HEPATITIS C VIRUS PERSISTENCE AND LYMPHOTROPISM

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BY

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A thesis submitted to the School of Graduate Studies

In partial fulfilment of the requirements for

The degree of Doctor of Philosophy

Faculty of Medicine

Memorial University of Newfoundland

St. John's, Newfoundland

September 28th, 2010

ABSTRACT

Hepatitis C virus (HCV) leads to chronic liver disease in up to 80% of those infected. While thought to be mainly hepatotrophic, HCV has been considered to be able to replicate in cells of the immune system. The extent and implications of viral replication in immune cells are unknown, however, this site may represent an important reservoir from which the release of infectious virions, leading to viral reactivation may take place.

We hypothesize that HCV-naive lymphocytes are susceptible to infection with wild-type HCV and are capable of supporting the full cycle of its replication, including the release of biologically competent virions. We also postulate that small amounts of virus, lingering for years after apparent complete clinical resolution of hepatitis C, retains its infectious potential.

This thesis is comprised of three related, but also stand-alone studies. In the first study, we established an *in vitro* infection system in which wild-type HCV was used as inoculum and primary human lymphocytes served as infection targets. Employing this system, we demonstrated that HCV can infect T lymphocytes, as it became apparent via detection of HCV genome replicative intermediates, non-structural proteins, and the appearance of unique variants in infected cells. We further identified and characterized the biophysical properties of virion particles by sucrose gradient centrifugation and by immune electron microscopy with HCV-specific antibodies directed against virus envelope (E2) protein, and by examining the *in vitro* infectivity of secreted virions by their serial passage in virus-naïve lymphocytes.

In the second study, we revealed that *in vitro* infection of T cells with HCV can lead to an altered T cell subset distribution characterized by an enrichment of $CD8^+$ T cells that

appears to be a result of an inhibition of $CD4^+$ cell proliferation but not virus-related apoptotic death of the cells.

In the third study, by utilizing the system established in the first study, we revealed for the first time that small amounts of HCV persistently circulating after apparent complete clinical resolution of hepatitis C due to antiviral therapy can infect HCV naïve human T cells. This finding may have significant pathogenic (residual liver disease) and epidemiological (infectivity) consequences.

Overall, our findings provide conclusive evidence that cells of the immune system can act as reservoirs of HCV and that virus replication in this compartment leads to the production of infectious virions. Furthermore, we described that virus exposure can alter the proliferative ability of lymphoid cells, possibly impairing their response to virus and slowing viral clearance *in vivo*. Finally, we documented the infectious potential of residual virus that lingers in essentially asymptomatic patients after resolution of chronic hepatitis C. While the clinical significance of our findings are currently under investigation, the *in vitro* system established, capable of supporting the entire cycle of HCV replication in the natural cell milieu, may serve as a valuable tool to study poorly understood aspects of HCV infection, including factors determining cell susceptibility and virus-induced cytopathic mechanisms induced by the virus, as well as being applicable for evaluations of novel anti-HCV agents.

ACKNOWLEDGEMENTS

There are many people I wish to thank for their support during my Ph.D. studies and without whom this would not have been possible. Firstly, I would like to acknowledge and sincerely thank my supervisor Dr. Thomas Michalak. I have enjoyed my time in your lab and have a list of lessons as long as my arm that will follow me for the rest of my life. I truly appreciate your friendship and the amount of time and effort that you dedicate to the success of your students. I hope to be able to pass that gift on in the future.

I wish to extend my sincere thanks to the members of my supervisory committee, Dr, Michael Grant and Dr. Kensuke Hirasawa for their guidance and encouragement throughout my studies.

I would like to acknowledge the Canadian Liver Foundation and the National CIHR Research Training Program in Hepatitis C for their financial support of my studies.

To Tram, my "HCV buddy" many thanks for everything. I have learned from your work ethic and I cherish your friendship.

Many thanks to Shashi, who introduced me to the Michalak lab, for your advice on my projects and your cheery attitude each day in the lab, you are a true friend.

To the rest of the members of the lab, Trish, Norma, Cliff, Adam, Mohammed, Brad, Georgia, Danielle and Colleen and the members of the immunology group, thanks for all your help and friendship. I will miss the discussions, laughs, words of wisdom, potlucks, softball games and most importantly, the stock tips!

To my brothers Rory, Redmon and Ronan and my friends from outside the lab, you guys are the greatest, thanks for all the support.

I wish to thank my sister, Natasha and her husband Joe for their fierce loyalty, the late night pep-talks during my course work, the candy bouquets while studying for my comprehensives and for bringing my adorable little nephews Declan and Aiden into my life.

Words cannot express my gratitude to Peter for your unwavering love and support. I am truly the luckiest.

Finally, I would like thank my loving mother Elena, who has always conveyed the importance of hard work and perseverance. To her I dedicate this thesis.

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ABBREVIATIONS

AH	acute hepatitis
OAS	2'-5' oligoadenylate synthetase
ALT	alanine aminotransferase
Alexa 488	Alexa Fluor 488
APC	allophycocyanin
anti-HCV	antibodies against HCV
AST	aspartate aminotransferase
BNX	beige/nude/X-linked immunodeficient
CFSE	carboxyfluorescein succinimidyl ester
CsCl	cesium choride
CHC	chronic hepatitis C
cDNA	complementary DNA
Con A	concanavalin A
C	core
CTL	cytotoxic T cells
DC	dendritic cells
DNA	deoxyribonucleic acid
EVR	early virological response
ER	endoplasmic reticulum
E1	envelope 1
E2	envelope 2
FACS	fluorescence activated cell sorting
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAG	glycosaminoglycan
GFP	green fluorescence protein
HAV	hepatitis A virus
HEV	hepatitis E virus
HBV	hepatitis B virus
HCVcc	cell culture-derived JFH-1 virus
HCVpp	hepatitis C virus pseudoparticles
HCV	hepatitis C virus
HDV	hepatitis delta virus
HGV	hepatitis G virus
HCC	hepatocellular carcinoma
HOMA-IR	homeostasis model assessment of insulin resistance
Huh	human hepatoma
HIV	human immunodeficiency virus

HVR	hypervariable region
ISG	IFN-stimulated genes
IEM	immunoelectron microscopy
Ig	Immunoglobulin
IRS	insulin receptor substrate
IFN/RBV	interferon alpha and ribavirin combination therapy
IFN-β	interferon beta
IFN-γ	Interferon gamma
IFN-λ	interferon lambda
IRF	interferon regulatory factors
IL	interleukin
IRES	internal ribosomal entry site
IDU	intravenous drug usage
JFH-1	Japanese fulminant hepatitis-1
Kb	kilobase
LDLR	low density lipoprotein receptor
MGN	membranoproliferative glomerulonephritis
MC	mixed cryoglobulinemia
MMLV	Moloney murine leukemia virus,
mAb	monoclonal antibody
NK	natural killer
NS	nonstructural
NHP	normal healthy plasma
NAH	nucleic acid hybridization
OCI	occult infection
OLT	orthotopic liver transplants
PAMP	pathogen-associated molecular pattern
PerCP	peridinin chlorophyll protein complex
PBMC	peripheral mononuclear cells
PTA	phosphotungstic acid
PE	phycoerythrin
PHA	phytohemagluttinin
PWM	poke wheat mitogen
PCR	polymerase chain reaction
PCT	Porphyria cutanea tarda
PD-1	programmed death receptor-1
PDL-1	programmed death receptor-ligand 1
PKR	protein kinase R
RVR	rapid virological response

rHCV UTR-E2	recombinant HCV UTR-E2 fragment
Tregs	regulatory T cells
RT	reverse transcriptase
RNA	ribonucleic acid
RdRp	RNA-dependent RNA polymerase
SRB1	scavenger receptor class B type 1
SCID	severe combined immunodeficiency disease
SOCS	suppressor of cytokine signaling
SVR	sustained virological response
sRNA	synthetic RNA
TIM-3	T cell immunoglobulin and mucin domain-containing protein 3
Th-1	T-helper type 1
TLR	Toll-like receptor
TMA	transcription mediated amplification
TNF-α	tumour necrosis factor alpha
IFN-3	type III interferon
UTR	untranslated region
Alb/uPA	urokinase plasminogen activator transgene driven by the albumin promoter
vge	virus genome equivalent

THESIS CO-AUTHORSHIP STATEMENT

This thesis is comprised of 6 chapters. Chapter 1 is an introduction and provides the background and rationale for the studies carried out during this project. Chapters 2 to 4 contain original data collected during each of the segments. Chapter 5 is a discussion which connects findings from all three projects while Chapter 6 relays the overall conclusions of the work and the future directions of each study.

The large majority of the work described in this thesis was carried out by the author. The role of the author included contributing to the design of the experiments, establishment of techniques, data collection and analysis, and manuscript preparation. The work described in Chapter 2 was published as a first authored paper entitled "***De novo* infection and propagation of wild-type hepatitis C virus in human T lymphocytes *in vitro***" in *Journal of General Virology,* 2006, volume 87, pp. 3577-3586. The study described in Chapter 4 was published as a first authored paper entitled "**Hepatitis C virus persisting after clinically apparent sustained virological response to antiviral therapy retains infectivity *in vitro***" in *Hepatology*, 2009; volume 49, pp.1431-1441. The author acknowledges the contributions made by others: all evaluations of HCV RNA negative strand in Chapters 2, 3, and 4 were performed by Dr. Tram N.Q. Pham, staining of cells for confocal microscopy in Chapter 2 was carried out by Dr. Shashi A. Gujar. Dr. Cliff S. Guy provided a contribution to the evaluation of HCV inoculum infectivity carried out in Chapter 4.

Chapter One: Introduction:

1.1. VIRAL HEPATITIS

Hepatitis, from the Greek words *hepato,* meaning 'liver' and *itis,* reflecting 'inflammation', refers to inflammatory disease of the liver that is characterized by the presence of hepatocyte injury and lymphomononuclear cell infiltrates. Viral infections are one of the major causes of hepatitis, referred to as viral hepatitis. The causative agents include hepatitis A through G viruses. Thus, hepatitis A virus (HAV) is an RNA virus and a member of the *Picornaviridae* family that is transmitted by the fecal-oral route. HAV infection has an acute course with no tendency to progress to chronic hepatitis (Melnick, 1995). A vaccine exists for HAV that provides lifelong protection from infection (Wiedermann et al., 1997). Hepatitis B virus (HBV) is a highly infectious, deoxyribonucleic acid (DNA) virus and member of the *Hepadnaviridae* family. HBV is transmitted by bodily fluids and induces a persistent infection which may result in both acute and chronic hepatitis, as well as asymptomatic occult long-term virus carriage (Mulrooney-Cousins and Michalak, 2007; Liang, 2009). Presently, 2 billion people are thought to be infected with HBV worldwide with at least 350 million individuals having symptomatic chronic hepatitis B (Lavanchy et al., 1999). Currently, a vaccine that confers lasting immunity is widely available, however, new infections in vaccine non-protected individuals and in children born to infected mothers are still a great global health problem (Hoofnagle, 2006). Hepatitis D virus (HDV) is a negative sense, single-stranded, closed circular ribonucleic acid (RNA) virus of the genus *Deltavirus*. HDV is a

satellite virus for HBV in that it requires coinfection with HBV to infect, assemble and release virions (Farci, 2003). Hepatitis E virus (HEV) is a small non-enveloped single-stranded positive-sense RNA virus. It is the sole member of the genus *Hepevirus* and is mainly transmitted by the fecal-oral route (Mushahwar, 2008). Hepatitis G virus (HGV) is an RNA virus of the *Flaviviridae* family which appears to be closely related to hepatitis C virus (HCV), replicates primarily in lymphocytes, and does not appear to cause liver disease (Kleinman, 2001). HCV and infection caused by this virus are the subject of this dissertation and will be discussed in detail in the subsequent Sections.

1.2. EPIDEMIOLOGY OF HCV INFECTION

Identified in 1989 as the causative agent of non-A, non-B hepatitis (Kuo et al., 1989), HCV is a small single-stranded RNA virus that belongs to the *Flaviviridae* family and is a member of the hepacivirus genus. HCV is a highly genetically variable virus with 6 major HCV genotypes, as currently recognized, and over 100 subtypes that are classified based on viral nucleotide sequence heterogeneity (Simmonds, 1999; Ramia and Eid-Fares, 2006). The main target and the site of HCV replication is the hepatocyte, although HCV has been shown to replicate in extrahepatic sites, particularly lymphoid cells, including B and T lymphocytes and dendritic cells (DC) (Bronowicki et al., 1998; Radkowski et al., 2000; Goutagny et al., 2003; Pham et al., 2004; Pham et al., 2008). In the steady state of HCV infection, hepatocytes contribute the majority of HCV virions found in circulation (Pawlotsky, 2006), although this may not be the case following liver transplantation due to HCV-induced, end-stage liver disease where the majority of the virus might be derived from infected lymphoid cells (Pal et al., 2006).

The course of disease observed in HCV infection is characterized by an acute phase with 15-25% of infections resulting in spontaneous resolution of hepatitis and with the remaining 75-85% leading to chronic hepatitis C (CHC) defined as HCV RNA positivity for at least 6 months (Hoofnagle, 2002). The course of acute and chronic hepatitis C, as well as spontaneous resolution of hepatitis C will be discussed in detail in Sections 1.5.1 and 1.5.2., respectively.

HCV is considered to be a non-cytopathic virus. Thus, liver injury occurs as a result of the virus-specific host immune response to infection of hepatocytes (Guidotti and Chisari, 2006). At the same time, *in vitro* studies examining the effect of HCV on primary human hepatocytes showed that HCV may also have a direct cytopathic effect (Lazaro et al., 2007). Of those patients with CHC, between 5 and 25% will develop liver cirrhosis (Mattson et al., 1993). The main cause of HCV-induced cirrhosis is the long-term inflammation and the replacement of injured hepatocytes by connective tissue and abnormal hepatic tissue nodules (Pol et al., 2004). The resulting cirrhosis is the cause of approximately 50% of orthotopic liver transplants (OLT) (Prieto et al., 1998). Furthermore, approximately 5% of cirrhotic patients will eventually develop hepatocellular carcinoma (HCC) (Llovet, 2005; Thomas and Seeff, 2005). The genotype of HCV appears to play a role in disease outcome in that those infected with genotype 1b have a consistently greater risk of developing HCC (Bruno et al., 1997). As well, those infected with genotype 3 are at higher risk for steatosis (Rubbia-Brandt et al., 2000). While genotype is a major predictor of antiviral treatment success, studies have found no

clear relationship between genotype and liver disease progression (Poynard et al., 1997; Puoti et al., 1997).

1.2.1. Global Prevalence and Endemic Areas

It is estimated that 170,000,000 people worldwide (Wasley and Alter, 2000) and approximately 300,000 individuals in Canada with symptomatic HCV infection accompanied by CHC (Fischer et al., 2006). Egypt has the highest prevalence for HCV infection with up to 20% of the population being chronically afflicted (Mohamed et al., 1996; Habib et al., 2001). This unusually high rate of infection is believed to be due to parenteral antischistosomal mass-treatment campaigns which were discontinued in the 1980's. This may represent the greatest incident of iatrogenic transmission of a blood-borne pathogen worldwide (Frank et al., 2000).

As mentioned in Section 1.2.1, HCV is classified into 6 major HCV genotypes and over 100 subtypes according to variations in the nucleotide sequence of the genome (Simmonds, 1999; Ramia and Eid-Fares, 2006). In terms of classification, nucleotide sequences of HCV strains of the same subtype can differ by as much as 5% to 15%, while differences between subtypes range from 10% to 30% and genotypes by 30% to 50% (Hoofnagle, 2002). HCV genotypes are known to have a geographical distribution (Mellor et al., 1995; Wasley and Alter, 2000). Genotypes 1 through 3 can be found worldwide, although 1b is the most common strain overall (Simmonds, 2004). Genotype 1a is the main virus type prevailing in Europe and North America, while 1b is predominant in Japan (Greene et al., 1995; Simmonds et al., 2005). Genotype 2 is mainly occurring in the Mediterranean, Western Africa, and the Far East (Mellor et al., 1995;

Simmonds, 2004). Genotype 3 is found in Southeast Asia, India and Australia, whereas genotype 4 is the predominant strain in Egypt, northern Africa and the Middle East (Ramia and Eid-Fares, 2006). In South Africa, genotype 5 is the most prevalent, while type 6 occurs in Southeast Asia and Australia (Mondelli and Silini, 1999; Simmonds, 2004; Simmonds et al., 2005). Previously, at least 11 genotypes have been described (Bukh et al., 1993; Simmonds, 1999), but these genotypes were later re-classified into 6 major genotypes due to the similarity in nucleotide sequences (Simmonds et al., 2005).

1.2.2. Transmission Routes and Risk Factors

HCV is a highly infectious blood-borne virus. The major route of transmission is by blood-blood contact (Conry-Cantilena et al., 1996) and the major risk factor associated with HCV transmission is intravenous drug use (IDU) (Alter et al., 1999). It is estimated that as much as 80% of new HCV infections are due to IDU (Law et al., 2003) and in active IDU, the prevalence of HCV can be as high as 95% (Fischer et al., 2004). Sexual contact has been found to be a risk factor for HCV transmission, although less than 3% of monogamous partners of HCV-infected individuals in the United States have been found to be infected (Terrault, 2002). Other risk factors include intranasal cocaine use, hemodialysis, tattooing, piercing and needlestick injuries (Kiyosawa et al., 1991; Eyster et al., 1991; Niu et al., 1993; Thomas et al., 1995; Conry-Cantilena et al., 1996; Lavanchy et al., 1999; Firestone et al., 2007). Available data for the United States collected by the CDC's Sentinel Counties Study of Viral Hepatitis between 2001 and 2004, list the following risk factors: IDU (39%), occupational exposure (4%); household exposure (3%), transfusions (2%), sexual transmission (16%) and no indentified risk (33%) leading

to HCV infection (Weinbaum, 2009). Mother-to-infant transmission of HCV has been found in 4 to 7% of cases of babies born to mothers with a high level of viremia although the mode of transmission is unknown (Roberts and Yeung, 2002).

1.3. Flaviviridae Family

The *Flaviviridae* family of viruses includes the *Flavivirus*, *Hepacivirus* and *Pestivirus* genera. The *Flaviviridae* family consists of viruses which have positive-sense, single-stranded RNA genomes ranging from 9.5 kilobase (Kb) to 12.5 Kb that are translated as a single polyprotein. Virion particles of the *Flaviviridae* family are enveloped and spherical (Lindenbach et al., 2007b). The *Flavivirus* genera is made up of viruses such as dengue virus, West Nile encephalitis virus, tick-borne encephalitis virus, Murray Valley encephalitis virus, St. Louis encephalitis virus, Japanese encephalitis virus, and yellow fever virus (Lindenbach et al., 2007b). The *Pestivirus* genera includes viruses such as bovine viral diarrhea virus, border disease virus and classical swine fever (hog cholera) virus (Collett et al., 1988). HCV is the sole member of the *Hepacivirus* genera (Lindenbach et al., 2007b). It has been postulated that *Flaviviridae* viruses use the low density lipoprotein receptor (LDLR) to enter host cells (Agnello et al., 1999).

1.4. HEPATITIS C VIRUS

1.4.1. Genome Organization

HCV is comprised of a 9.6-Kb RNA genome which is flanked by highly structured 3'- and 5'-untranslated regions (UTR) (Figure 1.1.). The 5'-UTR contains an internal

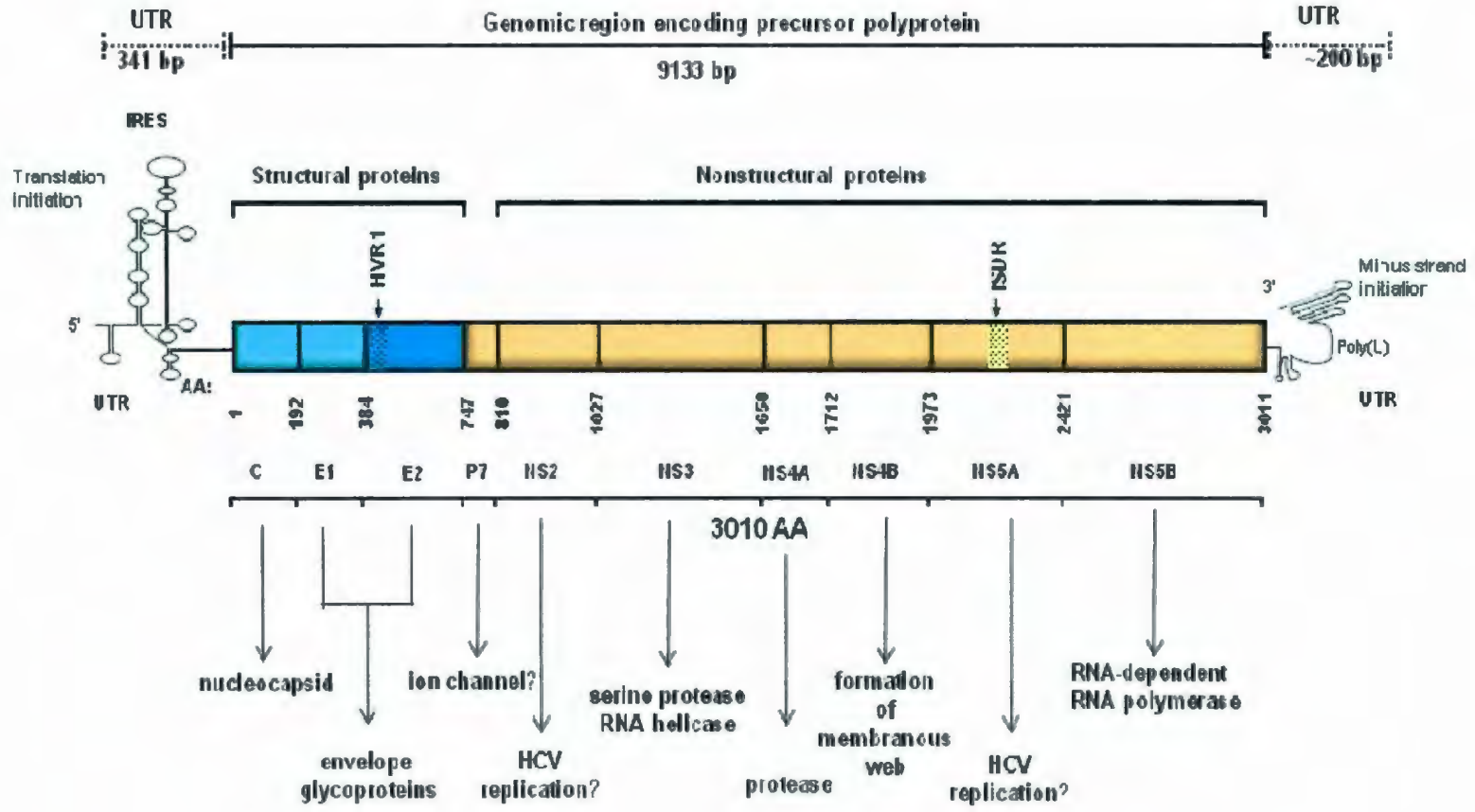
ribosomal entry site (IRES) for initiation of viral protein translation and structured RNA elements required for virus replication and viral protein translation (Honda et al., 1996; Lindenbach et al., 2007b).

1.4.2. HCV Lifecycle

1.4.2.1. Entry and Uncoating

HCV particles circulate in the blood in association with lipoproteins (Burlone and Budkowska, 2009). The viral E1 and E2 envelope glycoproteins are necessary for virus entry (their functions will be discussed in detail in Section 1.4.3) (Suzuki et al., 2007). HCV binding and entry is thought to be mediated by a number of molecules such as: (1) LDLR, which has been shown to enhance infection of normal human hepatocytes with serum-derived HCV *in vitro* (Agnello et al., 1999; Molina et al., 2007); (2) Glycosaminoglycans (GAG), which have been found to be involved in the binding of serum-derived HCV to vero cells (Germi et al., 2002); (3) Scavenger receptor B1 (SRB1), a molecule that binds to soluble HCV E2 proteins *in vitro* (Scarselli et al., 2002) and is required for the *in vitro* entry of HCV pseudoparticles (HCVpp) (described in Section 1.9.2.2.) (Bartosch et al., 2003c) and particles derived from a genotype 2a Japanese fulminant hepatitis (JFH)-1 HCV isolate (JFH-1 isolate described in Section1.9.2.3) (Catanese et al., 2010); (4) CD81, a tetraspanin that has been shown to bind with high affinity to recombinant HCV E2 glycoprotein and to viral particles from HCV-infected patients *in vitro* (Pileri et al., 1998; Petracca et al., 2000) and (5) Claudin-1 and occludin, which are tight junction proteins found on hepatocytes that have been

Figure. 1.1. HCV genome organization. The HCV genome is a single-stranded RNA of approximately 9.6 Kb. It contains one long open reading frame that encodes a large polyprotein precursor of ~3000 amino acids (AA). The polyprotein is translated after initiation via the internal ribosomal entry site (IRES) found in the 341 bp 5'-untranslated region (UTR). The HCV polyprotein is co- and post-translationally cleaved by host and viral proteases to yield 10 structural and nonstructural (NS) proteins. These proteins are: core (C), envelope 1 (E1) and envelope 2 (E2), p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. At the N-terminus of the E2 region, from amino acid 384 to 410, there lies a hypervariable region (HVR1). As well, in the NS5A region, the putative interferon sensitivity determining region (ISDR) located at amino acids 2209 to 2248, has been recognized. The 3'-UTR is located at the C-terminus of the HCV genome, contains a poly(U) sequence and is thought to have a role in the initiation of minus strand RNA synthesis. Putative functions of individual viral proteins are indicated.


UTR
341 bp
Genomic region encoding precursor polyprotein
9133 bp
UTR
~200 bp
IRES
Translation initiation
Structural proteins
Nonstructural proteins
HVR1
ISDR
Minus strand initiation
3'
5'
Poly(U)
UTR
AA:
UTR
1
192
384
747
810
1027
1658
1712
1973
2421
3011
C
E1
E2
P7
NS2
NS3
NS4A
NS4B
NS5A
NS5B
3010 AA
nucleocapsid
envelope glycoproteins
ion channel?
HCV replication?
serine protease RNA helicase
protease
formation of membranous web
HCV replication?
RNA-dependent RNA polymerase

seen to play a role in the entry of JFH-1 and HCVpp into murine cells and human hepatoma cell lines *in vitro* (Evans et al., 2007; Ploss et al., 2009).

HCVpp and JFH-1 particles have been observed to be internalized in clathrin-coated pits by clathrin-mediated endocytosis *in vitro* (Blanchard et al., 2006). The fusion of HCVpp and JFH-1 particles with the cellular membranes of Huh-7 cells *in vitro* has been found to occur in the early endosome, a process that is thought to be driven by the acidification of these vesicles (Blanchard et al., 2006). When fusion is completed, uncoating and release of the HCV genome in the cytoplasm takes place (Dubuisson et al., 2008).

1.4.2.2. Polyprotein Translation and Processing

Translation of the HCV long open reading frame yields a single polyprotein of approximately 3,000 amino acids (Choo et al., 1991). During this process, in the first step, the HCV RNA genome binds to the ribosome. Translation is a cap-independent process mediated by an IRES located in the domains II through IV of the 5'-UTR (Bartenschlager et al., 2004). The generated polyprotein is then targeted to the endoplasmic reticulum (ER) where it is co- and post-translationally cleaved by host and viral proteases to yield 10 viral structural and nonstructural (NS) proteins. These proteins are: core (C), envelope 1 and envelope 2 (E1 and E2), p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (see Figure 1.1.) (Bartenschlager et al., 2004; Penin et al., 2004).

1.4.3. Functions of Viral Proteins

The HCV virion is made up of structural proteins which include core, E1 and E2. The core or capsid protein is released from the polyprotein by host signal peptidases yielding the mature protein which binds RNA and is targeted to the cytoplasmic surface of the ER (Dubuisson, 2007). E1 and E2, which are also released from the polyprotein by host signal peptidases, are transmembrane glycoproteins that constitute the virus envelope and are required for viral entry into cells. E2 has been found to bind to many putative HCV receptors (see Section 1.4.2.1.). The E2 protein sequence contains a hypervariable region (HVR) which is a target for neutralizing antibodies and variations in this region are thought to be responsible for virus immune escape (also see Section 1.8.2)(McAllister et al., 1998). p7 is a short membrane peptide which is cleaved from the polyprotein by host signal peptidases and appears to have ion channel features (Pavlovic et al., 2003). It is thought to be required for HCV infectivity in chimpanzees (Sakai et al., 2003), as well as being required for assembly and release of infectious virions (Steinmann et al., 2007a). The 3-dimensional structure of p7 has recently been revealed and this protein is thought to be a viroporin (Luik et al., 2009). Additionally, the introduction of mutations in the coding region of p7 has led to inhibited replication of HCV in Huh-7 cells as well as HCV particle release from the same cells (Steinmann et al., 2007b). Unlike the structural proteins, the NS proteins are cleaved from the polyprotein by the NS2-3 and NS3-4 viral proteases. NS2 is a membrane spanning protein (Santolini et al., 1995) and in the HCV genotype 2a JFH-1/Huh-7-based cell culture system, which will be described in detail in

Section 1.9.2.3, it has been shown that adaptive mutations in the NS2 alone can lead to enhanced production of HCV (Russell et al., 2008).

The NS3 protein includes a serine protease domain, which is located at the N-terminus of the protein, and a helicase domain, at the C-terminus. The serine protease is known to cleave the HCV polyprotein at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B sites.

NS4A is the smallest of the HCV NS proteins and is part of the NS3-4A protease, which is involved in cleavage of the polyprotein (Lindenbach et al., 2007a). The NS4B is an integral membrane protein that associates with the ER membranes. Data has shown that the expression of NS4B induces the formation of the membranous web within the cytoplasm which is involved in HCV replication (Penin et al., 2004). Replication-enhancing mutations have been identified in NS4B in the JFH-1-based cell culture system (HCVcc) which will be described in Section 1.9.2.3 (Pietschmann et al., 2009).

The NS5A protein is a membrane-associated phosphorylated multidomain protein that has been found to play a role in HCV replication (Lindenbach et al., 2007b). In the JFH-1 Huh-7 system, adaptive mutations in NS5A confer more robust HCV replication (Dubuisson, 2007). As well, NS5A has been shown to contain an interferon sensitivity determining region (ISDR) found at amino acids position 237-276. Mutations in this region can result in higher interferon sensitivity and lower HCV RNA loads in hepatoma cell lines *in vitro* (Fukuma et al., 1998).

The NS5B protein is an RNA-dependent RNA polymerase (RdRp), the enzyme which drives HCV replication. NS5B is post-translationally inserted into the ER membrane, an association that has been found to be required for RdRp activity. Because of its well determined role in HCV replication, NS5B is a target for potential HCV-specific antiviral

therapies (Pockros et al., 2008; Kneteman et al., 2009), which will be discussed in greater detail in Section 1.10.2.

1.4.4. Replication Strategy

Plus-stranded RNA viruses, such as polio virus (Bienz et al., 1992), mouse hepatitis virus (Gosert et al., 2002) and flaviviruses (Westaway et al., 1999), are known to reproduce via a replication complex which is comprised of viral proteins, replicating RNA and altered cellular membranes. The existence of an HCV replication complex has been clearly shown in cell culture models of HCV (Gosert et al., 2003). Studies employing the replicon system have shown that in HCV-transfected Huh-7 cells, all of the HCV NS proteins, as well as HCV RNA plus-strand, coexist in the cytoplasm in association with a specific membrane ultrastructural alteration, referred to as the membranous web (Gosert et al., 2003). HCV propagation occurs via synthesis of the HCV RNA negative strand, which serves as a template for synthesis of the HCV RNA positive strand. Therefore, the detection of this strand is accepted as an indicator of actively progressing replication. The HCV RdRp, discussed above, is the enzyme responsible for this process (Lohmann et al., 1997; Moradpour et al., 2003). Due to the lack of 3'-exonuclease proofreading activity, the RdRp inserts mutations into the genome during replication (Bartenschlager and Lohmann, 2000). The HCV quasispecies, defined as a swarm of closely related but genetically distinct variants, is a direct consequence of a high rate (10^{12} virions per day) of error-prone replication which results in the generation of a multiplicity of viral variants. Thus, the error rate of HCV during replication is 1.5-2 $x10^{-3}$ base substitutions

per genome site per year (Neumann et al., 1998). The role of the HCV quasispecies in viral immune evasion will be discussed in detail in Section 1.8.2.

1.4.5. Virion Ultrastructure and Biophysical Properties

The virion of HCV is a spherical 40-60-nm particle that is made up of a detergent-sensitive lipoprotein envelope with spike-like projections. This envelope encapsulates a spherical nucleocapsid that exhibits polyhedral symmetry (Kaito et al., 1994; Heller et al., 2005). The non-enveloped HCV nucleocapsid ranges in size from 25-50 nm and have been seen to band at approximate 1.24 g/mL in a sucrose gradient and 1.33 g/mL in cesium chloride (Miyamoto et al., 1992; Kanto et al., 1994; Trestard et al., 1998; Maillard et al., 2001) . The density of enveloped HCV virions has been found to be heterogeneous with reported densities ranging from 1.04 to 1.11 g/mL in a sucrose density gradient and between 1.18 and 1.25 g/mL in a cesium chloride gradient (Hijikata et al., 1993; Kanto et al., 1994; Diaz et al., 2006). Low density particles have been shown to be associated with lipoproteins (Diaz et al., 2006), while high density particles, with immunoglobulins (Hijikata et al., 1993).

1.5. NATURAL HISTORY OF HCV INFECTION

1.5.1. Acute Hepatitis

The first 6 months following HCV invasion is considered as the acute phase of infection. During this time period, viral RNA titers rise rapidly with the viral load doubling as quickly as every 10.8 hours (Glynn et al., 2005). HCV RNA can be detected in the serum within 7-21 days after exposure using polymerase chain reaction (PCR)-based assays

(Farci et al., 1991), while it takes on average 50 days for HCV-specific antibodies to be detected (Orland et al., 2001; Cox et al., 2005). In the majority of HCV-infected patients, acute infection is asymptomatic and frequently not diagnosed (Marcellin, 1999). In 15-30% of HCV-infected patients, symptomatic acute hepatitis (AH) is identified (Busch and Shafer, 2005). The symptomatic phase is thought to occur between 2 and 12 weeks after exposure and can last up to 12 weeks thereafter (Orland et al., 2001). During AH, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, which indicate hepatocyte injury, can be as high as 10 times the normal level (Thomas and Seeff, 2005). The HCV RNA load in patients during AH has been seen to range from 10^4 to 10^7 genome copies or virus genome equivalents (vge) /mL (Alter M.J et al, 1992; Glynn et al., 2005). In patients who experience symptomatic acute infection, the frequency of spontaneous, apparent complete clearance of viremia is higher than that in those with asymptomatic infection (Gerlach et al., 2003; Santantonio et al., 2003).

1.5.2. Spontaneous Resolution of Hepatitis C

AH can be spontaneously resolved in 15-25% of cases (Thimme et al., 2001; Cox et al., 2005). In children, this clearance can occur in as few as 6% of HCV RNA-seropositive individuals, as was seen in a study of 224 European children (Jara et al., 2003). In terms of immunological determinants, a robust and vigorous response including HCV-specific $CD4^+$ and $CD8^+$ T cell reactivity against multiple viral epitopes is associated with spontaneous recovery (Thimme et al., 2001; Gerlach et al., 2003; Santantonio et al., 2003). Among genetic factors, a single nucleotide polymorphism upstream of the interleukin (IL)-28B gene encoding the type III interferon (IFN-3 or IFN-λ), has been

recently found in individuals with European and African backgrounds to be associated with enhanced resolution of hepatitis C (Thomas et al., 2009). The rates of spontaneous clearance in IDU who have been re-infected can be as high as 83%, further emphasizing the role of the adaptive immune response in apparent complete resolution of viremia (Osburn et al., 2010). It has also been found that patients infected with genotype 3 have a greater frequency of resolution of hepatitis C than those afflicted with genotype 1 (Lehmann et al., 2004).

1.5.3. Chronic Hepatitis

Chronic hepatitis C is characterized as HCV RNA persistence in serum (plasma) for 6 months or longer post-exposure and can occur in up to 85% of patients infected (Thomas and Seeff, 2005). During chronic symptomatic infection in adults, HCV RNA loads are lower than in AH and they generally plateau at levels between 10^4 and 10^6 IU/mL (approximately $5x10^4$ and $5x10^6$ vge/ mL) (Fabrizi et al., 2000). In a proportion of patients, serum HCV RNA and ALT levels can fluctuate (Inglesby et al., 1999). Patients with CHC can be asymptomatic for many years although some may experience fatigue, right upper quadrant pain, nausea, dark urine, itching and anorexia (Hoofnagle, 1997). In CHC, the measure of disease progression is the degree of liver necroinflammatory lesions, which is characterized by portal and intralobular lymphomononuclear cell infiltration, hepatocyte necrosis and fibrosis, as determined by histological examination of liver biopsy.

1.5.4. Factors Affecting Progression to Liver Disease

Factors affecting the progression of CHC and cirrhosis in individual patients appear to be both host and virus related. In a study which examined development of liver disease in 2235 HCV-infected patients by examining the METAVIR score (a standardized scoring system which measures both the degree of inflammation and fibrosis), the median rate of progression to fibrosis was 0.133 fibrosis units per year, with fibrosis progression per year being defined as the ratio between fibrosis stage in METAVIR units and the time-span of infection (Poynard et al., 1997). Several independent host factors have been found to be associated with a faster rate of liver injury progression, such as: (1) male sex (possibly related to a protective effect of estrogen) (Di Martino et al., 2004); (2) alcohol consumption (greater than 50 g/day) (Schiff and Ozden, 2003), and (3) age of acquisition of infection (those who are infected after the age of 40 show more severe disease). The median time from the acquisition of HCV infection to cirrhosis is estimated to be 30 years. However, men infected after the age of 40 have been seen to progress to cirrhosis with a median of only 13 years, while women who did not consume alcohol progress to cirrhosis in a median of 42 years (Poynard et al., 1997).

Additional factors promoting progression of CHC include duration of infection, immunosuppressive therapy and low $CD4^+$ T cell count due to human immunodeficiency virus (HIV) infection, HBV infection or schistosomiasis coinfection, non-alcoholic steatohepatitis and non-responsiveness to antiviral therapy (Thomas and Seeff, 2005). Excessive iron load in the liver has also been suggested to accelerate progression to cirrhosis. Mutations in the hemochromatosis gene, which causes higher ferritin and serum

iron in heterozygous patients, has been seen to be an independent risk factor for progression to liver fibrosis and, subsequently, cirrhosis in HCV-infected patients (Erhardt et al., 2003; McCaughan and George, 2004).

