INFECTION AND PROPAGATION OF HEPATITIS C VIRUS IN HUMAN CD4$^+$ AND CD8$^+$ T LYMPHOCYTES *IN VITRO*

by

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ABSTRACT

Accumulated molecular and clinical evidence indicate that human immune cells can support replication of hepatitis C virus (HCV). The aim of this study was to investigate the ability of authentic, plasma-occurring HCV to infect human $CD4^+$ and $CD8^+$ T lymphocytes *in vitro*. For this purpose, we adopted the previously established *in vitro* HCV replication system in total T cells derived from cultured normal human PBMC by employing affinity-purified $CD4^+$ and $CD8^+$ T lymphocytes as targets. Using this system, we were able to demonstrate that molecularly intact HCV can infect and productively replicate in both $CD4^+$ and $CD8^+$ T lymphocytes, albeit at low levels, by documenting: (1) Presence of HCV positive and replicative (negative) strands in infected cells; (2) Intracytoplasmic localization of viral proteins; (3) Emergence of new HCV variants in the *de novo* infected cells, and (4) Release of HCV RNA-reactive particles from the infected cells with biophysical properties distinct from those of virions in inocula used to infect these cells.

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As I close this chapter of my life not only I feel privileged for everything I have gained academically during my years in Memorial, but I also hope that I have, in turn, contributed into making this University a better place for women, and students in general.

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ABBREVIATIONS

ACD-A	acid-citrate-dextrose anticoagulant, solution
ACK	ammonium-chlorate-potassium
ADAR1	RNA-specific adenosine deaminase-1
AHB	acute hepatitis B
AHC	acute hepatitis C
ALT	alanine aminotransferase
APC	Allophycocyanin
apoB	apolipoprotein B
apoE	apolipoprotein E
AT	ambient temperature
BCR	B-cell receptor
Bp	base pairs
BM	bone marrow
BMI	bone marrow intensity
BNX	beige/nude/X-linked
BSA	bovine serum albumin
CHV	canine hepacivirus
CHB	chronic hepatitis B
CHC	chronic hepatitis C

CLDN-1	claudin-1
CNS	central nervous system
CPM	counts per minute
CSF	cerebrospinal fluid
DAA	direct acting antivirals
DAPI	4', 6-diamidino-2-phenylindole
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
d.p.i.	days post-infection
DTT	Dithiothreitol
E1	envelope 1
E2	envelope 2
EDTA	Ethylenediaminetetraacetic acid
EB	elution buffer
ER	endoplasmic reticulum
EIA	enzyme immunoassay
EGFR	epidermal growth factor receptor
ESLD	end-stage-liver-disease
EPHA2	ephrin type A receptor 2
EHV	equine hepacivirus
EPgV	equine pegivirus

EVR	early virologic response
FDA	Food and Drug Administration, USA
FITC	fluorescein isothiocyanate
FKBP	FK506 binding protein
GAG	glycosaminoglycan
GBV-C	GB virus C
GBV-B	GB virus B
GHV	guereza hepacivirus
GDBS	global database on blood safety
GN	glomerulonephritis
H	Hour
HAV	hepatitis A virus
HBSS	Hank's buffered salt solution
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCVcc	cell culture-derived HCV
HCVpp	HCV pseudoparticles
HDP	healthy donor plasma
HDV	hepatitis D virus
HEV	hepatitis E virus
HGV	hepatitis G virus

HIV	human immunodeficiency virus type 1
HHpgV-1	human hepegivirus-1
HLA	human leukocyte antigen
HVR	hypervariable region
ICAM-3	intercellular adhesion molecule-3
IDU	intravenous drug users
IEM	immune electron microscopy
IFIH1	interferon-induced helicase
IFN	interferon
Ig	immunoglobulin
igVR	intergenotypic variable region
IL	interleukin
IOM	Institute of Medicine
IPS	promoter-stimulator protein 1
iPSCs	induced pluripotent stem cells
IRES	internal ribosomal entry site
IRF	interferon-regulatory factor
IRRDR	IFN/ ribavirin resistance-determining Region
ISDR	interferon-α sensitivity-determining region
ISGs	interferon-stimulated genes
ISGF3	ISG factor 3

IU	international unit
JEV	Japanese encephalitis virus
JFH-1	Japanese fulminant hepatitis-1
Kb	kilobase
KIR	killer cell immunoglobulin-like receptor
LB	Luria-Bertani
LCL	lymphoblastoid cell line
LDL	low density lipoprotein
LDL-R	low density lipoprotein-receptor
LMP	low melting point
L-SIGN	liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin
LN	lymph node
mAb	monoclonal antibody
MAVS	mitochondrial antiviral-signaling protein
MC	mixed cryoglobulinaemia
MDA-5	melanoma differentiation-associated protein 5
mDC	myeloid dendritic cell
MHC	major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
Min	minute

miR-122	microRNA -122
M-MLV	Moloney-murine leukemia virus
MPCCs	micropatterned cocultures
MTP	microsomal transfer protein
MVEV	Murray valley encephalitis virus
NANB	non-A non-B hepatitis
NAT	nucleic acid testing
NFAT	nuclear factor of activated T cells
NFKB	nuclear factor kB
NHL	non-Hodgkin's lymphoma
NIH	National Institutes of Health
NK	natural killer
NPC1L1	Niemann-Pick C1–like 1
NS	non-structural
OAS	2'5'-oligoadenylate-synthetase
OCI	occult hepatitis C infection
OCLN	Occludin
OLP	oral lichen planus
OLT	orthotopic liver transplantation
ORF	open reading frame
PAMPS	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells

PBS	phosphate-buffered saline
PCT	porphyria cutanea tarda
PD-1	programmed-death 1
pDC	plasmacytoid dendritic cell
PDU	pixel density unites
PE	Phycoerythrin
PEG-IFN-α	pegylated interferon alpha
PerCP	peridinin chlorophyll protein complex
PFA	paraformaldehyde
PHA	phytohemagglutinin
PHH	primary human hepatocytes
PKR	protein kinase R
PRRs	pattern recognition receptors
Rag2	recombination-activating gene 2
RBV	Ribavirin
RdRp	RNA-dependent RNA-polymerase
RIG-I	retinoic-acid inducible gene-I
RNA	ribonucleic acid
RT	reverse transcriptase
RTK	receptor tyrosine kinase
RHV	rodent hepacivirus
S	second

SCID	severe combined immunodeficient
SDS	sodium dodecyl sulfate
SPgV	simian pegivirus
SLEV	St. Louis encephalitis virus
SOCS3	suppressor of cytokine signalling 3
sRNA	synthetic RNA
SR-B1	scavenger receptor B1
SRCR	scavenger receptor cysteine-rich
SRFBP1	serum response factor binding protein 1
SSCP	single-stranded conformational polymorphism
STING	stimulator of interferon genes
SVR	sustained virological response
TE	1 mM EDTA in 10 mM Tris-HCl buffer, pH 8.0
TBK1	tank-binding kinase-1
TCAG	The Centre for Applied Genomics
TfR1	transferrin receptor 1
Th-1	T-helper type 1
Tim-3	T-cell immunoglobulin and mucin domain-containing molecule 3
TLR	toll-like receptors
TNF	tumor necrosis factor
TRIF	toll-IL1-receptor domain-containing adaptor inducing IFN-β
TTI	transfusion-transmissible infection

uPA	urokinase-type plasminogen activator
UTR	untranslated region
VGE	virus genomic equivalents
VLDL	very low density lipoproteins
WNV	West Nile virus
WHO	World Health Organization

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CHAPTER ONE: INTRODUCTION

1.1 Viral Hepatitis

Hepatitis, coined from the ancient Greek word hepar (ἦπαρ), meaning liver and the suffix –itis, indicating inflammation, is an inflammatory liver disease characterized by hepatocyte injury, including necrosis, associated with lymphomononuclear cell infiltration.

Viral infection is the most common cause of hepatitis. There are six viruses (hepatitis A through G viruses) capable of causing hepatitis. Hepatitis A virus (HAV) is a small, non-enveloped, single-stranded RNA virus, member of the *Picornaviridae* family. HAV is transmitted by the faecal-oral route and, in most cases, causes acute, self-limiting infection. An HAV vaccine has been available since 1992 and it is capable of conferring life-long protection (Orli and Arguedas, 2006).

Hepatitis B virus (HBV) is a partially double-stranded DNA virus of the *Hepadnaviridae* family, which is carried in the blood and other bodily fluids (***i.e.***, saliva, tears, semen, vaginal secretions) of the infected individuals. Therefore, it can be transmitted via sexual intercourse, the parenteral route, or from the infected mother to the baby. Acute hepatitis B (AHB) can progress to chronic hepatitis B (CHB) which, in up to 10% of individuals infected during adulthood, can lead to life-threatening liver cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC) (Ganem and Prince, 2004). HBV may also persist as occult, asymptomatic infection which is the consequence of resolved AHB or primary infection with very low virus doses (Michalak *et al*., 1994;

Michalak *et al*., 2004; Mulrooney-Cousins and Michalak, 2007). More than 780,000 individuals die every year due to the acute fulminant or chronic consequences of HBV infection (World Health Organization [WHO], 2014). An effective vaccine against HBV has been available almost for thirty years; however, the present estimated global vaccine coverage is 69% (Zanetti *et al*., 2008). Notably, an estimated 4.5 million individuals are infected every year worldwide (Goldstein *et al*., 2005).

Hepatitis D virus (HDV) is a small, single-stranded, negative sense RNA virus of the genus Deltavirus, which has not yet been classified to a family. HDV is a subviral RNA satellite virus that depends on HBV envelope for infection, virion assembly and transmission (Polish *et al*., 1993). Therefore, it has a similar transmission route as HBV. HDV can be acquired as either a coinfection with HBV or superinfection of HBV-infected individuals. Vaccination against HBV also confers protection against HDV.

Hepatitis E virus (HEV) is a small, non-enveloped, single-stranded RNA virus, member of the *Hepeviridae* family. HEV is usually transmitted via the faecal-oral route. Acute HEV infection is a self-limited disease with a case-fatality rate lower than 0.1%. In pregnant women, HEV infection is more life threatening as it leads to fulminant hepatic failure and death in 15% to 20% of cases (Patra *et al*., 2007). The first HEV vaccine, HEV 239 (Zhu *et al*., 2010) was approved in China in January 2012, but cost and its long term efficacy remain a concern.

Hepatitis G virus (HGV), currently referred to as GB virus C (GBV-C), is an enveloped, single-stranded, positive RNA virus, member of the *Flaviviridae* family and the *Pegivirus* genus. HGV is most commonly transmitted via the parenteral route and

generally causes a mild, self-limited infection. HGV is primarily lymphotropic (Handa and Brown, 2000) and it is the human virus most closely related to hepatitis C virus (HCV), which is the virus studied in this thesis.

1.2 HCV Infection

1.2.1 Epidemiology

The global prevalence of HCV is not well known due to frequently asymptomatic nature of acute infection. It is estimated by WHO that there are currently 130-150 million chronically infected individuals (WHO, 2014). These individuals have a high risk of developing liver cirrhosis and HCC.

Even though HCV is endemic worldwide, there is a great geographic variability in its distribution. The lowest HCV prevalence is reported in industrialized nations in Western Europe, North America and Australia, whereas the highest prevalence is observed in countries located in Asia and Africa (Shepard *et al*., 2005) with Egypt having the highest reported seroprevalence rate of 22% (Frank *et al*., 2000). This large reservoir of chronic HCV infection is believed to have been established during nationwide schistosomiasis parenteral treatment campaigns (Frank *et al*., 2000). The second highest seroprevalence rate is reported in Pakistan, where it ranges between 2.4% and 6.5% of the population, and it is followed by China with a seroprevalence rate of 3.2% (Shepard *et al*., 2005). In Canada, it is estimated that around 250,000 persons are chronically infected with HCV, with a prevalence rate of 0.78%, and at least 50,000 of them remain undiagnosed (Remis, 2007).

HCV is classified into six major genotypes (designated as 1-6), and more than 100 subtypes (designated as a, b, c, etc.), on the basis of genomic sequence heterogeneity (Simmonds *et al*., 1993). HCV genotypes are known to have unique patterns of geographical distribution. In particular, genotype 1a is distributed widely in North America and Europe, genotype 3a is found commonly in Europe, while genotype 1b is the commonest worldwide and it is responsible for up to 73% of HCV cases in Japan (Takada *et al*., 1993; Simmonds, 2004). Genotype 2 is mostly detected in Mediterranean countries, Western Africa and Far East (Mellor *et al*., 1995; Simmonds *et al*., 2004). Genotype 3 is endemic in South-East Asia, but is also distributed throughout India, the Far East and Australia (Tokita *et al*., 1994a, 1994b, 1995; Mellor *et al*., 1995; Simmonds, 2004). Genotype 4 is most widely distributed in Middle East and Africa, particularly Egypt, where it accounts for more than 90% of infections (Ramia and Eid-Fares, 2006). Infections caused by genotype 5 are common in South Africa (Cha *et al*., 1992). Finally, genotype 6 is mainly distributed in South-East Asia and Australia (Simmonds, 2004). Determining HCV genotypes is of great clinical significance, as they play a role in disease pathology and response to treatment (see Sections 1.6.3. and 1.11.).

1.2.2 Transmission Routes and Risk Factors

HCV is most commonly transmitted parenterally (Conry-Cantilena *et al*., 1996). The primary risk factor for HCV transmission in the developed world is intravenous drug use (IDU). In USA and Australia, 68% and 80% of current HCV infections, respectively, are caused by IDU (Alter, 2002; Dore *et al*., 2003). IDU has been identified as the major

risk factor in several European countries as well (Elghouzzi *et al*., 2000; Dalgard *et al*., 2003; Balogun *et al*., 2003). The role of IDU in HCV transmission in developing countries remains unclear due to the very limited availability of relevant data, however, unsafe therapeutic injection practices constitute a major risk factor for HCV infection in these countries (Frank *et al*., 2000). This is not the case for the developed world, with the exception of isolated outbreaks occurring mostly in nonhospital health care settings (Thompson *et al*., 2009; Fischer *et al*., 2010). In these settings the presumed mechanism of infection is patient-to-patient transmission due to negligence of health care personnel to comply with basic principles of aseptic technique and infection control (e.g. syringe reuse).

Another high risk factor for HCV infection, mostly in developing countries, is blood transfusion. WHO's Global Database on Blood Safety (GDBS) has estimated that 47% of blood donations in low-income countries are not screened for transfusion-transmissible infections (TTIs), including HCV (GDBS summary report, 2011). In developed countries, blood transfusion was a high risk factor before 1992 when HCV screening of blood donations was introduced. Since then, the risk of TTIs has been decreased so dramatically that classic methods of measurement of transfusion risk are no longer able to determine risk or identify transfusion-associated transmission events (Busch *et al*., 2003).

HCV can also be transmitted via sexual contact (Alter *et al*., 1989), but far less efficiently than other sexually transmitted viruses, including HBV and human immunodeficiency virus type 1 (HIV). More specifically, among people in long-term,

monogamous relationships, the risk of HCV acquisition ranges between 0-0.6% per year (Terrault, 2002) as determined by standard HCV RNA detection assays. Regarding HCV vertical transmission, the rate ranges between 4-7% for pregnant women with high level of viremia (Ohto *et al*., 1994; Roberts and Yeung, 2002). In the setting of HIV co-infection, the rate of vertical transmission increases by 4 to 5-fold (Zanetti *et al*., 1995). Other risk factors for HCV transmission include accidental sticks with contaminated needles, hemophilia, hemodialysis, organ transplants conducted before 1992, intranasal cocaine use, body piercing, and tattoos (Villena, 2006).

1.3 Flaviviridae Family

The *Flaviviridae* family of viruses is subdivided into *the* flavivirus, hepacivirus, pegivirus and pestivirus genera. The *Flaviviridae* family was named after the yellow fever virus (*flavus* in Latin means yellow). Viruses belonging to the *Flaviviridae* family are spherical with a diameter of 40-60 nm. They contain a single, positive-strand RNA genome which is enclosed within a nucleocapsid contained in a host-cell derived lipid envelope (Petersen and Roehrig, 2001). Their genome ranges between 9.5-12 Kilobases (Kb) in length, lacks both a 5'-cap structure and a 3'-poly-A tail, and it is flanked by 3'- and 5'-untranslated regions (UTR). Following infection, the genome is translated into a large polyprotein which is modified and cleaved to generate 10 proteins (3 structural and 7 nonstructural), common to all the flaviviruses (Lindenbach *et al*., 2007). The flaviviruses are associated with great global health and economic burdens. Other members of the *Flavivirus* genus include Dengue virus, Japanese encephalitis virus (JEV), Murray Valley

encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV), West Nile virus (WNV) and yellow fever virus. The *Pestivirus* genus includes bovine viral diarrhea virus 1 and 2, border disease virus, and classical swine fever virus. HGV is a member of the *Pegivirus* genus which includes but is not limited to human hepegivirus-1 (HHpgV-1), simian pegivirus (SPgV), and equine pegivirus (EPgV). HCV is a member of the Hepacivirus genus which also includes canine hepacivirus (CHV), equine hepacivirus (EHV), GB virus B (GBV-B), guereza hepacivirus (GHV), and Rodent hepacivirus (RHV).

1.4 Hepatitis C Virus

1.4.1 Genome Organization

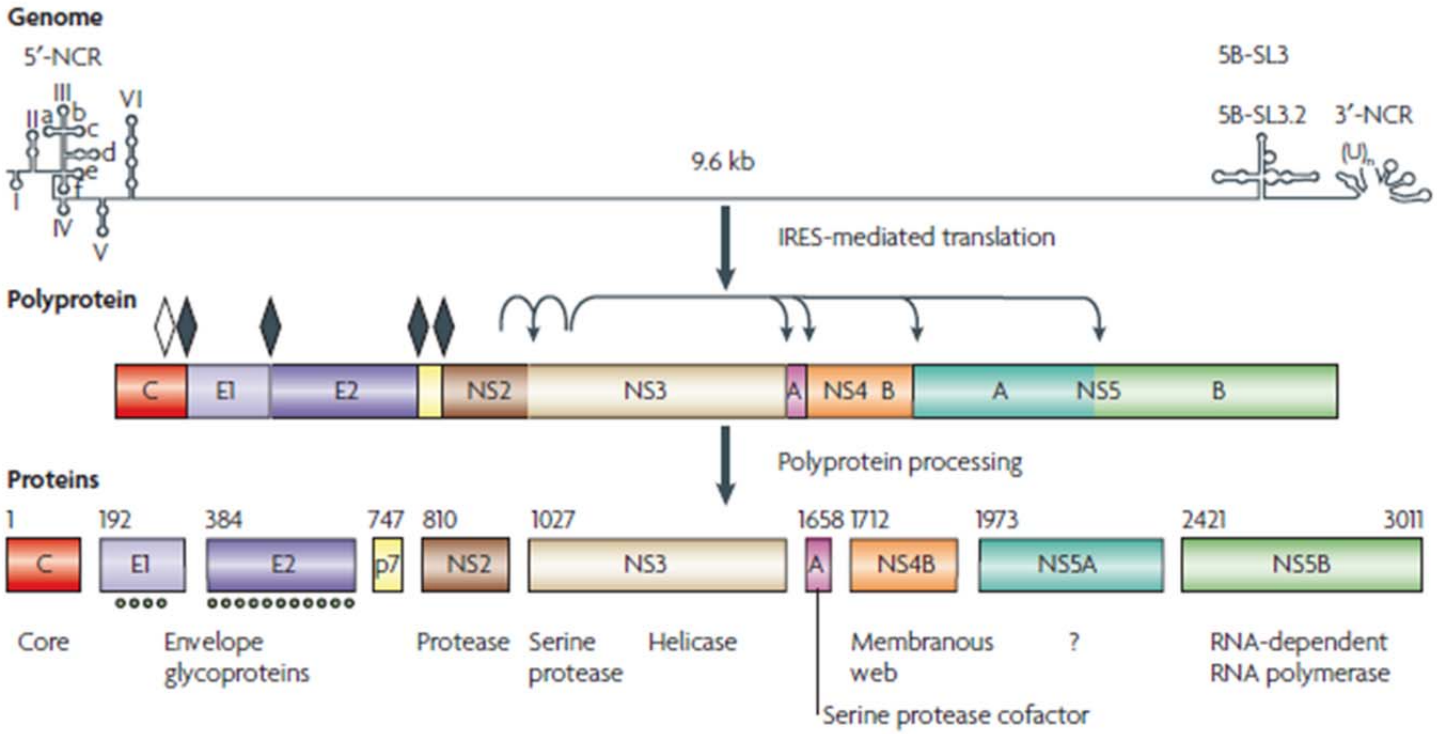
The HCV genome consists of a 9.6-Kb single-stranded, positive-sense RNA composed of a 5'-UTR, a long open reading frame (ORF), and a 3'-UTR (Figure 1.1) (Choo *et al*., 1989). The 341 nucleotide (nt)-long HCV 5'-UTR contains an internal ribosomal entry site (IRES) (Brown *et al*, 1992; Tsukiyama-Kohara *et al*., 1992), which is indispensable for cap-independent translation of HCV RNA (Honda *et al*., 1999) and is comprised of four highly structured domains (domains I-IV) (Brown *et al*., 1992). Apart from playing an important role in viral protein translation, the HCV 5'-UTR also contains *cis* elements critical for HCV RNA replication (Friebe *et al*., 2001). Furthermore, it has been found that the HCV 5'-UTR can bind to microRNA-122 (miR-122), leading in enhanced viral RNA translation and accumulation within infected hepatocyte-like Huh7 cells (Jopling *et al*., 2005; Wilson *et al*., 2011). The long ORF is translated directly to a large polyprotein precursor of about 3,000 residues (Choo *et al*., 1991), which is co- and post-

translationally processed by viral and cellular proteases to produce 10 structural and nonstructural (NS) proteins. The structural proteins include: core, envelope 1 (E1) and envelope 2 (E2), while the NS proteins are: NS2, NS3, NS4A, NS4B, NS5A and NS5B, and the peptide p7 which is not yet assigned to either category (Figure 1.1) (Bartenschlanger *et al*., 2004; Penin *et al*., 2004). The 200-235 nt-long HCV 3'-UTR contains a short variable region, a poly(U/UC) sequence, and a practically invariable nt sequence, named the X-tail or 3'X region (Kolykhalov *et al*., 1996). The X-tail together with part of the poly(U/UC) tract are essential for RNA replication, while the remainder of the 3'-UTR contributes to enhancement of replication (Friebe and Bartenschlager, 2002; Yi and Lemon, 2003).

1.4.2 Proteins and Their Function

The HCV core protein is the first protein to be encoded by the HCV ORF. After being cleaved from the polyprotein by host signal peptidases, it binds viral RNA (Santolini *et al*., 1994) and presumably assembles into HCV capsids at the cytoplasmic surface of the endoplasmic reticulum (ER) (Mizuno *et al*., 1995; Blanchard *et al*., 2002; Blanchard *et al*., 2003). The HCV core protein is mostly found in the cytoplasm, in association with the ER, membranous webs (see below; Section 1.5.2.), lipid droplets and mitochondria. However, in certain cases, it has also been detected in the nucleus (Suzuki *et al*., 1995). In addition to its main role, which is the formation of the capsid that will store and protect HCV RNA, core protein most likely plays an important role in viral replication, virion maturation and pathogenesis (Polyak *et al*., 2006). Furthermore, HCV

Figure. 1.1: Schematic representation of the HCV genome and its polyprotein processing. Image from Moradpour *et al*., 2007.


Genome
5′-NCR
5B-SL3
5B-SL3.2
3′-NCR
9.6 kb
IRES-mediated translation
Polyprotein
C
E1
E2
NS2
NS3
A
NS4 B
A
NS5
B
Polyprotein processing
Proteins
1
192
384
747
810
1027
1658
1712
1973
2421
3011
C
E1
E2
p7
NS2
NS3
A
NS4B
NS5A
NS5B
Core
Envelope glycoproteins
Protease
Serine protease
Helicase
Membranous web
?
RNA-dependent RNA polymerase
Serine protease cofactor

core protein modulates several host cell functions, such as gene transcription, lipid metabolism, apoptosis and various intracellular signaling pathways, by interacting with numerous cellular proteins (reviewed in Tellinghuisen and Rice, 2002).

E1 and E2 are highly glycosylated, transmembrane proteins, cleaved from the polyprotein by host signal peptidases, which form non-covalent, heterodimeric complexes that constitute the building blocks of the virion envelope (Lavie *et al*., 2007). E1 protein acts as the virion envelope-host membrane fusion subunit, while E2 recognizes and binds to various putative HCV cell receptors, as described in Section 1.4.3. The E2 protein sequence contains three highly variable regions: hypervariable region 1 (HVR1), HVR2, and the intergenotypic variable region (igVR) (Hijikata *et al*., 1991; McCaffrey *et al*., 2011).

The HCV p7 protein is a short protein, which is released from the polyprotein by host signal peptidases and possesses cation channel properties (Griffin *et al*., 2003; Pavlovic *et al*., 2003). Those properties, together with predicted structural and topological features, suggest that p7 belongs to the viroporin family of proteins (Harada *et al*., 2000; Premkumar *et al*., 2004). It has been shown that p7 is indispensable for productive infection *in vivo* (Sakai *et al*., 2003). It also plays an important role in virus particle assembly and release of infectious virions (Steinmann *et al*., 2007; Atoom *et al*., 2013). Finally, p7 may play an important role in viral envelopment by protecting E2 from premature degradation through an ion channel-like activity (Atoom *et al*., 2013). NS2 is a transmembrane protein encompassing part of the NS2-3 protease, also known as autoprotease, which is responsible for cis cleavage at the NS2-NS3 junction (Lorenz *et*

al., 2006; Santolini *et al*., 1995; Yamaga and Ou, 2002). NS2 is dispensable for HCV RNA replication and appears to be essential for completion of the HCV replication cycle both *in vivo* and *in vitro* (Kolykhalov *et al*., 2000; Pietschmann *et al*., 2006). Furthermore, it has been shown that NS2 interacts with structural and other NS proteins to coordinate virus assembly (Ma *et al*., 2010; Popescu *et al*., 2011).

NS3 is a multifunctional HCV protein with a serine protease activity located in its N-terminus and a NTPase/RNA helicase activity in its C terminus (Gallinari *et al*., 1998). The NS3 protease, with NS4A as a co-factor (Kim *et al*,, 1996), cleaves the HCV polyprotein at the NS3-4A, 4A-4B, 4B-5A and 5A-5B junctions (Bartenschlanger *et al*., 1993). The enzymatic activity of NS3 as a NTPase/RNA helicase renders NS3 indispensable for RNA replication. NS4B is an integral membrane protein that is co-localized with other NS proteins at the ER membrane (Hugle *et al*., 2001). The main role of NS4B is inducing the formation for the membranous web, a specialized membrane structure which is thought to be the HCV replication site (Egger *et al*., 2002; Gao *et al*., 2004).

The HCV NS5A protein is a membrane-anchored phosphoprotein which exists in several phosphorylation states. NS5A plays an important role in viral replication. Numerous cell culture-adaptive mutations located in NS5A have been found to enhance HCV RNA replication (Blight *et al*., 2000; Krieger *et al*., 2001; Lohmann *et al*., 1999). Many of these mutations affect hyperphosphorylation of NS5A, denoting that HCV replication efficiency might be affected by NS5A phosphorylation status. Furthermore, NS5A plays an important role in HCV infectious particle assembly (Appel *et al*., 2008).

In particular, domain III has been identified as the key determinant in NS5A for particle formation. NS5A also contains two regions, called interferon (IFN)/ribavirin (RBV) resistance-determining region (IRRDR) and interferon-α (IFN-α) sensitivity-determining region (ISDR) thought to play a role in the response to IFN treatment (Enomoto *et al*., 1996; Zeuzem *et al*., 1997; El-Shamy *et al*., 2008). It has been postulated that high number of mutations in these areas correlates with lower resistance to IFN-α and, therefore, higher IFN treatment response rates. NS5B is a tail-anchored protein, targeted post-transnationally in the cytoplasmic side of the ER. NS5B acts as an RNA-dependent RNA-polymerase (RdRp) which drives HCV replication. It is responsible for the synthesis of the negative (replicative) HCV RNA strand by using the genome as a template and the subsequent synthesis of the positive (genomic) RNA strand from this replicative intermediate (Behrens *et al*., 1996). Due to its central role in HCV replication, NS5B constitutes a promising target for antiviral agents (Harper *et al*., 2005; Murakami *et al*., 2007).

1.4.3 HCV Life Cycle

The first step in the HCV replication cycle is attachment of viral proteins, mainly E2, to host cell receptors. This interaction determines virus tissue tropism and will be discussed in detail in Sections 1.5.1. and 1.5.2. Attachment to the target cell is followed by virion internalization via clathrin-mediated endocytosis (Blanchard *et al*., 2006). Acidification of the endosomes leads to HCV glygoprotein–cell membrane fusion, which

is followed by uncoating and release of the HCV RNA into the cytoplasm (Blanchard *et al*., 2006).

Once inside the cell, the positive-sense HCV RNA is directly translated by a cap-independent IRES-mediated process (Wang *et al*., 1993). The translation process yields a large polyprotein which is co- and post-translationally cleaved by both host and viral proteases, as described in Section 1.4.2.

HCV replicates via synthesis of a negative-strand HCV RNA which serves as a template for HCV RNA positive strand synthesis. Thus, the detection of HCV RNA negative strand serves as an important marker of active replication. As mentioned in Section 1.4.2, NS5B catalyzes HCV RNA synthesis (Lohmann, 1997) and, since it lacks stringent template specificity and proofreading activity, it is characterized by a high error rate contributing to the generation of large pool of HCV quasispecies found in each host (discussed in Section 1.4.5). In human hepatoma Huh-7 cells bearing HCV subgenomic replicons, it was shown that the site of HCV RNA replication is an ER membrane alteration, called the membranous web (Gosert *et al*., 2003; Mottola *et al*., 2002). Studies have also shown that HCV RNA replication is influenced by cholesterol and lipid metabolism (Ye *et al*., 2004; Kapadia and Chisari, 2005; Sagan *et al*., 2006).

It has been suggested that HCV assembly and maturation takes place in the ER and post-ER compartments, and that virions exit through the secretory pathway. Studies suggest that lipid droplets (intracellular organelles used for neutral fat storage) interact with HCV core and together they play a key role in assembly and production of infectious virions (Miyanari *et al*., 2007). Furthermore, it was shown that HCV assembly and

maturation in Huh7 cells requires microsomal transfer protein (MTP) and apolipoprotein B (apoB), mimicking the synthesis of very low density lipoproteins (VLDL) (Gastaminza *et al*., 2007).

1.4.4 Virion Ultrastructure and Biophysical Properties

HCV particles are spherical ranging from 40-75 nm in diameter (Kaito *et al*., 1994; Wakita *et al*., 2005; Gastaminza *et al*., 2010). As previously mentioned, core together with E1 and E2 proteins constitute the main structural components of the HCV virion. In particular, E1 and E2 proteins are anchored in the host cell-derived lipid envelope which encapsulates an isocathedral nucleocapsid composed by multiple copies of the core protein and the genomic viral RNA (Kaito *et al*., 1994). HCV particles can circulate in various forms in sera of HCV-infected patients (reviewed in André *et al*., 2005). These forms include: free virions, virions bound to immunoglobulins, non-enveloped HCV nucleocapsids (also known as naked cores) which are not infectious, and particles physically associated with low-density lipoproteins (LDL) and VLDL, both of which represent the infectious fractions. This characteristic explains the great heterogeneity in HCV buoyant density. For example, using patient plasma, it was shown that the buoyant density of the non-enveloped particles in a sucrose gradient ranges from 1.23-1.27 g/mL, while the enveloped virions band at densities ranging from 1.05-1.11 g/mL (Kaito *et al*., 1994) with peak infectivity in the fractions ranging from 1.09-1.11 g/mL, as determined by challenging of chimpanzees with different gradient fractions (Bradley *et al*., 1985).

1.4.5 HCV Mutants and Quasispecies

HCV is a highly diversified virus with genetic variability existing at different levels. The genetic divergence is greatest between the 6 different genotypes which differ in 30% to 35% in nucleotide sequences (Simmonds *et al*., 1993). The greatest levels of genetic diversity between genotypes are observed in the genomic regions encoding the two envelope glycoproteins and mainly in the E2 protein, as previously mentioned (Section 1.4.2.). Areas, such as the core gene and the genes encoding the non-structural proteins, are more conserved. The most conserved area of the HCV genome is the 5'-UTR, which encompasses sequences and secondary structures necessary for the initiation of viral replication and translation (Bukh *et al*., 1995).

Each of the 6 HCV genotypes encompasses a group of more closely related subtypes, showing a difference in nt sequence ranging between 20% and 25% (Simmonds *et al*., 1993). Subtypes 1a, 1b and 3a, represent the major subtypes in the Western world (Simmonds *et al*., 2004).

HCV continuously diversifies within the host, forming groups of related, but genetically distinct variants that share sequence similarity, named quasispecies (Martell *et al*., 1992). With an RdRp that lacks proofreading (misincorporation frequencies averaging about 10^4 to 10^5 per nucleotide copied) and 10^{12} virions produced daily (Pawlotsky, 2003) every possible HCV mutant is produced several times a day within the infected host. HCV quasispecies play a very important role in liver disease progression, response to IFN therapy, and resistance to direct acting antivirals (DAA). More

specifically, patients who spontaneously resolve hepatitis C, harbor a more homogenous quasispecies population, while patients who progress to chronic hepatitis C (CHC) contain more diverse variant spectra (Farci and Purcell, 2000). Furthermore, patients with minimal HCV diversity have less chances of harboring treatment-resistant quasispecies and, therefore, are more likely to achieve clinically apparent sustained virological response (SVR) than patients with greater HCV genome variant complexity (Pawlotsky, 2003; 2006). Finally, all DDAs can select drug resistant variants. Resistance refers to the selection of pre-existing HCV variants that are resistant to a specific drug and are caused by amino acid substitutions found within or close to the region targeted by a given drug (Strahotin and Babich, 2012). As the nonresistant variants are eradicated by the use of a certain DAA, the resistant ones fill in the replication space.

1.5 HCV Tissue Tropism

1.5.1 HCV Hepatotropism

The main target of HCV and site of its propagation is the hepatocyte (Fournier *et al*., 1998; Rumin *et al*., 1999; Castet *et al*., 2002; Lazaro *et al*., 2007). However, *in vitro* infection of primary human hepatocytes (PHH) has been characterized by low levels of HCV replication. It has been suggested that the differentiated status of hepatocyes *in vitro* could be affecting their ability to support HCV replication (Farquhar and McKeating, 2008). As a result, the majority of studies have focused on the interactions between human hepatocarcinoma cell lines (mostly Huh-7 cells and their derivatives) and the HCV pseudoparticles (HCVpp) (described in Section 1.10.2.2) or the laboratory strain

Japanese fulminant hepatitis 1 (JFH-1) (described in Section 1.10.2.3) and JFH-1-derived clones. Using these *in vitro* systems, it was proposed that HCV entry into the hepatocytes is a complex multistep process, mediated by several molecules. Defined subgroups of these molecules possibly dictate HCV hepatotropism and host specificity *in vivo*.

The dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN or CD209) and the liver/lymph node-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing integrin (L-SIGN or CD209L) are C-type lectins thought to contribute to HCV capture and its persistence in the liver (Koppel *et al*., 2005). It was believed that binding of HCVpp to L-SIGN and CD-SIGN is mediated by interaction with the HCV E2 protein (Ludwig *et al*., 2004).

Initial attachment of HCV to cells takes place via low-affinity interaction of the virion-associated apolipoprotein E (apoE) with low-density lipoprotein receptor (LDL-R) (Agnello *et al*., 1999) and glycosaminoglycans (GAGs) (Germi *et al*., 2002). Even though LDL-R is dispensable for HCV entry, it might also be required for increased HCV replication (Albecka *et al*., 2012). Moreover, it has been documented that upregulation of LDR–Rs in Daudi cells (B lymphoblast cell line) was followed by increased HCV RNA positive strand expression in these cells (Burlone *et al*., 2009). With regards to the role of GAGs in HCV entry, it has been reported that HCV occurring in plasma binds GAGs expressed on the surface of African green monkey kidney Vero cells (Germi *et al*., 2002).

CD81, a ubiquitously expressed tetraspanin, was shown to be indispensable for HCV entry based on neutralization of HCV transmission using anti-CD81 antibodies (Wakita *et al*., 2005; Zhong *et al*., 2005; Lindenbach *et al*., 2005). The involvement of

CD81 in HCV entry was first hypothesized when it was found that it could bind to soluble E2 protein (Pileri *et al*., 1998). Its role was later confirmed using both HCVpp and cell culture-derived HCV (HCVcc) and Huh7.5 cells as targets (Bartosch *et al*., 2003a; Hsu *et al*., 2003; Cormier *et al*., 2004; Zhang *et al*., 2004). The role of CD81 in HCV entry requires interaction with another entry factor, claudin-1 (CLDN-1) (Harris *et al*., 2010). Furthermore, CD81 confers tropism of HCV for human cells as it was shown that mice expressing human CD81 in the liver were rendered permissive to HCV infection (Dorner *et al*., 2011; 2013). However, CD81 occurs on many cell types and, therefore, cannot alone determine hepatocyte tropism.

Scavenger receptor-B1 (SR-B1) was identified as an HCV receptor in the HepG2 hepatoma cell line due to its ability to bind recombinant HCV E2 via the HVR1 (Scarselli *et al*., 2002). It was later confirmed as an entry factor for both HCVpp (Bartosch *et al*., 2003b) and HCVcc JFH-1 (described in Section 1.10.2.3) (Catanese *et al*., 2010).

Claudin-1 (CLDN-1), a tight junction protein (Evans *et al*., 2007), is thought to be working together with CD81 to mediate HCVcc entry into Huh7.5 cells (Farkuhar *et al*., 2012). CLDN-6 and CLDN-9 were also found to play role in HCVpp entry into HEK-293 T cells as expression of either of these molecules mediated entry of HCVpp into these cells (Zheng *et al*., 2007).

Occludin (OCLN), another tight junction protein, has been identified as an HCV entry factor by rendering murine cells expressing human CD81, SR-B1 and CLDN1 infectable with HCVpp (Ploss *et al*., 2009). Together with CD81, OCLN confers HCV species tropism, as it was shown that mice engineered to express human CD81 and

OCLN1 in the liver were rendered susceptible to HCV infection (Dorner *et al*., 2011; 2013).

