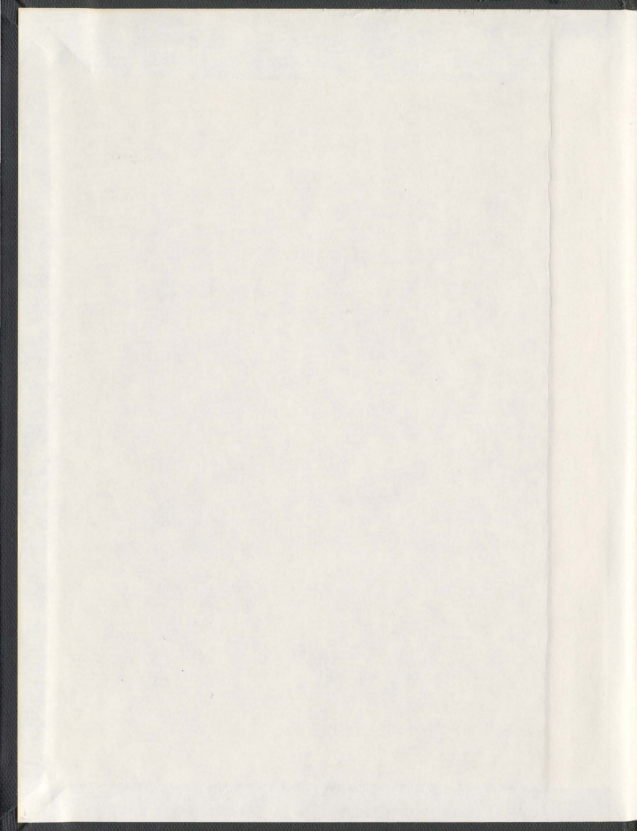# MECHANISMS OF HEPATITIS C VIRUS INFECTION
OF HUMAN T LYMPHOCYTES

MOHAMMED ABDULLAH RASHAD SARHAN

001311

MECHANISMS OF HEPATITIS C VIRUS INFECTION OF HUMAN T

LYMPHOCYTES

BY

Mohammed Abdullah Rashad Sarhan

A thesis submitted to the School of Graduate Studies

in partial fulfilment of the requirements for

the degree of Doctor of Philosophy

Faculty of Medicine

Memorial University of Newfoundland

ABSTRACT

Hepatitis C virus (HCV) induces one of the most prevalent, potentially fatal chronic viral infections worldwide. This virus symptomatically infects approximately 170 million people (2.5% of the global population). Although HCV is primarily a hepatotropic virus, accumulated experimental and clinical evidence indicates that HCV can also replicate in cells of the immune system. The ability of HCV to infect human cells is currently interpreted in the context of the interactions identified between the Japanese fulminant hepatitis-1 (JFH-1) strain of HCV or HCV pseudoparticles and human hepatoma cell lines, particularly Huh7.5 clone. The factors determining HCV lymphotropism remained unknown.

Based on previous observations demonstrating that wild-type HCV can infect and replicate in human T lymphocytes both *in vivo* and *in vitro*, we hypothesized that HCV utilizes certain T cell-specific molecules to enter these cells and, therefore, that HCV uses (at least in part) different strategy to infect immune cells than Huh7 cells or primary hepatocytes.

This thesis is comprised of three related studies. In the first study, we established an *in vitro* infection system applying readily available human T cell lines, such as Molt4 and Jurkat T cells, and naturally occurring wild-type HCV obtained from patients with chronic hepatitis C. Employing this system, we confirmed that HCV infects primary T lymphocytes as well as the mentioned T cell lines, as evident by detection of HCV RNA negative (replicative) strand and virus non-structural (NS5A) and structural (core) proteins. However, some T cell

i

lines were not susceptible to HCV infection. We found that cells prone to HCV infection are endowed with CD5, a scavenger receptor that belongs to the cysteine-rich superfamily. We documented that this molecule serves as HCV T cell receptor by multiple supplementing each other approaches.

In the subsequent study, we evaluated whether other molecules proposed to serve as HCV receptors based on the JFH-1-Huh7 cell studies may contribute to establishment of HCV infection in T lymphocytes. For this purpose, the mRNA and protein expression of the different HCV candidate receptors, such as CD81 (mRNA only), scavenger receptor class B type1 (SR-B1), occludin (OCLN), claudin-1 (CLDN-1), claudin-4 (CLDN-4) and claudin-6 (CLDN-6), in addition to CD5 molecule identified in the first study, were investigated. We examined T cell lines, peripheral blood mononuclear cells (PBMC) and affinity-purified primary human T cells and their CD4+ and CD8+ subsets, and compared to hepatoma Huh7.5 and HepG2 cell lines and primary human hepatocytes (PHH). We found that while CD5, CD81, and OCLN are likely involved in mediating T cell susceptibility to HCV infection, SR-B1 and CLDN-1 are not. This validated the concept that HCV uses different receptors to infect various cell types naturally targeted by this virus. In general, this study narrowed the range of factors utilized by HCV infecting T lymphocytes amongst those identified using HCV preparations and Huh7.5 cells.

In the third study, we tested the ability of the recombinant HCV JFH1$_T$ virus, a culture-adapted JFH-1 strain demonstrating significantly higher infectivity towards Huh7.5 cells than the classical JFH-1 strain, to infect T cell lines, human

PBMC and primary T cells, in comparison to the plasma-derived wild-type HCV. We also examined the susceptibility of Huh7.5 cells to the plasma-derived wild-type HCV. The results revealed that T cell lines, PBMC and primary human T cells were susceptible to the patient-derived wild-type HCV but not to $JFH1_T$ clone, while Huh7.5 cells were only prone to $JFH1_T$ but not to wild-type virus. This study demonstrated contrasting differences between wild-type HCV and JFH clones in terms of cell tropism and suggested that HCV-T cell infection system can be more representative of the actual (wild-type) virus behaviour than genetically modified and Huh7.5 cell culture-adapted viruses.

Overall, our findings have provided conclusive evidence that wild-type HCV can infect and replicate not only in primary human T lymphocytes but also in some readily available human T cell lines. We identified CD5, a glycoprotein belonging to the scavenger receptor cysteine-rich superfamily, as a T cell-specific molecule mediating the cell susceptibility to HCV infection. In addition, CD81 was found to act as a co-receptor, while OCLN is likely involved in infection of the virus-prone T cell lines but unlikely primary T cells.

In general, our research provided the first insight into the molecular mechanism determining HCV ability to infect cells of the immune system.

ACKNOWLEDGMENT

Table of Contents

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

5'-UTR	5'-untranslated region
aa	amino acids
AH	acute HCV infection
ALT	alanine aminotransferase
APC	allophycocyanin
ARFP	alternative reading frame protein
ASGP-R	asialoglycoprotein receptor
AST	aspartate aminotransferase
ATCC	American Type Culture Collection
BLYS	B-lymphocyte stimulator
BOC	boceprevir
C	core protein
CD	cluster of differentiation
CHC	chronic hepatitis C
CLDN	claudin
CMV	cytomegalovirus
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
d.p.i.	days post-infection
DAPI	4',6-diamidino-2-phenylindole
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
DMEM	Dulbecco's modified Eagle's medium
EBV	Epstein-Barr virus
EHM	extrahepatic manifestations
eIF	initiation factor
EL2	large extracellular loop 2
EMA	European Medicines Agency
ER	endoplasmic reticulum
EVR	early virological response
F	frame shift
FCS	fetal calf serum
FDA	Food and Drug Administration
FFU	focus-forming units
FITC	fluorescein isothiocyanate
GAGs	glycosaminoglycans
GFP	green fluorescent protein
HAV	hepatitis A virus

HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCVcc	cell culture produced JFH-1 particles
HCVpp	HCV pseudotype particles
HDL	high density lipoproteins
HDV	hepatitis D virus
HEK-293	human embryonic kidney 293 cells
HepG2	hepatoma G2 cells
HEV	hepatitis E virus
HGV	hepatitis G virus
HIV	human immunodeficiency virus
HPRT	hypoxanthine phosphoribosyltransferase
HSPGs	heparan sulfate proteoglycans
HSV	herpes simplex virus
Huh7.5	human hepatoma 7.5 cells
HVR	hypervariable regions
ICAM-3	intercellular adhesion molecule-3
IDU	injection drug user
IFN	interferon
IRES	internal ribosome entry site
IRF	interferon regulatory factor
Irr. shRNA	control irrelevant short interfering RNA
ISDR	interferon sensitivity determining region
ISGs	interferon stimulatory genes
JFH-1	Japanese fulminant hepatitis-1
$JFH1_T$	JFH-1 triple mutant
kb	kilobase
KDa	kilodalton
LDL-R	low-density lipoprotein receptor
LEL	large extracellular loop
L-SIGN	liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin
mAb	monoclonal antibody
MC	mixed cryoglobulinemia
MFI	mean fluorescence intensity
MOI	multiplicity of infection
MPCCs	micropatterened cocultures
NAH	nucleic acid hybridization techniques

TLR	toll-like receptor
TM	hydrophobic transmembrane regions
TNF-α	tumour necrosis factor alpha
UT	untreated
wHCV	wild-type HCV
vge	virus genome equivalents
ZO	zonula occludens

THESIS CO-AUTHORSHIP STATEMENT

This thesis is comprised of 8 chapters. Chapter 1 is an introduction and provides the background and rational for the studies carried out during this thesis. Chapter 2 demonstrates the purpose of our studies. Chapters 3 to 5 contain original data collected during each of three studies. Chapter 6 is a discussion which connects findings from all three projects while Chapter 7 relays the overall conclusions of the work and the future directions of each study. Then, chapter 8 list the references used in my thesis.

The large majority of the work described in this thesis was carried out by the author. The role of the author included contribution to the design of the experiments, establishment of techniques, data collection and analysis, and manuscript preparation. The work described in Chapter 3 was published as a first authored paper **entitled "Hepatitis C Virus Infection of Human T Lymphocytes is Mediated by CD5"** (J Virol. 2012 **86**:3723-35). The study described in Chapter 4 was submitted as a first authored paper entitled **"Differential Expression of Hepatitis C Virus Candidate Hepatocyte Receptors in Human T Lymphocytes Prone or Resistant to Infection with Wild-Type Virus"**. In our final study, described in Chapter 5 **"Wild-Type Hepatitis C Virus and HCV JFH-1 Clones Contrastingly Differ in Their Ability to Infect Human Lymphocytes and Hepatoma Cells"**, it was submitted as a first authored paper. The author acknowledges the contributions made by others: evaluations of HCV RNA negative strand in Chapters 3, 4, and 5 were performed

CHAPTER ONE:

INTRODUCTION:

1.1 VIRAL HEPATITIS

Hepatitis viruses comprise a group of diverse pathogens that primarily infect the liver and belong to different virus families with very different replication strategies. They are infecting hundreds of millions people, causing either acute or chronic infections. Some of these viruses cause diseases progressing to liver cirrhosis and hepatocellular carcinoma (HCC), which are the leading causes of death worldwide. Among these viruses, hepatitis A virus (HAV), hepatitis E virus (HEV) and hepatitis G virus (HGV) are transmitted via the oral-fecal route, whereas hepatitis B virus (HBV), hepatitis C virus (HCV) and hepatitis D virus (HDV) parenteraly.