Considering virus-related factors, it has been postulated that the expression of HCV proteins can affect the degree of liver fibrosis and disease progression. For example, HCV core protein has been shown to induce oxidative stress, alter mitochondrial function and increase the production of reactive oxygen species, which in turn promote apoptosis (Okuda et al., 2002). It has been reported that increased expression of activated caspases, which are mediators of apoptosis, in sera of patients with CHC, is associated with a higher degree of fibrosis in these patients. (Bantel et al., 2004).

Coinfection with HIV has been shown to augment progression to fibrosis in patients with CHC and is accompanied by higher serum levels of HCV RNA (Thomas et al., 1996; Benhamou et al., 1999). At the same time, evidence has shown that certain antiviral therapies can slow the progression of CHC to cirrhosis. HIV/HCV coinfected patients treated with protease inhibitors experienced a significantly lower rate of cirrhosis than patients not receiving this treatment (Poynard et al., 1997; Benhamou et al., 2001; Feld and Liang, 2006).

Nonetheless, so far, it is still not possible to accurately predict the rate of progression of CHC to fibrosis for individual patients based on known virus and host factors (Feld and Liang, 2006).

For children, as compared to adults, the course of CHC seems to be more mild and the rate of progression to fibrosis is much slower (Broide et al., 2004). Most children are diagnosed incidentally and rarely have symptoms. As well, children with CHC

spontaneously resolve hepatitis less frequently and rarely develop cirrhosis or hepatocellular carcinoma as well as extrahepatic complications. The explanation for a milder course of infection and slower progression to fibrosis could be related to an apparent immune tolerance of HCV at birth or early in life (Garcia-Monzon et al., 1998).

1.5.5. Occult Persistence

The introduction of highly sensitive nucleic acid amplification assays capable of detecting below 10 vge/mL or 2 IU/mL has changed our understanding of the natural history of HCV infection. These assays have allowed for the detection of very low levels of HCV RNA positive and negative (replicative) strands in individuals who have resolved CHC either spontaneously or due to antiviral therapy with IFN and RBV (Pham et al., 2004) .

Presently, standard clinical laboratory assays have sensitivities which range between 30 and 1000 vge/mL or 9.6 and 615 IU/mL (Pham and Michalak, 2008). Commonly, the predominant test being used in clinics is the VERSANT HCV RNA version 3.0 assay from Bayer with a sensitivity of approximately 1000 vge/mL or 615 IU/mL. The most sensitive of these tests involve transcription mediated amplification (TMA) assays such as the VERSANT HCV® RNA assay that can achieve a sensitivity of ~30 vge/mL or 9.6 IU/mL. However, this assay is not yet routinely used for clinical evaluation.

The detection of HCV RNA positive strands in the serum, and both positive and negative strands in peripheral blood mononuclear cells (PBMC) and/or liver of individuals who have been found to be repeatedly HCV RNA non-reactive by standard clinical laboratory tests and have repeatedly normal ALT levels is defined as occult

infection (OCI) (Pham et al., 2004; Radkowski et al., 2005a; Laskus et al., 2007; Michalak et al., 2007; Pham et al., 2008). This finding was first reported by this laboratory (Pham et al., 2004) and confirmed by other groups (Castillo et al., 2004; Radkowski et al., 2005a; Castillo et al., 2006). It has been found that applying sensitive detection techniques (sensitivity of 10 vge/mL or 2 IU/mL) uncovers HCV replication for years after apparent complete spontaneous or therapeutically-induced resolution of hepatitis (Pham et al., 2004). Additionally, HCV RNA has been reportedly detected in patients with elevated liver function tests of unknown etiology (Castillo et al., 2004).

Furthermore, HCV RNA positive and negative strands have been detected in $CD4^+$ and $CD8^+$ T cells, B cells and monocytes in individuals after clinically apparent resolution of hepatitis C as well as the appearance of distinct HCV variants in their immune cells (Pham et al., 2008).

In terms of duration of OCI, HCV RNA positive and negative strands have been detected in liver biopsy samples taken from patients that had apparently clinically cleared the virus up to 8 years earlier (Radkowski et al., 2005a). At this point, due to limited follow-up periods, it is not known whether occult HCV infection occurs for the entire life, like other persistent viruses, such as HBV (Michalak et al., 1994) and Epstein-Barr virus (EBV) (Babcock et al., 1998). It has been suggested that a major clinical consequence of occult infection are the public health implications in that patients, without clinical markers of infection, may carry a risk to the community with respect to blood donation and organ transplantation (Pham et al., 2004; Feld and Liang, 2005).

The presence of residual virus that has the ability to replicate may lead to HCV reactivation after apparent successful antiviral treatment. The rates of HCV recurrence,

after sustained virological response (SVR), which is defined as serum HCV RNA negativity by standard clinical laboratory RT-PCR assays for a minimum of 6 months after completion of antiviral treatment, have been reported to be as high as 20% (Di Liberto et al., 2006). A study following 97 HCV infected subjects after a response to IFN/RBV, in which testing for HCV RNA was carried out every 6 months over a 7 year follow-up period, revealed that 11of these patients (corresponding to 11.3%) showed viral recurrence (Ciancio et al., 2006). Viral reactivation has also been observed in HCV RNA- negative patients who were recipients of liver, kidney (Melon et al., 2005; Savas et al., 2007) or bone marrow transplants (Zekri et al., 2004), and it is suggested to occur as a result of immunosuppressive therapy (Lee et al., 2005).

The challenges to estimating the numbers of patients that experience HCV recurrence after SVR are: (1) The lack of sufficiently sensitive standard clinical HCV RNA detection assays; (2) The infrequency of testing of serial serum samples of patients after SVR and (3) Testing is limited to serum or plasma but not including PBMC or liver tissue.

Factors which can enhance the detection of OCI include testing of larger volumes of plasma or serum after ultracentrifugation (Bartolome et al., 2007) and stimulation of PBMC with mitogens if the naïve cells were found to be HCV non-reactive (Pham et al., 2005). In studies carried out in this lab in which both the sera and PBMC compartments were tested in individuals after resolution of hepatitis, HCV RNA positive and in some cases negative strands were detected in at least one compartment (either serum or PBMC) in the majority of cases tested (Pham et al., 2004). In later studies, it was also found that serial sampling of sera and lymphoid cells augmented the degree of detection of residual

virus (Pham et al., 2007). As well, a recent study examining 276 individuals with no indication of hepatic disease (HCV antibody and plasma HCV-RNA negative with normal liver enzyme levels) found that 3.3% of these patients carried HCV-RNA in their untreated PBMC (De Marco et al., 2009), supporting the notion of sampling multiple compartments when testing for HCV RNA.

Occult HCV persistence has been suggested to be responsible for HCV-specific cellular immune responses in the absence of clinical markers of viremia (Quiroga et al., 2006). To that end, it has been speculated that occult HCV persistence may be helpful to the host in that there is maintained antigenic stimulation in the host and by constant stimulation of HCV-specific T cells (Pham et al., 2008). The idea that residual HCV replication contributes to ongoing liver pathology was addressed by Castillo and colleagues in a study that found virus in 57 of 100 patients who had no detectable virus by standard clinical assays (Castillo et al., 2004). They also found that the same individuals who had residual viral replication also had persistently elevated liver enzymes and were more likely to have liver fibrosis and mild inflammation (Castillo et al., 2004). At the same time, in 172 HCV antibody-positive, HCV RNA-negative patients who had apparently resolved viremia and had normal liver functions, liver biopsies revealed that 82% had fibrotic changes with $CD8^+$ T cell infiltrates that were similar to that seen in viremic patients. These findings support the notion that a large number of patients that have been clinically found to have resolved CHC continue to carry HCV in the liver (Hoare et al., 2008).

From this laboratory, studies indicate that sensitivity of HCV detection assays should be <10 vge/mL and testing should include samples of all 3 compartments of virus

occurrence (i.e., plasma, PBMC and liver) and preferably serial samples (Michalak et al., 2007).

1.5.6. Extrahepatic Non-Lymphatic System Manifestations

Studies have shown an association of HCV with several diseases of organs other than the liver and the lymphatic (immune) system (which will be reviewed in Section 1.6.1). Membranoproliferative glomerulonephritis (MGN) has been associated with chronic HCV infection (Johnson et al., 1993; Davda et al., 1993). Although factors leading to the development of HCV-related MGN are unclear, deposition of immune complexes containing HCV antigens, immunoglobulin (Ig)G or IgM class antibodies to those antigens in the renal glomeruli are most likely the pathogenic factor (Johnson et al., 1993). It has also been suggested that B cell activation and antibody production, which will be discussed in Section 1.6.1., are a possible mechanism of the development of this pathology (Johnson et al., 1993). The notion that HCV may have a role in the development of MGN is supported by the fact that interferon alpha (IFN-α)-based antiviral therapy leading to a reduction of HCV RNA load in patients has also been shown to result in histological improvement of renal pathology (Yamabe et al., 1995).

Vasculitis, a potentially fatal disease characterized by the inflammatory destruction of blood vessels, veins and or arteries (McMurray and Elbourne, 1997), has been found in some cases to be associated with HCV infection (Marcellin et al., 1993). HCV-related vasculitic lesions have been observed in such places as the skin, muscle, lungs and intestine. The mechanism of development of HCV-related vasculitis is likely the same as MGN. Treatment of HCV-related vasculitis with immuosuppressive agents, such as

rituximab (an anti-CD20 antibody used to deplete B cells), has been shown to reduce pathology in 67% of patients although up to 95% of patients experience clinical recovery when rituximab was coupled with IFN/RBV therapy (Terrier et al., 2009). As well, all relapses of vasculitis in the rituximab/IFN/RBV-treated patients were associated with a failure of antiviral therapy to control HCV infection.

Increased development of insulin resistance and type 2 diabetes mellitus have been observed in patients with HCV (Mehta et al., 2003; Romero-Gomez, 2006). A retrospective study of patients with diabetes found that 4.2% of diabetic patients, were infected with HCV, however, 29% of those HCV-positive patients carried the genotype 2a virus (Mason et al., 1999). The mechanism of HCV-related insulin resistance is unclear. A study by Kawaguchi and colleagues (2004) found that increased fasting insulin levels were associated with the presence of HCV core in serum, liver fibrosis, and decreased expression of insulin signaling molecules, insulin receptor substrate (IRS) 1 and 2 respectively. In an *in vitro* study of human hepatoma cells expressing HCV core and in HCV core-transgenic mouse livers, it was found that expression of HCV core leads to degradation of IRS1 and IRS2. Resolution of HCV infection has been seen to improve insulin resistance and increase expression of IRS1 and IRS2 (Kawaguchi et al., 2005). While diabetes development has been seen to be related to liver fibrosis stage, it has been suggested that the association between stage of fibrosis and insulin resistance may be indirect in that factors that promote insulin resistance may also promote development to fibrosis (Negro, 2006). Additionally, in a study of 28 non-diabetic HCV patients, increased insulin resistance was observed, as measured by an increased homeostasis model assessment of insulin resistance (HOMA-IR) value, as well as greater

insulin secretion at baseline (Lecube et al., 2006). Higher levels of pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α) were found in patients with HCV, which suggests that this cytokine may be mediating insulin resistance.

Thyroid disorders, such as Hashimoto's thyroiditis (Agmon-Levin et al., 2009), autoimmune thyroid disease (Pateron et al., 1992) and hypo- or hyperthyroidism, have also been found at increasing rates in patients with CHC. The mechanism of development of thyroid disease in CHC is unknown (Fernandez-Soto et al., 1998). However, IFN-α treatment in patients with CHC who show thyroid disease, can lead to further thyroid gland dysfunction (Fernandez-Soto et al., 1998).

Porphyria cutanea tarda (PCT) is a disease caused by reduced uropophyrinogen decarboxylase activity and leads to cutaneous lesions, skin fragility, iron overload and chronic liver disease (Fargion et al., 1992). PCT has been found to be strongly associated with HCV infection (Herrero et al., 1993; Decastro et al., 1993). In a study of 20 Japanese patients with PCT, 85% were found to be HCV infected (Tsukazaki et al., 1998). The role of HCV in the development of PCT is unclear, although it is thought that infection triggers the development of the disease in genetically predisposed individuals (Zignego et al., 2007).

Other extrahepatic manifestations that occur with a greater frequency in HCV-infected individuals include lichen planus (Nagao et al., 2000), Sjogren's syndrome (Agmon-Levin et al., 2009) and chronic polyarthritis (Zignego et al., 2007).

1.6. HCV LYMPHOTROPISM

HCV lymphotropism refers to the propensity of HCV to infect and replicate in the cells of the immune system. HCV has been shown in several studies to infect lymphoid cells as evidenced by the detection of HCV RNA positive strand and its replicative intermediate, HCV RNA negative strand. Data exists indicating that in the absence of detectable HCV RNA in the serum, virus may be detected in the PBMC (Pham et al., 2004; Pham et al., 2005; De Marco et al., 2009; Pham et al., submitted).

Reports of lymphotropism include HCV infection of T cells and B cells (Shimizu et al., 1992; Zignego et al., 1992; Navas et al., 1994; Goutagny et al., 2003; Radkowski et al., 2004; Pham et al., 2004; Pham et al., 2005; Pal et al., 2006; Pham et al., 2008). HCV replication in lymphoid cells has been described during CHC as well as years after apparent complete therapy-induced or spontaneous resolution of hepatitis C (Pham et al., 2004; Radkowski et al., 2005a; Pham et al., 2008).

In a study which measured the presence of HCV RNA positive and negative strands in lymphoid cell subsets in patients with CHC and OCI, it was found that HCV was able to replicate in $CD4^+$ and $CD8^+$ T cells as well as in B cells (Pham et al., 2008). Additionally, in the same study, HCV NS5A protein was detected in the same cell subsets and HCV variants distinct from those occurring in plasma were also identified.

In terms of the HCV RNA loads in lymphoid cells, it has been shown that between 100 and 1000 vge/10^7 cells can be detected in approximately 30% of patients with OCI who have apparently resolved hepatitis, while approximately 10^4 vge/10^7 cells can be detected in patients with CHC (Pham et al., 2007). Meanwhile, HCV RNA levels

comparable to those occurring in immune cells from patients with CHC can be detected in ~10% of patients with OCI (Pham and Michalak, 2008). It is presently unclear whether HCV persists for life in the lymphatic system, but it is currently known that virus can linger in immune cells for at least 9 years after apparent complete recovery from hepatitis C (Radkowski et al., 2005a; Pham et al., submitted).

Whether or not HCV found in lymphoid cells represents a lymphotropic strain or whether HCV replication in the immune cell environment selects for specific viral variants is presently unclear. However, HCV viral sequences detected in lymphoid cells have been found to be distinct from the wild-type virus in circulation (Shimizu et al., 1997; Pham et al., 2004; Radkowski et al., 2005a). Replication of HCV in the lymphatic system has been proposed as a reservoir for reinfection of liver grafts post transplantation (Pal et al., 2006). HCV lymphotropism and extrahepatic replication has been implicated as the underlying cause of B cell lymphoproliferative disorders (Blackard et al., 2006) which will be discussed in detail in Section 1.6.1. In terms of impairment of lymphoid cell function, HCV-infected T cells and B cells have been found to be functionally impaired and this will be discussed in Sections 1.6.2 and 1.6.3, respectively. In this thesis, the lymphotropic nature and replication of HCV in T lymphocytes was investigated and will be discussed in detail in Chapters 2, 3 and 4.

1.6.1. B Cell Dysfunctions and Related Pathology

Disorders associated with HCV infection involving B lymphocytes include type II mixed cryoglobulinemia (MC)(Pawlotsky et al., 1995) and B-cell non-Hodgkin's lymphoma (Zignego et al., 1997; Ferri and Mascia, 2006).

1.6.1.1. Mixed Cryoglobulinemia

Approximately 80% of patients with type II mixed cryoglobulinemia are also infected with HCV (Agnello et al., 1992). This disease is associated with the accumulation of cryoglobulins, which are immunoglobulins that form solid deposits at 4°C. It has been found that up to 5% of patients with HCV-related cryoglobulinemia will develop vasculitis (Vassilopoulos and Calabrese, 2002). The mechanism of cryoglobulinemia in HCV infection is not clear, but is thought to be related to unregulated B cell proliferation. B cells have been shown to be stimulated to undergo proliferation and IgM production by direct stimulation of CD81 by HCV E2 glycoproteins (Fearon and Carroll, 2000; Curry et al., 2003). As well, in HCV infected patients with fibrosis and end stage cirrhosis, there is an expansion of $CD5^+$, $CD81^+$ B cells in the liver (Curry et al., 2003). The contribution of HCV to the appearance of MC is supported by the resolving effect that antiviral therapy has on this condition. Thus, it has been observed that treatment with IFN/RBV results in clinical recovery from HCV-associated MC in up to 67.5% of patients, suggesting that resolution of MC is linked to a decreased level of viremia (Saadoun et al., 2006).

1.6.1.2. B Cell Non-Hodgkin's Lymphoma

Lymphotropic HCV infection, identified by the detection of HCV RNA in circulating lymphoid cells, has been found in the majority of HCV patients with non-Hodgkin lymphoma (Ferri et al., 1997). B-cell non-Hodgkin's lymphoma is defined as an uncontrolled proliferation of B lymphocytes associated with a high incidence of circulating monoclonal B cells. As mentioned in Section 1.6.1.1, it has been suggested

that the interaction between HCV and CD81 on B cells may result in stimulation of B cells, leading to uncontrolled B cell proliferation.

1.6.2. T Cell Dysfunctions

As will be discussed in Section 1.7.3, HCV clearance is associated with a robust and multi-specific $CD4^+$ and $CD8^+$ T cell response. In patients chronically infected with HCV, T cells have been found to be functionally exhausted and display defects in production of interferon-gamma (IFN-γ) and IL-2 (Semmo et al., 2007), as well as impaired proliferation in response to stimulation with HCV-specific antigens (Golden-Mason et al., 2009). In T cells, exposure to HCV *in vitro* resulted in impaired IFN-γ signaling in that in HCV-infected T cells, Stat-1 and T bet mRNA levels were suppressed after stimulation with IFN-γ compared to uninfected T cells (Kondo et al., 2007). An additional functional defect observed in T cells of patients with CHC is reduced cytotoxicity (Thimme et al., 2001). A possible mechanism of T cell exhaustion is the higher expression of certain inhibitory receptors compared to healthy counterparts. In this regard, programmed death receptor-1 (PD-1) is a major inhibitory receptor on T cells. Binding of PD-1 by its ligand programmed death receptor-ligand 1 (PD-L1) results in the inhibition of T cell proliferation and cytokine production (Blank et al., 2005). It has been demonstrated that PD-1 expression on total T cells and $CD8^+$ T cells in the peripheral blood and in the livers of patients chronically infected with HCV is significantly higher than that of those who spontaneously resolve (Golden-Mason et al., 2007). It has been further shown that blocking the PD-1/PDL-1 interaction with antibodies to PD-L1 and/or PD-L2 leads to recovery of proliferation, IFN-γ secretion and

IL-2 production in previously dysfunctional, HCV-specific CTLs (Golden-Mason et al., 2007; Nakamoto et al., 2008). In addition to PD-1, T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) interaction with its ligand galectin-9 has been implicated as another possible mechanism of T cell exhaustion in CHC. The binding of TIM-3 to galectin-9 has been found to result in negative regulation of T-helper type 1 (TH1) responses and the induction of peripheral tolerance (Sabatos et al., 2003; Zhu et al., 2005). In this regard, in HIV infection, expression of TIM-3 has been found to be elevated on both $CD4^+$ and $CD8^+$ T cells compared to uninfected individuals or those who were able to control infection (Jones et al., 2008). As well, it has been shown that TIM-3 expression is upregulated on $CD4^+$ and $CD8^+$ T cells in CHC patients (Golden-Mason et al., 2009). Further, the greatest proportion of PD-1/TIM-3-dual expressing T cells was found in the liver of CHC patients. Blocking of TIM-3 and TIM-3 ligand interaction rescued proliferation of T cells and IFN-γ production (Golden-Mason et al., 2009).

1.6.3. HCV-Related Dysfunction of NK Cells

Natural killer (NK) cells belong to the first line of defence against viral infections and are rapidly activated to acquire cytotoxic activity and to produce inflammatory cytokines such as IFN-γ and TNF-α (Biron, 1999). The innate immune response is characterized by a considerable amount of cross-talk between NK cells, NK T cells and DC (Biron, 1999; Heath et al., 2004; Kanto and Hayashi, 2007). It is thought that during HCV infection, the failure of the host immune response may be due to virally inhibited cross-talk between these cells which leads to decreased priming of the adaptive immune response.

As mentioned previously, HCV particles and recombinant HCV E2 proteins bind to CD81 (Pileri et al., 1998). *In vitro*, ligation of CD81 by HCV E2 protein has been shown to directly block NK cell function, as evidenced by inhibited IFN-γ and TNF-α production, as well as diminished cytotoxic granule release and decreased CD25 expression (Crotta et al., 2002; Tseng and Klimpel, 2002).

1.6.4. Factors Augmenting HCV Replication in Immune Cells

Coinfection with other viruses may augment HCV replication in lymphoid cells. For example, Molt-4 cells infected with murine retroviruses have been shown to be more readily infected with HCV as compared to their counterparts who were free of retroviruses. Active replication in these cells was evidenced by the detection of HCV RNA negative strand (Shimizu et al., 1992). In patients coinfected with HCV and HIV, circulating levels of HCV RNA in the sera have been found to be higher than in HCV-monoinfected patients (Thomas et al., 2001) suggesting that perhaps HCV RNA levels in the PBMC may also be higher.

In addition, the removal of certain PBMC subsets, such as IFN-γ-producing $CD8^+$ T cells, has been shown to increase HCV replication in $CD8^+$-depleted PBMC compared to those not depleted of the T cell subset (Li et al., 2005b). This is thought to be due to the anti-viral properties of IFN-γ as the addition of $CD8^+$ T cell-conditioned medium to Huh-7 cells has been shown to result in decreased HCV replication (Li et al., 2004). As well, addition of $CD8^+$ T cells to $CD8^+$ T cell-depleted PBMC led to inhibition of HCV replication (Li et al., 2005b). Mitogens such as phytohemagluttinin (PHA), concanavalin A (Con A) and poke weed mitogen (PWM), have been utilized to augment HCV

replication in PBMC from individuals with OCI (Pham et al., 2005). Mitogen treatment led to increased HCV replication and, in consequence, virus genome detection in circulating immune cells (Pham et al., 2004; Pham et al., 2005).

1.7. HCV-INDUCED IMMUNE RESPONSES

1.7.1. Innate Immunity

1.7.1.1. Cellular Components

1.7.1.1.1. Natural Killer Cells

NK cells are part of the first line of defence against HCV (Kanto and Hayashi, 2007). Under normal circumstances, the interaction of NK cells with DC leads to IFN-γ production and NK-mediated cytolysis (Yu et al., 2001). In HCV infection, the function of NK cells appears to be impaired in terms of decreased cytolysis and IFN-γ production. *In vitro*, in human hepatic cell lines, such as Huh-2, Huh-7 and Huh-7-derived lines, NK cell-conditioned medium high in IFN-γ has been shown to inhibit HCV replication, while incubation of NK cells with anti-CD81 antibodies resulted in decreased IFN-γ production and a lessened anti-HCV effect (Li et al., 2004). NK cells from CHC patients have also been seen to secrete immunosuppressive cytokines, such as IL-10, and transforming growth factor β which can downregulate DC functions (Zignego, 2004). In HCV-infected individuals, proportions of NK cells in circulation has been found to be similar in numbers, as compared to uninfected patients, however, the cells cytolytic activity is 4-fold lower than that of healthy individuals (Corado et al., 1997). As well, the same study

showed that *in vitro* infection of PBMC with HCV resulted in decreased NK cell cytotoxic capacity. Further, successful antiviral therapy in patients chronically infected with HCV leads to recovery of NK cell function (Bonavita et al., 1993).

1.7.1.1.2. Natural Killer T Cells

During the normal response to viral infection, NKT cells are thought to activate DC and NK cells through secretion of IFN-γ. It has been observed that NKT cells in chronic HCV infection are biased towards Th2-type response (Kanto and Hayashi, 2007). As well, it has been identified that the proportion of Valpha24/Vbeta11 double positive NKT cells are lower in chronically infected patients compared to controls (Lucas et al., 2003).

1.7.1.1.3. Dendritic Cells

The role of DCs in the innate response to viral infection involves the detection, processing and presentation of viral antigens. The major DC subsets are myeloid DC, which mainly secrete IL-12 and TNF-α and drive a Th1 response. On the other hand, plasmacytoid DC secrete IFN-α and drive a Th2 response (Liu, 2001). DC in patients with CHC, have an impaired response to HCV antigens, such as HCV core and E1, while maintaining their ability to respond to non-HCV antigens, such as tetanus toxoid (Sarobe et al., 2002). Additionally, DC from HCV-infected patients are unresponsive to exogenous IFN-α *in vitro* (Kanto and Hayashi, 2007).

1.7.1.2. Interferon Induction

Innate immune components are the first responders in a virally infected host. The innate response is triggered by the recognition of a pathogen-associated molecular pattern (PAMP), which binds its specific PAMP receptor. In HCV infection, double-stranded RNA is bound by two PAMP receptors, Toll-like receptor (TLR) 3 and retinoic-acid-inducible gene I (RIG-I). Engagement of PAMP receptors result in the downstream activation of interferon regulatory factors (IRF), such as IRF-3, IRF-5 and IRF-7, as well as nuclear factor κB (NFκB) (Malmgaard, 2004). This results in the secretion of IFN-beta (IFN-β), which binds to the IFN-α/β receptor leading to JAK-STAT signaling and subsequent activation of IFN-stimulated genes (ISG). In the chimpanzee model of HCV infection, the expression of interferon response genes involved in the type 1 IFN signaling pathway can be induced as early as 2 days after infection (Bigger et al., 2001; Thimme et al., 2001). The observed early induction of IFN does not appear to be related to or predict resolution of hepatitis (Thimme et al., 2001; Thimme et al., 2002) which may be explained by subversion of the IFN response by viral components, a topic that will be discussed in Section 1.8.1.

1.7.2. Adaptive Immunity

1.7.2.1. Humoral Immunity

On average, by 50 days after HCV exposure, patients seroconvert and produce antibodies against HCV proteins (anti-HCV) (Orland et al., 2001; Cox et al., 2005). These antibodies are not thought to be broadly neutralizing, which may be attributed to the high

sequence variation in the HCV genome and the generation of escape mutants (Farci et al., 1994). The role of the humoral response in the clearance of HCV is not clear as apparent HCV clearance has been observed in the absence of HCV-specific antibody production (Post et al., 2004). The appearance of anti-HCV antibodies in patients and in experimentally infected chimpanzees does not appear to protect against reinfection with the same strain or a heterologous strain of HCV (Farci et al., 1992). However, using chimeric mice with humanized livers as a model of HCV infection, human monoclonal antibodies against HCV E2 have been identified to be able to protect against challenge with a heterologous HCV quasispecies. *In vitro*, this antibody was able to neutralize HCV particles released from Huh-7 cells *in vitro* infected with JFH-1 (system described in Section 1.9.2.3) and HCVpp expressing E1 and E2 from several HCV genotypes (Law et al., 2008). Recent studies have confirmed this finding with anti-E2 antibodies showing neutralizing abilities against pseudoviruses carrying E1 and E2 glycoproteins from genotypes 1a, 1b, 2b, 3a and 4a (Broering et al., 2009).

1.7.2.2. Cellular Immune Responses

Throughout the course of HCV infection, spontaneous decline in viremia in the acute phase is associated with strong, HCV-specific, $CD4^+$ and $CD8^+$ T cell responses, directed against multiple HCV epitopes (Gerlach et al., 1999).

HCV-specific $CD4^+$ T cell reactivity that develops in the acute phase of infection are associated with a sustained Th1 response, characterized by the induction of type 1 cytokines, such as IFN-γ and TNF-α, and with viral control (Gerlach et al., 1999). $CD8^+$ cytotoxic T cells (CTL) contribute to viral elimination in HCV infection through direct

killing of infected cells and the induction of antiviral cytokines, such as IFN-γ and TNF-α. Both $CD4^+$ and $CD8^+$ T cell activation is typically required for resolution of hepatitis (Rehermann and Nascimbeni, 2005), a finding that has been very well documented in the chimpanzee model. In one such study, $CD4^+$ T cells were depleted by antibody before infection of chimpanzees. In the presence of a functional memory $CD8^+$ T cell response, the elimination of $CD4^+$ T cells resulted in poor viral control and persistent viremia (Grakoui et al., 2003). In another study, chimpanzees that had been shown to control HCV challenge and clear the viremia in two previous HCV infections, were depleted of $CD8^+$ T cells before a third exposure to virus. Even in the presence of previously primed $CD4^+$ T cells, the absence of $CD8^+$ T cells resulted in prolonged viremia until $CD8^+$ T cells recovered (Shoukry et al., 2003).

$CD4^+$ and $CD8^+$ T cell responses in chimpanzees infected with HCV are seen to be delayed with the approximate time to intrahepatic, HCV-specific T cell activation being 4 to 8 weeks after onset of viremia (Thimme et al., 2001; Thimme et al., 2002). As well, the induction of HCV-specific peripheral blood T cell responses in chimpanzees appears to be delayed until 8 weeks, reflecting that seen in the liver (Shin et al., 2008). Meanwhile, in chimpanzees, the appearance of HCV-specific T cell responses and IFN-γ production occurs directly before a decline in serum HCV RNA load (Thimme et al., 2001).

In terms of epitopes recognized by HCV-specific T cells, in HLA-A2, HCV-infected patients, several CTL epitopes, in regions such as core, E2, NS3, NS4 and NS5, have been identified. Although no clear immunodominant epitopes have been found, it has been shown, using MHC-class 1 tetramers, that a CTL epitope within NS3 (1073–81) is

frequently recognized in HLA-A2 positive patients (Takaki et al., 2000). NS3 (1073-81)-specific T cell responses are found to be associated with control of virema (Takaki et al., 2000), although recognition of this epitope alone is thought to be inadequate for viral control (Urbani et al., 2005). HCV-specific $CD4^+$ T cell epitopes have been uncovered and, in the case of NS3 (1248-1261), they can be recognized by patients with at least 5 different HLA-DR backgrounds (Diepolder et al., 1997). In IDU, $CD4^+$ T cell responses were investigated in terms of the magnitude and the HCV epitopes targeted in infected patients that spontaneously cleared viremia versus those who developed CHC. It was observed that there is a slight, non-statistically significant difference in the magnitude of HCV-specific $CD4^+$ T cells between the patient groups although patients that went on to resolve hepatitis had $CD4^+$ T cell responses mainly directed to the non-structural proteins, while those acquiring CHC were observed to have $CD4^+$ T cell responses predominantly targeting the HCV core protein (Ruys et al., 2008).

As far as a memory T cell response to HCV infection, patients who were exposed to HCV eighteen years earlier and have undetectable anti-HCV antibodies, continued to have HCV-specific $CD4^+$ and $CD8^+$ T cell responses (Takaki et al., 2000), although this control could be due to the presence of OCI (Pham and Michalak, 2008). As well, in chimpanzees, prior exposure to HCV has been shown to result in a shorter course of infection with reduced peaks of viremia in re-challenged animals (Bassett et al., 2001). In humans, in studies examining re-infection of IDU with continuing exposure to HCV, those who had previous HCV infection followed by spontaneous resolution of hepatitis, had a 4-fold reduced likelihood of developing viremia following re-exposure (Grebely et al., 2006). At the same time, HCV-specific $CD4^+$ and $CD8^+$ T activation appears to occur

in all recovered patients as such responses have been observed in both patients who have spontaneously resolved hepatitis as well as those who have achieved an SVR after IFN/RBV therapy (Klade et al., 2009).

Regulatory T cells (Tregs) are known to maintain self-tolerance and control deleterious immune responses (Sakaguchi et al., 2006). The role of Tregs in the immune response to HCV infection remains unclear. *In vitro*, co-culture of HCV-specific $CD8^+$ T cells with $CD4^+CD25^+$ Tregs led to decreased IFN-γ production (Sugimoto et al., 2003). It has been observed that removal of $CD4^+CD25^+$ Tregs from peripheral blood resulted in rescued HCV-specific $CD4^+$ and $CD8^+$ T cell proliferation and IFN-γ production (Cabrera et al., 2004). However, in the chimpanzee model, there appeared to be no difference in the quantities of Tregs in the liver and circulation in animals that had chronic hepatitis as compared to those that resolved hepatitis (Manigold et al., 2006).

1.8. HCV ESCAPE FROM ANTIVIRAL IMMUNE RESPONSES

1.8.1. Interference with the Innate Immune Response

As mentioned earlier, there is a strong innate activation due to recognition of HCV double-stranded RNA that leads to the stimulation of the interferon pathway resulting in the induction of ISG. As well, propagation of subgenomic replicons and full-length HCV clones have been shown to be inhibited in the presence of IFN-α *in vitro* (Frese et al., 2001; Lindenbach et al., 2005). However, early induction of IFN-α in the chimpanzee model of hepatitis C does not predict viral clearance (Thimme et al., 2001). This observation has led to studies of the ability of HCV proteins to directly or indirectly interfere with the interferon induction cascade. As outlined in Section 1.7.1., IRF-3 is part

of the signalling cascade implicated in the innate response to HCV RNA. NS3/4A protease was found to be an antagonist of this response and blocks IRF-3 activation through cleavage of the adaptor protein TRIF (Ferreon et al., 2005; Li et al., 2005a). NS3/4A protease has also been shown to block RIG-I signaling and subsequent IRF-3 activation via cleavage of the adaptor protein IFN promoter-stimulator 1 (Loo et al., 2006). As well, HCV is known to disrupt the IFN-α signaling pathway through disruption of protein kinase R (PKR) signaling. The HCV E2 and NS5A proteins can both bind and inhibit PKR-PKR phosphorylation (Gale et al., 1997; Taylor et al., 1999), although it has been shown that expression of NS5A alone can lead to cells becoming resistant to the effects of IFN-α against viruses, such as interferon-sensitive encephalomyocarditis virus (Polyak et al., 1999). An additional viral strategy of evasion of the IFN response is through the production of IL-8, which is induced by the interaction of HCV NS5A protein with 2'-5' oligoadenylate synthetase (OAS) (Polyak et al., 2001). As discussed in Section 1.7.1.2, ligation of the IFN-α/β receptor during the host response to the detection of double-stranded viral RNA, leads to JAK-STAT signaling and subsequent activation of ISG. HCV core protein has been shown to induce the expression of suppressor of cytokine signalling (SOCS) proteins 1 and 3, which blocks JAK-STAT signaling through the IFN-α/β receptor and inhibits downstream ISG expression (Bode et al., 2003; Vlotides et al., 2004).

1.8.2. Quasispecies Expansion and Immune Escape

Host selective immune pressure has been shown to drive HCV quasispecies expansion (Manzin et al., 2000). One of the examples is that patients that are immunosuppressed

have a less evolved viral population (Lawal et al., 1997). Immune pressure on HCV is exerted by: (1) the humoral response which includes neutralizing antibodies directed specifically against the dominant neutralizing epitope contained in the HVR1 of the E2 and (2) the cellular response, in which CTLs are directed against conserved regions of the genome, such as the NS3 protein. In a study of children with perinatal HCV infection, it was observed that HCV-infected newborns, in the presence of high levels of viral replication, had a single dominant variant or a small number of closely related viruses that remained stable for several weeks while the humoral and cytotoxic host immune responses were immature. The quasispecies in these newborns was observed to diverge and expand around 6 to 7 months of age. In contrast, acutely infected adults are seen to have rapid viral evolution after infection, likely due to the presence of an active virus-specific immune response (Manzin et al., 1998; Manzin et al., 2000).

Studies have shown that the CTL response can "select" for HCV variants that have mutations in the conserved regions which can render the virus unrecognizable to virus-specific CTLs. These changes in viral sequence are known as virus escape mutants because they can evade or subvert host immunosurveillance (Weiner et al., 1995). These mutations, however, can occur at the expense of virus fitness in that the mutated viruses may be less replication competent. Thus, the survival of such mutations and their subsequent stabilization hinges on whether the immune escape mutants will be able to replicate efficiently. If the mutated virus is as fit or replication competent as the wild-type virus, it may be 'fixed' in the quasispecies in the absence of immune pressure. However, if the escape mutant has reduced replication efficiency, in the absence of host immune pressure, the wild-type virus, which replicates more robustly will prevail and

will drown out the escape mutant. This event is known as reversion. In this regard, in chimpanzees, virus isolated from an animal in the acute phase of HCV infection was used to infect an HCV-naïve animal and the presence of neutralizing antibodies in the plasma of the infected animal was evaluated over an 11-year period against the virus used for inoculation. While plasma obtained 2 years after infection neutralized the original inoculum, plasma taken from the same animal 11 years after inoculation was no longer neutralizing for this same inoculum. This suggested that the quasispecies had gone through significant evolution with respect to the virus in the original inoculum (Farci et al., 1994).

In humans, mutants in the HCV NS3 region have been found that confer escape from $CD8^+$ T cell responses in patients with HLA-B8 background (Timm et al., 2004). Interestingly, when virus carrying the HLA-B8-associated escape mutation was transmitted to an HLA-B8 negative individual, the mutation was subsequently lost, suggesting a reversion of mutations in the B8-associated epitope to the fitter wild-type in the absence of HLA-B8 restricted CTL pressure

1.9. MODELS OF HCV INFECTION

1.9.1. Animal Models

The main challenge in the attempt to understand the natural history and pathogenesis of HCV infection is the lack of a convenient and reliable animal model. Some of the models currently employed are: (1) Chimpanzees (Farci et al., 1992); (2) the *Scid*-Alb/uPA mouse (Kneteman et al., 2006; Kneteman et al., 2009); (3) the HCV trimera

mouse (Eren et al., 2006; Galun et al., 2007); (4) the *Tupaia belangeri* ; (5) the NOD/Scid mouse model, and (6) transgenic mice expressing HCV proteins. Each of the above mentioned animal models have advantages and disadvantages which are related to: (1) differences in the course of disease compared to humans; (2) the reproducibility of the model; (3) the availability of animals, and (4) the cost of the model. Specific aspects of the most widely used animal models are discussed below.