The iron uptake receptor, transferrin receptor 1 (TfR1), which is widely expressed in mammalian cells, including hepatocytes, has also been identified as an HCV entry factor (Martin and Uprichard, 2013). Clinical observational data suggesting that an iron accumulation disorder often occured in the liver of patients with CHC (Fujita *et al*., 2007) prompted investigation for the role of TfR1 in HCV entry.

Niemann-Pick C1–like 1 (NPC1L1) cholesterol uptake receptor has also been identified as an HCV entry factor (Sainz *et al*., 2012). NPC1L1 is expressed in the apical canalicular surface of polarized hepatocytes (Jia *et al*., 2011). Antibody-mediated blocking of NPC1L1 was shown to inhibit HCV entry to Huh7.5 cells in a way similar to that of CD81 antibodies (Sainz *et al*., 2012). Furthermore, the use of Ezetimibe, an FDA-approved NPC1L1-specific inhibitor used to treat hypercholesterolemia, was found to block HCV entry and cell-to-cell transmission *in vitro* (Sainz *et al*., 2012). In the same study, the use of Ezetimibe *in vivo* delayed establishment of HCV genotype 1b infection in mice bearing human liver grafts.

Two receptor tyrosine kinases (RTKs), epidermal growth factor receptor (EGFR) and ephrin type A receptor 2 (EPHA2) were also identified as molecules involved in HCV entry (Lupberger *et al*., 2011). EGFR and EPHA2 promote CD81-CLDN1 interaction (Lupberger *et al*., 2011). Two anticancer compounds, erlotinib and dasatinib, which inhibit EGFR and EPHA2, respectively, were able to block HCV entry in hepatoma cells and PHHs. Erlotinib was also shown to be effective at preventing HCV

infection in human liver chimeric, severe combined immunodeficient (SCID) mice which overexpress a urokinase-type plasminogen activator (uPA) transgene in the liver (Lupberger *et al*., 2011).

Finally, serum response factor binding protein 1 (SRFBP1) was recently found to be involved in HCV cell entry (Gerold *et al*., 2015). It is thought that SRFBP1 forms a complex with CD81 and coordinates host cell penetration.

1.5.2 HCV Lymphotropism

Even though HCV is considered to be mainly a hepatotropic virus, accumulating molecular and clinical evidence indicates that it also invades and propagates in the cells of the immune system, a property referred to as lymphotropism.

Studies from different groups have shown that HCV replicates in the peripheral blood mononuclear cells (PBMC) of chronically infected patients, as evidenced by detection of HCV RNA positive, and its replicative intermediate, HCV RNA negative strand (Willems *et al*., 1994; Lerat *et al*., 1996; Blackard *et al*., 2006; Pham *et al*., 2008). Furthermore, detection of viral RNA in the immune compartment, was not limited to patients with CHC, but was also observed in patients who had achieved spontaneous or treatment-induced resolution of HCV infection (Pham *et al*., 2004; Radkowski *et al*., 2005; Pham *et al*., 2005, 2008; MacParland *et al*., 2009; Pham *et al*., 2012).

Immune cell subsets supporting HCV replication *in vivo* include $CD4^+$ and $CD8^+$ T cells, B cells, monocytes and possibly dendritic cells (Pham *et al*., 2004, 2008, Radkowski *et al*., 2005; Pal *et al*., 2006; Pawelczyk *et al*., 2013; Dai *et al*., 2015). It is

worth noting that HCV infection, as evidenced by detection of both the negative stand and NS5A protein, was documented in $CD4^+$, $CD8^+$ T cells, monocytes and B cells of patients with both CHC and clinically resolved hepatitis C, called secondary occult HCV infection (OCI) (Pham *et al*., 2008). In the same studies, it was found that overall loads of HCV were significantly higher in the patients with CHC than OCI, with monocytes carrying greatest virus loads.

Further evidence of HCV lymphotropism is the detection of virus sequence variants in the PBMC and immune cell subsets that differ from those found in the serum and liver of HCV-infected patients (Maggi *et al*., 1997; Navas *et al*., 1998; Laskus *et al*., 2002; Ducoulombier *et al*., 2004; Roque-Afonso *et al*., 2005; Di Liberto *et al*., 2006; Pham *et al*., 2008, 2012; MacParland *et al*., 2009; Durand *et al*., 2010, Chen *et al*., 2013, 2015). In one of these studies, it was uncovered that HCV variants residing in B cells of patients with OCI, exhibited different translational efficiency in B cells than in Huh7.5 cells, suggesting that those B cell variants were better adapted to propagate in B cells (Durand *et al*., 2010). Overall, these results indicate that either lymphotropism is a propensity of certain HCV variants or that infection of immune cells is a factor that favors selection of unique HCV variants.

The lymphotropic nature of HCV is also in agreement with the greatest prevalence of certain lymphoproliferative disorders among HCV-infected patients, such as mixed cryoglobulinemia type II (MC type II), non-Hodgkins B cell lymphoma and mucosa-associated tissue lymphoma (Agnello *et al*., 1992; Ferri *et al*., 1993; Tkoub *et al*., 1998). Evidence of HCV involvement in the aforementioned disorders will be summarized in

Section 1.8.2. Apart from B cell dysfunction, there is evidence linking HCV with a number of functional abnormalities observed in other immune subsets which will be discussed in Section 1.8.2.3.

Taken together, the accumulated data indicate that the cells of the immune system constitute the site of active HCV propagation and can serve as a reservoir where the virus may persist indefinetely, and from where it can spread, as in the cases of reinfection following clinically apparent resolution of hepatitis C or after liver transplantation due to HCV-induced end-stage-liver-disease (ESLD). Strong evidence supporting the immune cell origin of the variants reinfecting the transplanted liver is the detection of viral sequences in the plasma of transplanted patients that are identical to those harbored in the pretransplant PBMC (Laskus *et al*., 2002). The ability of HCV to infect lymphocytes is further supported by data generated from numerous *in vitro* studies, which will be summarized in Section 1.10.2.5.

Interestingly, lymphotropism is a propensity of naturally-occurring, patient-derived HCV, but not laboratory constructed or Huh7 cell culture-adapted HCV clones, such as JFH-1 (further discussed in Section 1.10.2.3). More specifically, it has been shown that the JFH-1 clone is unable to infect human lymphoid cells *in vitro* (Marukian *et al*., 2008; Murakami *et al*., 2008). Recent studies completed in this laboratory, clearly documented that JFH-1 and related $JFH1_T$ clone were not able to establish *in vitro* infection in primary T cells, PBMCs, or T cell lines in contrast to authentic, patient-derived HCV (Sarhan *et al*., 2012b).

The increasing body of data on HCV lymphotropism generated interest on the mechanism and molecules used by HCV to infect human lymphocytes. Another study performed in this laboratory, revealed that CD5, a glycoprotein that belongs to the scavenger receptor cysteine-rich (SRCR) family of receptors, is essential for infection of human T lymphocytes with naturally occurring HCV (Sarhan *et al*., 2012a). It is of note that CD5 is a molecule displayed by T cells (Cantor *et al*., 1975; Ledbetter *et al*., 1979; and on a small population of B cells (Caligaris-Cappio *et al*., 1982; Antin *et al*., 1986). Therefore, it is the first HCV receptor identified that is target cell-specific. This is in contrast to all other molecules identified as those potentially mediating HCV hepatotropism which are ubiquitously expressed on many cell types and not just on hepatocytes. Further, studies were performed investigating association between T cell susceptibility and the molecules found to mediate infection of human hepatoma-derived Huh7 or Huh7.5 cell lines by HCV JFH-1 and HCVpp. In this regard, expression of CD5 and proposed HCV hepatocyte receptors, such as CD81, SR-B1, OCLN, CLDN1 and CLDN4 was evaluated in HCV-resistant and susceptible T cell lines, PBMCs, primary T cells, JFH-1-prone Huh7.5 and HCV-resistant HepG2 cell lines (Sarhan *et al*., 2013). The results showed that: (1) Both CD5 and CD81 expression is concomitant with susceptibility of T lymphocytes to infection; (2) Susceptibility of HCV-prone T cell lines, but not primary T cells, coincides with high levels of OCLN expression, and (3) No association was found between SR-B1, CLDN-1 or CLDN-4 expression and T cell susceptibility to infection with authentic HCV (Sarhan *et al*., 2013).

1.6 Natural History of HCV Infection

1.6.1 Acute Hepatitis

The first 6 months (mo) after exposure to HCV are conventionally regarded as the acute phase of hepatitis C. Acute hepatitis C (AHC) is rarely diagnosed due to its frequently asymptomatic nature. The majority of newly infected individuals either do not experience any symptoms or develops mild, nonspecific signs of infection, such as fatigue, arthralgias, itch, anorexia and epigastric pain. Only 15%-30% of acutely infected patients experience more severe symptoms (Busch and Shafer, 2005), usually starting 3 to 12 weeks upon exposure (Alter and Seef, 2000). Those symptoms typically last for 2-12 weeks (Orland *et al*., 2002; Marcellin, 1999) and may include jaundice, low grade fever, and nausea. Two to 8 weeks post-infection, serum alanine aminotransferase (ALT) begins to increase and may often reach levels 10-fold higher than normal (Chen and Morgan, 2006). HCV RNA is detectable in serum/plasma 2 to 14 days post exposure with the use of modern nucleic acid testing (NAT) methods. The sensitivity threshold of clinically available quantitative NATs is 10 to 15 International Units/mL (IU/mL) with 1 IU ranging from 2 to 7 virus genomic equivalents (vge), depending on the assay (Pawlotsky 2000; Germer and Zein, 2001; Mulrooney-Cousins and Michalak, 2016). HCV genome load increases continuously and may reach 10^5 to 10^7 IU/mL immediately before serum ALT rise and symptoms appear. Anti-HCV antibodies can be detected between 20-150 days post-infection (Busch and Shafer, 2005) using third-generation enzyme immunoassays (EIA). Symptomatic AHC is associated with higher rates of apparent HCV clearance as measured by clinical laboratory assays and probably signify a

more robust and effective immune response against the virus (Gerlach *et al*., 2003). Fulminant hepatic failure during AHC is rare and occurring at <1% of infected individuals (Farci *et al*., 1996).

1.6.2 Spontaneous Resolution of Hepatitis C

Spontaneous clinical resolution of hepatitis C, which is not equivalent with HCV molecular clearance (Pham *et al*., 2004; Chen *et al*., 2015), occurs only in 25% of acutely infected individuals (Grebely *et al*., 2007). As discussed in detail in Section 1.8.2, vigorous and virus multispecific $CD4^+$ and $CD8^+$ T cell responses, accompanied by a type 1 pattern of cytokine production, correlate with spontaneous resolution of hepatitis (Thimme *et al.*, 2001). Factors associated with a self-limiting course of AHC infections include female sex (Yamakawa *et al*., 1996), younger age at time of infection (Zhang *et al*., 2006), aboriginal ethnicity (Grebely *et al.*, 2007), a history of jaundice (Gerlach *et al.*, 2003), and coinfection with HBV and HAV viruses (Piasecki, *et al.*, 2004; Wietzke-Braun *et al.*, 2007). A genetic factor associated with spontaneous resolution, that has attracted much attention is a polymorphism found in the region of the IL28B gene encoding IFN-lambda-3 (IFN-λ-3). This single nucleotide polymorphism (SNP (rs12979860), defines two alleles C and T, from which the C-allele is associated with a greater likelihood of spontaneous clearance of hepatitis C and increased responsiveness to IFN/RBV therapy (Ge *et al*., 2009). Recently, it was also revealed that polymorphic haplotypes in the interferon-induced helicase (IFIH1) gene, which encodes the melanoma

differentiation-associated protein 5 (MDA-5), strongly correlate with resolution of HCV infection (Hoffmann *et al*., 2015).

1.6.3 Chronic Hepatitis C

CHC develops in 75% to 85% of acutely infected individuals and is defined by persistence of HCV RNA in serum for at least 6 mo upon newly acquired infection (Chen and Morgan, 2006). During CHC, in contrast to AHC, serum ALT and HCV RNA levels stabilize reaching 10^4 to 10^6 IU/mL and they rarely fluctuate more than 1 $\log_{10}$ (Fabrizi *et al*., 2000; Rehermann, 2009). CHC can be asymptomatic for years with some people experiencing nonspecific signs, such as fatigue and arthralgia. Lack of more severe symptoms can continue even after development of liver cirrhosis (replacement of liver tissue by scar tissue) and HCC. Among patients with CHC, 5% to 20% will develop cirrhosis after 20 years. Once cirrhosis is established, the risk of the development of HCC is 1% to 4% per year (Lauer and Walker, 2001).

Liver injury in HCV infection is an immune-mediated process driven by continuous cycles of inflammation and necrosis, which lead to fibrosis and finally cirrhosis. The rates of liver disease progression may vary greatly between patients (Lauer and Walker, 2001). More specifically, female sex and younger age at time of HCV acquisition decrease the risk of progression to advanced liver disease, while increased alcohol consumption, obesity, African-American ethnicity, immunosuppression and other co-comorbidities (*e.g.*, HBV, HIV co-infection) increase the risk of liver disease progression. In addition, infection with HCV genotype 3 is associated with increased risk

of cirrhosis and HCC development compared to genotype 1, while patients infected with genotype 2 have a lower risk of cirrhosis and HCC compared to those infected with HCV genotype 1 (Kanwal, *et al.*, 2014). Patients with a favorable risk profile may not develop serious liver disease even for 30 years after infection (Poynard *et al*., 1997). Some of the consequences of liver cirrhosis are: (1) Portal hypertension which manifests by ascites, esophageal varices, encephalopathy; (2) Defective liver protein synthesis and metabolism in general, and (3) Development of HCC.

1.6.4 Persistent Occult HCV Infection

OCI is defined as the presence of HCV RNA at low levels in plasma (typically < 100 to 200 vge/mL, PBMC and/or liver (usually around 10 to 100 vge/μg of total RNA for both compartments), which are not detectable by the current clinical laboratory assays. This is accompanied by HCV replication in PBMC and liver for many years after having achieved clinically apparent SVR (Pham *et al*., 2004; Radkowski *et al*., 2005; Castillo *et al*., 2006; Ciancio *et al*., 2006). This type of infection likely also occurs after an asymptomatic exposure to very small doses of HCV. As previously indicated, HCV has been found to replicate in the same immune cell subsets in OCI as in CHC, with B cells carrying highest virus loads in OCI (Pham *et al*., 2008; Chen *et al*., 2013, 2015). Furthermore, it has been revealed that immune cells in OCI harbor HCV sequence variants that differ from those circulating in the plasma (Di Liberto *et al*., 2006; Pham *et al*., 2008). Also, it was uncovered that OCI is characterized by immune cell cytokine expression profiles that differ from the ones observed in CHC, as well as in healthy

individuals (Pham *et al*., 2009), suggesting that OCI is not disregarded by the immune system. Additionally, HCV-specific T cell responses have been identified in patients years after apparent recovery from HCV infection (Quiroga *et al*., 2006a; 2006b; Veerapu *et al*., 2011) and that was associated with HCV persistence in the liver (Quiroga *et al*., 2006a; 2006b).

OCI was discovered only after nucleic acid amplification assays of increased sensitivity were developed for the detection of the HCV RNA positive and negative strand. The assays developed and used in this laboratory for detection of HCV RNA positive strand have a sensitivity level ≤ 10 vge/mL (≤ 3 IU/mL) or ≤ 5 vge/μg (≤ 1.5 IU/μg) total RNA (Pham *et al*., 2004). Factors contributing to the extremely intricate task which is the successful and consistent detection of OCI include: (1) Use of HCV RNA detection assays comprised by two rounds of cDNA amplification by PCR (direct and nested) after reverse transcription (RT), which are followed by detection of amplicons via nucleic acid hybridization (NAH) (*i.e*., RT-PCR/NAH assay); (2) Upregulation of HCV RNA expression in PBMC via *ex vivo* stimulation with mitogens (Pham *et al*., 2004, 2005); (3) Analysis of greater volumes of plasma or higher numbers of cells; (4) Serial testing of samples of plasma and lymphocytes from patients examined; (5) Screening both PBMC and individual immune cell subsets isolated from these PBMC; (6) Extremely careful handling of samples to avoid contaminations, and (7) The use of optimized RNA extraction protocols that guarantee isolation of a maximum amount of high quality HCV RNA (Pham *et al*., 2010).

The clinical consequences of OCI are not yet well defined, since, in most cases,

HCV RNA detection in patients who achieve SVR is not performed in the clinical setting using highly sensitive assays. As a result, the persistence of HCV as OCI and its potential clinical implications await for verification. However, the reactivation of virus persisting in the PBMC or liver of patients with clinically apparent SVR is entirely possible, especially in cases of immunosuppression either iatrogenic or related to comorbidities (reviewed in Pham *et al*., 2010). It is worth mentioning cases of relapse of hepatitis C in patients who had received liver transplants following clinically apparent SVR (Zhou *et al*., 1996; Chartlton *et al*., 1998; Nudo *et al*., 2008). There are also reports associating OCI with progressive liver disease. More specifically, one study showed that the percentages of patients with OCI (as evidenced by detection of HCV RNA in liver tissue) that had inflammation, fibrosis, or cirrhosis, were greater than the percentages observed in patients with the absence of HCV RNA detected in their livers (Castillo *et al*., 2004). Furthermore, there are reports associating OCI with development of lymphoproliferative disorders (Fowell *et al*., 2008; Youssef *et al*., 2012; Farahani *et al*., 2013).

Several studies implied association of OCI with HCC development. It has been reported that 2.3-3.5% of patients with SVR developed HCC (Makiyama *et al*., 2004; Kobayashi *et al*., 2007). Furthermore, HCC relapse has been documented in patients with SVR who have received curative hepatectomy (Sanefuji *et al*., 2009). Also, development of well-differentiated HCC has been reported in a patient 13 years after achievement of SVR (Mashitani *et al*., 2009). It is important to study the contribution of OCI to the perpetuation and reactivation of liver injury. There is evidence, including data from this laboratory, implying that individuals with SVR should be regularly monitored even after

what appears to be successful virus elimination (Makiyama *et al*., 2004; Tsuda *et al*., 2004; Kobayashi *et al*., 2007; Chen *et al*., 2013, 2015).

With regards to the infectious potential of the virus persisting after clinically apparent SVR, a study performed in this laboratory demonstrated that traces of HCV which lingered in the PBMC of patients even 6 years post-SVR were able to productively infect naïve human T cells *in vitro* (MacParland *et al*., 2009). In addition, trace amounts of HCV RNA detected in the plasma of patients with SVR was found capable of infecting HCV-naive chimpanzees (Veerapu *et al*., 2014).

1.7 Immune Responses to HCV Infection

1.7.1 Innate Immune Responses

Innate immune responses constitute the first line of defense against pathogens. IFNs are key molecules in inducing an antiviral state in the cells exposed to virus and in activating as well as modulating the cells of the innate immune system.

Immediate and strong induction of type I IFNs in the liver is a predominant characteristic of acute HCV infection (Bigger *et al*., 2001; Su *et al*., 2002). Innate immune defenses are triggered by recognition of viral macromolecular motifs known as pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (Akira *et al*., 2006). In HCV infection, the innate immune response is thought to be initiated by infected hepatocytes through two PRRs, toll-like receptor 3 (TLR3) and retinoic acid inducible gene-I (RIG-I). TLR3 recognizes double stranded RNA (dsRNA) in the endosomes (Li *et al*., 2012), while RIG-I recognizes the 3'-NTR poly-U/UC region

in the cytoplasm (Saito *et al*., 2008). Once activated, TLR3 recruits adapter molecule Toll-IL1-receptor domain-containing adaptor inducing IFN-β (TRIF), while RIG-I recruits the adapter molecule IFN-β promoter stimulator protein 1 (IPS-1) (Saito *et al*., 2008). These interactions activate a downstream intracellular signaling cascade resulting in activation of nuclear factor kB (NFKB) and IFN-β secretion. Secreted IFN-β then induces an anti-viral state which expands to the uninfected neighboring cells by binding to the IFN-α/β receptor and activating the JAK/STAT pathway (Aaronson and Horvath, 2002). Activation of this pathway leads to induction of the IFN-stimulated genes (ISGs) (Stark *et al*., 1998) and IFN-α subtype diversification (Gibbert *et al*., 2012). In chimpanzees, transcriptome analysis revealed induction of ISGs early in the acute phase (Su *et al*., 2002). Replicon-based studies have identified 4 of these ISGs as having an active role against HCV: protein kinase R (PKR), P56, 2'5'-oligoadenylate synthetase (OAS), and RNA-specific adenosine deaminase-1 (ADAR1) (Pflugherber *et al*., 2002; Hui *et al*., 2003; Guo *et al*., 2004; Taylor *et al*., 2005). Furthermore, recent findings suggest that MDA-5 is involved in induction of IFN production from HCV infected cells (Israelow *et al*., 2014; Cao *et al*., 2015; Hiet *et al*., 2015). As previously mentioned (Section 1.6.2), SNPs in the gene encoding MDA-5 strongly correlate with resolution of HCV infection (Hoffmann *et al*., 2015) implying that MDA-5 plays an important role in the natural course of HCV infection.

Rapid induction of IFN-α/β in chimpanzees upon exposure does not, however, control the infection (Thimme *et al*., 2002), which is explained by the numerous strategies that HCV uses to attenuate the innate immune response. *In vitro* studies have shown that

HCV NS3/4A protein blocks TLR3 and RIG-I signaling by cleaving adapter molecules TRIF (Li *et al*., 2005) and IPS-1 (Foy *et al*., 2003). Furthermore, it was found that NS3/4A inhibits IFNα/β production, by binding to tank-binding kinase-1 (TBK1) and preventing it from phosphorylating and activating IFN-regulatory factor 3 (IRF3) (Breiman *et al*., 2005), an important molecule in the cascade leading to IFNα/β induction. In addition, it has been shown that HCV NS4B blocks IFN production by disrupting the stimulator of interferon genes (STING) interaction with mitochondrial antiviral-signaling protein (MAVS) and TBK1 (Nitta *et al*., 2013). HCV core protein also plays an important role in compromising the innate immune responses by interfering with the JAK/STAT pathway in multiple ways: (1) It binds directly to STAT1 leading it to degradation (Lin *et al*., 2006); (2) It activates suppressor of cytokine signaling 3 (SOCS3), a JAK/STAT signaling inhibitor (Bode *et al*., 2003); (3) It upregulates protein phosphatase 2A (PP2A) resulting in decreased transcriptional activity of ISG factor 3 (ISGF3) (Heim *et al*., 1999), and (4) It inhibits binding of ISGF3 to IFN-stimulated response elements. Further, NS5A inhibits PKR by direct binding (Gale *et al*., 1997) and interacts with 2'5'OAS leading to interleukin (IL)-8 induction, which attenuates ISGs overall expression (Polyak *et al*., 2001). Finally, HCV E2 directly binds to and inhibits PKR activity (Taylor *et al*., 1999).

Accumulating data indicate that type III IFNs (IL28 A/B and IL29, also known as IFN lambda 2, 3 and 1, respectively) play a very important role in HCV infection. Type III IFNs are induced in response to TLR3 and RIG-I triggering and exert their antiviral activities by inducing ISGs via the JAK/STAT pathway (Balagopal *et al*., 2010). They

bind, however, to receptors other than those of type I IFNs, which have different cellular distribution. Published data suggest that type III IFNs are the major cytokines produced in the liver of HCV-infected humans and chimpanzees, as well as in PHH *in vitro* infected with JFH-1 (Thomas *et al*., 2012; Park *et al*., 2012). Additionally, a polymorphism located in IL28B gene is associated with both spontaneous resolution of hepatitis C and response to IFN treatment (Ge *et al*., 2009), further highlighting the important role of IFN-III in innate immune responses against HCV.

Important cellular components of the innate immune response again HCV are natural killer (NK) and NK T cells. During acute infection, those key players are frequently found in the liver, where they recognize infected hepatocytes via an antigen-independent manner and respond by exerting cytotoxicity and releasing large amounts of IFN-γ (Ahlenstiel *et al*., 2010; Amadei *et al*., 2010). It has been suggested that NK and NK T cells play a more important role in HCV containment than HCV-specific T cells (Amadei *et al*., 2010). It is worth mentioning that genetic studies of individuals exposed to HCV have shown that certain combinations of a killer cell immunoglobulin-like receptor (KIRs) genotype and HLA type correlate with resolution of hepatitis C (Khakoo *et al*., 2004). Moreover, a recent study suggests that KIR2DL3^{+}NKG2A^{-} NK cells may control early HCV infection prior to seroconversion leading to a state of "natural resistance" in persons who inject drugs (Thoens *et* al., 2014). Once NK and NK T cells are activated they produce IFN-γ which activates Kupffer cells and leads to production of proinflammatory molecules, such as TNF-α, galectin-9, IL-18 (Mengshol *et al*., 2010; Chattergoon *et al*., 2011). IL-18 seems to be the first cytokine produced in response to

HCV, marking the acute phase of HCV infection (Chattergoon *et al*., 2011). Nonetheless, the role of IL-18 in HCV infection needs to be further studied. SNPs in the promoter and the coding region of the IL-18 binging protein have been associated with resolution of infection in certain patient groups (An *et al*., 2008; Mosbruger *et al*., 2010).

With regards to cellular innate immunity, the main producers of IFNs among the cells of the immune system are the plasmatocytoid and myeloid dendritic cells (pDCs and mDCs, respectively). Both of these cell types contain high levels of interferon regulatory factor 7 (IRF7) and are, therefore, capable of rapid production of high levels of IFN-α. pDCs are highly specialized cells accumulating in the peripheral lymph nodes during an infection. They express TLR7 and TLR9 as sentinels for viral infections and are capable of producing up to a 1000-fold more IFN-α than other cell types, driving the innate response. It has been shown *in vitro* that short range exosomal transfer of HCV RNA from infected cells to pDCs triggers IFN-α production via TLR7 engagement (Dreux *et al*., 2012). mDCs, on the other hand, migrate to the lymphoid tissue where they link the innate and adaptive immune response arms. Studies have shown that the frequency of DCs in the circulation during HCV infection is lower and that their function is compromised (Decalf *et al*., 2007; Dolganiuc *et al*., 2006). The HCV-mediated impairment of DC functions, as well as NK and NK T cells, will be discussed further in Section 1.9.1.

1.7.2 Adaptive Immune Responses

1.7.2.1 Humoral Immune Responses

HCV-specific antibodies are detectable 8-20 weeks upon infection (Thimme *et al*., 2001 and 2002). A defect in the priming of HCV-specific B cells has been suggested as the reason underlying this delay, as there is no general immunosuppression observed in HCV acute infection (Rehermann, 2009). Other effects of HCV infection on B cell number, function and phenotype leading to the development of certain extrahepatic disorders (*e.g*., type II MC) will be discussed in Section 1.8.2.1. Doubts for the importance of the role of humoral response in resolution of hepatitis C have stemmed from reports of immunocompromised patients clearing HCV infection (Christie *et al*., 1997), as well as patients clearing infection before emergence of neutralizing Abs (nAbs). Earlier studies suggested that HCV-specific antibodies emerge during the acute phase at low titers and they are virus isolate-specific, while their titers and breadth of specificity increases with progression to CHC (Logvinoff *et al*., 2004; Cox *et al*., 2005). Another study, however, showed that nAbs emerging during the acute phase exert pressure on viral variants, driving their sequence evolution (Dowd *et al*., 2009). In the same study, it was reported that spontaneous resolution of hepatitis C was accompanied by high levels of nAbs which peaked at the time of clearance. On the same note, a strong nAb response developed in a patient with CHC led to a decrease in serum HCV RNA to undetectable level by a clinical assay and overturning of T-cell exhaustion (Raghuraman *et al*., 2012). Several other studies also suggest that B cells and neutralizing antibodies play a

significant role in spontaneous resolutions of HCV (Giang *et al*., 2012; Osburn *et al*., 2014).

1.7.2.2 T Cell Responses

HCV-specific T cells are considered to be the major mediators of HCV clearance (Lechner *et al*., 2000; Thimme *et al*., 2001). This is evident by the fact that decrease in viral load is concomitant with emergence of HCV-specific T cells and IFN-γ detection in the liver (Shin *et al*., 2011). T cell responses are detectable 5-9 weeks post-infection (Thimme *et al*., 2002). As with humoral immunity, this delayed onset has been related to defective priming (Rehermann, 2009).

Spontaneous resolution of acute hepatitis C is characterized by the presence in the circulation of robustly proliferating HCV-specific $CD4^+$ T cells (Diepolder *et al*., 1995; Missale *et al*., 1996) that produce high levels of type I cytokines, such as IFN-γ, IL-2 and TNF-α (Gerlach *et al*., 1999). On the contrary, HCV-specific $CD4^+$ T cell responses are undetectable or weak during the acute phase of hepatitis in patients who progress to CHC. Additionally, retreat of originally robust HCV-specific $CD4^+$ T cell responses is correlated with loss of viral control and relapse of viremia (Gerlach *et al*., 1999; Nascimbeni *et al*., 2003).

On the other hand, HCV-specific $CD8^+$ T cells are present in the blood of acutely infected patients, independently of the disease outcome (Kaplan *et al*. 2007). Until the appearance of HCV-specific $CD4^+$ T cells, $CD8^+$ T lymphocytes exhibit a stunned phenotype, *i.e*., decreased proliferation, IFN-γ production and cytotoxicity, and increased

expression of programmed death-1 (PD1) molecule (Thimme *et al*., 2001; Lechner *et al*., 2000; Urbani *et al*., 2002; Kasprowicz *et al*., 2008). The functional impairment of $CD8^+$ T cells is reversed and memory $CD8^+$ T cells emerge upon appearance of HCV-specific $CD4^+$ T cells and reduction in viral load (Urbani *et al*., 2006). The timing between emergence of HCV-specific $CD8^+$ T cells and a decrease in virus load to levels undetectable by clinical laboratory assays highlights the important role of $CD8^+$ T cells in containing HCV infection (Shoukry *et al*., 2003; Neumann-Haefelin and Thimme, 2011). Activation of both HCV-specific $CD4^+$ and $CD8^+$ T cells is indispensable for resolution of hepatitis C. The requirement for $CD4^+$ T cells was demonstrated in the study in which chimpanzees were reinfected with HCV after antibody-mediated depletion of this cell subset. However, in spite of the presence of functional memory $CD8^+$ T cell responses, viral control was incomplete (Grakoui *et al*., 2003). Similarly, the role of $CD8^+$ T cells was shown in chimpanzees which were exposed to HCV after *in vivo* depletion of $CD8^+$ T cells. Again, the presence of memory $CD4^+$ T cells was not enough to contain viral infection, which was inhibited only upon $CD8^+$ T cell restoration (Shoukry *et al*., 2003).

With regards to memory T cell responses in HCV infection, studies have shown that, they do not confer sterilizing immunity. Studies of chimpanzees that were re-exposed to HCV after spontaneous resolution of hepatitis C revealed that the majority of reinfected animals exhibited a vigorous proliferation of T cells, accompanied by clinical resolution of infection (Bassett *et al*. 2001). Furthermore, a study on reinfection in IDUs showed that the rates of HCV infection were lower in those who had been re-exposed to the virus in the past (Grebely *et al*., 2006). Finally, another study analyzing memory immune

responses in re-infected IDUs, showed that protective immunity was characterized by increased proliferation of HCV-specific T cells, as well as increased breath of T cell and nAb responses, followed by rapid decrease of viral load (Osburn *et al*., 2010).

In CHC, HCV-specific T cells are constantly stimulated, yet all their effector functions are compromised. $CD4^+$ T cells produce less IL-2 and IFN-γ and this decrease correlates with cytotoxicity impairment and overall dysfunction of $CD8^+$ T cells (Francavilla *et al*., 2004). In the absence of $CD4^+$ T cell help, $CD8^+$ T cells exhibit an exhausted phenotype characterized by increased levels of PD-1 and T-cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3; a negative immune regulator), and low levels of CD127 (Golden-Mason *et al*., 2009; Kroy *et al*., 2014). Overall, exhaustion of T cells seems to be conferred by expression of a panel of several different inhibitory receptors, rather than a single one (Bengsch *et al*., 2010), which explains the reason behind the limited efficacy of anti-PD-1 immunotherapy in both humans and chimpanzees with CHC (Fuller *et al*., 2013; Gardiner *et al*., 2013). Failure of $CD8^+$ T cells to control HCV replication is accompanied by appearance of resistant HCV variants bearing mutations in HLA class I-restricted virus epitopes (Grakoui *et al*., 2003). As a result, $CD8^+$ T cells in CHC are either unable to recognize newly emerged epitopes or, even when their cognate epitopes are present and unaltered, they are unable to exert their effector functions as a result of expression of several different inhibitory receptors.

1.8 Extrahepatic Manifestations of HCV Infection

1.8.1 Non-Lymphatic System Manifestations

Even though the liver is the main source of clinical manifestations, HCV infection coincides with a number of non-hepatic symptoms and disorders. Some of these manifestations have a strong and/or well documented association with hepatitis C, for example: porphyria cutanea tarda, lichen planus, certain types of nephropathy, while others, for example: thyroid disorders, diabetes mellitus type 2, need to have their association with HCV infection further confirmed and documented.

Porphyria cutanea tarda (PCT) is caused by decreased activity of the enzyme uroporphyrinogen decarboxylase, leading to defective porphyrine metabolism and, as a result, to the induction of development of skin erythema, vesicles and bullae due to sun exposure. A strong correlation between sporadic PCT and HCV infection was uncovered when several studies showed the prevalence of chronic HCV infection in about 50% of patients with sporadic PCT (Piperno *et al*., 1992; Ferri *et al*., 1993). Since no significant defect was found in the porphyrine metabolism in patients with CHC not suffering from PCT, it is possible that HCV infection acts as an accelerator, rather than a causal factor for the emergence of PCT (Hussain *et al*., 1996; O'Reilly *et al*., 1996).

Oral lichen planus (OLP) is a skin disorder characterized by degeneration of the skin deep basal epithelium mediated by inflammatory infiltrates, mainly $CD4^+$ T cells. It is affecting 1% of the general population. An association between HCV infection and OLP has been established by studies estimating the prevalence of HCV infection among these patients at 27% (Carrozo *et al*., 1996; Bagan *et al*., 1998). This association was

further solidified by detection of HCV RNA positive and negative strands in biopsies of oral mucus membrane from OLP patients (Arrieta *et al*., 2000) and by reports showing OLP improvement in HCV-infected patients receiving IFN-α therapy (Nagao *et al*., 1999).

The association between HCV infection and glomerulonephritis (GN) has been well documented (Johnson *et al*., 1994; Daghestani and Pomeroy, 1999). Amongst the different types of nephropathy associated with HCV infection, a clear association has been established for cryoglobulinaemic or MC-related nephropathy. Involvement of HCV in the pathogenesis of this disease has been proven by the detection of immunocomplexes of HCV antigens with anti-HCV antibodies. Deposition of immunocomplexes in glomerular basement membranes is known to induce damage of glomeruli (Horikoshi *et al*., 1993).

Several thyroid disorders (*i.e*., hypo- or hyperthyroidism, Hashimoto's thyroiditis coinciding with the presence of anti-thyroid autoantibodies), often accompany chronic HCV infection, particularly in female subjects of older age (Preziati *et al*., 1995; Fernandez-Soto *et al*., 1998; Huang *et al*., 1999). Detection of anti-thyroid peroxidase Abs is the most common in patients with CHC and it is observed in around 14% of women with CHC (Marazuela *et al*., 1996). Therapy with IFN-α has been associated with the development of thyroid disorders in HCV-infected patients (Preziati *et al*., 1995; Fernandez-Soto *et al*., 1998) and also with the induction, precipitation and aggravation of thyroid disorders in individuals treated for hepatic diseases other than CHC (Hsieh *et al*., 2000). Taken together, these data suggest a synergistic effect between HCV infection and

treatment with IFN-α in the induction of thyroid gland diseases. Finally, cancer of thyroid gland is often observed in patients chronically infected with HCV (Antonelli *et al*., 2007).

Higher prevalence of type-2 diabetes mellitus has been shown in patients with CHC (Simo *et al*., 1996). It is thought that this disorder is associated with high insulin-resistance (Petit *et al*., 2001), which has been found to be likely induced by HCV core protein (Shintani *et al*., 2004). It has been postulated that diabetes mellitus can increase the risk of development of HCC and overall rate of mortality (El-Serag *et al*., 2004).

1.8.2 Lymphatic System Manifestations

1.8.2.1 Mixed Cryoglobulinemia

Type II MC is the most documented extrahepatic manifestation of HCV infection. Type II MC is characterized by accumulation of immunocomplexes which consist of a monoclonal IgM-rheumatoid factor plus polynclonal IgG. These immunocomplexes are called cryoglobulins since they typically precipitate below normal body temperature. Circulating cryoglobulins are deposited in small and medium size vessels causing vasculitis in 2-3% of HCV-infected patients with cryoglobulinemia (Sansonno *et al*., 1996; Adinolfi *et al*., 1996; Mazzaro *et al*., 2005).

Up to 80% of patients with type II MC can be infected with HCV (Agnello *et al*., 1992), while the prevalence of this disorder among patients chronically infected with HCV ranges between 19% and higher than 50% (Lunel *et al*., 1994; Wong *et al*., 1996). The involvement of HCV in the pathogenesis of this disorder is further supported by

detection of both HCV RNA and anti-HCV Ab in the cryoprecipitates, and also from the effect of antiviral treatment on the course of this disease. More specifically, regression of MC is strongly associated with a decrease in serum HCV RNA load and recurrence of the disease coincides with a relapse of HCV infection (Martyak *et al*., 2009).

The mechanism underlying pathogenesis of HCV infection-associated MC is complex and seems to be related with unregulated B cell proliferation. More specifically, it has been shown that there is an expansion of $CD5^+$ B cells, known to produce low-affinity IgM, in the peripheral blood of patients with CHC (Curry *et al*., 2000). Expansion of $CD5^+$ B cells expressing high levels of the HCV co-receptor CD81 was reported in the HCV-infected livers of patients with CHC (Curry *et al*., 2003). It was also reported that binding of a combination of HCV E2 protein and anti-CD81 mAb to CD81 on human B cells induced proliferation of naïve B cells and that E2-CD81 binding lead to phosphorylation of protein tyrosine and hupermutation of the immunoglobulin genes in B cell lines (Cocquerel *et al*., 2003; Rosa *et al*., 2005; Machilda *et al*., 2005). Furthermore, it was shown that $CD5^+$ B cells are more resistant to apoptosis than $CD5^-$ B cells in patients with CHC but not in healthy individuals, and that increased levels of anti-apoptotic cytokines in the serum of patients with CHC protected $CD5^+$ B cells from apoptosis (Mizuochi *et al*., 2009). It has also been demonstrated that E2-CD81 interaction protects human B lymphocytes from Fas (CD95)-mediated apoptosis (Chen *et al*., 2010). Finally, a recent collaborative study between the Department of Medicine, University of Maryland, USA and this laboratory showed that HCV NS3/4A induced upregulation of host B-cell receptor (BCR) signaling in HCV-infected B cells (Dai *et al*.,

2015), thus revealing a putative molecular mechanism underlying HCV-associated B-cell lymphoma.

