [116]. This interaction is crucial for virus particle production [153]. In infected cells, a large number of cLD are found in a close proximity to the membranes of the membranous web, and this is believed to create a suitable microenvironment for assembly [151].

Besides core protein and NS5A, many other viral proteins have been reported to play a role in the assembly process. As mentioned previously, NS2 plays a central role in the

process. The NS3 helicase domain and the linker region are also important for the assembly, but it is not known whether the enzymatic activity of the helicase domain is required [154,155]. NS4B and NS5B have been also reported to contribute in the virus assembly [156,157]. According to the available complex data, it is hypothesized that the viral RNA is shuttled from the replicase site to the assembly space between the cLD and the ER membrane by NS3 and NS5A. Core proteins travel from the cLD membrane to the ER membrane, which contains E1 and E2 proteins. Then, core protein, in combination with the RNA, build the nucleocapsid while budding into the ER lumen [152,158]. Accumulating evidence suggests that the assembly process is tightly linked to the VLDL pathway [159-161]. Newly formed virions are then transported via the secretory pathway to the Golgi apparatus, were E1 and E2 undergo further modifications, before the virus particle is released through budding from the cell membrane (Fig. 1.1) [162,163].

1.3 Studying hepatitis C virus.

1.3.1 Cell culture models.

After its discovery in 1989, the ability to study HCV was hampered by the lack of effective cell culture systems in which the virus could undergo its entire life cycle. Early studies reported success in infection of immortalized cell lines and primary hepatocytes from

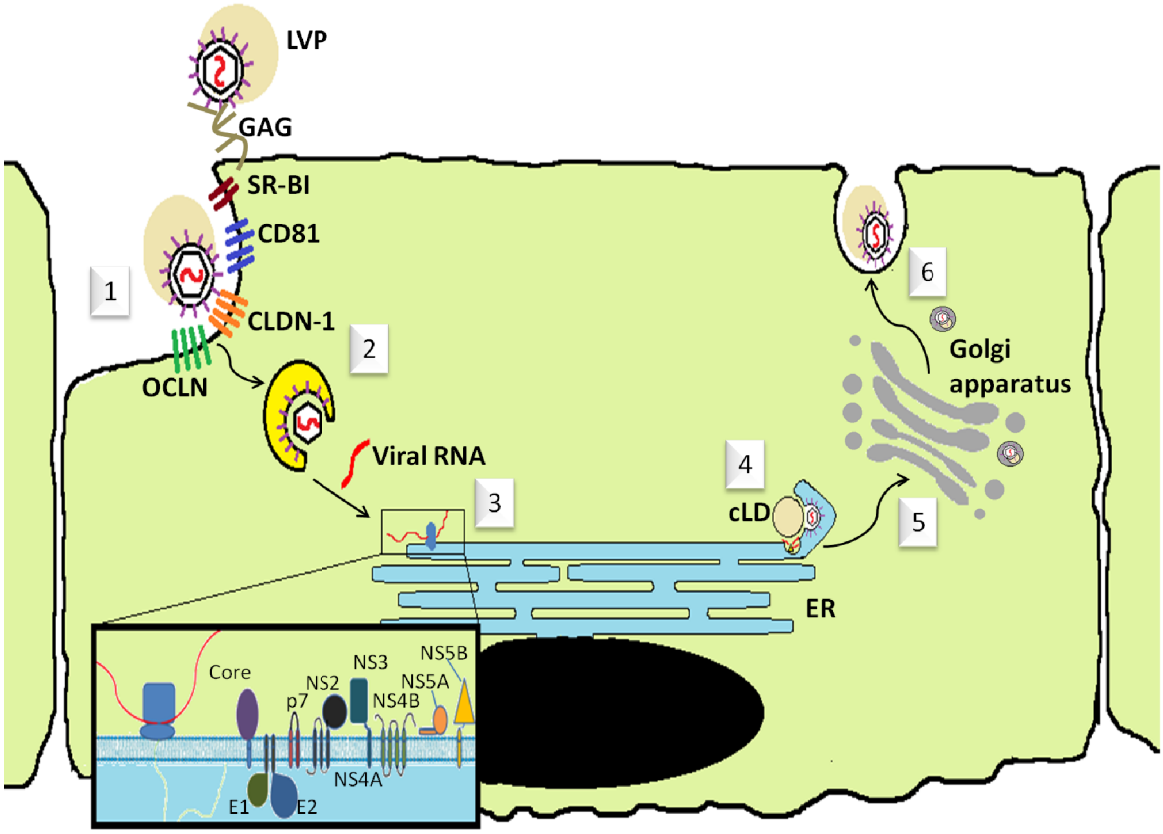


Figure 1.1 HCV life cycle.

1. HCV entry is mediated by the interaction of the LVP with a group of receptors including GAGs, CD81, SR-BI, CLDN-1 and OCLN. **2**. HCV particles are then internalized by clatherin-dependent endocytosis followed by the the fusion between the viral and endosomal membranes and the uncoating of the viral RNA. **3**. HCV RNA is then translated directly into the polyprotein, which is cleaved to generate the ten viral proteins. **4**. The non-structural proteins modify ER membranes to form the membranous web where viral RNA replication and the assembly of the new virions occurs. **5**, **6**. Progeny virions are transported via the secretory pathway to the Golgi apparatus and are released at the cell membrane.

human and chimpanzee by direct inoculation of serum from HCV infected patients [164-170]. However, the replication reported by these studies was poor, variable, and hard to reproduce. Furthermore, viral proteins could not be detected using available assays, and viral RNA was only detectable by reverse transcription polymerase chain reaction (RT/PCR) [171,172]. This prompted the researcher community to design alternative systems to study certain parts of the HCV life cycle or even simply one or more viral proteins independently.

The first breakthrough in this field was the development of the subgenomic replicon system by Lohmann *et al*. in 1999 [83]. This replicon system was generated by transfecting the human hepatoma cell line Huh-7, with a bicistronic RNA construct composed of an HCV IRES followed by a selection gene (neomycin phosphotransferase), followed by a second cistrone containing the genomic region comprising the HCV non-structural proteins NS3-NS5B as well as the viral 3' UTR driven by an Encephalomyocarditis virus IRES. Drug selection for cells carrying the viral replicon RNA generated cell lines that were able to persistently replicate the replicon RNA. The replication efficiency of the first subgenomic HCV replicon was low and limited to a single strain of HCV, the genotype 1b Con-1 strain isolate. However, more efficient replicons and several other genotypes were later developed (reviewed in [173,174]). The main advantage provided by this system is the availability of different clones that encompass a wide range of HCV genotypes. The main disadvantage of this system is that it only allows the study of non-structural protein processing and RNA replication.

However, subgenomic replicons provided a significant contribution to the study of HCV and played an important role in the discovery of direct-acting antivirals.

Studying the entry step continued to be a challenge because replicon systems were not able to recapitulate it. The first major breakthrough in studying this step was the development of HCV pseudoparticles (HCVpp) in 2003 [175,176]. HCVpp are generated by co-transfecting 293T cells with two or three plasmid combinations that include the HCV E1 and E2 genes, retroviral *gag-pol* genes from human immunodeficiency virus (HIV) or murine leukemia virus (MLV), and a reporter gene (Green fluorescent protein gene (*GFP)* or Luciferase gene). The transfected cells produce HCVpp, which are composed of HCV envelope glycoproteins and an HIV or MLV nucleocapsid and contains a reporter gene. These particles can be used to infect permissive cells, such as human hepatoma cells (i.e. the Huh-7 cell line and derivatives thereof). Since the entry step is mediated by HCV envelope proteins, and the infected cells can be easily identified by the expression of the reporter gene, this system provides a useful tool to study HCV entry [173,174]. The HCVpp system is also the main tool for studying neutralizing antibodies [177,178]. An HCVpp library, which is composed of 19 distinct variants of genotype 1 has been used to test for broadly neutralizing antibodies [179]. The main drawback of using this system is the fact that these particles are produced in 293T cells, which is a kidney cell line. This can result in differences in the glycosylation of the envelope proteins in HCVpp compared to the natural virus. Furthermore, since 293T cells are unable to produce lipoproteins, the resulting HCVpp are not associated with the lipoproteins in the same way HCV particles are under physiologic conditions.

In 2005, Wakita *et al*. described the first fully infectious HCV cell culture (HCVcc) system [41]. They used the full-length RNA of the JFH1 strain, which is a genotype 2a strain isolated from a Japanese patient with fulminant hepatitis [180]. When they transfected Huh-7 cells with this full-length RNA, virus particles were produced and were able to infect naïve cells, recapitulating the entire HCV life cycle in cell culture. The drawback in using this strain was the low titer of virus obtained. However, this system provided the basis for future optimization.

Several approaches were used to improve the HCVcc system and to increase viral titers in cell culture. Firstly, infecting Huh-7.5 cell line, which is a cured clone of Huh-7 cells harboring subgenomic HCV replicon, improved the titer, probably because of the defective RIG-I and higher levels of CD81 in Huh-7.5 cells [181-184]. Another group generated a chimeric virus composed of core-NS2 from the HCV J6 strain, which is also a genotype 2a strain, and NS3-NS5B from JFH1. This chimeric virus generated significantly higher titers than the wild-type JFH1 [181]. Additional intragenotypic and intergenotypic chimeras were generated later by Pietschmann and his colleagues [185]. To generate these chimeras they used a fusion point just after the first transmembrane domain of NS2. This allowed them to produce a more robust chimera of J6-JFH1, which became known as Jc-1. In addition, they were able to generate intergenotypic chimeras representing genotypes 1a, 1b, 2a, and 3b. However, with the exception of Jc-1, which is an intragenotypic chimera, these other chimeras all generated lower titers than wild-type JFH1. In another study, passaging the virus for three weeks in Huh-7.5.1 cells (a

derivative of Huh-7.5 cells) resulted in the appearance of cell culture-adapted variants that gave a 200-fold increase in titer compared to JFH-1 [186].

In 2008, Russell *et al*. reported three cell culture-adapted mutations in E2, p7, and NS2 (N417S, N765D and Q1012R, respectively). The adapted strain containing these three mutations, later called $JFH1_T$, was reported to generate 1000-fold more infectious virus than wild-type JFH1 [187]. Since the replication rate of JFH1 is much lower than the levels observed in HCV-infected individuals, $JFH1_T$ may better represent natural HCV infection. For this reason, we use this strain to model for HCV infection herein.

The latest progress in the field was published recently by Saeed *et al.* in which they reported the development of the first pan-genotypic cell culture system that allowed replication of different genotypes of virus including isolates from patient sera [188]. This was achieved by generating Huh-7.5 cells that express SEC14L2, which is suggested to sustain HCV replication by enhancing a vitamin E-mediated protection against lipid peroxidation.

1.3.2 Animal Models.

Chimpanzees can be infected with HCV and it was the first animal model to be used for studying this virus. The use of this model began about ten years before the discovery of HCV itself to confirm that NANB hepatitis could be transmitted to chimpanzees upon inoculation of serum from an infected human [4,5]. Since then, the use of this animal model added great contributions to the field, which began with the discovery of the virus, and continued with the understanding of many aspects regarding HCV biology, including

the pathogenic mechanisms, the cellular immune responses against the virus, as well as antiviral and vaccine efficacy studies [189-192]. However, unlike the natural course of infection in humans, the majority of infected chimpanzees rapidly cleared the infection, and even in the few that develop chronic infection, no signs of fibrosis have been observed and only one case of HCC has been reported [192,193]. Although the use of chimpanzees in HCV research was invaluable, these studies were limited due to the high cost and limited availability of facilities able to carry out this research. In 2013, the use of chimpanzee in research was banned due to ethical issues, with chimpanzee studies now limited to vaccine trials in most countries [173].

Interestingly, besides humans and chimpanzees, the tree shrew (*Tupaia belangeri*) was found to be the only non-primate that is permissible for HCV infection [194]. Infection in tree shrews results in intermittent viremia during the acute phase, and evidence of chronic infection was observed and long-term histological analysis showed the development of HCV-induced liver disease in HCV-infected animals [195,196]. Despite these attractive features of this model, the use of tree shrew in HCV research is still very limited, probably due to the low and variable infection rates and the difficulties in breeding this animal in captivity [173,191,193].

In contrast, the mouse is a very attractive animal model for virological research. However, its use in HCV research was hampered by the fact that mice are not permissive to HCV infection. In order to overcome this barrier, three approaches were followed to modify either the virus itself or the mouse in a way that supports infection (reviewed in [192,193]). The first approach was to allow the virus to adapt to mouse CD81 and mouse

OCLN, both of which cannot be utilized by HCV envelope proteins for entry [140]. The adaptation to mouse CD81 was achieved by passaging the virus for extended periods in the presence of mouse CD81 [197]. This adapted virus was reported to replicate and produce infectious particles in a mouse liver cell line defective in innate immune pathways [198].

The second approach was to genetically manipulate the mouse in order to express human receptors such as CD81 and OCLN (humanized mouse model). This was achieved either by adenovirus delivery or by transgenic expression of the receptors. The mouse expressing human CD81 and OCLN was found to support HCV entry [199]. In a later report, the same group showed that blunting the innate antiviral response of transgenic mice expressing the four human HCV receptors (CD81, SR-B1, CLDN-1 and OCLN) supported virus production as viremia was detected in this mouse for several weeks, meaning the entire HCV life cycle was recapitulated in this model [200].

The third approach was to directly humanize the mouse liver by xenotransplanting human hepatocytes. In this model, severely immunosuppressed mice (e.g. Severe combined immunodeficiency [SCID] mice) were used to prevent the rejection of human liver cells by the mouse immune system [201]. Primary human hepatocytes (PHH) were injected intrasplenically where they will engraft in the liver and repopulate most of the mouse liver [202]. Cell injury was induced in the murine hepatocytes to provide a proliferation signal to the engrafted human hepatocytes and to give them a growth advantage over the murine hepatocytes [173]. The most commonly used way to induce cell injury in murine hepatocytes is by the transgenic expression of urokinase

plasminogen activator (uPA) [203,204]. The human liver chimeric mouse model contributed to better understanding of many aspects of HCV including the neutralizing antibodies and testing new antiviral drugs [205-209]. The main limitation of this model is the absence of an adaptive immune responses in the host. To overcome this limitation, an immunocompetent mouse model was recently developed by double humanizing the mouse with human immune progenitor cells (e.g. CD34+ human hematopoietic stem cells) and human hepatocyte progenitors [210-212].

1.4 HCV immunology.

1.4.1 Innate immune responses against HCV.

The induction of type I and type III interferons (IFNs) is one of the first defence mechanisms that can restrict viral infection. Type I IFN includes IFN-β, which is encoded by a single gene, and IFN-α, which is encoded by a cluster of 13 genes. Type III IFNs include three subtypes of IFN-λ: IFN-λ1, IFN-λ2, and IFN-λ3 (also known as IL-29, IL-28A, and IL-28B, respectively) [213,214]. Type I and Type III IFNs binds to different receptors on target cells, but they are believed to induce the same intracellular signaling pathway and stimulate the expression of the same genes [215,216].

In order for the IFN response to be initiated, the virus must be recognized by at least one of a group of receptors, known as the pathogen recognition receptors (PRRs). These receptors recognize specific characteristics of the virus, i.e., the PAMPs. The PRRs are classified into three classes, RIG-I-like receptors (RLRs), toll-like receptors (TLRs) and Nod-like receptors (NLRs).

RIG-I is the main PRR that recognizes HCV [182,217]. It is an RNA helicase that is composed of three domains: a C-terminal regulatory domain, a DExD/H box type RNA helicase domain in the centre and two tandem caspase activation and recruitment domains (CARDs) in the N-terminus [59,218-221]. The C-terminal regulatory domain and the RNA helicase domain are involved in the detection of viral RNA [221]. The ligands recognized by RIG-I are the cytosolic 5' triphosphate containing dsRNA and the 3' UTR that is rich in poly-U/UC [217,221,222]. Binding of HCV RNA to RIG-I induces conformational changes in RIG-I that leads to oligomerization and translocation of RIG-I from the cytosol into intracellular membranes, which results in the interaction of the activated RIG-I, through its CARD domains, with an adaptor protein called MAVS located on the mitochondria, peroxisomes and mitochondria-associated membranes (MAMs) of the endoplasmic reticulum [223-226]. This process requires the interaction of RIG-I with two proteins, the chaperone protein 14-3-3ε and the E3 ubiquitin ligase TRIM25 [227,228]. TRIM25 in turn requires another ubiquitin ligase, Riplet, to be able to associate with and activate RIG-I [229]. Interaction between RIG-I and MAVS results in the activation of a downstream cascade that results in the activation of two transcription factors: IFN regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Once activated by phosphorylation and dimerization, IRF3 is translocated to the nucleus where it plays a major role in activating the IFN-β promoter (reviewed in [230-232]).

TLR3 is also able to sense HCV, but its exact role in the immunity against it is still not clear. TLR3 is an intraendosomal sensor that is composed of an ectodomain that

recognizes the RNA ligand, connected by a transmembrane region to a Toll/IL-1 receptor (TIR) domain, which is responsible for initiating the downstream signaling [233,234]. The HCV PAMP that is recognized by TLR3 is the dsRNA intermediate that accumulates in the cell during replication [235]. Binding of TLR3 to its ligand results in the dimerization of the TIR domains. This in turn recruits the adaptor protein TRIF and results in the activation of IRF-3 and NF-κB, which subsequently induces the expression of IFN-β (reviewed in [231,232,236]).

Once expressed, IFN-β will bind to its cognate receptor in an autocrine and paracrine manner. This leads to the activation of a Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway, which subsequently results in the expression of hundreds of genes collectively known as IFN-stimulated genes (ISGs). One of the known ISGs is IRF7, which is a transcription factor for IFN-α promoters, thus results in more type-I IFN production and the positive feedback amplification of the response [237,238]. Other ISGs encode several proteins with known antiviral activities, which restrict HCV accumulation and infection of neighbouring cells. In contrast, other ISGs, such as ISG15, have been reported to promote HCV activity [239].

Interestingly, Protein kinase R (PKR), a protein known for its antiviral functions by inhibiting host mRNA translation, was identified later as a third PRR for HCV [240]. However, unlike the other two PRRs, PKR was reported to induce a pathway that supports HCV infection. Binding of PKR to dsRNA was found to initiate, in a kinase-independent way, a signaling pathway that involves MAVS and IRF3, but not RIG-I. This pathway results in the rapid induction of 49 genes, including ISG15. ISG15 inhibits RIG-I

at early stages of infection by blocking its TRIM25-mediated ubiquitination, thus preventing the induction of IFN-β [240].

Despite the early activation of the IFN response following HCV infection, this innate immune response fails to clear the majority of new infections [241,242]. This failure is the result of the development of multiple strategies by the virus to evade the innate immune responses. Different evasion strategies target each of the three steps of the IFN response by disrupting PRR signaling, inhibiting JAK/STAT signaling, and antagonizing the antiviral functions of the ISGs (reviewed in [59]).

The best-characterized and most important strategy followed by HCV to evade the induction of the IFN response is the cleavage of MAVS by NS3-4A protease activity. MAVS molecules are anchored in membranes and the cleavage of MAVS liberates them, resulting in a block in the RIG-I signaling pathway [99,243]. The HCV NS3-4A protein targets MAVS located on MAMs, which is sufficient to block RIG-I signaling, regardless of intact MAVS located on the mitochondrial membrane [225]. More recently, Riplet was reported to be a second cleavage victim of NS3-4A within the RIG-I pathway, cleavage of which abrogates the association between RIG-I and TRIM25 and inhibits the signaling pathway [229]. Furthermore, NS3-4A protease activity is also able to attenuate TLR3 signaling by cleaving TRIF [100]. Since MAVS is also involved in PKR signaling, its cleavage by NS3-4A is also expected to inhibit this pathway [232].

JAK/STAT signaling was also reported to be blocked by HCV. Core protein was reported to have a central role by inhibiting this pathway at different steps (reviewed in

[59,244]). Other HCV proteins have been reported to antagonize additional ISGs. For example, NS5A was reported to antagonize the antiviral function of 2′–5′ oligoadenylate synthetase (2–5 OAS), an ISG product that induces the degradation of viral RNA [245,246]. The kinase activity of PKR was reported to be inhibited by two viral proteins, E2 and NS5A [247,248]. This prevents PKR's inhibitory effects on cellular mRNA translation, thus reducing the cellular factors necessary for virus replication. However, the exact effect of inhibiting the kinase activity of PKR on HCV infection is not clear.

Natural killer (NK) cells form another important arm of innate immunity against viral infections. NK cells restrict viral infection by performing two important functions: killing virally infected cells and secreting cytokines (such as IFN-γ) that can control viral infection. Activation of NK cells is controlled by a balance between activating and inhibitory signals that result from the interactions between a variety of NK cell receptors and their ligands on the surface of target cells [249]. NK cells are enriched in the liver, accounting for 25%-40% of the lymphocytes present [250]. This has led many to believe that NK cells play a pivotal role in controlling HCV infection prior to the onset of adaptive immune responses. In line with this, NK cells are activated during acute HCV infection [251,252]. An epidemiological study reported that a specific NK cell receptor-ligand combination is associated with the clearance of viral infection [253]. Furthermore, a strong line of evidence suggests that progress of HCV infection to chronicity is associated with changes in the NK cell subsets and receptor expression [254-258]. The interaction between CD81 on NK cells and the viral E2 protein was reported to inhibit NK cell functions [259,260]. In agreement with this, a recent publication from the Grant

lab showed that co-culturing NK cells with HCVcc-infected Huh-7.5 cells resulted in a downregulation of NKp30, an activating receptor of the natural cytotoxicity receptor family, and resulted in inhibiting NK cell cytotoxicity and cytokine secretion [261].

1.4.2 Adaptive immune responses against HCV.

Unlike innate immune responses, the adaptive immune response to HCV is active late after the primary infection, at approximately 6-8 weeks post-infection [262,263]. All of the components of the adaptive immune response, including both humoral and cellular responses (CD8+ and CD4+ T cells), have been shown to play a central role in determining the outcome of the infection and participate in HCV-pathogenesis [264,265].

In most of the cases, acute HCV infection induces the production of antibodies directed against different epitopes located within the structural or the non-structural viral proteins [263]. Some of these antibodies have the ability to prevent virus binding, entry, or uncoating and are called neutralizing antibodies. For example, antibodies directed against the hypervariable region-1 (HVR-1) of the E2 protein have been found to be neutralizing [266,267]. However, the role of the neutralizing antibodies in determining the outcome of HCV infection is not clear as controversial findings have been reported by several groups. Some studies reported that the generation of neutralizing antibody is not associated with spontaneous clearance of HCV in chimpanzees and humans [268,269]. In contrast, a cohort study of a single source outbreak reported an association between early induction of neutralizing antibodies and viral clearance [270]. Moreover, a recent study reported that spontaneous clearance of viral infection is associated with the rapid development of a broadly neutralizing antibody response [179]. During chronic infection,

neutralizing antibodies apply a selection pressure on the existing HCV infection causing continuous generation of escape variants [177].

Unlike the humoral immune response, the important role of both CD8+ and CD4+ T cell responses in controlling HCV infection is well established. Antibody-mediated depletion of CD8+ T cells in a previously protected chimpanzee resulted in a persistent infection until the recovery of HCV-specific CD8+ T cells in the liver [271]. Similarly, antibody depletion of CD4+ T cells in a previously protected chimpanzee was also found to cause persistent infection [272]. Several other studies have shown a correlation between HCV-specific CD8+ T cell responses and viral clearance in humans [273-276]. Other groups also reported the importance of the CD4+ T cell response in controlling HCV infection. A strong, broad, and persistent CD4+ T cell response was found to associate with the resolution of the infection [277-280]. Furthermore, certain human leukocyte antigen (HLA) class I and class II alleles were found to associate with virus clearance, thereby emphasizing the roles of CD8+ and CD4+ T cells in controlling HCV infection [281].

1.5 Programmed cell death.

Historically, the term programmed cell death (PCD) was first used in 1965 to describe the organized death of certain larva muscle cells during the process of transformation into moths [282]. In 1972, the term apoptosis was first proposed by Kerr *et al*. to describe a morphologically distinct form of cell death [283]. Afterwards, cell demise was divided into two major forms: necrosis and apoptosis. Apoptosis was described as a programmed, energy dependent cell death, while necrosis was considered as an accidental, passive, and

unwanted form of cell death. However, under certain conditions, necrosis was found to be induced in a well-orchestrated manner as a backup mechanism for apoptosis [284,285]. This led many to reclassify necrosis as an additional form of programmed cell death (PCD). In addition, other forms of programmed cell death were characterized later, increasing the number of PCD pathways. In this section, the major forms of PCD will be described, with an emphasis on apoptosis and pyroptosis, the two forms of PCD related to the findings described herein.

1.5.1 Apoptosis.

Since its discovery, apoptosis has gained a lot of interest from different biological and medical fields. The induction of apoptosis was found to be an integral part of an enormous number of vital biological processes including fetal development, haemostasis, regulation of the immune system and defence against intracellular pathogens. Defects in this process lead to serious complications including autoimmunity and cancer [286].

1.5.1.1 Hallmarks of apoptosis.

In tissues, apoptosis usually involves single cells or small clusters of cells [287]. One of the earliest morphological features of apoptosis is the compaction and segregation of nuclear chromatin to form fine granules that become marginated against the nuclear membrane followed by the condensation of the cell with preservation of the organelles [287-289]. As apoptosis proceeds, the plasma membrane blebs and forms fragments known as apoptotic bodies in a process called budding, and the nucleus becomes fragmented [287,288,290]. Apoptotic bodies are immediately engulfed by nearby phagocytic cells without inducing an inflammatory response.

One of the most important hallmarks of apoptosis is the fragmentation of cellular DNA. This process occurs as a result of the activation of DNA degradation enzymes, such as DNase, in the apoptotic signaling cascade. These enzymes cleave the cellular DNA at the linker regions between nucleosomes generating fragments of DNA each containing a single nucleosome or oligoneucleosomes [291]. However, as will be discussed later in this section, other forms of PCD can also induce DNA fragmentation.

Another hallmark of apoptosis is the externalization of phosphatidyl serine (PS), which is normally sequestered to the inner leaflet of the plasma membrane, to the outer leaflet of the plasma membrane [292,293]. The exposed PS on the surface acts as the key "eat me" signal on the surface of the apoptotic cell, which facilitates its engulfment by phagocytic cells [294]. Since the externalization of PS occurs early in apoptosis, this provided the bases for assays designed to detect apoptosis [295].

The process of apoptosis is dependent on the activation of a group of proteolytic enzymes called cysteinyl aspartate specific proteases (caspases). Caspases are widely expressed in cells as inactive proenzymes, with different stimuli able to result in their cleavage and activation. Once activated, they cleave other caspases and initiate a cascade of events that leads to cell death. To date, 18 caspases has been identified in mammals but only few of them are involved in the apoptotic pathway [296]. The members of the caspase family are divided into upstream, or initiator caspases (caspases-1, -2, -8, -9, -10, -11 and -12), and downstream, or executioner caspases (caspases-3, -6, -7 and -14) (reviewed in [296,297]). Moreover, caspases can perform other, non-apoptotic functions

in the cell, including cell-cell communication, cytokine maturation, inflammatory responses, spermatogenesis, and neuronal differentiation [297,298].