1.9.1.1. The Chimpanzee Model

The chimpanzee has been used to study the course of HCV infection, the pathogenesis of HCV as well as prevention and management strategies against HCV. Chimpanzees have been employed to investigate several aspects of hepatitis C immunopathogenesis including: (1) the role of $CD4^+$ and $CD8^+$ T cells in viral clearance (Shoukry et al., 2003; Grakoui et al., 2003); (2) the lack of immune protection following reinfection (Farci et al., 1992); (3) the efficacy of vaccination (Esumi et al., 1999; Puig et al., 2006; Rollier et al., 2007; Elmowalid et al., 2007), and (4) the infectivity of lymphocyte-derived virus (Shimizu et al., 1998). It has been demonstrated that approximately 20 copies of HCV can transmit infection to chimpanzees (Katayama et al., 2004a). Chimpanzees produce virions at high levels, i.e. 10^5 to 10^7 vge/ mL (Bukh, 2004). In experimentally infected chimpanzees, development of chronic hepatitis has been observed to occur in approximately 60% of animals (Bigger et al., 2004), which is comparable to that seen in humans. In terms of reproducibility, the course of infection in each chimp, similar as in humans, cannot be predicted (Rosenberg, 2001). The use of chimps is very limited due to high costs of animal maintenance (approximately $30,000 per animal), restricted

availability and ethical issues tied to the fact that these animals are endangered. With these issues in mind, most studies including chimpanzees have very few animals. There are a wide variety of tools available to examine the immunopathogenesis of infection and to test the efficacy of antivirals in the chimp model due to the fact that many human reagents, i.e. antibodies, oligonucleotide primers and assays, are directly applicable to investigations in chimpanzees. However, the chimpanzees are not an optimal choice to test classical antiviral agents, such as IFN-α. Thus, in studies using IFN-α with or without ribavirin, as well as adenovirus-based gene therapy to upregulate expression of IFN-α in the liver, treatment failed to decrease HCV load despite the fact that high levels of IFN-α were found to be circulating in treated animals (Lanford et al., 2006). On the other hand, there are studies showing that the HCV-NS5B inhibitor A-837093 successfully inhibited HCV replication in the chimpanzee model (Wagner et al., 2006) although due to availability of very few animals, dose-dependent studies are difficult.

1.9.1.2. The *Scid*-Alb/uPA Mouse Model

The beige *Scid*-Alb/uPA mouse is the most successful small animal model of HCV infection. In this model, severe combined immunodeficient (SCID) mice are generated that express a urokinase plasminogen activator transgene driven by the albumin promoter (Alb/uPA). The damaged mouse livers are then repopulated with a xenograft of human hepatocytes that are functional and produce human albumin. Repopulated livers can then be infected with HCV (Mercer et al., 2001). The *Scid*-Alb/uPA mouse has been utilized in the testing of antiviral therapies, such as IFN-α and inhibitors blocking NS3 protease or NS5B polymerase activities (Kneteman et al., 2006; Kneteman et al., 2009).

However, due to the fact that these mice are immunodeficient, this is not a model for testing HCV vaccines. Another restriction is that the mice are susceptible only to HCV doses greater than or equal to 10^5 copies per mouse (Mercer et al., 2001), which makes it infeasible to test the infectivity of low-copy numbers of virus occurring in OCI. Finally, while this animal model is less expensive than the chimpanzee model, it is labour intensive and requires the availability of high quality human hepatocytes for mouse liver repopulation.

1.9.1.3. The *Scid*–BNX Trimera Mouse Model

The *Scid*–BNX "trimera" mouse is a chimeric mouse model in which beige/nude/X-linked immunodeficient (BNX) mice were sublethally irradiated and reconstituted with bone marrow from *Scid* mice. The animals are then able to accept fragments of HCV-infected liver from patients with chronic HCV. The trimera mouse model has been employed to test the efficacy of neutralizing antibodies in inhibiting HCV infection in human liver grafts (Eren et al., 2006; Galun et al., 2007) and antiviral agents (Ilan et al., 2002). The limitations of the *Scid*-BNX mouse model is that only approximately 50% of mice become infected with HCV after grafting and that this infection only lasts for 10-50 days post-transplant (Galun et al., 1995).

1.9.1.4. The Nod/*Scid* Mouse Model

The NOD/*Scid* mouse is a model in which NOD/SCID mice are engrafted with human liver tissue which can then be subsequently infected with HCV from infected patients.

When infection is successful, viremia can last for up to 4 weeks. However, this model suffers from a low rate of infection (Maeda et al., 2004).

1.9.1.5. The Tupaia Belangeri Model

The *Tupaia belangeri* has been shown to be susceptible to HCV infection (Xie et al., 1998). *In vitro*, primary hepatocytes from the tupaia have been infected with HCV and are capable of producing virions that can *de novo* infect HCV-naïve hepatocytes (Zhao et al., 2002). In a study looking at the role of SRBI, *in vitro* pre-incubation of primary Tupaia hepatocytes with anti-SRBI antibodies led to inhibition of HCV particle or E2 protein binding, suggesting this model can be useful in investigating the interaction between E2 and SRBI (Barth et al., 2005). In this model, *in vivo* susceptibility of the tupia to HCV is greater after whole-body irradiation, which would make vaccine studies unlikely (Xie et al., 1998).

1.9.1.6. Transgenic Mice

Several laboratories have generated transgenic mice expressing HCV proteins, such as core, E1, E2, NS3, NS4 and NS5A either alone (Kawamura et al., 1997; Majumder et al., 2002; Kawamura et al., 2006; Chang et al., 2008) or together (Pasquinelli et al., 1997; Matsuda et al., 1998; Frelin et al., 2006). These mice have been used to investigate the role of HCV proteins in the immunopathogenesis of liver injury in the induction of steatosis, the development of HCC, and modulation of apoptotic cell death. Using this model, several observations on the affects of viral proteins on liver pathology were made, among others that HCV core protein can induce lipid accumulation in hepatocytes and

that the expression of this protein may result in the development of cancer (reviewed in (Barth et al., 2008).

1.9.2. Cell Culture Models

1.9.2.1. The Replicon Systems

The replicon system is an *in vitro* virus culture method that was first described in 1999 (Lohmann et al., 1999). This approach was based on the transfection of cloned selectable viral RNA which replicates autonomously in the target cells. Replicons have been developed for several viruses, such as HCV, bovine diarrhoea virus, Lassa virus and Sindbis virus (Frolov et al., 1997; Behrens et al., 1998; Hass et al., 2004). The HCV subgenomic replicon was first created with a 5'-HCV IRES, a neomycin phosphotransferase gene, which determines G418 resistance, a second IRES from encephalomyocarditis virus, which directs processing of the polyprotein, and the HCV sequence from the beginning of NS2 or NS3 to the end of the 3'- UTR (Lohmann et al., 1999). Cell culture adaptive mutations indentified in the non-structural gene encoding regions, such as in the interferon sensitivity determining region found in the NS5A, which increased replicative ability of the replicon (Blight et al., 2000). As well, adaptive mutations in the NS5B gene improved the efficiency of colony formation (Lohmann et al., 2001). In general, the replicon systems have advanced our understanding of the role of individual viral proteins in virus and served as a tool for the testing of antiviral therapies potentially targeting HCV. Among others, the first clinically tested NS3 protease inhibitor BILN 2061 was tested in Huh-7 cells carrying the HCV 1b subgenomic replicon (Lamarre et al., 2003).

1.9.2.2. Pseudoparticle System

The HCV pseudoparticle (HCVpp) system is a cell culture system employing retroviral and lentiviral core particles expressing HCV E1 and E2 glycoproteins This system can be used to study infectivity and the early stages of viral infection (Bartosch et al., 2003b). Due to packaging of the particle with a green fluorescence protein (GFP) marker gene, this system has been employed to show that primary hepatocytes and hepato-carcinoma cells were infected *in vitro* and that this infection required the presence of El and E2 proteins, and was neutralized by anti-E2 monoclonal antibodies (mAb) (Bartosch et al., 2003a; Owsianka et al., 2005). In addition, HCVpp have been employed to investigate putative HCV co-receptors, such as LDLR (Bartosch et al., 2003b), CD81 (Cormier et al., 2004; Owsianka et al., 2006), SRB1 (Voisset et al., 2005), claudin-1 (Evans et al., 2007) and occludin (Ploss et al., 2009). In addition, the HCVpp system is a tool to indentify factors which may enhance infectivity such as addition of apolipoprotein C1 to target cells (Meunier et al., 2005).

1.9.2.3. The HCVcc System

The JFH-1 strain of HCV was isolated from a 32 year old Japanese male with fulminant hepatitis. It is a genotype 2a strain of virus which replicates efficiently and supports secretion of infectious viral particles in cell culture, known as (HCVcc), after transfection of full-length JFH-1 RNA into a human hepatoma (Huh)7.5 cell line, without requiring cell culture adaptive mutations (Zhong et al., 2005; Lindenbach et al., 2005; Wakita et al., 2005). The major improvement of this strain over the previously described HCV replicon system is that the JFH-1 viral clone is able to support virus assembly and the secretion of

infectious particles into culture supernatant enabling the study of the full HCV life cycle. The generated particles, which bind to anti-core, E1 and E2 antibodies, display biophysical properties of complete HCV virions of 50-65nm in diameter and a buoyant density of 1.01 to 1.17g/mL with the peak of infectivity between 1.09-1.11 g/mL in sucrose (Zhong et al., 2005; Lindenbach et al., 2005; Wakita et al., 2005). JFH-1-derived virus has been shown to propagate in primary human hepatocytes although the level of replication observed is presently unclear (Molina et al., 2008). On the other hand, it has been observed that JFH-1 particles do not infect or replicate in primary lymphocytes, which the authors suggest may be due to the fact that, unlike virus particles found in patient sera, JFH-1 is a cloned virus without quasispecies properties, and may therefore lack the presence of lymphotropic variants (Marukian et al., 2008). JFH-1 particles have *in vivo* infectivity in chimpanzees, although infection was associated with low virus titres of approximately 10^3 vge/mL, a transient 9-week course of viremia, and no seroconversion (Kato et al., 2008). However, in the above described, JFH-1-infected chimpanzees, adaptive mutations in NS2 developed that resulted in greater virus replication. Although not thought to be required for replication, cell culture adaptive mutations have been described in the NS2 and p7 proteins that independently lead to 3 to 4 log higher virus titres in *in vitro* infected Huh-7.5 cells (Russell et al., 2008).

Originally, only the JFH-1 HCV strain was able to infect and propagate in Huh-7.5 cells which are deficient in RIG-I and TLR3, limiting this system to the study of the full cycle of HCV genotype 2a infection in this specific hepatoma line (Zhong et al., 2005; Lindenbach et al., 2005; Wakita et al., 2005). However, infectious strains of full length virus have been developed that are intergenotypic and intragenotypic chimeras of JFH-1

nonstructural proteins with structural elements from other 2a virus strains and other HCV genotypes including 1a, 1b 2a, 3a as well as the J6CF 2a strain (Pietschmann et al., 2006; Mateu et al., 2008). These HCV chimeras replicate at different efficiencies in Huh-7 cells with some requiring adaptive mutations for infectivity (Yi et al., 2007). Chimeric viruses also have different specific infectivities suggesting that the presence of JFH-1 non-structural proteins alone does not confer robust replication, rather there is an interaction between structural and non-structural elements which determine replication and particle formation.

1.9.3. *In Vitro* Infection of Primary Hepatocytes

In vitro infection of primary human hepatocytes with serum-derived HCV has been described in some studies (Fournier et al., 1998; Lazaro et al., 2007; Buck, 2008). The potential benefit of such a system is testing the susceptibility to infection with different genotypes (Lazaro et al., 2007). *In vitro* infection of chimpanzee hepatocytes with wild-type HCV has also been reported (Lanford et al., 1994). Compared to the replicon system, which is restricted to specific virus sequences and employs hepatoma-derived Huh-7 cells, this system can employ wild-type virus and allows for investigation of the early steps of HCV infection and replication (Fournier et al., 1998). Nonetheless, the data reporting the use of primary hepatocytes as HCV targets are limited due to difficulties in obtaining primary human hepatocytes and challenges in maintaining hepatocytes in culture.

1.10. THERAPY OF HCV INFECTION

1.10.1. Approved Therapies and Treatment Strategies

From the initial observation that IFN-α could be employed in the treatment of non-A, non-B hepatitis (Hoofnagle et al., 1986), antiviral therapy for HCV has been advanced. Numerous multicenter studies evaluated the efficacy of treatment with different preparations of IFN-α and in treatment regimes with and without RBV (McHutchison et al., 1998; Poynard et al., 1998; Manns et al., 2001; Fried et al., 2002). This led to the presently accepted treatment for CHC with IFN/RBV. Thus, IFN is administered intravenously as pegylated IFN-α2a (180 μg/week) or pegylated IFN-α2b (1.5 μg/kg/week). RBV is administered orally at doses of 0.8 to 1.2 g/day depending on body weight (Heathcote et al., 2000; Fried et al., 2002). The current treatment efficacy is approximately 80% in patients with genotypes 2 and 3 infection and approximately 45% in patients with HCV genotype 1 infection (Heathcote et al., 2000; Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004). Therefore, identification of the HCV genotype prior to therapy is a key element in determining the course of antiviral treatment. Since genotypes 1, 4, 5 and 6 are less responsive to IFN/RBV therapy, the treatment for these patients is 48 weeks, whereas for those carrying genotypes 2 and 3 the treatment is 24 weeks long. Monitoring the reduction in serum HCV RNA allows for determination of the response to therapy very early in treatment. Data has shown that the kinetics of the reduction of viremia is biphasic (Zeuzem, 1999). Phase 1 consists of the first 24 hours after administration of IFN/RBV. In this period, there appears to be a rapid dose-dependent reduction in serum viral load. In Phase 2, which is the time period

beyond day 2, a slower decline in viremia is observed. The response in this phase can predict overall treatment outcome and the possibility of an SVR (Zeuzem et al., 2001). HCV RNA testing at week 4 and week 12 after treatment initiation is used to determine whether an early virological response (EVR), which is defined as a 2-$\log_{10}$ viral reduction or apparent complete clearance of HCV RNA at 12 weeks, or rapid virological response (RVR), defined as undetectable serum HCV RNA at week 4, has occurred. Measurement of the RVR and EVR allows for early prediction of antiviral treatment efficacy and decisions of whether to stop or continue the treatment. Data have shown that the course of IFN/RBV therapy for patients who achieve an RVR can be shortened to between 12 and 16 weeks for genotypes 2 and 3 and to 24 weeks for genotype 1 (Heathcote, 2007; Poordad et al., 2008). If a patient carries genotypes 1, 4, 5 or 6, and the HCV load does not drop by 2 $\log_{10}$ or more by 12 weeks of therapy, then the patient has only a 0-3% chance of achieving SVR after 48 weeks of treatment. In the case of patients who respond poorly to IFN/RBV therapy in the first 4 weeks of administration, a prolonged, 72-week course of treatment has been shown to increase SVR rates (Heathcote, 2007).

1.10.2. Future Therapies

There are several challenges in the development of future HCV therapies. These challenges include: (1) A need to develop modalities targeting HCV (they include agents specifically directed against HCV enzymes, such as HCV protease, helicase and RNA dependent RNA polymerase); (2) Improving treatment efficacy (this may require agents targeting sites of extrahepatic virus replication; (3) Minimalization of the side effects of therapy and (4) Management of resistant viruses (this may include development of

antivirals against resistant HCV strains which are unresponsive to the current IFN-α/RBV therapy (Pawlotsky, 2004; Koike, 2006).

The development of HCV-specific therapies is an important area of research and several promising agents are under development. Among them, VX-950, also known as telaprevir, which is an oral HCV protease inhibitor that has been shown to decrease the HCV load in patients who had previously not responded to classical IFN-α/RBV therapy (Reesink et al., 2006; Hezode et al., 2009). In 2003, Lammare and colleages developed an HCV NS3 protease inhibitor, BILN 2061, which showed a potent effect on HCV replication as evidenced by the sharp virus load decline observed in patients treated with this drug. The study of the efficacy of this drug was halted in phase II clinical trials due to cardiac cytotoxicity (Lamarre et al., 2003). Reports have also shown that SCH 503034, a potent oral HCV protease inhibitor, when given in combination with PEG-IFN-α-2b, is able to reduce viremia in hard to treat non-responders (Sarrazin et al., 2007). Polymerase inhibitors, such as HCV 796 and R1262 (Pockros et al., 2008; Kneteman et al., 2009), which target the activity of HCV NS5B, have also been generated and have shown some efficacy in suppressing viral replication. One of the main challenges of antiviral treatment is adherence to therapy (Broers et al., 2005) which can be poor in patients receiving IFN/RBV therapy due to side effects. In a study treating patients who had failed traditional IFN-α treatment, an altered form of IFN-α, albumin-interferon alpha (Albuferon) was recently employed. This drug combines the potency of IFN-α and the stability of human serum albumin, with the half-life of Albuferon being 18 times longer than IFN-α and its clearance rate is 140 times slower. In patients treated, none discontinued therapy due to side-effects of the drug (Balan et al., 2006).

The issue of the emergence or selection of resistant viral strains is an important consideration in the development of new antiviral treatments. In a study comparing 8 patients treated with telaprevir alone to 8 patients receiving combination therapy of telaprevir and IFN-α over a period of 14 days, resistant viral variants were found in 6 of the 8 patients on the monotherapy regime while only 2 of 8 patients on combination therapy showed them. This suggests that combination therapy may be necessary to prevent the development of drug resistance (Kieffer T et al, 2006). However, while the combination of telaprevir and IFN-α are effective in suppressing HCV replication, whether this will result in an SVR remains unknown (Lang, 2007).

1.10.3. Orthotopic Liver Transplantation and Recurrence

Cirrhosis resulting from HCV-induced CHC, and HCC, are the main causes in approximately 50% of liver transplantations (LT) in Western countries (Prieto et al., 1998). High-level HCV viremia occurs almost invariably by one month after liver transplant (Gretch et al., 1995; Testa et al., 2000). Progression to fibrosis has been observed with up to 50% of patients developing chronic active hepatitis in liver allografts during the first year following OLT (Gretch et al., 1995; Berenguer et al., 2000). Explanations for increased progression to fibrosis after LT appear to be related to: (1) the age of the liver donor; (2) differences in the virus virulence; (3) genetic differences between recipients, and 4) the amount of immunosupression or alcohol consumption following LT (Berenguer et al., 2000). With respect to faster progression to fibrosis, it has been clearly shown that the use of immunosuppressive therapies, such as cyclosporine and tacrolimus, is associated with higher HCV replication. In recent years,

the immunosuppressive agents used following LT has changed from cyclosporine to tacrolimus, in some treatment regimes, although studies show little or no difference in the level of recurrent HCV observed regardless of the agent (Ghobrial et al., 1999; Martin et al., 2004; Hilgard et al., 2006).

1.11. PURPOSE OF THE STUDY

Prior to these studies, it was known that HCV RNA positive and negative (replicative) strands and HCV proteins can be detected in the lymphocytes of patients chronically infected with HCV, as well as in those who have achieved a clinically apparent SVR. However, the infectivity of virus replicating in and the biophysical and ultrastructural properties of virions secreted by lymphocytes of patients with progressing symptomatic or clinically resolved HCV infection, remains unknown. Replication of HCV in cells of the immune system, similarly as in other long-term viral infections, could have implications in terms of viral interference in immune responses, immune escape and HCV persistence.

It has also been previously shown that HCV replication in lymphoid cells of patients who resolved CHC can be significantly upregulated after mitogen stimulation. Based on our hypothesis that mitogen stimulation may both enhance HCV replication efficiency, as well as increase lymphocyte susceptibility to virus infection, we designed a preliminary study testing this possibility. In these preliminary experiments, unstimulated lymphoid cells were compared to those stimulated with a T cell mitogen. We found that stimulated cells could be more readily infected with wild-type HCV than non-stimulated cells.

Therefore, using these pre-treated lymphoid cell targets, the central objectives of the current studies were:

1. To establish an *in vitro* infection system in which plasma or lymphoid cell-derived HCV can be used as an inoculum and normal human lymphoid cells as infection targets, and in which infectious HCV virions will be propagated. Specifically, we will explore the *in vitro* susceptibility of virus-naïve, mitogen-pre-treated, total PBMC to infection with molecularly unmodified HCV, their ability to support HCV replication, to express viral proteins, and the assembly of complete HCV virions. We will test whether virion particles released in cell culture by *de novo* infected lymphoid cells can transmit HCV infection to naïve cells, and whether HCV replication can be inhibited by treatment with IFN-α 2b and prevented by antibodies directed against the HCV envelope or a postulated virus receptor, CD81. Finally, we will investigate ultrastructural properties of HCV particles released into culture supernatant by *in vitro* infected lymphoid cells.

2. To assess whether exposure of T cells to wild-type HCV influences the cell phenotype, proliferation, and cell survival in *in vitro* cultures. In this regard, we will examine modifications to the $CD4^+$ to $CD8^+$ ratio following exposure to HCV and changes to the proliferative capacity and susceptibility to apoptosis of CD4-positive and CD8-positive T cells. We will also test whether *in vitro* exposure of naïve lymphocytes to HCV may have an effect on the expression profile of selected cytokines in these cells.

3. To determine whether small amounts of HCV lingering in the circulation long after therapeutically-induced clinically apparent resolution of CHC remain infectious. For this purpose, we will employ the *in vitro* HCV replication system established in aim 1. HCV *de novo* infection will be identified by detecting HCV RNA negative strand,

intracytoplasmic NS5A protein and the appearance of HCV variants in infected T cells. Also, neutralization of infection will be assessed using anti-E2 mAb, blocking with anti-CD81 mAb, and treatment of the target cells with IFN-α 2b. We also will compare biophysical properties, i.e., buoyant density and sedimentation velocity, of the virus produced by *in vitro* infected cells with those of virions occurring in the plasma or released by patients' PBMC and used as inocula. Finally, an attempt will be made to visualize HCV virions circulating in patients with persistent asymptomatic HCV infection as well as those secreted by *de novo* infected T cells using immunoelectron microscopy.

The establishment of a system for *in vitro* infection of normal, non-tumorous human cells with molecularly unmodified, wild-type HCV, will be of significant importance for studies of the complete cycle of HCV replication, identification of factors determining host's susceptibility to HCV, particularly molecules serving as HCV receptors, and for investigations on possible cytopathic consequences of wild-type HCV infection. Such a system will also be applicable for pre-clinical testing of the potential suitability of novel agents against HCV.

Chapter Two: *De Novo* Infection and Propagation of Wild-Type Hepatitis C Virus in Human T Lymphocytes *In Vitro*

This study has been published in Journal of General Virology in December, 2006, volume 87, pp. 3577-3586.

2.1. SUMMARY

While exploring previous findings that *ex vivo* treatment of lymphoid cells from HCV-infected individuals with T cell-stimulating mitogens augments detection of the residing virus, an *in vitro* HCV replication system was established, in which mitogen-induced T cell-enriched cultures served as HCV targets and the derived T cells multiplied virus during repeated serial passage. HCV replication was ascertained by detecting HCV RNA positive and negative strands, HCV NS5A and E2 proteins, release of HCV virions and nucleocapsids (confirmed by immunoelectron microscopy) and *de novo* infection of mitogen-induced T cells prepared from healthy donors. Further, affinity-purified normal human T lymphocytes were also susceptible to HCV infection *in vitro* and HCV replication was detected in pure T cells isolated from a patient with chronic hepatitis C. These results document that T cells can support propagation of HCV both *in vivo* and *in vitro*. The infection system established offers a valuable tool for *in vitro* studies on the entire cycle of HCV replication, virus cytopathogenicity and evaluation of antiviral agents against wild-type HCV in the natural host-cell milieu.

2.2. INTRODUCTION

Hepatitis C virus is a single-stranded RNA virus with a genome of 9,600 base pairs in length which encodes for a single polypeptide subsequently co-translationally and post-translationally cleaved to at least ten structural and nonstructural proteins (Bartenschlager and Lohmann, 2000). HCV is thought to propagate via synthesis of the so-called "negative strand". Although considered to be primarily hepatotropic, accumulated evidence indicates that HCV also replicates in the lymphatic system (Lerat et al., 1996; Shimizu et al., 1997; Laskus et al., 1998). Its replicative intermediate has been demonstrated in PBMC from patients with progressing CHC (Laskus *et al.,* 1998; Okuda *et al.,* 1999; Willems *et al.,* 1994) and individuals with apparent complete resolution of the disease (Pham et al., 2004; Pham et al., 2005; Radkowski et al., 2005a; Radkowski et al., 2005b). HCV replication has been documented in T lymphocytes (Zignego et al., 1992), B cells (Zignego et al., 1992; Morsica et al., 1999; Bare et al., 2005), monocytes (Radkowski et al., 2004), and dendritic cells (Goutagny et al., 2003) in CHC patients. In addition, recent studies from this laboratory have shown that *ex vivo* mitogen treatment of PBMC from patients with CHC or those with occult HCV infection augmented HCV replication in this compartment (Pham et al., 2004; Pham et al., 2005; Pham and Michalak, 2006).

Further support for the notion that lymphotropism is a natural propensity of HCV has stemmed from work with transformed or immortalized lymphoid cell cultures. For instance, HCV propagation has been demonstrated in EBV-transformed B cells isolated from PBMC of patients with CHC (Sung et al., 2003). The presence of HCV RNA and

proteins has also been reported in *in vitro* infected human T cell lines, such as MOLT-4 coinfected with human T cell lymphotropic virus (Shimizu et al., 1992). However, many of these investigations relied on the target cells being coinfected with other viral pathogens and overall, were difficult to reproduce. The lack of an adequate understanding of the mechanisms of HCV infection and a possible role of co-infecting viruses in promoting lymphoid cell susceptibility to HCV raises a concern that the initiation of HCV infection and propagation of the virus in these systems might have been altered.

Considerable efforts to establish HCV replication in hepatocyte cultures have recently succeeded with the system in which transfection of Huh-7 hepatoma cells with a full-length HCV RNA led to secretion of infectious viral particles (Zhong et al., 2005; Lindenbach et al., 2005; Wakita et al., 2005). However, this system is presently robust only for replication of HCV genotype 2a viral clones.

The present study was undertaken in an effort to establish an *in vitro* infection system in which: (1) plasma or lymphoid cell-derived wild-type HCV can be used as an inoculum; (2) virus-transformed or -immortalized cells will not be used as infection targets, and (3) infectious HCV will be propagated. Over the course of this work, we designed conditions allowing for infection of human T cell-enriched cultures with wild-type HCV and for its productive replication in the derived T cells. The system created should be of value for studies on the complete cycle of HCV replication, factors determining the host's susceptibility to HCV, and cytopathic consequences of wild-type HCV infection. It could also be utilized for testing the efficacy of anti-HCV agents in the natural host cell milieu.

2.3. MATERIALS AND METHODS

2.3.1. Plasma-Derived HCV inocula

Plasma from patient N07/M with serologically and histologically documented CHC was used as the main source of wild-type HCV. This inoculum, designated HCV N07/M, carried HCV genotype 1a at $1x10^5$ vge/mL, as determined by real-time RT-PCR (Pham et al., 2004). To establish whether the inoculum would be able to infect lymphoid cells, PBMC isolated from N07/M were examined and found to be positive for both HCV RNA strands by RT-PCR/nucleic acid hybridization (RT-PCR/NAH) assays (Pham et al., 2004). The estimated HCV load was approximately 10^5 vge per 10^7 cells. Plasma samples from five CHC patients were also used as sources of wild-type HCV: N08/M plasma contained HCV genotype 1b at $5x10^6$ vge/mL; C07/F, genotype 1a at $1.6x10^5$ vge /mL; C32/M, genotype 1a at $8.5x10^6$ vge/mL; C34/M, genotype 1a at $2.6x10^6$ vge/mL; and N23/M, genotype 1b at $1.9x10^4$ vge/mL. HCV replication in PBMC was evident in these patients (not shown). The study was approved by the local Human Investigation Committee and samples were collected after informed consent had been obtained.

2.3.2. Preparation of Lymphoid Cells

Lymphoid cells serving as HCV infection targets were isolated from a healthy donor (A/M) with no clinical history or molecular indication of HCV exposure, as confirmed by RT-PCR/NAH assay (sensitivity of $\leq$10 vge/mL) (Pham et al., 2004), and who was

seronegative for anti-HCV (enzyme immunoassay; Abbott Diagnostics). For some experiments, lymphoid cells from two other HCV RNA- and anti-HCV-negative individuals, B/M and C/F, were utilized. For direct infection or serial passage of HCV, PBMC were isolated from 40 mL blood. Monocyte-depleted cells were resuspended in culture medium at $1x10^6$ cells/mL (Pham et al., 2004). For some experiments, T lymphocytes were affinity-purified from PBMC of donor A/M. In parallel, T cells from a CHC patient (N09/F), who carried genotype 1a at $2x10^6$ vge/mL, were isolated by negative selection using MACS magnetic beads (Miltenyi Biotec) (Pham et al., 2005). T cells were 97 % pure by flow cytometry.

2.3.3. Direct Infection with Plasma-Derived HCV

Monocyte-depleted lymphoid cells were exposed to 5 µg phytohaemagglutinin (PHA; ICN Biomedicals)/mL for 48 h prior to inoculation with HCV (Pham et al., 2004). For direct infection, PHA-treated cells ($1x10^6$ cells/mL) were exposed to 250 µL heat-inactivated (30 min at 56°C) plasma containing approximately $2.5x10^4$ vge. In parallel, the cells were exposed to 250 µL plasma from donor B/M for mock infection. Inocula were removed after 24 h, then the cells were washed and cultured with 20 U recombinant human interleukin-2 (IL-2; Roche Diagnostics)/mL for 72 h (phase A). At 4 days post-infection (p.i.), supernatant was collected and the cells were cultured with 5 µg PHA/mL and 20 U IL-2/mL (designated PHA/IL-2) for the next 72 h (phase B). At 7 days p.i., supernatant was harvested and the cells were again cultured for 3 days in medium with IL-2 (phase C). The culture supernatant collected at 10 days p.i. was centrifuged at 400 *x*

g for 30 min and used as the source of HCV for the serial-passage experiment. In a supplementary experiment, affinity-purified T cells from donor A/M were infected with N07/M or N08/M inoculum and cultured for 10 days p.i. as described above.

2.3.4. Serial Passage of HCV in T Cell Culture

PHA-stimulated target cells were incubated with clarified supernatant after phase C from the direct infection, normally containing approximately 10^5 vge HCV. After 24 h, the cells were collected, washed and stimulated with IL-2 or PHA/IL-2 (phases A, B and C) as described above. At day 10 p.i., supernatant was harvested and cells were cultured for 96 h with PHA/IL-2 (phase D). The culture ended at 14 days p.i. with collection of the supernatant and cells. A sample of the phase D supernatant was preserved for analysis, whereas the remainder was used to infect fresh PHA-induced lymphoid cells. This was repeated 14 consecutive times, spanning a total of 28 weeks. The phase D supernatants were examined for completeness of cell removal by phase-contrast microscopy. Randomly selected phase D supernatants were amplified for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by using primers and PCR conditions reported previously (Hodgson and Michalak, 2001) and no signals were detected. To determine whether HCV produced after the multiple passage in A/M T cells could infect cells from other healthy donors, PHA-pretreated lymphoid cells from B/M and C/F were incubated with pooled supernatant after phases A–C of passage 11 and cultured as described for the direct-infection experiment.

2.3.5. Blocking of HCV Infection in T cells with Anti-CD81

The experiment was carried out with anti-CD81 mAb JS81 (Pharmingen) at 1 : 20 and 1 : 100 dilutions in 50 μL containing $5x10^6$ mitogen-induced A/M T cells by using a previously published protocol (Zhong et al., 2005). T cells preincubated with an isotype-control mAb and inoculated with the same HCV served as controls. The cells were cultured for 14 days before analysis.

2.3.6. Treatment with Alpha Interferon (IFN-α).

To reaffirm that active HCV replication was established in T cells, the cells were treated in triplicate with 1000, 100 or 10 U recombinant human IFN-α 2b (Research Diagnostics)/mL at the time of HCV inoculation. The same IFN- amounts were added to the culture each time that the medium was changed (phases A–D). The cells were cultured for 14 days as described above. IFN-α concentrations were non-toxic to lymphoid cells in an MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay (data not shown). Cells inoculated with HCV, but not treated with IFN-α served as controls.

2.3.7. Ultracentrifugation and Buoyant Density Gradients

To determine HCV RNA in T cell culture supernatants, samples (5 mL) were centrifuged at 400 *x g* for 30 min and then at 150 000 *x g* for 22 h at 4 °C in an SW50.1 rotor (Beckman Instruments). RNA was extracted from the pellets and analysed by RT-PCR/NAH. To examine ultrastructural features of the released viral particles, samples

(5 mL) of passage 5, 8 and 11 supernatants were clarified, ultracentrifuged and analysed by electron microscopy. As controls, the supernatant from A/M cells not infected with HCV, but cultured under identical conditions, was prepared. To analyse properties of HCV RNA reactive particles further, 15 mL pooled supernatant obtained after phases A–C from passage 8 was clarified and ultracentrifuged as described above. The resulting pellet was resuspended in 800 µL 10 mM Tris/HCl buffer (pH 7.2) with 0.15 M NaCl and 10 mM EDTA and overlaid onto a 12 mL discontinuous gradient of 1.1–1.6 g caesium chloride (CsCl)/mL prepared in the same buffer. In parallel, plasma samples (800 µL) from patient C26/F carrying $1.1x10^6$ vge/mL and from a healthy donor were fractionated. After centrifugation at 200 000 ***x g*** for 48 h at 10 °C in a Beckman SW41 rotor, fractions (750 µL) were collected from the top of each gradient and analysed for HCV RNA and by electron microscopy.

2.3.8. RNA Extraction and RT-PCR/NAH.

Total RNA was extracted by using TRIzol (Invitrogen) from $1x10^7$ cells (which usually yielded approx. 15 µg RNA) or from 100 µL of the pellet suspension obtained by ultracentrifugation of culture supernatants. RNA was also isolated from samples (650 µL) of the CsCl fractions by using TRIzol LS (Invitrogen). RNA (1–4 µg) was transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase for HCV RNA positive-strand detection or with recombinant *Tth* DNA polymerase for virus RNA negative-strand detection (Pham et al., 2004). PCR was carried out by using primers and conditions reported previously (Pham et al., 2004). A water sample and a mock-treated

test RNA were always included as contamination controls. Complementary DNA (cDNA) from PHA-treated A/M cells exposed to B/M plasma (mock infection) and cultured as the infected cells served as a negative control, whilst 10-fold dilutions of recombinant HCV UTR-E2 fragment (rHCV UTR-E2) acted as quantitative standards. Specificity of the detection and validity of controls were confirmed routinely by Southern blot hybridization (Pham et al., 2004). Detection of HCV synthetic RNA (sRNA) positive and negative strands was not affected by the presence of cellular RNA from healthy PHA-stimulated lymphoid cells. HCV RNA in plasma was quantified by real-time RT-PCR using LightCycler Fast Start Master hybridization probes (Roche Diagnostics) (Pham et al., 2004).

2.3.9. Detection of HCV NS5A and E2 Proteins

For Western blotting, proteins of T cells infected with HCV and recovered after passages 3, 11 and 14 were separated on 10 % sodium dodecyl sulfate-polyacrylamide gels and blotted as reported previously (Michalak et al., 2000). Blots were probed with anti-HCV NS5A mAb (Biodesign) or anti-HCV E2 (ALP98) mAb (provided by Dr A. Patel, Institute of Virology, University of Glasgow, UK). HCV replicon cell line AB12-A2FL, containing full-length HCV genotype 1b (provided by Dr C. Richardson, Ontario Cancer Institute, University of Toronto, Canada), was used as a positive control. A/M T cells not exposed to HCV, but cultured under the same conditions, were a negative control. Reactions were visualized by a horseradish peroxidase-conjugated secondary antibody and chemiluminescence. To assess intracellular expression of HCV E2 protein in *in vitro*-

infected T cells, confocal immunofluorescence microscopy was carried out by using AB12-A2FL Huh-7 cells, naïve Huh-7 cells and HCV-naïve T cells as controls. Cells were grown overnight on polylysine-coated 16-well glass slides (Nalge Nunc International), fixed with 4 % paraformaldehyde and permeablized with 0.5 % Triton X-100. HCV E2 protein and tubulin were identified with anti-HCV E2 (AP33) mAb and rat anti-tubulin (Chemicon), respectively. Cy2-labelled donkey anti-mouse or Cy5-labelled donkey anti-rat antibodies (both from Jackson ImmunoResearch) were used in the second layer. Cells were examined in a FluoView FV300 confocal system (Olympus).