1.8.2.2 Non-Hodgkins B Cell Lymphoma

HCV infection is also strongly associated with non-Hodgkins B cell lymphoma (NHL). HCV infection of PBMC has been reported in the majority of HCV-positive patients with NHL (Ferri *et al*., 1997). This lymphoproliferative disorder is characterized by an unregulated proliferation of B lymphocytes leading to an increased number of monoclonal B cells in the circulation. The suggested mechanisms underlying HCV induced-unregulated proliferation of B cells are outlined in Section 1.8.2.1.

1.9 Alterations in Immune Cell Function Coinciding with HCV Infection

1.9.1 NK and NK T Cell Function Disfunctions

A number of reports have shown that HCV directly targets NK cell functions, although the possibility of HCV replication in these cells has not yet been investigated. With regards to NK cells, *in vitro* studies have shown that binding of HCV E2 to CD81 leads to direct inhibition on NK cell function (Tseng and Klimpel, 2002; Crotta *et al*., 2002), however, this is not the case when E2 is part of complete virions (Yoon *et al*., 2009). *In vitro* studies have also revealed that HCV core protein upregulates major histocompatibility complex (MHC) class I expression on hepatocytes and that it stabilizes expression of human leukocyte antigen (HLA)-E by DCs leading to impaired NK cell

cytolytic activity (Herzer *et al*., 2003; Natterman *et al*., 2006). Furthermore, it was shown that NK cells exhibited decreased cytotoxicity and cytokine production concomitant with a downregulation in NKp30 cell surface expression, in the presence of HCV-infected cells (Holder *et al*., 2013). In Addition, it has been reported that decreased expression of MHC class I polypeptide-related sequence A and B (MICA and MICB, respectively) on DCs in the setting of CHC further lowers NK cell levels of activation (Jinushi *et al*., 2003). Furthermore, it has been observed that the absolute number and percentage of peripheral blood NK cells are decreased in patients with CHC compared to healthy individuals (Meier *et al*., 2005). It was shown that this abnormality is connected with decreased levels of IL-15 (*i.e.*, an important cytokine in NK cell maturation, frequency and function) in patients with CHC (Meier *et al*., 2005). Since it has been documented that DCs have impaired ability to produce IL-15, it is possible that a decreased number of NK cells in those patients is a downstream effect of DC impairment (Jinushi *et al*., 2003). Additionally, it has been reported that NK cells in patients with CHC are impaired in their ability to produce IFN-γ (Dessouki *et al*., 2010) and produce more Th2 cytokines, such as IL-10 and TGF-β and the chemokine IL-8 (Crotta *et al*., 2010). This abnormal NK cell cytokine profile leads to polarization of T cell differentiation towards a Th2 response, which is associated with HCV persistence, and further compromises DC functions (Zignego, 2004). A recent report (Serti *et al*., 2014) suggests that the attenuated IFN-γ production by NK cells in the setting of CHC is caused by the decreased ability of monocytes to respond to HCV infected cells by producing IL-18 which would in turn stimulate NK cell cytokine production.

The impairment of NK T cells function in the context of HCV infection needs to be further investigated. So far, it has been reported that the frequency of invariant NK (iNK) T cells is comparable between healthy individuals and patients infected with HCV. However, iNK T cells in CHC produce higher levels of Th2 cytokines upon activation contributing to a cytokine milieu that promotes virus persistence (Kanto and Hayashi, 2007).

1.9.2 Alterations in DC Function

As far as pDCs are concerned, it has been shown that their frequency in the circulation of patients with CHC is reduced (Decalf *et al*., 2007) and that there is a decreasee in their ability to produce IFN-α after *in vitro* stimulation (Dolganiuc *et al*., 2006). *In vitro* studies have shown that HCV core and NS3 proteins trigger TNF-α production via TLR-2 engagement, leading to inhibition of IFN-α production and to pDC apoptosis (Dolganiuc *et al*., 2003). Additionally, it has been reported that HCV JFH-1 inhibits TLR7 ligand-induced pDC CD40 expression, which in turn impairs the cell ability to activate naïve CD4 T cells (Liang *et al*., 2009).

With regards to mDCs, it has been reported that in the setting of HCV infection they are characterized by defective maturation and functional differentiation accompanied by decreased production of IL-12 and increased release of IL-10 (Auffermann-Gretzinger *et al*., 2001; Dolganiuc *et al*., 2003). *In vitro* studies have demonstrated that HCV core binding to complement receptor of macrophages and DCs lead to decreased production of IL-12 (Eisen-Vandervelde *et al*., 2004). Data regarding the effect of HCV on the

allostimulatory capacity of DCs are so far contradictory (Wedemeyer, *et al*., 2002; Bain *et al*., 2001; Longman *et al*., 2004).

1.9.3 Alterations in T Cell Functions

The alterations of T cell functions in the context of HCV infection will be discussed in Chapter 5.

1.10 Models for Study of HCV Infection

1.10.1 Animal Models

1.10.1.1 The Chimpanzee Model

Chimpanzees have played a pivotal role in the discovery of HCV, the study of HCV infection and characteristics of the elicited innate and adaptive immune responses. The major contributions of the chimpanzee model to HCV and hepatitis C research include: (1) Discovery of HCV (Choo *et al*., 1989). Molecular clones of HCV were first isolated from a cDNA expression library prepared with mRNA from a chimpanzee challenged with non-A, non-B hepatitis (NANBH)-infected serum; (2) Testing the infectivity of different HCV clones (Kolykhalov *et al*., 1997; Yanagi *et al*., 1997; Shimizu *et al*., 1998; Sakai *et al*., 2007); (3) Defining the role of $CD4^+$ (Skoukry *et al*., 2003) and $CD8^+$ T cells (Grakoui *et al*., 2003) in control of HCV; (4) Uncovering the role of memory T cells ($CD4^+$ and $CD8^+$) in re-infection with HCV (Farci *et al*., 1992; Nascimbeni *et al*., 2003; Bassett *et al*., 2001); (5) Testing efficacy of potential vaccines

against HCV (Esumi *et al*., 1999; Puig *et al*., 2006; Rollier *et al*., 2007), and (6) Testing the anti-HCV efficacy of antiviral agents (Olsen *et al*., 2004; 2011; Carroll *et al*. 2009). In many countries, invasive research in chimpanzees has been banned. A report published in 2011 from the Institute of Medicine (IOM) addressing the guidelines for use of the chimpanzees, concluded that most research performed on chimpanzees is unnecessary. In 2015, the National Institutes of Health (NIH) announced that it would no longer support biomedical research on chimpanzees.

1.10.1.2 The Scid-Alb/uPA Mouse Model

The Scid-Alb/uPA mice are SCID mice which overexpress a uPA transgene in the liver under the control of the albumin promoter (Alb) leading to degeneration of their hepatocytes (Mercer *et al*., 2001). Being SCID, their livers can be repopulated with PHH and can subsequently be infected with HCV. Scid-Alb/uPA mice have been used extensively to study anti-HCV treatments, such as IFN-2α and DAAs, including anti-NS3 and anti-NS5B proteases (Kneteman *et al*., 2006; Kneteman *et al*., 2009). The antiviral effects of telaprevir were studied by utilizing this model (Kamiya *et al*, 2010). A major disadvantage of the Scid-Alb/UPA model, as with any immunodeficient animal model, is the inability to study the host's immune responses, which are of pivotal importance to the pathogenesis and outcome of liver disease in HCV infection.

1.10.1.3 The Fah$^{-/-}$Rag2$^{-/-}$IL2rg$^{-/-}$ (FRG) Mouse Model

The FRG mice lack the fumaryl acetoacetate hydrolase gene (Fah), the recombination activating gene 2 (Rag2), and the γ-chain of the receptor for IL-2 (Il-2rγ) (Bissig *et al*., 2010). Lack of Fah causes degeneration of their hepatocytes. Being severely immunocompomised their livers can be repopulated by PHH which can be subsequently infected with HCV. Advantages of this model are that the liver can be engrafted up to 95% with human hepatocytes and that degeneration of the hepatocytes can be inhibited by an orally administrated drug. A major contribution of this model was its use for testing the efficacy of Debio 025, an HCV inhibitor targeting the host factor cyclophilin A (Bissig *et al*., 2010).

1.10.1.4 The SCID-BNX Trimera Mouse Model

For the generation of this model, mice of a beige/nude/X-linked (BNX) immunodeficient background were reconstituted with SCID mouse bone marrow cells after total body irradiation. The mice were then grafted at extrahepatic sites with *ex vivo* infected liver tissue fragments (Galun *et al*., 1995). The trimera mouse model was used for evaluation of a DAA and a anti-HCV putative monoclonal Ab (Ilan *et al*., 2002). Major drawbacks of the system include low levels of viremia as well as a high frequency of graft loss.

1.10.1.5 The AFC8-hu HSC/Hep Mouse Model

In this model, liver cell death is induced by expression of a fusion protein of the FK506 binding protein (FKBP) and caspase 8, which is driven by the albumin promoter (AFC8) (Washburn *et al*., 2011). Subsequently, $CD34^+$ human hematopoietic stem cells and hepatocyte progenitors are transplanted simultaneously, leading to immune reconstitution with human leukocytes and liver repopulation with human hepatocytes. The mice can then be infected with sera from HCV-infected patients. This model allows study of HCV-specific T cell responses and liver pathogenesis; however, limitations include lack of detection of HCV RNA in the blood and impaired generation of HCV-specific Abs.

1.10.1.6 Mouse Models Expressing Proposed Human HCV Receptors

In these mouse models, immunocompetent mice were genetically engineered in order to express the human CD81, OCLN, SR-B1 and CLDN1 (Dorner *et al*., 2011). The HCV genome used for infection of these mice was also genetically modified in order to induce luciferase expression in the infected mice. Since the mice used were immunocompetent, HCV replication was very low and transient. However, that model was used to study passive immunization against HCV using anti-CD81 and anti-E2 antibodies, as well as the efficacy of a recombinant vaccine eliciting anti-HCV nAbs. Building on this work, mice expressing human CD81, OCLN, SR-B1 and CLDN1, were crossed with mouse strains deficient in several genes involved in innate immunity (Dorner *et al*., 2013). Blunting innate immune pathways resulted not only in measurable

persistent viremia, but also in production of *de novo* infectious viral particles from the liver of infected mice providing evidence for recapitulation of the entire HCV life cycle in this mouse model.

In a similar approach, mice with intact immune system and expressing both human CD81 and OCLN genes, were shown to be permissive to infection by HCVcc and HCV positive sera (Chen *et al*., 2014). In this mouse model, the full HCV life cycle was recapitulated, and viremia persisted for more than 12 months accompanied by fibrotic and cirrhotic progression.

1.10.1.7 The Tupaia Belangeri (Tree-shrew) Model

Tupaia belangeri are small mammals native to Southeast Asia. Initially, it was shown that *in vitro* infection of tupaia primary hepatocytes with serum or plasma from HCV-infected humans resulted in productive infection and release of virions capable of infecting naïve tupaia hepatocytes (Zhao *et al*., 2002). Later on, it was also shown that HCV-infected tupaia developed liver pathology and that their serum could be used as inoculum inducing HCV infection in HCV-naïve tupaia (Amako *et al*., 2010). More recent studies showed that transfection of the four tupaia orthologs of CD81, CLDN-1, OCLN and SR-B1 in HCV-resistant mouse cell lines rendered them susceptible to HCVpp or HCVcc (Tong *et al*., 2011). Low HCV viremia is a major limitation for this, otherwise, promising animal model.

1.10.2 Cell Culture Models

1.10.2.1 Replicon System

The replicon system, developed in 1999, is a cell-culture system based on the selection of cells supporting stable replication of cloned, sugbenomic, autonomous replicating HCV RNAs. The original subgenomic replicon consisted of a genetically modified, self-replicating genotype 1b HCV RNA, which contained a selection marker (neo) under the control of HCV 5'-UTR-IRES, and the HCV non-structural genes (NS2-NS5 or more often NS3-NS5) under the control of an IRES from encephalomyocarditis virus that directed replication and polyprotein cleavage (Lohmann, 1999). Over the past years, it became clear that certain cell culture adaptive mutations dramatically enhanced RNA replication. These mutations are mostly located in the center of NS5A encoding region (Krieger, 2001). Since the first generation of replicons, a number of different replicon constructs have been created, including subgenomic or genomic, bi- or monocistronic, replicons expressing luciferase, subgenomic replicons of genotypes 1a, 2a, and, more recently, 3a and 4a (Saeed *et al*., 2012). The replicon system enabled the study of the HCV replication process, the role of viral proteins, and facilitated discovery of anti-HCV drugs by allowing high throughput screening of HCV inhibitors that target either viral genes or host factors (*e.g*. cyclophilins). An example is the discovery of telaprevir (VX-950) (Lin *et al*., 2006) using Huh7 cells bearing the subgenomic replicon as targets. The replicon system, however, does not recapitulate the entire HCV life cycle and, therefore, it cannot be used to study viral entry, assembly or egress. More importantly, the majority of research using the replicon system has been conducted in Huh7 cells, a

hepatocyte-like cell line derived from HCC with many unique features that do not occur in PHH.

1.10.2.2 HCV Pseudoparticles (HCVpp)

The HCV pseudoparticles are lenti- or retroviral core particles expressing the HCV glyproteins E1 and E2 on their surface (Bartosch *et al*., 2003a; Drummer *et al*., 2003). Originally, HCVpp expressed chimeric HCV E1 and E2 proteins, but a second generation of HCVpp has been developed using the unmodified HCV glycoproteins. The development of HCVpp allowed studies on HCV entry that started by confirming that both E1 and E2 proteins are required in this step (Bartosch, *et al*., 2003b). It has since been used to identify and confirm HCV candidate receptors and co-receptors, as well as host factors affecting HCV infectivity (reviewed in Wilson and Stamataki, 2012). Furthermore, studies employing HCVpp confirmed the hypothesis that HCV enters the cells via clathrin-mediated endocytosis (Hsu *et al*., 2003). The development of HCVpp bearing envelope proteins of genotypes 1a, 1b, 2a, 3a, 4a, 5a and 6a have enabled studies on cross- and genotype-specific neutralization (Bartosch *et al*., 2003a; Meunier *et al*., 2005). An important drawback of this system is the fact that it does not give information on entry for viral particles associated with either lipoproteins or immunoglobulins which represent the majority of particles circulating in patient sera, and whether the entry facilitated or not productive virus replication. Finally, the use of Huh7 cells as targets in this system limits the *in vitro* to *in vivo* correlation and translation.

1.10.2.3 Cell Culture-Derived HCV (HCVcc)

In 2005, it was reported that an HCV strain could replicate and release infectious particles (HCVcc) upon transfection of Huh7 cells (Zhong *et al*., 2005; Lindenbach *et al*., 2005; Wakita, 2005). That strain was cloned from a genotype 2a virus isolated from a Japanese patient with fulminant hepatitis and was, therefore, referred to as JFH-1. A unique feature of JFH-1 is that it can replicate efficiently (50-fold more efficiently than adapted subgenomic replicons) in Huh7 cells without adaptive mutations (Kato *et al*., 2003). Furthermore, hepatoma cells infected with JFH-1 released viral particles that infect naïve Huh7 cells *de novo*. JFH-1 is also capable of infecting PHH (Molina *et al*., 2008; Helle *et al*., 2013). Another important characteristic of JFH-1 is that it can infect chimpanzees, although at very low efficiency, as well as, although with significant difficulty, mice transplanted with human hepatocytes (Lindenbach *et al*., 2006; Kato *et al*., 2008). It is incapable, however, to infect human primary lymphocytes and T cell lines unlike authentic, plasma-derived virus (Sarhan *et al*., 2012b). Further advancements of the HCVcc include the generation of HCVcc inter-genotypic chimaeras (Scheel *et al*., 2008) and the $JFH1_T$ strain (Russell *et al*., 2008). The $JFH1_T$ was generated by cell-culture adaptive mutations and is characterized by a 100- to 1000-fold greater production of virus particles compared to the parental JFH-1 (Russell *et al*., 2008). A fully replicating genotype 1a strain (H77-S) was also generated, but it was found to be of very low infectivity to Huh7 cells (Yi *et al*, 2006). The HCVcc system enabled extensive research in HCV biology and allowed screening for drugs targeting every aspect of HCV. Those drug screens, however, only provide information for genotype 2a, which is a rare

and easy genotype to treat, and reflect virus drug susceptibility in hepatoma Huh7.5 cells which may not necessarily mirror susceptibility of hepatocytes infected *in situ* with naturally occurring HCV.

1.10.2.4 *In Vitro* Infection of Primary Human Hepatocytes (PHH)

Some studies have documented successful *in vitro* infection of PHH with HCV-positive patient serum (Fournier *et al*., 1998; Rumin *et al*., 1999; Castet *et al*., 2002; Lázaro *et al*., 2007; Gondeau *et al*., 2014) and release of infectious virus capable of *de novo* infection of virus-naïve PHH (Lázaro, 2007). The later represents the closest model of HCV replication in the liver studied so far. Infection of PHH with patient serum represents a most physiologically relevant system that has provided researchers a great insight on the interactions between native HCV and hepatocyte lipoprotein receptors (reviewed in Farquhar, 2008). Furthermore, this system allowed testing PHH susceptibility to different HCV genotypes (Lázaro, 2007). However, PHH are extremely hard to establish and maintain in culture, and the cells only show low level, inconsistent HCV replication, which has led researchers to explore other alternatives, such as micropatterned cocultures (MPCCs) of PHH (Ploss *et al*., 2010) and hepatocyte-like cells derived from human induced pluripotent stem cells (iPSCs) (Schwartz *et al*., 2012; Wu *et al*., 2012).

1.10.2.5 *In Vitro* Infection of Primary Human T Lymphocytes and T Cell Lines

The first *in vitro* infection system using wild-type, plasma-occurring HCV and primary human T lymphocytes was developed in this laboratory (MacParland *et al*., 2006). This system was based on the previous studies demonstrating that phytohaemagglutinin (PHA), as well as other mitogens, can induce upregulation of HCV replication in human lymphocytes (Pham *et al*., 2004, 2005). Subsequently, PHA-pretreatment of primary T cells was used to increase their susceptibility to patient-derived, native HCV. Utilizing this system, it was found that HCV can infect PBMC-derived T cells, as evidenced by expression of HCV replicative strand, non-structural proteins and the identification of unique HCV variants in the target cells. In addition, it was shown that the virions secreted by the *in vitro* infected T cells were able to *de novo* infect mitogen-induced, HCV-naïve lymphocytes. The biophysical properties of the produced virions were characterized by sucrose gradient centrifugation and immune electron microscopy (IEM). The results showed that the viral particles released by the *in vitro* infected cells had similar physical and structural properties to those of complete HCV virions. Furthermore, using this *in vitro* infection system, CD5 was found to be essential for infection of human T lymphocytes with naturally occurring HCV (Sarhan *et al*., 2012a).

Other *in vitro* studies have shown that authentic, patient-derived HCV can propagate in human T cell lines (Shimizu *et al*., 1992; Sarhan *et al*., 2012a, 2012b). Importantly, the virus produced by the *in vitro* infected T cell lines was also found to be infectious to chimpanzees (Shimizu *et al*. 1998). Finally, it has been documented that

HCV secreted from a B-cell line (SB cells) was able to infect primary human $CD4^+$ T lymphocytes *in vitro* (Kondo *et al*., 2010).

1.10.2.6 *In Vitro* Infection of Other Immune Cell Types

Numerous studies have documented the ability of HCV to infect immune cell types other than T lymphocytes *in vitro*. A study published by Bertolini *et al*. in 1993 documented the ability of wild-type, plasma-occurring HCV to infect the human bone-marrow-derived B-cell line CE *in vitro*. Furthermore, it has been shown that, TOFE, a human lymphoblastoid B cell line (LCL), supports long-term viral replication of wild-type, plasma-occurring HCV (Valli *et al*., 1995; Iacovacci *et al*., 1997; Serafino *et al*., 2003). Sung *et al*., 2013 established three *in vivo* HCV-infected B-cell lines directly from patients with CHC. One of these B-cell lines (SB) persistently produced HCV virions in culture and was able to infect PBMC and Raji B-cell line *in vitro*. Apart from B cells, human monocyte/macrophages were also found to be susceptible to *in vitro* infection with wild-type HCV (Caussin-SchwemLing *et al*., 2001; Radkowski *et al*., 2003), as well as DC (Navas *et al*., 2002).

1.11 Therapy of HCV Infection

1.11.1 Standard Anti-HCV Therapy

The goal of treatment for hepatitis C patients is SVR, which is defined by clinical assays as undetectable serum or plasma HCV RNA 6 months after completion of therapy.

This status is associated with less than 5% chance of recurrence of a symptomatic disease (Shiffman, 2006). The first treatment option for patients with CHC was IFN-α (Hoofnagle *et al*., 1986). The success rates of which ranged between 6-12%. When IFN-α was combined with ribavirin (RBV) treatment success rates increased to 38% and reached 55% when pegylated-IFN-α (PEG-IFN-α) and RBV were employed (Poynard *et al*., 1995; McHutchinson *et al*., 1998; Manns *et al*., 2001; Fried *et al*., 2002). PEG-IFN-α and RBV is the standard of care since 2002 (Poynard *et al*., 1995; McHutchinson *et al*., 1998; Manns *et al*., 2001; Fried *et al*.,, 2002). The response to PEG-IFN/RBV depends greatly on the HCV genotype with the SVR rates for patients infected with HCV genotype 1 ranging between 41-52% in comparison to 76-84% for genotypes 2 and 3 (Manns *et al*., 2001; Fried *et al*., 2002; Shiffman *et al*., 2007). Genotype 4, 5 and 6 on the other hand are associated with lower responsiveness, but still higher than that of genotype 1 (Manns *et al*., 2001; Fried *et al*., 2002; Jacobson *et al*., 2007; Shiffman *et al*., 2007). More specifically, the SVR rates for genotype 4 range between 65% and 72% (Alfaleh *et al*., 2004; Hasan *et al*., 2004; Khuroo *et al*., 2004. Thus, determining genotype prior to treatment is of great clinical importance. It defines treatment duration which for patients with HCV genotype 2 and 3 receiving PEG-IFN/RBV is 24 weeks, in contrast to patients infected with HCV genotype 1, 4, 5 and 6 who are being treated for a total of 48 weeks.

In 2011, the first DAAs, boceprevir and telaprevir, both protease inhibitors, were approved by the Food and Drug Administration (FDA) and Health Canada and have since been a part of the standard of care for the treatment of genotype 1. More specifically, administration of boceprevir begins 4 weeks after PEG-IFN/RBV treatment initiation and

continues for the remainder of the 44 weeks, while telaprevir administration is initiated with the start of PEG-IFN/RBV and ends 32 weeks prior to PEG-IFN/RBV completion. Incorporation of either of these protease inhibitors in triple therapy has brought the SVR rates up to 70-80% for HCV genotype 1 patients, both treatment naïve and those who had previously failed to achieve SVR with IFN/RBV treatment (Shiffman, 2011). Those two protease inhibitors have, therefore, revolutionized HCV therapy, making for the first time CHC cure more likely for patients infected with genotype 1. Unfortunately, triple therapy as well as PEG-IFN/RBV alone is accompanied by numerous side effects, such as anemia, insomnia, anxiety, depression, IFN-thyroid disease, nausea, diarrhea, anal-rectal discomfort, rash, pruritus and others, making adherence to therapy extremely challenging.

In 2013, two new DAAs were approved by FDA and Health Canada, simeprevir (TMC435) and sofosbuvir (PSI-7977). Simeprevir is a NS3/4A protease inhibitor (Raboisson *et al*., 2008) which is taken orally once a day and is generally well tolerated. Phase III trials have demonstrated that it is much superior compared to PEG-IFN/RBV alone and that it exhibits high efficacy in treating genotype 1a and 1b infections in treatment-naïve patients and in patients who have been previously treated with IFN-based therapies (You and Pockros, 2013). Overall, up to 80% of clinical trial participants with HCV genotype 1 infection achieved SVR upon completion of therapy that included simeprevir (Traynor, 2014). For patients with HCV genotype 1 without cirrhosis and without HIV-1 co-infection, 12 weeks of dual therapy with simeprevir and sofosbuvir is recommended. For cirrhotic patients infected with HCV genotype 1 and without HIV-1 co-infection, duration of dual therapy with simeprevir and sofosbuvir is extended to 24

weeks. Finally, for patients infected with HCV genotype 1 or 4, with or without cirrhosis, the recommended treatment is simeprevir in combination with PEG-IFN/RBV for 12 weeks, followed by an additional 12 or 36 weeks of PEG-IFN/RBV depending on prior response status and presence of HCV-1 co-infection.

Sofosbuvir is a prodrug acting as an NS5B polymerase inhibitor (Murakami *et al*. 2010; Sofia *et al*. 2010). It is taken orally once a day and it is generally well tolerated. Clinical trials have shown that 90% of patients with HCV genotype 1a infection receiving sofosbuvir in combination with PEG-IFN/RBV achieve SVR 12 weeks after treatment discontinuation (Koff, 2014). Patients with HCV genotype 2 infection receiving sofosbuvir with RBV alone also achieve SVR 12 weeks after treatment discontinuation at a rate of at least 90% with little effect from coinciding cirrhosis. For HCV genotype 3 infected patients 24 weeks of dual therapy with sofosbuvir and RBV is recommended (Koff, 2014). Patients with HCV genotype 4 infection receiving sofosbuvir in combination with PEG-IFN/RBV achieve SVR in 12 weeks at a rate of 96% (Lawitz *et al*., 2013). The efficacy of sofosbuvir in combination with PEG-IFN/RBV or with RBV alone signifies a new era in HCV treatment. In 2014, FDA approved the use of sofosbuvir in combination with ledipasvir, an NS5A inhibitor (previously GS-5885) (Link *et al*., 2014). The sofosbuvir and ledipasvir combination product, called Harvoni, is indicated, with or without RBV, for the treatment of genotype 1, 4, 5 or 6 CHC infection. In 2014, FDA approved Viekira Pak which includes ombitasvir (ABT-267) an NS5A inhibitor, paritaprevir (AB-450), an NS3/4A protease inhibitor, ritonavir (ABT-450/r) a CYP3A inhibitor and dasabuvir (ABT-333), a non-nucleoside NS5B palm polymerase

inhibitor (Deeks *et al*., 2015). Viekira Pak, with or without RBV, is indicated for the treatment of patients with genotype 1 CHC infection, including those with compensated cirrhosis. In 2015, ombitasvir, paritaprevir and ritonavir were approved by the FDA for the treatment of patients with genotype 4 CHC infection without cirrhosis, used in combination with RBV, providing the first IFN-free treatment option for patients with genotype 4 infection.

Daclatasvir (BMS-790052), an NS5A inhibitor (Lemm *et al*., 2011), was approved by the FDA in 2015 for use, in combination with sofusbuvir, for the treatment of HCV genotype 3 infections. In 2016, its use in combination with sofusbuvir, with or without RBV, was expanded for the treatment of HCV genotypes 1 and 3.

Finally, in 2016, FDA approved elbasvir (MK-8742), an NS5A inhibitor (Coburn *et al*., 2013), in combination with grazoprevir (MK-5172), an NS3/4A protease inhibitor (Harper *et al*., 2012), for the treatment of genotype 1 or 4 chronic HCV infections, with or without the use of RBV.

When treatment has failed or emergence of decompensated liver disease has preceded CHC diagnosis, orthotopic liver transplantation (OLT) is performed as the last resort. In the United States, decompensated cirrhosis from CHC is the main reason for OLT with 40-45% of all OLT performed because of HCV-induced ESLD (Davis *et al*., 2003; El Serag, 2004). Recurrence of HCV infection is nearly universal in pre-OLT viremic patients, leading to diminished graft and patient survival (Féray *et al*., 1992; Wright *et al*., 1992; Rosen *et al*., 1998). In fact, within days post-OLT, serum HCV RNA titers may reach levels similar to those observed before operation and peak 1-3 months

later, reaching levels several-fold higher than prior to transplant (Wiesner *et al*., 2003; Charlton *et al*., 2003). As a result, there is acceleration of histological progression of hepatitis, which can lead to rapid development of cirrhosis and graft failure (Gane *et al*., 1996; Forman *et al*., 2002; Chopra *et al*., 2003). Therefore, it is critical to continuously treat these patients with highly effective antiviral agents.

1.11.2 Factors Affecting and Predicting Antiviral Therapy Outcome

Close monitoring of viral kinetics throughout anti-HCV therapy have revealed various treatment outcomes in CHC patients. As previously mentioned, SVR is defined as undetectable HCV RNA by the current laboratory assays, up to 24 weeks after completion of therapy. However, SVR is very rarely achieved as shown when assays of enhanced sensitivity are being used for HCV RNA detection (reviewed in Pham *et al*., 2010; Chen *et al*., 2013). Rapid virologic response (RVR) is defined as an undetectable, by the current assays, serum HCV RNA at treatment week 4. Early virologic response (EVR) consists of an HCV RNA reduction > 2 log in comparison to baseline viral titers by 12 weeks of therapy. Nonresponse is defined as < 2 log reduction of HCV RNA levels after 12 weeks of therapy compared to baseline titers, while partial response consists of an EVR which is not followed by viral titer decrease to undetectable levels at week 24 of therapy.

` Predicting probable treatment outcomes is of critical significance for identification of ideal duration of treatment and optimization of therapy in general. Several HCV and host factors can predict treatment response. Among viral factors, HCV genotype is a

critical baseline predictor for response to PEG-IFN/RBV, with genotype 1 patients showing the poorest response to treatment and patients infected with genotype 2 and 3 achieving the highest response rates.

HCV quasispecies variation can also predict treatment outcome, even though this factor is not routinely used as a predictor in a clinical setting. Patients with minimal HCV genomic variant complexity have less chances of harboring treatment-resistant quasispecies and, therefore, are more likely to achieve SVR (Pawlotsky, 2003 and 2006). Additionally, mutations within certain HCV genomic regions (*i.e.*, NS5A region; see Section 1.4.2) have been found to be associated with higher SVR rates.

HCV plasma baseline viral load is another important viral pre-treatment predictor of response to therapy. Patients with low baseline HCV load (*i.e.*, <600,000 – 800,000 IU/L) have increased chances of achieving SVR, regardless of HCV genotype (Poynard *et al.*, 1998; Manns *et al.*, 2001; Zeuzeum *et al.*, 2006), while patients with high pre-treatment viral load (*i.e.*, >800,000 IU/L) are more resistant to therapy and generally have worse long-term outcomes. In this regard, it has been documented that pre-treatment HCV RNA loads in PBMC of non-responders to PEG-IFN/RBV were significantly higher than those of responders (Pham *et al.*, 2013).

Viral kinetics during PEG-IFN/RBV therapy is also considered valuable indicator of treatment outcome. Patients presenting an RVR have >85% chances of achieving SVR (Martinot-Peignoux *et al*, 2009), while not achieving EVR by week 12 is associated with 97-100% chances of failure to secure SVR (Davis *et al.*, 2003; Ferenci *et al.*, 2005).

Finally, no viral titer decrease throughout treatment means that patients will not be able to respond to therapy (Martinot-Peignoux *et al*., 1998; 2009).

With regards to host predictors of treatment outcome, advanced age (Manns *et al*., 2001), male gender (Poynard *et al*. 2007) and African-American ethnicity (McHuthchison *et al*., 2000; Jeffers *et al*., 2004) are factors associated with poor response to PEG-IFN/RBV. Lower SVR rates are also associated with obesity (body mass index (BMI) > 25 kg/m^2) (Ortiz *et al*., 2002), insulin resistance (Romero-Gomez, 2005), as well as certain liver histological parameters (*i.e.*, advanced fibrosis, cirrhosis and steatosis, Poynard *et al*., 2000; Everson *et al*., 2006; Leandro *et al*., 2006). Comorbidities, such as HIV or HBV co-infection, excessive alcohol consumption, and drug use also compromise response to IFN/RBV treatment (Alberti *et al*. 2009).

Furthermore, ISG baseline expression pattern has been shown to predict treatment outcome. More specifically, in studies with HCV-infected chimpanzees, it was reported that high levels of ISGs in the liver correlate with IFN treatment failure (Lanford *et al*., 2007). In studies conducted in liver biopsies of individuals with CHC, it was found that high ISG expression in hepatocytes is associated with poor response to treatment, while high ISG expression in liver infiltrating monocytes correlates with improved treatment response (McGilvray *et al*., 2012). To this regard, a study reported in 2013 by Pham *et al*. showed that the baseline expression levels of IL-8, ISG15, OAS and TLR-4, -5, and 7 in the PBMC of non-responders to treatment were 3- to 10-fold higher than those of responders. After treatment, non-responders persistently expressed 6- to 20-fold higher levels of IL-8, ISG15 and OAS in their PBMC compared to responders. In the same

study, it was documented that the expression levels of IFN-α, IFN-γ and IFN-λ in the PBMC of responders were higher before and after treatment compared to those of non-responders.

Finally, a polymorphism found in the region of the IL28B gene is a strong predictor of treatment outcome with IFN-α/RBV as summarized in Section 1.6.2. This SNP (rs12979860) is located near the IL28B gene, encoding IFN-λ-3, and defines 2 alleles C and T. The C allele is associated with an almost two-fold increase in response to IFN/RBV treatment, among patients of European ancestry and African-Americans. Furthermore, the C-allele is associated with a greater likelihood of spontaneous resolution of hepatitis C (Ge *et al*., 2009).

CHAPTER TWO: HYPOTHESIS AND AIMS OF THE STUDY

The ability of HCV to replicate in $CD4^+$ and $CD8^+$ T cells *in vivo* has been shown by a study where HCV RNA positive and negative strands as well as HCV NS5A protein were detected in the immune cell subsets of patients with CHC and OCI (Pham *et al*., 2008). In the same study, it was shown by clonal sequencing that HCV variants harbored in the *in vivo* infected cells differed from those carried in the plasma of these patients, providing supporting evidence of active HCV replication in these immune cells.

Furthermore, an *in vitro* HCV replication system was previously established in which mitogen-induced total T cell cultures derived from PBMC served as targets for plasma occurring, wild-type HCV (MacParland *et al*., 2006), as summarized in Section 1.10.2.5. However, it remained unidentified if both $CD4^+$ and $CD8^+$ T cells were susceptible to infection with native HCV. It was expected that a significantly different intracellular anti-viral cytokine milieu in $CD4^+$ and $CD8^+$ T lymphocytes may predispose them to different susceptibility to HCV infection.

To investigate the ability of authentic HCV to infect $CD4^+$ and $CD8^+$ T lymphocytes *in vitro* and to characterize the molecular and biophysical properties of the virus propagating in these subsets, we adopted the previously established *in vitro* HCV replication system in total T cells derived from normal human PBMC by employing affinity-purified, normal human $CD4^+$ and $CD8^+$ T lymphocytes as targets. Thus, the aims of our study were:

1. To establish, based on the previous acquired expertise, an *in vitro* infection model in which patient-derived HCV infects normal human primary $CD4^+$ and $CD8^+$ T lymphocytes.

2. To determine whether productive replication of HCV takes place in the *in vitro* infected $CD4^+$ and $CD8^+$ T cells by detecting HCV RNA negative (replicative) strand and intracellular localization of HCV structural and non-structural proteins.

3. To characterize the biophysical properties of HCV RNA-reactive particles released by *de novo* infected $CD4^+$ and $CD8^+$ T cells and compare them to those of virions occurring in the plasma serving as HCV inocula to infect these cells.