1.5.1.2 Apoptotic pathways.

Two pathways can induce apoptosis: the extrinsic (death receptor) pathway and the intrinsic (stress/mitochondrial) pathway. The two apoptotic pathways are depicted in Fig 1.2. The extrinsic pathway is initiated by the interaction between a cell surface death receptor and its ligand. Known death receptors: tumor necrosis factor (TNF) receptor-1 (TNFR-1), Fas (also known as APO-1/CD95), death receptor (DR) 3, TNF-related apoptosis inducing ligand-receptor 1 (TRAIL-R1), TRAIL-R2 and DR6 [286,299-301]. These receptors belong to the TNF receptor gene superfamily and contain a cysteine-rich extracellular domain that interacts with the death ligand, and a cytoplasmic domain called death domain (DD), which is responsible for the initiation of intracellular signaling [299,302,303]. The interaction of these receptors with their ligands results in their oligomerization (mostly trimerization). This will result in the recruitment of the DD-containing adaptor proteins Fas-Associated protein with death domain (FADD) or Tumor necrosis factor receptor type 1-associated death domain protein (TRADD) through the homotypic interaction of the DDs on both molecules. FADD contains a second domain called the death effector domain (DED), which recruits other DED-containing proteins including procaspase-8 and procaspase-10 to form a death-inducing signaling complex (DISC) [303-305]. The DISC will then mediate the autocatalytic cleavage and activation

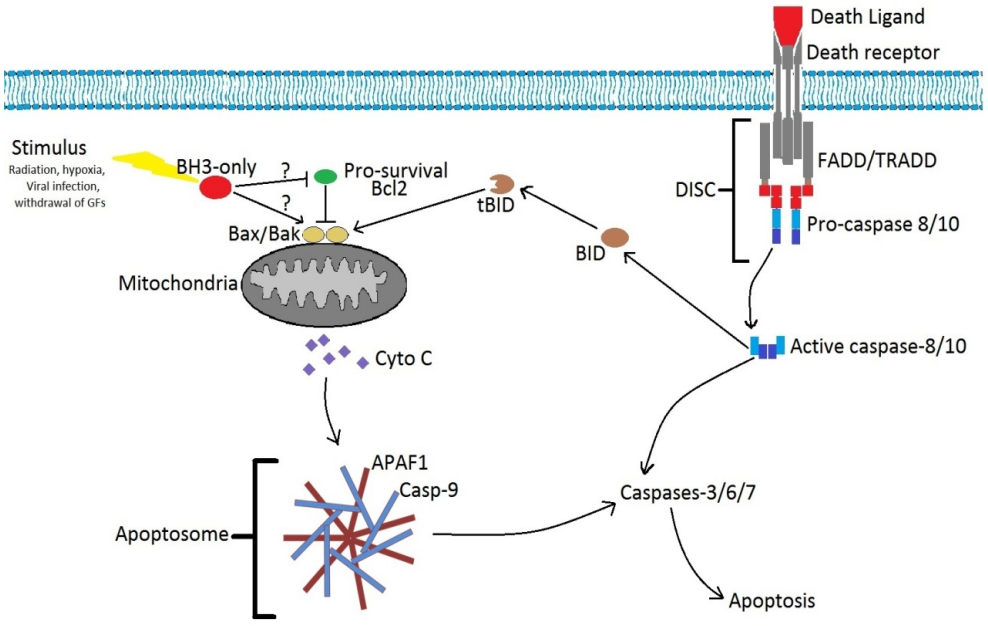


Figure 1.2 The intrinsic and the extrinsic apoptotic pathways.

Schematic representation of the extrinsic and the intrinsic (stress/mitochondrial) apoptotic pathways. The interaction between the death ligand with its cell surface receptor result in the recruitment of the adaptor proteins FADD/TRADD and procaspase-8/10 to the cytoplasmic domain of the receptor forming DISC. This interaction results in the cleavage and activation of caspase-8. Active caspase-8 cleaves and activates caspase-3/6/7 and BH3 interacting-domain death agonist (BID). Activation of BID amplifies the signal by activating the mitochondrial pathway. The intrinsic pathway is initiated by disrupting the balance between the pro- and anti-apoptotic Bcl-2 family members. This leads to mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome C to induce the oligomerization of APAF1 and the formation of the apoptosome, which activates caspase-3 and the other executioner caspases.

of caspase-8/ 10 [303]. The procaspase-8 molecule contains two DEDs and two catalytic subunits, p18 and p10. The activation of caspase-8 at the DISC occurs by the dimerization and several cleavages that eventually result in the formation of a heterotetramer $(p10/p18)_2$ [306,307]. Unlike the other death receptors, TNFR1 and DR3 DDs recruit TRADD [308]. TRADD is another DD-containing adaptor protein that can form two types of complexes, complex I and complex II. Complex I results from the recruitment of RIP-1, TRAF2, cellular inhibitor of apoptosis (cIAP) 1 and cIAP 2. This complex initiates a signaling pathway that ends with the activation of NF-κB and the p38 and c-Jun N terminal kinase (JNK) MAPK pathways [301,309]. Complex II is formed when TRADD recruits FADD, procaspase-8/10 and RIP-3. Complex II is a DISC that results in the cleavage and activation of caspase-8 [309,310]. The cell can control the activation of the extrinsic pathway by producing an inhibitory protein called cellular caspase-8/FADD-like IL-1β-converting enzyme (FLICE) inhibitory protein (c-FLIP). Like procaspase-8/10, this protein contains a DED domain, which allows it to interact with FADD, but it lacks the enzymatic activity found in caspase-8/10 [311,312].

It is worth mentioning here that formation of complex I by the TNF-α-TNFR interaction can activate two different pathways, each of which results in a different outcome regarding the fate of the cell. Complex I can result in the activation of JNK, which in turn activates the transcription factor activator protein-1 (AP-1). Complex I can also activate the transcription factor NF-κB (JNK and NF-κB activation pathways by complex I are reviewed in [309]). Activation of the JNK pathway contributes to TNF-α-induced cell death by activating E3 ubiquitin ligase Itch, which in turn ubiquitinates

cFLIP and targets it for proteasomal degradation [313]. Activation of NF-κB, on the other hand, results in the transcription of a group of genes including inflammatory chemokines and pro-survival proteins such as Bcl-xL, XIAP and cFLIP (reviewed in [314]).

Once activated, caspase-8/10 cleaves and activates the executioner caspases. In certain cell types (such as thymocytes), this signal by itself is enough to execute apoptosis. These cells are designated “type I”. In the cell types, amplification of the death signal by activation of the mitochondrial pathway is necessary for the induction of apoptosis [315,316]. These cell types are designated “type II” and include hepatocytes, which are the cell type of interest herein. Activated caspase-8 has the ability to initiate this second signal by inducing the cleavage of Bid (a Bcl-2 family protein). Truncated Bid (tBid) then translocates to the mitochondrial membrane and activates the mitochondrial apoptotic pathway [317].

The mitochondrial (intrinsic/stress) pathway is initiated intracellularly by several stimuli such as radiation, hypoxia, viral infections or simply the withdrawal of important growth factors. These stimuli initiate a series of events that induces MOMP and results in the release of cytochrome C (Cyt C) and other apoptotic factors from the intermembrane space of the mitochondria into the cytosol (reviewed in [318,319]). A family of 25 proteins, known as the B-cell lymphoma (Bcl)-2 family, controls the integrity of the outer mitochondrial membrane and the balance between them is what determines whether the cell will survive or undergo apoptosis (reviewed in [319,320]). Members of this family are divided into three subfamilies based on the function and the presence of some or all of the four conserved amphipathic regions called Bcl-2 homology (BH) 1-4 domains

[321,322]. The first subfamily contains pro-survival (anti-apoptotic) proteins that contain all of the four BH domains. Members of this subfamily includes Bcl-2, Bcl-2 like protein X (Bcl-xL), myeloid cell leukemia-1 (Mcl-1) [321]. The second subfamily contains a group of pro-apoptotic proteins that contain the first three BH domains (BH1-BH3), this subfamily includes Bcl-2-associated protein X (Bax) and Bcl-2 homologous antagonist killer (Bak) proteins. The last subfamily also contains pro-apoptotic proteins, but these contain only the BH3 domain, hence this group is designated the "BH3-only subfamily". Members of the BH3-only subfamily include BH3 interacting domain death agonist (Bid), bcl-2 interacting mediator of cell death (Bim), Bcl-2-associated death promoter (Bad) , p53 upregulated modulator of apoptosis (Puma) [321,323].

Activation of Bax and Bak results in conformational changes that lead to the formation of a homo-oligomer that is inserted into the mitochondrial membrane that leads to the release of Cyt C into the cytosol [324-327]. The activation of Bax and Bak is tightly controlled by the balance between the pro-survival Bcl-2 subfamily proteins and the pro-apoptotic BH3-only subfamily proteins. The mechanism by which these different subfamilies interact to protect or permeabilize the mitochondrial membrane is still has not been fully elucidated. Letai *et al* proposed that different BH3-only proteins could initiate the mitochondrial apoptotic pathways by acting as either activators or sensitizers of Bax/Bak [328]. They reported that some BH3-only proteins, like Bid and Bim, could directly activate the oligomerization of Bax/Bak. Others, such as Bad and Bik, act indirectly by binding to the pro-survival Bcl-2 subfamily, which sensitizes Bax/Bak to activation by the other BH3-only proteins. However, later reports supported an alternative

model in which all of the BH3-only proteins bind exclusively to the pro-survival Bcl-2 proteins. Bcl-2 proteins sequester Bax/Bak, and alternatively, Bax/Bak binding to BH3-only proteins results in their release and activation [329-332].

Once in the cytosol, Cyt C interacts with a protein known as apoptotic protease activating factor-1 (APAF-1). This interaction induces the oligomerization of APAF-1 which forms a wheel-like structure of seven APAF-1 molecules known as the "apoptosome". The apoptosome binds to and activates caspase-9, the initiator caspase for the intrinsic pathway, which in turn cleaves and activates the executioner caspases (formation and role of the apoptosome is reviewed in [333]).

Besides Cyt C, MOMP results in the release of several other apoptotic proteins. The first of these proteins is called Second mitochondria-derived activator of caspase (Smac), also known as direct inhibitor of apoptosis-binding protein with low pI (DIABLO). Once released into the cytosol, Smac/DIABLO interacts with and inhibits a group of proteins that belong to a family called inhibitor of apoptosis proteins (IAPs) [334]. Members of the IAP family, such as the x-linked IAP (XIAP) and the cellular IAPs (c-IAP1and c-IAP2), strongly inhibit caspases-9, -3 and -7 [335-337]. The interaction of Smac/DIABLO with the IAPs prevents their inhibitory activity on caspases and sensitizes the cell to apoptosis. The same mechanism is used by the serine protease high-temperature requirement A2 (HtrA2) that is also released from the mitochondria as a result of MOMP and is able to bind, cleave and inhibit XIAP [338,339]. Apoptosis-inducing factor (AIF) is another apoptotic factor released as a result of MOMP. This protein contains two functional domains, an oxidoreductase enzymatic activity domain

that was shown to perform pro-survival functions and a DNA binding site that catalyzes large-scale DNA fragmentation and chromatin condensation (reviewed in [340]). Finally, Endonuclease G is another apoptotic factor that, once released from the mitochondria, induces caspase-independent nucleosomal DNA fragmentation [341,342].

Activation of either the extrinsic or intrinsic pathways eventually results in the activation of the executioner caspases (caspases-3, -6 and -7) (reviewed in [287]). Following their activation, the executioner caspases cleave many substrates and cause the final events of cell death. Most of the execution functions are carried out by caspase-3, while caspases-6 and -7 play a less important role [343]. Active caspase-3 cleaves inhibitor of caspase-activated DNase (ICAD) to release the active caspase-activated DNase (CAD), which in turn causes DNA fragmentation and chromatin condensation [344]. Caspase-3 also cleaves and inactivates the DNA repair enzyme poly (ADP-ribose) polymerase (PARP) [345]. The Nuclear Mitotic Apparatus protein (NuMA) is another victim of caspase-3 whose cleavage results in nuclear disintegration [346,347]. Furthermore, caspase-3 cleaves gelsolin and the product of this cleavage, in turn, cleaves actin filaments causing severe morphological changes in the cell that can be easily observed *in vitro*, such as rounding up, detachment from the plate and fragmentation of the nucleus [348,349].

1.5.2 Pyroptosis.

Cookson and Brennan were the first to propose the term pyroptosis in 2001 to describe a caspase-1-dependent inflammatory form of programmed cell death that occurs in *Salmonella*-infected macrophages [350]. The name was derived from the Greek roots

pyro and *ptosis* (where *pyro* means fire or fever and *ptosis* means falling) to denote that this is an inflammatory form of death. The first observation of pyroptosis was in *Shigella flexneri*-infected macrophages returns back 1992, 9 years before the characterization of this form of cell death [351]. Other earlier studies revealed that these *Shigella flexneri*-infected macrophages died by a distinctive, caspase-1-dependent programmed cell death [352,353]. This caspase-1-dependent form of cell death was also observed in the macrophages infected with *Salmonella spp* [354,355]. However, all of these early reports referred to this form of death as apoptosis, based on its dependence on a caspase, which at the time was considered an exclusive hallmark of apoptosis. In addition to Salmonella and Shigella, pyroptosis was reported in many other bacterial infections including, but not limited to, *Listeria monocytogenes*, *Francisella tularensis* and *Yersinia spp* [356-358].

Pyroptosis is a pro-inflammatory form of cell death. Although they share some common features, pyroptosis has distinctive morphologic and mechanistic characteristics that differentiate it from apoptosis. As mentioned earlier, pyroptosis is dependent on the activation of caspase-1. The apoptotic caspases, caspases -3, -6 and -8 do not play any role in pyroptosis (reviewed in [359]). Pyroptotic cells are executed by formation of pores in the cell membrane, through which ions and water enter, resulting in cell swelling, and eventually cell lysis. This results in the release of their cellular contents into their surroundings [360]. Despite the fact that both pyroptosis and apoptosis share the ability to cause DNA fragmentation [358,361]. The mechanism of DNA fragmentation in pyroptotic cells is still not clear. Unlike apoptosis, pyroptosis does not cause the activation of ICAD/DFF45, and it does not cause MOMP or the release of AIF and

Endonuclease G from the mitochondria [356,358,360]. Finally, accumulating evidence suggests that active caspase-1 can cleave and activate caspase-7, providing a mechanism that links apoptotic and pyroptotic pathways [362,363].

PARP cleavage is widely used as a specific marker for apoptosis. However, there are contradicting reports regarding PARP cleavage in pyroptotic cells have been published. One report suggests that PARP remains in its active, intact form during pyroptosis in *Salmonella*-infected macrophages and Inhibition of PARP by a specific inhibitor did not prevent Salmonella-infected macrophages from undergoing cell-lysis [360,361]. In contrast, at least one report has suggested caspase-1-mediated PARP cleavage in pyroptosis [364]. Furthermore, activation of caspase-1 was reported to induce the cleavage of PARP directly, or indirectly by cleaving caspase-7 first, which in turn can cleave PARP [362,363,365]. In light of these reports, the use of PARP cleavage as an apoptosis-specific marker should be reconsidered. For this reason, the results of PARP cleavage experiment herein were confirmed by testing for caspase-3 cleavage directly.

Pyroptosis is initiated by the binding of several stimuli to a receptor. Unlike the extrinsic apoptosis pathway, pyroptosis is initiated by a group of PRRs that are located in the cytosol and belong to the NLR family. Different members of this family recognize different stimuli, but all result in the activation of caspase-1. The NLR family CARD domain-containing protein 4 (NLRC4/also known as Ipaf) was found to detect several bacterial components, such as flagellin of *Salmonella typhimurium* and of *Legionella pneumophila*, and the basal body rod component of the type III secretion apparatus of several gram negative bacteria (*Salmonella typhimurium*, *Burkholderia*

pseudomallei, Escherichia coli, Shigella flexneri, and *Pseudomonas aeruginosa*) [366-368]. NACHT (NAIP, CIITA, HET-E, TP1), leucine rich repeat (LRR) and pyrin domain (PYD)-containing protein-1 (NLRP1/also known as NALP1) was reported to detect *Bacillus anthracis* lethal toxin and induces pyroptosis in the affected macrophages [369]. Absent in melanoma 2 (AIM2) is a member of the hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats (HIN-200) receptor family that detects foreign cytosolic DNA [370]. AIM2 was found to be responsible for the activation of caspase-1 in *Francisella tularensis* infections and to stimulate pyroptosis in *Listeria monocytogenes*-infected cells [371,372]. Finally, NLRP3 (also known as NALP3) can detect a wide range of PAMPs and DAMPs (danger-associated molecular patterns); these include toxins, extracellular ATP, uric acid, bacterial PAMPs, fungal PAMPs, and most importantly to this thesis, viral DNA and RNA including HCV RNA (reviewed in [359]).

Detection of the PAMPs or DAMPs by any of the previously mentioned receptors will result in their self-oligomerization and recruitment of caspase-1 and the adapter protein apoptosis-associated speck-like protein containing a CARD (ASC) to form a multiprotein complex known as the inflammasome [373-375]. The inflammasomes then act as a platform for caspase-1 activation and the maturation of the inflammatory cytokine IL-1β [373,376]. ASC is comprised of two domains: a PYRIN domain and a CARD domain. ASC and the receptor interact through their PYRIN domains, and ASC and caspase-1 interact through their CARD domains. [375]. The NLRC4 protein contains a CARD domain that allows it to interact directly with caspase-1 without the need for an

ASC molecule [377]. The assembly of any of these two forms of the inflammasome results in the activation of caspase-1.

Caspase-1 is present in the cytosol as an inactive 45kDa precursor protein. This precursor can be processed in the inflammasome to give the active form, which is comprised of a tetramer of two 20kDa and two 10kDa polypeptides [378,379]. This active caspase-1 can perform a wide range of functions. In one study, 41 proteins involved in many vital pathways were identified as substrates for active caspase-1 [380]. Multiple steps of the glycolysis pathway are targeted and cleaved by caspase-1 (e.g. aldolase and glyceraldehyde-3-phosphate dehydrogenase). Caspase-1 also targets protein precursors that affect cytoskeletal architecture, translation, ATP metabolism and many chaperone proteins. As mentioned previously, active caspase-1 was also reported to target caspase-7 [362]. Finally, the most well-known targets for active caspase-1 are a group of pro-inflammatory proteins, including the inflammatory cytokines pro-IL-1β and pro-IL-18, as well as the autocleavage of caspase-1 [381]. Cleavage of pro-IL-1β and pro-IL-18 results in their maturation and secretion from the cell, which in turn results in the recruitment of inflammatory cells and increases the production of inflammatory cytokines [382]. Although the activation of pro-inflammatory cytokines is not required for the execution of pyroptosis; as pyroptosis was detected in IL-1β/IL-18 double knockout mice [383]. Caspase-1 performs a dual function in the pyroptotic pathway by acting as an initiator and an executioner caspase. However, the pathways downstream of caspase-1 activation that result in cell death are not completely understood.

Recent studies in mice reported that in addition to the previously described canonical inflammasomes, pyroptosis can also be induced by non-canonical inflammasomes. This pathway is activated by lipopolysaccharides (LPS) within the cytosol and relies on caspase-11 activation, rather than the activation of caspase-1 (reviewed in [384]). Interestingly, caspase-11 itself was reported to acts as a sensor for the detection of LPS in the cytosol and there are two homologs of caspase-11 in humans: caspase-4 and caspase-5 [385]. Similarly to caspase-11, caspase-4 and caspase-5 were reported to regulate the activation of non-canonical inflammasomes in response to LPS alone [385,386].

1.5.3 Other forms of cell death.

1.5.3.1 Autophagic cell death.

Autophagy (self-eating) is a process in which the cell digests its cytosolic proteins and organelles by surrounding them with a double membrane to form a vesicle known as a phagosome. The phagosome then fuses with a lysosome that delivers the necessary enzymes to digest the contents of the phagosome (reviewed in [387]). Autophagy acts as a pro-survival mechanism under stress conditions, such as starvation. Conversely, autophagy was reported to cause autophagic cell death (or type II cell death, where type I is apoptosis), which is a cell death associated with the accumulation of autophagosomes in the dying cell [388,389]. Nevertheless, the role of autophagy in this form of cell death is controversial. While some studies have reported that autophagosomes are associated with, rather than the cause of, cell death [390,391]. Others have suggested that autophagy is indeed the cause of cell death as autophagic cell death, in certain animals, is not affected by inhibiting or mutating apoptotic caspases. These studies also demonstrated

that this form of cell death does not occure in animals with defective autophagy proteins [392,393].

1.5.3.2 Necrosis and necroptosis.

Necrosis (type III cell death) has often been described as a passive, accidental, and uncontrollable form of cell death that occurs due to severe environmental stress, such as ischemia, physical trauma, or bacterial infections [394]. However, under certain conditions, necrosis can occur in a highly regulated manner. This form of cell death was termed “necroptosis” (reviewed in [391,394]). Necroptosis is induced in some cases as an alternative mechanism to trigger cell death during caspase inhibition. For example, interaction of Fas-FasL under the conditions of caspase inhibition results in the activation of the necrotic pathway [395]. Binding of TNF-α to its ligand can also induce necroptosis in the target cell. The necroptotic signaling pathway depends largely on proteins known as receptor interacting protein kinases 1 & 3 (RIPK1 & RIPK3). Binding of the TNF receptor to its ligand leads to the recruitment of TRADD, RIPK1 and RIPK3 to the cytoplasmic domain of the receptor. This leads to the interaction between RIPK1 and RIPK3 and subsequently results in the formation of a complex known as the necrosome (pathway is reviewed in [391,394]). It is still not clear how necroptosis executes the cell, but depletion of intracellular ATP was reported as a potential execution mechanism for necroptosis [396]. Furthermore, reactive oxygen species (ROS) were produced in RIPK3-regulated TNF-induced necroptosis [397]. Morphologically, necrotic cells increase in volume, the organelles swell, and eventually the plasma membrane ruptures, releasing all of the cellular contents [398].

1.5.3.3 Ferroptosis.

Ferroptosis is a recently described form of cell death that is dependent on intracellular iron [399]. This form of cell death is caused by the iron-dependent accumulation of lethal lipid ROS. Morphologically, no cell membrane rupture or blebbing and no chromatin condensation or changes in nuclear size have been observed in ferroptosis. This form of cell death is characterized by morphological changes in the mitochondria, which includes mitochondrial shrinkage, an increase in the density of the mitochondrial membrane and the decrease or vanishing of the crista (reviewed in [400]). Ferroptosis can be induced by several molecules including erastin, acetamenophen and sulfasalazine (a drug used in the treatment of inflammatory diseases) [399,401,402].

1.6 HCV and apoptosis.

The ability of HCV to modulate different apoptotic pathways has been studied extensively. However, the controversy among these reports is so extensive that it is difficult to draw conclusions from these reports. The reason for this controversy could be attributed in part to the use of different systems to test the effect of HCV on apoptotic pathways. These systems include liver biopsies obtained from HCV infected patients, expression of single HCV proteins in cell culture, the replicon system, transgenic mice, humanized mice and HCVcc. In addition, the various studies tested different apoptotic markers, each representing the activation of a single apoptotic pathway, or parts of it, but not the other pathways. Furthermore, some studies focused on testing the effect of HCV infection or HCV proteins on externally-induced apoptosis, i.e. apoptosis induced by different apoptotic ligand-apoptotic receptor interactions. Others tested the direct

induction or inhibition of apoptotic proteins or pathways by HCV. Overall, each individual HCV protein and the whole replicating virus were reported to have a both pro-apoptotic and anti-apoptotic effects by various groups. This extensive controversy around the relationship between HCV infection and apoptosis was the justification for the work described in this thesis. To set the stage for this project, some of the key studies in this area will be reviewed.

Testing the expression of apoptotic markers in liver biopsies obtained from chronically infected individuals was one of the earliest tools used to study HCV-induced apoptosis. However, the use of liver biopsies is limited by sample availability and the invasiveness of the procedure. In addition, the differentiation between immune system-induced apoptosis and direct virus-induced effects are difficult to discern *ex vivo*. For these reasons, only a few groups have used this system to study HCV-induced apoptosis. In one such study, apoptosis was detected in the liver sections obtained from chronically HCV-infected patients, and the apoptotic index correlated with histological activity grading [403]. A second group detected activation of caspase-3 and -7 in liver biopsies samples obtained from chronically infected individuals [404]. This caspase activation correlated with the degree of inflammatory injury in the liver. Recently, another group reported apoptosis, autophagy and unfolded protein response (UPR)/ER stress in liver tissue obtained from biopsies performed on HCV-infected individuals [405]. In contrast, at least one report suggests a pro-survival effect during HCV infection where a decrease in the levels of Bid were observed in non-cirrhotic, HCV-linked tumor biopsies [406]. This mechanism might play a role in HCV-induced tumorigenesis.

Several groups have also used single protein expression as a tool to test the pro-/anti-apoptotic effects of individual HCV proteins. The HCV core protein has been the most extensively studied with several groups reporting contradicting findings regarding its effect on apoptotic pathways. Expression of core protein caused ER stress and ER calcium depletion in Huh-7 and HepG2 cells [407]. ER stress stimulated the expression of a protein called C/EBP homologous protein (CHOP), which is a pro-apoptotic protein that activates the mitochondrial apoptotic pathway. In a second report, the expression of core protein in 293T cells triggered them to undergo apoptosis [408]. Analysing the mechanisms responsible for this induction revealed that the core protein interacts with a pro-survival protein called 14-3-3ε. This interaction was proposed to release Bax from the Bax/14-3-3ε complex, allowing it to induce the mitochondrial apoptotic pathway. Moreover, C-terminally truncated core was reported to translocate to the nucleus and induce PKR-dependent apoptosis in transfected PHH [409]. In addition, Core protein was reported to contain a BH3 domain through which core can interact with the pro-survival Mcl-1 protein, preventing it from inhibiting the pro-apoptotic Bad protein, thus inducing apoptosis [410]. Furthermore, in a stably transfected osteosarcoma cell line, HCV core protein was reported to induce caspase-independent apoptosis-like cell death [411].

In contrast, the expression of genotype 3a HCV core protein caused pro-survival effects by downregulating a group of pro-apoptotic proteins including multiple caspases, cytochrome C, and p53 [412]. It also increased the level of phosphorylated-Akt (a pro-survival protein) and increased the viability of transfected cells [412]. Likewise,

expression of genotype 1b core protein by Huh-7 or Hela cells inhibited ROS-induced apoptosis by increasing Bcl-xL and decreasing Bax levels [413].

The effect of HCV core expression on death ligand-induced apoptosis is also controversial. Expression of the core protein was reported to sensitize the cell to Fas-mediated apoptosis without affecting the level of Fas expression [414]. Core protein was also reported to bind to the DD of TNF receptor 1 (TNFR1), sensitizing the cell to TNF-induced apoptosis [415]. The core-induced sensitization to TNF-α was also reported to be attributed to the induction of ROS as a result of the interaction between core and the heat shock protein (Hsp60). This interaction is thought to impair the function of Hsp60 as a regulator of ROS production [416]. Finally, core protein sensitizes Huh-7 cells to TRAIL-induced apoptosis by augmenting Bid cleavage by caspase-8, which activates the mitochondrial apoptotic pathway and amplifies the TRAIL-induced death signal [417]. In contrast, one group reported an anti-apoptotic effect of the core protein on death ligand-induced apoptosis, and its expression in MCF-7 cells was also reported to inhibit TNF-induced apoptosis [418]. In a second report for the same group, core protein was found to inhibit the TNF-induced apoptosis in HepG2 cells, and this inhibition was caused by the stimulation of c-FLIP expression in core-expressing cells [419].