1.1.1. Hepatitis A virus

HAV belongs to the genus hepatovirus within the *Picornaviridae* family. It is a nonenveloped agent with an icosahedral capsid of ~30-nm in diameter containing a positive single-stranded (ss) RNA genome of 7.5 kilobase (kb) (112, 114). The genome open reading frame (ORF) encoding a polyprotein of 2225 amino acids (aa) is preceded by a 5'-untranslated region (5'-UTR) and followed by a much shorter 3'-UTR. The translated polyprotein is cleaved into 11 proteins through a cascade of proteolytic events mediated mainly by the viral 3C protease (365). HAV infection has an acute course and does not progress to chronic hepatitis (265). Vaccines against HAV provide lifelong protection from infection

(410). HAV is primarily transmitted by the fecal–oral route (288) via contaminated water and food, and fomites act as vehicles of transmission (4). Virus occurs in the feces of infected patients at very high concentration (up to 10^{11} genome copies/g) for 2 weeks after the onset of symptoms and lasts at lower levels for at least 4 more weeks (78).

1.1.2. Hepatitis B virus

HBV is an enveloped DNA virus and is the smallest mammalian DNA virus known (diameter of 42 nm). It has an icosahedral nucleocapsid of approximately 30nm in diameter that encloses the viral partially double-stranded DNA genome and viral DNA polymerase. The complete genome encodes viral proteins through four highly overlapping (67%) ORFs called S, C, P, and X. HBV belongs to genus hepadnavirus within the family *Hepadnaviridae*. HBV infects as a symptomatic, serologically detectable infection more than 370 million people, while more than 2 billion people worldwide show serological and/or molecular evidence of exposure to this virus. Virus may cause acute and chronic liver disease, as well as asymptomatic occult long-term infection (291). Acute HBV infection is self-limited in 95% of infected adults and in 10 - 70% in children and young adults. However, virus clearance may fail especially in newborns of HBV-infected mothers (288, 399). Up to 30% of patients with chronic hepatitis B progress to liver cirrhosis (LC) and HCC (WHO, 2000a). Lifelong persistent occult infection appears to be a common consequence of resolution of acute hepatitis B (276, 341, 272, 275, 341). In this regard, investigations in the woodchuck model of hepatitis B were

essential in recognition of this pathogenically and epidemiologically important form of hepadnaviral infections (272). Currently, vaccines that confer lasting immunity against HBV are widely available. However, new infections in vaccine non-protected individuals and in children born to HBV-infected mothers are still a great global health problem (175).

1.1.3. Hepatitis D virus

HDV is a negative sense, single-stranded, closed circular RNA virus of the genus deltavirus. HDV is a satellite virus for HBV in that it requires coinfection with HBV to infect, assemble and release virions (107). There are eight genotypes of HDV. The infection of HDV can occur simultaneously with HBV or as superinfection of a chronic HBV carrier. The former pattern can be associated with "eradication" of both agents, whereas superinfection mostly evolves to HDV chronicity. HDV can enhance progression of chronic hepatitis to cirrhosis (305).

1.1.4. Hepatitis E virus

HEV is a small non-enveloped single-stranded positive-sense RNA virus. It is the sole member of the genus herpevirus and is mainly transmitted by the fecal-oral route (293). HEV has four genotypes but only one serotype. Genotypes 1 and 2 exclusively infect humans, whereas genotypes 3 and 4 infect pigs and several other mammalian species. The disease usually affects young adults, and is particularly severe among pregnant women and persons with pre-existing liver cirrhosis (9).

1.1.5. Hepatitis G virus

HGV was isolated from the plasma of a HCV-infected patient. It is a single stranded, positive-sense RNA virus belonging to the *Flaviviridae* family. HGV has a close sequence homology and genomic organization to HCV (38). However, unlike HCV, HGV is neither hepatotropic nor causes acute or chronic hepatitis (217). The virus replicates in the lymphatic system, including the spleen, bone marrow (38) and peripheral blood mononuclear cells (PBMC), including CD4+ T cells (418). No disease has been associated with HGV infection, but co-infection of individuals infected with human immunodeficiency virus (HIV) decreases morbidity and mortality and slows progression to acquired immunodeficiency syndrome (38).

1.2 HEPATITIS C VIRUS

HCV is the subject of this dissertation and will be described in detail in this and following sections.

1.2.1. Epidemiology

HCV infection is a worldwide endemic disease and is characterized by a high degree of geographical variation in its distribution. It is estimated that 123-170 million people are infected with HCV (14, 368, 407). However, this number is likely under-estimated considering the sensitivity of the current clinical assays available for detection of HCV RNA and anti-HCV antibodies. In the United States, HCV infection is currently the most common chronic blood-borne infection with an estimated 3.2 million individuals chronically infected (19). Due to lack of

vaccine or effective treatment and post-exposure prophylaxis, prevention and control of hepatitis C is the main point of focus for health authorities in many countries. The prevalence of HCV in industrialized countries is relatively low (0.01-0.5%) and is mainly attributed to injection drug users (254, 368). In Canada, approximately 300,000 individuals are chronically infected with HCV (Fischer *et al.*, 2006). In Northern Africa and Asia, higher prevalence of HCV infection has been reported (39, 368). In developing nations, such as Egypt, Hubei, Mongolia and Pakistan high prevalence of HCV infection (17-26%) has been reported (106, 132, 407). HCV infections in these countries are largely attributed to unsafe medical procedures and blood transfusions (331, 368) and to incomplete or lack of screening of donated blood (331). Although transmission of HCV via sexual, occupational (e.g., in health care personnel) and perinatal exposures can occur, this mode of virus spread is not well documented compared to percutaneous exposure (1, 331, 368). It is estimated that 75-85% of individuals infected with HCV develop asymptomatic chronic hepatitis C (CHC) depending on virus genotype (1, 39). Between 5 and 25% of patients with CHC develop LC (258), and approximately 5% of cirrhotic patients will eventually progress to HCC (233).

Initially, 11 genotypes were described (55, 379), which were later reclassified into 6 major genotypes due to the similarity in nucleotide sequences (368, 381). These different genotypes have variable geographical distribution (264, 407), where genotypes 1 through 3 occur worldwide and genotype 1b is the most common strain overall (380). Genotype 1a is the main virus type prevailing

in Europe and North America, whereas 1b is predominant in Japan (160, 381). Genotype 2 occurs frequently in the Mediterranean region, Western Africa, and the Far East (264, 380). Genotype 3 is common in Southeast Asia, India and Australia, and genotype 4 is the predominant strain in Egypt, Northern Africa and the Middle East (343). In South Africa, genotype 5 is the most prevalent, while type 6 occurs in Southeast Asia and Australia (283, 380, 381).

1.2.2. Transmission Routes and Infection Risk Factors

HCV is a highly infectious blood-borne virus (75). The major risk factor associated with HCV transmission is intravenous drug use (IDU) (15). It is estimated that as much as 80% of new HCV infections are due to IDU (218) and the prevalence of HCV can be as high as 95% in active IDU (121). The risk of HCV transmission by sexual contact is less than 3% among individuals with single partners, however, the risk is higher in homosexuals and among those with multiple partners (389). Other more common risk factors include: intranasal cocaine use, haemodialysis, tattooing, piercing and needle stick injuries (75, 104, 120, 132, 198, 298, 393). Approximately six out of every 100 infants born to HCV-infected mothers become symptomatically infected, which occurs predominantly during or near delivery. However, in the case of maternal HCV viremia at delivery or HIV-HCV coinfection, the risk of transmission is 2–3 times greater. HCV transmission through breast milk has not been reported; however, HCV-positive mothers should consider abstaining from breastfeeding if their nipples are cracked or bleeding (415).

1.3   FLAVIVIRIDAE FAMILY

Flaviviridae family includes three genera: the flaviviruses (from the Latin *flavus*, yellow), the pestiviruses (from the Latin *pestis*, plague), and the hepaciviruses (from the Greek *hepatos*, liver). The flavivirus genus includes dengue virus, West Nile virus, tick-borne encephalitis virus, Murray Valley encephalitis virus, St. Louis encephalitis virus, Japanese encephalitis virus, and yellow fever virus (228). The Pestivirus genus includes viruses such as bovine viral diarrhea virus (BVDV) and border disease virus, and classical swine fever (hog cholera) virus (74). The hepacivirus genus includes only HCV (228).