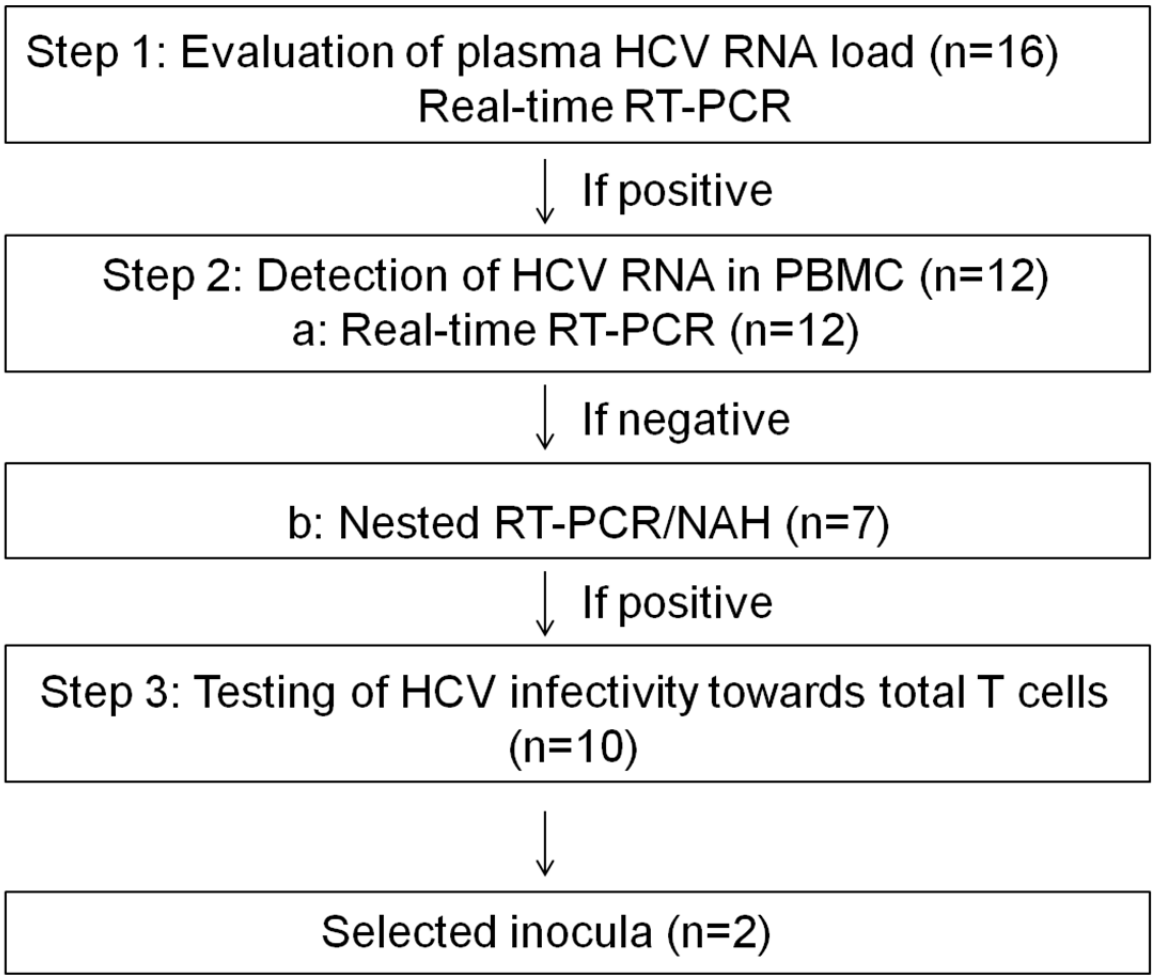
4. To assess the existence of HCV variant sequences in the *de novo* infected $CD4^+$ and $CD8^+$ T cells and determine if they differ from those occurring in the plasma used as HCV inocula.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Donors of HCV Inocula

The expertise previously gained in this laboratory that applied the HCV replication system in T cells revealed that the following conditions can lead to the identification of HCV inocula most suitable for infection experiments: (1) Treatment-naïve patients with CHC should serve as donors; (2) Their plasma should carry a high HCV RNA load, preferable more than 10^4-10^5 vge/mL; (3) HCV RNA should be detectable in the donors PBMC, and (4) Their plasma should be capable of *in vitro* infecting normal PBMC-derived T cells using the conditions previously established in this laboratory (MacParland *et al*., 2006). Therefore, identifying donors of HCV inocula for the *in vitro* infection of affinity-purified $CD4^+$ and $CD8^+$ T cells in this study was a three-step process (see Figure 3.1). At step one, the HCV RNA load in the plasma of 16 patients with clinical diagnosis of CHC was evaluated by real-time RT-PCR. At the second step, real-time RT-PCR was used again to examine the presence of HCV RNA in the PBMC of patients with detectable HCV RNA in their plasma. In cases where HCV RNA in the PBMC was undetectable by real-time RT-PCR, we employed the nested-RT-PCR/NAH assay the sensitivity of which is approximately 10-fold greater than that of the former (Pham *et al*., 2004). At the final step, plasma carrying high HCV RNA load from patients with detectable HCV RNA in their PBMCs were used for *in vitro* infection experiments of PBMC-derived T cells using the cell culture conditions established previously (MacParland *et al*., 2006). Plasma found capable of in vitro infecting PBMC-

Figure 3.1. Overall approach to selection of HCV-positive inocula for T lymphocyte infection experiments and the numbers of the inocula examined at each stage**.**


Step 1: Evaluation of plasma HCV RNA load (n=16)
Real-time RT-PCR
If positive
Step 2: Detection of HCV RNA in PBMC (n=12)
a: Real-time RT-PCR (n=12)
If negative
b: Nested RT-PCR/NAH (n=7)
If positive
Step 3: Testing of HCV infectivity towards total T cells (n=10)
Selected inocula (n=2)

derived T cells, as evidenced by detection of HCV RNA positive and negative strands, served as inocula for the *in vitro* infection experiments with affinity-purified $CD4^+$ and $CD8^+$ T cells as targets.

3.2 HCV Immune Cell Targets

Monocyte-depleted PBMC (see Section 3.5), as well as $CD4^+$ and $CD8^+$ T cells affinity-purified from PBMC of two healthy individuals (A/M and B/F) served as targets for HCV *in vitro* infection experiments. As indicated in Section 3.1, monocyte-depleted PBMCs were used as targets for HCV infection experiments aiming to identify the most infectious and suitable inocula used in the main infection experiments with affinity-purified $CD4^+$ and $CD8^+$ T cells. The donors of PBMC had no clinical history of HCV exposure and their sera was negative for HCV RNA, as evidenced by RT-PCR/NAH assay (sensitivity ≤ 10 vge/mL or 3 IU/mL) (Pham *et al*., 2004) and for anti-HCV antibodies (enzyme immunoassay; Abbott Molecular, Mississauga, Ontario, Canada). Affinity-purified $CD4^+$ and $CD8^+$ T cells from healthy controls were also exposed, in some experiments, to healthy donor plasma (HDP), as controls.

3.3 Plasma Collection and PBMC Isolation

Blood between 20 and 130 mL, depending on the type of experiment, was collected in Vacutainer tubes containing acid-citrate-dextrose anticoagulant, solution A (ACD-A) (Becton Dickinson, Franklin Lakes, New Jersey). PBMC isolation was performed immediately after blood collection to avoid potential RNA degradation.

Initially, the blood was centrifuged at 800 x *g* for 30 minutes (min) at ambient temperature (AT). The supernatant was then carefully removed, aliquoted in 1.8 mL poly-propylene precooled tubes (Nunc CryoTubes; Sigma-Aldrich, Oakville, Ontario, Canada) and stored at -80˚C. Following plasma removal, the remainder blood was diluted with phosphate-buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA) (1:1 final dilution), carefully layered over Ficoll-Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) in a 1:1 ratio, and centrifuged at ~400 x *g* for 30 min at AT. The buffy coat (thin white interface containing PBMC) was carefully collected, diluted with PBS-EDTA in a ~ 1:1 ratio, and centrifuged at 200 x *g* for 10 min at AT. The supernatant was collected and the cell pellet was treated with 5-10 mL of ammonium-chlorate-potassium (ACK) buffer for 15 min to lyse contaminating red blood cells. After ACK treatment, 5-10 mL of PBS-EDTA were added and the cells were centrifuged at 200 x *g* for 10 min at AT. The supernatant was removed and the resulting pellet diluted with PBS-EDTA to allow accurate cell counting and estimation of viability using a benchtop automated cell counter (Countess Automated Cell Counter; Invitrogen Life Technologies, Burlington, Ontario, Canada).

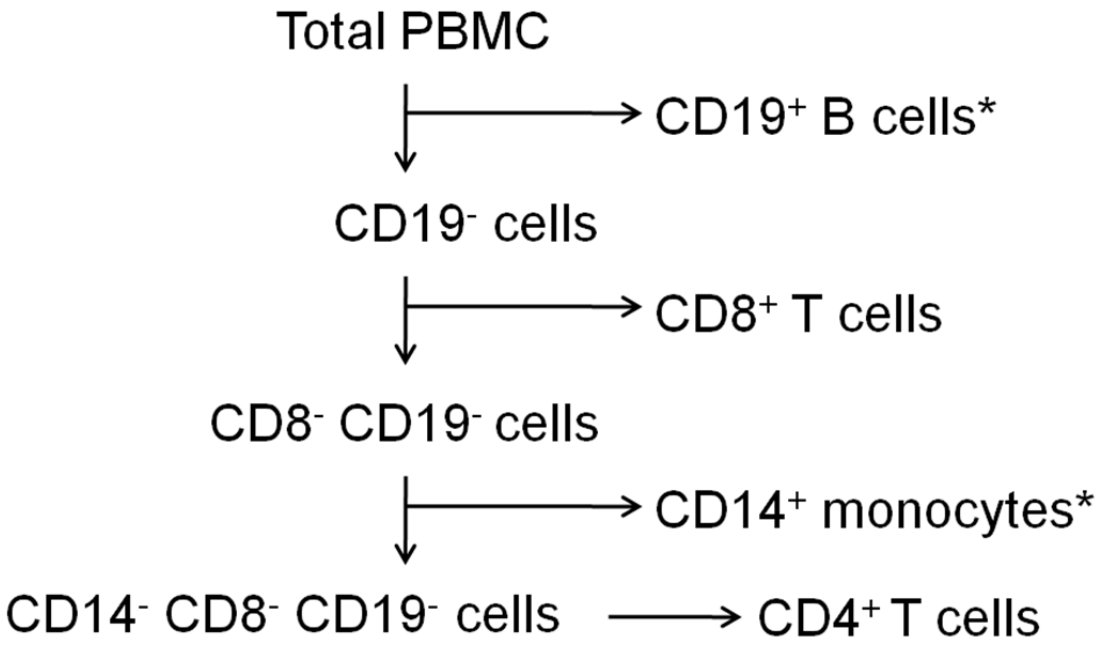
3.4 T Lymphocyte Subsets Isolations

$CD4^+$ and $CD8^+$ T cells were affinity-purified from total PBMC of healthy donors by positive selection using a stepwise approach and an automatic bench-top seperator (AutoMACS, Miltenyi Biotech Inc., Auburn, California). B cells and monocytes were also isolated during this process for use in other ongoing experiments not related to this

study. Sequential sorting of cell subsets was performed in a specific order (see Figure 3.2) and following a protocol developed in this laboratory aiming to maximize cell recovery and purity of multiple immune cell types from the same PBMC sample (Corkum *et al*., manuscript in preparation).

In the first step, isolation of $CD19^+$ positive cells was performed. For this purpose, total PBMC were centrifuged at 350 x *g* for 15 min at 4°C, and cells were resuspended in 80 µL of precooled MACS running buffer (solution containing PBS, pH 7.4, 0.5% bovine serum albumin (BSA), and 2 mM EDTA) per 10^7 cells. 20 µL of CD19 microbeads (Miltenyi Biotech Inc.) were added and the cells were incubated for 15 min on ice. Then, cells were washed with adding MACS running buffer, bringing total volume to 10 mL, and mixed by gently inverting the tube containing cell suspension. The cells were spun down at 350 x *g* for 15 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 2 mL of MACS running buffer. At this point, the cell suspension was ready for magnetic separation. $CD19^+$ cells were isolated using a positive selection in standard mode on two subsequent columns. After isolation was completed cells were counted in the $CD19^+$ positive and negative fractions, and cells were cryopreserved. The $CD19^-$ fraction was used for subsequent subsets isolations. Thus, $CD8^+$ T cell affinity purification was done next, but using $CD8^+$ microbeads. After obtaining the $CD8^+$ positive fraction, $CD8^+$ T cells were counted, their viability measured, and they were immediately used for HCV infection experiments. After $CD8^+$ positive selection, we proceeded to $CD14^+$ isolation prior to $CD4^+$ selection. We followed this

Figure 3.2. Separation of different immune cell subsets from total PBMC by sequential positive selection using macrobeads coated with antibodies against individual immune cell markets and autoMACS Myltenyi Pro Separator. Initially, separation of $CD19^+$-positive B cells was done that was followed by positive removal of $CD8^+$ T lymphocytes, as both of these cell populations represent relatively small percentages of the total PBMC population. Subsequently, positive selection of $CD14^+$ (monocytes) was done prior to $CD4^+$ T cell selection to avoid contamination of $CD4^+$ T cells with $CD14^+$ cells since some them also express CD4. * $CD19^+$ and $CD14^+$ cells were not investigated in this study (Corkum *et al*. manuscript in preparation).


Total PBMC
CD19+ B cells*
CD19- cells
CD8+ T cells
CD8- CD19- cells
CD14+ monocytes*
CD14- CD8- CD19- cells
CD4+ T cells

order of separation since some macrophages are known to express low levels of $CD4^+$ and we wanted our $CD4^+$ population to have the highest purity possible. For positive selection of $CD14^+$, we followed the same procedure as for $CD19^+$ and $CD8^+$ cells with the only difference being the separation program applied. We used the possel program, which performed a positive selection in standard mode for isolation of cell populations with frequencies higher than 5%. The isolated $CD14^+$ cells were cryopreserved and the $CD19^-CD8^-CD14^-$ negative fraction was used for positive selection of $CD4^+$ cells. For this purpose, we followed the procedure used for $CD14^+$ isolation. After isolation of $CD4^+$ cells, the cells were counted and used as targets for infection with HCV.

3.4.1 Assessment of Cell Purity by Flow Cytometry

Purity of isolated $CD4^+$ and $CD8^+$ T cell subsets was evaluated using flow cytometric analysis immediately following isolation. For each labelling, 5×10^5 cells were collected into FACS tubes where MACS running buffer was added to bring volume to 100 μL. Cell subsets were incubated with peridinin chlorophyll protein complex (PerCP)-conjugated anti-human CD4, fluorescein isothiocyanate (FITC)-conjugated anti-human CD8, allophycocyanin (APC)-conjugated anti-human CD14, and phycoerythrin (PE)-conjugated anti-human CD20. Antibodies to CD20 were used instead of CD19 since, after CD19 microbead labeling of cells, CD19 staining can be weak. Parallel incubations with appropriate isotype controls were performed using PerCP-conjugated mouse IgG2a, FITC-conjugated mouse IgG2a, APC-conjugated mouse IgG2a, and PE-conjugated mouse IgG1. All antibodies and isotype controls were purchased from

Miltenyi Biotec. Incubation took place in the dark at 4°C for 30 min. The cells were then washed with 2 mL of 0.25% Tween-20 (Sigma-Aldrich) in PBS-EDTA by centrifugation at 300 x *g* for 10 min at 4°C, fixed in 2% paraformaldehyde (PFA) in PBS-EDTA, washed again, and examined with a FACSCalibur cytometer (Becton Dickinson Biosciences, Mountain View, CA). Analysis of the data was performed using FlowJo v.X.0.7 software (Tree Star, Ashland, Oregon). Using forward versus side scatter, lymphocytes were selected. Gates for determining positivity were established using isotype controls so that ~99.0 % of events were negative.

3.5 HCV Infection System

For infection experiments with T cell-enriched PBMC as targets, 4-6 x 10^7 total PBMC were placed into flasks with complete AIM-V medium (Invitrogen) and left at 37°C for 2.5 hours (h) to deplete monocytes via adhesion to plastic. Subsequently, the supernatant from the flask was transferred into 50 mL conical tubes and centrifuged at 400 x *g* for 15 min. After centrifugation, the supernatant was discarded and the cell pellet resuspended with appropriate volume of PBS-EDTA to achieve a cell concentration that would allow accurate cell counting and measurement of viability using the Countess. After counting, monocyte-depleted PBMC were used as targets for infection with HCV (MacParland *et al*., 2006; see Section 3.5).

Thus, monocyte-depleted PBMC, or afffinity-purified $CD4^+$ or $CD8^+$ T cells were exposed to 5 μg/mL of phytohemagglutinin (PHA; ICN Biomedicals Inc., Aurora, Ohio) for 48 h at 37°C, prior to inoculation with HCV (Pham *et al*., 2004). PHA-pretreated

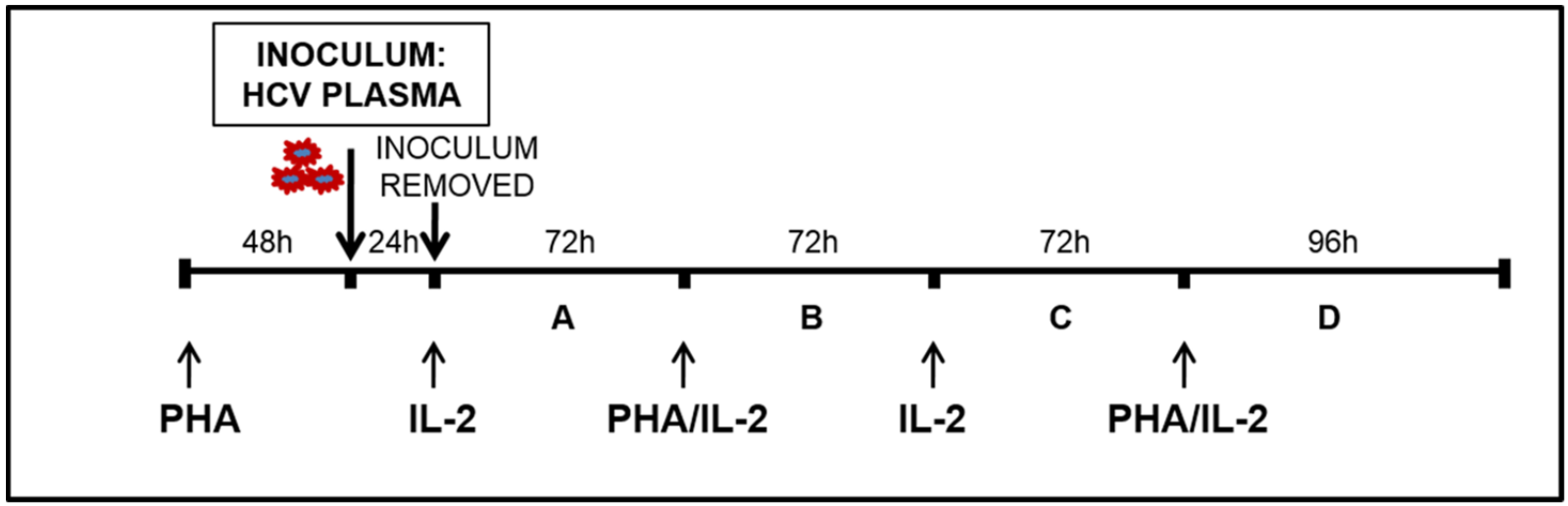
cells were exposed to HCV infectious plasma at different virus/cell ratios (see Table 4.2). Prior to inoculation the HCV infectious plasma was cleared at 400 x *g*, 4°C for 30 min and heat-inactivated at 56°C for 30 min. After 24 h inoculation the HCV inoculum was removed, cells were washed three times, counted and cultured under alternating stimulation with PHA and/or interleukin-2 (IL-2; 20 U/mL; Roche Diagnostics, Quebec, Canada) for 14 days post-infection (d.p.i.), as described before (MacParland *et al*., 2006) (see Figure 3.3). More specifically, after removal of the inoculum cells were cultured with IL-2 for 72 h (phase A), followed by a 72 h stimulation with IL-2 and PHA (phase B). Then, cells were cultured with IL-2 alone for another 72 h (phase C), and finally with IL-2 and PHA for 96 h (phase D). At the end of each phase, 10^6 cells/mL were put back in culture after being washed three times. The remaining cells were used for HCV RNA positive and negative strand analysis and for identification of NS5A and core proteins by confocal microscopy. Cell culture supernatants were also collected at the end of each phase. Collected supernatants were stored at -80°C until use for characterization of the biophysical properties of HCV RNA-reactive particles via buoyant density gradient analysis.

3.6 Analysis of HCV Biophysical Properties

3.6.1 Iodixanol Gradient

To examine the buoyant density of the viral particles released from the *in vitro* infected cells and compare it to that of the virions carried in the plasma used as inoculum, iodixanol gradient ultracentrifugation was performed. For this purpose, we used culture

Figure 3.3. Schematic representation of HCV-infection cell culture system used in this study. Monocyte-depleted PBMC or affinity-purified $CD4^+$ and $CD8^+$ T cells were exposed to 5 μg/mL of PHA for 48 h prior to inoculation with HCV. PHA-treated cells were exposed to HCV inoculum at different virus: cell ratios as shown in Materials and Methods and Results. After 24-h inoculation, the inoculum was removed, cells washed and cultured under alternating stimulation with PHA and/or IL-2 for 14 days post-infection (d). The 3-day period between 1 d and 4 d was designated as phase A, between 4 d and 7 d as phase B, between 7 d and 10 d as phase C, and between 10 d and 14 d as phase D.


INOCULUM:
HCV PLASMA
INOCULUM
REMOVED
48h
24h
72h
72h
72h
96h
A
B
C
D
PHA
IL-2
PHA/IL-2
IL-2
PHA/IL-2

supernatants (10 mL) collected from timepoints of experiments where HCV RNA negative strand had been detected after infection of $CD4^+$ or $CD8^+$ T cells. More specifically, from experiment III (Table 4.3), we used supernatants collected from 10 d.p.i. as well as 14 d.p.i. from infected $CD4^+$ T cells, and 7 d.p.i. from $CD8^+$ T cells. We also included the inoculum HCV-11/M used in this experiment. Thus, 200 µL of HCV-11/M plasma in 9.8 mL PBS was used as a control. From experiment VI (Table 4.3), 10 mL of culture supernatants collected from 14 d.p.i. from $CD4^+$ T cells and 10 d.p.i. from $CD8^+$ T cells infected with inoculum HCV-16 were used. Again, we included 200 µL of plasma 16 in 9.8 mL PBS. All supernatants were initially pre-clarified at 400 x *g* for 30 min at 4°C in the presence of a protease inhibitor cocktail (Sigma-Aldrich) in a 1 to 200 ratio. Subsequently, they were concentrated by ultracentrifugation at 150,000 x *g* for 22 h at 4°C in a TH-641 rotor using Sorvall Discovery 100SE ultracentrifuge (Mandel Scientific Company Inc., Guelph, Canada). After removal of supernatant, the pellets were resuspended in 500 µL of culture medium (AIM-V). In parallel, 50 µL of plasma were diluted in 450 µL of medium (AIM-V). Resupended pellets and diluted plasma were layered separately over a 4.5 mL continuous 10 to 50% iodixanol gradient (prepared using OptiPrep Density Gradient Medium [Sigma] and Hanks' balanced salt solution [HBSS; Invitrogen]). A gradient consisting solely of culture medium (*i.e.*, 500 µL of AIM-V medium layered over a 4.5 mL continuous 10 to 50% iodixanol) was also prepared following the procedure described above. After ultracentrifugation at 100,000 x *g* for 16 h at 4°C in a Beckman SW55 Ti rotor (Beckman Coulter Inc, Pasadena, California) (Jones *et al*., 2011), sixteen 300 µL fractions were collected starting from the

top of each tube. The fractions obtained from the gradient consisting only of culture medium were used for measuring iodixanol density using a refractometer (UriSystem refractometer; Fisher Scientific International Inc., Hampton, New Hampshire).

3.7 RNA Extraction

For total RNA extraction from cells, 1 mL of TRIzol Reagent (Invitrogen) was added to ~ 1 x 10^7 cells. For RNA isolation from plasma or iodixanol gradient fractions, 250 µL or 300 µL of each aliquot was supplemented with 750 µL or 700 µL, respectively, of TRIzol LS (Invitrogen). In all cases, 200 µL of chloroform were added to each sample following 30 min incubation at AT. After vigorous shaking for 30 seconds (s), samples were incubated at ambient temperature for 2 to 3 min and centrifuged at 12,000 x *g* for 15 min at 4˚C. After centrifugation, the aqueous phase of each sample was transferred to a fresh tube where 500 µL of isopropanol were added. Samples were vigorously shaken again for 30 s and left at -20 ˚C overnight. After being retrieved from -20˚C, the samples were centrifuged at 12,000 x *g* for 10 min at 4˚C, the supernatant removed and pellets washed with 75% ethanol. The samples were centrifuged again at 7,500 x *g* for 5 min, the supernatant removed and the resulting RNA pellets left to air dry for no more than 5 min. Finally, the pellets were suspended in appropriate volume of RNase-free TE buffer (1 mM EDTA in 10 mM Tris-HCl buffer, pH 8.0; Ambion by Life Technologies), and the concentration and quality of the RNA was evaluated using spectrophotometric analysis of OD_{260}. 1 x 10^7 PBMC or T cells would yield 20 to 30 µg of RNA. RNA was aliquoted to 5 µg per vial and stored at -80 ˚C until use. A mock sample containing UltraPure

DNase/RNase-free distilled water (Invitrogen) added in TRIzol in place of *in vitro* infected sample or plasma was always included as a contamination control. When cell numbers allowed, RNA was also extracted from affinity-purified $CD4^+$ (experiments I, III and VI) and/or $CD8^+$ T cells (experiment III) exposed to HDP as additional contamination and infection controls.

3.8 cDNA Synthesis

For cDNA synthesis, 1 to 4 μg of RNA extracted from HCV-exposed and control T-cell enriched PBMC and affinity-purified $CD4^+$ and $CD8^+$ T cells and all RNA extracted from 250 μL of plasma or 300 μL of iodixanol fractions were transcribed using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Invitrogen). Thus, a 20 μL volume reaction was assembled on ice by mixing 1-4 μg of RNA template with 4 μL of 5X First Strand buffer (375 mM KCl, 15 mM $MgCl_2$ and 250 mM Tris-HCl buffer, pH 8.3), 1 μl (200 units) of M-MLV RT, 0.25 μL of RNaseOUT Recombinant Ribonuclease Inhibitor (10 units) and 2 μL of each of the following: 0.1 M dithiothreitol (DTT), 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH), and 100 ng/μL random primers (hexamer oligonucleotides). If necessary, the volume of the reaction was brought up to 20 μL by adding appropriate volume of UltraPure DNase/RNase-free distilled water. All reagents above were purchased from Invitrogen. Reaction, which included 1-h incubation at 37°C, followed by 5 min at 95°C took place in a PTC-200 thermocycler (MJR Research, Watertown, Massachusetts). A mock extraction was always included as contamination control. RNA prepared from a mock infection

(*i.e.*, cells exposed to HDP) was used as negative control. Synthetic RNA positive strand at 10^6 copies/μL (Section 3.10.2.) was always transcribed serving as a positive control.

f3.9 Recombinant HCV 5'-UTR-E2 Plasmid (rHCV UTR-E2)

The rHCV UTR-E2 fragment cloned into the dual promoter PCRII plasmid vector (Invitrogen) was used as positive control in our real-time and end-point RT-PCR. The excised rHCV UTR-E2 fragment was also used as a template for the synthesis of the synthetic HCV RNA positive and negative strand (see Sections 3.10.1 and 3.10.2, respectively), and, after labeling with ^{32}P, as a probe for NAH.

The rHCV UTR-E2 fragment of 1,821 bp was previously generated in this laboratory by transcribing RNA isolated from a patient chronically infected with HCV genotype 1a and cloning into the dual promoter PCRII plasmid vector using the TOPO-TA cloning system (Invitrogen) (Pham *et al*., 2004) .

In order to produce new stock of rHCV 5'-UTR-E2 plasmid for use in this study, One Shot TOP10 *E. coli* cells containing the rUTR-E2 fragment were retrieved from -80 °C and seeded on Luria-Bertani (LB) agar plates (for reagents and procedures pertaining to cloning and sequencing see Section 3.15). After overnight incubation at 37°C, 10 single colonies were selected and each colony was cultured in 15 mL conical tubes containing 3 mL of LB medium. After incubation in a rocking incubator at 200 rpm for 18 h at 37 °C, plasmid DNA was extracted from each colony using the QIAprep Spin Miniprep Kit (Qiagen, Venlo, Limburg, Netherlands), as described in detail in Section 3.15. Plasmid DNA from each colony was subsequently analyzed by restriction digest

using *Escherichia coli* restriction endonuclease I (*Eco*RI) and positive colonies were sequenced (see Section 3.15). The sequencing results confirmed the sequence and size of the rHCV 5'-UTR-E2 fragment and also revealed that the rHCV UTR-E2 fragment was cloned into the vector in a 5'- to 3'- orientation. We then transferred 20 µL from each of the 3 mL LB cultures containing positive colonies into 100 mL of LB. 100 mL cultures were placed on a rotating shaker at 200 rpm for 18 h at 37 °C to expand. The following day, plasmid DNA was extracted using the PureLink HiPure Plasmid DNA Maxiprep Kit (Invitrogen). For this purpose, the cultures were centrifuged at 4000 × *g* for 10 min. The resulting pellet was resuspended with 10 mL of R3 buffer containing 50 mM Tris-HCl buffer, pH 8.0, 10 mM EDTA, 20 mg/mL RNase A, until homogeneous. Then, 10 mL of lysis buffer (0.2 M NaOH, 1% [w/v] sodium dodecyl sulfate; SDS) was added. The mixture was gently inverted and incubated at AT for 5 min. 10 mL of precipitation buffer containing 3.1 M potassium acetate, pH 5.5, was subsequently added and the tube was inverted several times until the mixture became homogeneous. The mixture was then centrifuged at 12,000 × *g* for 10 min at AT. The resulting supernatant was loaded onto the provided equilibrated column and the column was allowed to drain by gravity flow. Then, the column was washed with 60 mL of wash buffer containing 0.1 M sodium acetate, pH 5.0, and 825 mM NaCl. The solution in the column was again allowed to drain by gravity flow. The flow-through was discarded. 15 mL of elution buffer (100 mM Tris-HCl buffer, pH 8.5, and 1.25 M NaCl) was added to the column and purified DNA was eluted into a sterile 30 mL centrifuge tube placed under the column. 10.5 mL of isopropanol was added to the elution tube and after mixing well the tube was

centrifuged at 12,000 × *g* for 30 min at 4°C. After discarding the supernatant, the DNA pellet was resuspended in 5 mL 70% ethanol and the elution tube was centrifuged again at 12,000 × *g* for 5 min at 4°C. The supernatant was removed and the pellet was air dried for 10 min. The DNA pellet was resuspended in 200 µL TE buffer and the concentration and quality of the purified DNA was evaluated using spectrophotometric analysis and stored at -20 °C.

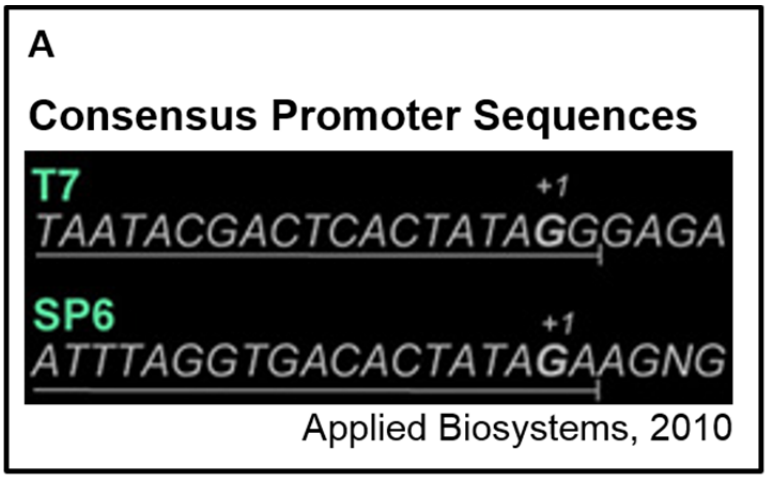
3.10 Preparation of HCV RNA Synthetic Strands

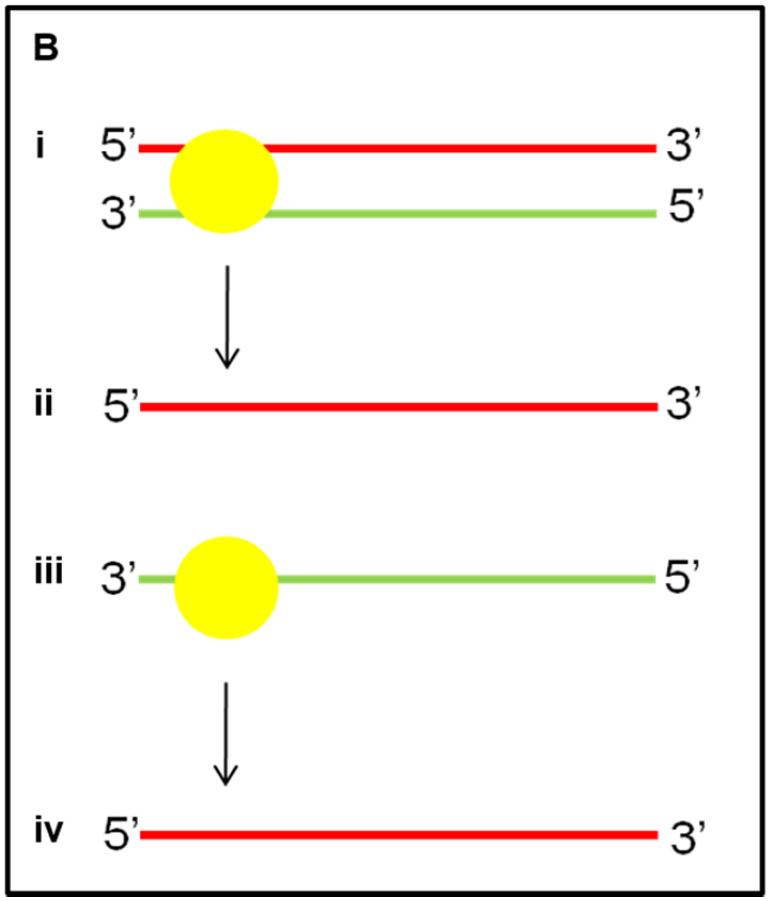
3.10.1 Synthetic HCV RNA Positive Strand

As a template for the synthesis of the HCV RNA positive strand, which was routinely used as a positive control for cDNA synthesis and as a specificity control for detection of the HCV RNA replicative (negative) strand, we used the dual promoter PCRII plasmid vector (Invitrogen) containing the rHCV UTR-E2 1821-bp (see Section 3.9). As seen in Figure 3.4, the PCRII vector contains an SP6 RNA polymerase promoter site at the 5'-end (upstream) of the cloned sequence that we wanted to transcribe. As mentioned in Section 3.9, sequencing confirmed that the rHCV UTR-E2 fragment was cloned into the vector in a 5'- to 3'-end orientation. To produce sense (positive strand RNA), we needed to transcribe, using the RNA polymerase corresponding to the phage promoter at the 5'-end of the cloned sequence, using in that case the SP6 RNA polymerase. Therefore, for synthesis of the HCV RNA positive strand, we used the MEGAscript SP6 transcription kit (Ambion, Life Technologies).

In the first step, we linearized the plasmid in the polylinker at the 3'-end

Figure 3.4. Synthesis of HCV RNA positive and negative strands. (A) Minimal promoter sequence requirements of the RNA phage polymerases T7 and SP6 used for *in vitro* synthesis of HCV RNA negative and positive strand, respectively. (B) Mechanism of action of the RNA phage polymerases. (C) Depending on the orientation of cDNA sequence to be transcribed relative to the promoter, the template may produce sense or antisense strand RNA. In this Figure, in order to produce sense RNA, we would have to transcribe using RNA polymerase recognizing promoter 1 at the 5'-end of protein coding region. To synthesize antisense RNA, we would have to transcribe using RNA polymerase recognising promoter 2 at the 3'-end of protein coding region. (D) The PCRII-TOPO vector containing the rHCV UTR-E2 fragment in a 5'- to 3'-end orientation. The vector contains an SP6 and a T7 RNA polymerase promoter site at the 5'- and 3'-end of the cloned sequence that we wanted to transcribe, respectively.


A
Consensus Promoter Sequences
T7
+1
TAATACGACTCACTATAGGGAGA
SP6
+1
ATTTAGGTGACACTATAGAAGNG
Applied Biosystems, 2010


B
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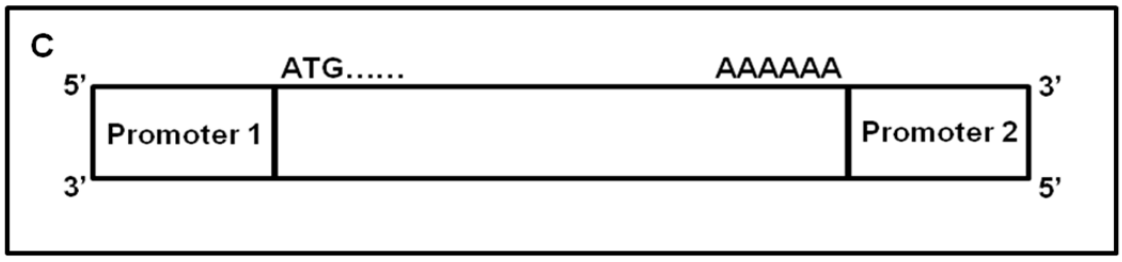
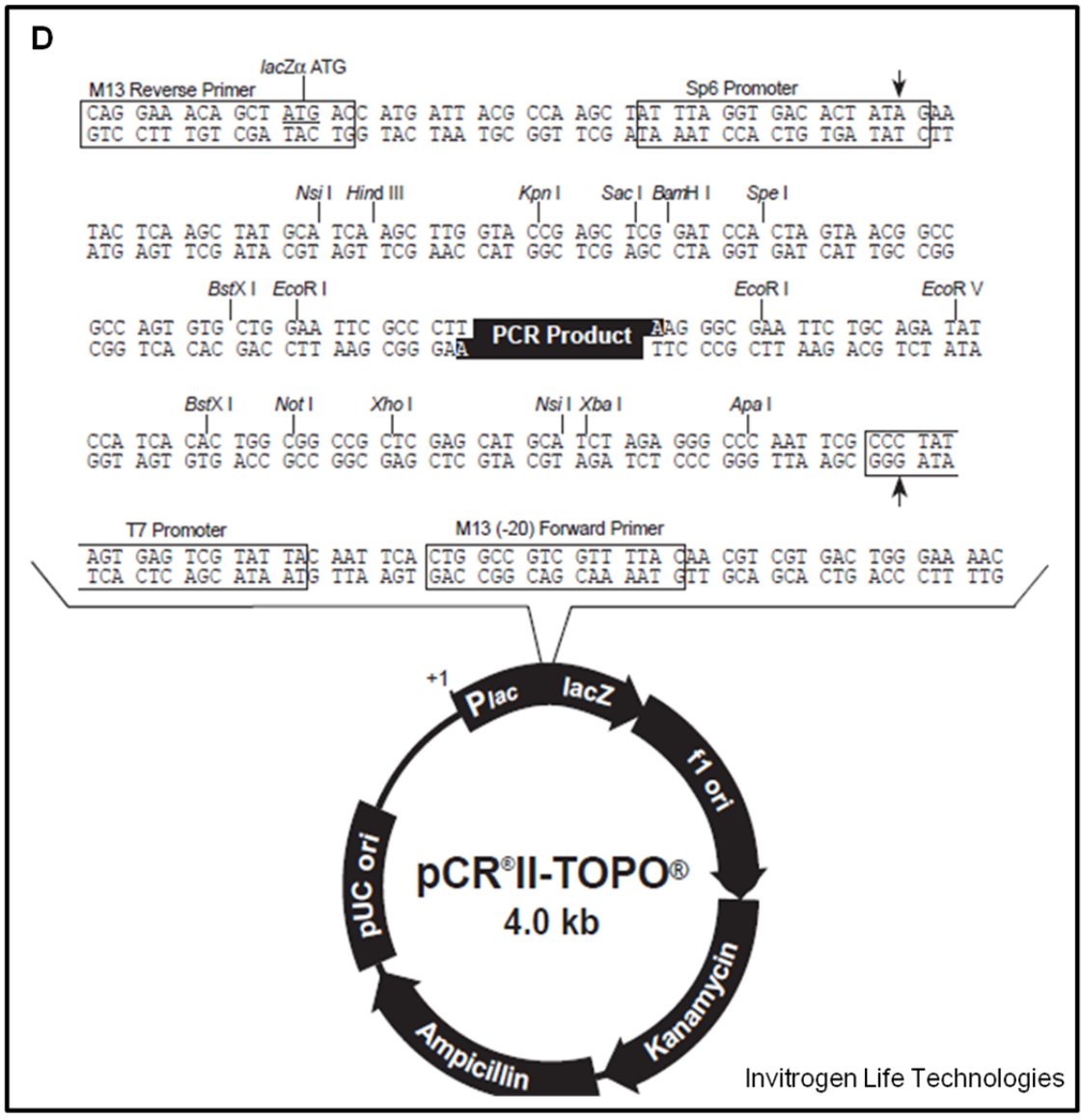

C
ATG……
AAAAAA
5'
3'
Promoter 1
Promoter 2
3'
5'


D
lacZα ATG
M13 Reverse Primer
Sp6 Promoter
CAG GAA ACA GCT ATG ACC ATG ATT ACG CCA AGC TAT TTA GGT GAC ACT ATA GAA
GTC CTT TGT CGA TAC TGG TAC TAA TGC GGT TCG ATA AAT CCA CTG TGA TAT CTT
Nsi I Hind III Kpn I Sac I BamH I Spe I
TAC TCA AGC TAT GCA TCA AGC TTG GTA CCG AGC TCG GAT CCA CTA GTA ACG GCC
ATG AGT TCG ATA CGT AGT TCG AAC CAT GGC TCG AGC CTA GGT GAT CAT TGC CGG
BstX I EcoR I EcoR I EcoR V
GCC AGT GTG CTG GAA TTC GCC CTT PCR Product AAG GGC GAA TTC TGC AGA TAT
CGG TCA CAC GAC CTT AAG CGG GAA TTC CCG CTT AAG ACG TCT ATA
BstX I Not I Xho I Nsi I Xba I Apa I
CCA TCA CAC TGG CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC AAT TCG CCC TAT
GGT AGT GTG ACC GCC GGC GAG CTC GTA CGT AGA TCT CCC GGG TTA AGC GGG ATA
T7 Promoter
M13 (-20) Forward Primer
AGT GAG TCG TAT TAC AAT TCA CTG GCC GTC GTT TTA CAA CGT CGT GAC TGG GAA AAC
TCA CTC AGC ATA ATG TTA AGT GAC CGG CAG CAA AAT GTT GCA GCA CTG ACC CTT TTG
+1
Plac
lacZ
f1 ori
pCR®II-TOPO®
4.0 kb
pUC ori
Kanamycin
Ampicillin
Invitrogen Life Technologies

(downstream) of the cloned sequence. For this purpose we performed restriction digest using *Xanthomonas badrii* restriction endonuclease I (*Xba*I). A total of 5 separate restriction digest reactions were performed. Each 50 µL total volume reaction contained 5 µL of DNA template (1 µg/µL), 5 µL (75 units) of *Xba*I, 5 µL of 10X Buffer H (100 mM Tris-HCl buffer, pH 7.5, 500 mM NaCl, 100 mM $MgCl_2$, and 10 mM DTT), 5 µL of 0.1% BSA, and 30 µL of sterile water. The mixture was incubated at 37°C overnight. A reaction with no added *Xba*I was included as an "uncut" control. All reagents required for restriction digest were purchased from Invitrogen. 3 µL from each restriction digest reaction were analyzed by gel electrophoresis on 1% agarose gel containing ethidium bromide (EtBr). Bands were visualized under UV light.