2.3.10. Transmission and Immune Electron Microscopy

For routine microscopy, aliquots of the pellets recovered after ultracentrifugation or fractions from CsCl gradients and respective controls were applied onto Formvar–carbon-coated 200-mesh microscopic grids. The grids were washed and stained negatively with 1% phosphotungstic acid (PTA). To precipitate HCV cores, aliquots of the pellets were incubated with 20 μg anti-HCV core IgG2a mAb (Virogen)/mL or with an isotype-control mAb for 1 h at ambient temperature and then overnight at 4 °C. Precipitates were loaded on grids. Alternatively, grids were incubated with anti-HCV core mAb or a control mAb for 10 min, washed, incubated with test samples for 10 min and washed. All grids were counterstained with PTA. To confirm that HCV virions were secreted by T cells, immunogold staining of the pellets recovered by ultracentrifugation from selected culture supernatants was done by using anti-E2 (AP33) mAb, as described by Owsianka *et al.* (2001). This was followed by incubation with anti-mouse IgG conjugated with

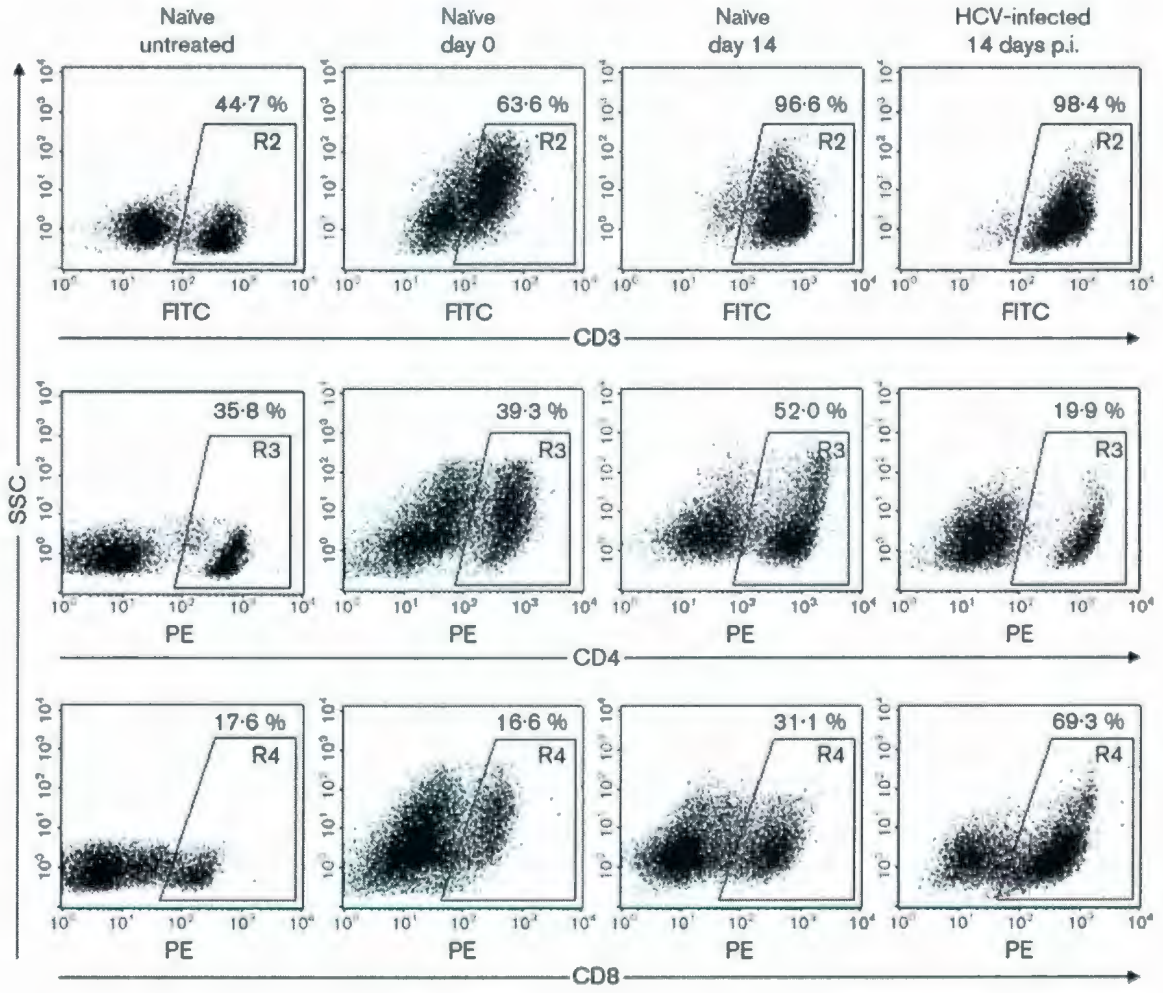
12 nm gold particles (Jackson ImmunoResearch) and counterstaining with 1 % PTA. Examinations were carried out with a JEM 1200 EX (JEOL) microscope.

2.4. RESULTS

2.4.1. Selective Expansion of T Cells in Culture

Anticipating that the intracellular environment supportive of HCV replication elicited by PHA (Pham et al., 2004) may also enhance lymphoid cell susceptibility to virus, monocyte-depleted cells were PHA-treated prior to HCV exposure. After 48-h culture, the cells expanded by 20% to 45%. Approximately 64% of them were CD3-positive (T cells), with a CD4- to CD8-positive cell ratio of 2.4:1 (Fig. 2.1), 24% were CD40-reactive (B cells, macrophages and/or dendritic cells), 16% were CD19-positive (B cells), and <1% CD14-positive (monocytes) (data not shown). After alternating stimulation of both virus-naïve and HCV-exposed cells with PHA and/or IL-2 for 14 days, 96.6% of the cells were CD3-positive (Fig. 2.1). Interestingly, while the ratio of CD4-positive to CD8-positive cells was 2:1 for uninfected T cells, that for the infected was 1:3.4. Less than 1% CD19-reactive cells were detected in HCV-infected cultures (data not shown). These data showed that T cells were essentially the only cell type supporting HCV propagation at the phase D of the passage from which supernatant was used to transmit infection.

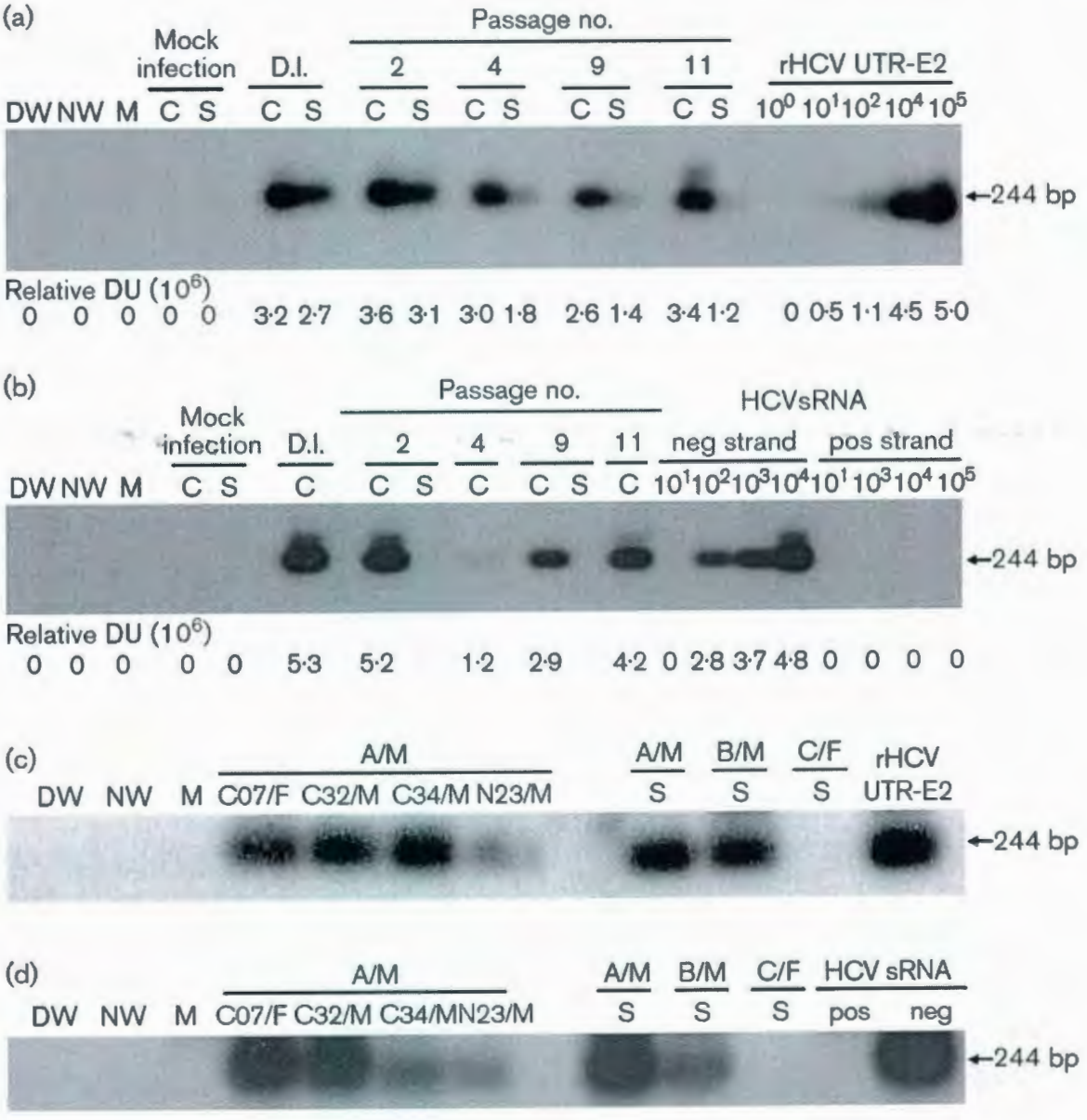
Figure. 2.1. Phenotypic characterization of lymphoid cells before and after infection with HCV. Monocyte-depleted lymphoid cells from donor A/M were untreated (Naïve untreated), treated with PHA for 48 h (Naïve day 0) or cultured for 14 days after PHA treatment (Naïve day 14). In addition, A/M cells recovered following 11 passages with HCV N07/M inoculum were examined (HCV-infected 14 days p.i.). Cells were incubated with fluorescein isothiocyanate (FITC)–anti-CD3 or with phycoerythrin (PE)–anti-CD4 or –anti-CD8 mAb, or with an appropriate immunoglobulin isotype control, and analysed by flow cytometry. Gates were set up on isotype controls. Percentages indicate positive cells.


Naïve
untreated
Naïve
day 0
Naïve
day 14
HCV-infected
14 days p.i.
44·7 %
R2
63·6 %
R2
96·6 %
R2
98·4 %
R2
FITC
FITC
FITC
FITC
CD3
35·8 %
R3
39·3 %
R3
52·0 %
R3
19·9 %
R3
SSC
PE
PE
PE
PE
CD4
17·6 %
R4
16·6 %
R4
31·1 %
R4
69·3 %
R4
PE
PE
PE
PE
CD8

2.4.2. HCV Genome Expression During *De Novo* Infection and Serial Passage in T Cell Cultures.

As shown in Figure 2.2A, HCV RNA positive strand was detected in both cells and culture supernatant after the direct infection with HCV N07/M, while the mock-infected cells were negative. HCV load was ~10^5 vge/10^7 cells. Comparable results were obtained when HCV-positive plasma from C32/M, C34/M and N23/M patients were used for direct infection of A/M cells (Figs 2.2C and 2.2D). These results implied that infection of the T cell cultures was not related to the origin of HCV inoculum. Heat inactivation of plasma did not influence HCV infectivity, although it occasionally improved cell survival. Estimated levels of HCV RNA positive strand in T cells after passages 2, 4, 9 and 11 (Fig. 2.2A) ranged between 10^5 and 10^6 vge per 10^7 cells, while the amount of HCV released during each serial passage was ~10^4 vge/mL. Taken together, the total HCV produced during the entire passage experiment was estimated to exceed 10^7 vge. Given that the HCV N07/M inoculum carried 2.5 x 10^4 vge and that 1.4 x 10^4 vge was recovered in culture medium after the inoculation, at least 1000-fold enrichment in the virus was achieved. Also, HCV RNA negative strand was found in T cells collected after direct infection and all passages analyzed, but not in phase D supernatants (Fig. 2.2B). Semi-quantitatively, there were ~10^4 vge of the negative strand for 10^7 cells.

Figure. 2.2. Detection of HCV RNA positive and negative strands in T-cell cultures after direct infection and during serial passage of HCV. In (a) and (b), A/M T cells (C) and their culture supernatants (S) were obtained after direct infection (D.I.) or indicated passages of HCV N07/M inoculum. A/M T cells exposed to normal human plasma and cultured as infected cells (mock infection) served as a negative control. Synthetic HCV RNA (HCV sRNA) positive and negative strands were used to confirm specificity of the detection and as quantitative standards. DU, Density units. In (c) and (d), A/M T cells were exposed to HCV-positive plasma from different CHC patients, or T cells from healthy donors A/M, B/M and C/F were inoculated with cell-free supernatant (S) obtained after passage 11 shown in (a). HCV RNA-positive (a, c) and -negative (b, d) strands were detected by specific RT-PCR/NAH. Water instead of cDNA amplified in direct (DW) and nested (NW) reactions, as well as a mock-treated test RNA (M), were included as contamination controls. Positive samples show the expected 244 bp amplicons.

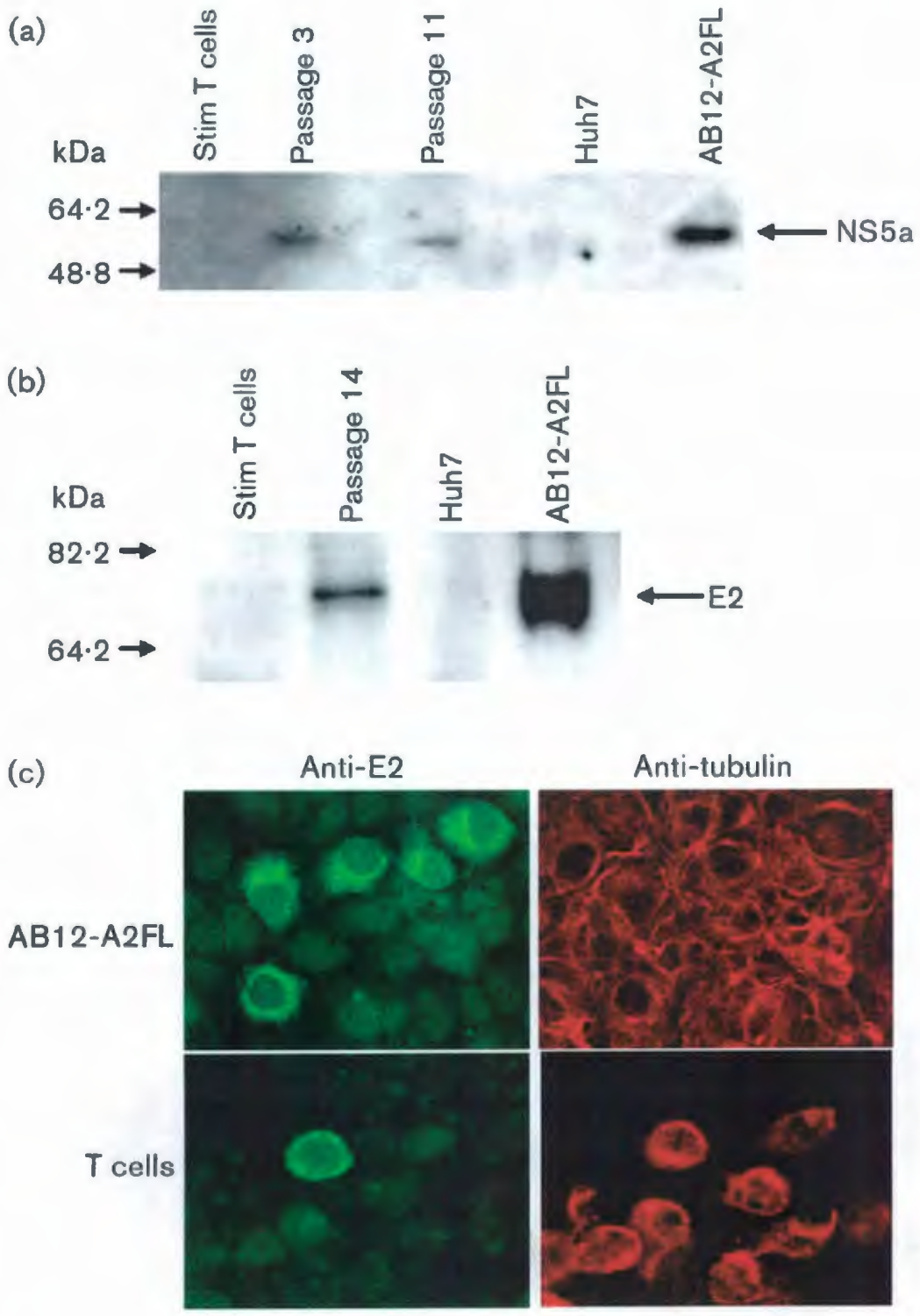

(a)
Mock infection
D.I.
Passage no.
2
4
9
11
rHCV UTR-E2
DW NW M C S C S C S C S C S C S 10^0 10^1 10^2 10^4 10^5
←244 bp
Relative DU (10^6)
0 0 0 0 0 3·2 2·7 3·6 3·1 3·0 1·8 2·6 1·4 3·4 1·2 0 0·5 1·1 4·5 5·0
(b)
Mock infection
D.I.
Passage no.
2
4
9
11
HCVsRNA
neg strand
pos strand
DW NW M C S C C S C C S C 10^1 10^2 10^3 10^4 10^1 10^3 10^4 10^5
←244 bp
Relative DU (10^6)
0 0 0 0 0 5·3 5·2 1·2 2·9 4·2 0 2·8 3·7 4·8 0 0 0 0
(c)
A/M
C07/F C32/M C34/M N23/M
A/M S
B/M S
C/F S
rHCV UTR-E2
DW NW M
←244 bp
(d)
A/M
C07/F C32/M C34/M N23/M
A/M S
B/M S
C/F S
HCV sRNA
pos neg
DW NW M
←244 bp

To determine whether the virus obtained after multiple serial passages in A/M T cells could infect T cells of other healthy individuals, PHA-treated cells from donors B/M and C/F were exposed to pooled supernatants from passages 11 and 12. After the direct infection, cells from B/M carried HCV RNA positive strand at ~10^5 vge/10^7 cells and negative strand at ~10^4 vge/10^7 cells. Interestingly, although cells from C/F proliferated to the same extent as those from A/M and B/M donors, they remained HCV RNA negative (Figs. 2.2C and 2.2D). This variation might be of biological significance and will be investigated in the future.

2.4.3. HCV Protein Display in *In Vitro* Infected T Lymphocytes.

To determine whether HCV infection was accompanied by synthesis of viral proteins, the presence of HCV NS5A and E2 proteins was examined by Western blotting. A protein band of ~56 kDa was detected in T cells after passages 3 and 11 when probed with anti-NS5A mAb (Fig. 2.3A). Also, a band of approximately 70 kDa, representing the E2 protein, was evident in passage 14 (Fig. 2.3B). Bands of comparable molecular sizes were detected in Huh-7 cells transfected with HCV AB12-A2FL replicon, but not in naïve Huh-7 cells or in normal T cells cultured under identical conditions as infected T cells. A confocal microscopic analysis suggested that the E2 protein has intracytoplasmic and plasma membrane-associated localization in infected T cells (Fig. 2.3C).

Figure. 2.3. Detection of HCV NS5A and E2 proteins in *in vitro*-infected T cells. A/M T cells after passages 3, 11 and 14 of HCV N07/M inoculum were lysed, then proteins were separated at 30 µg per lane and probed with (a) anti-NS5A or (b) anti-E2 (ALP98) mAb. The 56 kDa NS5A and 70 kDa E2 protein bands are marked on the right and molecular mass markers on the left. A/M cells not exposed to HCV, but cultured as infected T cells, and naïve Huh-7 cells were used as negative controls. In (c), T cells after direct infection with HCV N23/M inoculum were double-stained with anti-E2 (AP33) and anti-tubulin mAbs and analysed by confocal microscopy. Huh-7 cells transfected with the HCV AB12-A2FL replicon served as a positive control.


(a)
Stim T cells
Passage 3
Passage 11
Huh7
AB12-A2FL
kDa
64·2
48·8
NS5a
(b)
Stim T cells
Passage 14
Huh7
AB12-A2FL
kDa
82·2
64·2
E2
(c)
Anti-E2
Anti-tubulin
AB12-A2FL
T cells

2.4.4. Inhibition of HCV Infection by Anti-CD81 and Treatment with IFN-α

Pre-incubation of T cells with two concentrations of anti-CD81 mAb, but not with an isotype control, blocked HCV RNA expression (Fig. 2.4). To provide further evidence that active HCV replication was supported by T cells and to recognize applicability of our system for testing susceptibility of wild-type HCV to antivirals, the effect of IFN- α on the outcome of *de novo* infection in T cells was examined. The results showed a dose-dependent antiviral effect of IFN-α (Fig. 2.5). HCV replication was abrogated in cells treated with 1000 U/mL, but not inhibited uniformly by lower doses.

2.4.5. HCV Infection in Affinity-Purified T Cells

To further confirm natural susceptibility of T cells to HCV infection, affinity-purified T cells from healthy A/M donor were infected with HCV N07/M or HCV N08/M. In parallel, pure T cells from N09/F patient with CHC were cultured exactly as those from A/M donor. At 10 d.p.i., HCV RNA positive strand was detected at ~10^5 vge per 10^7 T cells infected with N07/M and N08/M inocula (Fig. 2.6A). Interestingly, T cells purified from N09/F patient carried HCV RNA positive strand at a similar level as those infected with HCV N07/M (Fig. 2.6A). The level of HCV RNA negative strand was ~10^4 vge/10^7 cells infected with N07/M and N08/M inocula, and in T cells from N09/F patient (Fig. 2.6B).

Figure. 2.4. Inhibition of HCV infection in T cells with anti-CD81 mAb. A/M T-cells pre-treated with anti-CD81 mAb at 1 : 20 or 1 : 100 were exposed to HCV-positive N23/M plasma (see Methods) and, after 14 days culture, were examined for HCV RNA. T cells pre-treated with an appropriate isotype control (ISO) at 1 : 20 served as controls. For (a), other controls were as described in the legend to Fig. 2.2 (b) Plot presentation of densitometric values obtained for (a).

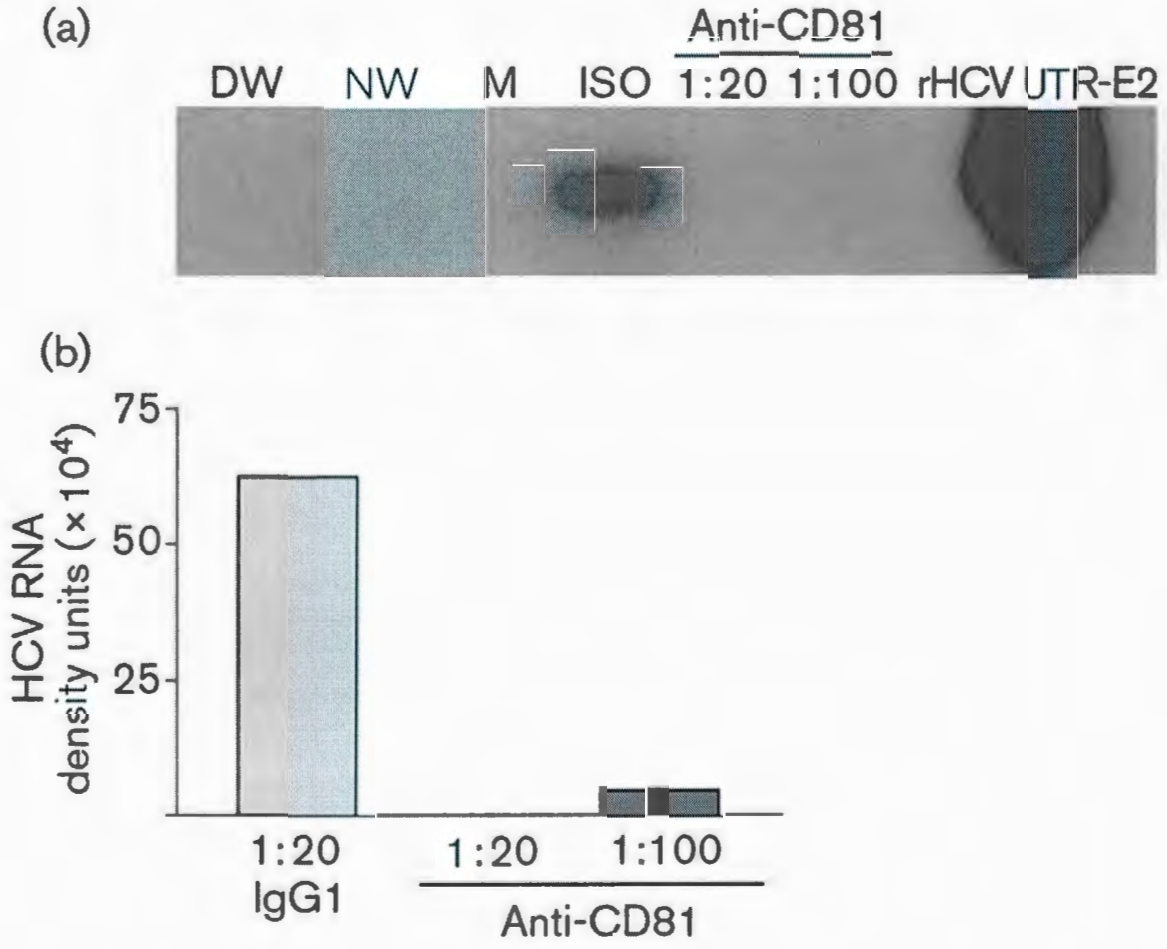

(a)
Anti-CD81
DW
NW
M
ISO
1:20
1:100
rHCV UTR-E2
(b)
HCV RNA
density units (×10⁴)
75
50
25
1:20
IgG1
1:20
1:100
Anti-CD81

Figure. 2.5. Effect of IFN-α treatment on detection of HCV RNA in *in vitro*-infected T cells. A/M T cells were exposed to HCV N07/M inoculum in the absence (HCV-UT) or presence of indicated concentrations of IFN-α in triplicate and cultured for 14 days. RNA was analysed for HCV RNA positive (a) and negative (b) strands by RT-PCR/NAH assays. Specificity and contamination controls were as described in the legend to Fig. 2.2.

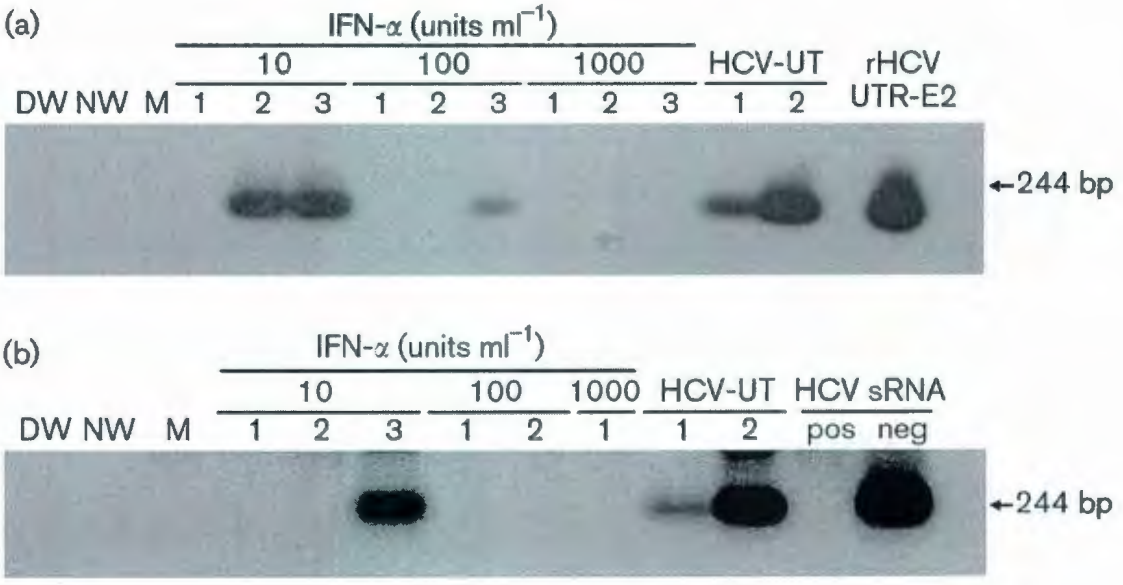
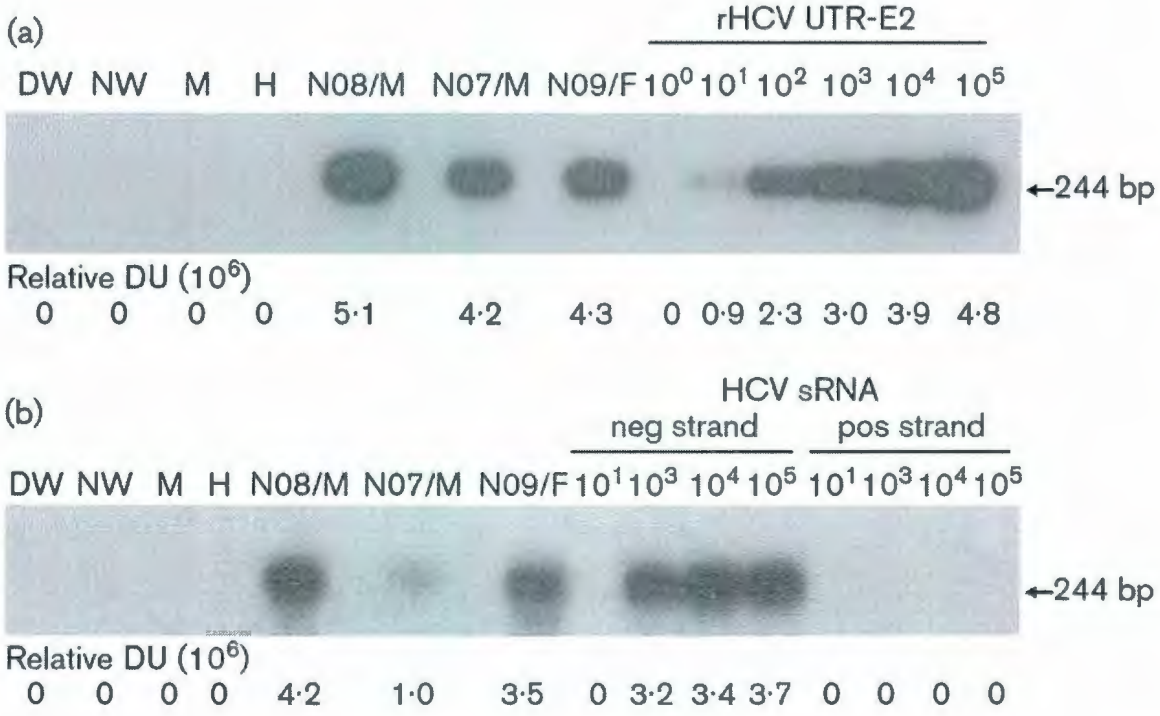

(a)
IFN-α (units ml^{-1})
10
100
1000
HCV-UT
rHCV UTR-E2
DW NW M 1 2 3 1 2 3 1 2 3 1 2
←244 bp
(b)
IFN-α (units ml^{-1})
10
100
1000
HCV-UT
HCV sRNA
DW NW M 1 2 3 1 2 1 1 2 pos neg
←244 bp

Figure. 2.6. Expression of HCV RNA in affinity-purified T lymphocytes infected with HCV either *in vitro* or *in vivo*. Affinity-purified T cells from donor A/M pre-treated with PHA were exposed to HCV N07/M or HCV N08/M inoculum and cultured (see Methods). In parallel, purified T cells from patient N09/F (CHC) were cultured under similar conditions. RNA was analysed for HCV RNA positive (a) and negative (b) strands. RNA from affinity-purified T cells of donor A/M, which were not exposed to the virus but cultured similarly, was included as a negative control (H). Contamination and specificity controls and quantitative standards were as described in the legend to Fig. 2.2.

Figure. 2.7. Isopycnic banding of HCV RNA in the supernatant obtained after eight consecutive passages of HCV N07/M inoculum in T cells. An aliquot of the concentrated supernatant pool from passage 8 (see Methods) and plasma from patient C26/F (CHC) and a healthy A/M donor were centrifuged through 1.1–1.6 g CsCl/mL gradients; 750 µL fractions were collected. HCV RNA was examined by RT-PCR/NAH. Depicted are HCV RNA levels (vge) in fractions from the passage 8 supernatant (●) and plasma of a chronic HCV carrier (○), and the CsCl density values (▲; g/mL). An arbitrary scale from – (absence) to +++ (several dozen particles per microscopic grid) was used to estimate the number of HCV virion (VP) and core (CP) particles encountered in individual fractions by electron microscopy. NT, Not tested.


(a)
rHCV UTR-E2
DW NW M H N08/M N07/M N09/F 10^0 10^1 10^2 10^3 10^4 10^5
←244 bp
Relative DU (10^6)
0 0 0 0 5·1 4·2 4·3 0 0·9 2·3 3·0 3·9 4·8
(b)
HCV sRNA
neg strand
pos strand
DW NW M H N08/M N07/M N09/F 10^1 10^3 10^4 10^5 10^1 10^3 10^4 10^5
←244 bp
Relative DU (10^6)
0 0 0 0 4·2 1·0 3·5 0 3·2 3·4 3·7 0 0 0 0

2.4.6. Buoyant Density of HCV RNA-Reactive Particles Released by *In Vitro* Infected T Cells.

To recognize properties of HCV particles produced by T cells, culture supernatant obtained after passage 8 was subjected to isopycnic centrifugation. The fractions collected were examined for HCV RNA by RT-PCR/NAH and virus particles by electron microscopy. As shown in Figure 2.7, the main peak of HCV RNA reactivity was identified at buoyant densities 1.16 - 1.19 g/mL (fractions 5 to 7; peak 1). There was also a peak at 1.26 - 1.32 g/mL (fractions 9 to 11; peak 2) and some minor increase in HCV RNA positivity at other densities. The greatest HCV copy numbers estimated were 1.4 x 10^5 vge/mL for fraction 6 (density 1.18 g/mL) of peak 1 and 0.75 x 10^5 vge/mL for fraction 9 (density 1.26 g/mL) of peak 2. Analysis of the fractions from C26/F plasma also showed HCV RNA peaks of distinct buoyant density (Fig. 2.7), confirming that the gradient was capable of separating a heterogenous population of HCV particles. Similar analysis of the supernatant from non-infected but cultured A/M T cells did not reveal viral signals (not shown).

2.4.7. Ultrastructural Features of HCV Particles Produced by T Cells.

The vast majority of HCV particles detected by electron microscopy after direct ultracentrifugation of T cell culture supernatants were nonenveloped cores (Fig. 2.8A i-iii). Their diameters ranged between 25 and 50 nm (mean 36.5 nm). These particles could be precipitated with anti-HCV core mAb (Fig. 2.8A, iv-vii), but not with an isotype control mAb (not shown). Fractions collected after a CsCl gradient showed the presence

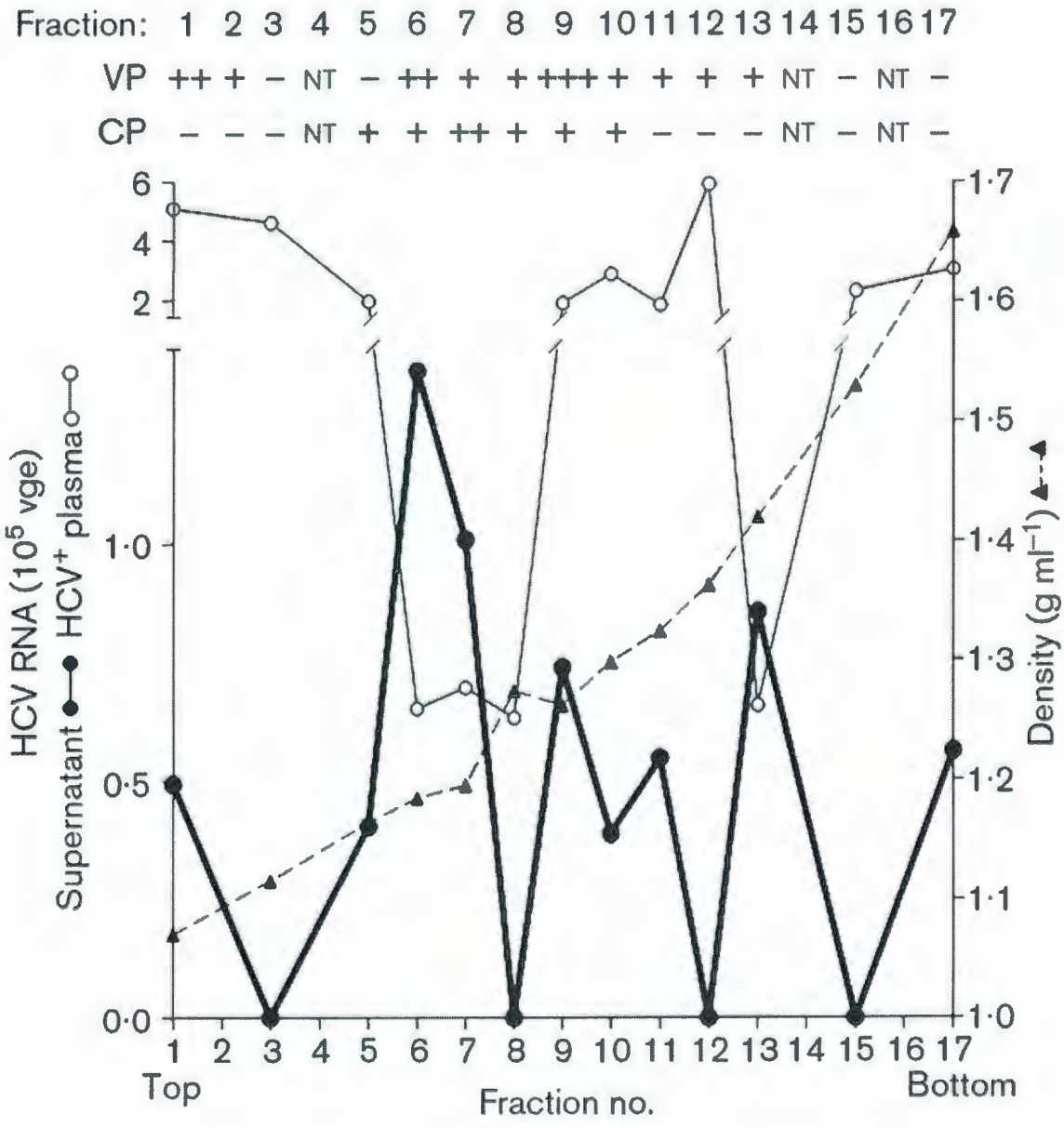
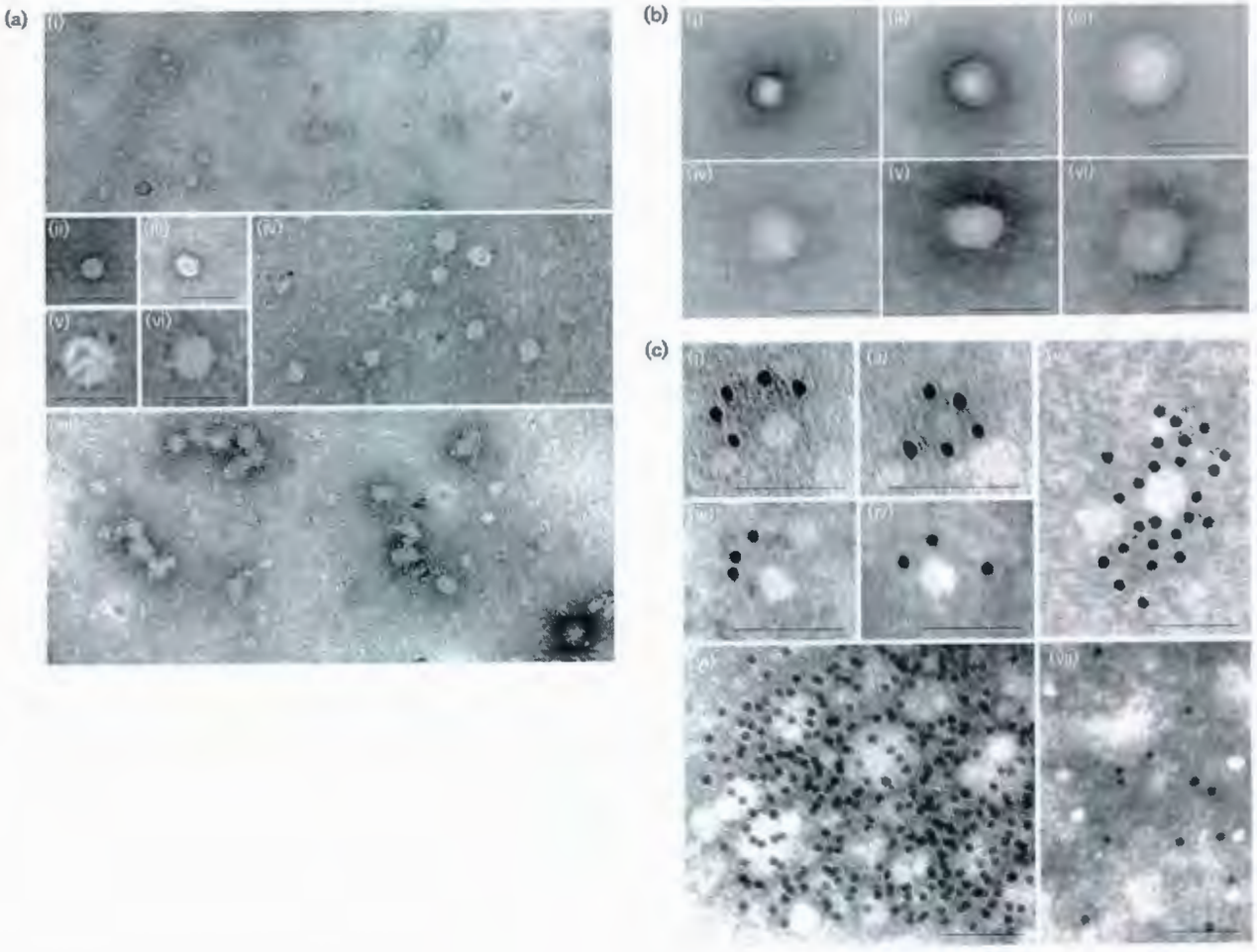

Fraction: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
VP ++ + – NT – ++ + + +++ + + + + NT – NT –
CP – – – NT + + ++ + + + – – – NT – NT –
HCV RNA (10^5 vge)
Supernatant ●—● HCV+ plasma ○—○
Density (g ml^{-1})
6
4
2
1·0
0·5
0·0
1·7
1·6
1·5
1·4
1·3
1·2
1·1
1·0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Top
Fraction no.
Bottom

Figure. 2.8. Ultrastructural identification of HCV virion and core particles in culture supernatants obtained after serial passage of HCV in T cells. (a) HCV core particles recovered after direct centrifugation of supernatants after passages 5, 9 and 11 of HCV N07/M inoculum. (i–iii) Examples of HCV core particles after passages 5 and 11. (iv–vi) Nucleocapsid particles from passage 9 detected by immunoelectron microscopy using anti-core mAb. Note visible spikes given by extended antibody molecules. (vii) Aggregates of HCV core particles immunoprecipitated with anti-core antibody. Bars, 50 nm. (b) HCV virion-like particles detected in fraction 6 (density, 1.18 g/mL) after CsCl gradient fractionation of the culture supernatant from passage 8 (i–iii) and C26/F plasma (iv–vi). Bars, 100 nm. (c) Detection of HCV virions produced by *in vitro*-infected T cells by immunoelectron microscopy with anti-HCV E2 (AP33) mAb. (i–iv) Single HCV virions decorated with colloidal gold particles in C32/M plasma (i–ii) and in the T-cell supernatant pooled after passages 10 and 13 of HCV N07/M (iii–iv). (v–vi) Examples of the medium-size (v) and large (vi) aggregates of HCV virions. The same culture supernatant pool exposed to the isotype-control mAb served as a negative control (vii). Bars, 100 nm. Preparations were counterstained with 1 % PTA.