The *Flaviviridae* family members share a number of common structural and virological characteristics. Thus, all possess envelopes constituted by a lipid bilayer that surrounds the nucleocapsid, which is composed of multiple copies of a small basic protein called core, and contains the RNA genome. The genome is positive-strand RNA, ranging in size from 9.6 to 12.3 x10^3 nucleotides (nt), with a singular ORF encoding a 3000-aa or longer polyprotein (228).   The structural proteins of the virus are encoded by the N-terminal part of the ORF, whereas the remaining portion of the ORF codes for the nonstructural proteins (NS) (Figure 1.1). Sequence motif-conserved RNA protease-helicase and RNA-dependant RNA polymerase (RdRp) are found at similar locations in the polyproteins of all flaviruses (277). In addition, *Flaviviridae* share similar polyprotein hydropathic profiles, with flaviviruses and hepaciviruses being closer to each other than to pestiviruses (68). The ORF is flanked by 5'- and 3'-UTR of 95–555 and 114–624-

nt in length, respectively (Figure 1.1) (395). Viruses of *Flaviviridae* bind to one or more cellular receptors organized as a receptor complex and appear to trigger receptor-mediated endocytosis. Fusion of the virion envelope with cellular membranes delivers the nucleocapsid to the cytoplasm. After decapsidation, translation of the viral genome occurs in the cytoplasm, leading to the production of a precursor polyprotein, which is then cleaved by both cellular and viral proteases into structural and NS proteins. Replication of the viral genome is carried out by the viral replication complex which is associated with cellular membranes. Viral replication occurs in the cytoplasm *via* the synthesis of full-length negative-strand RNA intermediates. Assembly of virions occurs in cytoplasmic vesicles by budding through intracellular membranes. Then, mature virions are released into the extracellular milieu by exocytosis. Despite the above mentioned similarities with the members of *Flaviviridae* genera, HCV does exhibit a number of differences (see also Sections 1.2.1 and 1.2.2) Flaviviruses translation is cap-dependent, *i.e.*, mediated by a type I cap structure located in the 5'-UTR (48). In contrast, the HCV 5'-UTR is not capped and, like that of pestiviruses and HGV virus, folds into a complex secondary RNA structure. This structure is forming, together with a portion of the core-coding domain, an internal ribosome entry site (IRES) that mediates direct binding of ribosomal subunits and cellular factors, and subsequent genome translation. The 3'-UTR of *Flaviviridae* viruses is highly structured, while that of HCV is relatively short, less structured, and contains a polyuridyl tract that varies in length. Unlike HCV, which has a narrow host specificity and tissue tropism and is transmitted exclusively through

direct contact with human body fluids, flaviviruses are mainly transmitted by arthropods, such as mosquitoes or ticks, and can infect a broad range of vertebrate animals, with humans being a dead-end host. Further, pestiviruses cannot infect humans and no known insect vector transmitting these viruses has been identified. In contrast to HCV, infections caused by flaviviruses are acute and self-limited in vertebrates. Furthermore, HCV induces immune responses that frequently fail to prevent infection and do not confer protection against reinfection with other virus strains. On the opposite, strong humoral and cellular immune responses are induced by infections with flaviviruses and pestiviruses that lead to self-recovery (108).

1.4 HEPATITIS C VIRUS GENOME AND PROTEINS

1.4.1. Genome Organization

HCV possesses a 9.6-Kb RNA genome which is flanked by highly structured 5'- and 3'-UTRs.

1.4.1.1 ORF

The HCV ORF contains 9024 to 9111 nt depending on the virus genotype. The ORF encodes 11 proteins, including 3 structural proteins, 6 NS proteins and the frame shift "F" protein (see Section 1.4.2), in addition to p7 protein which function has not yet been clearly defined (see Section 1.4.3).

Figure 1.1

10

Figure 1.1. Schematic representation of the HCV genome and encoded viral proteins. The boxed area corresponds to the single open reading frame of the HCV genome. The stem-loop structures represent the 5′ and 3′ UTR regions, including the IRES and 3′X regions. C–E1, E1–E2, E2–p7 and p7–NS2 junctions are cleaved by a cellular signal peptidase(s) to yield structural proteins. Interferon (IFN)-sensitivity-determining region (ISDR), has been linked to the response to IFN-α therapy in some strains of HCV. ARFP/F, alternative reading-frame protein/frameshift protein; LDLR, low-density lipoprotein receptor; RdRp, RNA-dependent RNA polymerase. Image from reference (385)

1.4.1.2   5'-UTR

The HCV 5'-UTR contains 341 nt located upstream of the ORF translation initiation codon. It is the most conserved region of the HCV genome and displays nt sequence identity of ~ 60% with HGV and approximately 50% with pestiviruses (68, 165). The 5'-UTR contains four highly structured domains, numbered I to IV, containing numerous stem-loops and a pseudoknot (50, 406). Domains II, III and IV, together with the first 12 to 30-nt of the core coding region, constitute the IRES (173). The HCV IRES has the capacity to form a stable pre-initiation complex by directly binding the 40S ribosomal subunit without the need of canonical translation initiation factors, an event that likely constitutes the first step of HCV polyprotein translation. Tissue compartmentalization of HCV with different IRES sequences was reported in several studies (215, 223, 294, 371). Among others, infection of lymphoid cell lines with HCV genotype 1a H77 strain led to the selection of a quasispecies with nucleotide substitutions within the 5'-UTR (223, 243). Furthermore, different translation efficiencies of HCV quasispecies variants isolated from different cell types in the same patient were observed, suggesting cell type-specific IRES interactions with cellular factors may also modulate polyprotein translation (127, 213, 223).

1.4.1.3   3'-UTR

The 3'-UTR is approximately 225-nt long and contains three regions. These regions, from the 5'- to 3'-end, consist of a variable region of approximately 30-40 nt, a long poly(U)-poly(U/UC) tract, and a highly conserved 3'-terminal stretch of 98 nt (3'X region) that includes three stem-loop structures

SL1, SL2 and SL3 (202, 386, 387). The 3'-UTR interacts with the NS5B RdRp and with two of the four stable stem-loop structures located at the 3'-end of the NS5B-coding sequence (66, 133, 182, 220, 421). The 3'-X region and the 52-nt fragment upstream of the poly(U/C) tract were found to be essential for RNA replication, whereas the remaining sequence of the 3'-UTR appears to enhance viral replication (133, 182, 421, 422).

1.4.2. Polyprotein Translation and Processing

As with other plus-strand RNA viruses, HCV replication starts with synthesis of a complementary negative strand RNA using the genome as a template. Then, positive-strand RNA is produced from a negative-strand RNA template, catalyzed by the NS5B RdRp. The positive-strand RNA progeny is transcribed at a level 5- to 10-fold greater than that of negative-strand RNA. HCV genome is translated through a 5'-cap independent pathway, utilizing the IRES within the 5'-UTR. This IRES binds the 40S ribosomal subunits directly, bypassing the need for pre-initiation factors, and inducing an mRNA-bound conformation in the 40S subunit (383). The IRES–40S complex then recruits eukaryotic initiation factor (eIF) 3 and joins the ternary complex of Met-tRNA–eIF2–GTP to form a non-canonical 48S intermediates. Association with 60S ribosomal subunit allows transition to the translationally active 80S complex that initiates viral protein synthesis (114, 185, 311). The generated polyprotein is then targeted to the endoplasmic reticulum (ER) where it is co- and post-translationally cleaved by host and viral proteases into 10 viral proteins (Figure 1.1). The amino-terminal one-third of the polyprotein encodes three HCV structural proteins: core

(C) protein, and the glycoprotein envelops E1 and E2. The p7 protein, a small integral membrane protein, seems to function as an ion channel (162). The rest of the genome encodes the NS proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B, which control the intracellular processes of the virus life cycle. The structural proteins mature by signal peptidases. HCV also encodes a small protein, called F (frame shift) or ARFP (alternative reading frame protein), that can be produced by ribosomal frame shifting into an alternative reading frame within the core gene (47, 229).

1.4.3. Functions of Viral Proteins

HCV El and E2 proteins are transmembrane glycoproteins that constitute the virus envelope and are required for viral entry into cells. E2 has been found to bind many putative HCV receptors (see Section 1.5). Highly heterogeneous hypervariable regions (HVR) have been identified in the aa sequence of the E2 envelope glycoprotein. HVR1 is constituted by the first 27 aa of the E2 ectodomain. The variability of the HVR1 region seems to be driven by selection of immune-escape variants and is responsible for significant inter- and intra-individual variation of the infecting virus, which may represent an important pathogenic mechanism leading to immune escape and persistent infection (91, 408).

Another short membrane peptide produced by cleavage of the polyprotein by host signal peptidases that appears to have ion channel features is the p7 protein (309). This protein is essential for HCV infectivity of chimpanzees (356), as well as for assembly and release of infectious virions (384). Recent data

suggest that p7 could be a viroporin (239). Additionally, mutations in the coding region of p7 led to inhibition of HCV replication and release from Huh-7 cells (384).

The NS proteins are proteolytically processed by the NS2-3 and NS3-4 viral proteases. NS2 is a membrane spanning protein (359). It has been shown that adaptive mutations in the NS2 alone can lead to enhanced production of HCV (352). The NS3-4A serine protease cleaves all downstream sites at NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B (228). NS4A is a small protein (54-aa) that anchors NS3 to cellular membranes through an N-terminal hydrophobic peptide (414). NS4B, an integral membrane protein, associates with the ER membranes and induces the formation of the membranous web necessary for HCV replication (310). Replication enhancing mutations have been identified in NS4B in the JFH-1-based cell culture system which will be described in Section 1.10.5.3 (321). The NS5A protein is a membrane-associated phosphorylated multidomain protein that has been found to play a role in HCV replication (228). In the JFH-1 Huh-7 infection system, adaptive mutations in NS5A confer more robust HCV replication (91). Several cellular proteins are capable of interacting with NS5A. In addition, NS5A has been shown to contain an interferon sensitivity determining region (ISDR) found at aa position 237-276. Mutations in this region can result in higher interferon sensitivity and lower HCV RNA loads in hepatoma cell lines *in vitro* treated with alpha interferon (IFN) (136). To date, several small molecule compounds targeting the HCV NS proteins, including protease, polymerase and NS5A, have been developed and are at various stages of clinical development

(18, 141). The NS5B protein is a RdRp, the enzyme which drives HCV replication. NS5B is post-translationally inserted into the ER membrane, an association that has been found to be required for RdRp activity. Inhibitors of the HCV NS5A protein and NS5B polymerase are potentially active across different HCV genotypes and have shown promising antiviral efficacy in early clinical studies (325, 326), which will be discussed in details in Section 1.10.1.5.

1.5   HCV LIFECYCLE

1.5.1 Virus Attachment

HCV entry and infection of target cells appears to be a highly orchestrated multistep process that is mediated by interaction of several viral and cellular factors. These molecules seem to function by initially concentrating the virus on the surface of the target cells to enable virions to come in contact with other receptors and co-receptors that further facilitate virus cell entry. Most of the HCV candidate receptors were identified mainly by using in vitro systems such as, virus-like particles produced by expression of structural HCV proteins in insect or mammalian cells, liposomes containing HCV E1-E2 proteins, HCV pseudotype particles (HCVpp) as well as the JFH-1-Huh7 system that will be described in details in Section 1.10.1.5.

1.5.2. Postulated HCV Receptors

Several cell surface molecules have been proposed to mediate HCV cell binding and internalization.

1.5.2.1   DC-SIGN and L-SIGN

The dendritic cell-specific intercellular adhesion molecule-3-grabbing noninategrin (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 that have been proposed as capture receptors for HCV. DC-SIGN is expressed by dendritic cells while, L-SIGN is expressed by sinusoidal endothelial cells in the liver and lymph nodes (144, 236, 327). However, these lectins are not expressed on hepatocytes; therefore, they are not considered HCV receptors for hepatocytes but rather may contribute to HCV capture and persistence in the liver or eventually other organs (204). The capture of HCVpp by both DC-SIGN and L-SIGN is believed to be mediated via binding to HCV E2 protein (238). However, neither L-SIGN nor DC-SIGN can be used for HCV entry of hepatocytes (235).