Once successful linearization of plasmids was confirmed, we treated the DNA with 200 µg/mL of proteinase K (Invitrogen) and 0.5% SDS for 30 min at 50°C. Following proteinase K, we performed DNA extraction. Thus, an equal volume of phenol/chloroform/isoamyl alcohol was added to DNA template and the mixture was left on a circular rotator for 10 min at AT. Thereafter, the mixture was incubated for 3 min at AT and span down for 3 min at 17,900 x *g*. The aqueous phase was transferred to a new Eppendorf tube and sodium acetate and ethanol (1/10 and 2.5-fold of the volume of the aqueous phase, respectively) were added and mixed by inverting the tube. After incubation at -20°C for 1 h, the tube was centrifuged at 17,900 x *g* for 10 min at 4°C. The supernatant was discarded and the pellet air dried for 15 min and resuspended in TE buffer. The concentration and quality of the purified DNA was evaluated by spectrophotometric analysis of OD_{260} and DNA was stored at -20 °C.

Synthesis of the HCV RNA positive sense was done using the MEGAscript SP6 transcription kit (Invitrogen). The DNA was diluted to a concentration of 0.8 μg/μL. A 20 μL volume reaction was assembled at AT and it contained: 2 μL of each of the rNTPs (50mM each), 2 μL of the 10X reaction buffer, 1.25 μg of DNA template, 2 μL of SP6 enzyme mix, and appropriate volume of nuclease-free water. The reaction was incubated at 37°C for 4 h. A control reaction including 2 μL of the control template provided with the kit (pTRI-Xef, 0.5 mg/mL) was performed in parallel. After incubation, DNase digestion was performed in order to remove residual DNA template. More specifically, one μL of TURBO DNase (2 U/ μL) was added into the newly transcribed RNA, and after mixing the reaction was incubated at 37°C for 15 min. All reagents mentioned above where provided within the MEGAscript kit.

Following DNase treatment, the transcribed RNA was purified using the Ambion MEGAclear kit. Initially, the volume of the newly transcribed RNA was brought to 100 μL with elution solution (provided with the kit) and mixed gently. 350 μL of binding solution concentrate was added and mixed. Then, 250 μL of 100% ethanol were added and the sample was applied to a filter cartridge supplied. The RNA mixture was pipetted onto the filter cartridge and centrifuged for ~15 s to 1 min at 10,000 × *g*. The flow-through was discarded. The collection/elution tube was reused for the washing steps. To wash the RNA, 500 μL of wash solution in 100% ethanol was applied onto the filter cartridge and the wash solution was drawn through the filter as in the previous step. The wash was repeated with a second 500-μL aliquot of wash solution. Then, the cartridge was centrifuged at 10,000 × *g* for 10–30 s to remove the last traces of wash solution. To

elute the purified RNA, 50 µL of elution solution pre-heated at 95°C was added to the center of the filter cartridge applied over a new tube. The tube was centrifuged for 1 min at 10,000 x *g* at AT. This elution step was repeated with a second 50 µL aliquot of elution solution. The eluate was collected into the same tube. To precipitate the RNA, 0.1 volume of 5 M ammonium acetate and 2.5 volumes of 100% ethanol were added. The mixture was incubated at –20°C overnight. The following day, the tube containing RNA was microcentrifuged at 16,000 x *g* for 15 min at 4°C. The supernatant was carefully removed and the pellet was washed with 500 µL 70% cold ethanol. After repeating centrifugation at 16,000 x *g* for 15 min at 4°C, the 70% ethanol was discarded. To remove the last traces of ethanol, the tube was quickly re-spun and residual fluid was aspirated with a very fine tipped pipette. The pellet was air dried for 5 min at AT and was resuspended in DNase/RNase-free distilled water. Concentration and quality of the purified RNA was evaluated using spectrophotometric analysis of OD_{260} and RNA was stored at -80°C. To confirm that HCV RNA positive strand was produced and that it was not contaminated with any residual DNA the following reactions were performed using different dilutions of the HCV RNA positive strand as template: (1) Nested endpoint-PCR/NAH without the reverse transcription step; (2) Nested endpoint-RT-PCR/NAH, and (3) HCV RNA negative strand synthesis followed by nested endpoint-PCR/NAH.

3.10.2 Synthetic HCV RNA Negative Strand

For the synthesis of the HCV RNA negative (replicative) strand, which was used as a positive control for detection of HCV RNA replicative strand, we used the same

template as for the synthesis of HCV RNA positive strand, *i.e.*, the dual promoter PCRII plasmid vector (Invitrogen) containing the rHCV UTR-E2 fragment (see Section 3.9). As shown in Figure 3.4 the PCRII vector contains a T7 RNA polymerase promoter site at the 3'-end (downstream) of the cloned sequence that we wanted to transcribe. To produce antisense (negative strand RNA), we needed to transcribe using the RNA polymerase corresponding to the phage promoter at the 3'-end of the cloned sequence, in that case the T7 RNA polymerase. Therefore, we used the MEGAscript T7 transcription kit (Ambion, by Life Technologies).

First, we had to linearize the plasmid in the polylinker at the 3'-end (upstream) of the cloned 5'-URT-E2 sequence. For this purpose we performed restriction digest using the *Haemophilus influenzae* restriction endonuclease III (*Hind*III). A total of 5 separate restriction digest reactions were performed. Each 50 μL total volume reaction contained: 5 μL of DNA template (1 μg/μL), 5 μL (50 units) of *Hind*III, 5 μL of 10X Buffer H (100 mM Tris-HCl buffer, pH 7.5, 500 mM NaCl, 100 mM $MgCl_2$, and 10 mM DTT), and 35 μL of DNase/RNase-free distilled water. The mixture was incubated at 37°C overnight. A reaction with no added *Hind* III was included as an "uncut" control. 3 μL from each restriction digest reaction were analyzed by gel electrophoresis. Bands were visualized under UV light. All subsequent steps, *i.e.*, proteinase K treatment, DNA extraction, HCV negative strand synthesis using the MEGAscript T7 transcription kit, and RNA purification using the Ambion MEGAclear kit were performed exactly as described in Section 3.10.1.

To confirm that HCV RNA negative strand was produced and not contaminated

with any residual DNA, the following reactions using serial dilutions of the newly synthesized HCV RNA negative strand as template were performed: (1) Nested endpoint-PCR/NAH without the reverse transcription step; (2) Nested endpoint-RT-PCR/NAH, and (3) HCV RNA negative strand synthesis followed by nested endpoint-PCR/NAH.

3.11 HCV RNA Quantification by Real-Time RT-PCR

Enumeration of HCV RNA copy numbers in the plasma and PBMC of patients with CHC was performed by real-time RT-PCR, as previously reported (Pham *et al*., 2004; Sarhan *et al*., 2012a; Chen *et al*., 2013). For this purpose, 50 ng of cDNA transcribed, as described in Section 3.8, from total RNA or from all RNA recovered from plasma or gradient fractions was amplified using SsoFast™ EvaGreen Supermix (Bio-Rad Laboratories, Inc., Hercules, California) in a LightCycler 480 (Roche Diagnostics) for 45 cycles (denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and final extension at 72°C for 5 s). Reactions were always performed in triplicates in the final volume of 10 µL containing 2 µL of SsoFast EvaGreen Supermix (Bio-Rad; 2x real-time PCR mix which contains dNTPs, Sso7d fusion polymerase, $MgCl_2$, EvaGreen dye and stabilizers), 2 µL of cDNA at 25 ng/µL and 5 pmoL of each primer: 5'-GCAGAAAGCGTCTAGCCAT (sense; UTR4) and 5'-CTCGCAAGCACCCTATCAG (antisense; RTU3), as described in Pham *et al*., (2004). Enumeration of viral load in test samples was based on ten-fold serial dilutions of the rUTR-E2 fragment which was synthesized as described in Section 3.9. A mock extraction and a water sample were always included as contamination controls, while synthetic HCV RNA positive strand at

10^6 copies/μL was used as positive control. The sensitivity of the assay ranged between 10 to 100 vge per reaction. In some cases, the specificity of the signal detection and validity of controls were confirmed by NAH with ^{32}P-labeled rHCV UTR-E2 as a probe, as described in Section 3.14.

3.12 Detection of HCV Genome by End-Point RT-PCR

HCV RNA positive strand was detected using cDNA transcribed from 1 μg or, in case of negative results, from 3 μg of total RNA. HCV cDNA was amplified employing an in house developed PCR technique which consisted of a direct and a subsequent nested round of PCR amplification. Primers designed to amplify the HCV 5'-UTR, cycling conditions, and controls used were previously described (Pham *et al*., 2004). More specifically, for both the direct and nested PCR, amplification took place for 35 cycles (denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min) in a PTC-200 thermocycler (MJR Research). Each reaction had a final volume of 100 μL containing 10 μL of 10X PCR buffer containing 200 mM Tris-HCl buffer, pH 8.4, 500 mM KCl, 8 μL of 2.5 mM dNTP mixture, 3 μL of 50 mM $MgCl_2$, 1 μL of each of the 10 pmol/μL primers (forward and reverse), 0.4 μL of *Taq* DNA polymerase (2 Units), cDNA template, and appropriate volume of sterile bidistilled water. For the direct PCR, 1 or 3 μg of cDNA were used as template. For the nested PCR, 10 μL of direct PCR product was used as template. The following primer pairs were utilized: 5'-CTGTGAGGAACTACT GTCTTC (sense; UTR1) and 5'-GCGGTTGGTGTTACGTTT (antisense; RTU1) for the direct round, and 5'-GCAGAAAGCGTCTAGCCATGGC

(sense; UTR2) and 5'-CTGCAAGCACCCTATGAGGCAGT (antisense; RTU2) for the nested round (Pham *et al*., 2004). All reagents used for end-point PCR were purchased from Invitrogen apart from the primers that were ordered from Integrated DNA Technologies, Inc. (Coralville, Iowa). The rHCV UTR-E2 fragment at 10^6 copies/μL was used as a positive control. A mock extraction and a water sample that had undergone reverse transcription were always included as contamination controls, while synthetic rHCV RNA positive strand at 10^6 copies/μL was used as positive control. The specificity of amplifications and validity of controls were confirmed by NAH with ^{32}P-labeled rHCV UTR-E2 as a probe, as described in Section 3.14. The sensitivity of this assay was ≤ 10 vge/mL (≤ 3 IU/mL) or ≤ 5 vge/μg of total RNA (Figure 3.5).

3.13 Detection of HCV RNA Replicative Strand

Detection of the HCV RNA negative (replicative) strand was performed by a strand-specific RT-PCR/NAH assay (Landford *et al*., 1999) using r*Tth* DNA polymerase (Promega Corp.). The r*Tth* DNA polymerase is a thermostable enzyme isolated from *Thermus thermophilus* HB-8. In the presence of manganese, r*Tth* DNA polymerase catalyzes the polymerization of nucleotides into DNA using an RNA template in the 5′→3′ direction. In the presence of magnesium, the same enzyme catalyzes the polymerization of nucleotides into duplex DNA in the 5′→3′ direction (Figure 3.6.). The negative strand-specific RT-PCR/NAH assay was performed as previously described (Pham *et al*., 2004; Sarhan *et al*., 2012a). More specifically, as RNA template, we used 1-4 μg of test RNA diluted in UltraPure DNase/RNase-Free distilled water (Invitrogen).

Figure 3.5. Assigned levels of HCV RNA positive strand detection by semi-quantitative RT-PCR/NAH. One round of the in-house RT-PCR (direct RT-PCR) detects approximately 10^4 or more vge/reaction when products are analyzed by electrophoresis on EtBr-agarose (assigned score: ++++). When direct RT-PCR is followed by EtBr-agarose electrophoresis and nucleic acid hybridization (NAH), the sensitivity of the signal detection increases by approximately 10-fold, reaching about 10^3 vge/reaction (assigned score: +++). By using 10 μL of the direct PCR product as a template for a second round of amplification (nested RT-PCR), the sensitivity of detection on EtBr-gel after electrophoresis further increases to around 10^2 vge/reaction (assigned score: ++). Finally, when nested RT-PCR is followed by NAH of the amplified products, the sensitivity of HCV RNA detection reaches below or equal to 10 vge/reaction (assigned score: +).

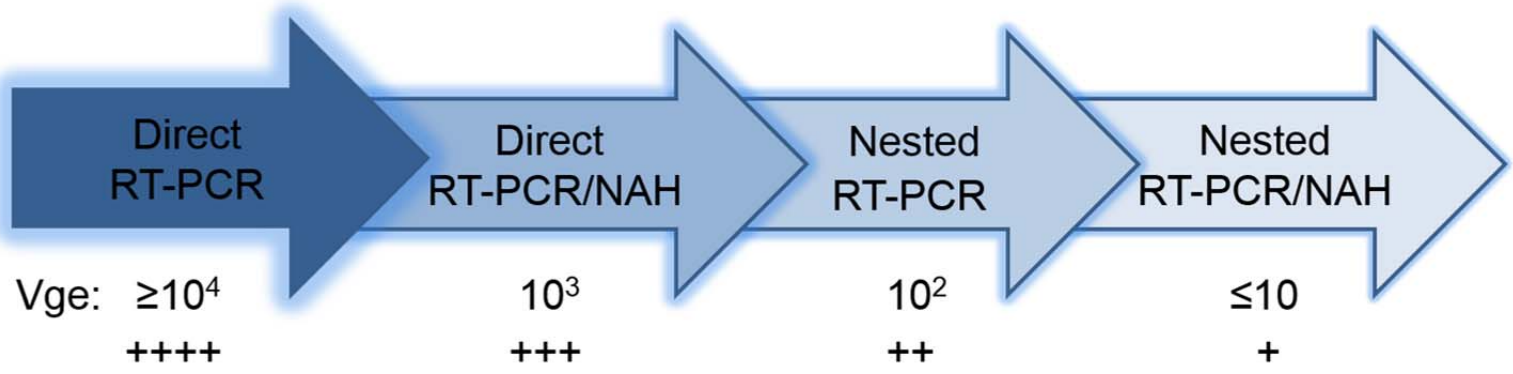
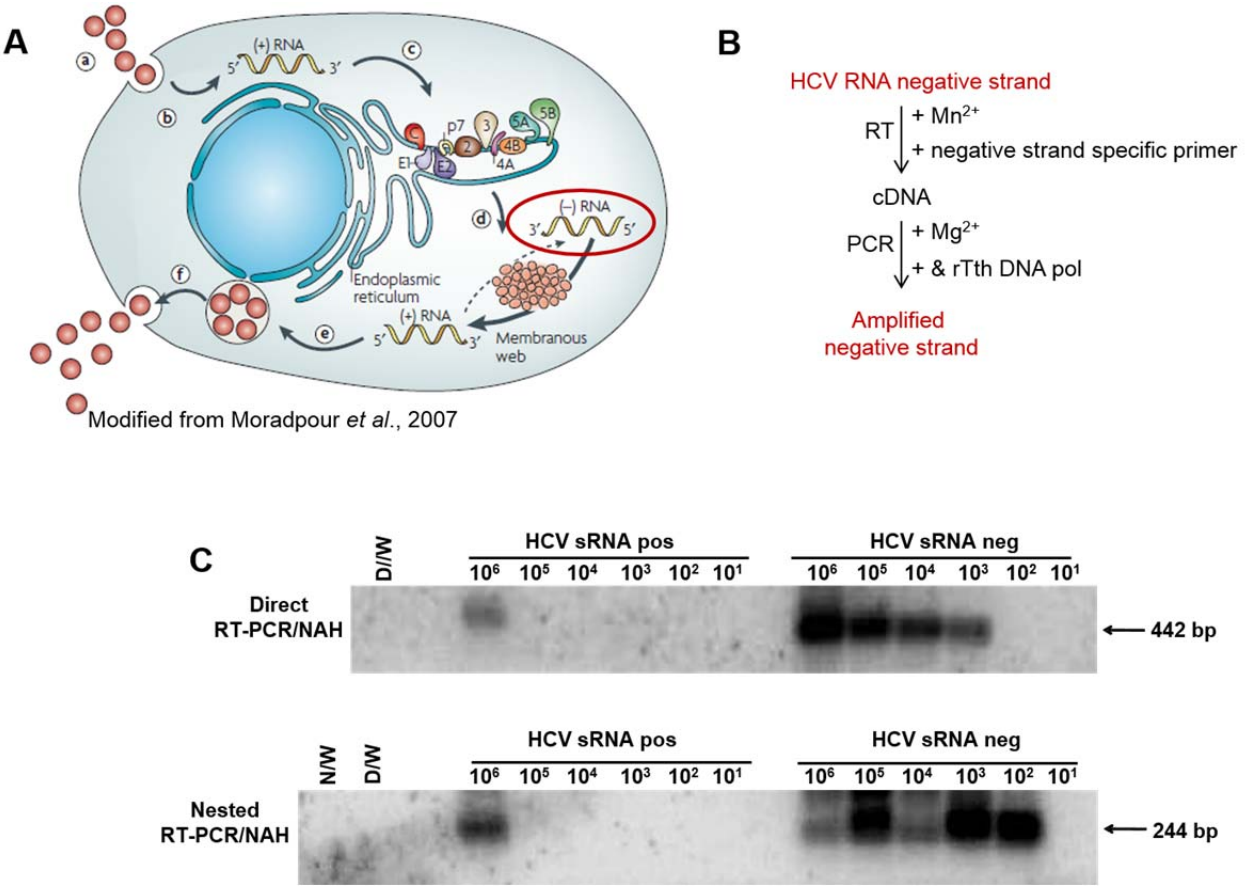

Direct
RT-PCR
Direct
RT-PCR/NAH
Nested
RT-PCR
Nested
RT-PCR/NAH
Vge: ≥10^4
10^3
10^2
≤10
++++
+++
++
+

Figure 3.6. Assay for HCV RNA negative (replicative) strand detection. (A) HCV replicates via synthesis of a negative-strand HCV RNA which serves as a template for HCV RNA positive strand synthesis. (B) Overall scheme of the HCV RNA negative strand detection by a RT-PCR/NAH assay using rT*th* DNA polymerase (see Materials and Methods). (C) As specificity control for HCV RNA negative strand detection, serial dilutions of synthetic HCV RNA positive strand were used (see Materials and Methods). As a sensitivity control, serial dilutions of synthetic HCV RNA negative strand were utilized. Using these standards it was shown that the negative-strand RT-PCR/NAH was capable of detecting as little as 10^2 copies/reaction of the correct (negative) strand, while nonspecifically identifying positive strand at concentrations $\geq 10^6$ copies. Water instead of cDNA amplified in direct (D/W) and nested (N/W) reactions were included as contamination controls. Positive signals showed the expected 442-bp (direct RT-PCR/NAH) or 244-bp (nested RT-PCR/NAH) nucleotide fragments. The specificity of the RT-PCR products was verified using a ^{32}P-labeled rHCV 5'-UTR-E2 fragment as a probe.


A
(+) RNA
5′
3′
a
b
c
d
e
f
C
p7
3
5A
5B
2
4B
4A
E1
E2
(–) RNA
Endoplasmic reticulum
Membranous web
Modified from Moradpour et al., 2007
B
HCV RNA negative strand
RT
+ Mn2+
+ negative strand specific primer
cDNA
PCR
+ Mg2+
+ & rTth DNA pol
Amplified negative strand
C
D/W
HCV sRNA pos
HCV sRNA neg
10⁶ 10⁵ 10⁴ 10³ 10² 10¹
Direct RT-PCR/NAH
442 bp
N/W
Nested RT-PCR/NAH
244 bp

The amount of template RNA used in this assay depended on the estimated copy number of the HCV RNA positive strand in the test sample. When the copy number of the HCV RNA positive strand was estimated to be 10-100 vge/mL, 4 μg of test RNA were used as a template, as it is known that the HCV RNA negative strand is 100-1000-fold less abundant than the positive strand in the infected cells (Komurian-Pradel *et al*., 2004). When the level of HCV RNA positive strand in the test RNA was estimated to be > 100 vge/mL, 1-2 μg of test RNA were used for this assay. It has been previously reported in this laboratory that a high concentration of HCV RNA negative strand can inhibit its detection by that assay.

In the first step, the 10 μL RNA was denaturated at 95°C for 1 min and then temperature was lowered to 70°C. Subsequently, 10 μL of preheated RT mix containing 2X RT buffer (20 mM Tris-HCl buffer, pH 8.3, with 180 mM KCl, 2 mM $MnCl_2$, 1.6 mM deoxynucleoside triphosphate mix, 15 pM sense UTR1 primer, and 5 U of rT*th* DNA polymerase) was added and cycled for 2 min at 60°C and 70°C for 15 min. The specificity of this assay derives from the RT step where only the sense UTR1 primer is added. After that step, the temperature was held at 70°C and 80 μL of preheated PCR mixture which contained a chelating buffer (10 mM Tris-HCl buffer, pH 8.3, with 100 mM KCl, 750 %M EGTA, 0.05% Tween 20, and 5% glycerol), 2.5 mM $MgCl_2$ and 15 pM of the antisense RTU1 primer were added. After 3 min at 94°C, amplification took place for 45 cycles (1 min at 94°C, 2 min at 60°C, and 3 min at 72°C), which was followed by a final extension step for 7 min at 72°C. 10 μL volumes of the direct PCR products were used as templates for the nested PCR, which was performed as described in

Section 3.12. PCR products were analyzed by gel electrophoresis and detected by NAH as described below (Section 3.14).

Serial dilutions of HCV RNA positive strand synthesized as described in Section 3.10.1 were used as specificity control for the assay. Serial dilutions of the synthetic HCV RNA negative strand prepared as reported in Section 3.10.2 were used as sensitivity control. Using these standards it was shown that the HCV RNA negative-strand RT-PCR/NAH was capable of detecting as low as 10^2 copies per reaction of the negative strand and that it maintained specificity of detection for up to 10^6 copies/reaction as shown in Figure 3.6.C. This was in accordance with previously reported data (Pham *et al*., 2004; Sarhan *et al*., 2012a). As negative controls, water samples were included.

3.14 Nucleic Acid Hybridization

In order to validate the specificity of the identified amplification signals and to enhance by approximately 10-fold the sensitivity of the signals' detection (Figure 3.6), NAH was done using the ^{32}P-labelled HCV UTR-E2 fragment as a probe.

3.14.1 HCV DNA Probe Labelling and Purification

To generate the probe, the rHCV UTR-E2 fragment was excised from the PCRII plasmid vector by restriction enzyme digestion with *Eco*RI (as described in Section 3.15). The digested products were then separated by electrophoresis in a 0.9% low melting point (LMP) agarose gel containing EtBr for approximately 40 min at 80 Volts. Subsequently, bands were visualized with UV light and desired products were excised using a sterile

razor blade. Agarose slices were then transferred into a 1.5 mL sterile microcentrifuge tube and incubated at 70 °C until the agarose gel was completely melted. Purification of amplicons was performed via a vacuum manifold using the Wizard PCR Preps DNA Purification System (Promega Corp.), as described in Section 3.15.

Radioactive labeling of HCV DNA was performed by random primed labeling of DNA using the Amersham rediprime II DNA labelling system (GE Healthcare). In this method, random sequence hexanucleotides are annealed to both strands of the denatured DNA template and Klenow fragment polymerase is used to extend the oligonucleotides. A non-radioactive nucleotide is replaced by the radiolabelled equivalent in the reaction mixture and, as a result, a uniformly labelled double-stranded probe is produced. More specifically, 25 ng of the purified HCV UTR-E2 fragment was diluted in 45 µL of TE buffer. DNA was denatured by boiling for 5 min and chilled for 2 min. 5 µL of ^{32}P – dCTP (PerkinElmer, Waltham, Massachusetts) were then added and the mixture was transferred to a tube containing an Amersham rediprime II DNA labelling system (GE healthcare) random-prime reaction (buffered solution of dATP, dGTP, dTTP, exonuclease-free Klenow enzyme and random primers). After repeated pipetting to dissolve the pellet, the mixture was incubated for 1 h at 37°C. Following ^{32}P labelling of the HCV UTR-E2 fragment, the newly synthesized probe was cleaned from the excess of ^{32}P-dCTP using Illustra nick columns Sephadex G-50 DNA grade (GE healthcare). Prior to use, the "nick column buffer" containing distilled water with 0.15% kathon CG/ICP Biocide was removed from the column and 3 mL of TE buffer was added. After shaking, the TE buffer was removed and another 3 mL of TE buffer was added, and left to elute

from the column to equilibrate it. 50 µL of the probe were added onto the column and left to elute into a 15 mL tube. 400 µL of TE buffer were then added to wash out unbound probe into the same 15 mL tube. The column was then mounted over a new 15 mL tube and 450 µL of TE buffer was added to elute the probe. The probe was transferred to a 1.5 mL Eppendorf tube and stored at 4°C. 2 µL of the probe was supplemented with 100 mL scintillation fluid (Perkin Elmer) and mixed. The specific activity of the probe was determined using the Beckman Coulter LS6500 scintillation counter and expressed as counts per minute (cpm).

3.14.2 Southern Blot Hybridization Analysis

As previously mentioned, PCR products were analyzed by gel electrophoresis on 1% agarose gels containing EtBr. Once electrophoresis was completed, the gel was soaked in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 30 min at ambient temperature and then in neutralizing buffer (1.5 M NaCl in 1M Tris-HCl buffer, pH 8.0) for another 30 min. Downword capillary transfer of DNA from the gel to a neutral nylon membrane (Hybond-N, GE Healthcare) took place according to Maniatis *et al*. (1982). More specifically, the gel was placed upside down on a bridge which consisted of a sheet of 3 MM filter paper (Whatman International Ltd, Maidstone, United Kingdom) placed on a glass surface and soaked in 10X standard saline citrate (SSC; diluted from 20X; 3M NaCl in 0.3 M sodium citrate dehydrate, pH 7.0). Parafilm was placed around the edges of the gel. The gel was covered with a nylon membrane, followed by 2 sheets of 3 MM filter paper soaked in 10X SSC, one dry sheet of 3 MM filter paper, and a 10-cm high

stack of dry paper towels. The whole set up was reversed and weight was placed over the glass surface of the bridge. After overnight transfer, the nylon membrane was baked at 80°C for 1 h under vacuum. The baked membrane was then transferred into a hybridization glass tube containing 5 mL of pre-hybridization buffer (4X SSC, 5X Denhart's solution, 0.5 % SDS, 100 µg/mL denatured [boiled and cooled] salmon sperm DNA; all from Invitrogen) and incubated at 65 °C for 1 h while constantly being rotated. Then, 6 X 10^6 cpm of probe were added directly into the glass tube that contained the hybridization buffer and the membrane was incubated overnight at 65 °C in a hybridization oven. The following day, the membrane was washed twice with 2X SSC and 0.1% SDS at AT and twice with 0.1X SSC and 0.05% SDS at 65 °C for 10 min. Then, it was air dried, covered with plastic wrap, and placed in a cassette (Bio-Rad Laboratories) with a phosphor screen (Kodak) for overnight at AT. The following day, image and density of the signals obtained were analyzed using a Pharos FX plus molecular imager system (Bio-Rad Laboratories) and expressed in pixel density units (PDU).

3.15 HCV Cloning and Sequencing

HCV genome amplicons detected in *in vitro* infected $CD4^+$ and $CD8^+$ T cells in Experiments I, III and V (see Table 4.3), and at time points where HCV replication has been confirmed, were analyzed by clonal sequencing. Those sequences were compared with the sequences harbored in the HCV inoculum used for *in vitro* infection of $CD4^+$ and $CD8^+$ T cells and in PBMC of the inoculum donor.

Initially, the amplicons were adenylated by addition of 1 μL of dATP (Invitrogen) and incubation at 72 °C for 10 min. Following 3'-adenylation, PCR products (~40 μL) were separated by electrophoresis in a 0.9% LMP agarose gel. Subsequently, bands were visualized with UV light and desired bands excised. Agarose slices were then transferred in a 1.5 mL sterile microcentrifuge tube and incubated at 70 °C until completely melted. Purification of amplicons was performed via a vacuum manifold using the Wizard PCR Preps DNA Purification System (Promega). More specifically, using this system, 1 mL of resin was mixed with each melted gel slice and subsequently pipetted into a syringe barrel. The syringe barrel was attached to a Wizard Minicolumn and the barrel/minicolumn assembly was connected to a vacuum manifold. Vacuum was applied to draw the resin/DNA mixture into the minicolumn. 2 mL of 80% isopropanol were added to each minicolumn and vacuum was applied to wash the minicolum. Vacuum was reapplied to dry the resin. The minicolumn was removed from the barrel, transferred to a 1.5 mL microcentrifuge tube and centrifuged at 10,000 x *g* for 2 min to remove residual isopropanol. Each minicolumn was then transferred to a new 1.5 mL microcentrifuge tube. 50 μL of water was added to each minicolumn. After 1 min, each minicolumn was centrifuged for 20 s at 10,000 x *g* to elute the DNA fragments. Eluted DNA was stored in the microcentrifuge tube at 4 °C or -20 °C for no more than 24-48 h before used for cell transformation.

Cloning was performed using the TOPO TA Cloning kit (Invitrogen). Initially, a 6 μL TOPO Cloning reaction was set for each PCR product and was left to incubate for 30 min at AT. Each reaction contained 4 μL of DNA template, 0.5 μL of TOPO vector

and 1 μL of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$). During incubation, One Shot TOP10 chemically competent *E. coli* cells were thawed on ice. For transformation of One Shot cells, the cloning reaction was added to the cells and kept on ice for 30 min. After 30 min, the cells were heat-shocked for 30 s at 42°C and then returned to ice.

After transformation, the cells were recovered and plated. For this purpose, 250 μL of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was added to each tube. The tubes were then placed in a rocking incubator for 1 h at 37 °C. After recovery, 100-150 μL of transformed cells were spread on prewarmed LB agar plates containing 50 μg/mL kanamycin. The plates were placed in the 37°C incubator for overnight. The following day, at least 20 single colonies were picked from each plate. Each colony was cultured in 15 mL conical tubes containing 3 mL of LB medium and incubated in a rocking incubator at 37°C for ~18 h.

After incubation, plasmid DNA was extracted from each colony using the QIAprep Spin Miniprep kit (Qiagen). More specifically, 1.5 mL of each inoculated culture was initially transferred to a new tube and spun for 5 min at 17,900 x *g* in a microcentrifuge. Supernatant was discarded and this process was repeated using the same tube until the entire culture had been transferred. Pelleted bacterial cells were resuspended in 250 μL of resuspension buffer (P1; 50 mM Tris-HCl buffer, pH 8.0, 10 mM EDTA, 100 μg/mL RNase A) and mixed by vortexing. 250 μL of lysis buffer (P2; 200 mM NaOH, 1% SDS) were then added and each tube was inverted gently 4-6 times to mix. 350 μL of neutralization buffer (N3; 3.0 M potassium acetate, pH 5.5) were

subsequently added and each tube was again inverted 4-6 times. Tubes were then centrifuged for 10 min at maximum speed in tabletop microcentrifuge. The supernatant (about 850 µL) from each tube was applied to the QIAprep column by pipetting and vacuum was applied. The QIAprep column was washed by adding 750 µL of neutralization buffer (PE; 10 mM Tris, pH 7.5, 80% ethanol). Vacuum was again applied to draw the wash solution through the column. The QIAprep column was transferred to a tube and centrifuged for 1 min at 17,900 x *g*. Each QIAprep column was then placed in a clean 1.5 mL microcentrifuge tube. To elute DNA, 50 µL of elution buffer (EB; 10 mM Tris-HCl buffer, pH 8.5) or water was added to the center of the column. After 2 min, the columns were centrifuged for 1 min and plasmid DNA isolation was completed.

Plasmid DNA from each colony was subsequently analyzed after restriction digestion with *E. coli* restriction endonuclease I (*Eco*RI). Thus, each 20 µL of reaction aliquot contained 10 µL of DNA template, 1 µL (15 units) of *Eco*RI, 2 µL of 10X Buffer H (0.5 M Tris-HCl buffer, pH 7.5, 1 M NaCl, 100 mM $MgCl_2$, 10 mM DTT), 0.25 µL of RNaseA (1 mg/mL) and 6.75 µL of sterile water, and was incubated at 37°C for 1 h. A reaction with no added *Eco*RI was included as an "uncut" control. All reagents required for restriction digest were purchased from Invitrogen. Each restriction digest product was analyzed by gel electrophoresis (120 Volts, 30 min) using 1% agarose gel containing EtBr. Bands were visualized under UV light. DNA from positive colonies was quantitated by Nanodrop. DNA from 20 clones derived from each 5'-UTR HCV amplicon was sent for sequencing at The Centre for Applied Genomics (TCAG) at The Hospital for Sick Kids in Toronto. For this reason, each sample was diluted to 43 ng/µL

and was sequenced bidirectionally using universal forward and reverse M13 primers and ABI 3730xl DNA Analyzer (Applied Biosystems by Life Technologies). Sequence analysis was performed using Sequencer software version 5.0 (Gene Codes Corp., Ann Arbor, Michigan).

3.16 Detection of HCV Proteins in De Novo Infected T Lymphocytes by Confocal Microscopy

3.16.1 Staining of HCV Core and NS5A Proteins

To detect HCV NS5A and core proteins, $5x10^5$ to $1x10^6$ B/F cells collected at 10 and 14 d.p.i., and exposed to HCV-11/M inoculum (experiment V; see Table 4.3) were fixed with 200 µL of 4% PFA for 10 min at AT and permeabilized with 200 µL of 0.1% saponin in PBS for 10 min at AT. Blocking of non-specific binding was performed with 5% normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania) in PBS (blocking buffer) for 1h at AT. Then, cells were incubated with mouse anti-HCV NS5A mAb (Chemicon International, Temecula, California) at 1:100 dilution in blocking buffer, or with mouse anti-HCV core mAb (Thermo Scientific by Thermo Fisher Scientific, Waltham, Massachusetts) at 1:200 dilution in blocking buffer. As controls, cells were exposed to mouse IgG1 K isotype control (eBioscience, Affymetrix, Santa Clara, California) at 1:100 dilution in blocking buffer. Staining with the primary antibodies took place overnight in the cold room on a tube rotator. The following day, cells were washed by centrifugation with PBS containing 0.25% Tween-20 and exposed to Cy3-conjugated donkey antibody to mouse IgG (Jackson

ImmunoResearch) at 1:500 dilution in PBS containing 0.25% Tween-20 for 1 h at AT in the dark. After cells were washed by centrifugation, the resulting pellet was resuspended with 20% glycerol in PBS. Cells were then mounted on slides and counterstained with 4', 6-diamidino-2-phenylindole (DAPI; 0.1 μg/mL) (Vector Laboratories, Inc., Burlingame, California). All steps, until mounting of cells on slides, were performed with cells being in suspension in 1.5 mL Eppendorf tubes (modified from Sarhan *et al*., 2012a). Slides were examined under an Olympus BX50W1 microscope with a FluoView FV300 confocal system (Olympus America Inc., Melville, New York). Approximately 1000 cells from each preparation were examined and HCV protein positive cells were counted. The same procedure was also performed for cells from donor B/F exposed to HDP collected at 10 and 14 d.p.i., which served as negative controls.