(a)
(b)
(c)

of both virion-like structures and non-enveloped cores (see Fig. 2.7). Virion-like particles were heterogeneous in diameter, which ranged between 51 and 74 nm (mean 67.4 nm), and envelope thickness between 6 to 15.6 nm (mean 11.4 nm). Figure 2.8B illustrates examples of HCV virion-like particles after CsCl fractionation of the passage 8 supernatant (Fig. 2.8B, i-iii) and C26/F plasma (Fig. 2.8B, iv–vi). Diameters of these virions were 62 - 85 nm (mean 77.6 nm) and the envelope thickness ranged from 9 to 27 nm (mean 20.6 nm). To further ascertain that HCV virions were released by the *in vitro* infected T cells, pellets recovered after ultracentrifugation of the T cell culture supernatants or C32/M plasma were stained by immunogold with anti-HCV E2 mAb. Singular enveloped virions dressed with gold particles were detected both in control C32/M plasma (Fig. 2.8C; i and ii) and in the pooled T cell culture supernatants recovered after passages 10 and 13 (Fig. 2.8C; iii and iv). Aggregates of gold-coated virions were also detected (Fig. 2.8C; v and vi). There were a few gold particles not associated with virions in the same supernatant after incubation with an isotype control mAb (Fig. 2.8C, vii).

2.5. DISCUSSION

In this study, lymphoid-cell cultures, established by *ex vivo* treatment of PBMC from healthy individuals with a T cell-inducing mitogen, were susceptible to wild-type HCV and capable of supporting its complete cycle of replication. Productive HCV replication was documented by detection of HCV RNA positive and negative strands, of NS5A and E2 proteins and by secretion of complete virions, which could infect T cells from healthy

donors *de novo*. The results also showed that circulating T cells in clinically evident HCV infection are an extrahepatic site of virus replication.

Data from the present and previous works (Pham et al., 2004; Pham et al., 2005), indicate that activation of lymphoid cells, which characterizes functioning, dividing and immature progenitor lymphoid cells, predisposes cells to HCV recognition and promotes an intracellular microenvironment supportive of virus replication. This may explain why HCV has been more readily identifiable in lymphoid-cell cultures or in PBMC from patients coinfected with other viral pathogens (Beld et al., 1998; Laskus et al., 2004) and why mature, mitogen-untreated lymphoid cells are poorly permissive to HCV infection *in vitro*. Our data raise a possibility that lymphoid organs, which embrace the most active proliferative expansion of lymphoid cells in adulthood, are sites of HCV propagation and long-term persistence of virus. The notion of lymphotropism is supported by data demonstrating HCV RNA in bone marrow, lymph nodes or spleens from patients with CHC or coinfected with human immunodeficiency virus type 1 (Laskus et al., 1998; Radkowski et al., 2000).

In our system, passage of HCV by infection of T cell cultures freshly prepared each time was associated with consistent detection of both HCV RNA positive and negative strands in the cells and of the positive strand in culture supernatants from all passages tested. The potency of the virus to infect the cells did not decrease during serial passage and the estimated amount of the virus produced appeared to be proportional to T cell proliferation. On average, 10^5 vge were detected per 10^7 infected T cells at the end of each passage. Interestingly, a comparable rate of approximately 10^5 vge per 10^7 cells was detected for T cells affinity-purified from patient N09/F with CHC.

HCV in *de novo*-infected T cells was susceptible to IFN-α treatment, with complete inhibition of virus replication observed at 1000 U/mL. This result resembles that reported for primary human hepatocytes infected *in vitro* with wild-type virus (Castet et al., 2002). In addition, as was shown for the JFH-1 full length viral clone in Huh-7 cells (Zhong et al., 2005), anti-CD81 mAb was able to inhibit HCV infection in our system.

To ascertain further that *in vitro*-infected T cells supported HCV replication and that the virus was assembled, ultrastructural characteristics of viral particles released by the cells were examined after either direct ultracentrifugation or fractionation in CsCl gradients. It was found that non-enveloped core particles were predominantly detected in the supernatants after direct centrifugation, whereas HCV virions were mainly seen in those fractionated throughout the density gradient, particularly in the fractions enriched with HCV RNA. These findings suggested that concentrating the virus without applying density equilibrium led to dissociation of viral particles and thus implied erroneously that HCV cores, but not complete virions, were mainly produced by infected T cells. In fact, depending on which method of HCV concentration is employed, separation of the viral envelope from the nucleocapsid has been observed (Fujita et al., 2001). Nevertheless, the HCV nucleocapsids detected in our T cell supernatants displayed the expected size and were immunoprecipitated with anti-HCV core mAb, as observed by others (Kaito et al., 1994; Maillard et al., 2001). Similarly, HCV virion-like particles detected after isopycnic banding showed heterogeneous properties, as reported previously (Hijikata et al., 1993; Kanto et al., 1994; Fujita et al., 2001; Pumeechockchai et al., 2002). Specificity of these particles was confirmed by immuno-gold staining with anti-E2 mAb, and both single, complete virions and immuno-aggregates of virions were detected. These results

documented conclusively that viral particles with physical and structural properties of complete HCV virions were released by *in vitro*-infected T cells.

Overall, our results from a multiparametric analysis demonstrated that mitogen-induced normal human T cells can support the complete cycle of HCV replication and produce infectious virions. This suggests that they are equipped not only with appropriate molecules capable of recognition and uptake of HCV, but also with the machinery to multiply the virus. This system should be of value for studies on recognition of cytopathic mechanisms of HCV infection, investigation of host factors determining susceptibility and the efficiency of virus replication, and for testing antiviral agents against wild-type HCV propagating in the natural host-cell milieu.

Chapter Three: Exposure of Lymphocytes to Wild-Type HCV *In Vitro* Inhibits $CD4^+$ but not $CD8^+$ T Cell Proliferation

3.1. SUMMARY

HCV has been shown to replicate in cells of the immune system. Previously, we established a culture system in which wild-type HCV productively propagates in mitogen-stimulated primary T cells. The aim of the current study was to determine whether exposure to HCV can lead to changes in T cell proliferation and eventually induce apoptosis, which may result in cell phenotypic changes in comparison to cells not exposed to virus. For this purpose, PBMC from a single healthy donor provided naïve T cells. The cells were exposed to HCV inocula from 3 patients with CHC carrying genotypes 2b, 3a and a mixture of 1a and 1b or to normal human plasma (NHP). Cells were analyzed prior to and up to 10 days post-exposure for HCV RNA positive and negative strands by specific RT-PCR/NAH assays. In addition, T cell proliferation was measured by the carboxyfluorescein succinimidyl ester (CFSE) assay, apoptosis by flow cytometry-based annexin-V/7-actinomycin D assay, and T cell phenotype determined by staining for CD3, CD4 and CD8 followed by flow cytometry. Two of three HCV inocula examined produced quantifiable intracellular HCV RNA signals, while HCV RNA negative strand, indicative of virus replication, was detected following exposure to one of the inocula. Two inocula containing HCV genotype 2b and 3a led to a shift in the T cell phenotype from predominantly CD4-positive towards CD8-positive at the end of the 10-day culture period. Interestingly, cultures exposed to these two inocula had the highest

levels of intracellular HCV RNA expression. This change in T cell subset distribution was associated with a statistically significant inhibition of lymphocyte proliferation, which appeared to be restricted to $CD4^+$ T cells. Furthermore, there were no significant differences in the rate of apoptosis during the 10-day culture period between T cells exposed or not to HCV. As well, expression of selected cytokines such as IFN-γ, TNF-α, and IL-2 did not vary noticeably in the cells cultured in the presence of virus or not. These results suggest that HCV, regardless of the genotype, may exert a direct effect on $CD4^+$, but not $CD8^+$, T lymphocyte proliferative capacity and that this effect may occur in the absence of detectable active virus replication within these cells. In consequence, this finding may imply that exposure to HCV is sufficient to modify T lymphocyte proliferation and to shift the T cell phenotype.

3.2. INTRODUCTION

HCV is a single-stranded RNA virus belonging to the *Flaviviridae* family that infects at least 170 million people worldwide (Wasley and Alter, 2000). In HCV-infected patients, viral replication, which is revealed through the detection of HCV RNA positive strand and the HCV replicative intermediate, the RNA negative strand, has been demonstrated in cells of the immune system, such as B cells, monocytes, and $CD4^+$ and $CD8^+$ T cells (Pham et al., 2004; Radkowski et al., 2004; Pal et al., 2006; Kondo et al., 2007; Pham et al., 2008). In this laboratory, it was demonstrated that primary T lymphocyte cultures, generated by *ex vivo* treatment of PBMC from healthy individuals with PHA, a T cell-inducing mitogen, were susceptible to wild-type HCV and capable of supporting viral

replication. Furthermore, these cells were able to produce infectious virions that *de novo* infected naïve lymphocytes (Section 2).

HCV infection leads to chronicity in up to 85% of those afflicted, while acute HCV infection is thought to spontaneously resolve in 15-25% of cases (Cox et al., 2005; Thomas and Seeff, 2005). Resolution of hepatitis C appears to occur as a result of a robust HCV-specific T cell-mediated response. In HCV-infected chimpanzees, the closest animal model of human HCV infection, recovery from hepatitis and a drop in plasma HCV loads to levels undetectable by clinical RT-PCR assays requires the activity of both $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cell cells (Shoukry et al., 2003; Grakoui et al., 2003). In patients with CHC, their T cells appear to have markers of exhaustion and are defective in their ability to produce IFN-γ and IL-2 (Semmo et al., 2007). PD-1, a major inhibitory receptor on T cells that leads to inhibition of T cell proliferation and cytokine production upon PDL-1 binding, has been found to be expressed at significantly higher levels in the PBMC and in the livers of patients with CHC compared to those who spontaneously resolve hepatitis C (Golden-Mason et al., 2007). Furthermore, TIM-3, a negative regulator of Th1 responses, has been found to be upregulated on peripheral blood $CD4^+$ and $CD8^+$ T cells in CHC patients (Golden-Mason et al., 2009). *In vitro*, the functional consequences of T cell infection in Molt-4 and Jurkat T cell lines, and primary T cells have been studied to a small extent. Among others, it was revealed that in Molt-4 cells, HCV infection suppressed IFN-γ signalling through STAT-1 pathway (Kondo et al., 2007).

Stemming from the previous observations that exposure of naïve lymphoid cells to HCV *in vitro* and their subsequent culture can lead to changes in the $CD4^+$ to $CD8^+$ T cell

ratio (Section 2), in the current work we asked whether and how HCV may alter the behaviour of lymphoid cells. This was tested by examining HCV-induced changes to the cells' proliferation, apoptosis and phenotype, and expression of selected cytokines.

We were able to confirm that exposure of T lymphocytes to HCV indeed caused a shift in the $CD4^+$:$CD8^+$cell ratio. This appeared to be due to inhibited proliferation of $CD4^+$ T cells but not increased apoptotic death of these cells.

3.3. MATERIALS AND METHODS

3.3.1. Plasma-Derived HCV Inocula and Target Cells

Plasma from patients C26/F (CHC-1), C33/M (CHC-2), N28/F (CHC-3) with clinically and serologically documented CHC was used as the source of wild-type HCV. These 3 inocula carried different HCV genotypes and various HCV loads expressed in vge/mL (Table 3.1.). Thus, CHC-1 carried a mixture of genotype 1a and 1b at 1.1×10^6 vge/mL, CHC-2 had genotype 3a at $\geq 1 \times 10^4$ vge/mL, and CHC-3 carried virus of 2b genotype at 2.4×10^6 vge/mL. Plasma HCV RNA load was determined by HCV-specific real-time RT-PCR, as reported previously (Pham et al., 2004). Selection of plasma serving as HCV inocula was also based on the finding that PBMC from the cases examined were HCV RNA positive strand reactive (Table 3.1.). Lymphoid cells serving as HCV targets for *in vitro* infection experiments were isolated from a single healthy donor who had no clinical history or molecular indication of HCV exposure, as confirmed by highly sensitive RT-PCR/NAH assay (sensitivity of < 10 vge/mL or < 2 IU/mL) (Pham et al., 2004).

Table 3.1. Clinical and Virological Characteristics of Individuals with Chronic Hepatitis C Providing HCV Inocula

Inoculum	Case/Sex	HCV genotype	HCV RNA load	
			Serum[a] (vge/mL)	PBMC[b] (vge/µg total RNA)
CHC-1	C26/F	1a/1b	1.1×10^6	$\geq 10^2$
CHC-2	C33/M	3a	$\geq 10^4$	nt
CHC-3	N28/F	2b	2.4×10^6	$\geq 10^2$

[a]Serum HCV RNA load evaluated by real-time RT-PCR

[b]PBMC HCV RNA presence determined by nested RT-PCR/NAH

nt- not tested

3.3.2. *In Vitro* HCV Infection and Culture Conditions

In this study, *de novo* infection of lymphoid cells with HCV was accomplished following methodology established in my previous work (Section 2.3.3), in which alternating stimulation with PHA in the presence of IL-2 resulted in infection and detection of propagating virus (Section 2). The data from this previous study revealed a change in the T cell subset distribution in lymphocyte cultures exposed to HCV but not in those non-exposed to virus (Section 2, Fig. 2.1). In examining the cause of this phenotype shift, we observed augmented lymphocyte proliferation early after exposure to virus, i.e., at 4 d.p.i. We also found at later time points of culture, i.e., 7-10 d.p.i., a high rate of cell proliferation which was accompanied by increased cell apoptosis independent of whether HCV was detectable or not in cultured cells (data not shown). This outcome was most likely related to the repeated exposure of cells to mitogen (PHA). To minimize this effect, which was potentially masking the influence of virus on immune cell proliferation and apoptosis, we altered the cell culture protocol in the present study and we stimulated lymphoid cells with mitogen only once prior to infection without subsequent repeated restimulation. Thus, monocyte-depleted lymphoid cells from a healthy donor were treated with 5 μg/mL PHA for 48 h prior to infection (Section 2.3.3.). Following stimulation, 1×10^7 cells were exposed to 2.7×10^5 vge from CHC-1 or CHC-3 or 500 μL (~ 10^4 vge) of plasma from (CHC-2) in 9.5 mL of culture medium at a final concentration of 1×10^6 cells/mL. In parallel, the same number of target cells was exposed to 500 μL of normal healthy plasma (NHP) from 3 different healthy donors, as negative controls (mock infection). As an additional control, target cells were incubated with 9.5 mL of

culture medium without adding plasma. At the same time, for investigation of the effect of virus exposure on cell proliferation, triplicate 1-mL cultures of 1 x 10^6 PHA-stimulated, CFSE-labelled lymphocytes (methodology described in Section 3.3.4) were exposed to CHC-1, CHC-2 or CHC-3, NHP-1, NHP-2, NHP-3 or NP as indicated above. In all cases, inocula were removed after 24 hours and the cells were washed twice thoroughly prior to resuspension in 9.5 mL of complete AIM-V medium (Invitrogen), as described before (Section 2.3.3.). Culture supernatants were collected at 10 d.p.i. and stored at -80°C. Cell pellets were collected at -2, 0, 1, 7, 10 d.p.i. and cryopreserved for RNA analysis. Also, at least 2 x 10^5 lymphoid cells were collected at each time point for determination of cell surface phenotype and evaluation of proliferation and apoptosis (See below).

3.3.3. Determination of Cell Phenotype

To determine the phenotype of immune cells after *in vitro* exposure to HCV, cells cultured in the presence of NHP or medium alone (NP) and those exposed to virus were incubated with a cocktail containing an anti-CD3 antibody labelled with Alexa Fluor 488 (Alexa 488) (BD Pharmingen), an anti-CD4 antibody bound to peridinin chlorophyll protein complex (PerCP) (BD Pharmingen) and an anti-CD8 antibody conjugated to allophycocyanin (APC) (Ebiosciences, San Diego, CA) or with a control staining cocktail containing the appropriate isotype controls (Ebiosciences or BD Pharmingen) for 30 minutes at 4°C. Using forward versus side scatter, lymphocytes (gate R1) (See Fig. 3.1) were separated from debris by flow cytometric analysis using a FACSCalibur flow cytometer (Becton Dickinson). Then, lymphocytes were sub-gated on Alexa-488-

positive cells ($CD3^+$ T cells) (gate R2) to determine percentages of $CD4^+$ and $CD8^+$ reactive cells by detecting APC-positive cells ($CD8^+$) found in the upper left quadrant and PerCP-positive cells ($CD4^+$), located in the lower right quadrant (See Fig. 3.1). Quadrant markers were set up on background staining using appropriate isotype controls.

3.3.4. Flow Cytometry-Based T Cell Proliferation Assay

To quantify the level of proliferation in HCV-exposed and unexposed lymphocytes, cells were stained with carboxyfluorescein succinimidyl ester (CFSE), as previously described (Gujar and Michalak, 2005). Briefly, 2.3×10^7 PBMC suspended in 2.3 mL of PBS were stained with CFSE (Molecular Probes, Eugene, Oregon), at the pre-tested optimal concentration of 1 mM, at 37°C for 10 min in a 15-mL conical tube. Subsequently, cells were washed twice with 10 mL of 5% fetal calf serum (GIBCO-Invitrogen Corporation, Auckland, New Zealand) in PBS. To carry out triplicate infections of labelled targets with each of the three HCV inocula tested, the three NHP and the NP control, CFSE-labelled lymphocytes were resuspended in 1 mL cultures of 1×10^6 cells in complete AIM-V medium (GIBCO) supplemented with 10% FCS in 6-well plates (Becton Dickinson). As an additional control to determine the CFSE staining in unstimulated cells, CFSE-labelled PBMC cultured with AIM-V medium alone without stimulation were included. Proliferation in PBMC from triplicate wells were analyzed at -2, 0, 1, 4, 7 and 10 d.p.i. using a flow cytometer and using Cellquest Pro (Becton Dickinson) or ModFit LT (Verity Software House, Topsham, ME) analysis softwares. Using forward versus side scatter, lymphocytes (gate R1) were separated from debris. Proliferation represented by the mean percentage CFSE low in triplicate or single cultures measured at

different time points throughout the culture period. Percentage CFSE low was based on an unstimulated control with cells cultured in medium alone. For triplicate infections, cells collected at 4 d.p.i. (in triplicate) and for their singular samples collected at 6, 7 and 10 d.p.i., CFSE-labelled lymphoid cells were stained with anti-CD3-APC, anti-CD4-PerCP and anti-CD8-PE. Using forward versus side scatter, lymphocytes (gate R1) were separated from debris, gated on $CD3^+$ T cells and sub-gated on $CD4^+$ or $CD8^+$ T cells to determine the proliferation of individual T cell subpopulations. Proliferation was represented by the mean percentage CFSE low ± SEM. Mitogen unstimulated controls were routinely included, as described above. Additionally, dilution of CFSE fluorescence was analyzed using the proliferation wizard module of ModFit LT software showing daughter generations. P.I. (proliferation index) values were determined using unstimulated cells to define the parent generation.

3.3.5. Annexin V-PE-/7-AAD Assay for Detection of Cell Apoptosis

To determine the degree of apoptosis in lymphoid cells after exposure to HCV inocula, lymphoid cells were stained with annexin V conjugated with PE, or 7-aminoactinomycin D (7-AAD), as previously established (Gujar et al., in press). Briefly, 1x 10^5 cells were washed with annexin buffer containing 10 mM HEPES (Invitrogen), 5 mM NaCl, 5 mM KCl and 2 mM $CaCl_2$ and centrifuged at 1,500 rpm for 10 min at 4 °C. Then, a cocktail containing 50 µg/mL of 7-AAD (Invitrogen) and 50 µg/mL of annexin-V-PE (Invitrogen) were prepared in annexin buffer. Cells were resuspended in 100 µL of apoptosis cocktail and kept on ice for 30 min. After staining, cells were washed once with annexin buffer, centrifuged at 400 ***x g*** for 15 min and resuspended in 500 µL of annexin buffer to be

analyzed by flow cytometry, as described before (Gujar et al., 2010).

3.3.6. Quantification of Cytokine Expression

In lymphocyte cultures in which numbers of recovered cells permitted, RNA was extracted, treated with 2 U of DNase (Sigma) for 10 min at room temperature followed by incubation in a stop solution for 10 min at 70 °C. RNA was transcribed to cDNA using MMLV-RT (Invitrogen). Due to very limited amounts of RNA obtained, quantification of the level of expression of IFN-γ, TNF-α, IFN5α and IL-2 was evaluated only. This was accomplished using SYBR Green real-time RT-PCR, the Roche LightCycler (Roche Diagnostics), and amplification conditions previously established in this laboratory (Pham et al., 2007). Reactions were performed using 50 ng of cDNA in a total volume of 20 μL using: sense primer 5'-TCAGCTCTGCATCGTTTTGG and antisense primer 5'-TGTTTTAGCTGCTGGGCACA for IFN-γ, sense primer 5'-TCTTCTCGA ACCCCGAGTGA and antisense primer 5'-CCTCTGATGGC ACCACCAG for TNF-α, sense primer 5'-CAGCCTGAGTAACAGGAGGA and antisense primer 5'-GCAGATGA GTCCTTTGTGCT for IFN5α, and sense primer 5'-CCCAAGAAGGCCACAGAACT and antisense primer 5'-TGCTGATTAAGTCCCTGGGTCTTA for IL-2. As a control, actin was amplified using cDNA generated from tested samples employing sense primer 5'-ATCTCCTGCTCGAAGTCC and anti-sense primer 5'-CAACCGTGAGAAGAT GACC. Cytokine mRNA expression was normalized against actin. Specificity of amplifications was confirmed by a melting curve analysis.

3.3.7. Statistical Analysis

The data were analyzed and differences between HCV-exposed cultures and those not-exposed to virus were determined using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Statistical analyses were performed by applying the Mann-Whitney, non-parametric, two-tailed test. P values of ≤ 0.05 were considered as statistically significant.

3.4. RESULTS

3.4.1. HCV Expression in Lymphoid Cells Before and after Exposure to HCV

HCV RNA positive strand was detected in T cell cultures following exposure to all inocula tested (Table 3.2) but not in the cultures exposed to NHP or medium alone (data not shown). The level of HCV RNA detection in the cells examined varied depending on inoculum and time of cell collection following exposure to virus. Thus, HCV RNA load ranged from <50 vge/μg total RNA to >8000 vge/μg total RNA (Table 3.2). HCV RNA negative strand was detected at 7 and 10 d.p.i. for cell cultures exposed to CHC-1 inoculum that also induced the highest HCV RNA positive strand loads in lymphocytes amongst the inocula tested. It is of note that in the preliminary experiments, the HCV RNA loads detected in lymphoid cell cultures subjected to repeated mitogen stimulation were consistently greater than those found in lymphoid cells infected after the single mitogen stimulation only, as indicated above. Similarly, HCV negative (replicative) strand was more frequently indentified in the cells repeatedly mitogen stimulated during 14-day cultures after exposure to different HCV inocula (data not shown).

Table 3.2. HCV RNA Detection In Lymphocytes Exposed to HCV Positive Plasma

Inoculum	HCV RNA load (vge/ug RNA)					
	1 d.p.i.		7 d.p.i.		10 d.p.i.	
	Positive strand	Negative strand	Positive strand	Negative strand	Positive strand	Negative strand
CHC-1	8777	nt	1290	+	1010	+
CHC-2	746	nt	204	n.d.	n.d.	nt
CHC-3	<50	nt	<50	nt	<50	nt

nt - not tested
n.d.- not detected

3.4.2. Phenotype of Lymphoid Cells During Culture after Exposure to HCV

In preliminary experiments and in the study reported in Section 2, exposure of T cells to HCV inocula was followed by their culture in the presence of IL-2 under alternating mitogen stimulation. In the presence of PHA and IL-2, PBMC cultures that were originally 45 % CD3-positive became greater than 95% CD3-positive T cells after 14 days of culture after exposure to virus. Interestingly, at the end of culture period, $CD8^+$ T cells became evidently more prevalent than $CD4^+$ T cells in the above cultures (See Fig. 2.1). Similarly, in this study, PHA-stimulation prior to HCV exposure leads to an enrichment in CD3-positive T cells during culture (Fig. 3.1B). Exposure to 2 of 3 HCV inocula tested, i.e., CHC-1 and CHC-2, and culture without repeated mitogen stimulation resulted in an altered ratio of CD4 to CD8-positive T cells in that $CD8^+$ T cells became more prevalent (Figs. 3.1A and 3.1B). As also observed in the preliminary experiment, Figure 3.1A illustrates that the enrichment in $CD8^+$ T cells was evident as early as 4 d.p.i. in cells exposed to CHC-1 where the ratio of $CD4^+$ T cells to $CD8^+$ T cells was approximately 2:3. In the remaining cultures exposed to HCV inocula, NHP or medium alone, the ratio was close to 2.5:2 at 4 d.p.i. At 7 d.p.i., a change in the T cell subset distribution also appeared in cells inoculated with CHC-2. At this time point, the $CD4^+$ to $CD8^+$ T cell ratio for CHC-1 was 2:3 and for CHC-2 was 2.5:2.3, while for CHC-3, NHP-1, NHP-2, NHP-3 and medium alone, the ratio was close to 3:2. At 10 d.p.i., the $CD4^+$ to $CD8^+$ ratio for T cells exposed to CHC-1 and CHC-2 were 2.8:2.1 and 2.7: 2.2,

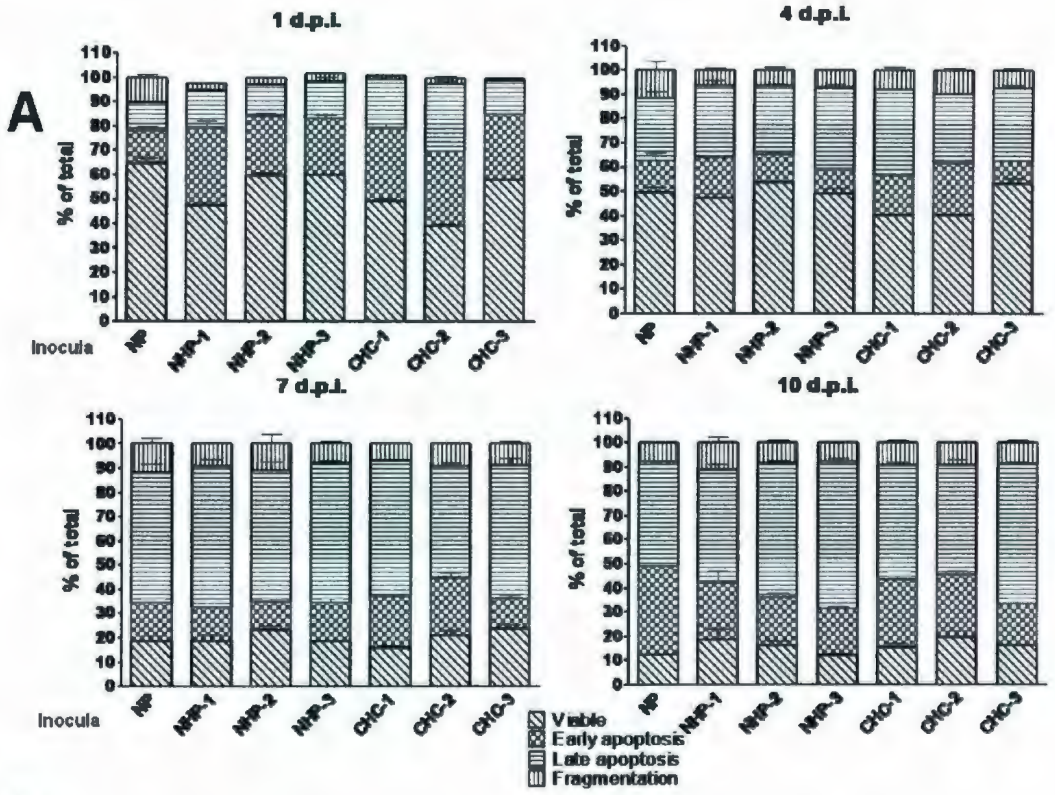
Figure. 3.1. Phenotypic characterization of cultured lymphocytes after exposure to HCV. Lymphoid cells from the same healthy donor were either exposed to medium alone (no plasma), (NP), 3 normal human plasma (NHP 1-3) or HCV inocula (CHC 1-3). Cells collected throughout the culture period at the time points indicated (d.p.i.) were stained with anti-CD3 Alexa-488, anti-CD4–PerCP and anti-CD8-APC mAbs (Section 3.3.1.), or with appropriate immunoglobulin isotype controls, and analysed by flow cytometry. (A) Graphical representation of flow cytometry data showing phenotype of T cells for each infection condition tested. $CD4^+$ T cells (solid black bars) and $CD8^+$ T cells (hatched bars) displayed as percentage of total $CD3^+$ T cells at 4 different time points throughout the culture period. (i.e., 1, 4, 7 and 10 d.p.i.). The detection of HCV RNA positive strand was shown for 1, 7 and 10 d.p.i. For HCV RNA positive strand detection, ++ indicates >1000 vge/ µg total RNA, + indicates between 50 and 1000 vge/µg total RNA, and +/-indicates <50 vge/µg total RNA. For negative strand detection, + indicates detection of the strand while – indicates no detection. Grey boxes indicate cultures in which the $CD4^+$ to $CD8^+$ ratio was altered when compared to cultures exposed to NP or NHP, n.a.- Not applicable, n.t.- not tested. (B) Determination of T cell phenotype in cell cultures exposed or not to HCV and cultured for 10 days post-exposure. Using forward versus side scatter, lymphocytes (gate R1) were separated from cellular debris. Lymphocytes were then sub-gated on Alexa-488 positive CD3 T cells (gate R2) for enumeration of $CD4^+$ and $CD8^+$ T cells by detecting APC positive $CD8^+$ T cells found in the upper left (UL) quadrant and PerCP-positive $CD4^+$ T cells in the lower right (LR) quadrant. Quadrant markers were set using cells exposed to the appropriate isotype controls. Numbers in the UL and LR quadrants indicate percentages of positive cells.

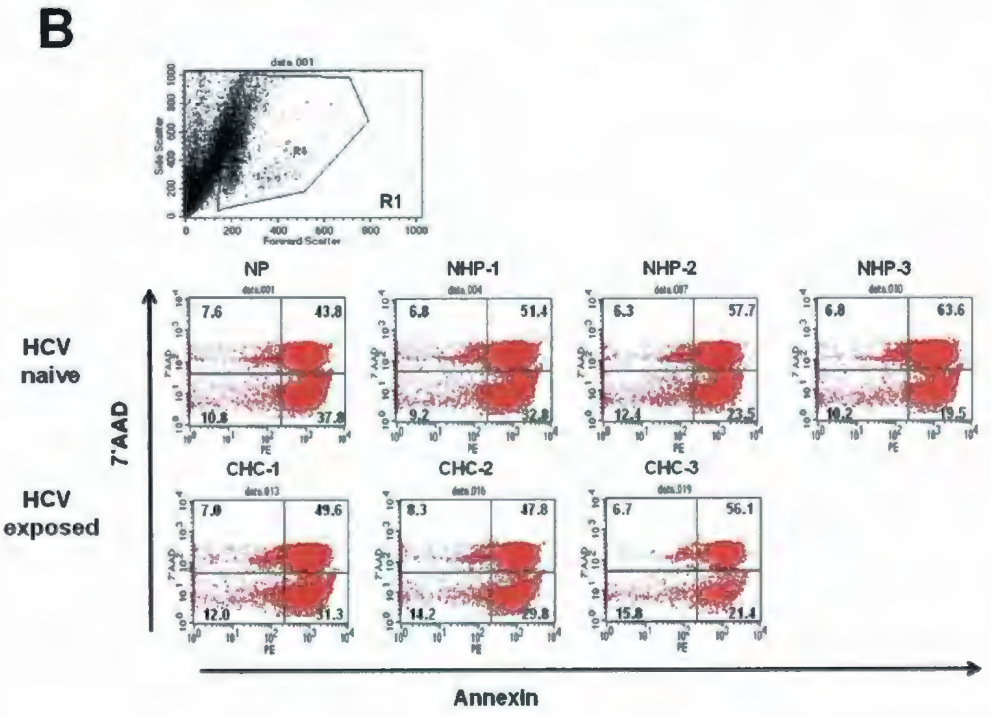
respectively, while the cultures not exposed to HCV, i.e., NP, NHP-1, NHP-2 and NHP-3, as well as those exposed to CHC-3 had very similar $CD4^+$ to $CD8^+$ ratios of approximately 3.5:1 (Fig. 3.1A). Finally, exposure of T cells to CHC-3 inoculum was found to have little effect on T cell phenotype. Interestingly, infection with this inoculum resulted in a detectable, but unquantifiable, level of HCV RNA positive strand throughout the culture period and no detection of HCV RNA negative strand (Table 3.2.). This finding may suggest that the level of HCV RNA expression in T cells was related to the observed change in T lymphocyte subset distribution.