The effect of E1, E2 and p7 on the induction of apoptosis has been studied less thoroughly than it has for the core protein. HCV E1 protein was shown to have pro-apoptotic functions since the expression of full-length or the transmembrane domain of E1 induced apoptosis in a hepatoma cell line [420]. Expression of the E2 protein has been reported to have both pro-apoptotic and anti-apoptotic effects in different studies.

Expression of E2 in Huh-7 cells resulted in the activation of the mitochondrial apoptotic pathway [421]. On the other hand, E2-containing replicons conferred protection to the cell from TRAIL-induced apoptosis [422]. The inhibition of TRAIL-induced apoptosis by E2 was also reported by a second group, who found that E2 induced the expression of a chaperone protein known as glucose regulated protein 94 (GRP94) [423]. This protein stimulated the pro-survival NF-κB pathway (discussed earlier), which confers resistance to TRAIL-induced apoptosis. Finally, the expression of the p7 proteins (genotypes 1b and 2a) in Huh-7.5 cells induced apoptosis; however this was not dependent on p7's ion channel activity [424].

The effects of the several non-structural proteins on the apoptotic pathways has also been the focus of several studies. NS2 was reported to perform an anti-apoptotic function by inhibiting a pro-apoptotic protein known as cell death-inducing DNA fragmentation factor-alpha (DFFA)-like effector-B (CIDE-B) [425]. This protein plays a role in promoting the formation of VLDL particles and it induces apoptosis by activating the mitochondrial apoptotic pathway [425-427]. NS3 was reported to interact with caspase-8, and this interaction induced caspase-8 mediated apoptosis in NS3-exepressing mammalian cells [428]. The capability of NS3 to interact with caspase-8 was independent from both its protease and helicase activities. In contrast, two other groups reported anti-apoptotic functions of NS3 [429,430]. The NS3 protein was reported to interact with the tumor suppressor protein p53, resulting in inhibition of actinomycin D-induced apoptosis [429]. Furthermore, NS3/4A expression was reported to protect the cell from TNF-α-induced apoptosis by inducing the expression of a mitochondrial receptor known as

translocase of outer mitochondrial membrane 70 (TOM70) [430]. TOM70 interacts with the pro-survival Bcl-2 family member protein Mcl-1, which targets Mcl-1 to the mitochondria [431]. While the expression of NS4A, in the absence of NS3, resulted in mitochondrial damage that induced apoptosis; Expression of both NS3 and NS4A was found to sensitize the cell to actinomycin D-induced apoptosis [432]. In addition, transfection of Huh-7 or 293T cells with NS4B resulted in the induction of the mitochondrial pathway of apoptosis and resulted in ER stress [433].

The effect of NS5A protein on apoptotic pathways has been studied extensively. With the exception of one report, in which the expression of NS5A in dendritic cells had a pro-apoptotic effect, NS5A has been reported to have multiple anti-apoptotic functions [434]. NS5A was reported to interact with p53 and its co-activator human TBP–associated factor (hTAF)II32, and this interaction inhibited the ability of p53 to induce apoptosis [435]. In addition, NS5A binds to the Src homology-3 domain (SH3) of the p85 subunit of phosphoinositide 3-kinase (PI3K). This binding activates PI3K, which in turn increases the phosphorylation and activation of the pro-survival enzyme Akt [436]. Akt in turn inhibits apoptosis by phosphorylating and inactivating the pro-apoptotic, Bcl-2 family member protein Bad [437]. Additionally, the SH3 binding motif of NS5A was reported to interact with the tumor suppressor bridging integrator 1 (Bin-1) to inhibit Bin-1-mediated apoptosis [438]. Furthermore, HCV NS5A was reported to interact with the 38kDa FK506-binding protein (FKBP38) [439]. The FKBP38 protein is known for its ability to interact with the pro-survival Bcl-2 family proteins, targeting them to the mitochondria and inhibiting apoptosis [440]. The interaction between NS5A and FKBP38 was reported

to increase the resistance of NS5A-expressing cells to drug-induced apoptosis [439]. NS5A expression was also reported to inhibit TNF-α-mediated apoptosis in Huh-7 cells by inhibiting a step upstream of caspase-8 activation [441]. Finally, only one study tested the effect of NS5B on the apoptotic pathways. In that study, NS5B was reported to have a pro-apoptotic effect by attenuating TNF-α-induced NF-κB activation and sensitizing the cell to TNF-α induced apoptosis [442].

The effects of HCV on apoptosis has also been studied in systems that include multiple HCV proteins. For example, in a cell line expressing a genotype 1a-derived replicon, it was found that HCV replication resulted in the upregulation of serine protease inhibitor Kazal (SPIK), which in turn rendered the cells more resistant to serine protease-dependent apoptotic death [443]. A second group used the replicon system to test the effect of HCV replication on TRAIL-induced apoptosis and found that HCV replication sensitized the cells to TRAIL-induced apoptosis by upregulating TRAIL receptors (DR4 and DR5) [444].

Mouse systems have also been used in studying the effect of HCV infection on apoptotic pathways. The effect of HCV proteins on Fas-induced apoptosis was tested a transgenic mice expressing core, E1, E2 and NS2 [445]. They found that the expression of HCV proteins suppressed Fas-induced apoptosis. Mechanistic analysis revealed that this inhibition was due to the inhibition of cytochrome C release from the mitochondria. In a second study, the hepatocytes of transgenic mice expressing the HCV polyprotein were found to be resistant to Fas-induced apoptosis [446]. This resistance was associated with the reduction of Bid protein levels in these cells. Opposing results were reported by

Joyce *et al*. who used SCID/Alb-uPA mice with humanized livers that support HCV infection and replication [447]. Infecting these mice with HCV was found to induce oxidative and ER stress, and to downregulate pro-survival NF-κB and Bcl-xL proteins, which ultimately resulted in the induction of apoptosis in the human liver cells [447].

After the development of the HCVcc system, many groups used it to study the induction/or inhibition of apoptosis by HCV infection. Unlike single protein expression experiments, this system better represents the natural course of HCV infection. Most studies that have used the HCVcc system have reported pro-apoptotic effects resulting from HCV infection. However, there are some discrepancies regarding the mechanism of apoptosis induction. In contrast, several groups have reported anti-apoptotic effects of virus infection on certain apoptotic pathways by using HCVcc.

Zhu *et al*. were the first to use the JFH1 strain to study apoptosis [448]. They developed a new human hepatoma cell line, LH86, which was more differentiated than the Huh-7.5 cell line. These cells supported JFH1 infection, but the efficiency of infection was low. Infection of these cell resulted in the induction of TRAIL and its receptors (DR4, DR5), which induced apoptosis. Mateu *et al*. also reported induction of apoptosis in Huh-7.5 cells infected with a chimeric virus containing the genome sequence from core to p7 of the J6 strain (a genotype 2a strain) and the reminder of the genome obtained from the JFH1 strain [449]. Furthermore, HCV-induced apoptosis was reported by Sekine-Osajima *et al*. in Huh-7.5.1 (a cell line derived from the Huh-7.5 GFP-HCV replicon cell line that is known to be highly permissive to HCV replication) infected with a tissue culture-adapted strain of JFH1 [450,451]. This adapted strain contained 9 amino acid

substitutions, 5 of which were located in the NS5B coding region. Apoptosis was caused by the induction of ER stress in these cells. In a second study, this group analysed the amino acid substitutions reported earlier and found that introduction of one or all of three mutations (C2441S, P2938S and R2985P located in NS5A and NS5B) into the parental JFH1 strain resulted in a higher replicative efficiency and resulted in increased cytopathic effects [452]. Furthermore, Deng L *et al*. reported an induction of apoptosis in Huh-7.5 cells infected with J6/JFH1 virus [453]. The apoptosis was induced by Bax activation and the activation of the mitochondrial apoptotic pathway. In contrast to previous findings. Deng L *et al.* reported that HCV infection does not cause ER stress. In a follow-up study, this group reported that the HCV-induced Bax activation is caused by the upregulation of the pro-apoptotic Bcl-2 protein family member Bim [454]. This upregulation is stimulated by the oxidative stress, and it required the activation of JNK pathway. Apoptosis of J6/JFH1 infected Huh-7.5 was also reported by Walters *et al.* [455]. Analysis of the transcriptional response to HCV infection in Huh-7.5, and in liver biopsies obtained from HCV-infected patients, revealed that the HCV-induced apoptosis is caused by the induction of cell cycle arrest. Further analyses showed that cell cycle arrest occurs at the G1 phase of the cell cycle in infected cells. In agreement with this, Kannan *et al.* also reported the induction of apoptosis in infected Huh-7.5 cells as a result of cell cycle arrest [456]. However, this group reported that the cell cycle arrest occurs at the interface of the G2 and mitosis phases of the cell cycle.

The HCVcc system was also used to study the effect of HCV infection on death ligand-induced apoptosis. This is particularly important to understand how HCV infection

affects the efficiency of the immune cells-mediated killing of infected hepatocytes. Lan *et al.* reported that infecting Huh-7.5 cells or PHH with JFH1 sensitizes them to TRAIL-induced apoptosis [457]. This sensitization was caspase-9 dependent and it was mediated by non-structural proteins. The same effect of HCV infection on TRAIL-induced apoptosis was also reported by Deng *et al.* who proposed that this effect was caused by the upregulation of TRAIL receptors DR4 and DR5 by a mitogen-activated protein kinase (MAPK)/ extracellular signal–regulated kinase (ERK) kinase 1 (MEK-1)-dependent pathway [444]. Finally, Park *et al*. reported that HCV infection sensitizes infected cells to TNF-α-induced apoptosis by inhibiting the pro-survival NF-κB pathway, which results in the downregulation of a group of pro-survival proteins including XIAP, Bcl-xL and cFLIP [442].

Anti-apoptotic effects of HCV infection have also been reported by using the HCVcc system. Liu *et al.* reported that HCV infection of Huh-7.5 cells caused a transient activation of the pro-survival PI3K-Akt pathway [458]. This activation was detectable only at the early stages of infection and resulted in enhancement of viral entry. Furthermore, HCV infection induced mitochondrial fission and mitophagy (the selective removal of defective mitochondria by autophagy) [459]. These two processes in turn protect the cell from undergoing apoptosis. Finally, Lee *et al.* demonestrated that infection with a luciferase gene-containing derivative of JFH-1 stimulated the expression of GRP94 in Huh-7 cells [423]. This resulted in activation of the pro-survival NF-κB pathway and inhibited TRAIL-induced apoptosis.

1.7 HCV pathogenesis and the possible role of programmed cell death.

HCV infection progresses to chronicity in 75%-85% of the cases [13]. Those chronically infected patients are at risk of developing severe liver diseases including liver fibrosis, cirrhosis and HCC [460]. The mechanism by which these liver diseases develop is poorly understood. This limits our ability to develop new treatment strategies to prevent or reduce the risk of the progression of HCV-induced liver diseases. This is particularly, important in light of the fact that the accessibility to DAAs is still very limited and that this treatment does not eliminate the risk of development of HCC [461]. An accumulating body of evidence suggest that the induction of programmed cell death in the HCV-infected liver plays a role in the pathogenic process. The proposed mechanisms by which apoptosis and pyroptosis contribute to the development of liver disease are discussed in more detail below and outlined in Fig. 1.3.

Liver fibrosis occurs as a response to chronic liver injury. Such injuries can result from drugs, autoimmunity or infection. If untreated, continuous liver fibrosis progresses to cause liver cirrhosis [462]. Liver cirrhosis is an advanced stage of liver fibrosis that is characterized by deformation of the liver vasculature and can lead to fatal complications including liver failure, esophageal varices and HCC [462-464]. Hepatic stellate cells (HSC) are the main contributor to fibrosis. These cells present normally in a quiescent state and play a role in vitamin A storage [465]. Once activated, HSCs undergo several changes including: the transformation to a myofibroblast-like phenotype, losing vitamin A storage, enhancing the expression of the pro-fibrogenic proteins (collagens) and starting to proliferate [466]. The production of large amounts of collagen-rich

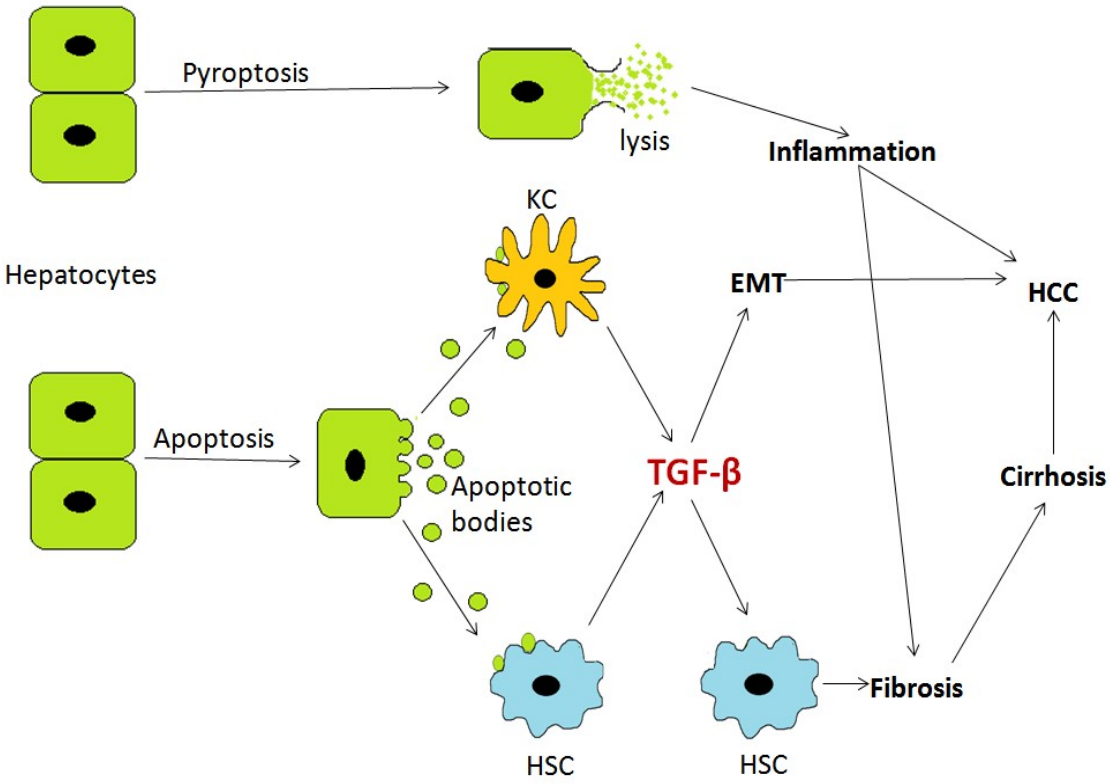


Figure 1.3 The proposed mechanisms for the role of apoptosis and pyroptosis in the development of progressive liver diseases.

Schematic representation of the proposed mechanisms for the role of apoptosis and pyroptosis in the development of the progressive liver disease. Apoptotic cells form apoptotic bodies, which are engulfed by kupffer cells (KC) and HSCs. This results in KC and HSC production of TGF-β, which plays a central role in liver pathogeneses. TGF-β stimulates the expression of the pro-fibrogenic genes in HSCs and induces EMT. Continued unresolved fibrosis progresses to cirrhosis, and EMT contributes in the development of HCC. Pyroptosis of the hepatocytes participates in creating an inflammatory environment in the liver, which also contributes to the progression to cirrhosis and predispose the liver to HCC.

extracellular matrix (ECM) by the activated HSCs causes fibrosis of the affected liver tissue.

Hepatocytes apoptosis can stimulate the liver fibrosis. As mentioned above (section 1.5.1.1), at late stages of the apoptotic pathway, the cells dissociate by forming apoptotic bodies. Engulfment of these apoptotic bodies by resident macrophages or HSCs has been reported to activate these cells to produce TGF-β [467-469]. TGF-β in turn, plays a central role in the activation of several pathogenic pathways. TGF-β is considered the most pro-fibrotic cytokine. It acts in an autocrine or a paracrine manner on HSCs to activate them and promote their production and deposition of collagen and other ECM, which hastens liver fibrosis [470,471]. TGF-β was also reported to induce a biological process known as epithelial-mesenchymal transition (EMT) in which the cells gradually lose their epithelial and hepatic cell markers and gain mesenchymal cell markers instead [472]. It is still not clear whether the EMT of the hepatocytes can contribute to the fibrogenic process. Hepatocyte EMT was reported by some groups to contribute to the process of fibrosis [473-475]. However, other reports contradict these findings, demonestrating that EMT by hepatocytes has no contribution to fibrosis [476].

HCC is the fifth of the most common type of cancer and the third leading cause of cancer-related death globally [477]. HCV-related HCC was reported to be the fastest-rising cause of cancer-related death in the USA [478]. Unlike the majority of other cancers, which develop in relatively healthy tissue, most of the HCV-related HCC cases develop in a background of advanced fibrosis and cirrhosis [477]. The mechanisms by which HCC develops in the HCV-infected liver are not understood. Persistent

inflammation and progressive fibrosis are believed to act in concert to create a pro-carcinogenic environment in the HCV-infected liver (reviewed in [479]). The apoptosis-induced TGF-β production in the HCV-infected liver can also play a role in HCC development. Apart from its pro-fibrogenic role, TGF-β was reported to have two opposing direct effects on cancer development at different stages of the disease. At the early stages, TGF-β acts as a tumor suppressor by causing cell cycle arrest and inducing apoptosis [472,480]. In contrast, at late stages, TGF-β-induced EMT increases cell survival and promotes invasiveness and metastasis of HCC [472,474,481].

Induction of pyroptosis could play a significant role in HCV-induced pathogenesis. The pro-inflammatory nature of this form of cell death contributes to HCV-induced chronic inflammation. DAMPs released from lysed pyroptotic cells can recruit immune cells and promote inflammation [482]. This chronic inflammation, in turn, is known to be an important driver for the pathogenic process in the HCV-infected liver. Activated inflammatory cells contribute to the generation of a pro-carcinogenic environment by the production of ROS and reactive nitrogen species, and cause lipid peroxidation [483]. Activation of the NF-κB pathway (a hallmark of the inflammatory response) can also be involved in fibrogenesis as well as in the initiation and progression of HCC in the chronically infected liver (reviewed in [484]). Several other reports showed an association between the degree of liver inflammation, and the development of HCC [477,485,486]. Besides that, inflammation and the release of ROS, inflammatory cytokines and chemokines by kupffer cells (KCs) are believed to induce the activation of HSCs, thus promoting fibrosis of the liver (reviewed in [487,488]).

1.8 Project design and research questions.

As demonstrated in the previous sections, induction of different forms of programmed cell death by HCV infection could be an important factor for the development of the pathogenic changes in the infected liver. The induction of apoptosis by HCV has been studied extensively. However, as described in section 1.6, within the literature there is considerable discrepancy regarding the effects of HCV infection on induction of apoptotic pathways. Furthermore, the mechanism by which this apoptosis is induced is still vague. Some of this discrepancy is attributed to the use of minimal systems that either do not reflect the complete life cycle of the virus, or rely on virus replication that is too low to accurately reflect-physiological HCV infection. We believe that the use of the tissue culture adapted $JFH1_T$ strain will better represent the natural infection, as this strain is a non-chimeric virus that can replicate to high levels comparable to physiological infection.

In the first part of this study, the ability of HCV to induce apoptosis, in the absence of any immune cells, was investigated by using an HCVcc system. Stocks of virus ($JFH1_T$ strain) were prepared and used to infect Huh-7.5 cells. The effect of HCV infection on several features related to apoptosis were tested, including viability, proliferation rate, DNA fragmentation and the cleavage/activation of caspase-3. The effect of caspase-3 inhibition on HCV-induced cell death was tested to confirm that the observed cell death was indeed due to apoptosis. The effect of HCV infection on the activation caspase-8 and the effect of inhibiting caspase-8 specifically on the HCV-induced apoptosis were also tested to investigate whether HCV can induce the activation of the extrinsic pathway.

In the second part of the project, the ability of HCV to induce apoptosis in neighbouring uninfected cells (bystander apoptosis) was investigated. This concept had not been studied before in the context of HCV infection. Bystander apoptosis has been described before in the context of HIV infection, where it is found to be responsible for CD4+ T cell depletion [489,490]. mechanism of programmed cell death in HCV might contribute to the overall pathogenesis of the virus. HCV might stimulate the infected cells to express death ligands or to produce death-inducing soluble mediators in an attempt to avoid being killed by immune cells. Such a mechanism might also affect neighbouring uninfected hepatocytes. To study the possibility of the induction of bystander apoptosis, a co-culture system containing Huh-7.5 cells and HCV non-permissive cells was designed. In this system, induction of apoptosis in the non-permissive cells is indicative of bystander apoptosis.

In the third part of this study we investigated the ability of HCV infection to induce pyroptosis in infected and neighbouring uninfected cells. Induction of the pro-inflammatory pyroptosis could have a significant impact on the overall pathogenesis of HCV infection, but has not been studied in the context of HCV infection to date. However pyroptosis has been demonstrated in HIV infection as well as in the closely related Dengue virus [491,492]. Pyroptosis was found to be induced in the hepatocytes of constitutively activated NLRP3 knock-in mice, and the activation of this pathway increased inflammation and activated hepatic stellate cells (HSCs), thus hastening fibrosis in the liver of the mice [493]. In this project, we exploited our adapted HCVcc system to study the induction of pyroptosis in infected hepatocytes in the absence of any effect of

the immune cells. This was done by measuring the activation of caspase-1 and measuring the effect of caspase-1 inhibition on HCV-induced cell death. We also studied the ability of HCV to induce bystander pyroptosis using a co-culture system.

1.9 Objectives.

The objectives of this project are:

1- To study the effect of HCV infection on the induction of apoptosis in infected cells in the absence of immune cells.

2- To study the ability of HCV infection to induce apoptosis in neighbouring uninfected cells.

3- To study the ability of HCV infection to induce pyroptosis in infected and neighbouring cells in the absence of immune cells.

4- To analyze the effect of HCV infection on different PCD pathways underlying HCV-induced apoptosis and/or pyroptosis.

Chapter 2: Materials and methods

2.1 Cell culture.

Infection, transfection and co-culture experiments were performed by using Huh-7.5, S29 and 293T cells [184,187]. All of these three types of cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen). The medium was supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) and referred to as complete medium. The cells were cultured in 15 cm dishes and incubated at 37°C in a 5% CO2 incubator. Most of the infection, transfection, and co-culture experiments were carried out in 10 cm dishes. The 10 cm dishes were seeded with 1,000,000 cells, 24 hours before the infection/transfection.

The cells were split every 72 hours up to 30-35 passages. Splitting the cells was performed by aspirating the complete medium and washing the cells 1X with 3 ml of trypsin (Invitrogen). Seven ml of trypsin were then added to cover the cells and incubated at 37°C for 5-6 minutes. Following that, the trypsin was inactivated by adding 10 ml of the complete medium to the plate then harvesting the cells to a 50 ml tube. A second wash of the plate was done with another 10 ml of complete medium to collect the remaining cells in the plate. The cells were then centrifuged and re-suspended in 20 ml medium. Finally, the cells were counted and 2,000,000 cells were returned to a new 15 cm plate containing fresh complete medium.

2.2 Plasmid preparation and viral RNA transfection.

Stock amounts of the $JFH1_T$ plasmid or the MLV-GFP plasmid were prepared by cloning into the commercially available DH-5α bacterial cells (Invitrogen) according to the manufacturer's instructions. The plasmids were then purified from the bacterial culture by using the commercially available Maxiprep Kit (Qiagen). $JFH1_T$ plasmid was linearized by the restriction enzyme XbaI (Invitrogen). HCV RNA was then generated by *in-vitro* transcribing the linearized plasmid by using the T7 Megascript kit (Ambion). The RNA was then transfected into Huh-7.5 cells grown on a 10 cm plates (prepared 24 hours in advance by seeding 1,000,000 Huh-7.5 cell/10 cm plate). The transfection was performed by using DMRIE-C reagent (Invitrogen) according to the manufacturer's instructions.

In the co-culture experiments, the MLV-GFP plasmid was transfected into S29 or 293T cells grown on 24-well plates (200,000 cell/well, plated 24 hours in advance) by using Lipofectamine 2000 kit (Invitrogen). The protocol for the 24-well plate provided by the manufacturer was followed with doubling the amount of plasmid used. This was found to give better transfection efficiency.

2.3 Generation of the virus stock, Infection and titer determination.

For this study, we used $JFH1_T$, a tissue culture-adapted strain of JFH-1 containing three adaptive mutations within E2, p7 and NS2 [187,494]. To generate virus stocks, 1 x 10^6 Huh-7.5 cells were seeded in 10 cm culture dishes and cultured overnight. The following day, cells were transfected with *in vitro*-transcribed viral RNA representing cell culture-adapted $JFH1_T$ using DMRIE-C reagent (Invitrogen) as previously described.

Virus-containing medium from transfected cells was collected three days post-transfection and virus titre was determined using a limiting dilution focus-forming assay described below [451]. Titred virus-containing medium was inoculated onto virus-naive Huh-7.5 cells for 3 hours at a multiplicity of infection (MOI) of 0.5. Following inoculation, culture medium was replaced with fresh complete medium and cells were cultured for three days. Virus-containing infection culture medium was then passaged on naïve Huh-7.5 for an additional round of infection in order to eliminate residual input RNA. Culture fluids were then harvested and clarified through Millex-HV 45μm filters (Millipore).

Virus titre was determined by performing a 10-fold serial dilution of the virus stock followed by infection in 8-well chamber slides that had been seeded with 50,000 Huh-7.5 cells/well on the previous day. Three days post-infection (p.i.), slides were stained with anti-HCV core antibody (B2, Anogen), followed by goat anti-mouse Alex Fluor® 488 (Invitrogen), and the number of foci in the highest positive dilution were counted. From this number the titre was expressed as focus forming units per millilitre (FFU/ml).

2.4 Immunostaining for indirect immunofluorescence.

Medium was aspirated from the wells of the 8-chamber slides and cells were washed by immersing the slide in 1X phosphate-buffered saline (pH=7.4; PBS) for 2 minutes. The cells were then fixed and permeablized by immersing the slide in 100% acetone for 2 minutes. For HCV core staining, the slides were covered with mouse monoclonal anti-HCV core antibody (B2, Anogen) diluted 1:200 in 5% BSA in PBS for 20 minutes. Slides were washed in PBS for 5 minutes, then incubated for 20 minutes with the secondary

antibody (goat anti-mouse Alexa Fluor® 488; Invitrogen) diluted 1:100 in PBS. The slides were then washed and mounted with Vectashield Hard Set mounting medium containing DAPI (Vector Laboratories). The slides were examined at 10X and 20X magnifications on a Zeiss Axio Imager.M2 immunofluorescence microscope.

In the cleaved caspase-8 experiment, the same protocol above was followed with the addition of an extra primary and an extra secondary antibody. The primary antibody solution contained two antibodies the HCV-core antibody described above and a rabbit monoclonal cleaved caspase-8 (Asp391) antibody (Cell Signaling) diluted at 1:100 in the primary antibody solution. In the secondary antibody step, a goat anti-rabbit Alexa Fluor® 594 IgG antibody (Invitrogen) was added diluted 1:200. The slides were examined at a 10X and 20X magnification on a Zeiss Axio Imager.M2 immunofluorescence microscope.