1.5.3.1   LDL Receptor

HCV particles in the blood were found to be associated with lipoproteins that facilitate uptake of lipoprotein-associated HCV into hepatocytes by low-density lipoprotein receptor (LDL-R) (56). The LDL-R is an endocytic receptor that transports lipoproteins, mainly the cholesterol-rich LDLs, into cells through receptor-mediated endocytosis (70). Upregulation of LDL receptors on the surface of Daudi cell lines, a B lymphoblastic cell line, upregulated the expression of HCV RNA positive strands in these cells (10). Also, free LDL was able to block HCV attachment to human fibroblasts (282). In addition, binding of infectious HCV particles recovered from plasma correlated with the density of LDL

receptors at the surface of Molt4 cells and fibroblasts, and binding was inhibited by LDL but not by soluble CD81 (417).

1.5.4.1 *ASGP Receptor*

The asialoglycoprotein receptor (ASGP-R) has been postulated to mediate binding and internalization of structural HCV core, E1, E2 and p7 proteins expressed in a baculovirus system. Transfection of 3T3-L1 mouse fibroblast cell line with the human hepatic ASGP-R major and minor subunits (H1 and H2) allowed binding and entry of these proteins *in vitro* (362). However, contribution of this receptor to HCV entry to hepatocytes remains hypothetical.

1.5.5.1 *GAGs and HSPGs*

Glycosaminoglycans (GAGs) are ubiquitously expressed by mammalian cells. They have been found to be involved in the binding of serum-derived HCV to Vero cells (147). E2, in particular its HVR-1, has been shown to bind heparan sulfate proteoglycans (HSPGs) with a stronger affinity than other envelope glycoproteins, other viruses, such as human herpes virus or dengue virus. However, it is conceivable that HSPG may serve as the initial docking site for HCV attachment and the virus is subsequently transferred to another high-affinity receptor (or receptor complex) triggering the entry of HCV (25).

1.5.6.1 *CD81*

Human CD81 is a 25-kDa molecule belonging to the tetraspanin superfamily. CD81 is the most extensively studied HCV receptor (322). It is expressed at the surface of numerous cell types and, therefore, cannot alone determine hepatotropic or lymphotropic nature of HCV. It has four hydrophobic

transmembrane regions (TM1 to TM4) and two extracellular loops, the small extracellular loop (SEL) and the large extracellular loop (LEL) of 28-aa and 80-aa, respectively. The intracellular and transmembrane domains of CD81 are highly conserved among different species. In contrast, the LEL sequence is variable, except between humans and chimpanzees, the only two species naturally permissive to HCV infection (248, 404). The CD81 LEL has been shown to mediate binding of HCV through its envelope glycoprotein E2 (322) (123). The E2 epitopes involved in CD81 binding remain controversial. Early studies suggested the involvement of aa 480-493 and 544-551 in the truncated soluble form of E2 (123), whereas a more recent study pointed to a role for two other domains, including aa 613-618 and a second domain spanning the two HVRs (aa 384-410 and 476-480) (348). Several studies showed that CD81 is necessary but not sufficient to initiate HCV infection and that factors other than CD81 are required. It is possible that the CD81 molecule could act as a post-attachment entry co-receptor and that other cellular factors may act together with CD81 to mediate HCV cell binding and entry (77).

1.5.7.1   SR-B1

The scavenger receptor B type 1 (SR-B1) is a 509-aa glycoprotein with a large extracellular loop anchored to the plasma membrane at both N- and C-termini by means of transmembrane domains with short cytoplasmic extensions (206). SR-B1 has been proposed as another candidate receptor for HCV (363). It is expressed at high levels in hepatocytes and steroidogenic cells (21, 206). SR-B1 was found to bind high density lipoproteins (HDL) which are internalized

through nonclathrin-dependent endocytosis. This process mediates cholesterol uptake and recycling of HDL apoprotein (377). HCV genotypes 1a and 1b recombinant E2 envelope glycoproteins were shown to bind HepG2 cells (a human hepatoma cell line that does not express CD81) by interacting with an 82-kDa glycosylated SR-B1 molecule (363). Binding of HCV E2 to SR-B1 appeared to be species specific, as transfection of rodent cells with human or tupaia SR-B1 (88% aa identity with human SR-B1) resulted in E2 binding, whereas neither mouse SR-B1 (80% aa identity) nor the closely related human scavenger receptor CD36 (60% aa identity) bound E2. The SR-B1 LEL appeared to be responsible for HCV binding to E2-HVR1 that was recently suggested to be facilitated by serum HDLs (28, 363, 402). However, the fact that antibodies directed against SR-B1 resulted only in a partial blockade of the binding suggests that SR-B1 is not the only cell surface molecule involved in HCV binding to hepatocytes (24). In addition, SR-B1 is expressed by many cell types implying an indirect role in HCV hepatotropism.

1.5.8.1 *Claudin-1*

Claudin-1 (CLDN-1) belongs to a family of highly conserved transmembrane proteins involved in the formation of tight junctions (TJ) (286). Claudins consist of short cytoplasmic amino and carboxy-termini, four membrane spanning domains, and a large (EL1) and a small (EL2) extracellular loop. They are expressed in most tissues, including the liver, and they interact with each other to form intercellular TJ strands (155). In addition, most claudins carry a protein-protein recognition module (PDZ)-binding motif at their C-terminus

through which they directly interact with the TJ-associated zonula occludens (ZO) proteins -1, -2, and -3 (183). Blocking this interaction results in the formation of aberrant TJ strands along the lateral cell membrane (259), suggesting that the interaction with ZO-1 protein and/or additional factors is essential for the correct incorporation of claudins into TJ structure. Furthermore, it has been shown that claudins are crucial for the barrier function of TJ, as mutations in CLDN-16 and CLDN-4 (paracellin-1) impairs the paracellular permeability to calcium, and magnesium and sodium, respectively (177, 382). Accordingly, CLDN-1 knockout mice die from dehydration within one day of birth due to defects in their epidermal barrier function (137).

Although, CD81 and SR-BI appear to be required for HCV entry, they do not confer susceptibility of HCV to non-hepatic cells (27, 428), suggesting that additional factor(s) are required to establish productive HCV infection. The study by Evans and colleagues (2007) demonstrated that non-permissive human embryonic kidney 293 cells (HEK-293T cells) that express CD81 and SR-BI, but not CLDN-1, become susceptible to HCVcc (cell culture produced JFH-1 particles) infection after ectopic expression of this claudin (102). This suggested that CLDN-1 is an essential HCV entry receptor, at least for hepatoma-derived Huh7 and related cell lines. In addition, it was proposed that virus receptor interactions prior to CLDN-1 engagement might trigger conformational changes in the TJ protein that allow HCV binding, similar to HIV, which interacts first with CD4 before binding to the CCR5 receptor (416).

1.5.9.1 Claudin-6 and -9

CLDN-6 and CLDN-9, which are expressed in the liver and PBMC, were proposed as additional co-receptors for HCV (429) as HEK-293T cells expressing CLDN-6 or CLDN-9, but not other claudins, become HCVpp permissive. Interestingly, however, not every CD81+/SR-B1+ non-permissive human cell lines become susceptible to HCV infection, even when ectopically expressing CLDN-1 (102, 323). This suggests that productive viral infection requires at least one or more human-specific viral entry factors.

1.5.10.1 Occludin

Occludin (OCLN) is another TJ protein that was proposed recently to be essential for HCV entry (323). The expression of OCLN allows nonpermissive murine and human cell lines to become susceptible to infection with HCVpp, whereas silencing of OCLN in permissive cells impaired HCV entry (323). On the other hand, a study by Hikosaka et al. (2011) found, contrary to the previous study, that expression of human CD81, CLDN-1, SR-B1, and OCLN receptors in mouse hepatocytes did not confer susceptibility to HCV infection (172).

1.5.2. Entry and Uncoating

Following viral attachment, the HCV nucleocapsid is released in the cytoplasm as a result of a fusion process between viral envelope and cellular membranes. Specialized viral proteins mediate fusion of HCV with the plasma membrane and viral entry into endosomes. This process is guided by viral glycoproteins and is pH-dependant (10). In general, there are two classes of fusion proteins (I and II) (224) and only class II fusion proteins enhance the

process of receptor-mediated endocytosis (228). Studies have shown that HCV envelope glycoproteins belong to class II fusion proteins (419) and they do not require cellular protease cleavage during their journey through the secretory pathway (303). In addition, E2 was shown to share structural homology with class II fusion proteins. The existence of a fusion peptide has been suggested in the ectodomain of E1 of HCV envelope (122, 350, 224, 419).

1.5.3. Replication Strategy

Several research groups have demonstrated that HCV replication is mediated through a replication complex (RC), which has been shown to mediate replication of plus-stranded RNA viruses, such as polio virus (40), mouse hepatitis virus (157), and flaviviruses (409). RC is comprised of viral proteins, replicating RNA and altered cellular membranes. The existence of an HCV RC has been clearly shown in cell culture models of HCV (156). Studies employing the replicon system have demonstrated that in HCV-transfected Huh7 cells, all of the HCV NS proteins, as well as HCV RNA plus-strand, coexist in the context of membranous web (156).

1.5.4. Assembly and Release

Studies on HCV assembly and release were limited until the discovery of the JFH-1 strain of HCV capable of producing infectious viral particles in cell culture. HCV core protein has been shown to self-assemble in yeast in the absence of viral RNA, generating virus-like particles with an average diameter of 35 nm (6, 7). Replication of HCV occurs at specialized sites on the

ER membrane termed, the membranous web (92, 95). Initiation of virion assembly appears to require the release of HCV genomes from such sites to allow contact with core protein, which forms the virion capsid. HCV core protein is located on the cytosolic side of the ER membrane where assembly is believed to start in the cytosol before further maturation. The release is initiated by transfer of nascent particles across the ER membrane to access the cell secretory pathways. Thus, assembly of infectious HCV particles has two interconnected phases: (1) an initiation phase, which takes place at the cytosolic side of the ER membrane and (2) maturation and release phases taking place at the luminal side of the ER membrane. Overall, particle formation might be initiated by the interaction of HCV genomic RNA with core protein which can bind positive-strand RNA through stem-loop domains I and III and nt 23-41 (375, 388). In the presence of nucleic acid, HCV core proteins can efficiently self-assemble to yield nucleocapsid-like particles with a spherical morphology and a diameter of 60-nm (208). Therefore, it is believed that core-RNA interaction plays a role in the switch from replication to packaging. Moreover, 45 to 60-nm virus-like particles were produced in HeLa and HepG2 cells transfected with a full-length of HCV RNA. These particles were found to be synthesized and assembled in the cytoplasm and budded into the ER cisternae to form coated particles (84, 279). Although much has been known about the HCV assembly and release, the mechanisms underlying exportation of mature virions in the pericellular space have yet to be understood.