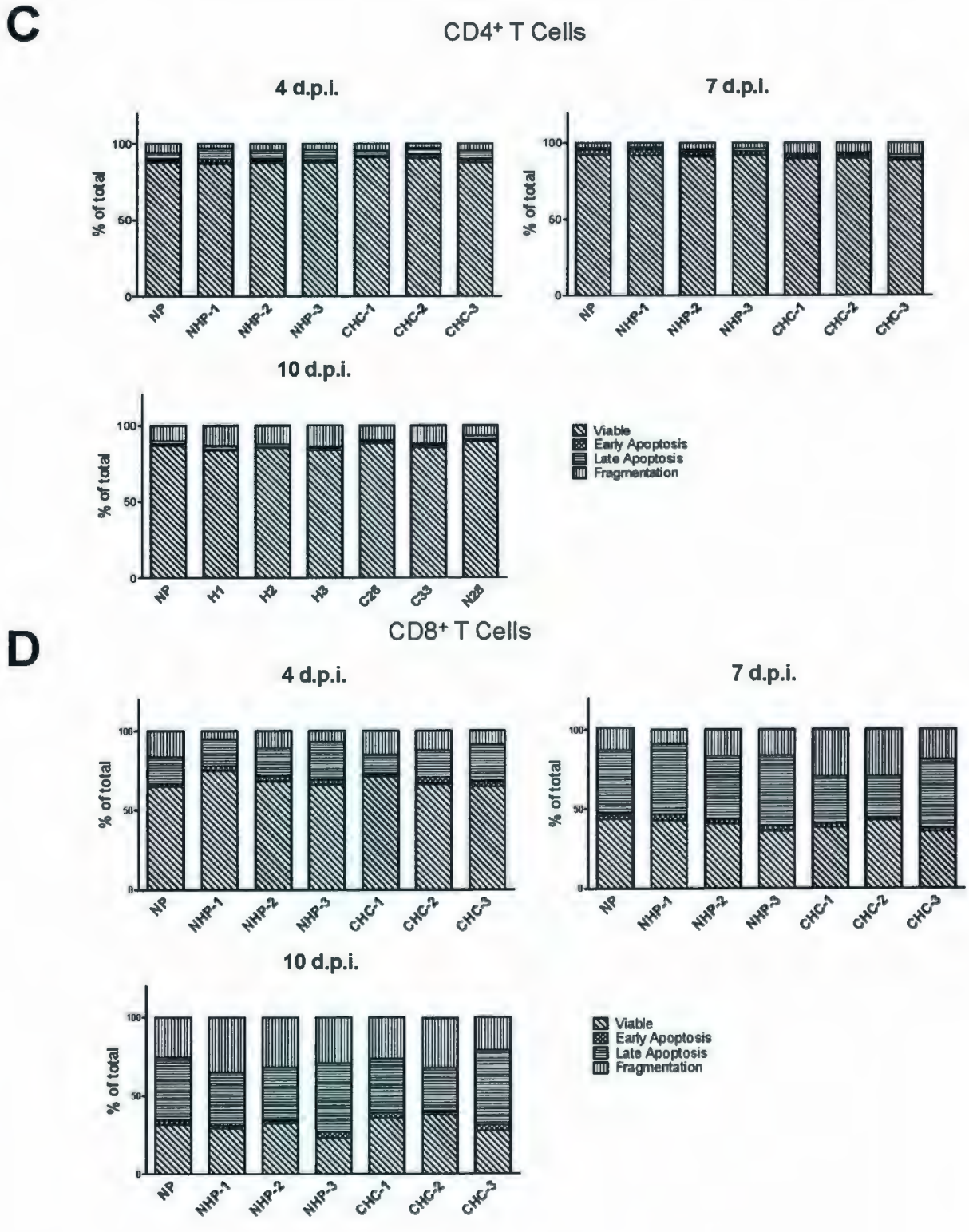
3.4.3. Apoptosis of Lymphoid Cells after Exposure to HCV and During Culture

To determine whether the observed apparent enrichment in $CD8^+$ T cells following exposure to HCV was due to enhanced cell apoptosis of $CD4^+$ T cells, we examined the degree of lymphoid cell death in triplicate cultures after exposure to a given HCV inoculum or to NHP. As seen in Figure 3.2., at all time points examined, the degree of apoptosis was similar regardless of whether the lymphoid cells were cultured in the presence of medium alone, NHP or wild-type HCV (Figs. 3.2A. and 3.2B.). Thus, under the conditions tested, HCV exposure did not appear to enhance apoptosis of T cells in culture. To further examine whether the alteration in phenotype may result from selective apoptosis of a certain T cell subset, we also examined the extent of death of $CD4^+$ and $CD8^+$ T cells separately. We observed that the degree of apoptosis of $CD4^+$ and $CD8^+$ T cells was not altered following exposure to HCV, as shown in Figs. 3.2C. and 3.2D., respectively.

Figure. 3.2. Determination of degree of lymphoid cell apoptosis by annexin-PE/7-AAD flow cytometry assay. (A) Graphical representation of degree of apoptosis detected in PHA treated lymphoid cell cultures exposed to NP, NHP 1-3 or CHC 1-3. Bars indicate the percentage of total cells in each phase of apoptosis. Graphs show data from triplicate experiments and are shown as mean ± SEM at 1, 4, 7 and 10 d.p.i. Bars with diagonal lines represent viable, checkered bars, early apoptotic, bars with horizontal lines, late apoptotic and vertical lines, fragmented cells. (B) Flow cytometric determination of stage of apoptosis and percentage of cells affected at 10 d.p.i following staining with annexin-PE and 7-AAD. Each quadrant represents a different phase of apoptosis. Lower left (LL)- viable cells, LR- early apoptotic cells, Upper right (UR)- late apoptotic and UL- fragmented cells. (C and D) Graphical representation of degree of apoptosis detected in lymphoid cell cultures exposed to NP, NHP or HCV inocula following subgating on $CD4^+$ T cells (C) or $CD8^+$ T cells (D). Bars indicate the percentage of total cells in each phase of apoptosis. Graphs show data from single experiments at 4, 7 and 10 d.p.i.


A
1 d.p.i.
4 d.p.i.
7 d.p.i.
10 d.p.i.
% of total
Inocula
NP
NHP-1
NHP-2
NHP-3
CHC-1
CHC-2
CHC-3
Viable
Early apoptosis
Late apoptosis
Fragmentation


B
R1
NP
NHP-1
NHP-2
NHP-3
HCV naive
CHC-1
CHC-2
CHC-3
HCV exposed
7'AAD
Annexin
7.6
43.8
10.8
37.8
6.8
51.4
9.2
32.8
6.3
57.7
12.4
23.5
6.8
63.6
10.2
19.5
7.9
49.6
12.0
31.3
8.3
47.8
14.2
29.8
6.7
56.1
15.8
21.4

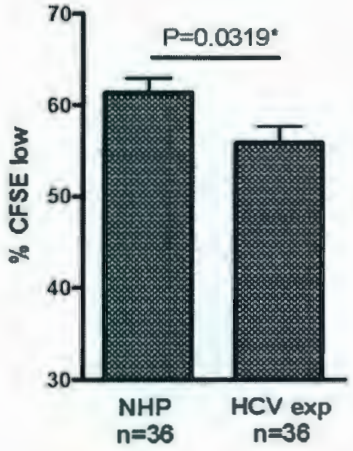

C
CD4+ T Cells
4 d.p.i.
7 d.p.i.
10 d.p.i.
% of total
100
50
0
NP
NHP-1
NHP-2
NHP-3
CHC-1
CHC-2
CHC-3
H1
H2
H3
C26
C33
N28
Viable
Early Apoptosis
Late Apoptosis
Fragmentation
D
CD8+ T Cells
4 d.p.i.
7 d.p.i.
10 d.p.i.
% of total
Viable
Early Apoptosis
Late Apoptosis
Fragmentation

3.4.4. Proliferation of Lymphoid Cells after *In Vitro* Exposure to HCV

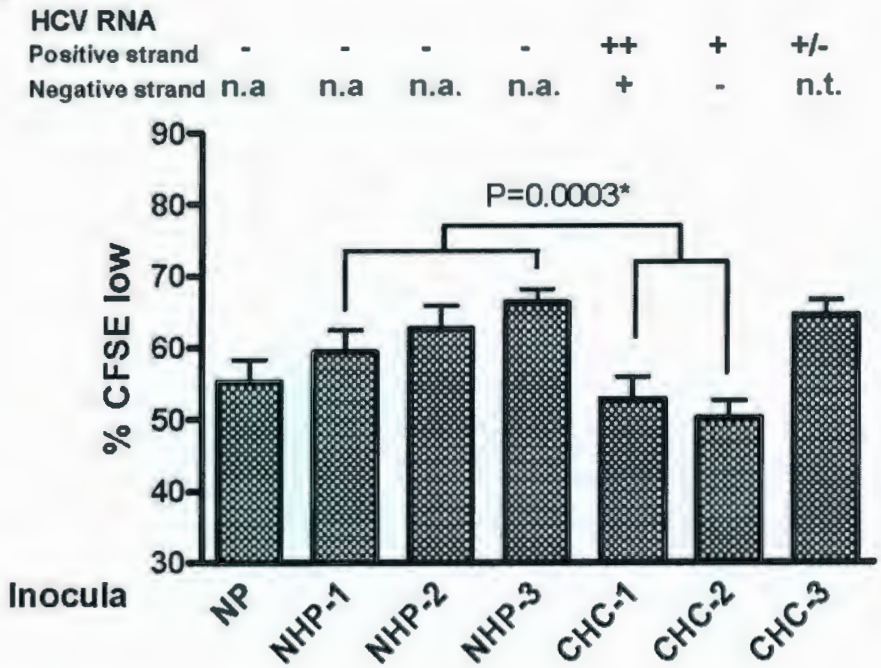
To assess the degree of proliferation of T cells after exposure to HCV or to control NHP, triplicate cell cultures were evaluated for proliferation using the flow cytometry-based CFSE proliferation assay. In preliminary experiments using repeatedly mitogen stimulated T cell cultures, HCV exposure led to inhibited T cell proliferation at 4 d.p.i (data not shown). At later time points, however, very high rates of proliferation in T cell cultures which were exposed or not to HCV were observed. This likely reflected a T cell response to repeated PHA stimulation, which is known to be a potent T cell stimulating mitogen (data not shown). Therefore, in the current study, we stimulated cells with mitogen prior to infection only and cultured for the remainder of the time in medium without additional PHA or IL-2. Elimination of repeated mitogen stimulation resulted in a significant decrease in total lymphoid cell proliferation both in the cultures exposed or not to HCV (Fig. 3.3A). Upon further examination of cultures exposed to individual HCV isolates, it was found that inhibition of T cell proliferation occurred after exposure to 2 of 3 inocula tested, i.e., CHC-1 and CHC-2 (Figs. 3.3B and 3.3C). Interestingly, exposure of cells to the CHC-3 inoculum did not induce inhibition of T cell proliferation. These cells, as indicated before, expressed very low levels of HCV RNA positive strand while HCV RNA negative strand was not detected. This may suggest that the level of HCV replication within T cells could influence the degree of inhibition of the cell proliferation.

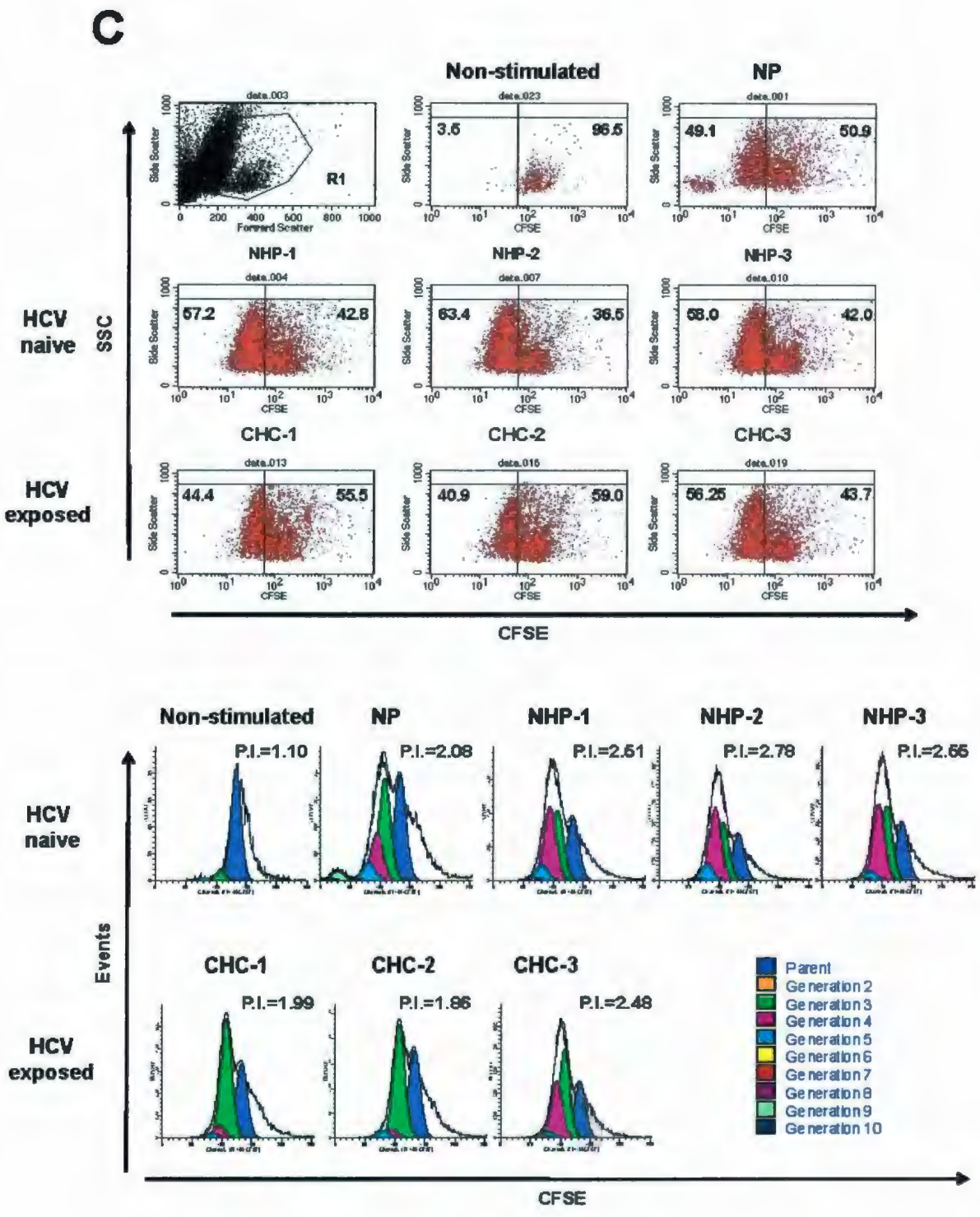
Figure. 3.3. Measurement of lymphoid cell proliferation after exposure to HCV or control normal human plasma. Lymphoid cells from the same healthy donor were stained with 1μM CFSE prior to PHA stimulation and infection, and proliferation was measured by flow cytometry. (A) Summary of proliferation in lymphoid cells exposed to NP, NHP 1-3 or CHC 1-3. Proliferation represented by the mean percentage CFSE low cells was measured in triplicate cultures at 4 time points throughout the culture period, percentage CFSE low was based on setting quadrant markers on an unstimulated control cultured with medium alone. (B) Graphical representation of lymphoid cell proliferation after exposure to HCV or NHP inocula. Lymphoid cells were either exposed to NP, NHP 1-3 or CHC 1-3. Proliferation was measured by flow cytometry and percentage CFSE low was determined based on unstimulated control cells cultured with medium alone. Proliferation represented by the mean percentage CFSE low and SEM measured in triplicate at 1, 4, 7 and 10 d.p.i. HCV RNA positive and negative strand detection are shown above proliferation (n.a.- Not applicable, n.t.- not tested). (C) As an example, Flow cytometric determination of proliferation at 7 d.p.i. for lymphoid cells exposed to NP, NHP 1-3 or CHC 1-3 using dot plots and Modfit analysis. Using forward versus side scatter, lymphocytes (gate R1) were separated from debris. Percentage CFSE low was determined based on an unstimulated control cells cultured with medium alone. Lower left and lower right quadrants represent cells with CFSE high (no proliferation) and CFSE low (ongoing proliferation) reactivity, respectively. Dilution of CFSE fluorescence was analyzed with ModFit LT showing daughter generations. P.I. values were determined by using unstimulated cells to define parent generation.

A


70
60
50
40
30
% CFSE low
P=0.0319*
NHP
n=36
HCV exp
n=36

B


HCV RNA
Positive strand - - - - ++ + +/-
Negative strand n.a n.a n.a. n.a. + - n.t.
90
80
70
60
50
40
30
% CFSE low
P=0.0003*
Inocula
NP
NHP-1
NHP-2
NHP-3
CHC-1
CHC-2
CHC-3


C
Non-stimulated
NP
NHP-1
NHP-2
NHP-3
CHC-1
CHC-2
CHC-3
HCV naive
HCV exposed
SSC
CFSE
R1
3.6
96.5
49.1
50.9
57.2
42.8
63.4
36.5
58.0
42.0
44.4
55.5
40.9
59.0
56.25
43.7
P.I.=1.10
P.I.=2.08
P.I.=2.51
P.I.=2.78
P.I.=2.65
P.I.=1.99
P.I.=1.86
P.I.=2.48
Events
Parent
Generation 2
Generation 3
Generation 4
Generation 5
Generation 6
Generation 7
Generation 8
Generation 9
Generation 10

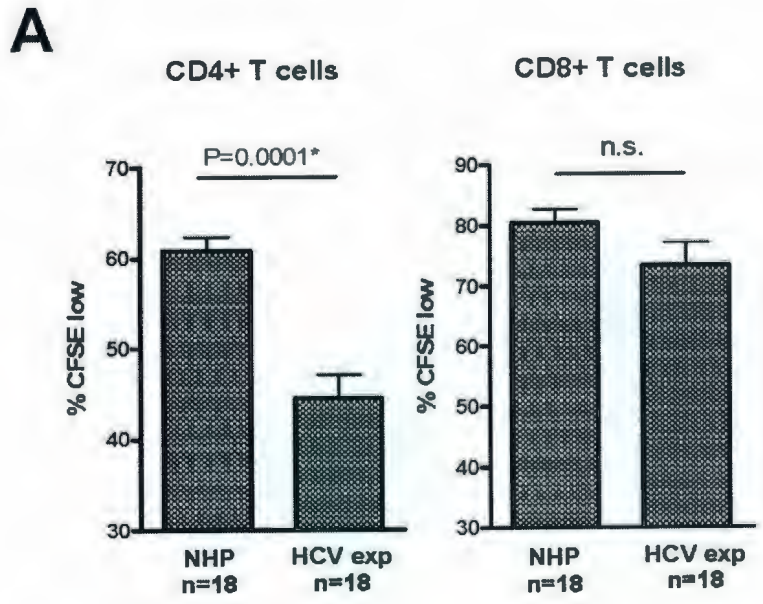
3.4.5. Proliferation of T Cell Subsets in Cultures after Exposure to HCV

To evaluate whether an inhibition in the proliferation of particular T cell subset may be responsible for the observed phenotype shift, we measured the proliferation of $CD4^+$ and $CD8^+$ T cells separately. When comparing HCV-exposed cultures to those exposed to NHP, we saw a significant decrease in $CD4^+$ T cell proliferation rate in cells exposed to HCV, while the proliferation of $CD8^+$ T cells was not affected regardless of whether the cells were exposed or not to HCV (Fig. 3.4A). Upon further evaluation of the level of proliferation of T cell subsets exposed to individual HCV inocula, it was evident that CHC-1 and CHC-2 plasma caused a significant decrease in the levels of $CD4^+$ T cell proliferation compared to those exposed to NHP (Fig. 3.4B). Interestingly, CHC-3 inoculum did not illicit a similar effect. Overall, no significant differences in $CD8^+$ T cell proliferation was seen in cells treated with HCV inocula compared to those exposed to NHP (Fig. 3.4B).

3.4.6. Expression of Cytokines in Cultures Exposed to HCV

To examine the effects of HCV on cytokine expression in lymphoid cells, we investigated the levels of IFN-γ, TNF-α, IFN-α5 and IL-2 mRNA in the cells exposed to three HCV inocula tested and three NHP. Due to limited RNA availability evaluation of all 4 cytokines in all cell cultures were not always possible. In this preliminary evaluation of cells stimulated with PHA prior to infection, there was an upregulation of IFN-γ mRNA during the 48-hour

Figure. 3.4. Evaluation of $CD4^+$ and $CD8^+$ T cell proliferation after HCV exposure. Lymphoid cells from the same healthy donor were stained with CFSE,PHA-stimulated and infected as described in Materials and Methods to measure proliferation of $CD4^+$ and $CD8^+$ T cell subsets. CFSE-labeled lymphoid cells were triple stained with anti-CD3-APC, anti-CD4- PercP and anti-CD8-PE, gated on CD3-positive T cells and subgated on $CD4^+$ or $CD8^+$ T cells. Proliferation was determined by flow cytometry. (A) Graphical representation of proliferation of T cell subsets after lymphoid cell exposure to three different NHP or HCV inocula. Proliferation represented by the mean percentage CFSE low ± SEM was measured at four different time points i.e., 4 d.p.i., in triplicate, and 6, 7 and 10 d.p.i. (B) Determination of the rate of proliferation in T cell subsets after exposure to different inocula. Lymphoid cells from the same healthy donor were CFSE stained prior to PHA stimulation and infection were exposed to NP, NHP 1-3 or CHC 1-3. Cells were stained as above, gated on CD3-positive T cells and sub-gated on CD4-positive and CD8-positive T cells. Proliferation was determined by flow cytometry and represented by the mean percentage CFSE low ± SEM measured at 3 different time points during the 10-day culture. HCV RNA positive and negative strand detection displayed above proliferation and is represented as described in the legend for Figure 3.1.


A
CD4+ T cells
CD8+ T cells
P=0.0001*
n.s.
% CFSE low
70
60
50
40
30
90
80
70
60
50
40
30
NHP
n=18
HCV exp
n=18
NHP
n=18
HCV exp
n=18

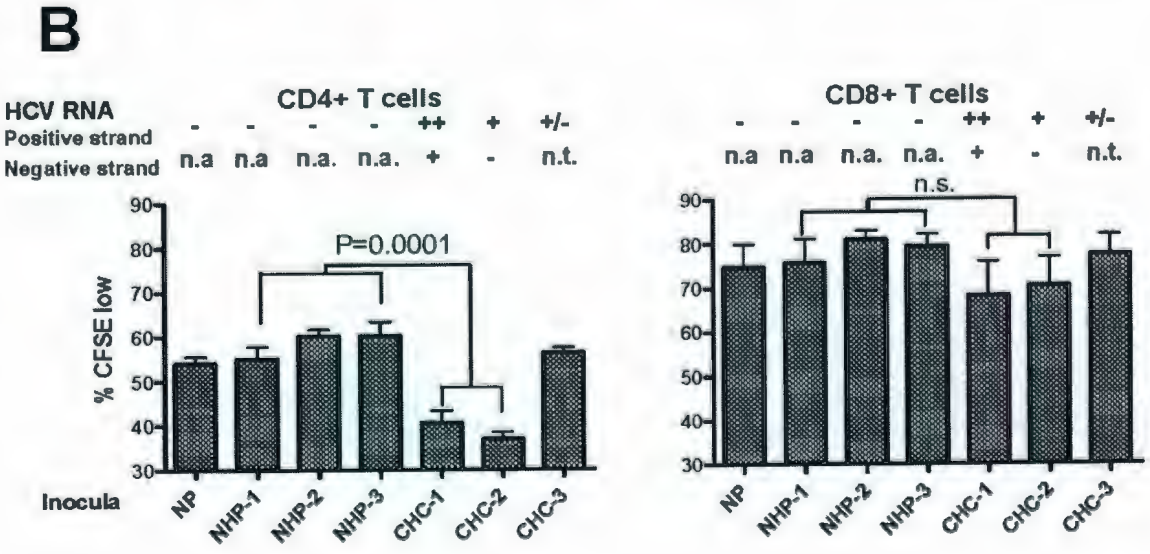
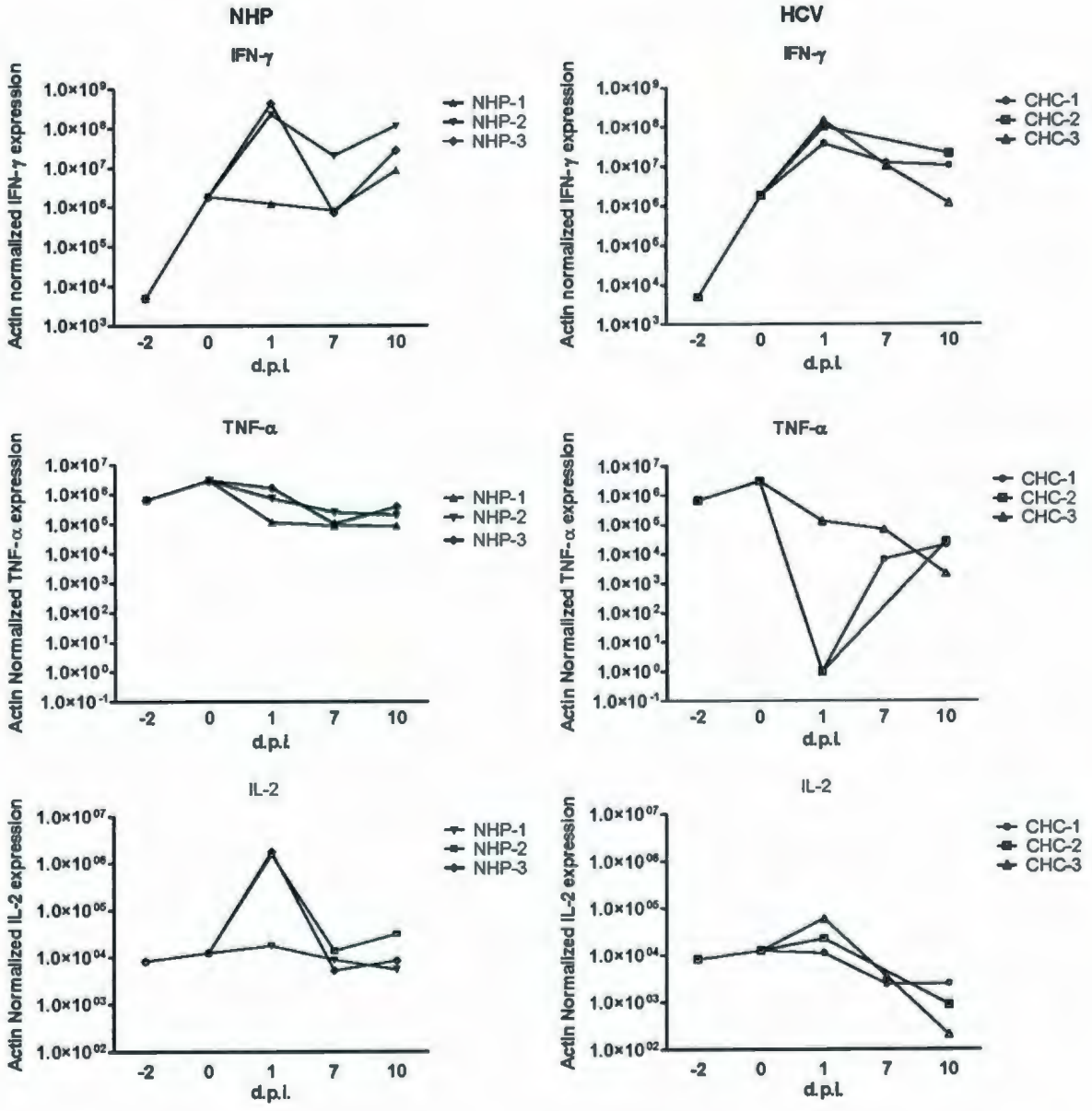

B
HCV RNA
Positive strand
Negative strand
CD4+ T cells
CD8+ T cells
- - - - ++ + +/-
n.a n.a n.a. n.a. + - n.t.
P=0.0001
n.s.
% CFSE low
90
80
70
60
50
40
30
Inocula
NP
NHP-1
NHP-2
NHP-3
CHC-1
CHC-2
CHC-3

Figure. 3.5. Expression levels of IFN-γ, TNF-α and IL-2 mRNA in T lymphocytes exposed to HCV-positive or normal human plasma. Lymphoid cells from the same healthy donor were treated with PHA for 48hr and exposed either to normal human plasma (n=3; NHP-1 to 3) or 3 different HCV inocula (CHC-1 to 3). DNase-treated RNAwas transcribed and amplified by real-time PCR using cytokine specific primers. IFN-γ, TNF-α and IL-2 expression was evaluated at 2 days prior to infection (-2) and at 0, 1, 7 and 10 d.p.i. Specificity of amplifications was confirmed by a melting curve analysis. Recombinant actin gene fragment was amplified in parallel and used as a loading control. Cytokine mRNA expression was normalized against actin. Contamination controls included water amplified instead of cDNA.


NHP
IFN-γ
Actin normalized IFN-γ expression
1.0×10⁹
1.0×10⁸
1.0×10⁷
1.0×10⁶
1.0×10⁵
1.0×10⁴
1.0×10³
-2
0
1
7
10
d.p.i.
NHP-1
NHP-2
NHP-3
HCV
IFN-γ
Actin normalized IFN-γ expression
CHC-1
CHC-2
CHC-3
TNF-α
Actin Normalized TNF-α expression
1.0×10⁷
1.0×10⁶
1.0×10⁵
1.0×10⁴
1.0×10³
1.0×10²
1.0×10¹
1.0×10⁰
1.0×10⁻¹
TNF-α
IL-2
Actin Normalized IL-2 expression
1.0×10⁰⁷
1.0×10⁰⁶
1.0×10⁰⁵
1.0×10⁰⁴
1.0×10⁰³
1.0×10⁰²
IL-2

stimulation period (Fig. 3.5). After PHA stimulation and until 1 d.p.i., IFN-γ induction was evident in cells exposed to NHP-2, NHP-3 or CHC-1-3. However, after this time point, no differences in expression were seen between cells exposed or not to virus. In the case of TNF-α, it was interesting that the expression of this cytokine was not detected at 1 d.p.i. in cultures exposed to CHC-1 and CHC-2 (Fig. 3.5). While the preliminary data indicated that there was no significant change in TNF-α expression between cells exposed or not to HCV, the two cultures which lacked TNF-α mRNA were also the cultures with the greatest level of HCV RNA positive strand detection throughout the culture period. In cells exposed to HCV inocula as well as those exposed to NHP, IFN-α5 expression was not detected by our otherwise sensitive real-time RT-PCR assay (sensitivity of 200 copies/50μg total). In terms of IL-2 mRNA levels, there appeared to be an upregulation in the IL-2 expression at 1 d.p.i. (i.e., 72 hours after PHA stimulation) in NHP-1 and NHP-2, although again there were no significant differences between cultures that were or were not exposed to HCV (Fig. 3.5).

3.5. DISCUSSION

In this study, we examined the effects of HCV-exposure on the T cell subset distribution, proliferative capacity and apoptosis of primary T lymphocytes during a prolonged culture period. Evident shifts were found in regard to the T cell phenotype in the cultures exposed to two (CHC-1 and CHC-2) of three inocula tested. This change was characterized by a shift from the predominantly CD4-positive T cell phenotype prior to HCV exposure to progressive enrichment of CD8-positive T cells during the 10 d.p.i. culture period. In both cases, this $CD8^+$ T cell enrichment was accompanied by a

significant reduction in $CD4^+$ T cell proliferation in HCV-positive cultures compared to those exposed to NHP or being HCV non-reactive. This effect appeared to be related to the level of HCV RNA positive strand detected in cells exposed, considering the fact that the inocula (CHC-1 and CHC-2), that led to the greatest changes in the $CD4^+$ to $CD8^+$ ratio, were the cultures that had readily quantifiable cellular levels of HCV RNA (Table 3.2.). Furthermore, in lymphoid cells exposed to CHC-1 inoculum, in which HCV RNA negative strand was detected at 7 and 10 d.p.i., indicating the highest level of HCV replication amongst the cultures examined, the inhibition in $CD4^+$ T cell proliferation appeared earlier (i.e., 4 d.p.i.), suggesting that the effect may be mediated by the presence of active viral replication. In the case of lymphoid cells exposed to CHC-2 inoculum, T cell phenotypic and proliferative changes were observed in the absence of detection of HCV replicative intermediates. It is almost certain that viral replication was taking place, however at the level that was below the limit of detection of the negative strand detection assay. It is of note, that the assay sensitivity for HCV RNA negative strand detection is approximately 100-fold less sensitive than our RT-PCR/NAH assay routinely used for HCV RNA positive strand identification and that the RNA negative strand occurs in infected lymphoid cells at quantities 100-fold lower levels than the viral RNA positive strand (Pham et al., 2004).

In cultures exposed to CHC-3 inoculum, the level of HCV RNA positive strand detected throughout the culture period was unquantifiable by a real time RT-PCR assay with sensitivity of 50 vge/μg total RNA. This indicates that this inoculum did not induce infection. Interestingly, there were also no changes in proliferation rate or the distribution T cell subsets in comparison to cells exposed to NHP. We have previously

observed that not all HCV inocula are able to infect pre-stimulated lymphoid cell targets from the same donors (see Section 2.4.2) (MacParland et al., 2009). The factors that determine infectivity of wild-type HCV in immune cells have yet to be recognized and they are under investigation in this laboratory.

In early experiments leading to this study, alternating stimulation with PHA and IL-2 was employed to upregulate lymphoid cell susceptibility to HCV infection and replication of virus during culture (Section 2.3.3). In the current work, stimulation with PHA after the initial 48 hr treatment was removed to prevent excessive cell activation that may mask the pro- or anti-proliferative effects of virus. The removal of this repeated mitogen stimulation resulted in a decreased viral replication in cells exposed to HCV inocula with reduced detection of both HCV RNA positive and negative strands (data not shown). This finding is consistent with previous observations in regard to HCV replication in *in vitro* cultures (Pham et al., 2005) and with findings from *in vitro* culture of other viruses (Chatterjee et al., 1985; Hyypia et al., 1985; Braun and Kirchner, 1986).

Previously, it has been shown that HCV-specific $CD4^+$ T cell responses in individuals chronically infected with HCV are poor or absent (Gerlach et al., 1999). In HCV-infected patients who transiently control viremia and have fluctuating plasma viral loads, including periods of HCV RNA negativity as determined by clinical assays of relatively low sensitivity, this apparent viral control is accompanied by increased virus-specific $CD4^+$ T cell response (Gerlach et al., 1999). In patients with CHC, T cells have been found to be impaired in the production of IFN-γ and IL-2 (Semmo et al., 2007). As well, studies investigating $CD4^+$ T cell function during a symptomatic persistent HCV infection have shown a significant loss of IL-2 secreting cells compared to individuals

who spontaneously resolved viremia, and weak HCV-specific IFN-γ production by $CD4^+$ T cells upon stimulation (Semmo et al., 2005). In the later study, HCV-specific IFN-γ production by $CD4^+$ T cells was rescued after *in vitro* culture with IL-2, but the effect of IL-2 on the proliferative capacity of $CD4^+$ T cells was not measured. These findings may suggest that the proliferative impairment in $CD4^+$ T cell observed in our study could be due to the impaired IL-2 secretion leading to decreased T cell proliferative capacity. Unfortunately, due to limitations in cell numbers, we were not able to perform evaluations by ELISA or ELISpot to examine if there was indeed impaired IFN-γ and IL-2 cytokine production by $CD4^+$ T cells. Preliminary data obtained in this study indicate that there were no significant differences between T cells exposed or not to HCV in terms of IFN-γ, TNF-α and IL-2 mRNA expression. On the other hand, it is possible the levels of HCV replication in the T cell cultures not subjected to repeated mitogen stimulation may not have been sufficiently high to induce detectable changed in transcription of these cytokines. Further investigations should be carried out to evaluate molecular gene expression as well as the level of display of cytokines and their receptors in the T cells exposed to HCV *in vitro*.

From our findings, we can postulate that HCV infection of T cells *in vitro* can lead to a cell phenotype shift characterized by an apparent enrichment of $CD8^+$ T cells, which is due to a relative decrease in proliferation of $CD4^+$ T cells. This proliferative effect appears to be enhanced in the presence of increased HCV replication. Furthermore, HCV exposure does not appear to augment apoptotic death of lymphocytes *in vitro*. It needs to be determined whether primary T cells derived from patients with CHC display similar characteristics *ex vivo*.

In summary, the results of this study provide further evidence that HCV infection may exert direct effects on the behaviour and function of T cells and raises the possibility that HCV may not need to be actively replicating in these cells to cause such effects.

Chapter Four: Hepatitis C Virus Persisting after Clinically Apparent Sustained Virological Response to Antiviral Therapy Retains Infectivity *In Vitro*

This study has been published in Hepatology 2009; volume 49 pp.1431-1441.

4.1. SUMMARY

Hepatitis C virus can persist in the liver, lymphoid cells and serum of individuals with apparent complete spontaneous or therapy-induced resolution of hepatitis C and can replicate *in vivo* and *in vitro* in human T cells. The current study aimed to assess the infectivity of HCV persisting at very low levels using the previously established HCV infection system in human T cells. Naïve lymphoid cells were exposed to plasma and/or supernatants from cultured PBMC from 9 individuals with apparent SVR after completion of antiviral therapy. Exposed cells were analyzed for HCV RNA positive and negative strands and, in selected cases, for HCV NS5A protein, appearance of HCV variants, and release of virions by immunoelectron microscopy. The results showed that 11 of the 12 cultures established became HCV RNA positive strand reactive, while 4 also expressed the virus replicative strand. NS5A protein was detected in the *de novo* infected cells and clonal sequencing revealed HCV variants not found in inocula. IEM demonstrated enveloped HCV particles in plasma used as inocula and in culture supernatant from T cells exposed to that plasma. Overall, HCV carried in 3 of the 9 individuals studied elicited productive infection *in vitro*. Conclusion: HCV persisting at

very low levels long after therapy-induced resolution of chronic hepatitis C can remain infectious. The retained biological competence of the virus might have implications with respect to the mechanisms of its persistence and epidemiology of HCV infection.

4.2. INTRODUCTION

Hepatitis C virus is a single-stranded RNA virus that chronically infects approximately 170 million people worldwide. Up to 85% of the infected individuals may CHC. HCV is infectious even at trace amounts, with approximately 20 virus copies capable of transmitting infection in chimpanzees (Katayama et al., 2004b). Recently, introduction of nucleic acid amplification assays detecting HCV genomes with enhanced sensitivity, reaching in our laboratory <10 vge/mL or <2 IU/mL, revealed that HCV can persist at low levels in individuals with apparently complete resolution of hepatitis C either spontaneously or due to antiviral therapy (Pham et al., 2004; Radkowski et al., 2005a; Castillo et al., 2006; Laskus et al., 2007; Pham et al., 2007; Pham et al., 2008). In general, occult HCV infection is considered when small quantities of HCV RNA are identifiable in serum (usually below 100 vge/mL), PBMC and/or liver in individuals who are repeatedly serum HCV RNA nonreactive by clinical laboratory tests with sensitivities ranging between 52 and 1000 vge or 10 to 615 IU per mL, and have no clinical or biochemical evidence of liver disease (Pham et al., 2004; Radkowski et al., 2005a; Laskus et al., 2007; Pham et al., 2007; Pham et al., 2008). In this silent form of HCV infection, detection of HCV RNA replicative (negative) strand is not uncommon, particularly when *ex vivo* activated PBMC are tested by sensitive HCV RNA negative strand-specific reverse transcription-nested polymerase chain reaction combined with

nucleic acid hybridization analysis of the resulting amplicons (Pham et al., 2004; Pham et al., 2005).

While originally thought to be strictly hepatotropic, HCV has been shown in numerous studies to also invade and replicate in cells of the immune system (MacParland et al., 2006; Pal et al., 2006; Blackard et al., 2006). In our recent work, conclusive evidence of HCV replication in $CD4^+$ and $CD8^+$ T lymphocytes, B cells and monocytes was presented (Pham et al., 2008). It has also been shown that the same immune cell subsets can be infected in both CHC and persistent low-level HCV infection continuing after resolution of CHC (Pham et al., 2008). Further, primary T lymphocytes from healthy individuals have been found to be susceptible to HCV infection *in vitro* (Section 2). Along this line, mathematical modeling has independently predicted that HCV originating from extrahepatic reservoirs, possibly the immune system, comprises approximately 3% of the circulating virus pool in CHC (Dahari et al., 2005). However, analysis of HCV quasispecies, which is the swarm of genetically related viral variants that circulate in HCV-infected individual, occurring in the liver, plasma, PBMC and lymphoid tissue of a patient with end-stage liver disease awaiting liver transplant demonstrated that extrahepatic variants may constitute more than 50% of those occurring in serum (Pal et al., 2006).