3.16.2 Double Cell Staining for Detection of HCV NS5A Protein and $CD4^+$ or $CD8^+$ T Cell Surface Markers

To detect CD4 and CD8 proteins as well as HCV NS5A protein, we used $5x10^5$ to $1x10^6$ cells from donor A/M collected at 10 and 14 d.p.i., and exposed to inoculum HCV-16 (experiment VI; see Table 4.3). For staining with the anti-HCV NS5A mAb and the Cy3-conjugated donkey anti-mouse antibody, we followed exactly the same procedure as described in Section 3.16.1. Prior to exposing the cells to the second primary mAb, blocking with 5% normal donkey serum in PBS for 1 h at AT was performed. Once blocking was completed, the antibody was added. Thus, cells were exposed to either rabbit anti-human CD4 mAb (Abcam, Cambridge, United Kingdom) at 1:250 dilution in

blocking buffer for 30 min on ice or to a rabbit IgG polyclonal isotype control (Abcam) at 1:100 dilution in blocking buffer. $CD8^+$ T cells were exposed to rabbit anti-human CD8 mAb (Abcam) at 1:400 dilution in blocking buffer or to a rabbit IgG polyclonal isotype control (Abcam) at 1:100 dilution in blocking buffer for 30 min on ice. After incubation, cells were washed by centrifugation with PBS containing 0.25% Tween-20 and exposed to Alexa 488-conjugated donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch) at 1:400 dilution in PBS containing 0.25% Tween-20 for 1 h in the dark at AT. After that step, cells were washed and processed as described in Section 3.16.1.

CHAPTER FOUR: RESULTS

4.1 Selection of HCV Inocula for $CD4^+$ and $CD8^+$ T Cell Infection Experiments

As previously indicated (Section 3.1), the selection of HCV inocula for the *in vitro* infection of affinity-purified $CD4^+$ and $CD8^+$ T cells was a three-step process (Figure 3.1). At step one, the HCV RNA load in plasma of 16 patients with clinically diagnosed CHC was evaluated by real-time RT-PCR. At the second step, real-time RT-PCR was used to examine the expression of HCV RNA in the PBMC of patients with the highest loads of HCV RNA in their plasma. In cases where HCV RNA in the PBMC was undetectable by real-time RT-PCR, we employed the nested-RT-PCR/NAH assay previously established in this laboratory (Pham *et al*., 2004). At the final step, plasma carrying high HCV RNA load and coming from patients with detectable HCV RNA in their PBMC were used for *in vitro* infectivity experiments of HCV-naïve PBMC-derived total T cells using the cell culture system developed in this laboratory (MacParland *et al*., 2006). Plasma found capable of *in vitro* infecting PBMC-derived total T cells, as evidenced by HCV RNA negative strand detection, served as inocula for the *in vitro* infection experiments using affinity-purified $CD4^+$ and $CD8^+$ T cells as targets.

4.1.1 HCV RNA in Plasma of Patients with CHC

As the first step in the inocula selection process, the HCV RNA load in plasma of 16 patients with CHC was evaluated by real-time RT-PCR (see Table 4.1). It was found

Table 4.1. Clinical Characteristics and Detection of HCV RNA in Plasma and PBMC of Patients with Chronic Hepatitis C

Case/ HCV Inoculum	Age/sex	Clinical diagnosis	HCV genotype[a]	HCV RNA load			Case overall HCV positivity[d]
				Plasma (vge/mL)[b]	PBMC (vge/µg)[b]	PBMC (vge/µg)[c]	
1/M	35/M	CHC	NT	$3.75x10^2$	ND	ND	+
2/F	51/F	CHC	1b	$1.9x10^3$	NA	NA	+
3/M	24/M	CHC	1a	$1.34x10^2$	NA	NA	+
4/M	NA/M	CHC	NT	$1.96x10^3$	ND	≤3.3	+
5/M	33/M	CHC	3a	<100	NA	NA	+
6/M	37/M	CHC	4	$1.16x10^4$	ND	≤3.3	+
7/F	NA/F	CHC	NT	$3.92x10^4$	ND	ND	+
8/M	NA/M	CHC	1a	$1.8x10^5$	<100	NT	+
9/M	43/M	CHC	1b	$5.95x10^2$	ND	≤3.3	+
10/M	57/M	CHC	1	$3.1x10^7$	$3.67x10^2$	NT	+
11/M	37/M	CHC	1a	$1.24x10^6$	$3.07x10^2$	NT	+
12/M	51/M	CHC	NT	$2.74x10^4$	<100	NT	+
13/M	40/M	CHC	NT	$9.69x10^4$	<100	NT	+
14/M	57/M	CHC	3a	$1.05x10^5$	ND	≤3.3	+
15/M	54/M	CHC	1a	$1.5x10^3$	ND	≤3.3	+
16	NA/NA	CHC	1a	$1.6x10^7$	NA	NA	+
HCV RNA positivity/ Total tested (%)				**16/16 (100%)**	5/12 (42%) **10/12 (83%)**	5/7 (71%)	**16/16 (100%)**

Abbreviations: F, female; M, male; NA, not available; ND, not detected; NT, not tested.

[a] Based on clinical laboratory data.

[b] HCV RNA load determined by real-time RT-PCR (sensitivity: 10-100 vge/reaction) using RNA extracted from 250 µL of plasma or total RNA (1 or 3 µg) extracted from PBMC.

[c] Estimated HCV RNA copy number detected by nested RT-PCR/NAH with 5-UTR-specific primers (sensitivity: ≤ 5 vge/µg RNA) using 3 µg of total RNA extracted from PBMC, as described in Materials and Methods.

[d] Overall positivity defined as the detection of HCV RNA reactivity in either serum or PBMC, or in both.

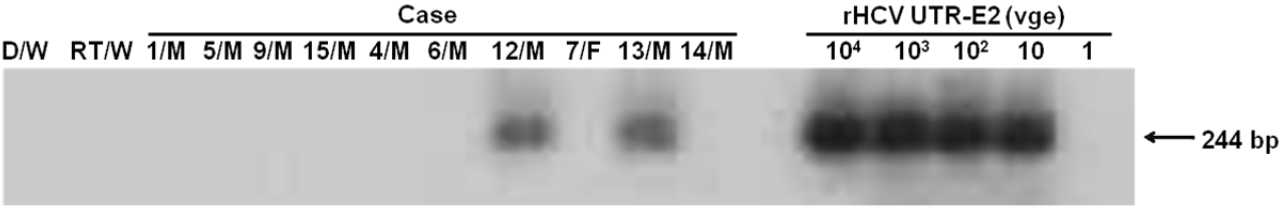
that HCV RNA positive strand was detectable in all individuals tested (n=16; 100%) and quantifiable in 15 of them. Thus, HCV RNA loads ranged from $1.34x10^2$ vge/mL (case 3/M) to $1.6x10^7$ vge/mL (case 16). HCV RNA load in plasma of patient 5/M was lower than 100 vge/mL; which was the assay lowest sensitivity level and, therefore, could not be quantified.

4.1.2 HCV RNA in the PBMC of Patients with CHC

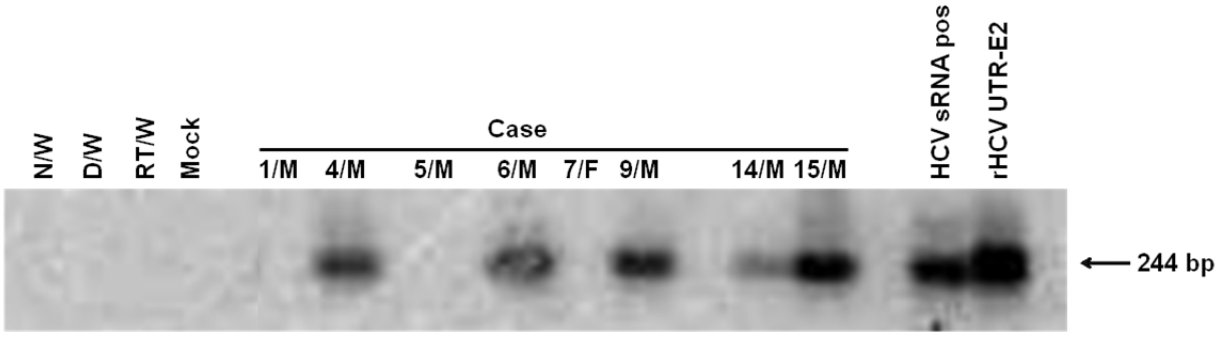
At the second step in selection of inocula, real-time RT-PCR was used to evaluate the presence of HCV RNA in the PBMC (if available) of patients with detectable HCV RNA in their plasma. PBMC samples were available from 12 out of 16 patients examined (Table 4.1). By employing real-time RT-PCR, HCV RNA was detected in PBMC of 5 out of these 12 patients (42%) and was quantifiable in two cases (10/M with $3.57x10^2$ vge/μg and 11/M with $3.07x10^2$ vge/μg). In cases where HCV RNA in the PBMC was undetectable by real-time RT-PCR (n=7), nested-RT-PCR/NAH was performed. HCV RNA was detected in 5 out of 7 individuals (71%) (Figure 4.1 B). The combined results from real-time RT-PCR and nested RT-PCR/NAH assays showed that HCV RNA was detected in PBMC of 10 out of 12 patients (83%) (Table 4.1). It is of note, that PBMC were tested without prior stimulation with mitogens which normally augments detection of replicating HCV in circulating lymphoid cells (Pham *et al*., 2004, 2005; Chen *et al*., 2013, 2015).

Figure 4.1. Comparison of HCV RNA detection in PBMC from CHC patients who provided HCV inocula tested by real-time RT-PCR/NAH and nested RT-PCR/NAH. RNA was extracted from PBMC and transcribed to cDNA. (A) Initially, cDNA equivalent of 50 ng of RNA from each sample were amplified by real-time PCR and the amplicons separated on agarose gel, transferred onto a nylon membrane and probed by NAH, as described in Materials and Methods. (B) In cases where HCV RNA in the PBMC was not detected by real-time RT-PCR/NAH, cDNA equivalent of 1 or 3 μg of RNA were amplified by nested-RT-PCR/NAH. Water added instead of cDNA (RT/W) and amplified by direct (D/W) and nested PCR (N/W), as well as RNA/cDNA-free mock (Mock) sample were used as negative controls. The positive controls included serial dilutions of the recombinant HCV UTR-E2 fragment (rHCV UTR-E2) as quantitative standards (vge) in (A) and a single concentration of HCV UTR-E2 (10^6 vge) in (B). Synthetic HCV RNA positive (sRNA pos) strand at 10^6 vge was also used as the positive and specificity control in (B). Positive signals showed the expected 244-bp 5'-UTR amplicons.

A Real-time RT-PCR/NAH



B Nested RT-PCR/NAH



4.1.3 HCV RNA Expression and Replication in Total T cells *De Novo* Infected with Selected HCV Inocula

At the final step of the inocula selection process, plasma carrying high HCV RNA load and coming from patients with detectable HCV RNA in their PBMCs were used for *in vitro* infectivity experiments of T cells derived from healthy human PBMC using the cell culture system previously developed (MacParland *et al*., 2006).

As shown in Table 4.2, HCV RNA positive strand was detected at variable levels in all the experiments where A/M served as donor of PBMC, and at different time points post-infection with various inocula tested. However, HCV RNA negative strand, indicative of active HCV replication, was only detected when A/M T lymphocytes were exposed to inocula from 11/M and 16 (Table 4.2 and Figure 4.2).

Using PBMC from B/F, HCV RNA positive strand was again detected at variable levels in all experiments performed, apart from experiments where inocula from 4/M and 9/M were used. With regards to HCV RNA negative strand in these experiments, the strand was only detected at 7 d.p.i. when plasma from patient 11/M was used as inoculum (Table 4.2).

Overall, the results showed that plasma from patients 11/M and 16 were capable of *in vitro* infecting PBMC-derived total T cells, as evidenced by HCV RNA negative strand detection (Figure 4.2). Therefore, plasma from these patients were selected as inocula for further infection experiments with affinity-purified $CD4^+$ and $CD8^+$ T cells.

Table 4.2. Detection of HCV RNA Positive and Negative Strands in PBMC-Derived T Cell Cultures Exposed to Patient-Derived HCV

Healthy donor PBMC[a]	Inoculum	HCV genotype	HCV load (vge/mL)	No. of target cells	HCV copies/cell ratio	HCV RNA					
						Positive strand (vge/µg)[b]			Negative strand (copies/µg)[c]		
						7 d	10 d	14 d	7 d	10 d	14 d
A/M	8/M	1a	$1.8x10^5$	$6.3x10^6$	1/140	10^3	≤3.3	≤3.3	ND	NT	NT
	10/M	1	$3.1x10^7$	$6.3x10^6$	1/1	ND	10^3	100	NT	ND	ND
	11/M	1	$1.24x10^6$	$6.3x10^6$	1/20	10^3	10^3	10^3	≤50	ND	ND
	16	1	$1.6x10^7$	$5x10^6$	1/10	NA	100	100	NA	ND	≤100
B/F	8/M	1a	$1.8x10^5$	$7x10^6$	1/160	300	100	ND	ND	ND	NT
	10/M	1	$3.1x10^7$	$7x10^6$	1/1	300	30	300	ND	ND	NA
	11/M	1	$1.24x10^6$	$7x10^6$	1/160	10^3	≤3.3	100	≤50	ND	ND
	4/M	NT	$1.96x10^3$	$5x10^6$	$1/10^4$	ND	ND	ND	NT	NT	NT
	6/M	4	$1.16x10^4$	$5x10^6$	1/1700	10^3	ND	≤3.3	ND	NT	NT
	7/F	NT	$3.92x10^4$	$5x10^6$	1/500	10^3	300	ND	ND	ND	NT
	9/M	1b	$5.95x10^2$	$5x10^6$	1/34000	ND	ND	ND	NT	NT	NT
	14/M	3a	$1.05x10^5$	$5x10^6$	1/200	≤3.3	ND	ND	NT	NT	NT
	15/M	1a	$1.5x10^3$	$5x10^6$	1/13000	300	300	ND	ND	ND	NT

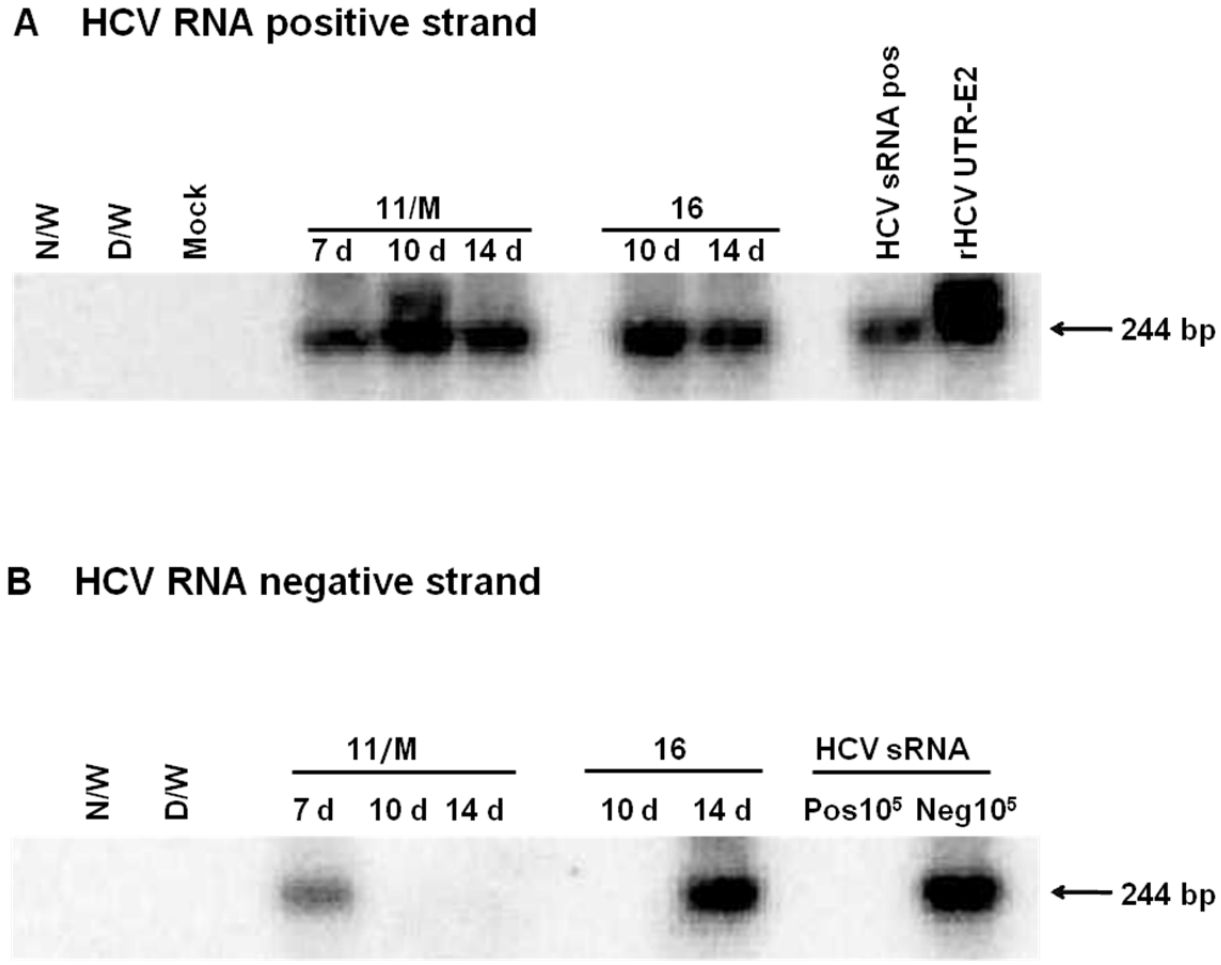
Abbreviations: F, female; M, male; d, days post-infection; NT, not tested; NA, not available; ND, not detected.

[a] Monocyte-depleted PBMC from donors A/M or B/F pre-stimulated with PHA were exposed to indicated inocula and cultured under alternating stimulation with PHA for 14 days, as described in Materials and Methods. Total RNA extracted from T cells harvested at 7, 10 and 14 days post-infection (d) was analyzed for HCV RNA positive and negative strands.

[b] HCV RNA positive strand detected by nested RT-PCR/NAH, as described in Materials and Methods.

[c] HCV RNA negative strand detected by strand-specific RT-PCR/NAH, as described in Materials and Methods.

Figure 4.2. Detection of HCV RNA positive and negative strands in PBMC-derived total T cells after their *in vitro* infection with authentic, patient-derived HCV. Monocyte-depleted PBMC from donor A/M pre-treated with PHA were exposed to HCV-11/M or HCV-16 (inocula) and cultured as described in Materials and Methods. RNA was analyzed for expression of (A) HCV RNA positive strand and (B) HCV RNA negative strand at different days post-infection (d). Synthetic HCV RNA (HCV sRNA) positive and negative strands were used to confirm specificity of the detections and as quantitative standards. The recombinant HCV UTR-E2 fragment (rHCV UTR-E2) served as additional positive control for detection of HCV RNA positive strand. Water instead of cDNA amplified in direct (D/W) and nested (N/W) reactions, as well as a mock-treated test RNA (Mock), were included as contaminations controls. Positive signals showed the expected 244-bp fragment.


A HCV RNA positive strand
N/W
D/W
Mock
11/M
7 d
10 d
14 d
16
10 d
14 d
HCV sRNA pos
rHCV UTR-E2
244 bp
B HCV RNA negative strand
N/W
D/W
11/M
7 d
10 d
14 d
16
10 d
14 d
HCV sRNA
Pos10⁵
Neg10⁵
244 bp

4.2 Purity of $CD4^+$ and $CD8^+$ T Cells Affinity-Purified from Healthy Donors

As previously mentioned (Section 3.4.), normal $CD4^+$ and $CD8^+$ T cells used as targets for *in vitro* infection with authentic HCV were isolated from total PBMC via positive selection using the autoMACSPro Separator. In order to evaluate the purity of the obtained subsets, flow cytometry analysis was performed, as described in Section 3.4.1. The phenotypic analysis of the affinity-purified $CD4^+$ and $CD8^+$ T cells demonstrated that the purity of the isolated subsets was higher than 96% and 97%, respectively. As an example, flow cytometry results for affinity purified $CD4^+$ and $CD8^+$ T cells used in experiment III are shown in Figure 4.3.

4.3 HCV Genome Expression and Replication in $CD4^+$ and $CD8^+$ T Cells *De Dovo* Infected with Wild-Type, Patient-Derived HCV.

As seen in Table 4.3, five experiments were performed where affinity-purified $CD4^+$ or $CD8^+$ T cells from donor B/F were separately exposed to inoculum HCV-11/M and cultured under conditions described in Section 3.5. In these experiments, HCV RNA positive strand was detected in $CD4^+$ T cells at all time points where RNA was available for analysis, apart from experiment I, 14 d.p.i.. Estimated HCV RNA loads in $CD4^+$ T cells ranged from 30 to 10^4 vge per μg of total RNA. With regards to $CD8^+$ T cells, HCV RNA positive strand was detected in all time points where total RNA was examined, apart from experiment III, 10 d.p.i. Estimated HCV RNA loads in $CD8^+$ T cells ranged from 25 to 10^4 vge per μg of total RNA.

With regard to expression of HCV RNA negative strand, the strand was detected

Figure 4.3. Purity of affinity-purified $CD4^+$ and $CD8^+$ T cells assessed by flow cytometry. (A) $CD4^+$ cell subset was incubated with PerCP-conjugated rabbit anti-human $CD4^+$ mAb or with rabbit IgG polyclonal isotype control, while (B) $CD8^+$ cell subset was stained with FITC-conjugated rabbit anti-human $CD8^+$ mAb or with rabbit IgG polyclonal isotype control. The samples were analyzed by flow cytometry (see Materials and Methods). Gates were set up on isotype controls.

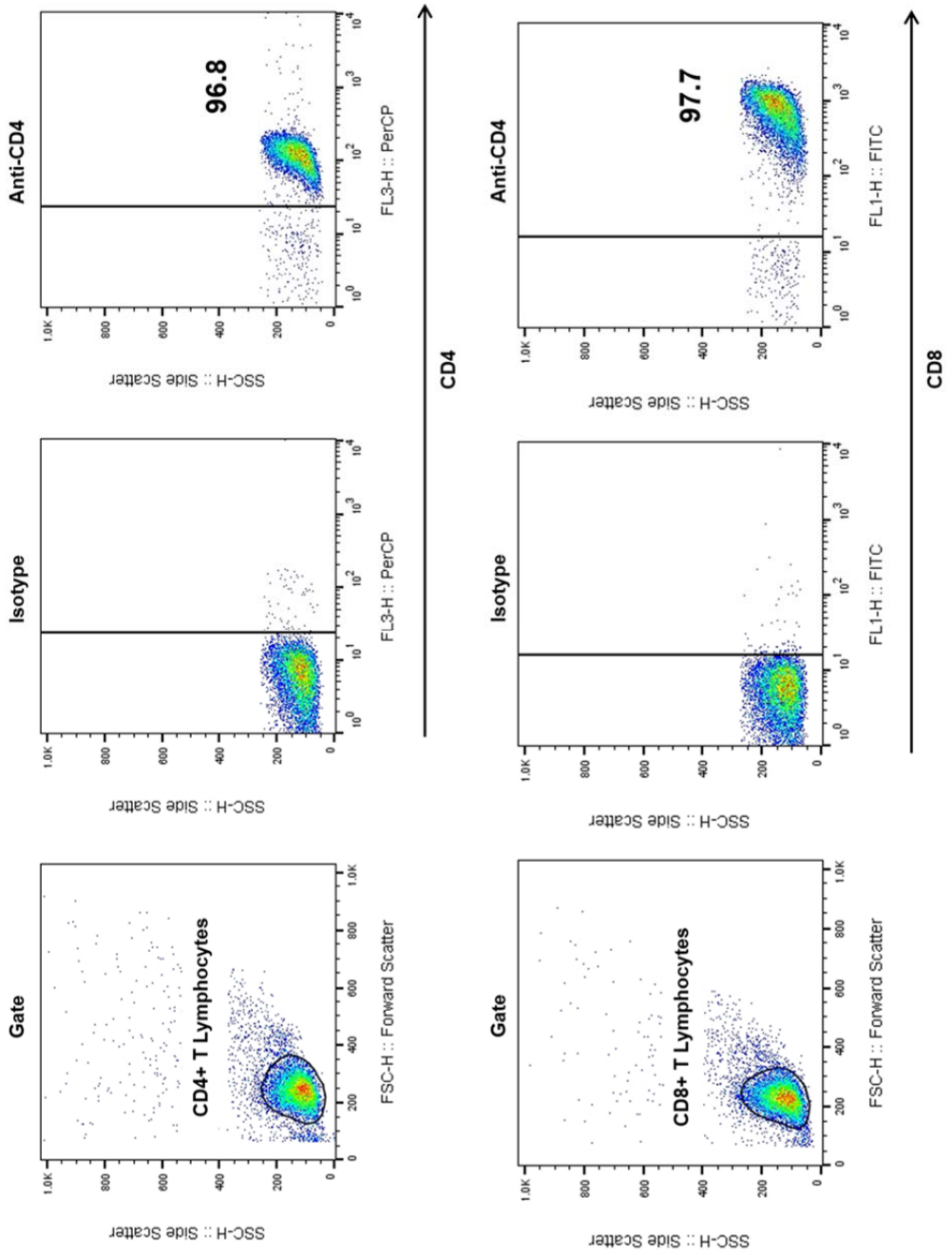

Gate
CD4+ T Lymphocytes
SSC-H :: Side Scatter
FSC-H :: Forward Scatter
Isotype
FL3-H :: PerCP
Anti-CD4
96.8
CD4
Gate
CD8+ T Lymphocytes
Isotype
FL1-H :: FITC
Anti-CD4
97.7
CD8

Table 4.3. Detection of HCV RNA positive and negative strands in affinity-purified CD4+ and CD8+ T cell cultures exposed to authentic, patient-derived HCV

Experiment	Healthy donor PBMC	Inoculum	T cell subtype[a]	No. of target cells	HCV copies/cell ratio	HCV RNA					
						Positive strand[b] (vge/µg)			Negative strand[c] (copies/µg)[d]		
						7 d	10 d	14 d	7 d	10 d	14 d
I	B/F	11/M	$CD4^+$	$5x10^6$	1/16	10^4	10^3	ND	ND	5000	NT
			$CD8^+$	$3.2x10^6$	1/10	10^4	10^4	10^4	ND	1000	ND
II	B/F	11/M	$CD4^+$	$3.5x10^6$	1/10	300	250	250	ND	≤25	ND
			$CD8^+$	$3.5x10^6$	1/10	NA	300	25	NA	≤25	ND
III	B/F	11/M	$CD4^+$	$5x10^6$	1/15	100	300	100	ND	≤25	≤25
			$CD8^+$	$5x10^6$	1/15	30	ND	30	≤25	NT	ND
IV	B/F	11/M	$CD4^+$	$5x10^6$	1/15	NA	30	100	NA	≤25	ND
			$CD8^+$	$5x10^6$	1/15	NA	30	300	NA	ND	250
V	B/F	11/M	$CD4^+$	$5x10^6$	1/15	1000	30	30	2500	ND	≤25
			$CD8^+$	$5x10^6$	1/15	NA	10^4	10^4	NA	ND	250
VI	A/M	16	$CD4^+$	$5x10^6$	1/1	NA	10^4	10^4	NA	ND	2500
			$CD8^+$	$5x10^6$	1/1	NA	10^4	10^3	NA	≤25	ND
HCV RNA positive or negative strand reactivity/ Total tested (%)					CD4:	4/4	6/6	5/6	1/4	4/6	3/5
					CD8:	2/2	5/6	6/6	1/2	3/5	2/6
					Overall:	6/6 (100%)	11/12 (92%)	11/12 (92%)	2/6 (33%)	7/11 (64%)	5/11 (45%)
							28/30 (93%)			**14/28 (50%)**	

Abbreviations: F, female; M, male; d, days post-infection; NA, not available (not sufficient RNA amount to be tested); ND, not detected; NT, not tested (not tested for HCV RNA negative strand because HCV RNA positive strand was not detected).

[a] Affinity purified $CD4^+$ and $CD8^+$ T cells from B/F and A/M healthy donors pre-treated with PHA were exposed to inocula HCV-11/M and HCV-16 and cultured as described in Materials and Methods. Exposure of B/F cells to HCV-11/M inoculum was repeated in 5 separate experiments. HCV RNA positive and negative (replicative) strands were evaluated as presented in Materials and Methods.

[b] HCV RNA positive strand detected by nested RT-PCR/NAH, as described in Materials and Methods..

[c] Estimated HCV RNA negative strand detected by strand-specific RT-PCR/NAH, as described in Materials and Methods.

[d] Estimated number based on testing of equivalent of 1 to 4 µg of RNA per reaction.

in both $CD4^+$ and $CD8^+$ T cells in all experiments performed. More specifically, in $CD4^+$ T cells, HCV RNA negative strand was detected at more than one time point in experiments III and V at estimated levels between 25 and 5,000 copies per μg of total RNA. In $CD8^+$ T cells, HCV RNA negative strand was also detected in all experiments and its loads ranged from 25 to 1,000 copies per μg of total RNA (Figure 4.4 A).

When target cells from donor A/M were exposed to inoculum HCV-16, HCV RNA positive strand was detected in both $CD4^+$ and $CD8^+$ T cells at all time points where total RNA was available for examination. Estimated HCV RNA load was 10^4 vge/μg of total RNA for $CD4^+$ T cells and ranged between 10^3 and 10^4 vge/μg of total RNA for $CD8^+$ T cells. In this experiment, HCV RNA negative strand was detected in both immune cell subsets; *i.e.*, 2,500 copies per μg at 14 d.p.i. for $CD4^+$ T cells and 25 copies per μg at 10 d.p.i. for $CD8^+$ T cells (Figure 4.4 B).

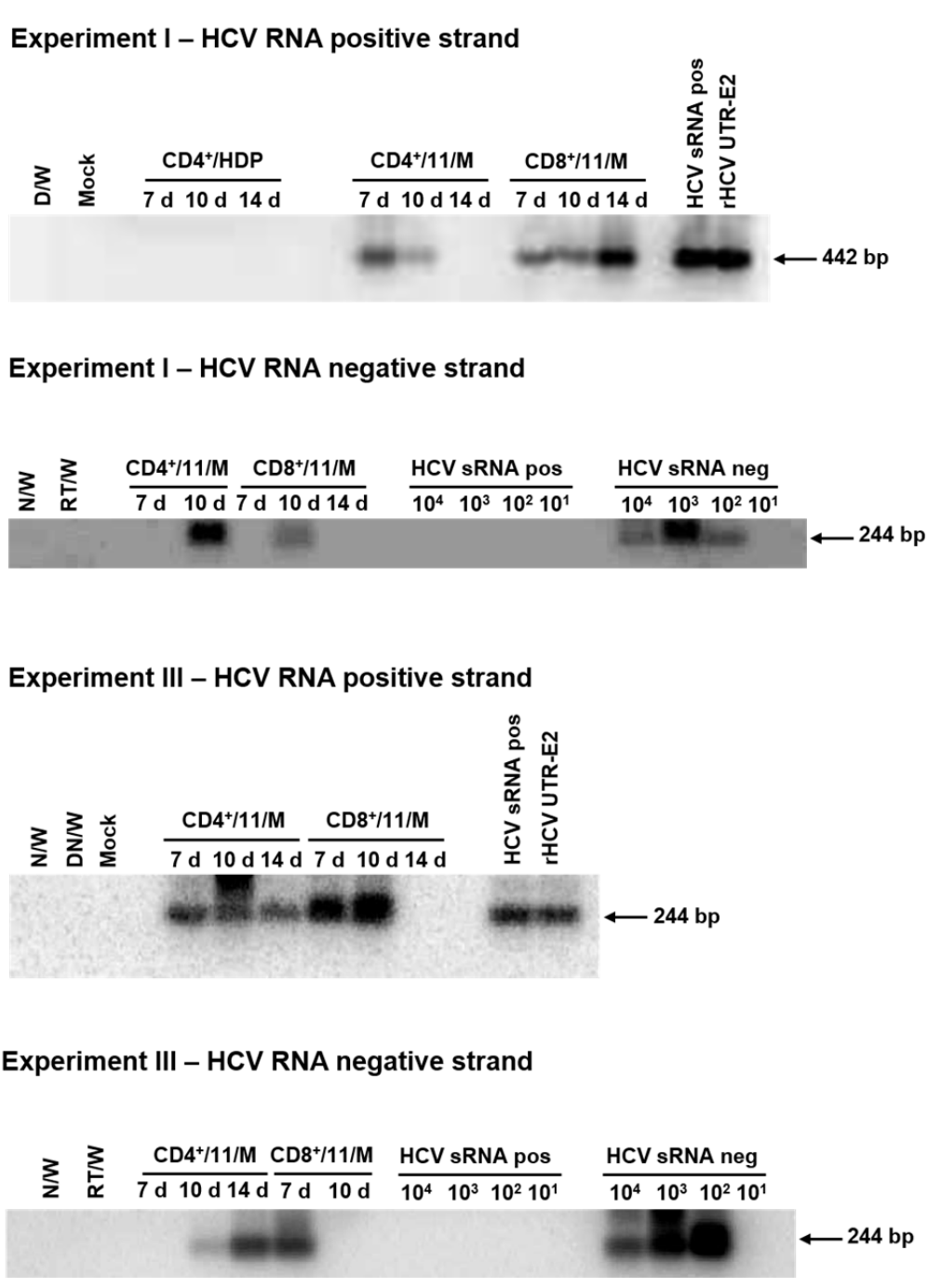
In conclusion, HCV RNA positive and negative (replicative) strands were detected in $CD4^+$ and $CD8^+$ T cells in all experiments performed. The positive strand was present at a higher percentage (100% at 7 d.p.i., and 92% at 10 and 14 d.p.i.; with 93% overall positivity) than the negative strand (33% at 7 d.p.i., 58% at 10 d.p.i., and 42% at 14 d.p.i.; with 50% overall positivity) (Table 4.3).

4.4 Identification of HCV NS5a and Core Proteins in *De Novo* Infected $CD4^+$ and $CD8^+$ T Cells.

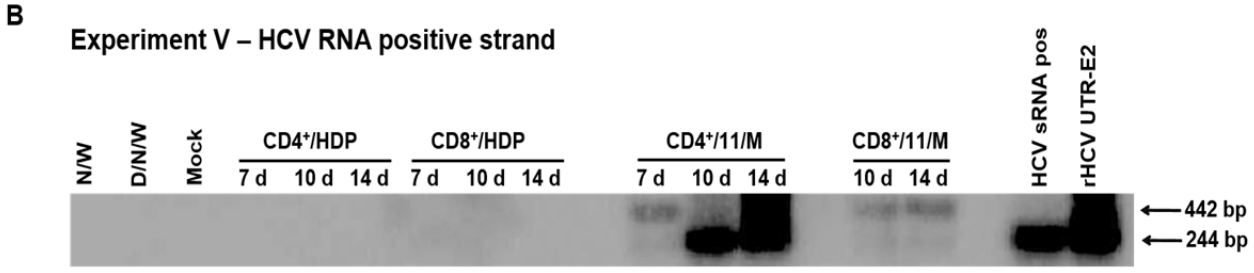
To determine whether detection of HCV RNA in infected $CD4^+$ and $CD8^+$ T cells was accompanied by synthesis of viral proteins, lymphocytes exposed to HCV-11/M

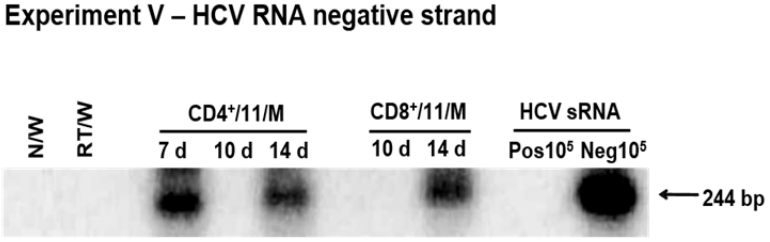
Figure 4.4. Detection of HCV RNA positive and negative strands in human $CD4^+$and $CD8^+$ T lymphocytes infected *in vitro* with patient-derived HCV. Affinity-purified $CD4^+$ and $CD8^+$ T cells from B/F and A/M healthy donors treated with PHA were exposed to HCV-11/M or HCV-16 or to HDP. (A) Cells in Experiments I and III infected with HCV-11/M. (B) Cells in Experiment V infected with HCV-11/M and in Experiment VI infected with HCV-16. Cells were cultured as described in Materials and Methods. RNA was analyzed for HCV RNA positive and negative strands (see Materials and Methods). Contamination and specificity controls are marked and they were as those outlined in the legends to Figures 4.1 and 4.2. As additional negative control, when the numbers of cells recovered allowed (Experiments I, V and VI), affinity-purified $CD4^+$ and $CD8^+$ T cells were exposed to healthy donor plasma (HDP) and cultured under the same conditions as HCV-infected cells (mock infection). Positive signals showed the expected 442-bp (direct RT-PCR/NAH) or 244-bp (nested RT-PCR/NAH) nucleotide fragments.