1.5.5. Virion Ultrastructure and Biophysical Properties

Recent studies have shown that both host and viral factors can affect the buoyant density profile of HCV particles. Host lipoprotein, immunoglobulins and HCV immune complexes, as well as the stage of infection are examples of these factors (171, 334). Electron microscopy studies revealed that HCV virions are spherical, ~40- to ~75-nm in diameter, pleomorphic particles. In addition, previous ultrastructural studies, using patient-derived plasma, reported particles with heterogeneous diameters ranging from 35 to 100-nm (189, 225, 246, 400). In Huh7.5 cells infected with JFH-1, two major HCV particles of ~60 and ~45 nm in diameter were identified. The 60-nm particles are characterized by a membrane bilayer (an envelope) and higher infectivity. Other studies have found that enveloped HCV virions display heterogeneous buoyant densities ranging from 1.04 to 1.11 g/mL in a sucrose density gradient and between 1.18 and 1.25 g/mL in a cesium chloride gradient (145, 171, 190). In contrast, the 45-nm particles lacking a membrane bilayer display a higher buoyant density and a lower infectivity (145). Similarly, studies have shown that the HCV nucleocapsid ranges in size from 25 to 50 nm with a density of 1.24 g/mL in a sucrose gradient and 1.33 g/mL in cesium chloride (190, 246, 400). In general, low density particles have been shown to be associated with lipoproteins, and found to be more infectious than high density particles associated with immunoglobulins (171). Importantly, the ratio between infectious and non-infectious HCV particles *in vivo* has been shown to range from 1:100 to 1:1000 (53).

1.6 HCV MUTANTS AND QUASISPECIES:

HCV is genetically diversified and exists in the host as a population of closely related variants that evolve in large part as a result of selective pressures exerted by host immune responses (252). This may have important implications in diagnosis, pathogenesis, treatment, and vaccine development (54). HCV replicates at very high levels with production of virions estimated at up to 10^{12} copies/day (252). This high rate of replication, combined with lack of an error correction mechanism and ongoing but variable immune selection, results in development of both synonymous and nonsynonymous mutations. In addition, immune selection puts pressure on key antigen-recognition sites and drives the emergence of a closely related virus family that can be identified the serum of infected patients. The dynamics and diversity of HCV viral population have predicted critical insights of HCV short-term outcomes including early spontaneous viral clearance (110), IFN-associated viral clearance, and HCV emergence following liver transplantation (336).

1.7 HCV TISSUE TROPISM

1.7.1 HCV Hepatotropism

Hepatocytes are considered the primary site of HCV infection. HCV hepatotropism has been explained by the higher expression of certain receptors, such as CD81, SR-B1, CLDN-1 and OCLN, as summarized in Section 1.5.2. However, most of these proposed HCV receptors were shown to be expressed by other HCV-nonsusceptible tissues or cells, which make it difficult to explain

how they mediate highly cell-restricted tropism of this virus. Furthermore, expression of all proposed HCV receptors in murine cells did not confer susceptibility to HCV infection (172).

1.7.2 HCV Lymphotropism

Much evidence accumulated in the recent years indicate that HCV can also invade and replicate in immune cells at levels, in some situations, comparable to that of primary human hepatocytes (PHH). In fact, HCV RNA negative-strand has been detected by reverse transcription (RT)-PCR in PBMC of infected individuals (44, 222, 374). Data also indicate that in the absence of detectable HCV RNA in the serum, virus can be detected in the PBMC and their subsets, such as CD4+ and CD8+ T cells, B cells and monocytes (85, 314, 315, 319). Further, *ex vivo* stimulation of PBMC with mitogens significantly augments HCV replication leading to detection of virus genomes in apparently HCV non-reactive cells (314, 315). On the other hand, *de novo* infection of T cells with plasma-derived HCV was associated with release of infectious HCV particles to the culture superanatant (243). HCV genome sequences detected in lymphoid cells have been found to be distinct from the virus in circulation (312, 315, 339, 371) supporting the existence of extrahepatic replication in immune cells. Shimizo and colleagues (1997) found that one out of thirteen patient-derived HCV inocula tested was able to induce infection persisting in human lymphocyte cell lines (371). In certain cases, HCV sequence polymorphisms within the IRES of 5'-UTR were identified which may promote HCV replication in immune cells but not in nonhaematopoetic cells (127, 212, 212). Analysis of HCV compartmentalization

27

in patients with CHC revealed the presence of replicating virus in T and B lymphocytes, as well as in monocytes (93, 314, 158, 315, 317, 319, 373, 437). Furthermore, it was uncovered that HCV not only replicates in lymphoid cells in patients with CHC but is also in individuals with apparent complete therapy-induced or spontaneous resolution of hepatitis C (315, 318, 339). In terms of the HCV RNA loads in intact lymphoid cells, it has been shown that between 100 and 1000 vge/10^7 cells can be detected in approximately 30% of patients with OCI who have apparently resolved hepatitis, while up to 10^4 vge/10^7 cells in patients with CHC were identified (314). Further, comparable levels of HCV RNA were found in immune cells in ~10% of patients with OCI (318). In regard to potential clinical consequences of HCV lymphotropism, replication of HCV in the immune cells may facilitate reinfection of liver grafts post-transplantation (304) and predispose to the development of lymphoproliferative disorders (42). Overall, evidence of active propagation of HCV has been uncovered in the main immune cell subtypes independent of whether infection is symptomatic or clinically silent, as well as in *in vitro* infection experiments with wild-type virus. However, factors mediating susceptibility of immune cells to HCV are yet to be identified. Due to the low virus load per cell and the high cell turnover per day, investigations on HCV lymphotropism remain challenging and require sensitive detection techniques and meticulous approaches.

1.8   NATURAL HISTORY OF HCV INFECTION

1.8.1 Acute Hepatitis C

The majority (60-70%) of infected individuals with acute HCV infection (AH) are asymptomatic. However, some may present with non-specific symptoms, such as jaundice, fatigue and flu-like symptoms (1, 335). The detection of HCV in the serum or liver is the first diagnostic marker of acute HCV infection and can be identified within one to 8 weeks after exposure to virus. Up to 50% of patients with AH show spontaneous resolution (271). Patients who contracted HCV infection at young age tend more likely to spontaneously clear symptoms than those who become infected at an older age (369). The HCV RNA load in patients during AH range from 10^4 to 10^7 genome copies or vge/mL (16, 152), with subsequent liver cells injury leading to high levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (392). Studies have shown that the frequency of spontaneous, apparent complete clearance of viremia in patients with asymptomatic acute infection, is lower than that in those with symptomatic infection (358).

1.8.2 Spontaneous Resolution of Hepatitis C

15-50% of cases with AH can spontaneously resolve hepatitis (271). On the other hand, only 6% of the children have spontaneous resolution of AH (184). Spontaneous recovery was found to be associated with a strong and vigorous immune response, including HCV-specific CD4+ and CD8+ T cell reactivity against multiple viral epitopes (358, 390). In addition, patients infected with genotype 3

have a greater frequency of resolution of hepatitis C than those with genotype 1 (221). Variation in genes involved in the immune response has already been linked to outcome of AH (391). However, most variability in spontaneous HCV clearance remains unexplained.

1.8.3 Chronic Hepatitis

CHC is defined as HCV RNA persistence in plasma for 6 months or longer post-exposure. Up to 85% of HCV-infected patients, depending on HCV genotypes, fail to clear the virus and develop chronic infection with persistent viremia (392). The majority of patients with CHC are asymptotic and 20% may experience nonspecific symptoms, such as fatigue, nausea and anorexia (174). Persistent normal liver enzymes is common in patients with CHC with high anti-HCV antibodies. Evidence of CHC is usually shown by necroinflammation on histological examination of liver biopsy. 10-15% of CHC patients will develop liver cirrhosis in 20-30 years (14, 392).

1.8.4 Factors Affecting Progression of Liver Disease

Several factors may affect liver disease progression in CHC and development of cirrhosis, which appear to be both host and virus related. Many independent host factors have been associated with a faster rate of progression of liver damage, among those are: (1) male sex (possibly due to a protective effect of estrogen in females) (87); (2) alcohol consumption (>50 g/day) (364), and (3) age of acquisition of infection (ages >40 at time of infection show more severe disease). The median time from the acquisition of HCV infection to cirrhosis is estimated to be 30 years. However, men infected after the age of 40

years have been seen to progress to cirrhosis with a median of only 13 years, while women who did not consume alcohol progress to cirrhosis in a median of 42 years (329). Furthermore, other factors, such as duration of infection, immunosuppressive therapy and low CD4+ T cell count due to infection with HIV, HBV infection or schistosomiasis coinfection, non-alcoholic steatohepatitis, and non-responsiveness to antiviral therapy can also progress faster to CHC (392). Iron overload in the liver and mutations of the hemochromatosis gene have also been suggested to accelerate progression to cirrhosis (99). In addition to the host factors mentioned before, virus-related factors, such as action of HCV proteins, can affect the degree of liver disease and fibrosis progression. For example, HCV core protein has been shown to induce oxidative stress, alter mitochondrial function and increase the production of reactive oxygen species, which in turn promote apoptosis (300). Expression of activated caspases, which are mediators of apoptosis, in sera of patients with CHC, was found to be associated with a higher degree of fibrosis (22). Coinfection with HIV has been shown to augment progression to fibrosis in patients with CHC and is accompanied by higher serum levels of HCV RNA (33). On the other hand, certain antiviral therapies, such as protease inhibitors appear to be associated with lower rate of cirrhosis when given to patients coinfected with HIV or HCV (34, 329). So far, to accurately predict the rate of progression of CHC to fibrosis for individual patients based on known virus and host factors is still not possible. The course of CHC in children seems to be milder with a slower rate of progression to fibrosis (49). Therefore, most children are diagnosed incidentally and rarely have symptoms. The

explanation for a milder course of infection and slower progression to fibrosis could be attributed to possible immune tolerance of HCV at birth or early in life (142).