The clinical relevance of low-level HCV carriage, including its potential ability to transmit infection, is yet to be determined. Nonetheless, HCV reactivation has been reported in patients with HCV RNA clearance confirmed by standard clinical laboratory assays prior to liver transplantation (Thomas et al., 2003; Forns et al., 2003). However, the opposite has also been described, albeit no details were given regarding assay

sensitivity and the quantity of template analyzed (Everson et al., 2005). Also, HCV RNA was identified in anti-HCV reactive patients receiving an HCV-negative bone marrow (Zekri et al., 2004) or kidney transplants (Melon et al., 2005). Taken together, a possibility exists that occult HCV infection could have both pathogenic and epidemiological importance.

At present, a sustained virological response is defined as serum HCV RNA negativity by clinical laboratory assays for at least 6 months after completion of antiviral therapy. However, given that the identification of low-level (occult) HCV infection was only made possible by employing research tests of a much greater sensitivity than that of those applied for clinical use, it is not surprising that low levels of HCV RNA are frequently escaping detection, giving conflicting results on the occurrence and infectivity of HCV persisting at trace levels. Implementation of assays detecting HCV RNA with a greater sensitivity (preferably <10 vge/mL) for clinical and population-based testing should meaningfully contribute to the identification of the scope of potential problems associated with low-level HCV infection (Michalak et al., 2007).

An HCV cell culture system allowing for authentic propagation of wild-type HCV in primary human T cells has previously been established in this laboratory (Section 2). In the current study, this system was employed to assess potential infectivity of HCV persisting at trace quantities for years in patients who achieved SVR after completion of IFN-α therapy with or without ribavirin. Our investigation has focussed on randomly selected cases which, although repeatedly serum negative by the clinical test, were found by RT-PCR/NAH to be positive for HCV RNA in sera and in PBMC after their *ex vivo* stimulation (Pham et al., 2004). We uncovered that the residual virus carried by some of

the individuals has the capacity to *de novo* infect and propagate in T cells, strongly arguing that the virus persisting as occult infection can retain its biological competence and, thus, be potentially infectious.

4.3. METHODS

4.3.1. HCV Inocula and Cell Targets

Nine patients who achieved SVR after completion of IFN-α or IFN-α/ribavirin therapy, as defined by repeated serum HCV RNA negativity by Roche Amplicor HCV version 2.0 assay (sensitivity 500 IU/mL or 1000 vge/mL; Roche Molecular Diagnostics, Pleasanton, CA) and normal liver function tests assessed at 6-12 month intervals, were investigated in this study (Table 4.1.). All patients were anti-HCV antibody positive by enzyme immunoassay (EIA; Abbott Diagnostics, Mississauga, Ontario, Canada). The follow-up period after SVR ranged between 24 to 72 months. All 9 individuals were found to carry HCV RNA at the time of this study when total RNA isolated from 500 μL of serum was assayed by highly sensitive RT-PCR/NAH (sensitivity of $\leq$ 10 vge/mL or $\leq$ 2 IU/mL) previously established (Pham et al., 2004). The estimated HCV RNA loads in the patients' sera ranged from <40 vge/mL to 400 vge/mL, with the exception of 6/F (59/F), who carried as much as 1.6×10^3 vge/mL (Table 4.1.). Also, while PBMC collected from the patients were seemingly HCV RNA nonreactive by the same highly sensitive assay, a 72-hour culture of the PBMC with phytohemagglutinin (PHA; 5 μg/mL; Sigma, Oakville, Ontario, Canada) and recombinant interleukin-2 (IL-2; 20 IU/mL; Roche) (Pham et al.,

Table 4.1. Clinical and Virological Characteristics of Individuals with Occult HCV Infection

Case	Age/ Sex	Route of Infection	Antiviral Treatment (Weeks)	HCV Genotype	Follow-Up Period After SVR (Months)	HCV RNA		
						Serum Load (vge/mL)*	PBMCs‡	
							HCV RNA–Positive Strand (vge/μg)	HCV RNA–Negative Strand
1/M‡	48/M	Unknown	IFN (24)	4	72	<40	300	+
2/M§	46/M	IVDU	IFN/R (24)	1a	60	400	50	ND
3/M‡	43/M	IVDU	IFN/R (48)	2a	60	40	<50	ND
4/F‡§	43/F	IVDU	IFN/R (48)	Unknown	60	40	ND	n.a.
5/F‡§	44/F	Inhalation	P-IFN/R (48)	1a	42	100	100	+
6/F‡	59/F	Unknown	P-IFN/R (48)	1b	36	1.6×10^3	ND	n.a.
7/F‡	48/F	Endemic	IFN/R (48)	1a	24	50	100	+
8/F‡	33/F	Inhalation	P-IFN/R (48)	1	24	100	<50	+
9/F‡§	45/F	IVDU	P-IFN/R (48)	3a	18	<50	<10	+

Abbreviations: F, female; HCV, hepatitis C virus; IFN, interferon alpha; IL-2, interleukin-2; IVDU, intravenous drug use; M, male; n.a., not applicable; NAH, nucleic acid hybridization; ND, not detectable; P-IFN, pegylated interferon alpha; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; R, ribavirin; RT-PCR, reverse-transcription polymerase chain reaction; SVR, sustained virological response; vge, virus genome equivalents.

*The serum HCV RNA load was determined by real-time RT-PCR.

†PBMCs were stimulated with 5 μg/mL PHA and 20 IU/mL IL-2, the HCV RNA-positive strand was measured by nested RT-PCR/NAH, and the RNA-negative strand was measured by strand-specific RT-PCR/NAH as described in the Patients and Methods section.

‡Plasma was used as the HCV inoculum for the *in vitro* infection.

§The supernatant from PBMCs after a 72-hour culture with PHA and IL-2 was used as the HCV inoculum for the *in vitro* infection.

2004) enabled detection of the virus genome in 7 individuals at estimated levels between <10 and 300 vge/μg total RNA (Table 4.1.). Furthermore, HCV RNA negative strand in PBMC was detected in 5 cases (Table 4.1.). Plasma from 8 patients was used for *in vitro* infection experiments (see Table 4.1.). In 4 cases (Table 4.1.), supernatant from PBMC cultured in the presence of PHA and IL-2 for 72 hours served as an inoculum for *in vitro* infection. Infectivity of both plasma and PBMC supernatants was examined in 3 cases (see Table 4.1.). Lymphoid cells serving as *in vitro* HCV targets were isolated from healthy donors who had no clinical history or molecular indication of HCV exposure, as confirmed by RT-PCR/NAH assay (Pham et al., 2004) and the absence of anti-HCV antibody by EIA (Abbott).

4.3.2. HCV Infection

Monocyte-depleted lymphoid cells from a healthy donor were treated with 5 μg/mL PHA for 48 h (Section 2.3.3.). Following stimulation, 7 x 10^6 lymphoid cells were exposed to 500 μL of test plasma in 6.5 mL of culture medium (Section 2.3.3.) or 7 mL of supernatant from *in vivo* infected PBMC which were cultured as indicated above and described in detail previously (Section 2.3.3.). In parallel, the same number of target cells was exposed to 250 μL of plasma from a patient with CHC carrying HCV genotype 1b at 7.3 x 10^5 vge/mL, as a positive control, and to 500 μL of plasma from a healthy donor, as a negative control (mock infection). Inocula were removed after 24 hours, the cells washed and cultured under alternating stimulation with PHA and IL-2 (phases A to D) for 14 days, as reported Section 2). It was previously established that after 14 days in culture ~98% of the cells were T cells (Fig. 2.1). Culture supernatants were collected at

1, 4 (phase A), 7 (phase B), 11 (phase C) and 14 (phase D) days post-infection (d.p.i.) and stored at -80 °C, while cells recovered at 14 d.p.i. (phase D) were cryopreserved for analysis.

4.3.3. Modification of HCV Infectivity by Anti-HCV E2, Anti-CD81 and IFN-α Treatments

Neutralization of HCV was carried out by incubating in duplicate 250 µL of 48/F plasma with an anti-HCV E2 mAb (AP33; provided by Dr. A. Patel, Institute of Virology, University of Glasgow, Glasgow, UK) for 1 h at 37 °C and then 1 hr at 4 °C prior to adding to T cell targets. Inhibition of infection was also done by pre-incubating T cell targets with anti-CD81 mAb (Pharmingen, San Diego, CA) before exposure to 44/F or 48/F plasma, as described previously (Section 2.3.5.). Appropriate isotype-matched mAbs were used in control experiments. To further reaffirm that active HCV replication was established in T cells, the cells were treated in duplicate with 1000 U/mL recombinant human IFN-α 2b (Research Diagnostics, Flanders, NJ) at the time of HCV inoculation, as reported (Section 2.3.6.). The cells exposed to the same amount of 44/F or 48/F plasma, but not treated with IFN-α, served as positive controls.

4.3.4. Ultracentifugation

To concentrate the virus and to recognize its general biophysical properties, 5 mL of plasma or 10 mL of pooled T cell culture supernatants collected after phases C and D, which were pre-clarified at 400 x ***g*** for 30 min in the presence of protease inhibitor

cocktail (1:200; Sigma), were layered onto 1-mL 30% sucrose cushions and centrifuged at 28,000 x *g* for 2.5 h at 4 °C in the TH641 rotor using Sorvall Discovery 100SE ultracentrifuge (Mandel Scientific Company Inc., Guelph, Canada). Based on the findings from preceding experiments (data not shown), 8.6 mL was removed from the top of each tube and the remaining 2.4 mL collected in 300-µl fractions (n=8) for evaluation of HCV RNA content and sucrose density. In some instances, two 10-mL samples of pooled T cell culture supernatant collected after phases A-D of the same infection experiment were concentrated as indicated above and the resulting equivalent fractions pooled and used for analysis.

4.3.5. RNA Extraction and RT-PCR/NAH Assays

Total RNA was extracted with Trizol (Invitrogen Life Technologies, Burlington, Canada) from ~1 x 10^7 cells (yielding ~10 µg RNA) or from 150 µL of the 300-µL sucrose fractions. RNA was reversely transcribed using Moloney murine leukemia virus reverse transcriptase (RT; Invitrogen). HCV RNA positive and negative strands were detected using cDNA derived from 1-2 µg or 2-4 µg total RNA, respectively, and primers, amplifications conditions, and controls exactly as reported in our previous studies (Pham et al., 2004; Pham et al., 2005). A water sample and a mock extraction were always included as contamination controls. cDNA prepared from the mock infection served as an additional RT-PCR negative control. Recombinant HCV 5'-UTR-E2 fragment (rHCV UTR-E2) served as a positive control. Specificity of the detection and validity of controls were routinely confirmed by NAH, *i.e.,* Southern blot hybridization, using ^{32}P-labeled rHCV UTR-E2 as a probe (Pham et al., 2004). Sensitivity of the RT-PCR assay

for HCV RNA positive strand was <10 vge/mL (<2 IU/mL) or 5 vge/μg total RNA, whereas that for HCV RNA negative strand detection 25-50 vge/μg total RNA (Pham et al., 2004). As a rule, HCV RNA negative strand was tested only in RNA samples from T cells which had been found reactive for HCV RNA positive strand.

4.3.6. Clonal Sequencing

The nucleotide sequences of 5'-UTR HCV amplicons detected in cultured T cells exposed to 44/F or 48/F plasma were compared to those amplified from respective plasma and from PBMC isolated from the patients who provided those plasma. The amplicons were cloned using the TOPO-TA cloning system (Invitrogen). Ten clones for each PCR product were sequenced in both directions using M13 primers and ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Streetsville, Canada). The resulting sequences were aligned with the help of Sequencher software version 4.7 (Gene Codes Corp., Ann Arbour, MI) (Pham et al., 2008).

4.3.7. Confocal Microscopy and Flow Cytometry

To detect HCV NS5A protein in *in vitro* infected T cells and to estimate the number of positive cells, confocal immunofluorescent microscopy and fluorescence activated cell sorting (FACS) were applied. For confocal microscopy, infected cells were fixed with 4% paraformaldehyde, permeablized with 0.5% Triton X-100, blocked with 10% normal goat serum, and double-stained with rat anti-tubulin (Chemicon International, Temecula, CA) and with either mouse anti-HCV NS5A mAb (Chemicon) or mouse isotype control (Pham et al., 2008). Then, cells were incubated with Cy2-labeled donkey anti-mouse and

Cy5-labeled donkey anti-rat antibodies (both from Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Cultured HCV-naïve T cells, Huh-7 cells, and Huh-7 cells carrying HCV AB12-A2FL replicon (provided by Drs C. Richardson and J. Wilson, Ontario Cancer Institute, Toronto, Canada) stained as above were used as controls. Cells were examined in a FluoView FV300 confocal system (Olympus America Inc., Melville, NY). Approximately 1000 cells per each preparation were examined and NS5A-positive cells counted. For FACS analysis, cells were fixed with 4% paraformaldehyde, permeablized with 0.5% saponin, and double-stained with anti-NS5A mAb and anti-tubulin, and then with Cy2- and Cy5-labeled secondary antibodies. Cells were examined by flow cytometry using a FACSCalibur cytometer (BD Biosciences Pharmingen, San Jose, CA) and the results analyzed by CellQuest Pro software (BD Biosciences).

4.3.8. Immunoelectron Microscopy

To determine whether complete HCV virions may circulate in individuals with clinically apparent SVR and were secreted by *de novo* infected T cell cultures, 500-µL of unfractionated 48/F plasma and culture supernatant from T cells exposed to that plasma was incubated with anti-E2 AP33 mAb, as reported (Section 2.3.10.). In addition, HCV RNA-positive fractions 4 and 7 (see Fig. 4.5A) obtained after centrifugation of 44/F plasma over sucrose were similarly incubated with anti-E2 mAb. Reacting particles were detected with anti-mouse IgG conjugated with 12-nm gold particles (Jackson ImmunoResearch) and counterstained with 1% phosphotungstic acid (PTA).

Examinations were carried out in a JEM 1200 EX microscope (JEOL Ltd., Tokyo, Japan).

4.4. RESULTS

4.4.1. HCV Genome Expression in T Cells Exposed to Plasma from Individuals with Clinically Apparent SVR

Using the previously established system allowing for *de novo* infection and propagation of wild-type HCV *in vitro* (see Section 2), infectivity of residual HCV occurring in plasma of individuals with clinically apparent SVR was tested. Pre-stimulated lymphoid cells exposed to plasma from 8 individuals and then cultured under alternate stimulation became reactive for HCV RNA positive strand in 7 of the cases (Fig. 4.1). HCV RNA negative strand, indicative of active virus replication, was evident in 3 of the 7 (42.8%) cell cultures that were positive for the virus positive strand. The HCV loads were estimated to be between $1 \times 10^3 - 5 \times 10^4$ vge/10^7 cells.

4.4.2. HCV NS5A Protein in *In Vitro* Infected T Cells.

To determine whether expression of HCV RNA in *in vitro* infected T cells was accompanied by synthesis of viral protein, cells exposed to 44/F and 48/F plasma were examined for HCV NS5A protein by confocal microscopy. As illustrated in Figure 4.2A, HCV NS5A occurred predominantly as granular intracytoplasmic deposits and at the plasma membrane of the positive cells. Percentages of NS5A reactive cells enumerated under a confocal microscope were between 0.78% and 1.35%. A flow cytometric analysis gave comparable results of 1.05% to 1.52% of HCV NS5A protein positive cells (Fig. 4.2B).

Figure. 4.1. Detection of HCV RNA–positive and HCV RNA–negative strands in T cell cultures after exposure to plasma from patients followed for up to 60 months after apparently complete clinical clearance of HCV. (A) HCV RNA–positive strand and (B) HCV RNA–negative (replicative) strand. HCV sRNA–positive and HCV sRNA–negative strands were used to confirm the specificity of the detections. Water instead of complementary DNA amplified in direct (D/W) and nested (N/W) reactions and a mock extraction (M) treated as test RNA were included as contamination controls. Positive samples showed the expected 244-bp amplicons. *A hybridization signal for 48/M with appropriate controls was overexposed to visualize its presence. Abbreviations: HCV, hepatitis C virus; HCV sRNA, synthetic hepatitis C virus RNA; rHCV UTR-E2, recombinant hepatitis C virus 5'-untranslated region E2 fragment.

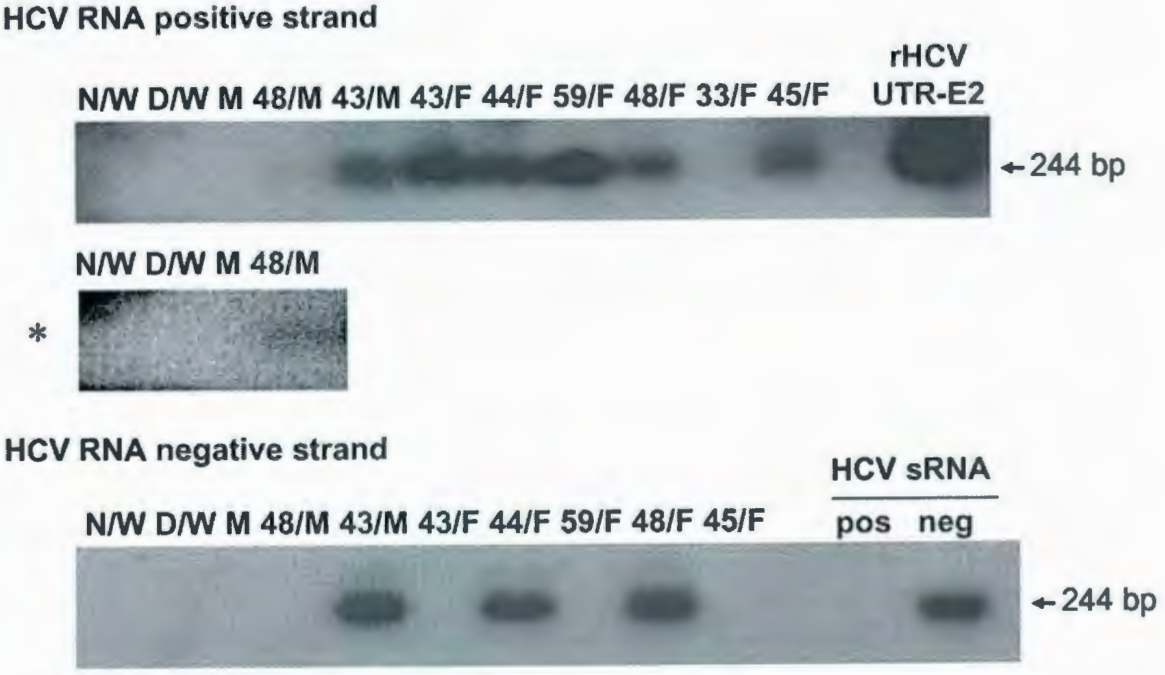
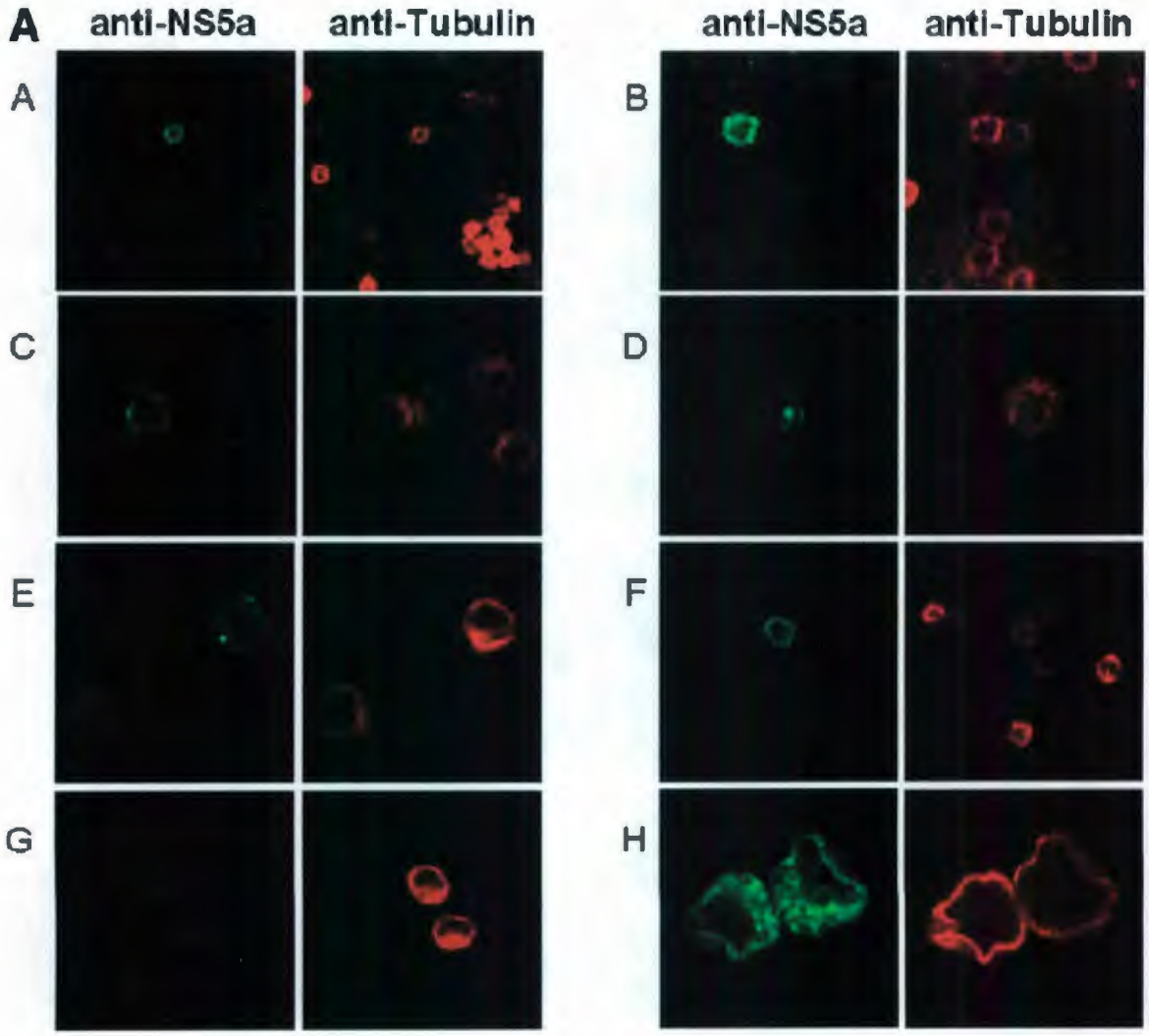
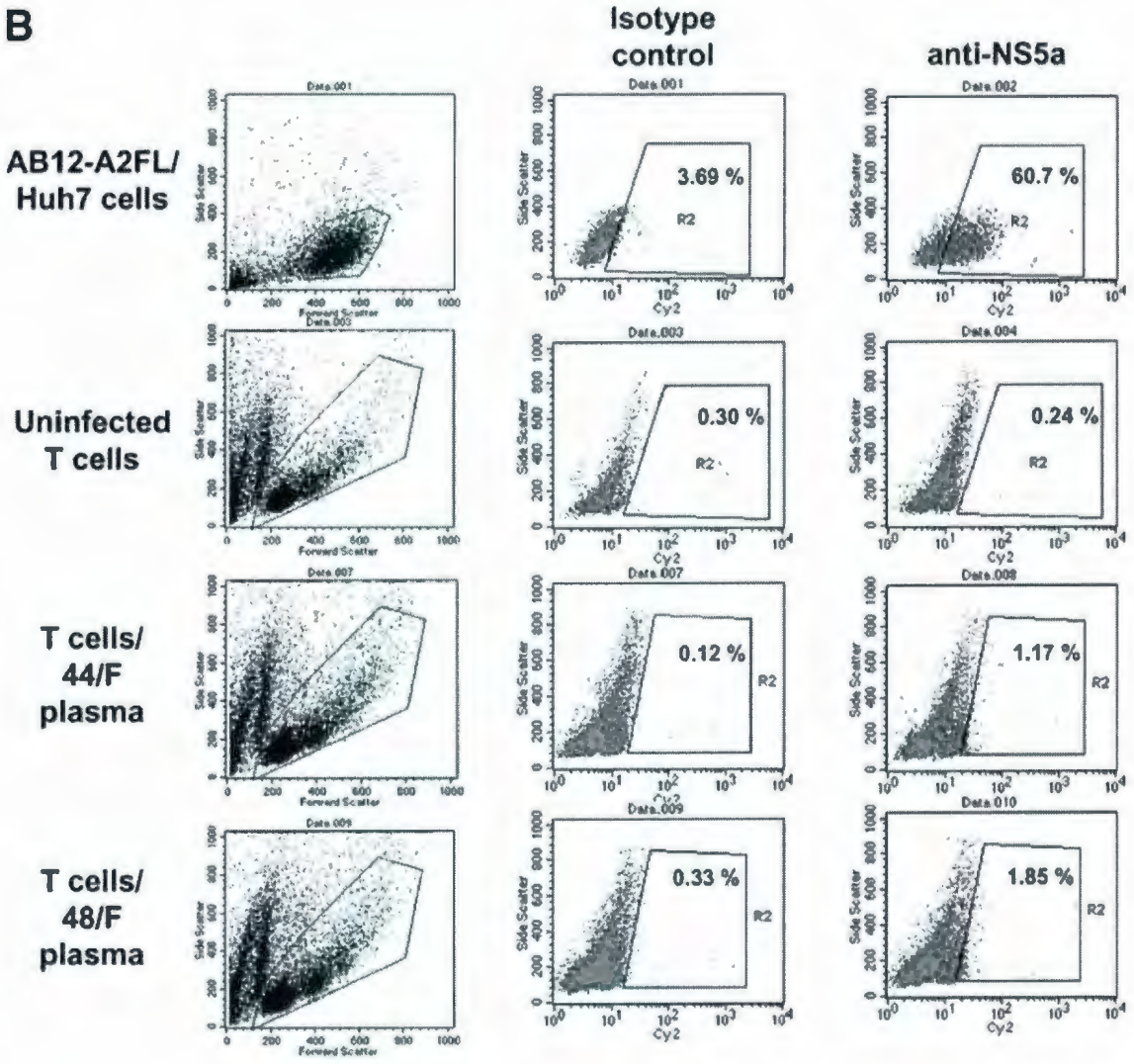

HCV RNA positive strand
rHCV
N/W D/W M 48/M 43/M 43/F 44/F 59/F 48/F 33/F 45/F UTR-E2
244 bp
N/W D/W M 48/M
*
HCV RNA negative strand
HCV sRNA
N/W D/W M 48/M 43/M 43/F 44/F 59/F 48/F 45/F pos neg
244 bp

Figure. 4.2. Expression of HCV NS5A protein in cultured T cells exposed to plasma from individuals with SVR. (A) Cultured T cells after exposure to (A,B) 43/M plasma, (C-E) 48/F plasma, and (F) plasma from a patient with chronic hepatitis C (positive control) were double-stained with anti-HCV NS5A and anti-tubulin mAbs and analyzed by confocal microscopy. (G) Cultured T cells exposed to HCV and stained with an isotype control antibody served as a negative control. (H) Huh-7 cells transfected with HCV AB12-A2FL full-length HCV replicon served as an additional positive control. Images were captured at 60 x magnification. (B) Flow cytometric quantification of HCV NS5A-positive T cells exposed to plasma from individuals with therapy-induced SVR. Prestimulated cells were incubated with 44/F or 48/F plasma and cultured as described in the Patients and Methods section. T cells were stained with anti-NS5A mAb or isotype control antibody. Gates were set on the basis of isotype controls. Stimulated, uninfected T cells similarly stained with anti-NS5A mAb served as a negative control. Huh-7 cells transfected with HCV AB12-A2FL replicon were used as a positive control. Percentages indicate positive cells. Abbreviations: HCV, hepatitis C virus; mAb, monoclonal antibody; NS5A, nonstructural protein 5A; SVR, sustained virological response.


A
anti-NS5a
anti-Tubulin
anti-NS5a
anti-Tubulin
A
B
C
D
E
F
G
H


B
Isotype control
anti-NS5a
AB12-A2FL/ Huh7 cells
Uninfected T cells
T cells/ 44/F plasma
T cells/ 48/F plasma
3.69 %
60.7 %
0.30 %
0.24 %
0.12 %
1.17 %
0.33 %
1.85 %
R2
Side Scatter
Forward Scatter
Cy2

4.4.3. Inhibition of HCV Infection by Anti-E2, Anti-CD81 and IFN-α

Pre-incubation of HCV present in 48/F plasma with anti-HCV E2 mAb, but not with an isotype control, neutralized the virus infectivity as evidenced by the absence of HCV RNA negative strand detection in the cells exposed to the treated inoculum (Fig.4. 3A). Similarly, pre-incubation of T cells with anti-CD81 mAb, but not with an isotype control mAb, blocked HCV replication, as indicated by a lack of HCV RNA negative strand detection, in experiments in which 44/F or 48/F plasma was used as inoculum (Fig. 4.3A). Furthermore, treatment with recombinant IFN-α prevented establishment of HCV replication in T cells, as shown in Figure 4.3B.

4.4.4. Unique HCV Variants in *De Novo* Infected T Cells

To assess whether HCV replication in *de novo* infected T cells led to appearance of variants distinct from those present in the plasma used for their inoculation, as it was observed in our previous study (see Section 2), 5'-UTR amplicons from 44/F and 48/F plasma, PBMC, and from cultured T cells exposed to these plasma were cloned, bidirectionally sequenced, and compared. As shown in Figure 4.4, clonal sequence analysis of the 147-bp fragment revealed a deletion at position 120 in all clones from PBMC and in 8 clones from *de novo* infected T cells, and a C to T change at position 249 in all clones from PBMC and in 7 clones from T cells when compared to the sequence amplified from 44/F plasma used as inoculum, indicating that unique HCV variants were present in the PBMC and, importantly, that the same variants emerged in the T cells exposed to the plasma. On the other hand, sequencing of the cloned 5'-UTR amplicons

Figure. 4.3. Effect of HCV neutralization with anti-E2 mAb, pre-incubation of target cells with anti-CD81 mAb, or their treatment with IFN-α on the replication of HCV in cultured T cells. (A) HCV contained in 48/F plasma was pretreated with anti-HCV E2 mAb in duplicate (ex 1 and ex 2) or an isotype immunoglobulin control mAb and then incubated with T cells, or T cell targets were first incubated with anti-CD81 mAb or an appropriate isotype control and then exposed to 44/F plasma (ex 1) or 48/F plasma in duplicate (ex 1 and ex 2). (B) T cell targets were exposed to 44/F or 48/F plasma in the presence or absence (UT) of 1000 U/mL recombinant human IFN-α 2b in duplicate (ex 1 and ex 2). RNA was analyzed for HCV RNA-positive and HCV RNA-negative strands after 14 days of culture. Specificity and contamination controls were as outlined in the legend to Fig. 4.1. Positive samples showed the expected 244-bp amplicons.
Abbreviations: HCV, hepatitis C virus; HCV sRNA, synthetic hepatitis C virus RNA; IFN-α, interferon alpha; mAb, monoclonal antibody; rHCV UTR-E2, recombinant hepatitis C virus 5-untranslated region E2 fragment.

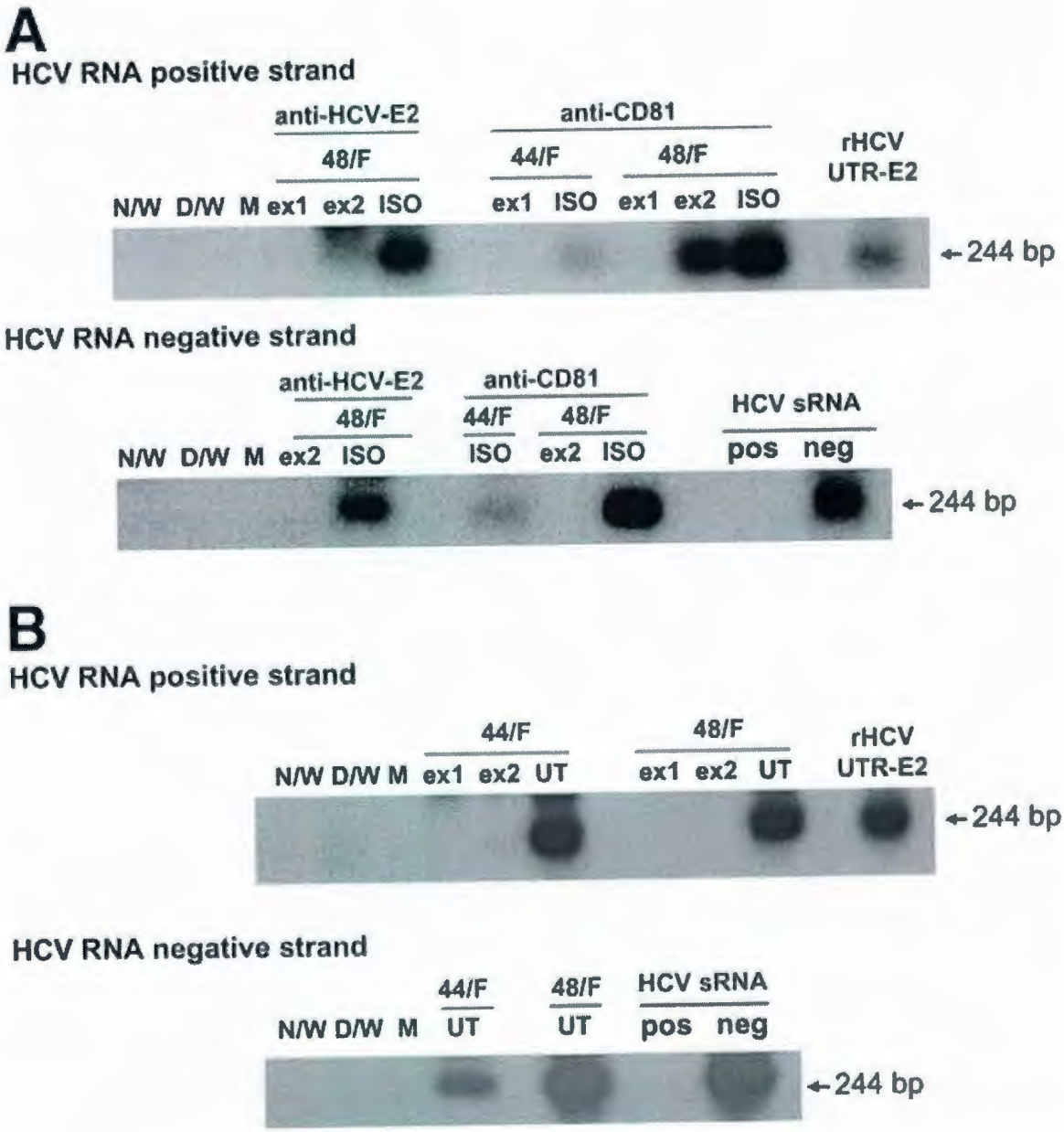
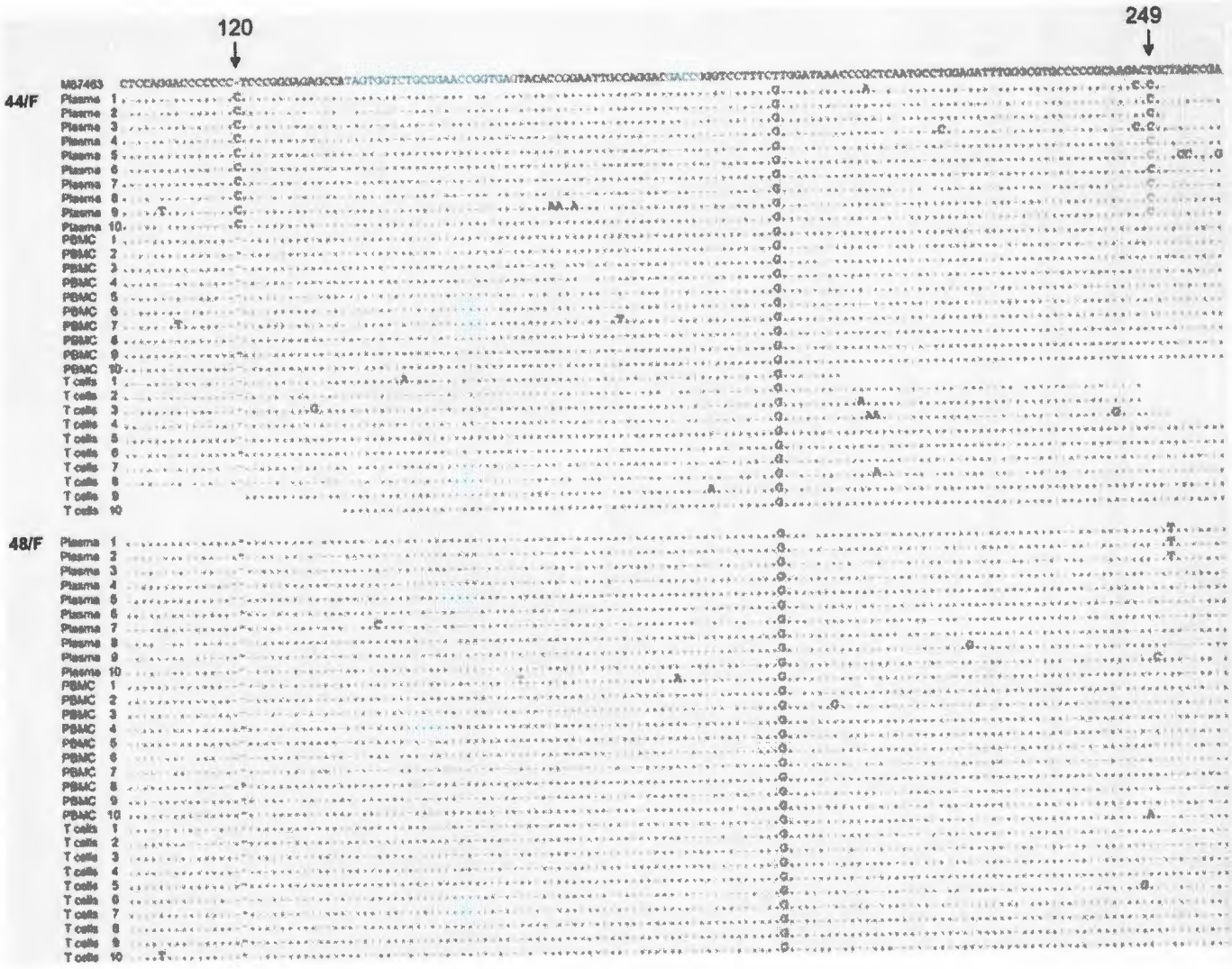

A
HCV RNA positive strand
anti-HCV-E2
anti-CD81
48/F
44/F
48/F
rHCV
UTR-E2
N/W D/W M ex1 ex2 ISO
ex1 ISO ex1 ex2 ISO
244 bp
HCV RNA negative strand
anti-HCV-E2
anti-CD81
48/F
44/F 48/F
HCV sRNA
N/W D/W M ex2 ISO
ISO ex2 ISO
pos neg
244 bp
B
HCV RNA positive strand
44/F
48/F
rHCV
UTR-E2
N/W D/W M ex1 ex2 UT
ex1 ex2 UT
244 bp
HCV RNA negative strand
44/F
48/F
HCV sRNA
N/W D/W M
UT
UT
pos neg
244 bp

Figure. 4.4. Nucleotide sequence alignment of clones derived from HCV 5'-UTR fragments amplified from 44/F and 48/F plasma, peripheral blood mononuclear cells, and cultured T cells exposed to that plasma. The 5'-UTR amplicons derived from each sample were cloned, and 10 randomly selected clones were sequenced bidirectionally. As a reference, the HCV genotype 1a sequence (GenBank accession number M67463) is shown on the top line. Nucleotides in the sequences identical to those of the reference are shown as dots, deletions are shown as dashes, and differences are shown as letters. Numbering of the nucleotides is according to the M67463 HCV genotype 1a sequence. Abbreviations: 5'-UTR, 5'-untranslated region; HCV, hepatitis C virus.