2.5 MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay.

In a 96-well plate, 5,000 Huh-7.5 cells were seeded in 100 µl of complete medium per well. The next day, cells were infected at different MOIs (1, 2, and 4) by aspirating the medium covering the cells and replacing it with 50 µl of the appropriate virus stock to give the desired MOI. A volume of 50 µl of complete medium was pipetted into the uninfected control wells. After 4 hours of incubation, the virus inocula were aspirated and replaced with 100 µl of fresh medium. The plates were then incubated at 37°C for 72 hours until the day of MTT assay. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) was prepared at 5 mg/ml PBS and diluted 1:10 in complete medium to generate the MTT working stock. Medium was removed from wells and replaced with

100 µl of MTT working stock, then incubated for 4 hours at 37°C. Following incubation, liquid was removed from the wells carefully so as not to disturb the formazan crystals that had formed at the bottom of the wells. These crystals were then dissolved by adding 100 µl of DMSO to each well and lightly shaking the plate for 10 minutes. The optical density of the solution in the wells was then read on a plate reader at 540 nm.

2.6 CFSE (Carboxyfluorescein succinimidyl ester) assay.

Huh-7.5 cells were seeded in 10 cm dishes at 1 x 10^6 cells/dish and infected on the next day at an MOI of 2 for 4 hours. The cells were then harvested and washed with complete medium, then incubated for 15 minutes in a pre-warmed (37°C) solution containing 10 µM Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) in PBS. The CFDA-SE solution was then removed and replaced by complete medium. Cells were then incubated for 30 minutes at 37°C then harvested for time 0. Alternatively, one sixth of the total number of cells was transferred to each of the wells of a 6-well plate. The cells were incubated at 37°C and harvested every 24 hours until 5 days. Cells were examined daily by light microscopy to ensure that the cells were not confluent. At each time point, cells were harvested, fixed with 2% paraformaldehyde in PBS, and washed with PBS. Finally, the CFSE intensity in the cells was measured by flow cytometer in the FL1-H channel of a Becton Dickinson FACS Calibur.

2.7 Propidium iodide (PI) staining and cell cycle analysis.

Huh-7.5 cells were seeded in 10 cm dishes at 1 x 10^6 cells/dish and infected on the next day at an MOI of 1. The PI staining protocol used here was adapted with minor modifications from the standard method reported by Riccardi and Nicoletti [495]. Briefly,

cells were harvested and fixed by resuspending them in 0.5 ml of PBS then adding 4.5 ml of cold 70% ethanol slowly to each of the tubes while on ice, then stored at -20°C until all cells were ready to be stained. To prepare for PI staining, the cells were centrifuged at 400 x *g* for 5 minutes and supernatants discarded. The cells were washed and resuspended in 0.5 ml of PBS, 0.5 ml of DNA extraction buffer was added, and incubated for 5 minutes. Next, cells were pelleted as above, supernatants were removed, and the cells were resuspended in 1ml of DNA staining solution and incubated for 30 minutes at room temperature. The DNA extraction buffer and the DNA staining solution were prepared as described in [495]. The DNA extraction buffer was prepared by mixing 192 ml of 0.2 M of Na_2HPO_4 with 8 ml of 0.1% Triton X-100 (pH=7.8). The DNA staining solution was prepared immediately before use by dissolving 200 μg of PI in 10 ml of PBS containing 2 mg of DNase-free RNase. PI intensity in the stained cells was measured using a Becton Dickinson FACS Calibur. Cellular debris and doublets were gated out during the analysis. Cellular debris were gated out during the analysis by excluding the cells with low forward scatter values. Doublets were also gated-out by plotting the FL2-A vs. FL2-W on a linear scale. Following that, the hypodiploid cells with an intensity lower than that of the diploid cells (G1) were counted.

2.8 DNA laddering assay.

Huh-7.5 cells were infected at an MOI of 1 as previously described. The DNA laddering assay procedure was adapted from the protocol reported by Gong *et al.* with some modifications [496]. Briefly, cells were harvested at time 0 (immediately after infection) and every 24 hours for 5 days. The cells were resuspended in 1 ml of PBS and

transferred to 10 ml of ice-cold 70% ethanol (Cells could be stored following this step at -20°C for weeks). Then, the ethanol-containing supernatants were removed by centrifugation and the cell pellets were resuspended in 40 µl of phosphate-citrate buffer (prepared by mixing 192 parts of 0.2M Na2HPO4 and 8 parts of 0.1 M citric acid, pH=7.8). The cells were incubated in the phosphate-citrate buffer for 30 minutes during which the tubes were vortexed every 10 minutes. The cells were then spin down at 1000 g and the supernatants were collected and transferred to new tubes. Three µl of 0.25 Nonide NP-40 and 3 µl of 1 mg/ml of RNase were added to each tube and incubated for 30 minutes at 37°C. Following that, 5 µl of 1 mg/ml of proteinase K were added to each tube and incubated for another 30 minutes at 37°C. The solutions were then mixed with the loading buffer, loaded on a 2% agarose gel and run at 4 V/cm for 4 hours. The DNA laddering was detected by ethedium bromide staining under UV light.

2.9 SDS-PAGE and western blotting.

The cell lysates were harvested from the infected (MOI of 1) or control cells at the specific time point by using the passive lysis buffer (Promega) and according to the manufacturer's instructions. Lysates was mixed at a 2:1 ratio with the 3X loading dye for SDS-PAGE. The lysates were run in 15% SDS-PAGE and then transferred onto nitrocellulose membranes using the western blotting system obtained from Bio-Rad® and according to the standard protocol. The membranes were incubated overnight with the specific dilution of the cleaved caspase-8 (Asp391)(18C8) rabbit monoclonal antibody (Cell Signaling) in TBS-T buffer. The specific dilution of the antibody recommended by the manufacturer was used.

2.10 Immunostaining for flow cytometry.

Cleavage of PARP and caspase-3 were detected using primary antibodies specific for the cleaved forms of these proteins [Cleaved PARP (Asp214) (D64E10) Rabbit mAb and Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb] using the staining protocol recommended by the manufacturer (Cell Signaling Technology). Briefly, infected or control cells were harvested, washed with 5 ml of PBS, then fixed by resuspending the cell pellet in 4% paraformaldehyde at 37°C for 10 minutes. The tubes were then chilled on ice for 1 minute and permeabilized by resuspending the cell pellet in ice-cold 90% methanol for 30 minutes. The cells were incubated on ice in this solution for 30 minutes or alternatively can be stored in this solution at -20°C for up to 4 weeks. Cells were then washed twice with the incubation buffer (0.5% BSA in PBS) and incubated for 1 hour at room temperature in the presence of the recommended dilution of primary antibody (1:800 for both). Next, the cells were washed with 3 ml of incubation buffer, then incubated for 30 minutes with Alex Fluor® 647 anti-rabbit secondary antibody (Cell Signaling Technology) diluted at 1:400. Cells are then washed and resuspended in 0.5 ml of PBS, then analyzed by flow cytometry using the Becton Dickinson FACS Calibur.

2.11 Caspase and NLRP3 inhibitors.

All caspase inhibitors were dissolved in DMSO according to the manufacturer's instructions (R&D Systems). The inhibitors used were Z-VAD-FMK (pan-caspase inhibitor), Z-IETD-FMK (caspase-8 inhibitor), Z-DEVD-FMK (caspase-3 inhibitor) and Z-WEHD-FMK (caspase-1 inhibitor). The NLRP3 inhibitor (MCC950; Cayman Chemical Company) was dissolved in DMSO to give a stock solution of 0.5 mg/ml. A

working stock of 0.1 μM was prepared fresh in complete medium on the day of the experiment. To ensure efficient inhibition over the extent of the four day experiment, at 48 hours p.i., medium was removed and replaced with medium containing freshly prepared inhibitors.

2.12 Measurement of active caspase-1.

To measure the levels of active caspase-1, FAM-FLICATM Caspase-1 Assay Kits were used (ImmunoChemistry Technologies). Briefly, Huh-7.5 cells were seeded in 6-well plates (150,000 cells/well) and infected at an MOI of 1. Four days later, the cells were harvested by trypsinization and stained with FAM-YVAD-FMK according to the manufacturer's instructions. In co-culture experiments where GFP was included, the FLICA® 660 Caspase-1 Assay Kit, which employs a far-red fluorescent caspase-1 inhibitor, was used.

2.13 Double staining with PI and anti-HCV core.

This protocol is a combination of the PI staining protocol described previously (section 2.7) and the HCV-core staining described by Kannan *et al.* with some modifications [456]. Briefly, infected (MOI of 1) or control cells were harvested by trypsinization and washed with PBS. The cells were fixed in ice-cold 70% ethanol then washed with PBS, then permeabilized using 0.2% Triton X-100. Next, the cells were resuspended in a solution containing mouse anti-HCV-core antibody (C7-50, Thermo scientific) diluted 1:400 in 1% BSA, 0.1% tween 20 in PBS and incubated for 1 hour. Following that, the cells were washed and reconstituted in the DNA extraction buffer for 5 minutes (section 2.7). The cells were then washed and reconstituted in the secondary

antibody (goat anti-mouse Alexa Fluor® 647, Invitrogen) diluted 1:400 in 1% BSA in PBS. Next, the cells were washed twice with PBS and resuspended in DNA staining solution (section 2.7). Finally, the cells were analysed using a Becton Dickinson FACS Calibur flow cytometer.

2.14 Co-culture assay.

On the day before initiation of co-culture, 293T or S29 cells (both non-permissive) were seeded in antibiotic-free medium at 2 x 10^5 cells/well in a 24-well plate. In parallel, 10 cm dishes were prepared each containing 1 x 10^6 Huh-7.5 cells (permissive). Next day, non-permissive cells were transfected with a GFP expression plasmid using Lipofectamine 2000 reagent (Invitrogen) and incubated for 4 hours. In parallel, permissive Huh-7.5 cells were infected with virus at an MOI of 1 and incubated for 3 hours. Immediately following transfection/infection, GFP-transfected non-permissive S29/293T cells were trypsinized, washed thoroughly with complete medium, then combined with infected or uninfected Huh-7.5 cells at a ratio of 1:5 (S29/293T:Huh-7.5). After four days of co-culture, cells were harvested and stained with cleaved PARP-specific antibody, cleaved caspase-3-specific antibody, or FAM-YVAD-FMK as described above.

To analyze contact dependence of HCV-induced cell death, co-culture experiments were performed in 10 cm transwell plates containing an inserts with a diameter of 7.5 cm and pore size of 0.4 μm (Corning). Huh-7.5 cells were seeded at 1 x 10^6 cells/dish and 2 x 10^5 S29 cells were placed in the insert. Huh-7.5 cells were infected at an MOI of 1 and then the S29-containing insert was placed in the dish. The cells were incubated for four

days, then the S29 cells were harvested and stained with either cleaved caspase-3-specific antibody or FAM-YVAD-FMK as described above. The schematic representation of the co-culture method is illustrated in (figure 2.1).

2.15 Lactate dehydrogenase (LDH) assay.

Huh-7.5 cells were seeded in 10 cm dishes at 1 x 10^6 cells/dish and infected on the next day at an MOI of 1. Four days later, 1 ml of the culture medium was collected and clarified. LDH activity was measured using the Pierce™ LDH Cytotoxicity Assay Kit (ThermoFisher Scientific) according to the manufacturer's instructions.

2.16 Statistical analysis.

The data was expressed as the mean +/- the standard deviation (SD). Statistical significance was analyzed using the paired Student's t-test. A *p* value of less than 0.05 was considered significant.

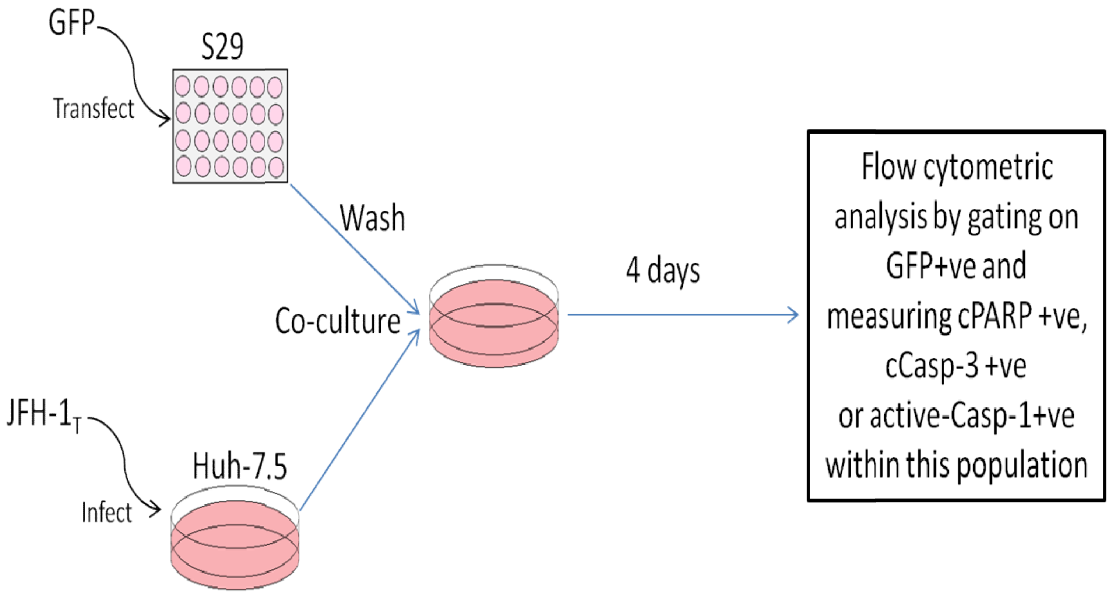


Figure 2.1 Schematic representation of the co-culture method for detecting bystander cell death.

S29 or 293T cells were seeded in 24-well plates then transfected with a GFP expression plasmid. Simultaneously, Huh-7.5 cells were infected at MOI of 1. Following the transfection, the S29/293T cells were trypsinized and washed twice, then co-cultured with the infected Huh-7.5 cells at a ratio of 1:5. Four days later, cells were harvested, stained and analysed by flow cytometry. Gating on the GFP positive cells was used to detect the effect specifically on the S29/293T cells.

Chapter 3: Results - HCV infection induces apoptosis

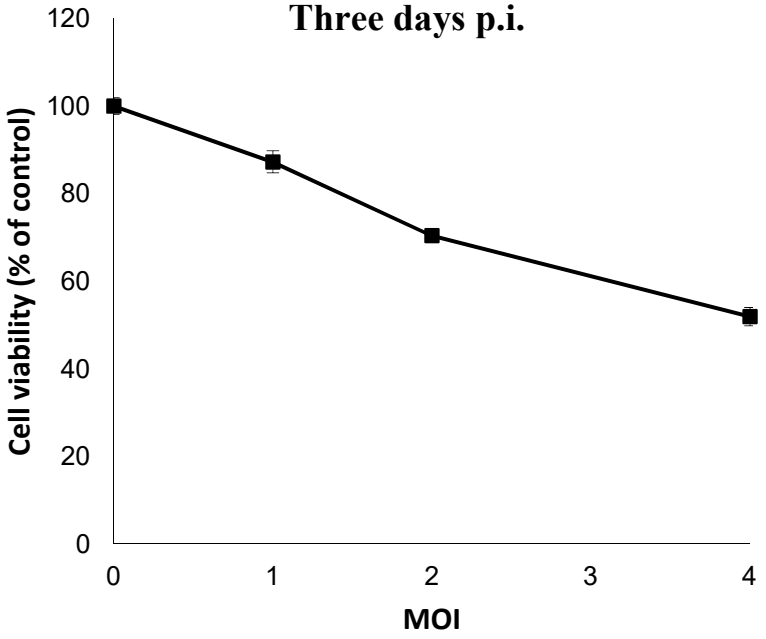
Some of the results described in this Chapter, along with additional data presented in Chapters 4 and 5, are included in a manuscript that is currently accepted pending revisions at *Scientific Reports*.

3.1 HCV infection reduced the viability of the Huh-7.5 cells.

We initially observed by microscopic examination that infecting Huh-7.5 cells with HCV ($JFH1_T$ strain) resulted in a reduction in the total number of cells growing in the dish (as the infected cells appeared less confluent). In addition, many of the cells in the dishes containing virus were floating in the medium instead of being attached to the plate as they are normally. These initial observations prompted us to investigate the reason for these observations and to test the cytopathic and/or cytostatic effects of HCV infection. To achieve this, we started our investigation by performing an MTT assay to test the effect of HCV infection on the total metabolic activity of the infected cell population, which reflects the total number of viable cells.

An MTT assay was performed at two time points, on days three and four p.i. To confirm that the observed effect was virus-specific, we infected Huh-7.5 cells at three different MOIs: 1, 2, and 4. The results of this experiment (Fig. 3.1) showed that HCV infection significantly reduced the total number of viable cells, and a greater reduction was observed on day four compared to day 3 p.i. The reduction in viability was associated with the MOI used, where higher MOIs caused greater reductions in the viability. This association was more obvious on day 3 post infection. On day 4 p.i., there was no significant difference between the reduction caused by the high MOIs (2 and 4). This is

A


Three days p.i.
Cell viability (% of control)
120
100
80
60
40
20
0
0
1
2
3
4
MOI

B

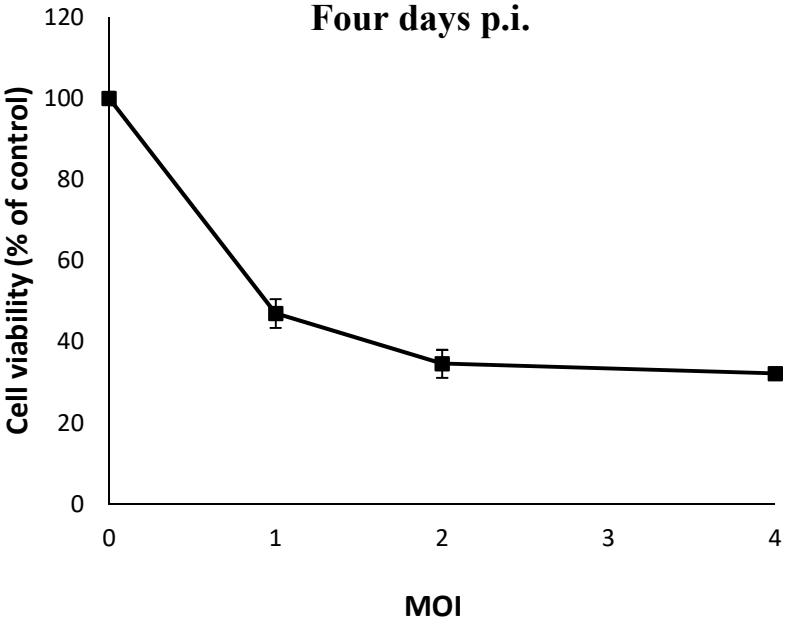

Four days p.i.
Cell viability (% of control)
120
100
80
60
40
20
0
0
1
2
3
4
MOI

Figure 3.1 HCV infection reduced the total viability of the infected cell population. Huh-7.5 cells were infected with $JFH1_T$ at MOIs of 1, 2 and 4. Three days p.i. (**A**) or four days p.i. (**B**) the total viability of the cells was tested by MTT assay. This data is representative of three independent experiments in (A) and two independent experiments in (B), and is expressed as mean cell viability compared to the uninfected control +/- SD from three replicates.

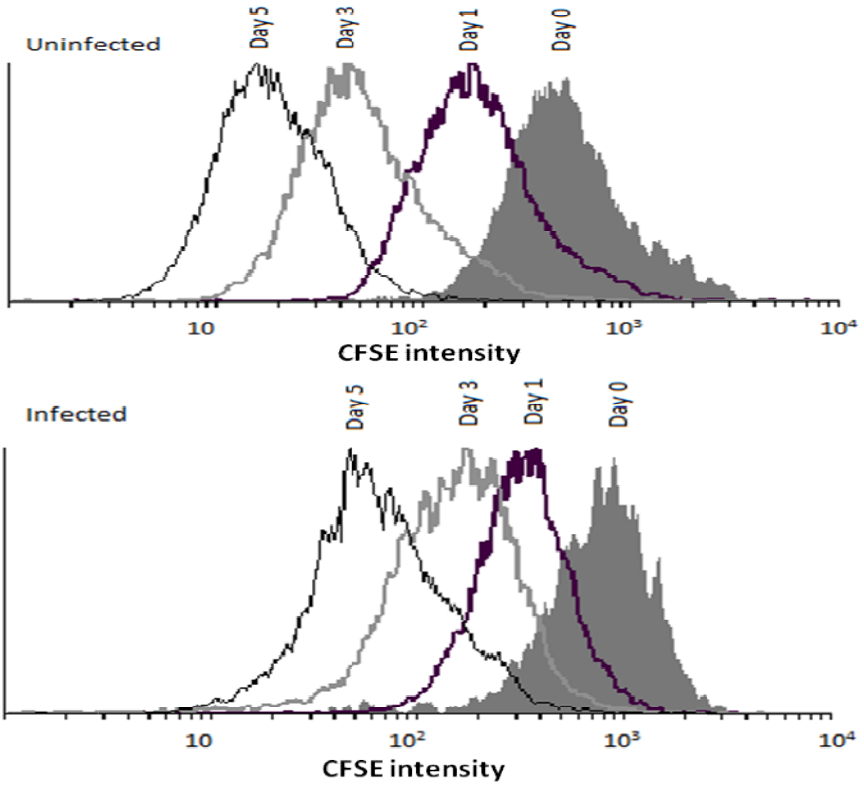
probably the result of achieving a very high percentage (close to 100%) of infected cells in advance of performing the MTT assay on day four, which could mask the effect of the differences between these very high MOIs. These lower levels of total cellular viability could result from either a reduction in the proliferation rate or increased cell death in the infected cell population. For this reason, both of these two possibilities were investigated independently.

3.2 HCV infection caused a reduction in the proliferation rate of the infected cells.

The reduction in the proliferation rate and the cell cycle arrest of the HCV-infected cells was reported previously by using other versions of HCVcc [455,456]. The possibility that a similar effect is occurring in our system and that this could be responsible, at least partially, for the observed reduction in the viability was tested using a CFSE dilution assay. Huh-7.5 cells were infected at MOI of 2 or left uninfected and then stained with the CFSE. As mentioned in detail in section 2.6, CFSE is a dye that is sequestered inside the cell by covalent coupling to intracellular molecules. Cell division results in splitting the cellular CFSE content between the two daughter cells and each subsequent round of division results in higher dilution of the cellular content of CFSE. Hence, monitoring the intensity of this dye in the cell population is a very useful tool for tracking cellular proliferation [497].

We investigated the effect of HCV infection on the proliferation rate by measuring the CFSE intensities in both the infected and uninfected cell populations at different time points. The time points chosen started from time zero (which is the mean intensity measured immediately after infection and labelling) and every 24 h for up to 5 days. We

A


Uninfected
Day 5
Day 3
Day 1
Day 0
10
10²
10³
10⁴
CFSE intensity
Infected
Day 5
Day 3
Day 1
Day 0
10
10²
10³
10⁴
CFSE intensity

B

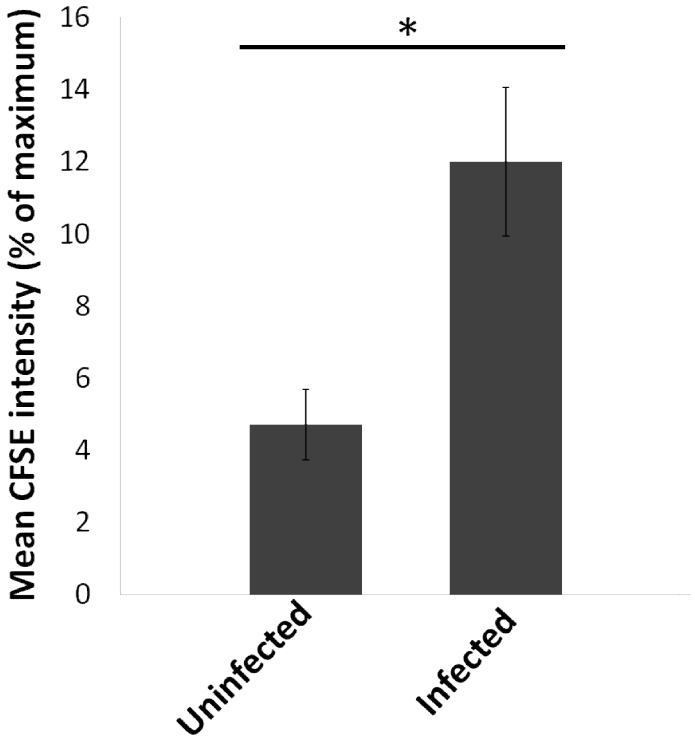

Mean CFSE intensity (% of maximum)
16
14
12
10
8
6
4
2
0
*
Uninfected
Infected

Figure 3.2 HCV infection caused a reduction in the proliferation rate.

Huh7.5 cells were infected at MOI of 2, then labelled with CFSE stain. Cells were harvested immediately after labelling (Day 0) and on each day for 5 days p.i. CFSE labelling intensity was measured by flow cytometry. **(A)** CFSE labelling intensity of infected and control cell populations on days 0, 1, 3 and 5. Data presented in this figure are representative of three independent repeats of this experiment **(B)** Mean CFSE intensities of the infected and control cell populations on day four p.i. Data is presented as the mean CFSE intensity of three independent repeats of the experiment, expressed as the percentage of CFSE intensity compared to the maximum intensity measured at time zero, +/- SD. * $P < 0.05$ (Student's t-test).

observed higher CSFE intensities in the infected populations than the uninfected (Fig. 3.2A). These differences became statistically significant on day four p.i. (Fig. 3.2B) when the mean CFSE intensity of the infected population was more than double that of the uninfected cell population. This result indicated that (on average) the infected cells had undergone one cycle of cell division less than did the control cells. This means that HCV infection causes a reduction in the proliferation rate, which is in agreement with previous reports [455,456].

3.3 HCV infection induced caspase-dependent, DNA fragmentation-inducing programmed cell death.

Concurrently, we tested the possibility that HCV induces programmed cell death (PCD) in the infected cell population. This can also cause a reduction in the total number of viable cells detected by the MTT assay. To achieve this, two features of PCD were tested: caspase activation and DNA fragmentation.