1.8.5 Occult HCV Persistence

The application of highly sensitive nucleic acid amplification assays based on RT-PCR/nucleic acid hybridization (NAH), capable of detecting below 10 vge/mL or 2.5 IU/mL allowed for the detection of very low levels of HCV RNA positive strand in individuals who have resolved AH spontaneously or CHC due to antiviral therapy with IFN-α and ribavirin (RBV) (315). Detection of HCV RNA positive strands in the serum, and both positive and negative HCV RNA strands in PBMC and/or liver of individuals who have been found to be repeatedly HCV RNA non-reactive by standard clinical laboratory tests and displayed repeatedly normal serum ALT levels is defined as occult infection (OCI) (273, 314, 315, 339). Persistence of HCV replication for years after apparent spontaneous or therapeutically-induced resolution of hepatitis C is now well documented (242, 312, 315). In addition, as indicated before (Section 1.7.2) HCV replication has been detected in different immune cell subsets, including T cells, B cells and monocytes (314). HCV RNA positive and negative strands have also been detected in liver biopsy samples taken from patients who had apparently cleared the virus for up to 8 years after achieving sustained virological response (SVR) (312, 339). Due to inadequate follow-up periods, it is not known whether or not occult HCV infection persists for the entire life, like other persistent viral infections, such as HBV (276), Epstein-Barr virus (EBV), herpes simplex virus

(HSV) and cytomegalovirus (CMV) (20). In a recent study, examining 276 individuals with normal liver enzyme levels, and negative anti-HCV antibody and plasma HCV RNA, 3.3% of them were found to carry HCV RNA in intact (mitogen unstimulated) PBMC (85), supporting the notion that sampling of multiple compartments is necessary when testing for OCI. Furthermore, detection of HCV-specific T cell immune responses in the absence of clinical markers of viremia appears to reflect persistence of HCV in this form of HCV infection (337). In addition to OCI progressing after resolution of AH or CHC, a low level HCV replication was detected in patients with ongoing mild liver pathology evidenced by persistently elevated liver enzymes. Thus, HCV RNA was detected in 57 of 100 patients who had no detectable virus by standard clinical assays (62, 63). Those patients were more likely to have liver fibrosis and mild liver inflammation (63).

Studies in this laboratory indicate that sensitivity of the assays used for detection of HCV RNA positive strands should be $\leq$10 vge/mL and testing should include samples obtained from all 3 compartments of virus occurrence, i.e., plasma, PBMC and liver, and preferably serial samples (273). Potential consequences of OCI are related to the risk of virus transmission by infected blood or organs donated by silently infected individuals (315). The presence of residual virus with ability to replicate may also lead to HCV reactivation after apparent successful antiviral treatment. Recurrence of HCV infection after SVR has been reported to be as high as 20% (86). The study of 97 subjects who achieved SVR, which were followed by HCV RNA testing every 6 months over a

33

7-year period, revealed that 11.3% of these patients developed viral recurrence (71). Recipients of liver, kidney (266) or bone marrow transplants (426) experienced HCV recurrence after being negative for HCV RNA by clinical laboratory assays, suggests that immunosuppressive therapy may contribute to reactivation of HCV infection (219).

1.9 EXTRAHEPATIC MANIFESTATIONS OF HCV INFECTION

HCV may trigger a wide spectrum of alterations in the host's immune system, possibly due to variable combinations of infectious, environmental and genetic factors. Extrahepatic manifestations (EHM) of HCV infection can involve the skin, eyes, joints, kidneys and the immune and nervous systems. Some of these conditions, such as cryoglobulinemia type 2 are more common and well-documented, while others are infrequent or their association with HCV infection has not yet been fully proven. Several studies have found that between 70-74% of patients with CHC experience EHM (58). In some cases, EHM could be the only presenting sign of HCV infection. EHM associated with HCV infection has been classified into four groups A-D which were defined according to the accumulated evidence of pathogenic associations between HCV and EHM. Thus, group A is defined as having the strongest evidence of association and includes mixed cryoglobulinemia type 2 and type 3 (MC) and B-cell non-Hodgkin's lymphoma (NHL), while group D includes only anecdotal observation (432). The infected extrahepatic tissues might act as reservoirs of HCV and play a role in both HCV persistence and reactivation of infection (138, 312).

1.9.1 *Mixed Cryoglobulinemia*

In 1990, Pascual and his colleagues were the first to describe an association between HCV and EHM, reporting two patients with MC (306). MC is the most commonly studied syndrome associated with HCV Infection (12, 278, 278, 278, 297). It is a benign B-cell proliferation that, in some cases, can switch to a frank lymphoma with different grades of malignancy (297). It was classified according to the immunoglobulin clonality into 3 types. HCV infection is strongly associated with MC type II and III (11). In some studies, up to 80% of patients with type II MC were found to be infected with HCV (11). The prevalence of MC increases with the CHC duration and only a small fraction of those patients with CHC who developed MC (less than 15%) have symptoms (138). Expansion of CD5+, CD81+ B cells in the liver of HCV-infected patients was observed, which is thought to be related to B cell proliferation and IgM production due to direct interaction between CD81 and HCV E2 glycoproteins (81, 113). Peripheral neuropathy is the most common complication of MC (117, 435) and renal involvement is one of the worst prognostic indices in the natural history of MC (119, 434). Several studies have shown an epidemiological association between MC and liver steatosis and severe liver damage (196, 240, 353). Current data suggest that antiviral therapy should be considered as the first choice treatment in MC and that combined pegIFN-α and RBV therapy is the most effective one (435). Recently, the introduction of Rituximab, a chimeric monoclonal anti-CD20 antibody, has been proven to be useful in improving most clinical manifestations and B-cell lymphoproliferation in the majority of MC patients (357).

1.9.2 B Cell Non-Hodgkin's Lymphoma

MC and the subsequent appearance of NHL represent two different clinical steps of the same disease process. B-cell NHL is defined as an uncontrolled proliferation of B lymphocytes associated with a high incidence of circulating monoclonal B cells (251, 263). HCV RNA in circulating lymphoid cells has been found in the majority of HCV patients with NHL (115, 378). However, a direct pathogenic role of HCV in NHL, if any, is not delineated.

1.9.3 Other Extrahepatic Disorders and Syndromes

Porphyria cutanea tarda and oral lichen planus are among EHM of HCV infection (79). However, these cutaneous disorders are only triggered by HCV infection in genetically predisposed individuals (435). Neurologic disorders, as well as nephropathies, have been associated with HCV infection in the absence of MC (11, 186, 226, 307). As well, HCV-related chronic polyarthritis can be observed in patients with CHC, both with and without MC (119). Intermittent oligoarthritis, generally not erosive and involving the big and middle articulations, was frequently observed in CHC (119). A Sjögren-like syndrome is also frequently observed in HCV-positive patients, mainly in the context of MC (164, 267). A high prevalence of diabetes mellitus type 2 associated with high insulin-resistance, as a part of a complex virus-induced dysmetabolic syndrome, including hepatic steatosis, were observed in patients with chronic HCV infection (17, 201). In addition, an association between aortic atherosclerosis and HCV infection has also been suggested (45, 181). The association between HCV infection and hypertrophic cardiomyopathy and dilated

cardiomyopathy has also been postulated. Additional potential associations include thyroiditis, erectile dysfunction (116) and psychopathological disorders (96, 128, 434).

1.9.4 Immune Cell Abnormalities Coinciding with HCV Infection

1.9.4.1   T Lymphocyte Dysfunctions

Dysfunction of the virus-specific T cells is a common feature in persistent viral infections, including infection with HCV. Resolution of hepatitis C requires a robust CD4+ and CD8+ T cell responses directed against multiple epitopes of HCV. It was observed that CD8+ T cells from patients with CHC overexpress various inhibitory receptors, such as programmed cell death 1 (PD-1) (90). The role of other inhibitory receptors, such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and T cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3), have also been demonstrated in T-cell dysfunctions occurring in the course of CHC (90, 344, 345). In addition, deficient IFN-gamma (IFN-γ) and IL-2 production by T cells (367) and their impaired proliferation in response to stimulation with HCV antigens have been identified (153). On the other hand, exposure of T cells to HCV *in vitro* resulted in impaired IFN-γ signalling, and suppression of STAT-1 and *T bet* mRNA levels after stimulation with IFN-γ compared to uninfected T cells (203). The higher expression of inhibitory receptors on HCV-specific T cells may account for the functional deterioration of T cells resulting in the inhibition of T cell proliferation and cytokine production (43). It has been demonstrated that PD-1 expression on total T cells and CD8+ T cells in the peripheral blood and in the livers of patients with CHC is significantly

higher than that in those who spontaneously resolve HCV (153). Furthermore, blocking the PD-l/PD ligand (PDL-1) interaction with antibodies to PD-L1 and/or PD-L2 led to recovery of proliferation, IFN-γ secretion and IL-2 production in previously dysfunctional, HCV-specific CTLs (153, 295). In addition, TIM-3 interaction with its ligand galectin-9 has been implicated as another possible mechanism of T cell exhaustion in CHC. Thus, binding of TIM-3 to galectin-9 results in negative regulation of T-helper type 1 (TH1) responses and the induction of peripheral tolerance (354, 431). TIM-3 expression has been shown to be upregulated on CD4+ and CD8+ T cells in CHC patients (153). In addition, the greatest proportion of PD-l/TIM-3-dual expressing T cells was found in the liver of CHC patients and blocking of TIM-3 and TIM-3 ligand interaction rescued proliferation of T cells and IFN-γ production (153). As indicated before (Section 1.7.2), HCV active replication, although at low levels, was found in both CD4+ and CD8+ T cells in CHC and persistent OCI. However, a potential direct effect of HCV infection of the phenotype and function of the effector cells remains unrecognized.

1.9.4.2   B Cell Dysfunction

In patients with CHC, B cells exhibit a nonspecific polyclonal activation with higher levels of the early activation marker, CD69, the costimulatory molecule, CD86, and the CCR5 chemokine receptor, CD195, when compared with B cells from healthy donors in response to stimulation with phytohaemagglutinin (PHA) (197, 338). Moreover, the B-lymphocyte stimulator (BLYS), known as tumour necrosis factor and Apo-L-related leucocyte-expressed

ligand-1 (TALL-1), was found to be up-regulated on the surface of B cells from HCV patients in response to PHA or HCV core antigen stimulation (105). This up-regulation of TALL-1 was associated with vigorous memory B-cell responses to viral antigenic stimulation. Additionally, suppressor of cytokine signalling-1 (SOCS-1), a negative feedback immunomodulator was inhibited in B cells from HCV-infected patients when compared to healthy individuals (284).