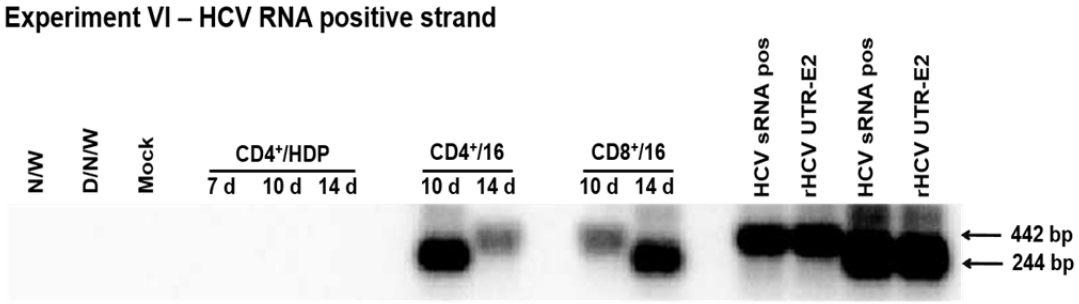
A

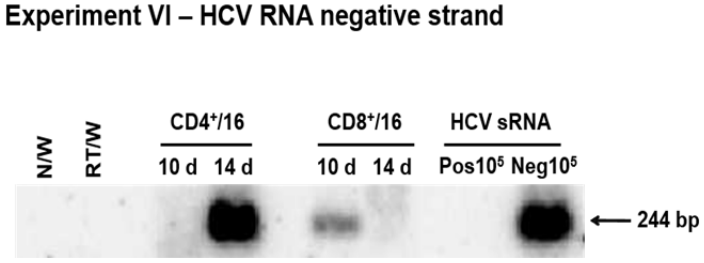

Experiment I – HCV RNA positive strand
D/W
Mock
CD4+/HDP
7 d 10 d 14 d
CD4+/11/M
7 d 10 d 14 d
CD8+/11/M
7 d 10 d 14 d
HCV sRNA pos
rHCV UTR-E2
442 bp
Experiment I – HCV RNA negative strand
N/W
RT/W
CD4+/11/M
7 d 10 d
CD8+/11/M
7 d 10 d 14 d
HCV sRNA pos
10⁴ 10³ 10² 10¹
HCV sRNA neg
10⁴ 10³ 10² 10¹
244 bp
Experiment III – HCV RNA positive strand
N/W
DN/W
Mock
CD4+/11/M
7 d 10 d 14 d
CD8+/11/M
7 d 10 d 14 d
HCV sRNA pos
rHCV UTR-E2
244 bp
Experiment III – HCV RNA negative strand
N/W
RT/W
CD4+/11/M
7 d 10 d 14 d
CD8+/11/M
7 d 10 d
HCV sRNA pos
10⁴ 10³ 10² 10¹
HCV sRNA neg
10⁴ 10³ 10² 10¹
244 bp

B


Experiment V – HCV RNA positive strand
N/W
D/N/W
Mock
CD4+/HDP
7 d 10 d 14 d
CD8+/HDP
7 d 10 d 14 d
CD4+/11/M
7 d 10 d 14 d
CD8+/11/M
10 d 14 d
HCV sRNA pos
rHCV UTR-E2
442 bp
244 bp


Experiment V – HCV RNA negative strand
N/W
RT/W
CD4+/11/M
7 d 10 d 14 d
CD8+/11/M
10 d 14 d
HCV sRNA
Pos10^5 Neg10^5
244 bp


Experiment VI – HCV RNA positive strand
N/W
D/N/W
Mock
CD4+/HDP
7 d 10 d 14 d
CD4+/16
10 d 14 d
CD8+/16
10 d 14 d
HCV sRNA pos
rHCV UTR-E2
HCV sRNA pos
rHCV UTR-E2
442 bp
244 bp


Experiment VI – HCV RNA negative strand
N/W
RT/W
CD4+/16
10 d 14 d
CD8+/16
10 d 14 d
HCV sRNA
Pos10^5 Neg10^5
244 bp

inoculum (Experiment V, 10 d.p.i. and 14 d.p.i.) were examined for the presence of HCV core or NS5A (Figures 4.5 A and B), while cells exposed to HCV-16 inoculum (Experiment VI, 10 d.p.i. and 14 d.p.i.) were examined for the presence of NS5A and $CD4^+$ or $CD8^+$ by double staining of each cell subtype for NS5A and either CD4 or CD8 (Figures 4.6. A and B). As shown in in Figures 4.5 and 4.6, both viral proteins were detected in the cytoplasm and close to the plasma membranes of $CD4^+$ and $CD8^+$ T cells. The pattern of NS5A or core protein staining was usually homogeneous throughout the cytoplasm. However, granular deposits with tendency to accumulate at the plasma membrane were also seen in both CD4+ and CD8+ cells (Figures 4.5 and 4.6). Percentages of NS5A and core reactive cells enumerated under a confocal microscope were 0.9% and 1.2% for $CD4^+$ and $CD8^+$ cells, respectively, and were comparable at all timepoints examined. $CD4^+$ and $CD8^+$ T cells exposed to HDP and stained for HCV NS5A and core proteins (negative controls), as well as $CD4^+$ and $CD8^+$ T cells exposed to HCV-11/M inoculum and stained with mouse IgG1 K isotype control showed no staining (Figures 4.5 and 4.6).

In summary, HCV core and NS5A proteins were produced in affinity-purified $CD4^+$ and $CD8^+$ T cells infected *in vitro* with authentic, patient-derived HCV. In addition, NS5A protein was detected in $CD4^+$ or $CD8^+$ T lymphocytes double stained with anti-NS5A mAb and respective cell surface markers.

Figure 4.5. Identification of HCV core and NS5A proteins in $CD4^+$ and $CD8^+$ T cells infected with authentic HCV by confocal microscopy. (A) Affinity-purified $CD4^+$ T cells and (B) $CD8^+$ affinity-purified T cells from B/F healthy donor were exposed to healthy donor plasma (HDP) or HCV-11/M inoculum and cultured as described in Materials and Methods. The cells were stained with either anti-HCV core mAb or anti-HCV NS5A mAb and counterstained with DAPI (see Materials and Methods). The images were captured at X 60 magnification. Positive cells clearly show cytoplasmic staining of either HCV core or NS5A protein, while T cells exposed to HDP were HCV core or NS5A negative.

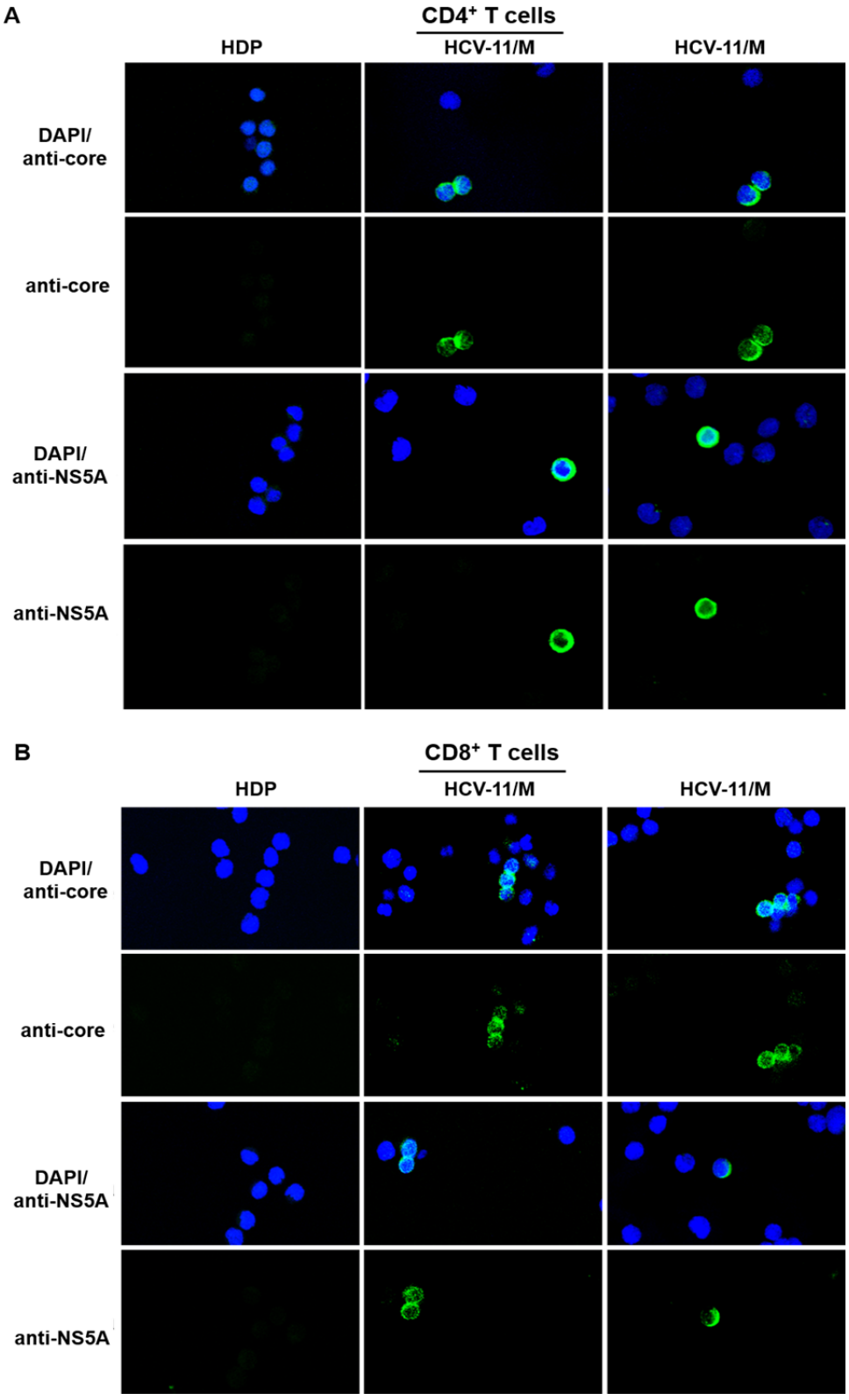
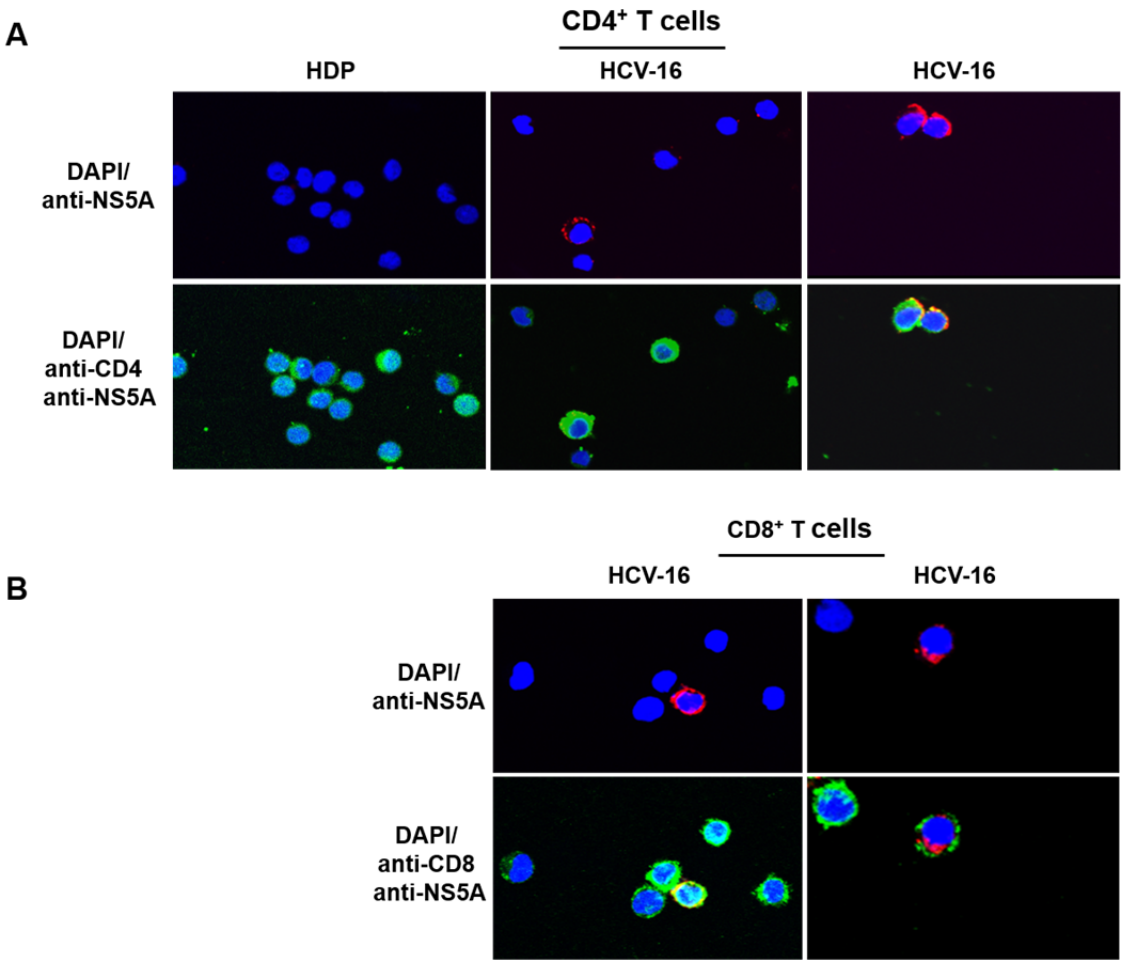

A
CD4+ T cells
HDP
HCV-11/M
HCV-11/M
DAPI/
anti-core
anti-core
DAPI/
anti-NS5A
anti-NS5A
B
CD8+ T cells
HDP
HCV-11/M
HCV-11/M
DAPI/
anti-core
anti-core
DAPI/
anti-NS5A
anti-NS5A

Figure 4.6. Double staining of $CD4^+$ and $CD8^+$ T lymphocytes *in vitro* infected with patient-derived HCV with anti-NS5A and anti-CD4 or anti-CD8 antibodies. (A) Affinity-purified $CD4^+$ cells and (B) affinity-purified $CD8^+$ T cells from A/M healthy donor were exposed to healthy donor plasma (HDP), as a negative control, or HCV-16 inoculum and cultured as described in Materials and Methods. The cells were stained with anti-CD4 or anti-CD8 mAb, counterstained with DAPI and subsequently incubated with anti-NS5A mAb (see Materials and Methods). The images were captured at X 60 magnification.

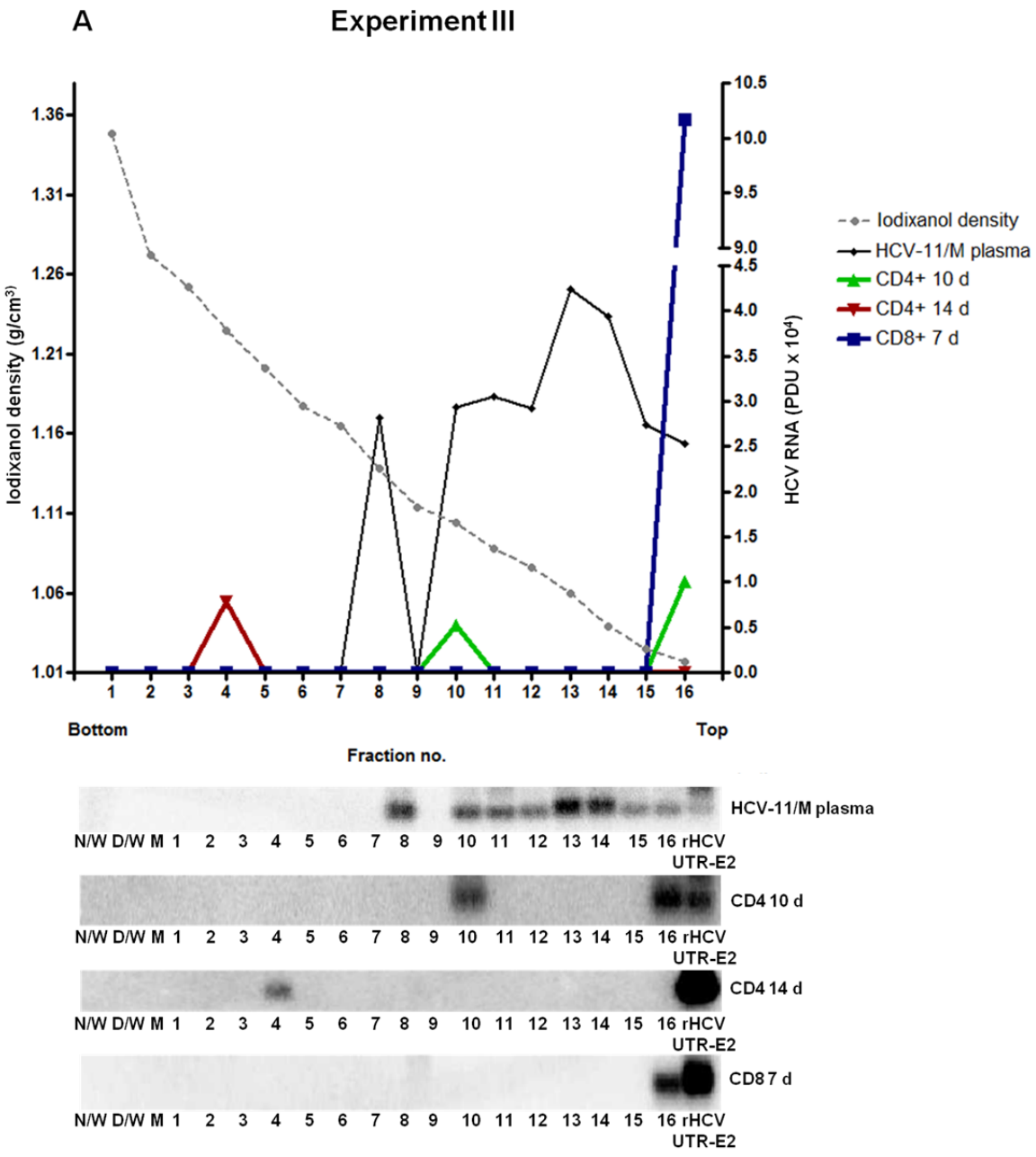

A
CD4+ T cells
HDP
HCV-16
HCV-16
DAPI/
anti-NS5A
DAPI/
anti-CD4
anti-NS5A
B
CD8+ T cells
HCV-16
HCV-16
DAPI/
anti-NS5A
DAPI/
anti-CD8
anti-NS5A

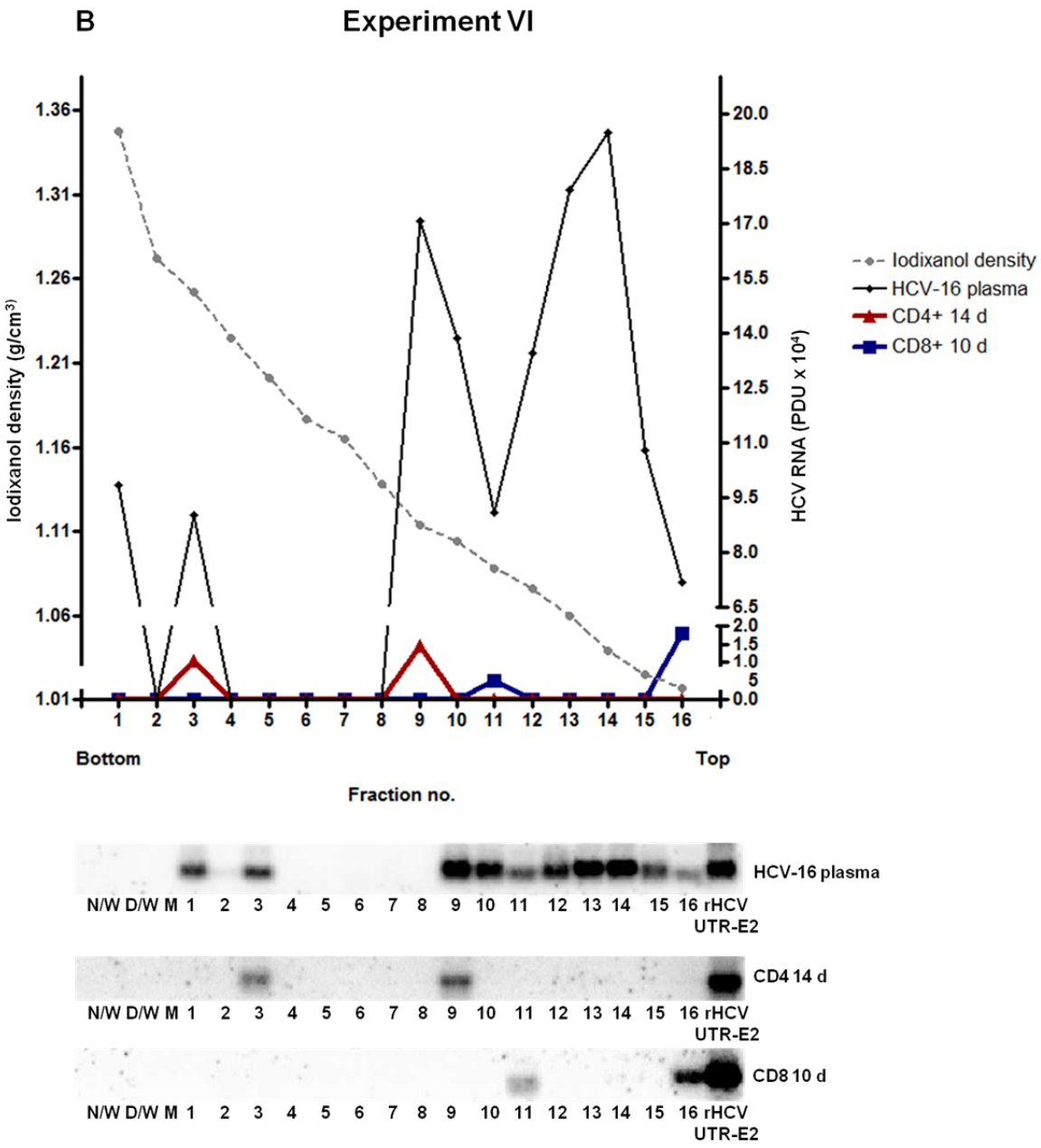
4.5 Buoyant Density of HCV RNA-Reactive Particles Released by *De Novo* Infected T Cell Subsets

To characterize the biophysical properties of HCV RNA-reactive particles released by *de novo* infected $CD4^+$ and $CD8^+$ T cells and compare them to those of virions occurring in the plasma serving as HCV inocula, supernatants collected from the infected $CD4^+$ and $CD8^+$ T cells and a sample of respective inoculum used for their infection were ultracentrifuged over an iodixanol gradient and 300 µL fractions were collected, as described in Section 3.6.1.

The analysis of the fractions obtained after density gradient ultracentrifugation of HCV-11/M inoculum (Experiment III) showed that HCV RNA-reactive particles occurred in fractions 8, 10, 11, 12, 13, 14, 15, and 16, corresponding to iodixanol densities of 1.138, 1.104, 1.088, 1.076, 1.060, 1.039, 1.025, and 1.017 g/cm^3, respectively (Figure 4.7 A). The peak of HCV RNA positivity was detected in fraction 13 and 14 at iodixanol densities of 1.060 and 1.039 g/cm^3 with PDU of 4.24 and 3.94, respectively (Figure 4.7 A). In the culture supernatant of $CD4^+$ T cells infected with HCV-11/M (Experiment III) and collected at 10 d.p.i., HCV RNA-reactive particles were found in fractions 10 and 16 at densities 1.104 and 1.017 g/cm^3 with the peak of HCV RNA positivity at fraction 16 with 1.00 PDU (Figure 4.7 A). In the same experiment, culture supernatant of $CD4^+$ T cells exposed to HCV-11/M and recovered at 14 d.p.i. demonstrated HCV RNA reactivity only in fraction 4 at a density of 1.225 g/cm^3 and 0.77 PDU. In the supernatant collected from HCV-11/M infected $CD8^+$ T cells obtained at 7

Figure 4.7. Buoyant density in a iodixanol gradient of HCV particles produced by *in vitro* infected $CD4^+$ and $CD8^+$ T cells. (A) Culture supernatants collected at 10 and 14 days post-infection (d.p.i.) from $CD4^+$ T cells and at 7 d.p.i. from $CD8^+$ T cells infected with HCV-11/M (Experiment III) and (B) at 14 d.p.i. from $CD4^+$ T cells and at 10 d.p.i. from $CD8^+$ T cells infected with HCV-16 inoculum (Experiment VI) were concentrated by ultracentrifugation (see Materials and Methods). The resulting pellets were suspended in AIM-V culture medium (500 μL) and layered over a 4.5 mL continuous 10-50% iodixanol gradient and ultracentrifuged as described in Material and Methods, in parallel with 50 μL heat-inactivated HCV-11/M (A) or HCV-16 in (B) diluted with 450 μL AIM-V medium. Sixteen 300 μL fractions were collected starting from the top of each gradient and all fractions were evaluated for HCV RNA by RT-PCR/NAH. In parallel iodixanol gradients, culture AIM-V medium alone (500 μL) was fractionated under identical conditions and collected fractions were used for measuring iodixanol density (g/cm^3). Contamination and specificity controls are marked and they were the same as those outlined in the legends to Figures 4.1 and 4.2. The density of the signals obtained from nucleic acid hybridization was enumerated using a Pharos FX plus molecular imager system and expressed in pixel density units (PDU).


A
Experiment III
Iodixanol density (g/cm³)
HCV RNA (PDU x 10⁴)
Iodixanol density
HCV-11/M plasma
CD4+ 10 d
CD4+ 14 d
CD8+ 7 d
Bottom
Top
Fraction no.
HCV-11/M plasma
CD4 10 d
CD4 14 d
CD8 7 d
N/W D/W M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 rHCV UTR-E2


B
Experiment VI
Iodixanol density (g/cm³)
HCV RNA (PDU x 10⁴)
Iodixanol density
HCV-16 plasma
CD4+ 14 d
CD8+ 10 d
Bottom
Top
Fraction no.
HCV-16 plasma
CD4 14 d
CD8 10 d
N/W D/W M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 rHCV UTR-E2

d.p.i., HCV particles were detected at fraction 16 at density 1.017 g/cm^3 and 10.16 PDU (Figure 4.7.A).

Analysis of HCV-16 inoculum (Experiment VI), showed that HCV RNA-reactive particles were present in fractions 1, 3, and 9 throughout to 16, corresponding to iodixanol densities of 1.348, 1.252, and 1.114 to 1.017 g/cm^3 (Figure 4.7 B). The peaks of HCV RNA positivity were detected in fractions 9, 13, and 14 at densities of 1.114, 1.060 and 1.039 g/cm^3, with PDU of 17.04, 17.90, and 19.46, respectively. In culture supernatant of CD4$^+$ T cells exposed to HCV-16 (Experiment VI) and collected at 14 d.p.i., HCV RNA reactivity was identified in fractions 3 and 9 at densities of 1.252 and 1.114 g/cm^3 with PDU of 1.04 and 1.46, correspondingly. With regards to culture supernatant of CD8$^+$ T cells exposed to the same inoculum (Experiment VI), obtained at 10 d.p.i., HCV RNA-positive particles were detected in fractions 11 and 16 at densities of 1.088 and 1.017 g/cm^3, and PDU of 0.49 and 1.79, respectively.

In summary, HCV RNA-reactive particles exhibited variable buoyant densities ranging from 1.017 to 1.348 g/cm^3 with the HCV RNA-positive particles released by *de novo* infected CD4$^+$ and CD8$^+$ T cells peaking at different densities in comparison to that of particles harbored in the plasma serving as HCV inocula. These differences in biophysical properties between HCV particles released by infected T lymphocytes and those occurring in inocula used to infect these cells were consistent with the *de novo* production of the virus.

4.6 HCV Genome Clonal Sequencing.

To determine whether HCV replication in the *in vitro* infected $CD4^+$ and $CD8^+$ T cells led to the emergence of unique HCV variants distinct from those occurring in the plasma used as inocula, 5'-UTR amplicons from plasma and PBMC of 11/M patient, and from affinity-purified $CD4^+$ and $CD8^+$ T cells infected with HCV 11/M were cloned, and 20 clones from each amplicon were bidirgenectionally sequenced and compared.

Clonal sequencing analysis of the 244-bp 5'-UTR fragment showed that apart from an insertion at position 126, which was found in both HCV-11/M (5% of clones) and HCV-11/M-infected $CD4^+$ T cells (Experiment I) obtained at 10 d.p.i. (5% of clones), the remaining (29) variants identified in infected $CD4^+$ and $CD8^+$ T cells represented unique nucleotide changes not found in HCV-11/M (Table 4.4).

Among 30 variants identified in infected $CD4^+$ and $CD8^+$ T cells, 17 were detected in $CD4^+$ T cells. Most variants (24 of 30) were identified in only 5% (1 out of 20) of clones tested, while in some cases, the percentage of clones carrying a unique variant (*i.e.*, SNP) was relatively high, *e.g.*, C to T change at position 115 was found in 40% (8 of 20) clones derived from $CD8^+$ T cells collected at 7 d.p.i. (Experiment III). Majority (27 of 30) of variants identified was found in one immune cell subtype with the exception of 3 SNPs which were common among $CD4^+$ and $CD8^+$ T cells. Thus, in Experiment V, a T to C change at position 144 was observed in both $CD4^+$ and $CD8^+$ T cells at 7 d.p.i., and an A to G substitution at position 252 was identified in both $CD4^+$ T cells and $CD8^+$ T cells collected at 7 and 14 d.p.i., respectively. Furthermore, a T to C

Table 4.4. Identification of Single-Nucleotide Polymorphisms (SNP) in the 5'-UTR Sequences Detected in CD4$^+$ and CD8$^+$ T Cells *In Vitro* Infected with Genotype 1a HCV-11/M

Cell type	Experiment	Days post-infection	Position and identity of nucleotide substitution[a]		
			68-150	151-220	221-311
CD4$^+$	I	10	126insC (10%); A134G (5%)		A260G (5%)
	III	10			T228C (5%); T282C (5%)
	III	14	A96G (5%); 126delC (5%); T149C (5%);	G153A (5%); T164C (5%); G189T (5%); T216C (5%)	T269C (5%)
	V	7	C112T (5%); T144C (5%)	G184A (5%)	
	V	14			A252G (5%)
CD8$^+$	I	10		T160C (5%); C183T (20%)	G286A (15%); T287C (15%)
	III	7	C115T (40%); A116G (5%)	C187T (5%)	C255T (5%)
	V	7	T144C (10%);	A165G (5%)	A252G(5%); C272T (5%); T282C (5%)

Abbreviations: del, deletion; ins, insertion.

[a] HCV sequence 244 nucloetides long amplified from HCV inoculum 11/M was used as a reference. The GenBank accession number for HCV genotype 1a prototype used for nucleotide position designation is M67463.

HCV 5'-UTR amplicons detected in CD4$^+$ and CD8$^+$ T cells infected with HCV 11/M (genotype 1a) from Experiments I, III and V at the time points where HCV RNA negative (replicative) strand was detectable were analyzed by clonal sequencing. In total 20 clones from each 5'-UTR amplicon were analyzed. The percentage of clones carrying a given mutation is noted in parenthesis.

substitution at position 282 was detected in $CD4^+$ T cells obtained at 10 d.p.i. (Experiment III) and also in $CD8^+$ T cells acquired at 7 d.p.i. (Experiment V).

Finally, no variants were identified amongst the clones of the amplicon from the *in vivo* infected PBMC of patient 11/M, as the sequence of all 20 clones was identical to the master sequence detected in HCV-11/M inoculum (plasma).

In summary, unique HCV variants were identified in *de novo* infected $CD4^+$ and $CD8^+$ cells at all timepoints tested and at variable percentages. Some of these variants were common in both $CD4^+$ and $CD8^+$ T cells in the same or different experiments. All of these variants, with the exception of an insertion at position 126, were unique to the *de novo* infected cells and not identified in the inoculum used for their *in vitro* infection.

CHAPTHER FIVE: DISCUSSION

Research on HCV lymphotropism has been ongoing for nearly two decades resulting in an accumulating amount of data through various *in vivo* and *in vitro* studies. As mentioned in Section 1.5.2, several studies have reported the *in vivo* susceptibility of total PBMC to HCV infection. These works have documented the detection of HCV RNA positive and negative strands, and/or HCV proteins in PBMC of patients with AHC or CHC (Willems *et al*., 1994; Lerat *et al*., 1996; Chang *et al*., 1996; Gong *et al*., 2003; Pham *et al*., 2008). The expression of HCV RNA negative strand was found to be greater in PBMC from CHC than ACH (Chang *et al*., 1996; Gong *et al*., 2003), implying that HCV replication in the lymphoid cells may be involved in supporting chronic infection. As previously discussed (Section 1.5.2), HCV infection of the PBMC has also been reported in patients with apparent spontaneous or treatment-induced resolution of hepatitis C (Pham *et al*., 2004; Radkowski *et al*., 2005; Pham *et al*., 2005, 2008; Callegos-Orozco *et al*., 2008; MacParland *et al*., 2009; Pham *et al*., 2012; Chen *et al*., 2013, 2015).

With regards to the possible propensity of HCV to preferentially infect certain PBMC subsets *in vivo*, several studies have found HCV replication to be more commonly observed in, if not restricted to, $CD19^+$ B cells (Lerat *et al*., 1998; Morsica *et al*., 1999; Pal *et al*., 2006). In other works, *in vivo* HCV replication extended among various immune cell subtypes, including $CD4^+$ and $CD8^+$ T cells, B cells, monocytes and dendritic cells (Pham *et al*., 2004; 2008; Pawełczyk *et al*., 2013) with levels of viral

replication varying among different cell types depending on the clinical status of the patients *i.e.*, CHC or OCI (Pham *et al*., 2008).

In vivo infection of immune cells is further supported by findings of numerous clonal sequencing analyses and by the results from examination of single-stranded conformational polymorphism (SSCP). Thus, it has been reported that HCV variants detected in PBMC were distinct from those in serum or liver of patients with CHC, which supports their extrahepatic origin (Maggi *et al*., 1997; Navas *et al*., 1998; Laskus *et al*., 2002; Ducoulombier *et al*., 2004; Roque-Afonso *et al*., 2005; Di Liberto *et al*., 2006; Pham *et al*., 2008, 2012; MacParland *et al*., 2009; Durand *et al*., 2010; Chen *et al*., 2013, 2015). Furthermore, non-random compartmental distribution of HCV quasispecies within PBMC subsets has been documented (Roque-Afonso *et al*., 1999), as well as HCV genotypic compartmentalization within immune cell subsets persisting for years, even after liver transplantation (Roque-Afonso *et al*., 2005). With regards to the effect of sequence variations on virus properties, it has been reported that B cells of patients with OCI harbor HCV IRES variants that are characterized by a lower translational efficiency in hepatocytes than plasma and liver-specific variants (Durand *et al*., 2010). Such findings not only suggest that the origin of these variants is extrahepatic, but also imply that there may be competition between hepatotropic and non-hepatotropic HCV IRES variants, which is driven at the translational level. In this regard, the notion of translational control of cellular tropism has been previously described for polioviruses (Kauder *et al*., 2004).

In patients with CHC, detection of HCV replication extends beyond the lymphoid

cells in circulation. Thus, HCV negative strand, as well as HCV core and NS3 proteins have been detected in biopsy specimens from perihepatic lymph node (LN) in patients with chronic HCV infection where B cells were identified as the main site of HCV replication (Pal *et al*., 2006). In the same study, quasispecies analyses showed that the HCV replication in the LN contributed greatly to the total level of viremia. In another study, HCV RNA presence has been documented in the cerebrospinal fluid (CSF) of patients co-infected with HCV and HIV (Bagaglio *et al*., 2005), as well as in autopsy brain tissue from HCV-positive patients with or without HIV coinfection (Radkowski *et al*., 2002). In the latter study, it was also shown that HCV variants harbored in the brain were different than those circulating in the serum and identical to these found in the LN. This study also implied that HCV can replicate in the central nervous system (CNS), probably in resident-macrophages of blood monocytic origin. HCV negative strand as well as HCV structural and NS proteins have also been detected in pluripotent hematopoietic $CD34^+$ cells purified from the bone marrow (BM) of patients with CHC (Sansonno *et al*., 1998). In the same study, it was observed that when these cells were cultured in the absence of growth factors there was a spontaneous increase in viral titers in both cell and their culture supernatants, indicating that $CD34^+$ cells and, therefore, BM in general can support the full HCV life cycle.

Apart from the large body of *in vivo* experimental and clinical evidence, accumulating data from numerous *in vitro* studies further lend support to the concept of HCV lymphotropism. It was shown that HCV virions released from SB B cell line, established from the splenocytes of an HCV-positive patient with type II MC and

monocytoid lymphoma, were able to infect primary human $CD4^+$ T cells (Kondo *et al*., 2007) as well as T cell lines (Molt-4 and Jurkat) (Kondo *et al*., 2009). In addition, several studies reported that serum containing HCV was able to infect T cell lines. Thus, it was shown that authentic, plasma occurring HCV could infect Molt-4 T cells (Shimizu *et al*., 1992; Sarhan *et al*., 2012a, 2012b), HPB-Ma T cells (Shimizu *et al*., 1993), H9 T cells (Nissen *et al*., 1994), and Jurkat T cells (Sarhan *et al*., 2012a, 2012b). As discussed in Section 1.10.2.5, research performed in this laboratory has shown that intact, plasma occurring HCV can infect PBMC-enriched T cells (MacParlant *et al*., 2006). Apart from T cells, it has been shown that HCV-positive sera can also infect DC (Navas *et al*., 2002), as well as human primary macrophages/monocytes (Caussin-Schwemlin *et al*., 2001; Laskus *et al*. 2004; Radkowski *et al*., 2004).

To our knowledge, *in vitro* infection of primary human $CD4^+$ and $CD8^+$ T lymphocytes with authentic, patient-derived HCV has not been documented prior to this study. For our purposes, we adopted the *in vitro* HCV replication system previously established in this laboratory in which mitogen-induced T cell cultures derived from total PBMC from healthy donors served as targets for wild-type HCV (MacParland *et al*., 2006), as summarized in Section 1.10.2.5. Instead of monocyte-depleted PBMC we used affinity-purified $CD4^+$ and $CD8^+$ T cells as targets aiming to document *in vitro* infection of these immune cell subsets by HCV. In this culture system, stimulation of target cells with PHA prior to exposure to HCV is crucial as data have shown that pre-treatment of cells with PHA increases their susceptibility to HCV infection, as well as expression of HCV negative strand, implying increased replication of the virus (Pham *et al*., 2004,

2005; MacParland *et al*., 2006). Another critical point in our system was the process applied for the identification of the HCV inocula that would be most suitable for *in vitro* infection of lymphoid cells (Section 3.1). Furthermore, we used highly sensitive assay techniques that allowed detection of low levels of HCV infection. In addition, it is noteworthy that we utilized several experimental approaches in order to document authentic HCV replication in $CD4^+$ and $CD8^+$ T cells. Thus, we considered the existence of productive replication only when HCV RNA negative (replicative) strand, as well as HCV structural and/or non-structural proteins, were detected. In addition, we strived to characterize the biophysical properties of HCV RNA-reactive particles produced by the *de novo*-infected $CD4^+$ and $CD8^+$ T cells and to compare them to those of virions circulating in the plasma serving as HCV inocula. Finally, we aimed to assess whether HCV variants harbored in the *de novo* infected $CD4^+$ and $CD8^+$ T cells differ from those occurring in the patient plasma used as HCV inocula.