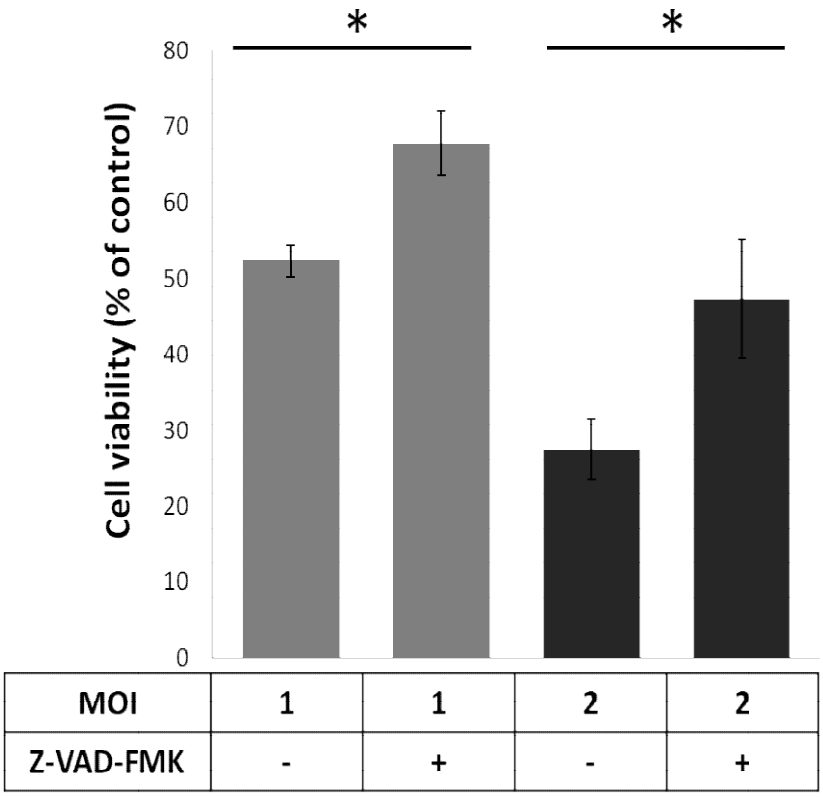
First, the role of caspase activation in the viability reduction seen in the HCV-infected population was tested. Apoptosis and pyroptosis are two types of PCD that are both dependent on activation of caspases. To test the possibility that the reduction in viability was caused by induction of one or both of these forms of PCD, we tested the effect of blocking the activation of caspases on the total viability of the HCV-infected Huh-7.5 cell population. Infected (MOIs of 1 and 2) or uninfected control cells were treated with the pan-caspase inhibitor Z-VAD-FMK. The effect of this treatment on viability was then measured by MTT assay. The results of this experiment showed that the inhibition of caspases by Z-VAD-FMK increased the total viability of the HCV-infected population of

cells (Fig. 3.3A). This effect was specific for the reduction in the viability caused by HCV infection as treating the uninfected cells with Z-VAD-FMK did not increase their viability. This result indicated that caspase-dependent PCD was induced and contributed to the reduction in viability observed in response to HCV infection.

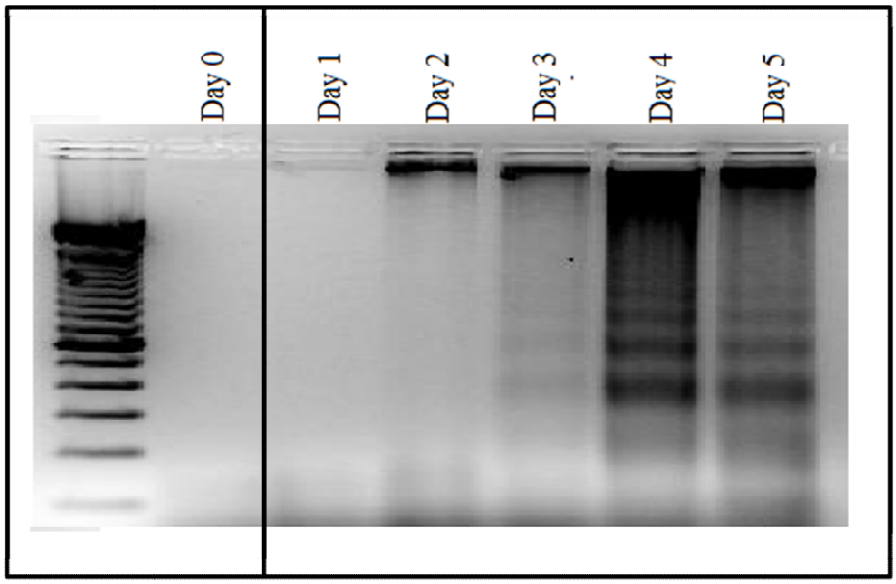
To confirm that apoptosis or pyroptosis are induced by HCV infection, we tested a second feature of those two forms of programmed cell death, which is the induction of DNA fragmentation. As described previously (section 1.5.1.1), internucleosomal DNA fragmentation is one of the hallmarks of apoptosis. As a result of this, the DNA of apoptotic cells becomes fragmented into segments equivalent to the DNA content of one nucleosome (180 bp) or oligonuecleosomes. Running the DNA of apoptotic cells on agarose gel electrophoresis gives characteristic bands at multiples of 180 bp [498,499]. Aside from the classical laddering assay, DNA fragmentation during apoptosis provides the basis for many other apoptosis detection tests, including the detection of the hypodiploid cells in a cell cycle analysis [495,500]. Pyroptosis can also result in formation of hypodiploid cells as it is also known to cause DNA fragmentation (described in section 1.5.2).

DNA fragmentation in the HCV-infected cell population was tested first by the classical DNA laddering assay. The low molecular weight DNA was extracted from HCV-infected Huh-7.5 cells immediately after the infection (day 0) and every 24 hours up to day 5 p.i. The results of this assay showed a faint laddering appearing first on day 3 p.i. The highest laddering intensity was seen on day 4 p.i. (Fig. 3.3B). Laddering was also detected on day 5 p.i., but the intensity of the bands were less than that seen on day 4 p.i.

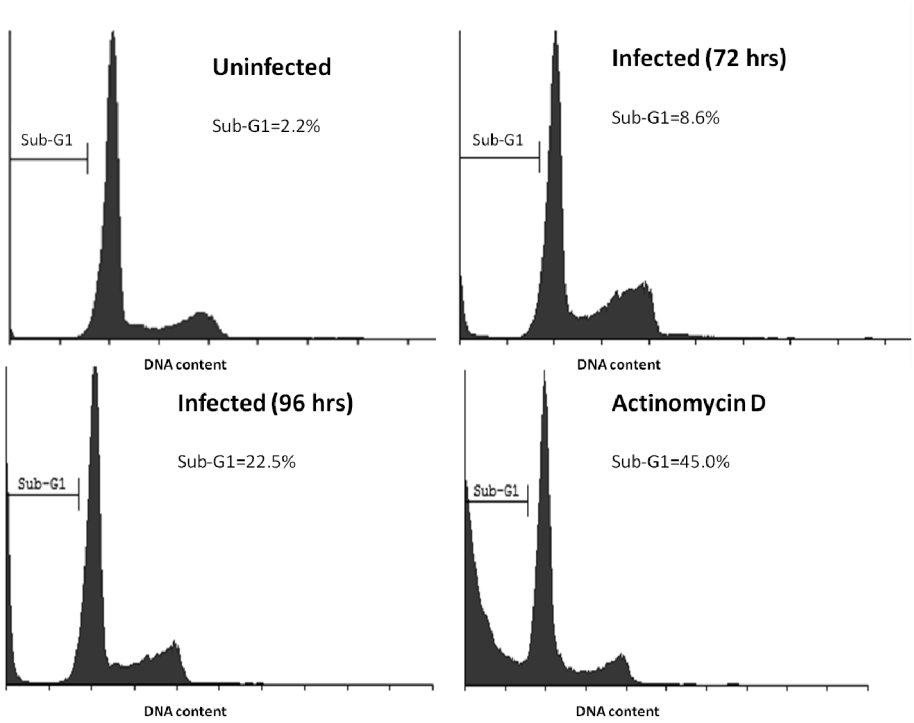
A


*
*
Cell viability (% of control)
80
70
60
50
40
30
20
10
0
MOI 1 1 2 2
Z-VAD-FMK - + - +

B


Day 0
Day 1
Day 2
Day 3
Day 4
Day 5

C


Uninfected
Sub-G1=2.2%
Sub-G1
DNA content
Infected (72 hrs)
Sub-G1=8.6%
Sub-G1
DNA content
Infected (96 hrs)
Sub-G1=22.5%
Sub-G1
DNA content
Actinomycin D
Sub-G1=45.0%
Sub-G1
DNA content

D

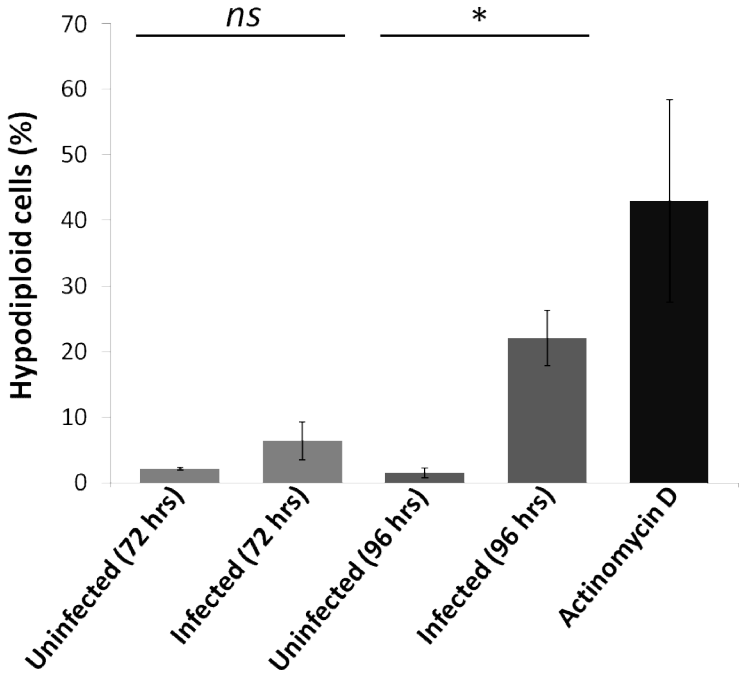

ns
*
70
60
50
40
30
20
10
0
Hypodiploid cells (%)
Uninfected (72 hrs)
Infected (72 hrs)
Uninfected (96 hrs)
Infected (96 hrs)
Actinomycin D

Figure 3.3 HCV infection induced a caspase-dependent, DNA fragmenting PCD.

(A) Huh-7.5 cells were infected at MOIs of 1 or 2 then incubated for 96 hours in complete medium containing either 20 μM of the pan-caspase inhibitor Z-VAD-FMK or an equivalent volume of DMSO as a control. Viability was measured on day 4 p.i. by MTT assay. The data presented in this figure is representative of two independent experiments in which each sample was tested in triplicate. The data is presented as the percentage of the viability compared to the uninfected control. **(B)** DNA laddering assay to detect DNA fragmentation. Huh-7.5 cells were infected at MOI of 1, low molecular weight DNA was extracted immediately after infection (day 0) and every 24 hours up to 5 days p.i. The data presented in this figure is representative of three independent experiments. Original gel image was spliced to remove an empty lane **(C) & (D)** Huh-7.5 cells were infected at MOI of 1 or left uninfected, the cells were harvested after 72 hours, or 96 hours p.i. Huh-7.5 cells incubated in complete medium containing 50 ng/ml of actinomycin D for 48 hours (due to potency) were used as a positive control. The harvested cells were permeabilized and stained with PI, then cell cycle analysis was performed and the percentage of hypodiploid cells was determined. The uninfected cell population presented in (C) was harvested 96 hours p.i. The data is presented in (D) as the mean percentage of the hypodiploid cells for four independent experiments +/- SD. *ns: Not significant,* * $P < 0.05$ (Student's t-test).

This is most probably due to the lower number of cells that remained intact until day 5 p.i due to the extensive cell death that occurred on day 4 p.i. No DNA laddering was detected in the uninfected cells even after 5 days of incubation (data not shown), meaning that this laddering is specifically induced by HCV infection. The induction of DNA fragmentation in HCV-infected cells was confirmed by analyzing the hypodiploid cells through cell cycle analysis. DNA fragmentation results in the formation of low molecular weight DNA fragments that can be extracted from fixed/permeabilized cells, leaving these cells with less DNA than a normal cell [495]. To test for DNA fragmentation, infected or uninfected cells were permeabilized and stained with propidium iodide (PI), which is a dye that binds to the DNA and gives fluorescence intensity that correlates with the DNA content of the nucleus. By performing a cell cycle analysis of these stained cells, the apoptotic/pyroptotic cells can be detected in the Sub-G1 region.

We were able to detect a slight increase in the number of hypodiploid cells in the infected Huh-7.5 population on day 3 p.i., however, this increase was not statistically significant (Fig. 3.3C & D). Significantly higher levels of hypodiploid cells were detected on day 4 p.i. in the infected, but not the uninfected, Huh-7.5 cells. This result confirmed that HCV infection induced DNA fragmentation in the HCV-infected population of cells. In summary, HCV infection was found to induce caspase-dependent, DNA fragmentation-inducing PCD. Both apoptosis and pyroptosis can be responsible for causing these effects. The ability of HCV infection to induce each one of these two forms of PCD was investigated separately and the results of this investigation will be presented in the next section and in Chapter 5.

3.4 HCV infection induced apoptosis.

First, we tested the ability of HCV infection to induce apoptosis by performing apoptosis-specific assays. As discussed in more detail in section 1.5, one of the main characteristics that differentiate between apoptosis and pyroptosis is the type of caspase that is responsible for their induction. Regardless of the pathway that initiates apoptosis, caspase-3 and other executioner caspases are always activated in order to induce the final events that cause the demise of the cell. Pyroptosis, on the other hand, is a caspase-3-independent form of PCD that depends solely on the activation of caspase-1 (reviewed in [359]).

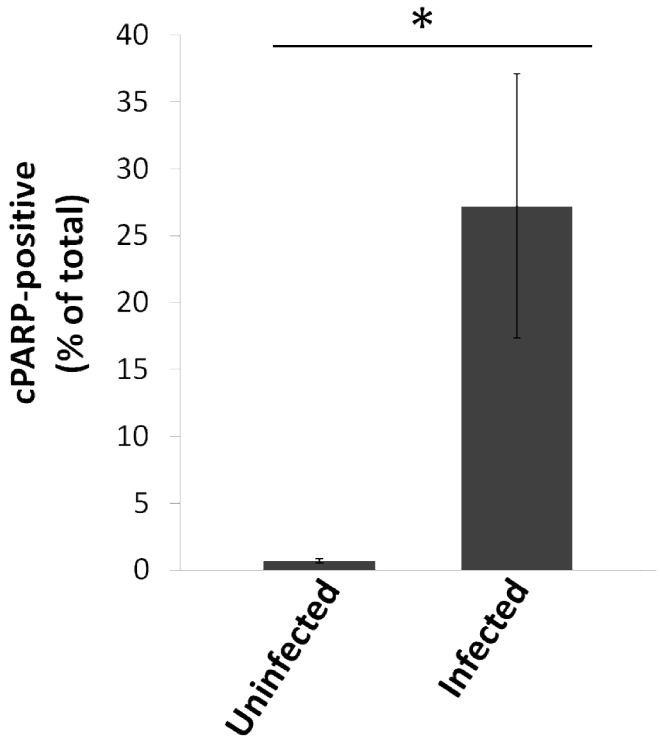
The activation of caspase-3 was tested first by measuring the cleavage of PARP, which is one of the main substrates of caspase-3 and its cleavage is commonly used as a marker for monitoring caspase-3 activation and apoptosis. Infected (MOI of 1) or uninfected control Huh-7.5 cells were harvested on day 4 p.i. and stained with an antibody that binds specifically to the cleaved form of PARP (cPARP), then analysed by flow cytometry. HCV infection was found to result in significantly higher numbers of cPARP-positive cells compared to the control (Fig 3.4A). However, and as discussed previously (section 1.5), some groups reported the possibility that caspase-1 activation can also cause the cleavage of PARP, which questions the specificity of this test for the detection of apoptosis. For this reason, caspase-3 activation was confirmed more specifically by staining cells with an antibody that binds to the active, cleaved form of caspase-3. In agreement with the cPARP analysis, HCV infection was found to result in significantly higher numbers of cleaved caspase-3-positive cells compared to uninfected

cells (Fig. 3.4B). These results suggested that HCV infection caused the induction of caspase-3 activation and apoptosis.

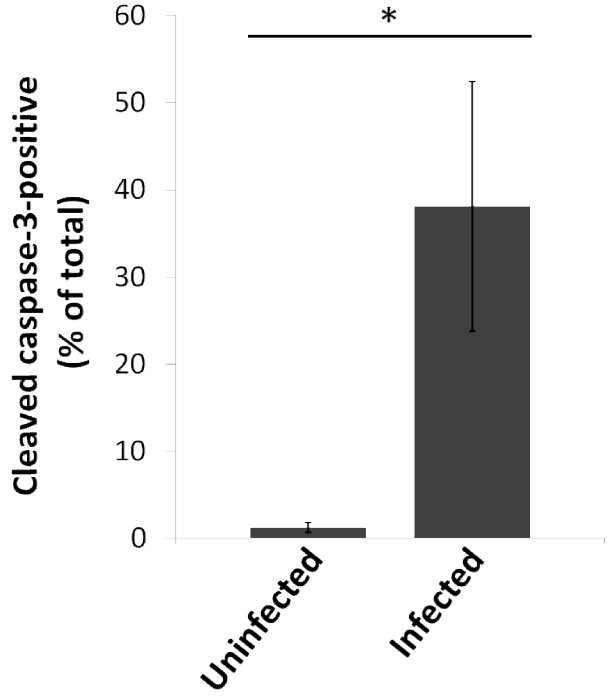
To confirm the induction of apoptosis and the role of caspase-3 activation in HCV-induced PCD (which in this case would be classified as apoptosis), we inhibited caspase-3 specifically in HCV infected Huh-7.5 cells and monitored the effect of this inhibition on the percentage of the cells undergoing PCD. This was performed by treating HCV-infected Huh-7.5 cells with Z-DEVD-FMK (a caspase-3-specific inhibitor) and monitoring the effect of this treatment on the number of hypodiploid cells present. The results of this experiment showed that the blockade of caspase-3 caused a small, but statistically significant reduction (~25%) in the percentage of hypodiploid cells contained within the target cell population (Fig. 3.4C). This result confirmed that apoptosis is induced in the HCV-infected population.

Finally, we also observed features of apoptosis in the target cell population by electron microscopy (EM) as cell condensation and plasma membrane blebbing were clearly evident (Fig 3.4D). However, plasma membrane disruption and cellular debris were also observed, indicative of a lytic form of cell death. These observations, in combination with the relatively moderate effect of inhibiting caspase-3 on reducing the number of hypodiploid cells prompted us to question whether HCV infection also causes pyroptosis, which is known to cause DNA fragmentation, but with lysis of affected cells [360,361].

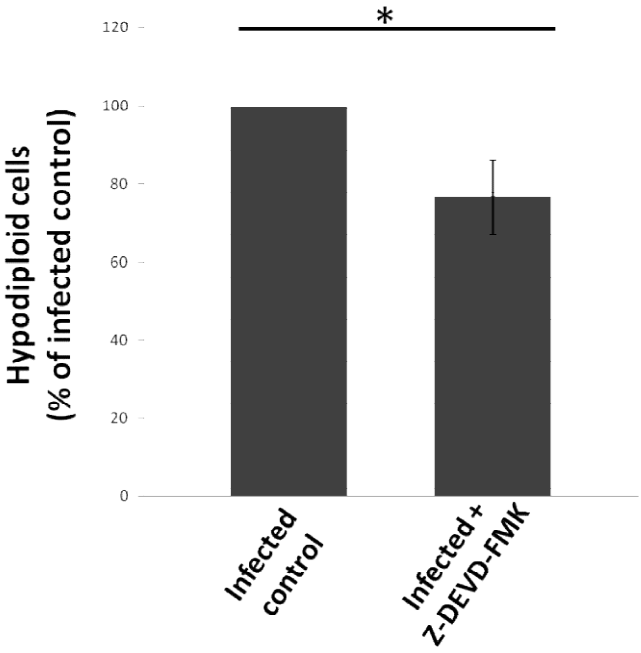
A


*
40
35
30
25
20
15
10
5
0
cPARP-positive
(% of total)
Uninfected
Infected

B


*
60
50
40
30
20
10
0
Cleaved caspase-3-positive
(% of total)
Uninfected
Infected

C


*
120
100
80
60
40
20
0
Hypodiploid cells
(% of infected control)
Infected control
Infected + Z-DEVD-FMK

D

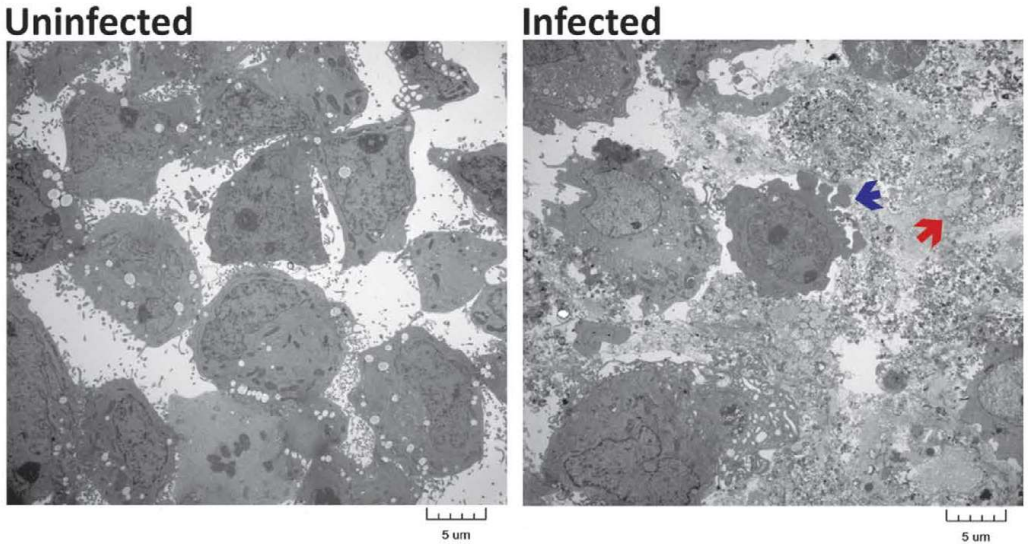

Uninfected
Infected
5 um
5 um

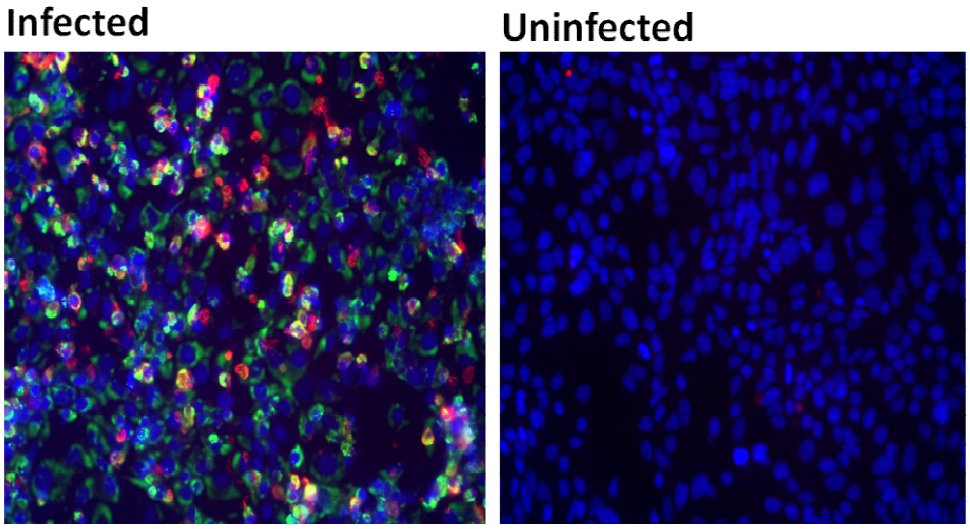
Figure 3.4 HCV infection induced apoptosis.

Huh-7.5 cells were infected at an MOI of 1 then harvested on day 4 p.i. The harvested cells were stained for cleaved PARP in **(A)** or cleaved caspase-3 in **(B)**. **(C)** Infected cells were grown in medium containing 100 μM of the caspase-3 specific inhibitor Z-DEVD-FMK or an equivalent volume of DMSO for 4 days, then harvested and stained with PI. **(D)** EM images of control or infected Huh-7.5 cells showing apoptotic features such as plasma membrane blebbing indicated by the blue arrow. The red arrow indicates cellular debris from a cell that had undergone lysis. Data are presented in A, B & C as the mean of three independent experiments +/- SD. Data presented in C represent the percentage of the hypodiploid cells compared to the untreated control. *$P < 0.05$ (Student's t-test).

3.5 HCV infection induced the activation of caspase-8, the initiator of the extrinsic pathway.

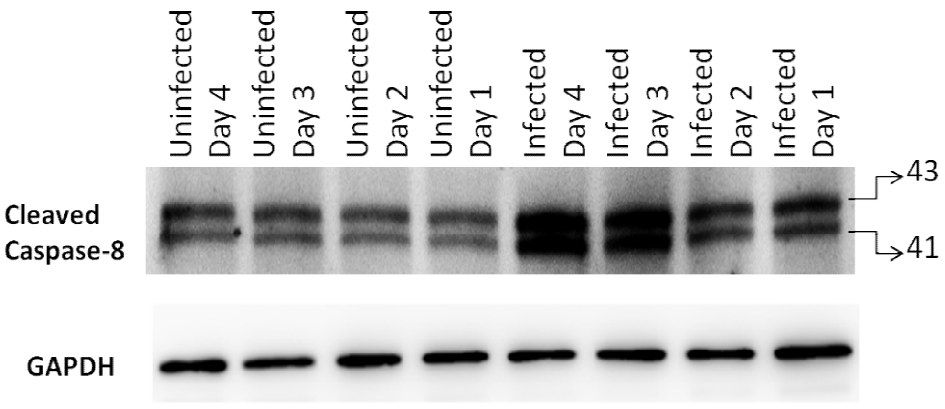
After showing that HCV-infection induces apoptosis, we asked how this apoptosis is induced and what apoptotic pathways are involved in the induction of this form of cell death. The activation of the mitochondrial pathway was reported and analysed previously [453,454]. However, the effect of HCV on the extrinsic pathway has not been studied before in the context of a fully infectious HCV. To test this effect, Huh-7.5 cells were infected at an MOI of 1, and then the activation of caspase-8 was analyzed using a specific antibody that binds to the active, cleaved caspase-8. By examining the stained cells under the fluorescent microscope, we were able to visualize active caspase-8 in the infected cell population but not in the control on day 4 p.i. (Fig. 3.5A). The same antibody was used in Western blotting to study the activation of caspase-8 at different time points following the infection. Cell lysates were obtained from infected or uninfected cells every 24 hours for 4 days and the level of active caspase-8 was compared. We detected higher levels of active caspase-8 in infected cells than in the control cells as early as 24 hours following the infection (Fig. 3.5B), and continued to increase until day 4 p.i. To test whether caspase-8 activation was important for the induction of apoptosis in the HCV-infected cell population, we specifically inhibited caspase-8 using Z-IETD-FMK and monitored the effect of this inhibition on the percentage of hypodiploid cells. We found that this treatment caused a significant decrease in the percentage of hypodiploid cells in the infected cell population (Fig. 3.5C). These results confirmed that caspase-8 is activated following HCV infection and it plays a role in the HCV-induced apoptosis.