1.9.4.3   HCV-Related Dysfunctions of NK Cells

NK cells are a key player in early immune response against pathogens and in the activation and maintenance of adaptive immune responses, with the ability to rapidly produce large amounts of cytokines, as well as, cellular cytotoxicity. NK cells promote the differentiation of adaptive T cells and their proliferation (438). Similarly, NK cells play an important role in the defence against viral infection (41) and surveillance of tumour micro environment (424). Morishima et al (2006) reported a decrease in the frequency of NK cells in both blood and livers of HCV-infected patients with liver cirrhosis (285). However, Corado et al. reported that while there were no differences in the frequency of CD3- CD56+ cells, persons with chronic HCV infection had a 4-fold lower level of spontaneous cytotoxicity than normal healthy individuals (76). In vitro, ligation of CD81 by HCV E2 protein has been shown to directly block NK cell function, as evidenced by inhibited IFN-γ and TNF-α production, as well as diminished cytotoxic granule release and decreased CD25 expression by NK cells (80, 401).

1.9.4.4 *Monocytes Dysfunctions*

HCV core protein interferes with toll like receptor (TLR2)-mediated monocyte activation and induces tumour necrosis factor-alpha (TNF-α) and IL-10 cytokines that lead to apoptosis of plasmacytoid DC and inhibition of IFN-α production (88). In addition, studies of patients with apparent spontaneous resolution of HCV infection identified low IL-10 and TNF-a production by monocytes in response to stimulation with HCV core protein (253).

1.10 HCV EXPERIMENTAL SYSTEM

1.10.1 Animal Models

The chimpanzee is the only reliable animal model for HCV (109). The lack of robust small animal models naturally susceptible to HCV infection has impaired the ability to study almost all aspects of HCV pathogenicity and HCV responses to preventive and therapeutic strategies. Recently, several small animal models for HCV infection have been evaluated, including the albumin (Alb)-urokinase plasminogen activator (uPA)-severe combined immunodeficiency disorder (SCID) mouse (199, 200); HCV trimera mouse (98, 139); the Tupaia belangeri; NOD/SCID mouse model, and transgenic mice expressing HCV proteins (244, 256, 397).

1.10.1.1 *The Chimpanzee Model*

The chimpanzee has been known to be susceptible to HCV (or the non-A, non-B hepatitis agent) and has long been used as a sole animal model for HCV infection (46). However, due to ethical reasons and vast costs, the use of this

animal for HCV research is strictly limited. Chimpanzees have been used to investigate hepatitis C immunopathogenesis, natural history and potential antiviral preventive and therapeutic strategies, including: (1) the role of HCV-specific CD4+ and CD8+ T cell responses in viral clearance (159, 376); (2) the infectivity of lymphocyte-derived virus (372); (3) the efficacy of vaccination and anti-HCV antibodies (100, 333, 349), and (4) anti-HCV efficacy of selected antivirals. It has been demonstrated that chimpanzees can produce HCV virions at high levels, between 10^5 to 10^7 vge/mL (53). Immunization of chimpanzees with virus-like particles induced an HCV-specific CD4+ or CD8+ T cells, suppressing the development of high viral loads in chimpanzees rechallenged with HCV (97). Chimpanzees inoculated with recombinant adenovirus vector encoding HCV NS proteins also induced HCV-specific immune T-cell response that subsequently inhibited the replication of HCV genotype 1a rechallenged chimpanzees (126). The development of CHC in chimpanzees ranges between 39% to 60% depending on study (29, 5, 126, 247), while in humans it is estimated to range between 45% and 85% depending on virus genotype (174). In addition, hepatitis is significantly milder in chimpanzees and liver fibrosis and cirrhosis have never been observed, while the development of HCC has only been reported in one animal (289). Studies using IFN-α, with or without RBV, as well as adenovirus-based gene therapy to upregulate expression of IFN-α in the liver, failed to decrease HCV load despite the fact that high levels of IFN-α were found to be circulating in the treated animals (210). On the other hand, silencing of miroRNA-122 in chronically infected chimpanzees lead to long lasting

suppression of HCV viremia with no evidence of viral resistance or side effects
(211).

1.10.1.2   *The Tupaia Model*

The tree shrew or tupaia (*Tupaia belangeri*) is a small, squirrel-like
mammalian that is related to primates (60). In a study by Tong *et al.* 2011, it was
demonstrated that tupaia CD81, SR-BI, CLDN-1, and OCLN can mediate entry of
HCVpp and HCVcc, with the latter resulting in the full cycle of virus replication
and appearance of infectious progeny virus. The tupaia and human OCLN aa
sequences shared 88% homology (397). The key functional region interacting
with HCV during virus entry was found to be located in the human OCLN large
extracellular loop (EL2) (323). Tupaia and human OCLN EL2 are 91%
homologous, with 4 of 45 residues in this region being different. Mouse and
human OCLN EL2 are 86% homologous, with six residues being different (397).

1.10.1.3   *Mouse Models*

1.10.1.3.1   *The Alb/uFA-SCID Mouse Model*

The Alb/uPA SCID-beige mouse containing a human-mouse chimeric liver
(200, 268) is currently the most reliable small animal model capable of supporting
HCV infection. In this model, among others, the genetic basis of the host
response to HCV infection and gene expression profiles from HCV-infected mice
and those from HCV-infected patients were investigated (244, 405). The results
demonstrated that host factors, such as the initial interferon response, the
differences in the level of activation of interferon stimulatory genes (ISGs) and the
duration of infection influence the host response to HCV infection. They also

42

showed that HCV mediates changes in the host gene expression, including genes regulating lipid metabolism (405). However, the model has a number of limitations, among which is the lack of pathogenic antiviral immune responses. In addition, mice infection with HCV are only induced by higher doses of HCV, i.e., greater than or equal to 10^5 copies per mouse (268). Consequently, infection with lower doses of HCV, characterizing OCI, cannot be investigated. Finally, this animal model is labour intensive and requires high quality PHH, which is hardly available even from specialized liver transplantation centers.

1.10.1.3.2   The Trimera Mouse Model

The HCV-trimera mouse model was developed by using lethally irradiated mice reconstituted with bone marrow cells from SCID mouse, in which human liver fragments infected *ex vivo* with HCV had been grafted. HCV viremia determined by detection of HCV RNA positive strand, peaked 18 days after transplantation of a liver fragment with 85% infection rate. Viral replication in liver grafts was evidenced by identification of HCV RNA negative (replicative) strand (179). The trimera mouse model has been employed to test the efficacy of neutralizing antibodies in inhibiting HCV infection in human liver grafts (98, 139) and evolution of antiviral agents (179).

1.10.1.3.3   The Nod/SCID Mouse Model

An alternative to the trimera model, are non-obese diabetic (NOD)/SCID mice engrafted with human liver tissue. Although very good engraftment (approximately 90%) was observed in these mice, inoculation of these animals with either HCV-positive human serum or culture media containing an infectious

43

HCVcc resulted in HCV very low titres being at the limit of detection by the PCR-based assay. HCV RNA sequences were detected in the engrafted liver tissue by *in situ* PCR. Overall, there is no robust animal model to study HCV replication or to be applied for evaluation of anti-HCV therapies (245).

1.10.1.3.4   Transgenic Mice

A number of transgenic mouse models have been developed to examine the potential pathogenic effects of the HCV core protein and/or the envelope glycoproteins on hepatocytes either alone (195, 249) or in combination with each other (257, 308). Thus, the model was mainly used to investigate the role of HCV proteins in the immunopathogenesis of liver injury, the modulation of apoptotic cell death, the development of HCC and the induction of steatosis. However, conflicting results have been reported and no conclusive evidence regarding a contribution of HCV structural proteins into the pathogenicity of liver injury was demonstrated (26).

All aforementioned models that were described depend on detection and quantification of HCV RNA by RT-PCR. The main advantage of these models is that HCV infection and replication occurs in human hepatocytes and represent a potentially less expensive *in vivo* model for studying HCV relative to the chimpanzee. Compared to the tissue culture replicon systems, adaptive mutations in the HCV genome do not seem to be required for replication in the xenograft models. However, due to the technical difficulty in creating and maintaining the mice, the limited availability of human hepatocytes, variable

human cell engraftment and inconsistent viral titters, none of these models has yet to gain widespread popularity (26).

1.10.2 Cell Culture Models

1.10.2.1   HCV Pseudoparticles

HCVpp were designed to study the early stages of viral life cycle. HCVpp are produced by transfecting two or three vectors in the HEK-293 cells. First vector encodes retroviral Gag and Pol proteins, which are responsible for particle budding at the plasma membrane and RNA encapsidation. Second vector encodes a reporter protein (Luciferase) or green fluorescent protein (GFP). Third vector encodes HCV glycoproteins E1 and E2, which were found to mediate tropism and fusion of HCVpp with target cell membrane. HEK-293 cells secreted HCVpp at an average of 10^5 particles/ml, which can be used to infect hepatoma-derived Huh7 cells. The infectivity can be evaluated by quantification of amount of luciferase or GFP expressed in HCVpp-positive Huh7 cells. These virus like particles can be neutralized with monoclonal antibody (mAb) against HCV glycoprotein E1 or E2 and sera from HCV-infected patient carrying anti-HCV and they offer a tool to identify inhibitors which block HCV entry (59, 178). In addition, HCVpp have been employed to investigate putative HCV co-receptors, such as LDL-R (28), CD81 (77), SR-B1 (402), CLDN-1 (102) and OCLN (323), as well as, a tool to indentify factors which may enhance HCV infectivity of target cells, such as apolipoprotein CI (270).

1.10.2.2 HCV Replicon Systems

The replicon system, which was based on the transfection of selected viral RNA clones that replicates autonomously in the target cells, was first described in 1999 (234). The replicon system has evolved to an efficient system allowing for forward and reverse genetic screens. The replicon has also served as a valuable tool for screening small antiviral molecules. The system has been established for several viruses, including HCV, bovine diarrhoea virus, Lassa virus, and Sindbis virus (30, 135, 166). The majority of HCV replicon research has been conducted on adapted subclones of human hepatoma-derived cell line, Huh7. Although this line has been incredibly useful in characterizing the different aspects of HCV biology, the cells are clearly different from the authentic PHH. In general, the HCV replicon systems have enriched our understanding of the HCV life cycle and allowed recognition of roles of individual HCV viral proteins in this cycle, as well as provided a tool for development of antiviral therapies potentially targeting HCV (209).