In this study, affinity-purified human primary $CD4^+$ and $CD8^+$ T lymphocytes exposed to native HCV were found to be susceptible to HCV infection. In particular, HCV RNA vegetative (positive) and negative strands were detected in these *in vitro* infected cells. Since HCV replicates via synthesis of the HCV RNA negative strand, detection of this replicative intermediate accounts for a direct evidence of active viral replication in the target cells. Thus, active replication was documented in both $CD4^+$ and $CD8^+$ T cells and in 50% of experimental timepoints (d.p.i.) tested. On the other hand, HCV RNA positive strand was detected in $CD4^+$ and $CD8^+$ T cells in the vast majority (93%) of timepoints (d.p.i.) examined. Such difference in detection of HCV RNA

positive and negative strand is expected, as it is known that the HCV RNA positive strand could be a 100-1000-fold more abundant than the negative strand in the infected cells (Laskus *et al*., 1998; Komurian-Pradel *et al*., 2004; Pawelczyk *et al*., 2013). These results can be further explained by the difference in sensitivity between the assay used for the detection of HCV RNA positive strand versus that used for HCV RNA negative strand identification, with the latter being approximately 10-100-fold less sensitive.

Moreover, the expression of HCV RNA negative strand did not always correlate with that of the positive strand at a certain timepoint. This observation may be reflecting virions attached to the cell surface. For this reason, we do not merely rely on detection of HCV RNA positive strand but always employed the negative strand-specific assay, which specifically detects this replicative strand. This assay maintains its specificity for up to 10^5 copies per reaction of the correct (negative) strand (Figure 3.5; Pham *et al*., 2004; Sarhan *et al*., 2012a).

It is notable that expression of both strands fluctuated not only between experiments, but also amongst timepoints of the same experiment. Such fluctuation can be explained by the low levels of HCV infection coinciding with HCV positive and negative strands expression at levels very close to the threshold of detection. Furthermore, our *in vitro* system used freshly isolated primary normal cells and authentic virus contained in patient plasma in place of cell line and a cloned virus aiming to reflect, as closely as feasible, the *in vivo* infection of $CD4^+$ and $CD8^+$ T cells.

Our finding that *de novo* infected primary human CD4+ T cells can support active replication of HCV, as evidenced by detection of virus RNA positive and negative strands

and proteins, has been supported by the findings reported by another group (Kondo *et al*., 2007). As previously mentioned, HCV RNA positive and negative strands have been detected in $CD4^+$ T cells isolated from PBMC of healthy donors which were exposed to HCV *in vitro* (Kondo *et al*., 2007). In this study, however, HCV virions released in culture from SB cells but not authentic, patient-derived virus were used to infect the $CD4^+$ T cells. Our results are also in agreement with previous findings from this laboratory (Pham *et al*., 2008), which showed that HCV RNA positive and negative strands and virus proteins were detected in CD4+ and $CD8^+$ T cells which were affinity purified from the PBMC of patients with CHC or OCI. It is evident from these combined data that HCV can replicate in $CD4^+$ and $CD8^+$ T cells both *in vivo* and *in vitro*.

Through our study, we further ascertained that the detection of HCV RNA in $CD4^+$ and $CD8^+$ T cells is indicative of active HCV replication by identification of HCV core and NS5A proteins via confocal microscopy in the *de novo* infected cells. Notably, as the NS proteins are not constitutive elements of HCV virions, the detection of the NS5A protein within the infected $CD4^+$ and $CD8^+$ T cells provides further compelling evidence of ongoing, productive HCV infection. As previously reported (Pham *et al*., 2008; Pawelczyk *et al*., 2013), no correlation was found between the intracellular display of HCV proteins and virus RNA negative strand expression, possibly owing to the low level of HCV replication. Our findings of HCV NS5A protein detection within $CD4^+$ and $CD8^+$ T lymphocytes are in accordance with the previous work from this laboratory demonstrating that HCV replication takes place in $CD4^+$ and $CD8^+$ T cells of patients

chronically infected with HCV or those with OCI (Pham *et al*., 2008). This provides further evidence that these immune cells constitute sites of persistent HCV propagation.

The ability of *de novo* infected $CD4^+$ and $CD8^+$ T cells to support infection of authentic HCV is further demonstrated in our study by comparing the biophysical properties of HCV RNA-reactive particles released by the infected cells versus those harbored in the inocula used for their infection. More specifically, ultracentrifugation of the inocula HCV-11/M and HCV-16 resulted in recovery of HCV RNA throughout a wide variety of densities. Such variability was expected as it has been extensively reported that buoyant densities of HCV RNA-reactive particles can vary greatly among patients (Nielsen *et al*., 2006; 2008). This heterogeneity can be due to the presence of variable ratios of virions and virus cores, and their association to variable degrees with immunoglobulins and lipoproteins (Nielsen *et al*., 2006). However, we observed a different and much less heterogeneous buoyant density profile of HCV RNA-reactive particles in the supernatants of the *de novo* infected cells than that of inocula used for their infection. Thus, most of the HCV RNA-reactive particles present in the inocula displayed buoyant densities not observed in T cells supernatants, while particles of other densities were more predominant in the supernatants than in plasma serving as inocula. Such difference of density profiles may imply that the viral particles released from the *de novo* infected T cells exhibited different biophysical properties compared to HCV particles in the plasma. This might be due to the possibility that HCV in plasma serving as inocula originated predominantly from hepatocytes and possibly to the interaction with different host protein and lipids. Similar studies have been previously performed in this

laboratory (MacParland *et al*., 2006, 2009). These studies came to the same conclusion, although they applied different fractionation procedures of HCV RNA-reactive particles. Overall, our observation that the HCV RNA-reactive particles released from the *de novo* infected lymphocytes differed in their biophysical profiles compared to those contained in inocula is in agreement with the conclusion that new HCV virions were produced.

Finally, the emergence of unique HCV variants in the sequence of the highly conserved 5'-UTR region of the HCV genomes detected in the *de novo* infected cells lends further support to the fact that primary $CD4^+$ and $CD8^+$ T cells are susceptible to infection with authentic HCV. The evolution in the virus sequence can take place only when the virus is replicating. Therefore, the appearance of unique SNPs in infected cells in our study reaffirms that there was active HCV replication. It is notable that some of the SNPs were observed in both T cell subtypes in either the same or different experiments, and other SNPs were present at relatively high frequencies. Our findings are in agreement with previous data originating from this laboratory where distinct variants appeared in enriched T cells derived from PBMC *de novo* infected with authentic HCV in culture under the same experimental conditions (MacParland *et al*. 2009).

It is notable that some of the variants that we observed have also been identified by others. Thus, the insertion of C at the nucleotide position 126 (126insC), which we observed in 5% of the clones derived from inoculum HCV-11/M as well as in 10% of clones in $CD4^+$ T cells at 10 d.p.i. in Experiment I, has also been identified in the LNs and cerebellum, but not in the serum, of a patient with CHC (Radkowski *et al*., 2002). Similarly to our work, the samples carrying this insertion were HCV RNA negative strand

reactive. Interestingly, 126insC has also been detected in the pretransplant PBMC but not serum samples of two patients with CHC (Laskus *et al*., 2002). In one of these patients, this mutation was seen in the post-transplant serum where it was maintained for up to 2 weeks post-liver transplantation, suggesting the extrahepatic origin of this variant. In the other patient, this mutation was lost from serum as early as one week post-liver transplantation. Furthermore, the substitution of C by T at nucleotide position 183 (C183T), which we found in 20% of CD8+ T cell-derived clones at 10 d.p.i. in Experiment I, has also been observed in monocytes, but not in the serum, of an HCV-positive patient with concomitant detection of HCV RNA negative strand in these cells (Radkowski *et al*., 2002). In another study (Laskus *et al*., 1998), C183T mutation was observed in the PBMC but not the serum of an HCV-infected patient. In the same study, C183T was found in matching serum and PBMC samples from two HCV-positive patients. Taken together, the variants identified both *in vivo* in the studies mentioned above and in our *in vitro de novo* infection system suggest that these variants may reflect evolution of HCV sequence over the course of replication in nonhepatic cells. Thus, they provide further evidence that $CD4^+$ and $CD8^+$ T cells support HCV replication.

Overall, we documented in this study that authentic, intact HCV can replicate in normal human primary $CD4^+$ and $CD8^+$ T cells by showing: (1) The expression of HCV replicative strand in the *de novo* infected cells, (2) Intracytoplasmic localization of virus-specific structural and NS proteins, (3) Emergence of new HCV variants in infected cells, and (3) Release of HCV RNA-reactive particles by the infected cells with distinct biophysical properties.

It would be reasonable to assume that HCV exposure and/or replication in $CD4^+$ and $CD8^+$ T cells could have an effect on their function. Research on the consequences of HCV infection on T cell function is very limited. It has been reported that *in vitro* infection of primary $CD4^+$ T cells with a lymphotropic HCV strain (SB-HCV) affected their IFN-γ /STAT-1/T-bet signaling leading to inhibition of IFN-γ production (Kondo *et al*., 2007, 2011). In the same study, it was shown that the SB-HCV replication in primary $CD4^+$ T cells suppressed their proliferation, as well as their development towards Th1 lineage. In another study, it was shown that infection of the MOLT-4 T cell line with SB-HCV inhibited cell proliferation and enhanced Fas-mediated apoptosis (Kondo *et al*., 2009). In addition, work from this laboratory (MacParland *et al*., 2015b) reported that authentic, patient-derived HCV inhibited $CD4^+$ but not $CD8^+$ T cell proliferation in the T cell infection model previously described (MacParland *et al*., 2006), which was also applied in the current study (Section 1.10.2.5). Interestingly, the results of the aforementioned study also implied that just exposure to HCV in the absence of detectable evidence of its replication was efficient to exert an inhibitory effect on $CD4^+$ T cell proliferation. Finally, it has been shown that HCV core protein upregulated the expression of anergy-related genes in Jurkat T cells stably expressing this protein (Dominguez-Villar et al., 2007). This expression profile was accompanied by activation of the NFAT transcription factor and suppression of the IL-2 promoter activity.

Apart from the studies mentioned, the expected direct effect of HCV replication on T cell cytokine profile, cell fate, and functions remains under investigation. Nonetheless, in the setting of CHC, HCV-specific $CD4^+$ and $CD8^+$ T cells are

characterized by impairment of their effector functions (Rehermann, 2009). $CD4^+$ T cells produce less IL-2 and IFN-γ and this decrease is concomitant with cytotoxicity impairment and overall dysfunction of $CD8^+$ T cells (Francavilla *et al*., 2004). It has been suggested that decreased production of IL-2 by $CD4^+$ T cells may be associated with compromised $CD8^+$ T cell cytotoxicity (Golden-Mason *et al*., 2007). Furthermore, as mentioned in Section 1.7.2.2, HCV-specific T cells express high levels of PD-1 (Radziewicz *et al*., 2007; Golden-Mason *et al*., 2007) with $CD8^+$ T cells also expressing increased levels of Tim-3 (a negative immune regulator), and low levels of CD127 (Golden-Mason *et al*., 2009). Binding of PD-1 to its ligand leads to inhibition of effector functions and apoptosis of HCV-specific T cells (Shin *et al*., 2006). It has been suggested that increased expression of PD-1 on HCV-specific T cells might be a result of chronic antigenic stimulation, since it subsides following mutation of virus epitopes recognized by T cells (Rutebemberwa *et al*., 2008). Functional deregulation of immune cell subtypes other than $CD4^+$ and $CD8^+$ T cells coinciding with CHC has been described in Section 1.9.3. The relationship between immune cell dysfunctions and HCV replication in these cell subsets warrants further investigation.

Apart from impairment of functionality, HCV infection of immune cells may have significant clinical implications. In particular, by targeting and establishing a low level infection in immune cells, the virus may be impairing the efficiency of immune responses preventing viral clearance and favoring chronicity. Therefore, infected immune cells may serve as viral reservoirs from where HCV may continue to replicate and where the emergence of distinct variants may be favored. It is conceivable that these strongholds of

infection may play a role in recurrence of symptomatic HCV infection after liver transplantation and spontaneous or treatment-induced resolution of hepatitis C (Pham and Michalak, 2008; Pham *et al*., 2010). Furthermore, HCV infection of immune cells could be playing a significant role in the several extrahepatic disorders of autoimmune and/or lymphoproliferative nature frequently observed in patients with CHC (Section 1.8).

In conclusion, our study lends further support to an increasing body of *in vivo* and *in vitro* data supporting the propensity of HCV to infect immune cells. It is of great importance to further investigate the molecular mechanisms underlying susceptibility of immune cells to HCV, as well as the consequences of HCV infection on functionality of these cells. Such studies could help us elucidate the role of immune cell infection in natural progression and pathogenesis of HCV infection. Furthermore, it would be of great value to identify whether elimination of the virus from the lymphoid compartment could be conducive to success of long-term outcomes of anti-HCV therapies.

CHAPTER SIX: SYMMARY AND CONCLUSIONS

In this study, we hypothesized that authentic HCV is capable of *in vitro* infecting primary human $CD4^+$ and $CD8^+$ T lymphocytes, which are the central cells in the adaptive, including antiviral, immune responses. For this purpose, we employed an *in vitro* HCV replication system previously established in this laboratory in which mitogen-stimulated T cell cultures derived from total PBMC served as targets for native, plasma occurring HCV (MacParland *et al*., 2006, 2009) (Section 1.10.2.5). In our studies, instead of monocyte-depleted PBMC as the source of total T cells, we used affinity-purified $CD4^+$ and $CD8^+$ T cells as targets of HCV infection.

A pivotal point in this culture system employed was the use of PHA to stimulate target cells, as it is known that PHA increases susceptibility of lymphocytes to HCV infection (Pham *et al*., 2004, 2005; MacParland *et al*., 2006, 2009, 2015b). Another crucial parameter in our study was the process of selecting the most suitable HCV inocula for our *in vitro* infection experiments (Section 3.1), as well as the use of highly sensitive HCV RNA detection techniques.

In our study, we were able to clearly demonstrate that native, molecularly intact HCV is capable of establishing infection of both $CD4^+$ and $CD8^+$ T lymphocytes by documenting: (1) Presence of HCV RNA positive and negative (replicative) strands in the cells from all experiments performed; (2) Expression of HCV core and NS5A proteins in the cytoplasm of the *de novo* infected cells; (3) Identification of HCV variants in the *de novo* infected cells not detected in the inoculum used for their infection (some of these

variants have been previously identified in the extrahepatic compartments), and (4) Release of HCV RNA-reactive particles from the *de novo* infected $CD4^+$ and $CD8^+$ lymphocytes exhibiting different biophysical properties from the particles contained in inocula used to infect the cells.

CHAPTER SEVEN: FUTURE DIRECTIONS

In our study, it was documented that authentic, intact, patient-derived HCV is capable of replicating in human primary $CD4^+$ and $CD8^+$ T lymphocytes by using a multiparametric detection approach.

It would be of great interest to compare the molecular and biophysical properties of the virions produced from the *de novo* infected T cells with that produced by B cells and monocytes. HCV variants emerging in the different immune cell subpopulations could be fully sequenced and their sequences compared to each other and to that contained in inocula used for their infection in order to identify mutations reflecting virus potential adaptation to a given lymphoid cell compartment. To get a more complete picture, these sequences should also be compared to variants isolated from the respective fractionated immune cell subtypes isolated from the donors providing these HCV inocula, as well as to variants circulating in plasma. Perhaps *ex vivo* stimulation of these immune cells with mitogens would provide a more relevant comparison, as it would upregulate HCV replication in these cells.

Furthermore, it would be intriguing to compare the ability of different HCV inocula to infect T cells. In particular, it would be interesting to uncover whether there is a difference between various HCV genotypes and subtypes or even between HCV inocula carrying different variants and their capacity to infect T cells.

Finally, it would be of great value to use our system in order to further study the direct effect of HCV replication on $CD4^+$ and $CD8^+$ T cell function. More specifically,

the expression of certain antiviral and functionally important genes could be evaluated via real-time RT-PCR or microarray analysis (MacParland *et al*., 2015a) as it was recently initiated in this laboratory. Similar evaluation should be performed on cells isolated from patients naturally infected with different HCV genotypes.

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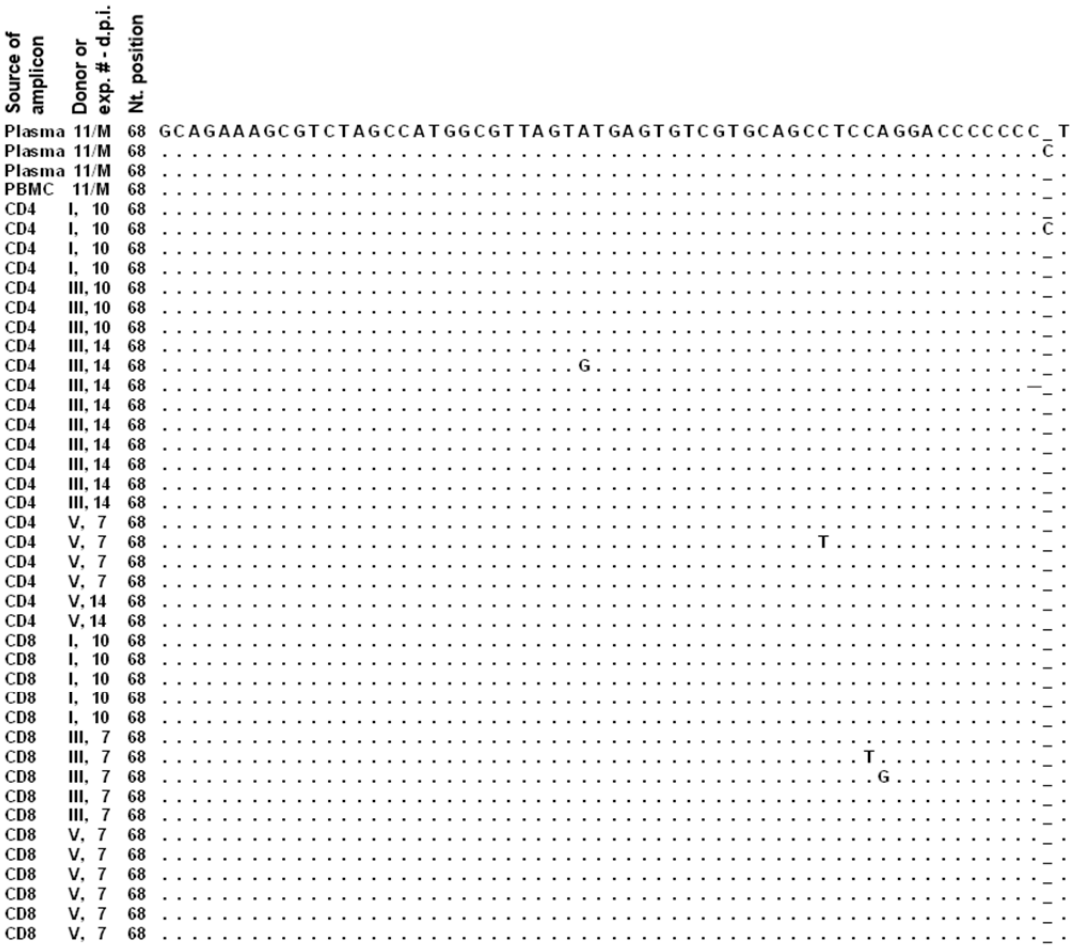
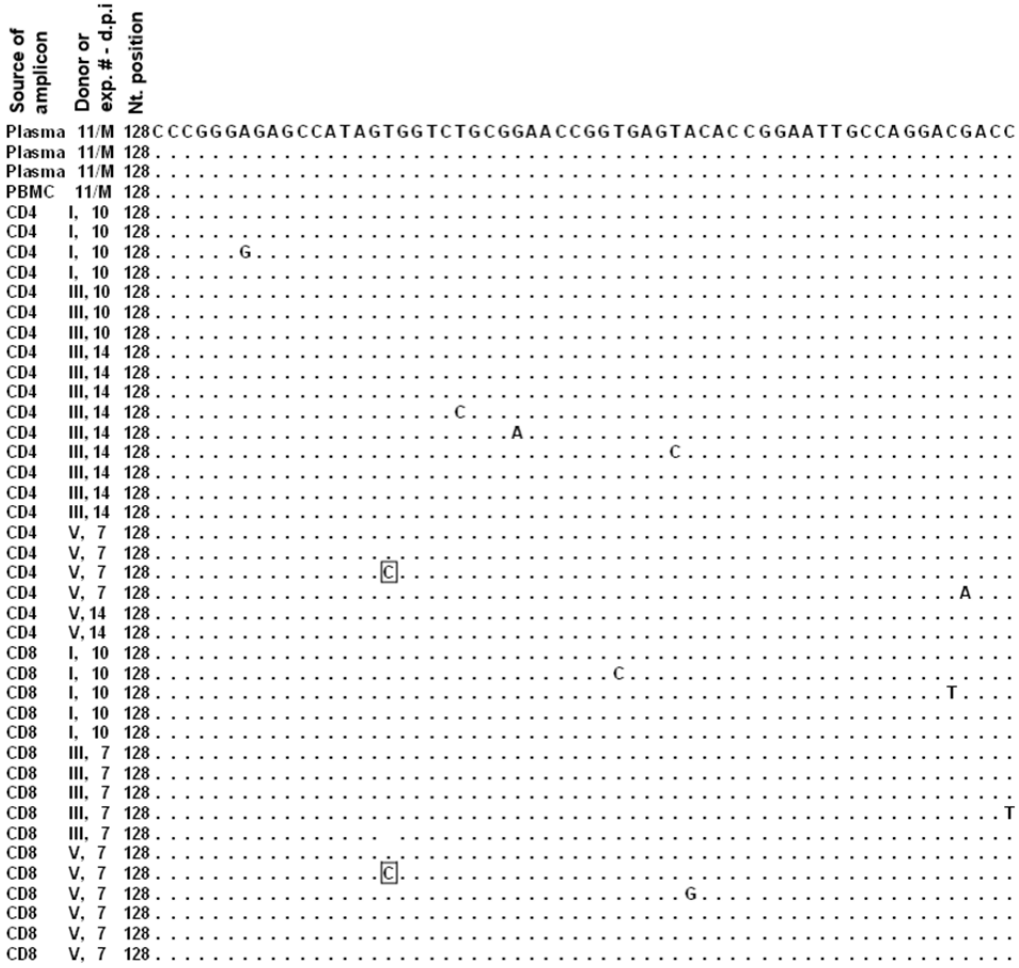
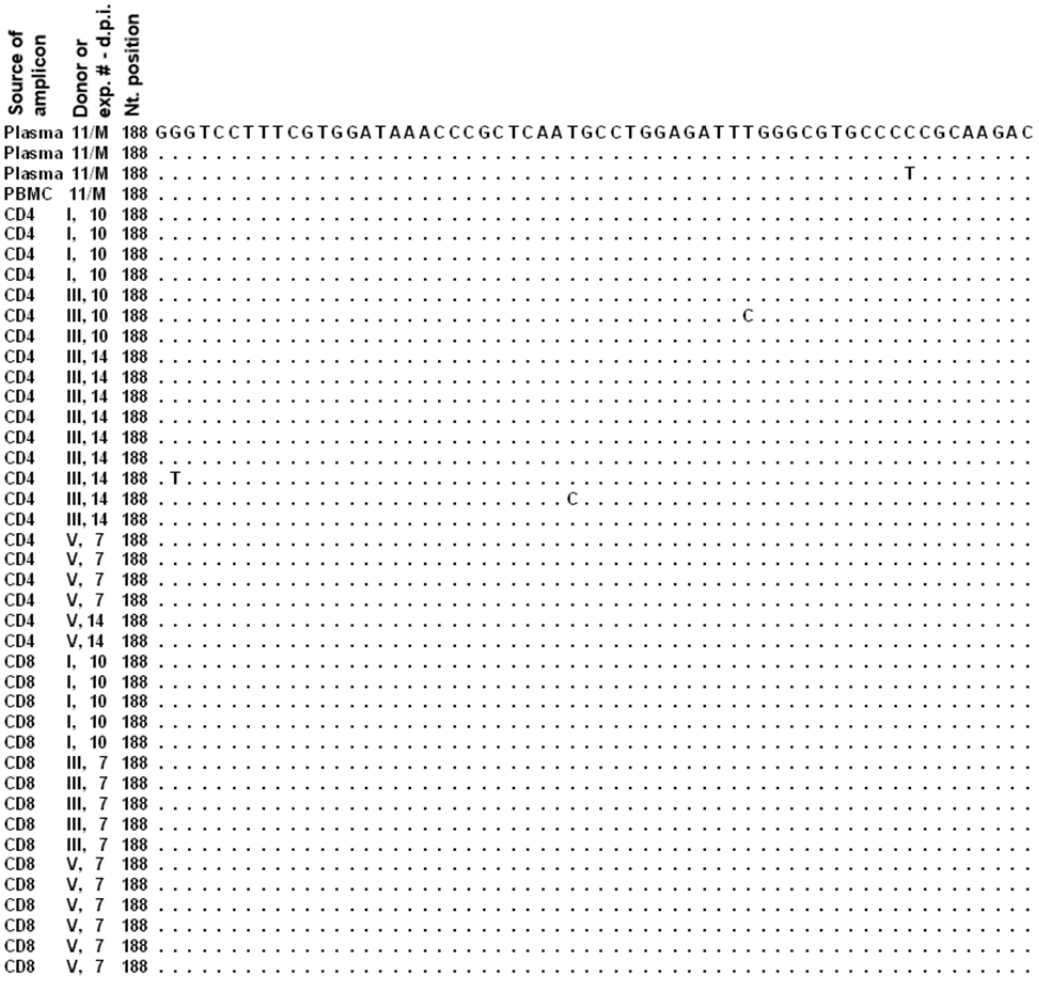
APPENDICES



Appendix 1. Nucleotide sequence alignment of the clones derived from HCV 5'-UTR fragments amplified from plasma and PBMC of donor 11/M, and from $CD4^+$ and $CD8^+$ T lymphocytes exposed to HCV occurring in that plasma. The 5'-UTR amplicons from each sample were cloned, and 20 randomly selected clones were sequenced bidirectionally. As a reference, sequences are compared with the genotype 1a prototype sequence (GenBank accession number M67463) shown at the top line. Representative sequences are shown and the number at the end of each sequence depicts the number of clones in which a given variant is found. Dots (.) indicate sequence identity with M67463, underlines (_) indicate gap introduced to preserve sequence alignment when an insertion occurs in one of the clones, dashes (—) indicate deletions, and differences are shown as letters. Single nucleotide polymorphisms (SNPs) that are common between $CD4^+$ and $CD8^+$ are denoted in a square. Abbreviations: Exp., experiment; d.p.i., days post infection; nt, nucleotide.


Source of amplicon
Donor or exp. # - d.p.i
Nt. position
Plasma 11/M 128 C C C G G G A G A G C C A T A G T G G T C T G C G G A A C C G G T G A G T A C A C C G G A A T T G C C A G G A C G A C C
Plasma 11/M 128
Plasma 11/M 128
PBMC 11/M 128
CD4 I, 10 128
CD4 I, 10 128
CD4 I, 10 128 G
CD4 I, 10 128
CD4 III, 10 128
CD4 III, 10 128
CD4 III, 10 128
CD4 III, 14 128
CD4 III, 14 128
CD4 III, 14 128
CD4 III, 14 128 C
CD4 III, 14 128 A
CD4 III, 14 128 C
CD4 III, 14 128
CD4 III, 14 128
CD4 III, 14 128
CD4 V, 7 128
CD4 V, 7 128
CD4 V, 7 128 C
CD4 V, 7 128 A
CD4 V, 14 128
CD4 V, 14 128
CD8 I, 10 128
CD8 I, 10 128 C
CD8 I, 10 128 T
CD8 I, 10 128
CD8 I, 10 128
CD8 III, 7 128
CD8 III, 7 128
CD8 III, 7 128
CD8 III, 7 128 T
CD8 III, 7 128
CD8 V, 7 128
CD8 V, 7 128 C
CD8 V, 7 128 G
CD8 V, 7 128
CD8 V, 7 128
CD8 V, 7 128


Source of amplicon
Donor or exp. # - d.p.i.
Nt. position
Plasma 11/M 188 GGGTCCTTTCGTGGATAAACCCGCTCAATGCCTGGAGATTTGGGCGTGCCCCCGCAAGAC
Plasma 11/M 188
Plasma 11/M 188 T
PBMC 11/M 188
CD4 I, 10 188
CD4 I, 10 188
CD4 I, 10 188
CD4 I, 10 188
CD4 III, 10 188
CD4 III, 10 188 C
CD4 III, 10 188
CD4 III, 14 188
CD4 III, 14 188
CD4 III, 14 188
CD4 III, 14 188
CD4 III, 14 188
CD4 III, 14 188
CD4 III, 14 188 T
CD4 III, 14 188 C
CD4 III, 14 188
CD4 V, 7 188
CD4 V, 7 188
CD4 V, 7 188
CD4 V, 7 188
CD4 V, 14 188
CD4 V, 14 188
CD8 I, 10 188
CD8 I, 10 188
CD8 I, 10 188
CD8 I, 10 188
CD8 I, 10 188
CD8 III, 7 188
CD8 III, 7 188
CD8 III, 7 188
CD8 III, 7 188
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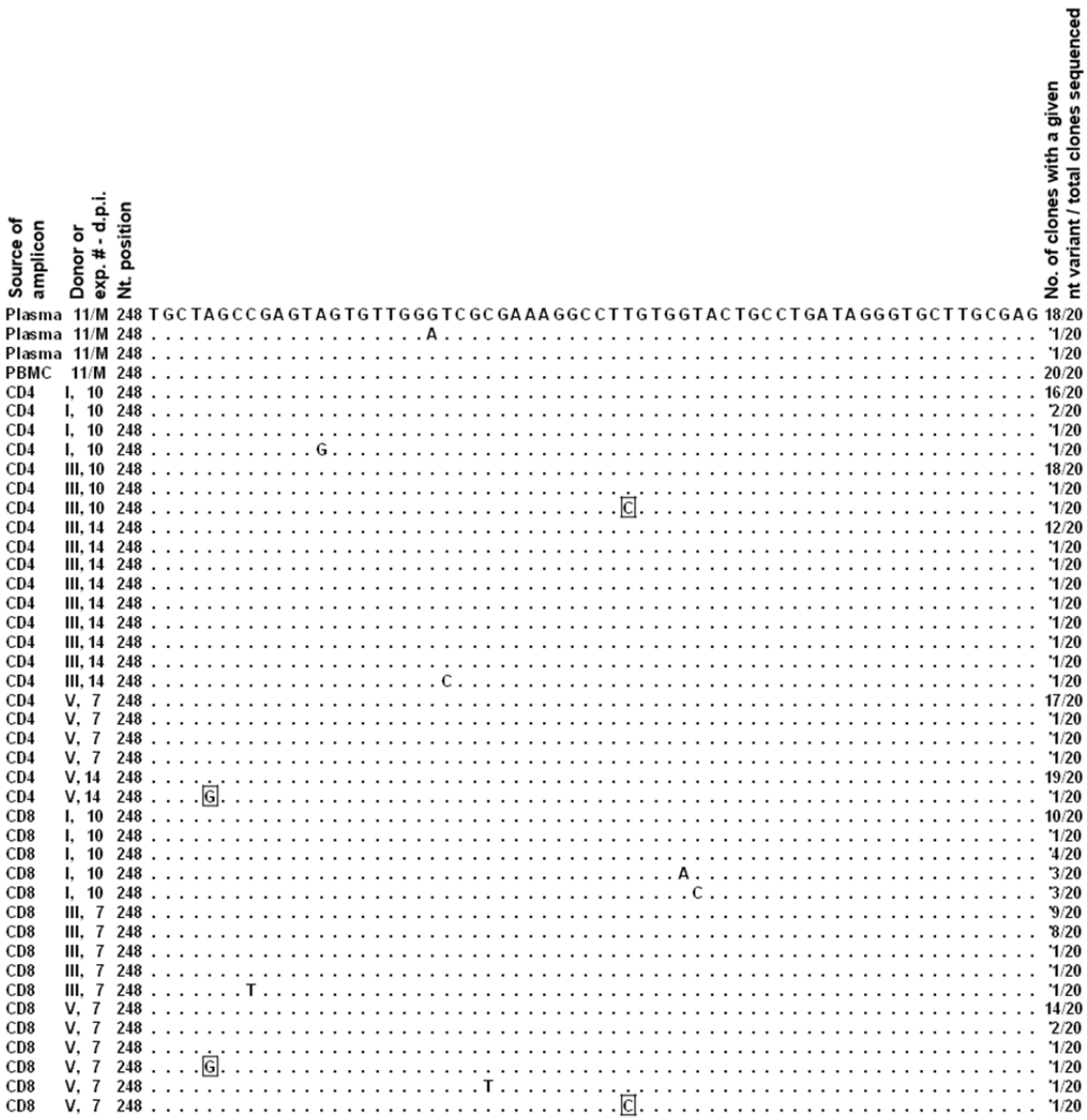
CD8 V, 7 188
CD8 V, 7 188
CD8 V, 7 188
CD8 V, 7 188


Source of amplicon
Donor or exp. # - d.p.i.
Nt. position
No. of clones with a given nt variant / total clones sequenced
Plasma 11/M 248 TGCTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAG 18/20
Plasma 11/M 248 A 1/20
Plasma 11/M 248 1/20
PBMC 11/M 248 20/20
CD4 I, 10 248 16/20
CD4 I, 10 248 2/20
CD4 I, 10 248 1/20
CD4 I, 10 248 G 1/20
CD4 III, 10 248 18/20
CD4 III, 10 248 1/20
CD4 III, 10 248 C 1/20
CD4 III, 14 248 12/20
CD4 III, 14 248 1/20
CD4 III, 14 248 1/20
CD4 III, 14 248 1/20
CD4 III, 14 248 1/20
CD4 III, 14 248 1/20
CD4 III, 14 248 1/20
CD4 III, 14 248 C 1/20
CD4 V, 7 248 17/20
CD4 V, 7 248 1/20
CD4 V, 7 248 1/20
CD4 V, 7 248 1/20
CD4 V, 14 248 19/20
CD4 V, 14 248 G 1/20
CD8 I, 10 248 10/20
CD8 I, 10 248 1/20
CD8 I, 10 248 4/20
CD8 I, 10 248 A 3/20
CD8 I, 10 248 C 3/20
CD8 III, 7 248 9/20
CD8 III, 7 248 8/20
CD8 III, 7 248 1/20
CD8 III, 7 248 1/20
CD8 III, 7 248 T 1/20
CD8 V, 7 248 14/20
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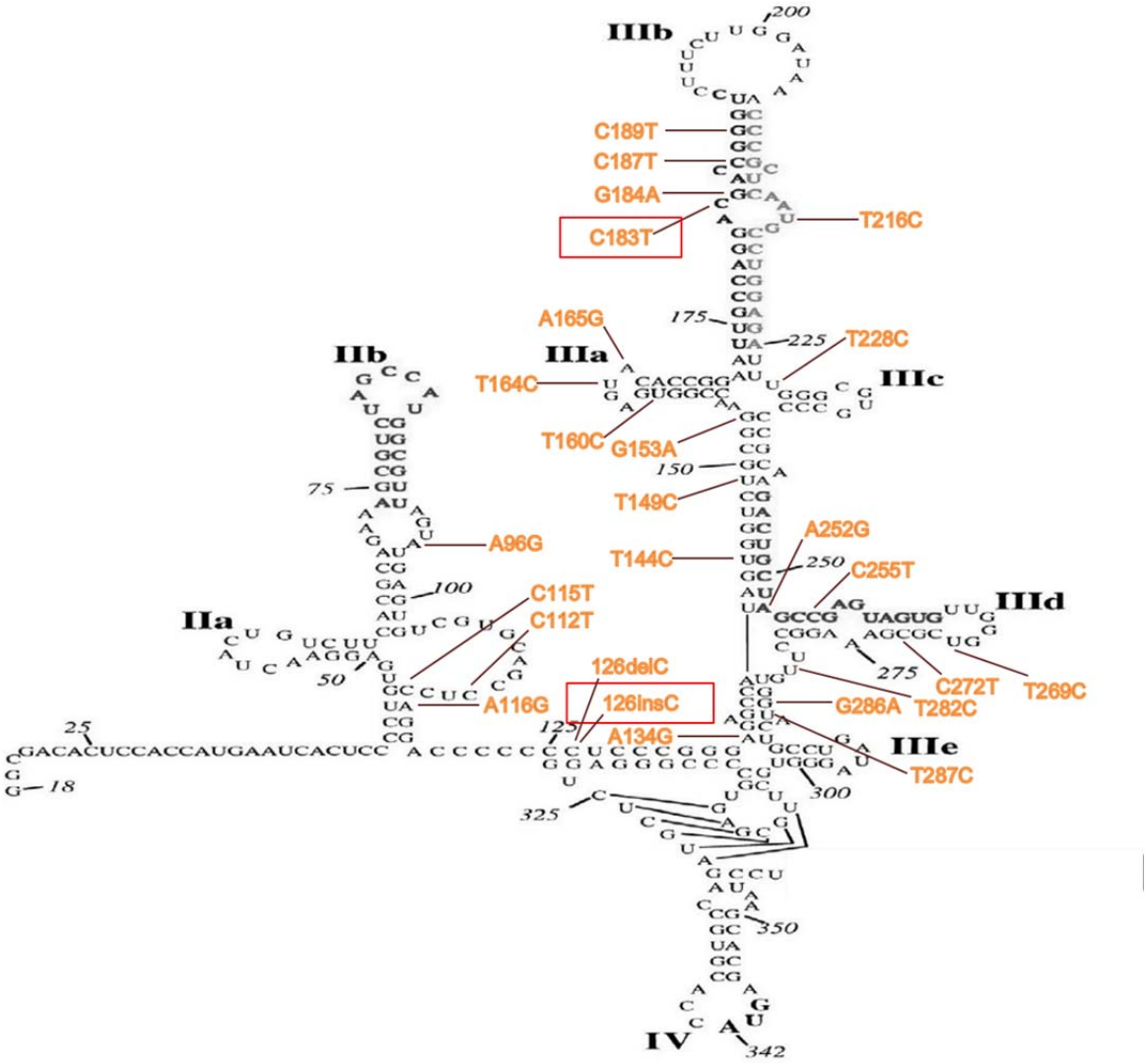
CD8 V, 7 248 1/20
CD8 V, 7 248 G 1/20
CD8 V, 7 248 T 1/20
CD8 V, 7 248 C 1/20



Appendix 2. Location of SNPs in the HCV 5'-UTR sequences detected in $CD4^+$ and $CD8^+$ T cells *in vitro* infected with genotype 1a HCV-11/M. The SNP positions and sequence changes are highlighted in orange. The SNPs also identified by others (Laskus *et al*., 1998, 2002; Radkowski *et al*., 2002) are denoted within red squares. The nucleotide positions numbered according to HCV genotype 1a with GenBank accession number M67463. Image modified from Prabhu R. *et al*., 2006.