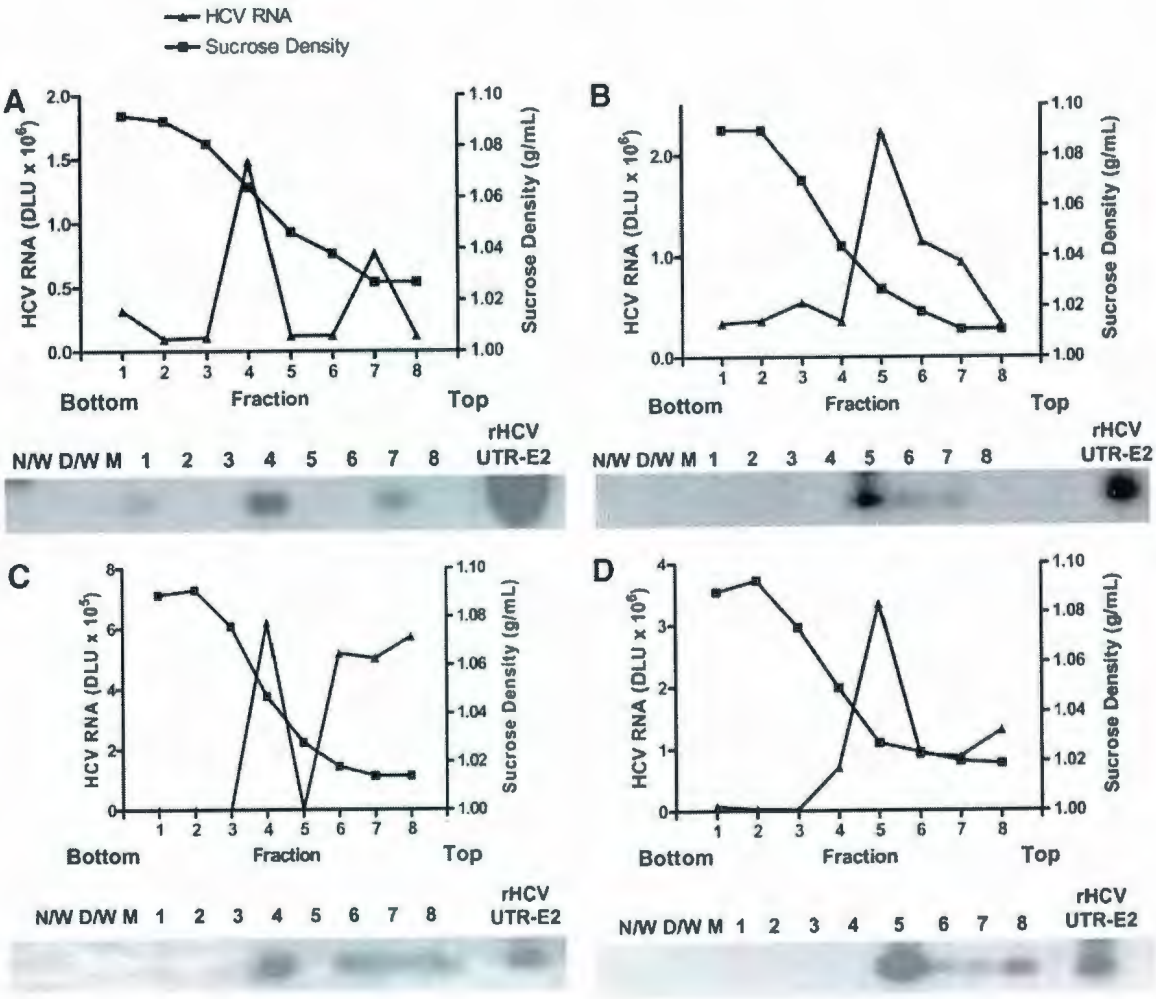

120
249
M67463
44/F
Plasma 1
Plasma 2
Plasma 3
Plasma 4
Plasma 5
Plasma 6
Plasma 7
Plasma 8
Plasma 9
Plasma 10
PBMC 1
PBMC 2
PBMC 3
PBMC 4
PBMC 5
PBMC 6
PBMC 7
PBMC 8
PBMC 9
PBMC 10
T cells 1
T cells 2
T cells 3
T cells 4
T cells 5
T cells 6
T cells 7
T cells 8
T cells 9
T cells 10
48/F
Plasma 1
Plasma 2
Plasma 3
Plasma 4
Plasma 5
Plasma 6
Plasma 7
Plasma 8
Plasma 9
Plasma 10
PBMC 1
PBMC 2
PBMC 3
PBMC 4
PBMC 5
PBMC 6
PBMC 7
PBMC 8
PBMC 9
PBMC 10
T cells 1
T cells 2
T cells 3
T cells 4
T cells 5
T cells 6
T cells 7
T cells 8
T cells 9
T cells 10

derived from 48/F plasma, PBMC and cultured naïve T cells exposed to 48/F plasma revealed random single nucleotide polymorphisms. There was no indication of the existence of lymphoid cell-specific HCV variants.

4.4.5. Physically Distinct HCV RNA-Reactive Particles Occur in Plasma and Culture Supernatants from T Cells Exposed to That Plasma

To gain an insight into general biophysical properties of HCV RNA-reactive particles occurring in plasma of individuals with SVR and those released into culture medium by T cells exposed to these plasma, samples of plasma and supernatants from the infected T cells were ultracentrifuged over sucrose and the 8 bottom fractions collected. The analysis of 44/F plasma showed that HCV RNA-reactive particles occurred in fractions 1, 4 and 7, corresponding to sucrose densities of 1.092, 1.064 and 1.027 g/mL, respectively, with apparent HCV RNA peak reactivity in fraction 4 (Fig. 4.5A). In the culture supernatant of T cells exposed to 44/F plasma, HCV RNA-positive particles were found in fractions 5-8 at densities between 1.024 and 1.011 g/mL with the peak RNA positivity in fraction 5 at density of 1.024 g/mL (Fig. 4.5B). HCV RNA-reactive particles after centrifugation of 48/F plasma banded at fraction 4 at sucrose density of 1.047 and in fractions 6-7 at densities 1.018-1.013 g/mL (Fig. 4.5C), while those occurring in the culture supernatant of T cells exposed to this plasma in fractions 5-8, having a sucrose density of 1.027 – 1.019 g/mL, with the HCV RNA peak reactivity in fraction 5 at density of 1.027 g/mL (Fig. 4.5D).

Figure. 4.5. Sedimentation velocity of HCV RNA-reactive particles in sucrose. Plasma samples from individuals 44/F and 48/F with clinically apparent SVR and supernatants from cultured T cells exposed to this plasma were separately layered onto 1-mL sucrose cushions and ultracentrifuged. Eight 300-L fractions collected from the bottom of each tube were assayed for HCV RNA-positive strands by reverse-transcription polymerase chain reaction/nucleic acid hybridization and for sucrose density. (A) 44/F plasma collected 3.5 years after SVR was achieved, (B) culture supernatant from T cells infected with HCV contained in 44/F plasma, (C) 48/F plasma collected 2 years after clinical SVR was achieved, and (D) culture supernatant from T cells infected with HCV contained in 48/F plasma. On the left side of each panel, the HCV RNA level is expressed in relative density units given by hybridization signals shown on the blot under each panel. On the blots, contamination and specificity controls are marked as in the legend to Fig. 4.1. Abbreviations: HCV, hepatitis C virus; rHCV UTR-E2, recombinant hepatitis C virus 5-untranslated region E2 fragment; SVR, sustained virological response.


HCV RNA
Sucrose Density
A
B
C
D
HCV RNA (DLU x 10^6)
HCV RNA (DLU x 10^5)
Sucrose Density (g/mL)
Bottom
Fraction
Top
N/W D/W M 1 2 3 4 5 6 7 8
rHCV UTR-E2

4.4.6. Infectivity of HCV Released By PBMC after Clinically Apparent SVR

To assess the infectivity of HCV found in *in vivo* infected PBMC of individuals with clinically apparent SVR, PBMC from 4 such patients followed for up to 5 years (Table 4.1.) were stimulated with PHA and IL-2 for 72 hours and the resulting supernatants used as inoculum to infect T cells. The data revealed that all cell cultures exposed to the PBMC supernatants acquired HCV RNA positive strand reactivity, while HCV RNA negative strand was detected in one of the cultures (Fig. 4.6).

4.4.7. Ultrastructural Identification of HCV Particles in SVR Plasma and Culture Supernatants of *De Novo* Infected T Cells

HCV particles were visualized with anti-E2 mAb by IEM (Fig. 4.7). Figure 4.7 A-C depicts HCV virions detected in unfractionated (total) plasma obtained from 48/F patient 24 months after achieving SVR. Figure 4.7. D-F shows HCV particles found in HCV RNA-reactive fractions 4 (panel D) and 7 (panels E and F) after fractionation of 44/F plasma collected 42 months after SVR. As shown in Figure 4.7. G-K, HCV virion particles, either singular or aggregates, were also detected in culture supernatants collected from T cells *in vitro* infected with virus carried in 44/F plasma. Particle sizes ranged from 50-75 nm in diameter.

Figure. 4.6. Detection of HCV RNA-positive and HCV RNA-negative strands in cultured T cells exposed to culture supernatants derived from *in vivo* HCV-infected, *ex vivo* stimulated peripheral blood mononuclear cells obtained from individuals with follow-up of up to 60 months after a clinical SVR. Contamination and specificity controls are marked as outlined in the legend to Fig. 4.1. Positive samples show the expected 244-bp amplicons. Abbreviations: HCV, hepatitis C virus; HCV sRNA, synthetic hepatitis C virus RNA; rHCV UTR-E2, recombinant hepatitis C virus 5-untranslated region E2 fragment.

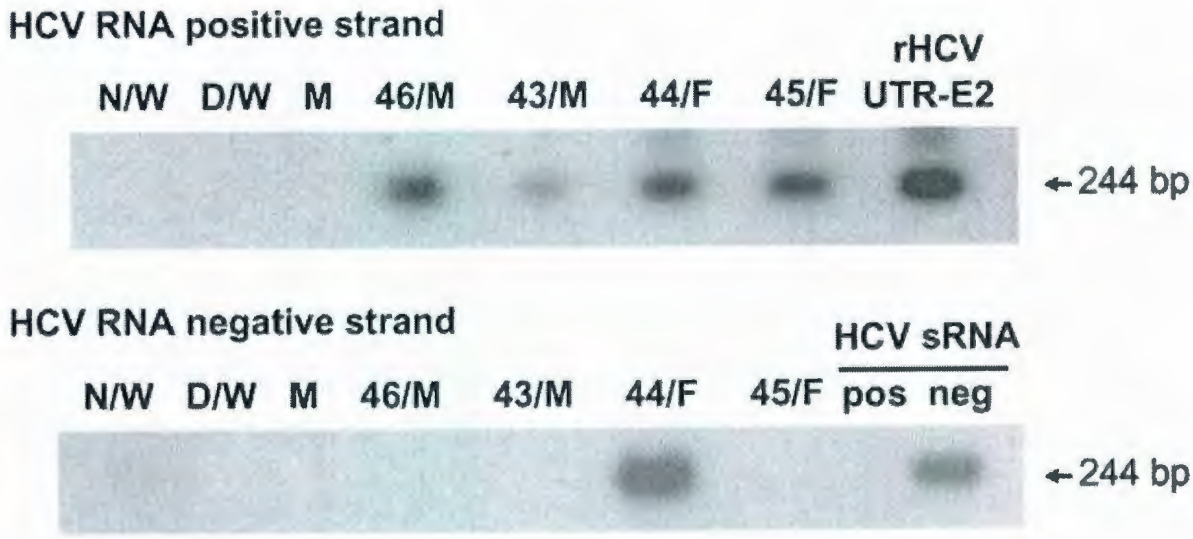
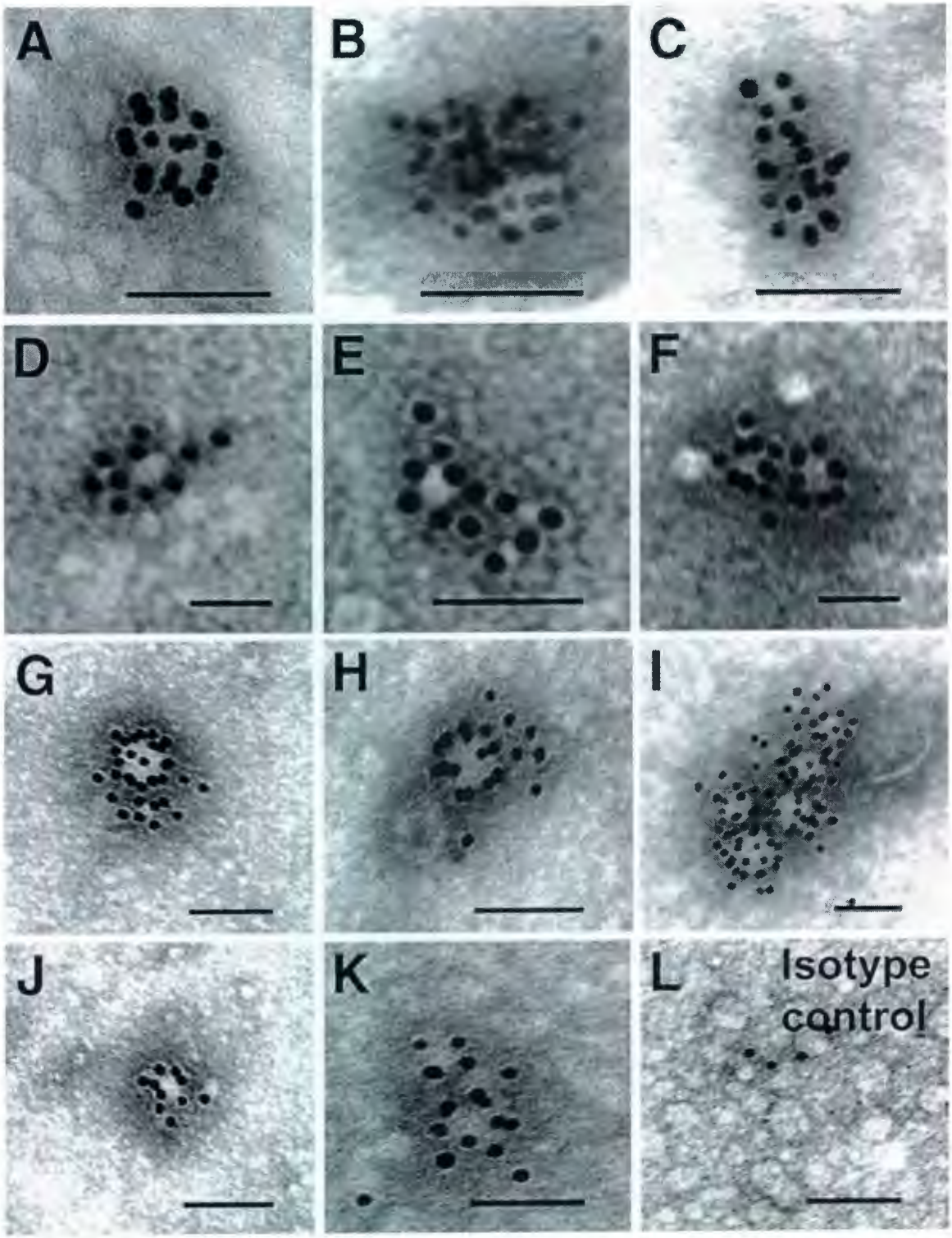

HCV RNA positive strand
rHCV
N/W D/W M 46/M 43/M 44/F 45/F UTR-E2
←244 bp
HCV RNA negative strand
HCV sRNA
N/W D/W M 46/M 43/M 44/F 45/F pos neg
←244 bp

Figure. 4.7. Ultrastructural features of HCV RNA-reactive particles in the plasma of individuals with clinical SVR and in the culture supernatant obtained from T cells exposed to one of the plasma samples as visualized by immunogold staining with anti-E2 mAb. (A-C) HCV virion particles in unfractionated plasma of patient 48/F. HCV virions in (D) fraction 4 (sucrose density, 1.064 g/mL) and (E,F) fraction 7 (sucrose density, 1.027 g/mL) of 44/F plasma. (G-K) HCV particles in the supernatant of cultured T cells infected with HCV contained in 44/F plasma. (L) The same culture supernatant pool shown in panels G to K exposed to the isotype control instead of anti-E2 mAb. Preparations were counterstained with 1% phosphotungstic acid. Bars indicate 100 nm. Abbreviations: HCV, hepatitis C virus; mAb, monoclonal antibody.


A
B
C
D
E
F
G
H
I
J
K
L
Isotype
control

4.5. DISCUSSION

These findings provide *in vitro* evidence that trace quantities of HCV persisting in the circulation for a long time after therapeutically induced resolution of CHC can remain infectious. The transmission of HCV infection was exemplified by detection of HCV RNA negative strand, NS5A protein and emergence of unique HCV variants in cultured T cells exposed to plasma from individuals with long-term follow-up after SVR. Furthermore, HCV replication in T cells was prevented following neutralization of virus with anti-E2 mAb, blocking with anti-CD81 mAb, and by treatment of the cells with recombinant human IFN-α 2b. In addition, HCV residing in PBMC after clinical resolution of infection was also found to be infectious. HCV virion particles specifically recognized by anti-E2 mAb were uncovered in plasma of these individuals and in the supernatant derived from *de novo* infected T cell cultures exposed to the plasma.

Similarly as in CHC (Pham et al., 2008), circulating lymphomononuclear cells have been found to be the site of active HCV replication in low-level infection continuing after resolution of hepatitis C, although *ex vivo* activation of the cells is usually required to uncover the virus presence (Pham et al., 2004; Pham et al., 2005). Among circulating immune cells, $CD4^+$ and $CD8^+$ T lymphocytes, B cells and monocytes were identified to be infected to a varying degree with HCV, but with overall viral loads greater in CHC than in occult infection (Pham et al., 2008). As with PBMC from occult infection, *ex vivo* stimulation of T cells affinity-purified from patients with low-level infection also significantly augmented HCV replication allowing for more ready detection of the residing virus, as the recent study showed (Pham et al., 2008).

It was also previously discovered that mitogen-activation of normal human T lymphocytes predisposes the cells to infection by wild-type HCV of different genotypes (MacParland et al., unpublished observations) (see Fig. 2.2). Productive replication of HCV in such treated cells, after exposure to either plasma from patients with CHC or culture supernatants from serial passage of wild-type HCV in T cell-enriched cultures, was shown by methods comparable to those used in the current study. Namely, by detection of HCV RNA negative strand, virus proteins (NS5A and E2) and HCV variants distinct from those occurring in respective inocula, and by identification of secreted complete virions, as evidenced by isopycnic banding and ultrastructural examinations (Section 2). In the present work, employing the same HCV replication system and similar evaluation criteria, it became evident that the virus occurring at low levels in 3 of 8 plasma collected 2 to 5 years after SVR (cases 43/F, 44/F and 48/F; Table 4.1.), was able to establish active HCV replication *in vitro*. In addition, HCV derived from *in vivo* infected lymphoid cells obtained from one (case 44/F) of 4 patients induced *de novo* infection in the culture system. Taken together, HCV carried by 3 out of the 9 individuals investigated in this study established infection *in vitro* which was confirmed by at least one criterion of active HCV replication, i.e., the appearance of HCV RNA negative strand. However, the infection initiated by the virus originating from two (cases 44/F and 48/F) of these 3 convalescent individuals was also confirmed by detection of viral protein, secretion of HCV RNA-reactive particles physically distinctive from those occurring in inocula, and identification of complete virions by IEM.

HCV RNA-reactive particles occurring in 44/F and 48/F plasma and those secreted by T cells exposed to that plasma displayed different sedimentation profiles after

ultracentrifugation over sucrose implying distinct biophysical properties. This further supported the conclusion that the virus released originated from the *de novo* infection process. In general, although the plasma virions, as confirmed by IEM, predominantly banded at higher sucrose densities (1.047-1.064 g/mL), those released from *in vitro* infected T cells tended to sediment at densities not exceeding 1.027 g/mL. Considering that HCV virions in plasma of patients with CHC have been shown to be of heterogenous densities, particularly when associated with immunoglobulins and lipids (Hijikata et al., 1993; Kanto et al., 1994; Diaz et al., 2006), that the majority of plasma virions should or do originate from infected hepatocytes, and that the viral particles found in culture supernatants in the current study were of low density and were exclusively produced by T cells, a possibility exists that virions assembled in hepatocytes and lymphoid cells could be biophysically distinct due to association with different host proteins and/or lipids giving, in consequence, different sedimentation profiles. Studies have yet to compare the biochemical properties of plasma virus in CHC and in persistent low-level HCV infection, as well as of virions produced by lymphoid cells in CHC and occult infection.

Infectivity of HCV traces persisting during the naturally acquired occult HCV infection has not yet been investigated. The present study, to our knowledge, is the first attempt in this regard. However, in early studies in chimpanzees, diluted plasma from a patient with acute post-transfusion hepatitis containing approximately 10 virions was capable of inducing infection which was characterized by elevated serum ALT and liver inflammation (Feinstone et al., 1981). More recently, as few as 20 copies of HCV RNA prepared by dilution of serum obtained during the pre-acute phase of hepatitis C of an infected chimpanzee has been demonstrated to cause HCV RNA-positive infection in the

absence of ALT elevation (Katayama et al., 2004b). However, since the sensitivity of the PCR assay used for detection of serum HCV RNA in the latter study appeared to be between 100 and 250 copies (Katayama et al., 2004b), a possibility remains that lower doses of HCV may also transmit infection in this model. Our present findings reveal that HCV circulating in some individuals with resolved hepatitis C is capable of inducing productive infection *in vitro* at doses of 20 to 50 copies. This can be interpreted as a strong indication of potential virus infectivity *in vivo*. In future studies, it would be of interest to determine the molecular mechanisms as to why HCV circulating in some individuals, but not in others, was infectious to T cells despite comparable levels of virus present.

In summary, the current study provides the first experimental evidence that HCV RNA detectable at low quantities for years after apparent complete resolution of CHC reflects the existence of traces of biologically competent virus, which in some situations, can retain infectivity.

Chapter Five: General Discussion

The main purpose of this project was to establish an *in vitro* system in lymphoid cells capable of supporting the complete replication cycle of wild-type HCV. We aimed to uncover conditions supportive of replication and confirm that this system was truly able to facilitate the entire HCV replication cycle by testing, among others, the ability of lymphoid-derived virus to *de novo* infect healthy lymphoid cells; and to determine the molecular and biophysical properties of virions produced by lymphoid cells *de novo* infected in culture. A further goal was to assess whether exposure of initially virus-naïve T lymphocytes to HCV in culture may alter the T cell phenotype, proliferative capacity, degree of apoptosis and expression of selected cytokines. Finally, we endeavoured to evaluate the infectivity of virus lingering in patients after apparently complete clinical recovery from infection following either spontaneous resolution of hepatitis C or achievement of a clinical SVR following antiviral therapy.

The system for *in vitro* infection of PBMC with wild-type HCV was based on previous findings that activated lymphoid cells are more capable of supporting propagation of HCV (Pham et al., 2005). The uniqueness of our studies in relation to previously reported work aiming at establishing with HCV infection *in vitro* lies in the fact that we utilized primary, normal human lymphocytes as the target cells and wild-type, molecularly intact HCV as the inoculum, while other studies have employed cloned recombinant and cell culture-adapted viruses (Marukian et al., 2008) and transformed or immortalized lymphocyte-derived cell lines as virus targets (Shimizu et al., 1992; Sung et al., 2003). The importance of using wild-type virus and primary lymphoid cells to study

in vitro infection is that, comparing to other HCV culture methods, this best mimics what would take place *in vivo* in an HCV-infected individual. In circulation, wild-type HCV exists as a swarm of genetically related viral variants. Recombinant cloned viruses, such as the JFH-1 viral clone, will gain the ability to mutate once they are replicating using the HCV NS5B polymerase but initially require transfection into Huh-7 cells as a single cloned viral sequence. In the current studies, we were interested in exposing our target cells to the entire quasispecies population existing naturally in an HCV-infected individual with the aim of sampling variants with different replicative abilities and possibly different lymphoid cell tropisms.

Many previous studies showing *in vitro* susceptibility of cultured cells to HCV infection have made use of cell lines either transformed by cancer, such as B cell lines from patients with non-Hodgkin's B-cell lymphoma (Sung et al., 2003), co-infected with other viruses, such as Molt-4 T cells co- infected with murine retroviruses (Shimizu et al., 1992), or hepatoma cell lines, such as Huh-7 and Huh-7.5 cells (Zhong et al., 2005; Lindenbach et al., 2005; Wakita et al., 2005). For the purposes of our investigations, we thought it important to look at primary lymphocytes as the targets. Previously, we and others have observed that persistent hepatitis viruses can replicate in lymphocytes in patients or animals without known co-infection with other viruses or the existence of cancers (Michalak et al., 1994) and we hypothesized that the same could be true for HCV.

The existence of HCV lymphotropism has ignited debate with the main question being whether detection of HCV reflects the existence of cell surface adhered free virus or truly

intracellular replicating virus. We investigated this question by examining if *de novo* infection of lymphocytes can lead to detectable production of the HCV genome replicative intermediate, the HCV RNA negative strand, and if protein components of the replication complex, i.e., the HCV non-structural proteins, can be detected along with HCV RNA. We further investigated whether secretion of enveloped virions is taking place and examined their presence in supernatants of *in vitro* infected cells and by serial passage of released virus in naive PBMC-derived T cells. We also confirmed active replication of HCV in *de novo* infected cells via treatment with interferon alpha.

Another question of high importance is whether virus infection of lymphoid cells can lead to pathological outcomes and modify the function of immune cells. It has been suggested that HCV infection can cause B cell dysfunction, as it has been found that close to 80% of patients with mixed cryoglobulinemia, characterized by an accumulation of cryoglobulins, are also chronically infected with HCV (Agnello et al., 1992). The notion of HCV-induced lymphocyte abnormalities is further supported by the finding that classical antiviral therapy for hepatitis infection results in clinical recovery from HCV-associated MC (Saadoun et al., 2006). Along the same lines, HCV-infected lymphoid cells have been detected in HCV patients with non-Hodgkin's lymphoma (Ferri et al., 1997). Alterations in T cell characteristics and function in HCV-infected individuals have also been described. In peripheral blood and intrahepatic T lymphocytes in patients with CHC, T cells have been found to display markers of exhaustion, such as the expression of inhibitory receptor PD-1 (Golden-Mason et al., 2007) and negative immune regulator TIM-3 (Golden-Mason et al., 2009)(see Section 1.6.2). As well, T cells

exposed to HCV *in vitro* show impaired IFN-γ signalling with suppressed levels of Stat-1 mRNA expression (Kondo et al., 2007). In our HCV-T cell infection system, we evaluated the phenotype, proliferation and degree of apoptotic death of HCV-exposed lymphoid cells and found that exposure to HCV selectively inhibits $CD4^+$ T cell, but not $CD8^+$ T cell proliferative capacity without having an influence on T cell apoptosis *in vitro*.

The data from our studies uncovered several previously unknown facts. It was discovered in the first study (Section 2) that T cell-enriched cultures derived from PBMC of healthy individuals are susceptible to infection with wild-type HCV following mitogen stimulation and support its replication when intermittently. This finding was supported by the detection of HCV RNA negative (replicative), detection of non-structural protein expression in infected cells, identification and characterization of the biophysical properties of enveloped virion particles by sucrose gradient centrifugation and by immune electron microscopy with HCV-specific antibodies against virus envelope (E2) protein, and by determining the *in vitro* infectivity of the secreted virus to virus-naïve lymphocytes. Subsequently, in the second study (Section 3), it was found that *in vitro* infection of T cells with HCV can lead to an altered T cell phenotype characterized by an apparent enrichment of $CD8^+$ T cells that occurs due to an inhibition of $CD4^+$ cell proliferation. As is known, the quantities of HCV remaining in circulation after apparent resolution of hepatitis C and control of viremia are quite low ($\leq$100 vge/mL). However, it was important to determine if these small amounts of virus can transmit infection to naïve human cells since it has been identified that even minute amounts (approximately

20 virus copies) of HCV can be infectious in a chimpanzee model of hepatitis C (Katayama et al., 2004b). In the third study (Section 4), it was established, for the first time, that small amounts of HCV RNA-containing virions which circulate or reside in peripheral lymphoid cells in patients after apparent complete resolution of CHC due to IFN/RBV therapy retains its infectivity and can propagate in mitogen pre-stimulated T cell cultures.

Our findings have several important implications. They conclusively document, using different investigative techniques, that HCV productively replicates in immune cells, such as T lymphocytes, and that these cells can be reservoirs of biologically competent, infectious virus. This may have implications with regard to viral persistence since virus residing in these cells may not be susceptible to the effects of antiviral therapy, including treatment with IFN/RBV (Blackard et al., 2006). HCV reinfection has been well described as occurring almost invariably after liver transplantation in patients with HCV-related end-stage liver disease (Testa et al., 2000). Furthermore, HCV recurrence has been reported in patients who had no detectable HCV RNA following antiviral therapy prior to liver transplantation (Thomas et al., 2003; Forns et al., 2003). Our findings suggest that lymphocytes harbouring replicating, virus may be a source of infectious virus capable to infect hepatocytes post-OLT.

The observed inhibition of $CD4^+$ T cell proliferation *in vitro* after exposure to HCV may have significance in that the virus may be directly affecting $CD4^+$ helper function which is required for resolution of hepatitis and clearance of viremia. Finally, the fact that minute amounts of HCV persisting in individuals after apparent resolution of

hepatitis C remains infectious to naïve lymphocytes *in vitro* implies that the virus retains its pathogenic competence in these patients.

The expected consequences of lymphotropism in terms of immune response to HCV remain unclear. The factors determining control of HCV are the balance between virus propagation and the strength of the immune response; in this regard, spontaneous resolution of hepatitis represents a predominance of the immune system over the virus. In patients with competent immune systems, virus persisting at low levels could provide protracted antigenic stimulation and priming of the immune system. This notion is supported in studies in which $CD8^+$ T cell infiltrates were observed in the liver of patients who apparently resolved viremia and had normal liver functions (Hoare et al., 2008) and HCV-specific T cells were found to be circulating in patients with no clinical signs of infection (Quiroga et al., 2006). Along the same line, HCV-specific $CD4^+$ and $CD8^+$ T cell responses have been described in patients who were exposed to HCV eighteen years earlier but had undetectable anti-HCV antibodies (Takaki et al., 2000). Recently, work from our lab has shown that in individuals with low-level (occult) HCV infection, either in the presence or absence of occult HBV co-infection, histological examination of livers biopsies obtained up to 5 years after clinical SVR revealed low grade inflammation in most cases although in almost all patients improvement in the degree of liver lesions was evident (Pham et al., manuscript submitted). The consequences of these intrahepatic immune infiltrates are unclear but the possibility exists that these T cells might be involved in a control of progressing viral replication while, at the same time, they may worsen liver injury and over many years perhaps lead to cirrhosis and HCC in some

cases. On the other hand, considering that in patients with CHC T lymphocytes can exhibit an exhausted phenotype, infiltrating lymphoid cells may be rendered functionally defective or exhausted which would lead to poor control of viremia and promote viral persistence.

In situations of immune compromise, the pool of virus persisting at low-levels could increase due to the absence of immune control and this may lead to reactivation of symptomatic infection. This has been observed in apparently HCV RNA-negative individuals receiving corticosteroid therapy (Lee et al., 2005), those treated with immunosuppressive agents after having undergone renal transplantation (Melon et al., 2005; Savas et al., 2007), and individuals receiving bone marrow transplants (Zekri et al., 2004).

Taking all of our findings into consideration, this work has furthered our understanding of the ability of cells of the immune system to act as a reservoir of HCV. Viral propagation in this compartment, occurring albeit at a low rate, is resulting in the production and assembly of infectious virions. We have, for the first time, shown clear evidence that these particles are able to *de novo* infect virus-naïve lymphoid cells. We showed that ongoing replication in this cell compartment may contribute to viral spread to naïve lymphocytes.

While the clinical consequences of the lymphatic (immune) system HCV infection are presently unclear, we believe that in certain cases, i.e., such as during immunosuppression, co-infection with other pathogens or toxic (anti-cancer) therapy, this residual virus can be reactivated. Overall, the model of infection and propagation of

wild-type HCV in normal human cells that was established and utilized in this work is a tool that can be employed to examine cytopathic mechanisms of HCV infection and can be used to uncover factors mediating susceptibility of host cells to this virus. Finally, at a time in which treatment modalities for HCV are shifting from IFN/RBV alone to incorporate HCV-specific drugs, such a system will be valuable to determine whether the virus can finally be eradicated not only from the liver but also from extrahepatic sites that can potentially serve as a source for reinfection of hepatocytes.

Chapter Six: Conclusions and Future Directions

Throughout these experiments, we examined how HCV infection in lymphoid cells may contribute to viral persistence. We investigated whether cells of the lymphatic system can be infected *in vitro* with HCV and whether these cells have the ability to produce complete infectious virions. We explored whether the functions of T cells could be altered following exposure to or infection with HCV. We further studied the infective potential of virus lingering at low levels after clinical resolution of hepatitis C using the *in vitro* HCV infection system established in the course of these works. The findings of these studies can be summarized and concluded as follows:

Study 1: The multiparametric analysis revealed that mitogen-stimulated T cells are susceptible and supportive of the complete cycle of HCV replication including the production of infectious virions capable of *de novo* infecting virus-naïve cells. The findings conclusively showed that T cells do not merely harbour adhered virus but can support propagation of biologically competent, infectious virus. The *in vitro* replication system created is also able to support replication of wild-type virus of different genotypes, although it is not as efficient, in terms of virus production, as recently established systems applying molecularly adapted HCV strains and Huh-7 hepatoma cells. This system represents a tool for investigations of factors that promote virus replication and determine susceptibility to infection, and can be employed to test the efficacy of novel antiviral treatment modalities.

Study 2: We found that *in vitro* infection of T cells with HCV can lead to a change in the T cell subset distribution, with an apparent enrichment of $CD8^+$ T cells. This phenotype shift appeared to be a result of an inhibition of $CD4^+$ T cell proliferation in the presence of virus. Meanwhile $CD8^+$ T cell proliferation and both $CD4^+$ T cell and $CD8^+$ T cell apoptosis were unaffected by virus exposure. As such, we conclude from this study that HCV infection or even exposure to virus may affect the function of $CD4^+$ T cells. The mechanism mediating this effect is yet unclear. It is presumed that a comparable event can occur *in vivo*, promoting virus persistence.

Study 3: For the first time, this study revealed that residual HCV, detectable at low quantities for years after apparent complete resolution of CHC due to standard antiviral IFN/RBV therapy, retains its infectious potential and is capable of producing infection in mitogen-induced T cells *in vitro.* This strongly suggests that HCV persisting at low-levels in the seemingly asymptomatic individuals can be a source of infectious HCV under certain conditions.

As far as the future directions of this work, the *in vitro* infectivity of lymphoid-derived HCV in primary human hepatocytes cultures has yet to be shown. This would further support the notion that viral replication in the immune cell compartment may have implications regarding HCV persistence and recurrence. Specific factors that mediate virus entry into T cells and facilitate replication of HCV in this compartment should be examined. As well, the infectivity of HCV in other lymphocyte populations, such as B cells or dendritic cells should be investigated. In T cell cultures, full sequencing of HCV RNA genomes propagated *in vitro* at different time points may reveal the appearance of

adaptive mutations which could be subsequently tested for *in vitro* infectivity in hepatocyte cultures. In terms of the second study, it remains to be determined why $CD4^+$ T cells are proliferating to a lesser degree than $CD8^+$ T cells and whether the same situation occurs in HCV-infected patients. To uncover whether the virus is acting directly on $CD4^+$ T cells or indirectly by acting on other cells, we would like to expose cultures of affinity purified $CD4^+$ T cells to HCV in parallel with total PBMC and measure T cell proliferation. As well, although our limited analysis of cytokine gene expression did not show a difference between cells exposed or not to HCV, it would be interesting to determine the levels of IL-2 secreted into culture supernatant by ELISA to determine the amount of IL-2 being secreted, and by ELISPOT to determine whether the frequency of IL-2-secreting cells is diminished. In addition, the question rises of whether treatment of HCV-infected $CD4^+$ T cells with IFN-α will rescue proliferation. Microarray analysis of RNA, confirmed by quantitative RT-PCR, may reveal gene expression changes in T cells after exposure to HCV.

Regarding our study on *in vitro* infectivity of low levels of HCV persisting in patients who resolved CHC, it would be highly valuable to investigate further this topic by employing an appropriate animal model. However, this in highly challenging due to the fact that chimpanzees are practically not available for HCV research and the *Scid*-Alb/uPA mouse model of infection requires approximately 10^5 vge in no more than 500 μL to infect a singular animal. To acquire this amount of virus would be very difficult, since individuals with OCI usually carry no more than 10^2 vge per mL of plasma. Thus, to infect one animal virus recovered from approximately 1 liter of plasma would be required.

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