A



Blue: DAPI/ Green: HCV core/ Red: cleaved caspase-8

B



C

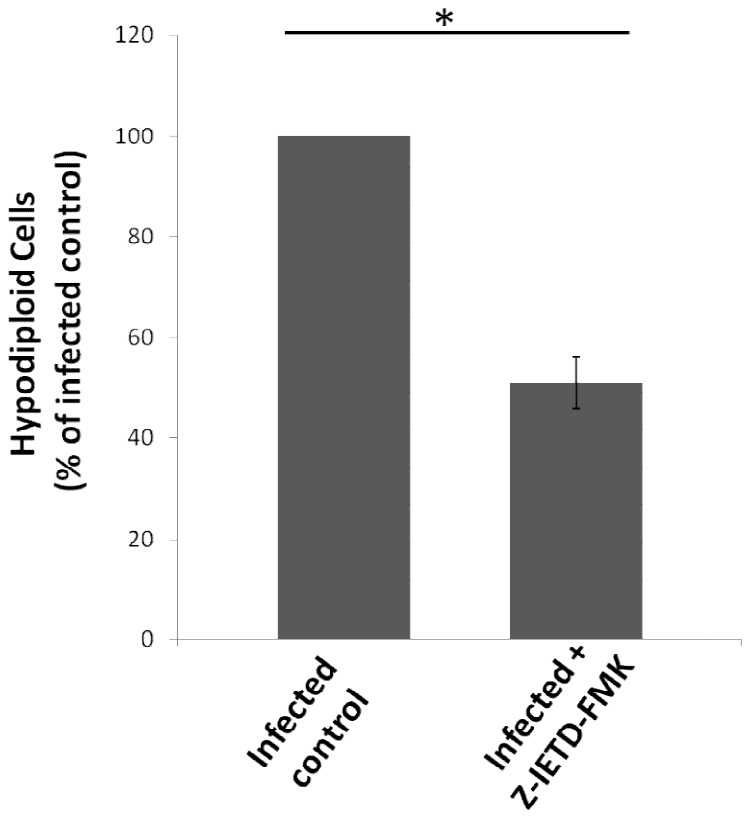


Figure 3.5 HCV infection induced the activation of caspase-8.

Huh-7.5 cells were infected at an MOI of 1. **(A)** The cells were stained 3 days p.i. for HCV core protein (green) and cleaved caspase-8 (red). **(B)** Cell lysates were harvested daily from control and HCV-infected cells up to day 4 p.i. Western blotting was performed to detect cleaved, activated caspase-8. **(C)** The effect of specific inhibition of caspase-8 on the apoptosis was tested by treating cells with 100 μM Z-IETD-FMK then measuring the effect of this treatment on the percentage of hypodiploid cells. Results shown in the graph are the mean percentage of the hypodiploid cells compared to the infected untreated control. The data is presented as the mean of three independent experiments +/- SD. ** $P < 0.005$ (student's t-test).

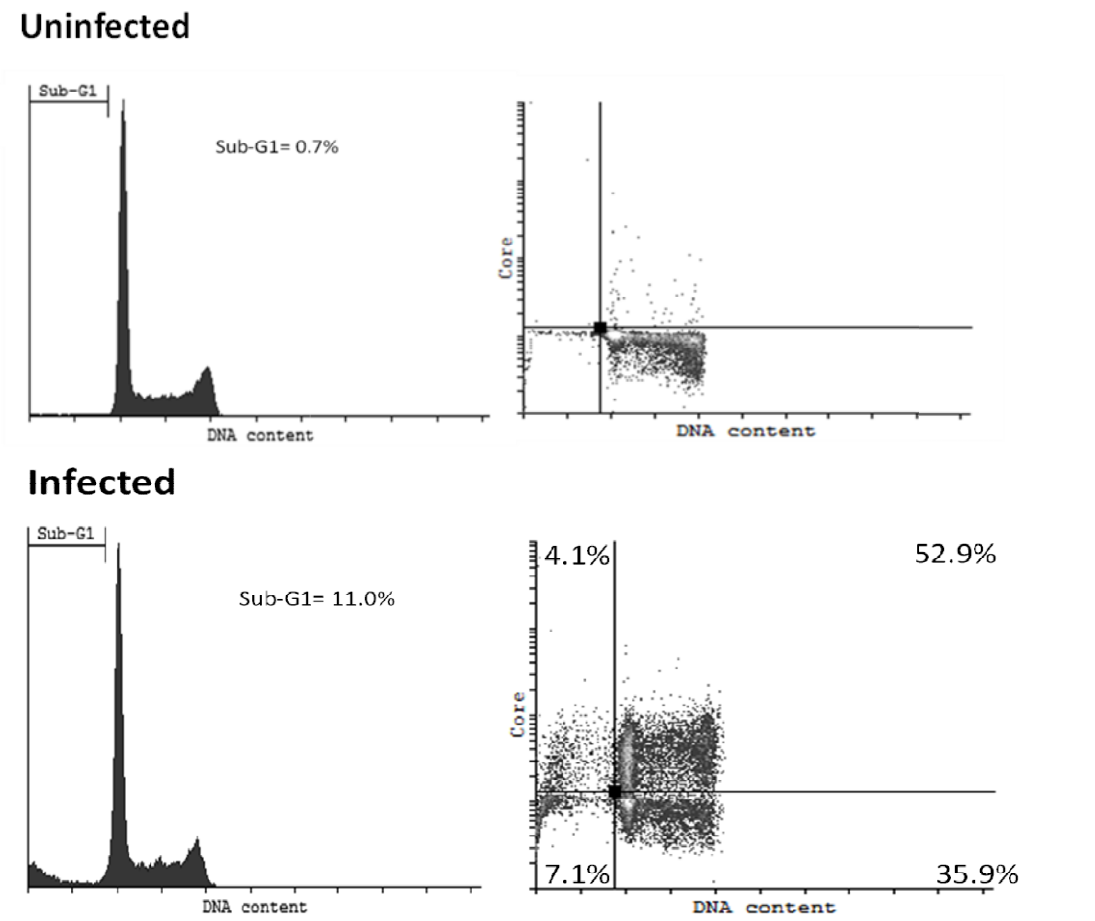
Chapter 4: Results - HCV infection induces a contact dependent bystander apoptosis

In the previous chapter, we demonstrated that HCV infection induces apoptosis in the infected cell population. However, the data presented in the previous chapter cannot distinguish whether the apoptosis is occurring exclusively in the infected cells, or whether it also affects the neighbouring uninfected cells, i.e., "bystander apoptosis". The concept of bystander apoptosis was reported previously in other viral infections. Bystander apoptosis is believed to be an important factor in the pathogenesis of cytomegalovirus retinitis [501,502]. Bystander apoptosis was also reported to be responsible for the CD4+ T cell decline in HIV-infected patients (reviewed in [503]). Furthermore, bystander apoptosis is induced in Ebola infection and causes massive lymphocyte death [504,505]. We are the first group to report the observation that HCV infection can induce bystander apoptosis, and the results of this investigation will be presented in this chapter.

4.1 HCV infection induced programmed cell death in both core-positive and core-negative cell populations.

In this experiment, infected (MOI of 1) or control cells were double stained with anti-HCV core antibody and PI to test whether high levels of intracellular core are required for the induction of apoptosis. The flow cytometric analyses of these cells showed a surprising result as hypodiploid cells were seen in both the core-positive and the core-negative populations (Fig. 4.1). This result means that DNA fragmentation-causing PCD is induced in Huh-7.5 cells regardless of their level of core protein, i.e., both infected and uninfected "bystander" cells. This prompted us to ask whether this observed death in the

A


Uninfected
Sub-G1
Sub-G1= 0.7%
DNA content
Core
DNA content
Infected
Sub-G1
Sub-G1= 11.0%
DNA content
4.1%
52.9%
Core
7.1%
35.9%
DNA content

B

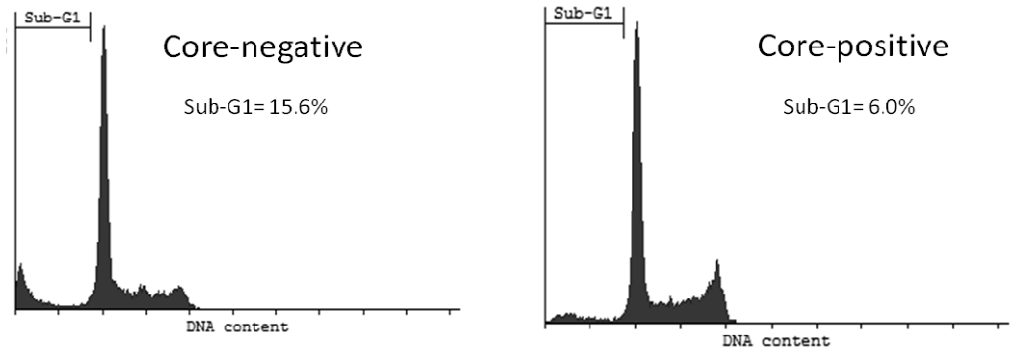

Sub-G1
Core-negative
Sub-G1= 15.6%
DNA content
Sub-G1
Core-positive
Sub-G1= 6.0%
DNA content

Figure 4.1 HCV infection induced DNA fragmenting PCD in neighbouring uninfected cells.

Huh-7.5 cells were infected at an MOI of 1. Three days p.i., cells were harvested and stained with anti-HCV core and PI. FACS analysis was used to determine the percentage of hypodiploid cells in the HCV core-negative and core-positive populations. **(A)** Percentage of hypodiploid cells in the infected and control cells in the total population of cells. **(B)** Percentage of hypodiploid cells after gating on either core-negative or core-positive sub-populations within the infected Huh-7.5 cells. The data presented in this figure is representative of three independent experiments.

core-negative cells is actually apoptosis and whether it can occur in the uninfected cells.

4.2 HCV infection induced bystander apoptosis.

To test for bystander apoptosis, we co-cultured Huh-7.5 cells with HCV-non-permissive cell lines and then monitored the effect of infecting the Huh-7.5 cell on the neighbouring non-permissive cells. The co-culture was performed at a ratio of five Huh-7.5 cells to one HCV-non-permissive cell. Firstly, we co-cultured Huh-7.5 cells with the closely related S29 cells. The S29 cell line is a sub-clone of Huh-7 cells that lacks CD81, the key receptor for HCV entry [187]. Unpublished data from our lab showed that CD81 is required for HCV infection and that the S29 cell line cannot be infected efficiently with HCV unless it is transfected with a CD81 expression plasmid.

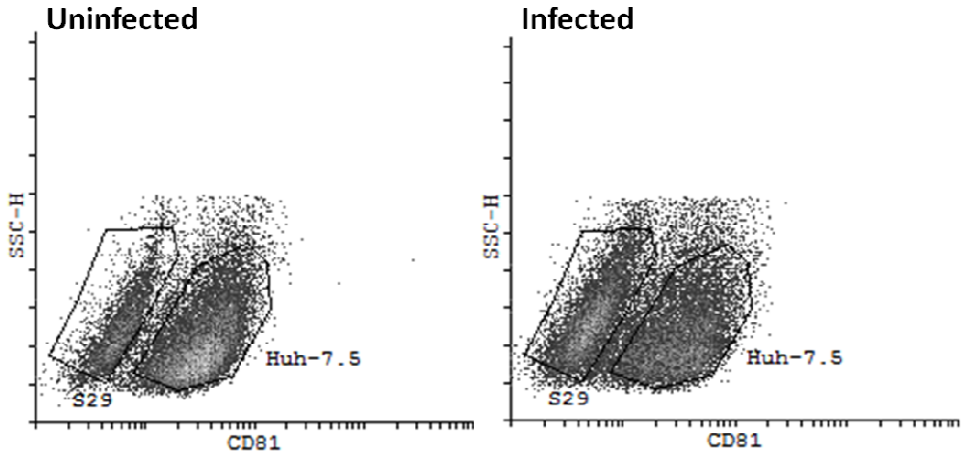
To differentiate between the two types of cells in the co-culture, we first stained the harvested cells with CD81 antibody, then gated on the CD81-positive or CD81-negative cell lines. We were able to see higher percentage of cPARP-positive cells within the CD81-negative population when it was co-cultured with infected Huh-7.5 cells than when it was co-cultured with uninfected control Huh-7.5 cells (Fig. 4.2A & B). However, we also noticed that HCV infection caused a reduction in the levels of CD81 expression. This is likely a strategy used by the virus to prevent super-infection, or possibly the virus causes infected cells to reduce the overall cellular protein expression, including CD81, in order to focus the protein synthesis machinery on viral protein synthesis. Regardless of the reason behind it, this reduction questioned the separation between the infected Huh-7.5 cells and the S29 cells based on the CD81 expression. To rule out the possibility that the reduction in CD81 expression was causing some of the infected Huh-7.5 cells to

appear in the CD81-negative gate and cause an increase in cPARP positive cells within this population we used a second approach to differentiate between the S29 cells and the Huh-7.5 cells in the co-culture.

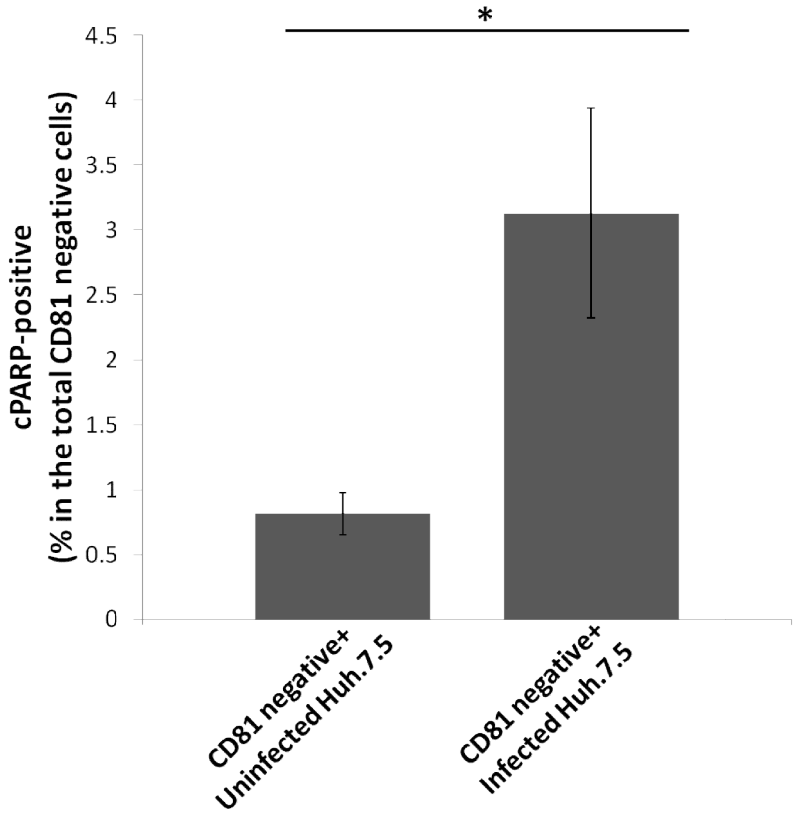
We labelled the S29 cells directly by transfecting them with a GFP-expressing plasmid. The transfected S29 cells were then co-cultured with either infected or control Huh-7.5 cells. At the end of the incubation period, the mixed population was harvested and stained with antibodies against cPARP or cleaved caspase-3 (see Fig. 2.1 for illustration of this strategy). Gating on the GFP-positive population provided us with a tool to detect caspase-3 activity specifically within the S29 cell population. Analysing the data obtained by this approach revealed that significantly higher percentage of cPARP-positive and cleaved caspase-3-positive cells were detected in the S29 cell population when they were co-cultured with infected Huh-7.5 cells (Fig. 4.2C, D & E). These results suggested that HCV infection induced bystander apoptosis.

Next, we asked whether the observed bystander apoptosis was specifically induced in neighbouring hepatocytes or does this bystander effect also extend to other cell types. To answer this, we co-cultured infected or control Huh-7.5 cells with 293T cells. The 293T cells were originally obtained from human embryonic kidney and they do not express CLDN-1, which is another key receptor for HCV. These cells are not permissive to HCVcc infection, and only very low susceptibility (1000-fold less than Huh-7.5) were reported in these cells when they ectopically express CLDN-1 [139]. We observed that,

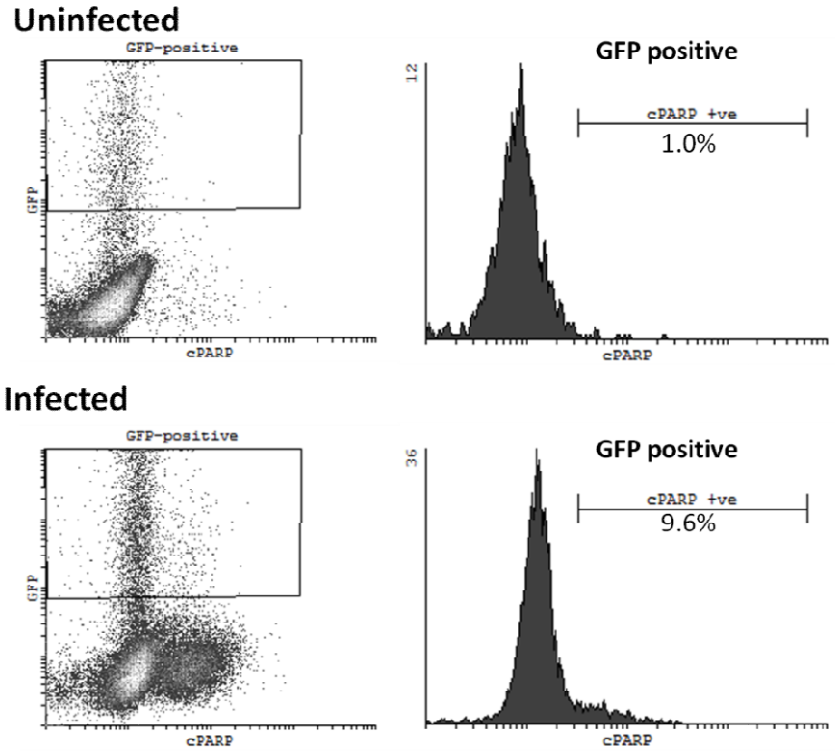
A


Uninfected
Infected
SSC-H
SSC-H
Huh-7.5
Huh-7.5
S29
S29
CD81
CD81

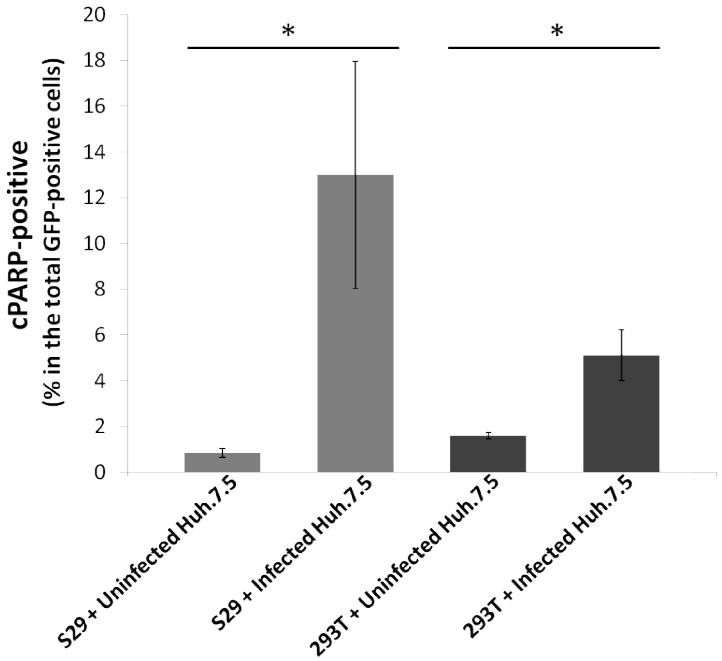
B


*
cPARP-positive
(% in the total CD81 negative cells)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
CD81 negative+
Uninfected Huh.7.5
CD81 negative+
Infected Huh.7.5

C


Uninfected
GFP-positive
GFP
cPARP
GFP positive
12
cPARP +ve
1.0%
cPARP
Infected
GFP-positive
GFP
cPARP
GFP positive
36
cPARP +ve
9.6%
cPARP

D


cPARP-positive
(% in the total GFP-positive cells)
20
18
16
14
12
10
8
6
4
2
0
*
*
S29 + Uninfected Huh.7.5
S29 + Infected Huh.7.5
293T + Uninfected Huh.7.5
293T + Infected Huh.7.5

E

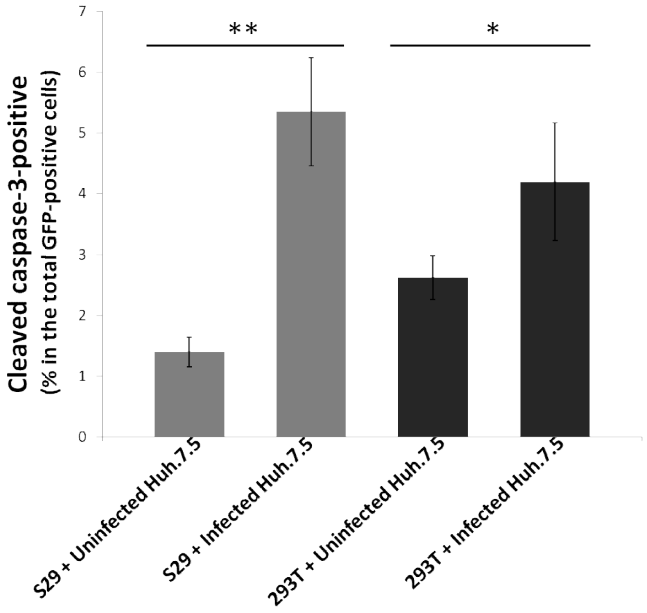


Figure 4.2 HCV infection induced bystander apoptosis.

Huh-7.5 cells were infected at MOI of 1 then co-cultured with either S29 cells or 293T cells at a ratio of 5:1 for 3 days in **(A & B)** or for 4 days in **(C, D & E)**. The S29 or the 293T cells were transfected with GFP-expressing plasmid prior to the start of the co-culture in **(C, D & E)**. Following the co-culture, the cells were harvested and stained with CD81 and cPARP antibodies in **(A & B)**, with cPARP antibody only in **(C& D)**, or cleaved-caspase-3 antibody only in **(E)**. The stained cells were analysed by flow cytometry and the S29 cells or 293T cells were differentiated in the analysis by gating on either the CD81-negative population in **(A & B)** the GFP-positive population in **(C, D & E)**. The data is presented as the percentage of cPARP or cleaved-caspase-3 positive cells among the S29 or the 293T cell population. Mean +/- SD is shown from at least three independent experiments. * indicates $p < 0.05$ and ** indicates $p < 0.005$ (Student's t-test).

similar to S29 cells, co-culturing the 293T cells with infected Huh-7.5 cells induced a higher percentage of caspase-3 activity among them (Fig 4.2D & E). This suggested that bystander apoptosis was induced in the 293T cells that were co-cultured with infected Huh-7.5 cells. However, the degree of induction of bystander apoptosis in the 293T cells was lower than that observed in the S29 cells. The possible explanations for this weak induction will be discussed in Chapter 6.

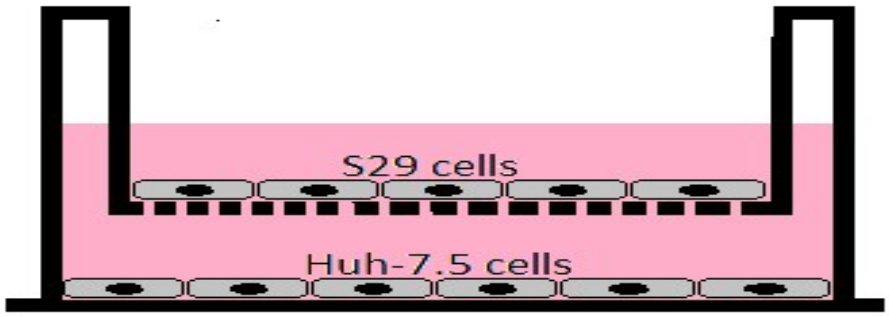
4.3 The induction of bystander apoptosis required cell-cell contact between the infected and the bystander cells.

After showing that HCV infection induces bystander apoptosis, we were interested in the mechanism by which this bystander apoptosis was induced. The infected cell could induce the death of its neighbouring cells by expressing certain death ligands that could interact with their cognate receptors on the adjacent cell to induce the extrinsic apoptotic pathway. Alternatively, the death signal could be transmitted via soluble mediators or exosomes. In the first scenario, cell-cell contact would be necessary for the induction of apoptosis. However, in the case of soluble mediators/exosomes-dependent transmission, cell-cell contact would not be required and the death could be induced even in cells that are relatively distant from the infected cell.

To determine which of these mechanisms is responsible for HCV-induced bystander apoptosis, we performed transwell assays. In these experiments, we prepared a co-culture of infected versus uninfected Huh-7.5 cells (lower chamber) with S29 cells (upper chamber) at the same ratio used in the previous section (5:1) as illustrated in (Fig. 4.3A). Inserts with a pore diameter of 0.4 μm were chosen to allow the exchange of soluble

material and exosomes, but prevent the movement of cells or even apoptotic bodies through them. Following a four day co-culture, the S29 cells were harvested and stained with an antibody specific for cleaved caspase-3 to detect apoptosis. Based on the very low levels of cleaved caspase-3-positive cells detected, the S29 cells did not undergo significant levels of apoptosis when co-cultured with infected or uninfected Huh-7.5 cells (Fig. 4.3B). The lack of induction of apoptosis in the bystander cells, despite the potential for passage of soluble molecules, viruses and exosomes, shows that cell-cell contact is required for the induction of bystander apoptosis.

A


S29 cells
Huh-7.5 cells

B

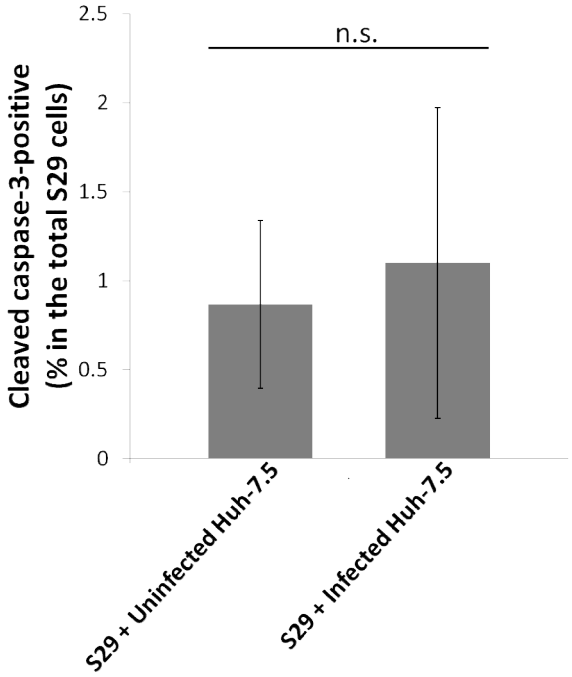

n.s.
Cleaved caspase-3-positive (% in the total S29 cells)
2.5
2
1.5
1
0.5
0
S29 + Uninfected Huh-7.5
S29 + Infected Huh-7.5

Figure 4.3 Bystander apoptosis required cell-cell contact to be induced.

(A) Infected or uninfected Huh-7.5 cells were co-cultured with S29 cells in a transwell plate at a ratio of 5:1 as illustrated. Four days p.i. S29 cells were harvested and stained with cleaved caspase-3 antibody. **(B)** The percentage of cleaved caspase-3-positive cells among the total S29 cells. The result is presented as the mean of three independent experiments +/- SD. *n.s.: Not significant* (student's t-test).

Chapter 5: Results - HCV infection induces pyroptosis and bystander pyroptosis

In chapter 3, we presented evidence suggesting that HCV infection induces apoptosis. However, some of the findings indicated the possibility that a second form of PCD also is induced in the context of HCV infection. For example, the EM images showed some features of a lytic form of death. Furthermore, other features such as DNA fragmentation and caspase activation are not specific for apoptosis and could be caused by a second form of cell death known as pyroptosis (described in detail in section 1.5.2). This led us to ask whether HCV infection affects the cells in a more complex way than we originally expected and is able to stimulate the cell to undergo more than one form of PCD. For these reasons, the possibility that pyroptosis is induced as a second form of PCD in the HCV-infected population was investigated and the results of this investigation will be presented in this chapter.

5.1 HCV infection caused cell lysis.

As we described previously, one of the main morphological features of pyroptosis is loss of the integrity of the plasma membrane, which leads to cell lysis and the release of cellular contents to the surroundings. Lactate dehydrogenase (LDH) is an enzyme that is normally sequestered inside the cell and is released extracellularly only in the event of cell lysis [506]. Measuring the activity of LDH in the medium of the cultured cells is a useful tool for detecting lytic cell death, including pyroptosis [506].

By measuring the activity of LDH in the medium of HCV-infected or uninfected Huh-7.5 cells, we found that HCV infection resulted in a significant increase of the LDH

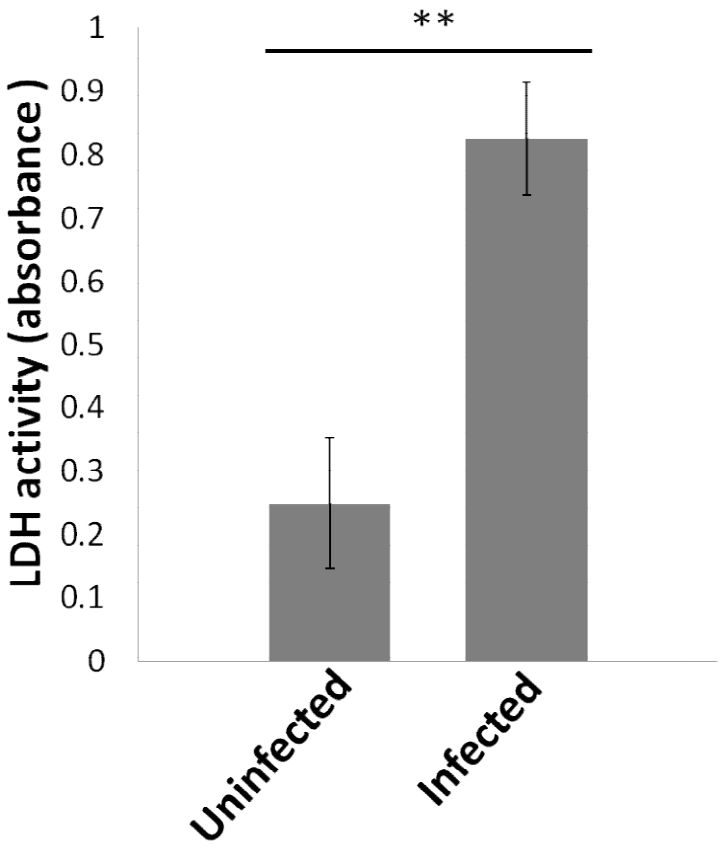


Figure 5.1 HCV infection increased the LDH activity in the supernatant.

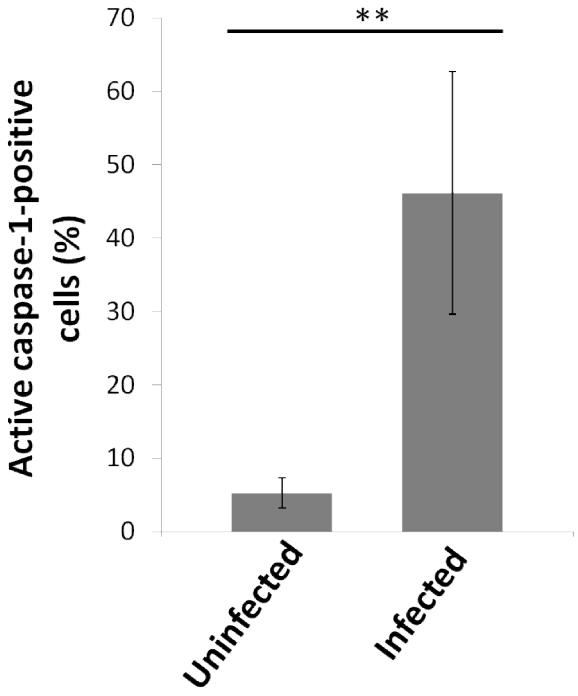
Huh-7.5 cells were infected at MOI of 1. The supernatants were collected four days p.i. and the LDH activities were measured in these supernatants. The absorbance value was calculated as the absorbance measured at 490 nm. Each bar represents the mean absorbance values of three independent experiments (each measured in triplicate) +/- SD. ** $P < 0.005$ (Student's t-test).

activity in the medium of infected Huh-7.5 cells (Fig. 5.1). However, it is important to point out here that the LDH release assay is not specific for pyroptosis. Other forms of lytic cell death, such as necrosis and necroptosis, can also result in an increase in the levels of LDH [507]. Moreover, during the late stages of apoptosis, and in the absence of scavenger cells, apoptotic cells undergo secondary necrosis, which also releases the cellular contents, including LDH, to the exterior of the cell [508]. These facts reduce the specificity of the LDH assay for the detection of pyroptosis. Despite this lack of specificity, LDH release is one of the predominant characteristics of cells undergoing pyroptosis, thereby supporting our theory that HCV infection induces pyroptosis. In the next sections, the results from more specific tests for pyroptosis will be presented.

5.2 HCV infection induced pyroptosis.

As we discussed previously, pyroptosis is a caspase-1-dependent form of PCD. The only exception for this role is LPS-induced pyroptosis, which can be induced under certain circumstances by a caspase-4/5-dependent pathway in human cells (discussed in more detail in section 1.5.2, the non-canonical inflammasomes). For this reason, we started our investigation by testing the effect of HCV infection on the activation of caspase-1. This was performed by infecting Huh-7.5 cells with HCV at an MOI of 1, then measuring the activity of caspase-1 in these cells four days p.i. The activity was measured by staining cells with FAM-YVAD-FMK FLICA probes. These fluorescent inhibitor-based probes enter the cells and bind covalently to the active form of caspase-1. We found that HCV infection caused a significant increase in the proportion of active caspase-1-postive cells (~45% compared to ~5%; Fig. 5.2A). This high level of

A


**
70
60
50
40
30
20
10
0
Active caspase-1-positive cells (%)
Uninfected
Infected

B

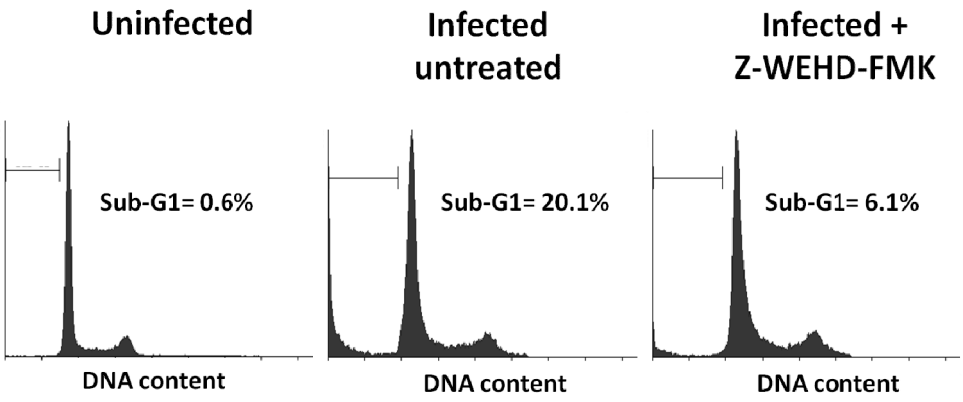

Uninfected
Infected untreated
Infected + Z-WEHD-FMK
Sub-G1= 0.6%
Sub-G1= 20.1%
Sub-G1= 6.1%
DNA content
DNA content
DNA content

Figure 5.2 HCV infection induced pyroptosis.

(A) Huh-7.5 cells were infected at MOI of 1 or left uninfected. On day 4 p.i., cells were harvested and stained with FAM-YVAD-FMK FLICA then analysed by flow cytometry to detect active-caspase-1-positive cells. Each bar represents the mean of the percentage of active-caspase-1-positive cells of six independent experiments +/- SD. **(B)** Huh-7.5 cells were infected at MOI of 1 or left uninfected then incubated in complete medium containing 100 μM of Z-WEHD-FMK or an equivalent volume of DMSO. Cells were harvested on day 4 p.i. and stained with PI. Cell cycle analysis was used to determine the percentage of hypodiploid cells in each of the populations. The results presented in this figure represent two independent experiments.

caspase-1 activation in combination with increased levels of LDH and the EM observations strongly suggested that HCV infection induces pyroptosis.

To further confirm the induction of pyroptosis by HCV infection, we tested whether this PCD could be specifically blocked by caspase-1 inhibition. This was done by treating the HCV-infected or control cells with the caspase-1-specific inhibitor Z-WEHD-FMK, and measuring the effect on the number of hypodiploid cells. Inhibiting caspase-1 rescued more than half of the cells undergoing HCV-induced PCD (Fig 5.2B), confirming that pyroptosis is induced in the HCV-infected cell population.

One of the major functions of the active form of caspase-1 is to induce the maturation of IL-1β by catalyzing the cleavage of its inactive cytoplasmic precursor (pro-IL-1β). We tested this function by measuring the levels of IL-1β in the supernatant of the infected cell population. Huh-7.5 cells were infected at MOI of 1, the supernatants were harvested on day 4 p.i. and the levels of IL-1β were measured by enzyme-linked immunosorbent assay (ELISA). Surprisingly, we could not detect IL-1β in the supernatant of the infected cells. Similar results had been published before by two groups [509,510]. The possible explanation for this result will be discussed in Chapter 6.

5.3 HCV infection induced pyroptosis through the activation of NLRP3 inflammasomes.

Caspase-1 can be activated by different types of inflammasomes depending on the nature of the stimuli that induce their activation (section 1.5.2). Viral RNA is known to induce the assembly and activation of NLRP3 inflammasomes [359]. This prompted us to

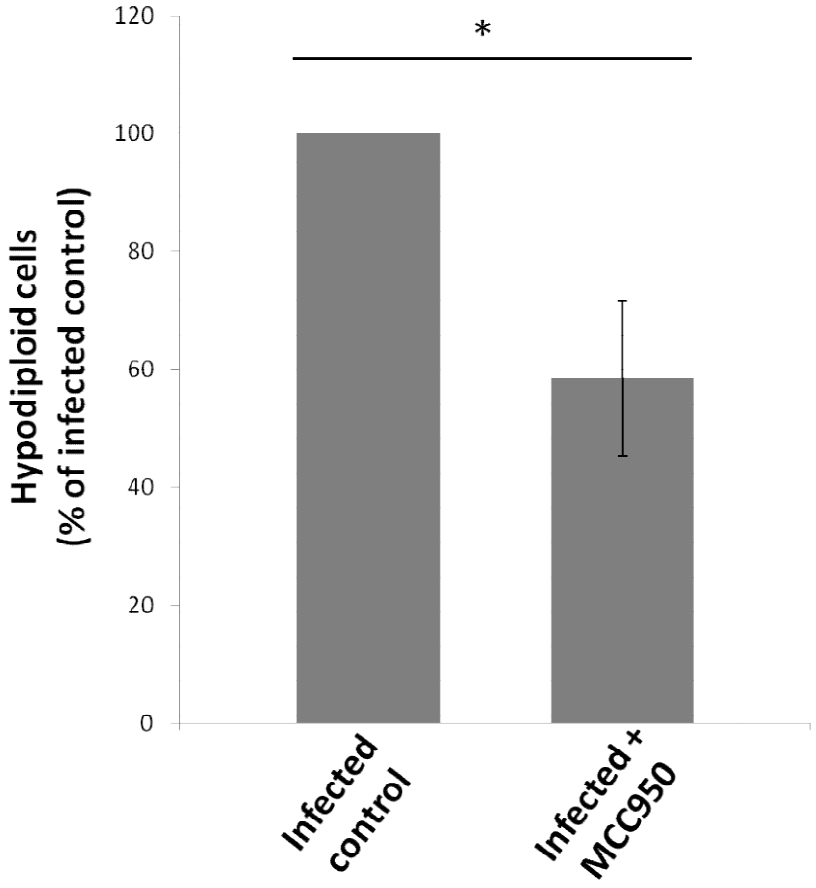


Figure 5.3 HCV induced pyroptosis through NLRP3 inflammasome activation.

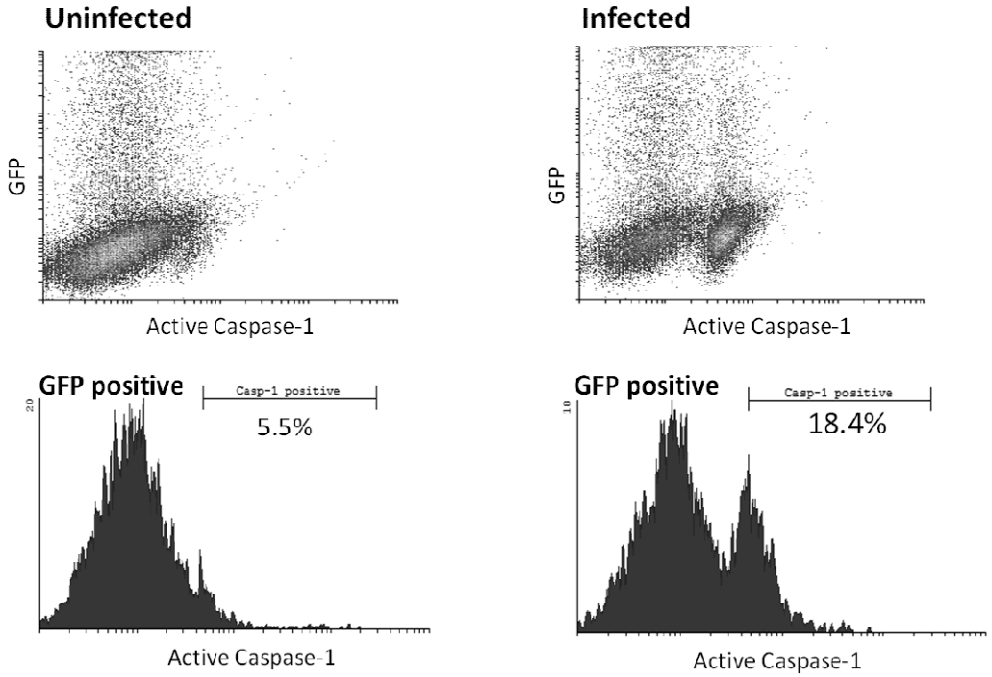
Huh-7.5 cells were infected at an MOI of 1 then incubated in complete medium containing 0.1 μM MCC950, a specific inhibitor for NLRP3. On day 4 p.i., the cells were harvested and stained with PI. Cell cycle analysis was performed to determine the percentage of hypodiploid cells in each of the populations. The data is presented as the mean percentage of the hypodiploid cells compared to the infected untreated control. The data is presented as the mean of at least three independent experiments +/- SD. * $P < 0.05$ (student's t-test).

ask whether HCV-induced pyroptosis is stimulated through NLRP3 inflammasome activation. To address this question we treated HCV-infected Huh-7.5 cells with a specific inhibitor for NLRP3 inflammasome activation and monitored the effect of this treatment on induction of cell death. The inhibitor we used was MCC950, which is a recently developed potent, selective, small molecule inhibitor for NLRP3 [511]. The infected cells were incubated in complete medium containing 0.1 μM of MCC950 for 4 days, and then harvested and stained with PI to detect the hypodiploid cells in the treated or the untreated populations. As expected, we found that inhibiting NLRP3 resulted in a significant decrease in the number of hypodiploid cells (Fig. 5.3). This inhibition confirms the role of NLRP3 inflammasome activation in HCV-induced pyroptosis.

5.4 HCV infection induced pyroptosis in neighbouring uninfected cells (bystander pyroptosis).

In the previous chapter, we presented evidence that HCV infection induces apoptosis in neighbouring uninfected cells. In section 4.1, we showed that hypodiploid cells can be detected in both the HCV-core positive and HCV-core negative populations (Figure 4.1). The presence of the hypodiploid cells within the HCV-core negative population can be the result of the bystander apoptosis. However, because pyroptosis can also cause DNA fragmentation and the formation of hypodiploid nuclei, it is possible that bystander pyroptosis is also induced in the HCV infected cell population. This might contribute to the total number of hypodiploid cells seen in the uninfected population.

A


Uninfected
Infected
GFP
Active Caspase-1
GFP positive
Casp-1 positive
5.5%
18.4%

B

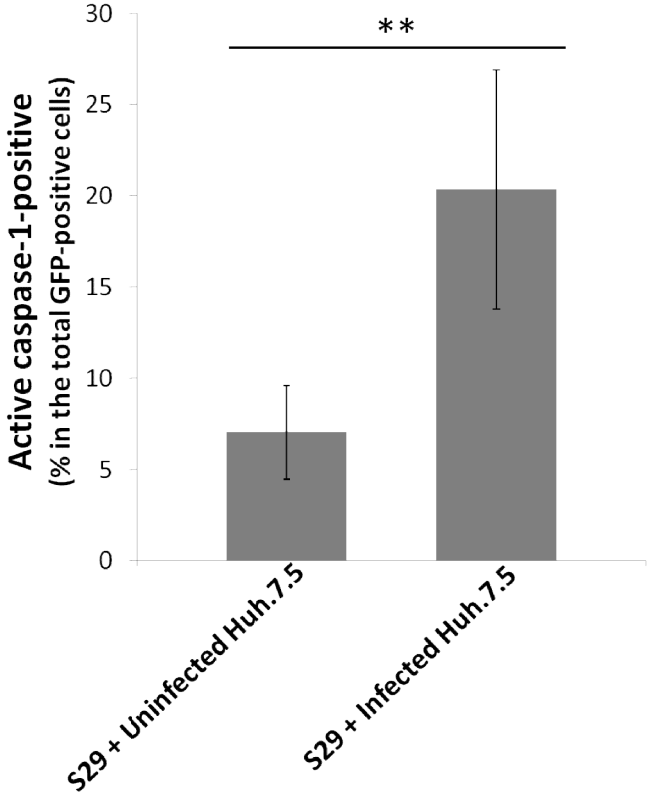

**
Active caspase-1-positive
(% in the total GFP-positive cells)
30
25
20
15
10
5
0
S29 + Uninfected Huh.7.5
S29 + Infected Huh.7.5

Figure 5.4 HCV infection induced bystander pyroptosis.

(A) Huh-7.5 cells were infected at MOI of 1 or left uninfected then co-cultured with GFP-transfected S29 cells at a ratio of 5:1. On day 4 p.i., the cells were harvested and stained with FAM-YVAD-FMK FLICA, then analysed by flow cytometry. S29 cells were differentiated in the analysis by gating on the GFP-positive population. Data from one representative experiment is shown. **(B)** Summary of mean percentages of active-caspase-1- positive cells among the S29 cell population of six independent experiments +/- SD. ** $P < 0.005$ (Student's t-test).

To test more specifically whether bystander pyroptosis was induced in the HCV-infected population, we co-cultured Huh-7.5 cells and GFP-transfected HCV-non-permissive cells as described for the detection of bystander apoptosis (section 4.2 and illustrated in Fig. 2.1). However, instead of staining with cPARP or cleaved caspase-3 antibody, the harvested cells were stained with FAM-YVAD-FMK FLICA to detect caspase-1 activity. By gating on GFP-positive cells, we found a significantly high percentage of active-caspase-1-positive cells in the S29 cell population that was co-cultured with infected Huh-7.5 cells, but not when they were co-cultured with uninfected Huh-7.5 cells (Fig. 5.4A & B). This observation suggests that the induction of pyroptosis by HCV infection was not limited to the infected cells, but also could be induced in bystander cells.

5.5 The induction of bystander pyroptosis did not require the cell-cell contact between the infected and the bystander cells.

Finally, we asked whether HCV-induced pyroptosis requires cell-cell contact between the infected and the bystander cell in order to be activated. In the previous chapter, we demonstrated that HCV-induced bystander apoptosis required cell-cell contact. To test whether bystander pyroptosis has the same requirement, we co-cultured infected or uninfected Huh-7.5 cells with S29 cells in different chambers of transwell plates. These plates will allow the exchange of soluble mediators and very small particles, such as virus particles and exosomes, between the two chambers, but will block cell-cell interaction. Despite the inability to interact physically, we found that co-culturing the S29 cells with infected Huh-7.5 cells resulted in higher numbers of active-caspase-1-positive cells than

when they were co-cultured with uninfected control cells (Fig. 5.6). This result indicated that, unlike bystander apoptosis, cell-cell contact is not necessary for the induction of bystander pyroptosis. The possible mechanisms for the induction of the bystander pyroptosis will be discussed in Chapter 6.

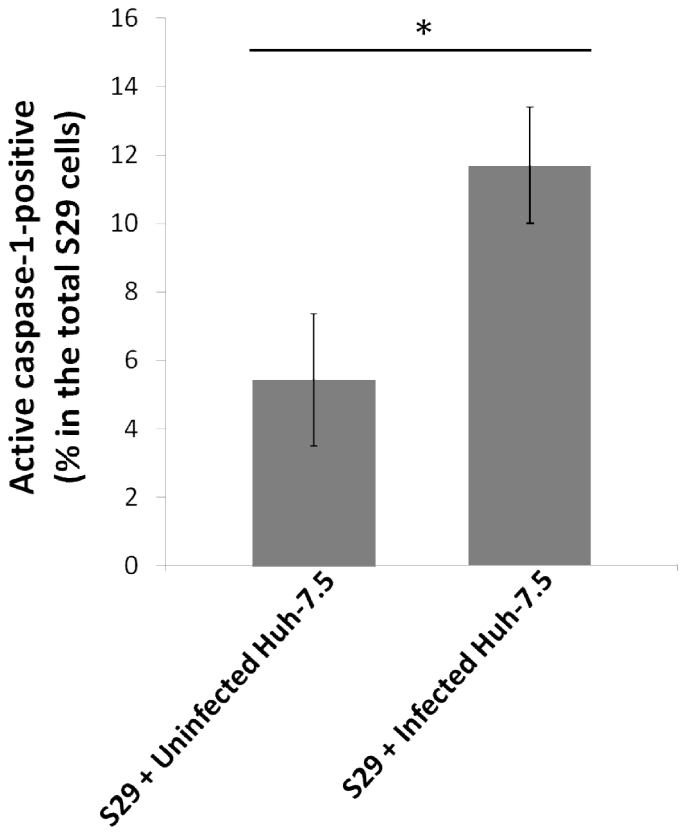


Figure 5.5 Cell-cell contact was not required for HCV-induced bystander pyroptosis.

Infected or uninfected Huh-7.5 cells were co-cultured with S29 cells in a transwell plate at a ratio of 5:1 as illustrated in (Fig. 4.3A). Four days p.i., S29 cells were harvested and stained with FAM-YVAD-FMK FLICA. Each bar represents the percentage of active-caspase-1-positive cells among the total S29 cells. The data is expressed as the mean of three independent experiments +/- SD. * $P < 0.05$ (student's t-test).

Chapter 6: Discussion

The gradual destruction of the liver that takes place in the context of HCV infection has been a matter of intense interest for many years. In this report, we investigated the possibility that HCV itself causes cytopathic effects in the absence of immune cells. We found that HCV infection indeed induced at least two forms of PCD, those being apoptosis and pyroptosis. We also demonstrated that both infected and neighboring uninfected (bystander) cells were induced to undergo each of these two forms of PCD. The bystander apoptosis we observed, but not the bystander pyroptosis, required direct contact between the infected and uninfected cells.

Our study employed a cell culture-adapted strain of HCV JFH-1 ($JFH1_T$) and human hepatoma-derived Huh-7.5 and related S29 cells. This system is one of the few non-chimeric, highly replicating HCV culture systems that allows a dynamic range of measurement sufficient for studies such as we have performed here. However, cell culture systems such as ours inherently possess caveats and limitations. For example, unlike all other patient isolates of HCV, the JFH-1 strain can propagate autonomously in cell culture without adaptive mutations or modification of target cells. This fact has led many in the field to question whether this strain actually represents natural HCV infection in patients. Additionally, the Huh-7.5 cells used by us and many others are cancer cell-derived and are known to have altered innate immune mechanisms. Given these caveats, any findings such as ours that might have pathogenic implications may not be directly extrapolated to persistent HCV infection of liver and should be confirmed in animal and infected human liver studies.

In a broader sense, it is important to consider whether or not the results presented here have relevance to the disease situation. Given the above-mentioned caveats of HCVcc systems, we cannot say at this point that apoptosis or pyroptosis is actually taking place in infected livers. However, apoptosis has been detected in the human liver cells of SCID/ALB-uPa humanized mice [447]. In human studies, a correlation between apoptotic index and histological grading in liver sections, and activation of caspase-3 and -7, has been demonstrated in liver biopsies from chronically infected individuals [403,404]. Based on these studies, it is possible that our results regarding apoptosis are physiologically relevant, but it will be important to revisit this topic in light of our new findings, and more importantly, the topic of pyroptosis has never been addressed in the context of HCV-infected individuals. It would be interesting now to test whether other subtypes/genotypes of HCV cause pyroptosis and bystander cell death because JFH-1 was originally isolated from a patient with fulminant hepatitis [180]. If these forms of PCD are specific to JFH-1 infection, one might speculate that perhaps only highly pathogenic strains of HCV can induce cell death by these mechanisms.

The pathogenic pathways responsible for development of HCV-associated liver disease are poorly understood, but induction of PCD in the infected liver may play an important role in disease progression. For example, induction of apoptosis activates a pro-fibrogenic pathway contributing to the overall pathogenesis associated with HCV infection [467,512]. Pyroptosis contributes to the creation of an inflammatory environment inside the infected liver, thereby promoting disease pathogenesis [477,482-488]. Various components of the innate and adaptive immune systems are known to

induce apoptosis of infected cells as part of their role in host defence against HCV. However, despite general agreement regarding the immunopathogenesis of HCV, there is considerable discrepancy in the literature regarding the effect of HCV on its host cell, specifically whether it is directly cytopathic.

In the pre-HCVcc era, many groups investigated effects of HCV infection on various apoptotic pathways using different approaches. However, contradictory findings within the published data make it difficult to discern the actual effect of HCV infection on programmed cell death. For example, almost every HCV protein has been reported to have both anti- and pro-apoptotic function depending on which study one reads (discussed in section 1.6). The reason for this controversy is most likely due to the use of different systems by different groups to test the pro- or anti-apoptotic effects of these proteins. Most of these reports relied on the expression of a single viral protein under the control of highly active promoters. This creates extremely high levels of that protein, far above their normal physiological levels. Furthermore, these systems failed to detect the effect of the interaction between the different viral proteins, which could also impact the pro- versus anti-apoptotic functions of different viral proteins. These facts make the relevance of these reports to the natural HCV infection highly questionable.

Studies employing HCVcc systems to specifically analyze the effects of HCV infection on apoptotic pathways have generated relatively consistent findings. For example, our results here are in agreement with those of other studies demonstrating of pro-apoptotic effects of HCV infection [448-450,453]. Still, other reports described the activation of certain pro-survival pathways following HCV infection in the context of

HCVcc (discussed in section 1.6). However, this reported activation was transient, detected early in the course of infection or activates certain pro-survival pathways but did not block HCV-induced apoptosis of the cell. Actually, activation of these pro-survival pathways might be responsible for extending the survival period of the infected cell enough to support viral replication.

The mechanism by which apoptosis is induced is still largely unclear. Different mechanisms were reported by different groups, as described in section 1.6. These discrepancies might be attributed to the use of different derivatives of the parental JFH-1 virus in executing their experiments. The use of the parental JFH-1 is criticized due to its extremely low efficiency of replication in cell culture. Some groups overcame this low replication efficiency by using the J6-JFH1 derivative, but this is actually a chimeric virus that does not exist in nature. The virus strain used in our project (the $JFH1_T$ strain) has high replication efficiency and contains only three adaptive point mutations compared to the parental JFH-1, which was isolated from an infected patient. Because of these facts, we believe that our system is superior to the previously used HCVcc systems, as it better represents natural HCV infection under the physiological conditions.

We presented here evidence that HCV infection reduces the proliferation rate of the infected cell population, which is in agreement with previous reports [455,456,513,514]. There was disagreement amongst these reports as to when in the cell cycle the arrest occurs. Some groups reported that the arrest occurs at the G1 phase [455,513], while others reported it at the G2/M phase [456]. An association between cell cycle arrest, P21 levels, and severity of fibrosis was reported [513]. However, it is not clear whether this

correlation is a result of the direct effect of the cell cycle arrest, or if it is caused by other factors, including the induction of PCD. Cell cycle arrest was not our interest in this project and therefore was not investigated beyond the point of showing that it was induced.

DNA fragmentation was tested by two different assays: DNA laddering assay and the detection of hypodiploid cells by cell cycle analysis of PI stained cells. Despite the fact that those two assays detect the same PCD hallmark, DNA fragmentation, they are actually different from each other by the feature used to detect the fragmentation. The DNA laddering assay detects the formation of low molecular weight fragments with sizes in multiples of 180 bp. These fragments result from the internucleosomal cleavage of the DNA because of caspase-3-mediated cleavage and activation of ICAD/DFF45 to release CAD/DFF40. This is an active DNase that catalyses the internucleosomal cleavage of DNA [344]. On the other hand, hypodiploid nuclei result from the loss of the low molecular weight fragments of DNA during the processing of the cell, regardless of the mechanism by which these low molecular fragments formed. Apoptosis is well-known to induce internucleosomal DNA fragmentation through caspase-3-mediated cleavage of the inhibitor ICAD/DFF45 [344]. As discussed earlier, pyroptosis is also known to induce DNA fragmentation. However, the DNase that is activated during pyroptosis is not known, and it is not clear whether it will cause internucleosomal cleavage and DNA laddering. One group reported that caspase-1 could cleave ICAD/DFF45, release the active DNase, and lead to DNA fragmentation *in vitro* [515]. In contrast, other groups could not detect any ICAD/DFF45 cleavage or DNA laddering in the macrophages

undergoing *Salmonella*-induced pyroptosis [355,360]. Since it is not clear whether the DNA laddering test is specific for the detection of apoptosis, we decided to be more cautious in interpreting the results of this test and we refrained from making apoptosis-specific conclusions depending on the results of this test. Detection of the hypodiploid cells in PI stained cells can result from both apoptosis and pyroptosis, and we confirmed this by showing that inhibiting caspase-3, caspase-8, or caspase-1 specifically resulted in a significant reduction in the number of the hypodiploid cells in our system.

As with the above-mentioned tests, many commonly used apoptosis detection assays should be revisited as they detect features shared with pyroptosis. DNA fragmentation-dependent assays such as the TUNEL assay should not be used for the identification of apoptosis unless pyroptosis is ruled-out. Moreover, Annexin V staining results should also be interpreted with more caution as the stain might enter the pyroptotic-ruptured cells and bind to phosphatidyl serine molecules in the inner leaflet. Combining PI stain with annexin V will resolve this issue as pyroptotic cells will allow the entry of both of these two molecules. Finally, despite its wide use, the interpretation of PARP cleavage results as an apoptotic marker must be reconsidered in the light of relatively recent publications in which caspase-1 activation was reported to cause similar effects (discussed in detail in section 1.5.2). In this project, cleavage of PARP was always supported by detecting the cleavage of caspase-3 to specifically indicate the induction of apoptosis.

We showed in section 3.5 that HCV infection induces the activation of caspase-8. This induction plays a role in the apoptotic pathway as inhibiting caspase-8 reduced the proportion of hypodiploid cells. Caspase-8 is the initiator caspase of the extrinsic

apoptotic pathway and is activated in the DISC following the binding of a death ligand to its cognate receptor (see section 1.5.1.2). However, an alternative pathway for the activation of caspase-8 was also reported. In this pathway, caspase-8 is processed and cleaved downstream of caspases-9, -3 and -6 [516,517]. Moreover, caspase-8 was reported to be activated by NLRP3 inflammasomes (more details regarding this pathway will be discussed below). For this reason, it is not clear to us which of these pathways are participating in the cleavage and activation of caspase-8 in our system and more investigation is needed to answer this question.

In Chapter 4, we presented evidence that apoptosis is not limited to the infected cells, but also is induced in neighbouring uninfected cells. As discussed earlier, bystander apoptosis has been described previously in the context of other viral infections, such as HIV, Cytomegalovirus (CMV) and Ebola virus, and is believed to contribute to the pathogenesis related to these viral infections [501-505]. To the best of our knowledge, our data here provide the first evidence of bystander apoptosis in response to HCV infection. Induction of bystander apoptosis could exacerbate the pathogenic process in the HCV infected liver. Such a mechanism of elimination of uninfected cells could help explain the massive destruction observed in the liver despite the belief that only a minority of hepatocytes (7%-20%) are actually infected with HCV in chronically infected patients [518]. Induction of apoptosis in adjacent uninfected cells, could theoretically amplify the pathogenic effect of HCV infection.

We presented evidence that the induction of bystander apoptosis requires cell-cell contact between the infected and the affected uninfected cells. The most attractive

candidate mechanism to explain this result is that the induction of bystander apoptosis occurs through the interaction between death ligands expressed on the surface of the infected cell and death receptors on the surface of neighbouring cells, which in turn causes the activation of the extrinsic apoptotic pathway in the neighbouring cell. This might be also the cause of the activation of caspase-8 observed in our system. The upregulation of death ligands such as Fas-ligand by HCV infection has been reported previously [519,520]. This upregulation of Fas-ligand induces apoptosis in activated CD4+ and CD8+ T lymphocytes [520]. Upregulation of Fas-ligand on the infected cell could be an evasion mechanism used by the virus to kill infiltrating activated lymphocytes. Consequently, this upregulation of death ligands could also induce apoptosis in neighbouring uninfected hepatocytes. Additionally, induction of death ligand expression might contribute to the development of HCC by providing transformed cells a tool to evade immune surveillance. Human hepatocytes are highly sensitive to Fas-ligand-induced apoptosis [521], however, human hepatoma cell lines, including Huh-7 cells (the parental cell line for the S29 cells used here), were reported to be resistant to Fas-mediated apoptosis [522]. Therefore, bystander cells would first need to be sensitized to Fas-induced apoptosis in order to be responsive to the Fas-ligand expressed on the neighbouring infected cells. It is plausible that such a mechanism may be at work in our system as in SCID/Alb-uPA mice, HCV infection causes an upregulation of Fas expression in both infected and neighbouring uninfected cells [447]. It is possible that a similar upregulation is occurring in our system leading to sensitization of neighbouring uninfected cells to FAS-induced apoptosis.

The expression of other death ligands by the infected cells might also be responsible for the induction of bystander apoptosis. For example, TRAIL expression was reported to increase significantly after HCV infection in a well-differentiated hepatoma cell line (LH86), and this increase was responsible for the induction of apoptosis in that system [448]. Treatment of Huh-7.5 cells with TRAIL induced apoptosis in these cells [457]. It is generally believed that TRAIL can induce cell death in cancer cells and spare most of the normal healthy cells. However, it has been reported that normal human, but not mouse, hepatocytes are actually sensitive to TRAIL-induced apoptosis [523,524].

Membrane bound TNF-α is another possible inducer of bystander apoptosis in a cell-cell contact-dependent manner. HCV infection results in an increase of serum levels of TNF-α. The major source of this cytokine in the liver is believed to be resident macrophages and T-cells [525]. However, TNF-α expression by hepatocytes was also observed in liver biopsies obtained from patients with chronic HCV infection [526]. Moreover, transfection of HepG2 cells with a full-length HCV DNA, or with HCV NS3 induced them to express high-molecular-weight TNF-α [527]. As we discussed earlier, TNF-α activates both pro- and anti-apoptotic pathways in different target cells. Expression of the membrane bound TNF-α by the kupffer cells was reported to induce cell-cell contact-dependent apoptosis in hepatocytes [528]. The possibility that expression of TNF-α by infected hepatocytes plays a role in the induction of bystander apoptosis in our system cannot be excluded. In summary, bystander apoptosis is most likely the result of the overexpression of one or more of the previously described death ligands on the surface of the infected cell and the sensitization of the neighbouring uninfected cell to

death ligand-induced apoptosis. Further investigation needs to be performed to test this hypothesis.

We tested the induction of bystander apoptosis in two different cell lines: S29 and 293T cell lines. The S29 cell line is a hepatoma cell line, while 293T cells are derived from embryonic human kidney. The 293T cells were used in this experiment to test whether the induction of bystander apoptosis occurs specifically in neighbouring hepatocyte-like cells or whether it can also affect other types of cells that encounter the infected hepatocytes. We chose 293T cells because they can be easily transfected with GFP, and most importantly, because they lack CLDN-1, which is one of the main receptors for HCV entry [139]. Using the 293T cell line will rule out the possibility that the observed apoptosis is caused by low levels of HCV infection. Although the induction of HCV-induced bystander apoptosis was observed in both the S29 and the 293T cells, the degree of induction of bystander apoptosis in the 293T cells was low in comparison to that seen in the S29 cells. This could be explained by the moderate responsiveness of the 293T cells to FAS-induced apoptosis and resistance to TRAIL-induced apoptosis [529,530]. Further reduction in the 293T cells responsiveness to the bystander apoptosis stimuli could be attributed to their reduced contact with the infected Huh-7.5 cells. This reduction in contact might be caused by the absence of CLDN-1, a tight junction protein, on the 293T cells.

We found that HCV induced pyroptosis in the infected cell population as a second form of PCD. Pyroptosis induction might have a significant effect on the pathogenesis of HCV as it participates in creating an inflammatory environment in which liver cirrhosis

and HCC develop (more details presented in section 1.7). Induction of pyroptosis was reported in other viral infections (discussed in Section 1.5.2) and this played a role in the pathogenesis of these infections. To the best of our knowledge, we are the first to report the induction of pyroptosis in HCV infections.

The production of mature IL-1β requires two signals: the first is delivered through recognition of the pathogen by PRRs, and leads to transcription of the pro-IL-1β gene; and the second is delivered by the induction of inflammasome assembly and results in cleaving pro-IL-1β and releasing the mature form [531]. Despite the clear activation of caspase-1 in infected and bystander cells, we could not detect IL-1β in the supernatant of these cells. Although surprising at first, this result is in agreement with previous reports in which three groups could not detect the induction of *pro-IL-1*β gene expression or secretion of mature IL-1β protein in response to HCV-infection of different hepatoma cell lines or immortalized PHH [509,510,532]. These groups showed that hepatic monocytes/macrophages are the only source of IL-1β in the infected liver. In contrast, a fourth group reported the detection of low levels of IL-1β in the supernatant of JFH-1-infected Huh-7.5 cells [533]. Although the exact cause of this controversy is not clear, the different strains of virus and the different clones of cell lines (JFH-1 vs. $JFH1_T$ and Huh-7.5 vs. Huh-7.5.1) might explain this discrepancy. The inability (or weak ability) of human hepatocytes to produce IL-1β is believed to be a regulatory mechanism aimed to prevent liver toxicity in response to the continuous exposure of hepatocytes to blood-borne pathogens [510]. Furthermore, the lack (or weak) induction of the *pro-IL-1*β gene in HCV-infected cells might be the result of the blockade in RIG-I and TLR-3 signaling

pathways caused by the viral NS3-4A (described in section 1.4.1). This in turn blocks the activation of NF-κB and the transcription of pro-inflammatory cytokines genes, including the *pro-IL-1*β.

Inhibiting the NLRP3 inflammasome rescued a significant number of cells from undergoing HCV-induced PCD. This result implicated the NLRP3 inflammasome in the induction of PCD in response to HCV infection. This result was not surprising to us since NLRP3 inflammasomes are known to be activated by many viruses, including both RNA and DNA viruses (reviewed in [534]). It was reported previously that HCV infection induces the activation of NLRP3 inflammasomes in hepatic macrophages/monocytes and hepatocytes [510,532,533]. In contrast, Chen *et al*. reported that there is no, or an extremely low, expression of NLRP3 in HCV-infected hepatocytes [532]. However, this group tested the expression of NLRP3 in JFH-1-infected Huh-7 cells. This system is known to have an extremely low infection efficiency in comparison to our system and natural HCV infection. To the best of our knowledge, we are the first group to report a role for the NLRP3 inflammasome activation pathway in HCV-induced cell death.

As with apoptosis, pyroptosis was not limited to infected cells, but also occurred in neighbouring uninfected cells. The concept of bystander pyroptosis was described previously in the context of HIV infection and is believed to play a major role in the depletion of the CD4+ T cells (reviewed in [535]). However, unlike bystander apoptosis, bystander pyroptosis did not require cell-cell contact. In other words, bystander pyroptosis seems to be induced by soluble mediators and/or exosomes. The mechanism of bystander pyroptosis induction is not clear, but lysed pyroptotic cells would release a

number of DAMPs including: High-mobility group box 1 (HMGB1), heat shock proteins, ATP, DNA and RNA [536]. Many of these DAMPs can induce inflammasome activation in affected cells. For example, extracellular ATP binds to purinergic receptor P2X7 (P2RX7), which opens ion channels leading to K^+ efflux, resulting in inflammasome activation [536]. HMGB1 stimulates a signaling pathway through TLR4 and receptor for advanced glycation end products (RAGE) that activates NLRP3 inflammasomes and pyroptosis in hepatocytes [537]. It is also possible that the transfer of viral proteins or viral RNA from the infected cells to neighbouring cells, either by the exosomes or through a non-productive entry of viral particles, might cause the activation of inflammasomes and the induction of pyroptosis in neighbouring uninfected cells even in the absence of viral replication. Lipoprotein lipase-treated virus particles have been shown to enter cells through a low-density lipoprotein receptor (LDLR)-mediated non-productive entry pathway [135]. This pathway leads to virus particle degradation and is likely responsible for the entry of Apo-E-depleted lipo-viro-particles in infected individuals. Abortive HIV infection was reported to cause bystander pyroptosis in CD4+ T cells [538]. A homologous mechanism in which the HCV RNA is detected in the cytosol leading to the activation of inflammasomes cannot be excluded.

Until our current investigation, induction of apoptosis in HCV-infected cells received the exclusive attention of all PCD-related studies in the field. In this project, we moved the understanding of this process to a new level by showing that the cytopathic effect of HCV infection is actually more complex than has been suggested. We showed that HCV infection causes the induction of at least two forms of PCD and this effect is not limited

to the infected cells, but it extends to the surrounding uninfected cells. How HCV can induce two distinct forms of PCD simultaneously remains to be determined. It is possible that different viral components are independently detected by separate sensors, or that different steps of the viral life cycle induce distinct cellular responses ultimately resulting in the independent induction of each form of PCD. Alternatively, perhaps the HCV-induced apoptotic and pyroptotic pathways share a common origin that activates both of them. Interestingly, recent evidence suggests that besides their classical role in activating caspase-1 and inducing pyroptosis, NLRP3 inflammasomes can also activate caspase-8, which in turn induces apoptosis [539-543]. This could be the case in HCV infection whereby activation of the NLRP3 inflammasome activates caspase-8 and caspase-1 to induce both apoptosis and pyroptosis, respectively. It is also possible that both apoptosis and pyroptosis are induced by the same cellular response to HCV infection. For example, in genetically obese mice, induction of ER stress in the hepatocytes was reported to activate NLRP3 and to induce both apoptosis and pyroptosis [544].

It is not clear at this point whether apoptosis or pyroptosis of infected versus bystander cells is induced by the same or different mechanisms. Presumably, certain responses, such as ER stress, occur solely in the infected cells, logically causing the infected cells to be more prone to apoptosis/pyroptosis than the bystander cells. Furthermore, the foci of infected cells typically observed in HCVcc-infected Huh-7.5 cells demonstrates that viral infection preferentially spreads to the closely neighbouring cells. This means that most infected cells are in close proximity to other infected cells and could, therefore, be easily affected by the same stimuli that induce the bystander

apoptosis/pyroptosis. Therefore, infected cells would be under higher pressure to undergo apoptosis/pyroptosis than the bystander cells. In support of this idea, regarding both apoptosis and pyroptosis, we observed generally higher levels of cell death in the overall population of cells than when we gated on only the uninfected cells. However, many viruses have evolved strategies for inhibiting cell death in its host in order to allow for the establishment and maintenance of virus replication (reviewed in [545]). HCV is no exception; almost every HCV protein has been reported to possess anti-apoptotic effects (section 1.6). We don't typically detect apoptosis in HCV-infected cells until day four post-infection. So it is possible that HCV somehow initially blocks apoptosis, and if so, this capability would confer a survival advantage to infected cells over bystander cells. At this point we can only hypothesize that the balance between pro- and anti-apoptotic/pyroptotic stimuli determines the fate of the infected/bystander cells.

Induction of PCD in response to viral infection is a well-established mechanism for restricting viral infections. It is widely accepted that various immune cells can induce apoptosis in infected cells, but PCD might also be induced by intracellular innate immune responses. However, the induction of these PCD pathways can be circumvented by numerous inhibitory viral proteins (reviewed in [545]). These opposing forces place the host cell into a continuous competition with the infecting virus over control of these cell death pathways. The results described here demonstrate that HCV infection induces two distinct forms of PCD: apoptosis and pyroptosis. The simultaneous induction of multiple forms of PCD in HCV-infected hepatocytes could be one of the strategies used by the host cell to overcome the inhibitory effect of the viral proteins. In this regard, HCV must

overcome a higher barrier by inhibiting two different death pathways in order to maintain the survival of its host cell and maintain its replication. Nevertheless, it is clear that HCV is a highly evolved pathogen that is able to maintain control over these cell death pathways until it has completed its life cycle and released new viral progeny to infect *naïve* hepatocytes.

The induction of apoptosis and pyroptosis in the HCV-infected liver seems to act like a double-edged sword. In the short-term, it might play a role in restricting HCV infection. However, induction of these two forms of PCD could be one of the major drivers of the pathogenic process after many years of the chronic infection. As we described in more detail in section 1.7 and illustrated in fig. 1.3, each of these two forms of PCD might have distinct contributions to liver disease progression. Induction of apoptosis in hepatocytes results in increasing the levels of TGF-β in the infected liver, which hastens the fibrotic process and induces EMT, leading in the long-term to liver cirrhosis and HCC. Pyroptosis creates an inflammatory environment that also hastens fibrosis and participates in predisposing the cells to malignant transformation and development of HCC. Induction of these forms of PCD in neighbouring uninfected cells might also exacerbate the pathogenic process. Recently identified direct-acting antiviral agents have shown amazingly high cure rates, but studies have shown that HCC can still develop even after elimination of virus from an infected individual [461]. We believe that understanding the exact mechanisms by which the virus stimulates these two forms of PCD in infected and bystander cells might provide us with targets for the development of novel treatments and

eventually a comprehensive therapeutic regimen that can both eliminate virus and prevent progression of HCV-related liver disease in HCV-infected patients.

In summary, the interaction between HCV and its host cell is complicated and results in stimulating multiple responses, many of which are capable of inducing PCD. As a result of this interaction, at least two forms of PCD are induced: apoptosis and pyroptosis. The exact mechanism by which HCV induces these two forms of PCD is not clear. Activation of NLRP3 inflammasomes plays a role in HCV-induced pyroptosis and possibly apoptosis. The induction of PCD is not limited to the infected cells, but also occurs in bystander cells. Bystander apoptosis, but not bystander pyroptosis, requires physical interaction between the infected and bystander cells in order to be induced. A schematic representation that summarizes our main findings is presented in Fig. 6.1.

Future directions:

This project reported for the first time the activation of multiple mechanisms of cell death during HCV infection. However, it also presented many questions that need more investigation. The induction of ER stress in HCV-infected cells has been reported previously [447,450]. However, the role of ER stress in the induction of each of those two forms of PCD needs to be studied further. This can be achieved by testing the effect of infection on the expression of a group of ER stress markers and testing the effect of inhibiting ER stress on the induction of apoptosis and pyroptosis. Inhibition of HCV-induced ER stress might be achieved by treating the infected cells with tauroursodeoxycholic acid (TUDCA), which is a chemical chaperone that is known to reduce ER stress [546]. The effect of this treatment on the number of cells undergoing

PCD and on the activation of caspase-3 and caspase-1 will demonstrate the importance of ER stress in the induction of each of the two forms of PCD.

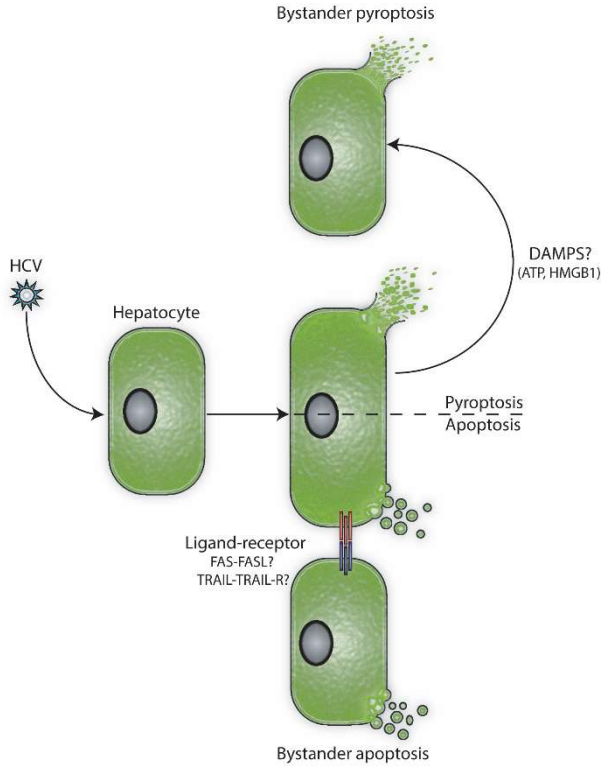


Figure 6.1 Proposed model for the induction of multiple forms of PCD in response to HCV infection.

Schematic representation of the proposed mechanisms for the induction of multiple forms of PCD in response to HCV infection. HCV infection induces at least two forms of programmed cell death in the infected cells: apoptosis and pyroptosis. Apoptosis is also induced in the neighbouring uninfected cells (bystander apoptosis). The induction of bystander apoptosis is cell-cell contact-dependent, and we propose that it is induced by an interaction between a death ligand expressed on the surface of the infected cell and a

death receptor expressed on the surface of the bystander cell. The induction of bystander pyroptosis does not depend on cell-cell contact. The exact mechanism by which bystander pyroptosis is induced is not yet known, but will be the focus of subsequent studies.

More investigation is required in order to determine the mechanism by which bystander apoptosis is induced. Based on the observation that bystander apoptosis is cell-cell contact-dependent, we hypothesize that bystander apoptosis is mediated by an interaction between a death ligand expressed on the surface of an infected cell and its receptor on the surface of a bystander cell. To confirm this mechanism of induction and to identify the responsible death ligand-receptor, the effect of HCV infection on the cell surface expression for each of the known death ligands could be tested, including Fas-Ligand, TRAIL and membrane bound TNF-α. The role of each of these ligand-receptor interactions can be further confirmed by testing the effect of specifically blocking their interaction on the induction of bystander apoptosis. The interaction between the death ligand and its receptor can be blocked by treating the cells with commercially available blocking antibodies. An increase of the expression of a death ligand and the inhibition of bystander apoptosis following the treatment with its specific blocking antibody will confirm the involvement of that specific ligand-receptor interaction in the induction of bystander apoptosis. The result of this experiment might also provide us with information regarding the source of caspase-8 activation in the infected cell population.

After showing that HCV induces pyroptosis as a second form of PCD in the infected cells, many questions emerged that warrant investigation. Firstly, what is the mechanism of induction of pyroptosis in the infected cells? Pyroptosis might be induced because of

direct recognition of viral RNA by NLRP3, or it could occur as a secondary response to the generation of DAMPs, or to other HCV-induced cellular responses. For example, HCV infection was reported to induce the production of ROS, which in turn have the capability to activate NLRP3 inflammasomes and pyroptosis [454,547]. Furthermore, and as we mentioned above, ER stress can induce NLRP3 inflammasome activation and pyroptosis. Further investigation is needed to answer this question.

More investigation is also needed to understand the factors that determine the fate of the infected cell and whether it will undergo apoptosis or pyroptosis. Answering this question seems to be complicated in light of the many pathways that are activated in these cells. In a previous DNA transfection study, apoptosis was found to be induced at lower transfected DNA concentrations, while induction of pyroptosis required a higher DNA concentration [540]. A similar mechanism may be at play in our system whereby the infected cells with high HCV RNA content undergo pyroptosis, while the more recently infected cells with lower levels of RNA might undergo apoptosis. More investigation is also needed to test whether the apoptotic and pyroptotic pathways are induced simultaneously in the same cell or if different cells induce only one of these two PCD pathways. In this case, activation of one of the two PCD pathways might inhibit the activation of the other, or as we mentioned above, each of these two pathways might be activated under different conditions, dependent on the level of various viral components. The possible role of NLRP3 inflammasome activation in the activation of caspase-8 and the induction of apoptosis in infected cells must also be tested. This can be done by

specifically inhibiting NLRP3 and examining the effect of this inhibition on caspase-8 and caspase-3 activation.

Further investigation is also needed to determine the mechanism by which bystander pyroptosis is induced. As discussed earlier, bystander pyroptosis might be induced by DAMPs released from infected cells, or by the non-productive uptake of the virus or viral components by the bystander cell. Many experiments need to be done to address this question. For example, induction of pyroptosis could be monitored in S29 cells incubated with medium containing lysates from Huh-7.5 cells. The lysates can be obtained from either uninfected cells or infected cells to determine whether the induction of bystander pyroptosis is HCV-specific or occurs as a general response to the release of neighbouring cells' contents into the microenvironment. It is also possible that the bystander pyroptosis-inducing factor is transferred by the exosomes from infected to neighbouring cells. The role of exosomes in the induction of bystander apoptosis might be tested by inhibiting exosome production in the infected cells by treating them with an exosome inhibitor such as GW4869.

Finally, the ultimate goal should be to confirm our findings in chronically infected patients' livers. Detecting the induction of apoptosis and pyroptosis as two forms of PCD occurring in hepatocytes in liver biopsies obtained from HCV-infected patients and showing that these forms of cell death occur in both the infected and neighbouring cells will be of great value and would represent a cutting edge finding in the field.

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