1.10.2.3 HCVcc System

JFH-1, a genotype 2a strain of HCV, was isolated from a 32 year old Japanese male with fulminant hepatitis. It replicates only efficiently in the human hepatoma Huh7 cells (230, 403, 430). Using Huh7 cells as host, JFH-1 clone is able to support virus assembly and secretion of infectious particles into the culture supernatant, enabling the study of the full HCV life cycle, which is considered a major improvement of this system in comparison to the HCV replicon system. However, Huh-7.5 cells are deficient in retinoic acid inducible

gene I (RIG-I) and TLR3. They are also characterized by abnormal proliferation, deregulated gene expression, dysfunctional mitochondria, and aberrant signalling and endocytosis pathways. These features are likely responsible for the cells high susceptibility to JFH-1 HCV (52, 188, 207). The JFH-1 particles, assembled in Huh7 cells, display biophysical properties compatible with complete HCV virions of 50-65-nm in diameter and a buoyant density of 1.01 to 1.17 g/mL with the peak of infectivity corresponding to a buoyant density between 1.09-1.11 g/mL (230, 403, 430).

In regards to attempts to infect PHH with JFH-1, Ploss *et al.* (2010) was able to express JFH-1 virus in microscale PHH using micropatterned cocultures (MPCCs), which sustained very low levels of viral presence for several weeks, as has been shown by employing a highly sensitive fluorescence- and luminescence-based reporter systems. However, neither HCV RNA positive and negative strands, nor HCV proteins were detectable by RT-PCR and Western blotting or immunofluoresence, respectively (324). On the other hand, JFH-1 particles do not seem to infect or replicate in lymphoid cells, but may support HCV JFH-1 translation and polyprotein processing (292). Although this strain replicates efficiently and produces infectious virus in Huh7 cell cultures, its replication capacity and pathogenicity *in vivo*, when injected into HCV-naive chimpanzees, were very low, as indicated by the luciferase activity measured in subgenomic replicons generated from JFH-1 strains isolated from infected chimpanzees (355). In addition, low-level of viremia without evidence of hepatitis, and no seroconversion were detected in chimpanzees (193). Isolates other than

47

HCV JFH-1 genotypes that efficiently replicate in cell culture have not yet been identified. However, infectious strains of full-length JFH-1 have been developed that are intergenotypic and intragenotypic chimeras of JFH-1 nonstructural proteins with structural proteins from other 2a strains and other HCV genotypes, including 1a, 1b and 3a (320). These HCV chimeras replicate at different efficiencies in Huh-7 cells and some require adaptive mutations for infectivity (423). In conclusion, high replication efficiency of JFH-1 strain in hepatoma Huh7 cells created opportunities for investigation of whole HCV life cycle. However, the system has a number of important limitations which may not properly reflect wild-type HCV replication in naturally occurring cell targets and need to be verified in future studies.

1.10.2.4   *In Vitro Infection of Primary Hepatocytes*

In vitro infection of PHH with serum-derived HCV or HCVcc derived from JFH-1 has been attempted (111). Nonetheless, establishing and maintaining cultures of PHH that sustain HCVcc replication has proven difficult (111). PHH appear to support HCV replication at very low levels which has been confirmed by detection of HCV RNA negative (replicative) strand (61, 131, 280, 281, 403). The rate of JFH-1/HCVcc replication in PHH appeared to be one order of magnitude greater than that observed after infection of the cells with serum-derived wild-type HCV, whereas it has one order of magnitude smaller than that observed for Huh7.5 cells (281). Anti-CD81 and soluble CD81 (sCD81) are both capable of inhibiting HCVcc infection of PHH. In contrast, serum-derived HCV infection of PHH could not be inhibited by sCD81, although the virus remained

48

sensitive to the neutralizing effects of anti-CD81 antibodies (37, 124, 425). In addition, exogenous HDL promotes HCVcc and HCVpp infection or interaction with PHH via binding to SR-B1(27, 89, 270, 402, 425), but has a minimal or no effect(s) on serum-derived HCV infection of PHH (280). The aforementioned observations suggest that wild-type HCV may significantly differ from HCVcc of JFH-1 (111).

1.11 THERAPY OF HCV INFECTION

1.11.1 Standard of care and Treatment Strategies

No vaccine against HCV is currently available and prophylaxis with immune globulin is not effective in preventing HCV infection after exposure. (415). In regard to treatment of CHC, it has early been established that IFN-α could be employed (176). Different IFN-α preparations with or without RBV are now used as the standard of care for patients with CHC (250, 261, 330). Standard regimen includes subcutaneously administered pegylated IFN-α 2a (180 µg/week) or pegylated IFN-α 2b (1.5 µg/kg/week) together with oral RBV at doses of 0.8 to 1.2 g/day, depending on body weight (168, 169) for 48 weeks in infections with genotype 1 and 4, and 24 weeks for other genotypes (101). The current treatment efficacy is approximately 80% in patients infected with genotypes 2 and 3, and approximately 45% in patients with HCV genotype 1 infection (168, 250). The strongest predictors of SVR are: (1) the recently identified genetic polymorphisms located in chromosome 19, close to the region coding for IL28B (or IFN-λ3) (146); (2) the HCV genotype, and (3) the stage of

fibrosis. Other predictors of response to IFN/RBV therapy include: baseline HCV RNA levels, the dose and duration of IFN therapy, body mass index, age, insulin resistance, gender, and the characteristics of liver disease (including levels of ALT, AST and GGT) and/or co-infection with another hepatotropic virus or with HIV (101, 250). Currently, patients infected with HCV genotype 1 who failed to diminish HCV viral load to an undetectable serum level after therapy with pegylated IFN/RBV can be re-treated with direct acting antivirals (e.g. HCV protease inhibitors) in combination with pegylated IFN/RBV, as will be detailed later. The first generation protease inhibitors, such as telaprevir (TLPV) and boceprevir (BOC) are not recommended for treatment of CHC caused by genotypes other than genotype 1 (101). Therefore, identification of the HCV genotype prior to initiation of therapy is a key element in determining the course of antiviral treatment. Measurement of the rapid virological response (RVR), undetectable serum HCV RNA, by standard clinical assay, at week 4, and the early virological response (EVR), a 2-log 10 viral reduction at week 12, allow for early prediction of antiviral treatment efficacy and decisions of whether to stop or continue the treatment (427). Data have shown that the course of IFN/RBV therapy for patients who achieve an RVR can be shortened to between 12 and 16 weeks for genotypes 2 and 3 and to 24 weeks for genotype 1, respectively (167). Patient infected with HCV genotypes 1, 4, 5 or 6 who did not experience 2 log or more drop in the viral load by 12 weeks of therapy has a 0-3% chance of achieving SVR after 48 weeks of treatment. However, patients who respond

poorly to IFN/RBV therapy in the first 4 weeks of administration, a prolonged, 72-week course of treatment has been shown to increase SVR rates (167).

1.11.2 Recent and Future Therapies

A large number of direct-acting antiviral drugs against HCV are at different stages of preclinical and clinical development (370). The aim of the new therapeutic strategies is to increase the efficacy, decrease the duration of treatment, improve tolerability and patient adherence, and facilitate easier administration. The European Medicines Agency (EMA) and Food and Drug Administration (FDA) approval have recently been reported for two NS3/4 protease inhibitors, TLPV and BOC, in combination with pegylated IFN/RBV in both naïve and non-responder patients infected with HCV genotype 1 (3, 148). Limited phase 2 testing has shown that TLPV also has activity against HCV genotype 2 infection, but not against genotype 3 (129, 130), while BOC has activity against genotype 2 and 3 (148). However, currently, neither drug should be used to treat patients with genotype 2 or 3 HCV infections. When either drug is administered as monotherapy, without IFN/RBV, resistant variants are selected leading to treatment failure (129). Because protease inhibitors have been developed using HCV genotype 1-based enzymatic assays, they have not been optimized against other genotypes. In a study done on intergenotype chimeras of JFH-1 clone, broader efficacy range of TLPV between genotypes was shown with genotypes 1 and 2 being most susceptible, and genotypes 4 and 5 most resistant (180). Other DAA drugs are at earlier stages of clinical development, including additional protease inhibitors, nucleoside/nucleotide analogues and non-

nucleoside inhibitors of the HCV RNA-dependent RNA polymerase, NS5A inhibitors, and cyclophilin inhibitors. IFN-sparing regimens, with or without RBV, are also currently being tested (101).

1.11.3 Liver Transplantation and Recurrence

HCV-related end-stage liver diseases, such as cirrhosis and HCC, are the most common indication for liver transplantation in western countries (332). However, universal reinfection of liver allografts with HCV (143) is a major problem (35, 143). Thus, *de novo* liver injury can be demonstrated by histology as early as 9 days post-transplant, but most typically develop after 3 months with heterogeneous clinical presentation, severity and outcome (140). This injury might be attributed to the host-mediated immune response that is enhanced by the increased viral load. In addition, donor age is an independent factor that determines the outcome of liver transplantation where older age of the donor is associated with greater disease severity and faster disease progression as well as poorer graft and patient survival (36, 411, 351). Other factors that modulate the outcome of liver transplantation include differences in the virus virulence, genetic differences between recipients and the type and amount of immunosupression or alcohol consumption following liver transplantation (35, 351). In fact, persistence of HCV in immune cells may act as an extrahepatic reservoir that is believed to be implicated in the reinfection of allografts and the degree of liver damage (8, 433), as well as the recurrence of CHC after liver transplantation.

CHAPTER TWO:

PURPOSE OF THE STUDY AND GENERAL APPROACH

Molecular and clinical evidence accumulated in the last decade indicates that HCV is not only hepatotropic but also lymphotropic virus. Several well designed and executed studies convincingly documented that HCV can infect different cell subsets of the host's immune system, including T lymphocytes. In this regard, both T cells in HCV-infected patients with CHC and asymptomatic persistent infection, as well as cultured primary T cells from healthy donors infected *ex vivo* were found to support active replication of biologically competent HCV. Further, the current understanding of the nature of the HCV ability to infect human hepatocytes and the mechanism of virus entry has been almost entirely based on the results from studies investigating interactions between a highly unique HCV JFH-1 strain, related replicon-based viruses or HCV pseduoparticles and a specific clone of human hepatoma Huh7 cells displaying an immuodeficient phenotype. These studies generated a plethora of notable data, however they failed, so far, to identify molecule/s determining HCV hepatocyte-specific tropism or to confirm that the receptors uncovered by using the JFH-1-Huh7 cell model in fact contribute to infection of normal human hepatocytes by naturally occurring HCV *in vivo*. On the other hand, molecules determining susceptibility of T lymphocytes to infection with wild-type HCV have not yet been investigated and, therefore, remain unknown. In this context, the objectives of our studies were:

1. To establish a reproducible culture system capable of supporting HCV replication by applying readily available human T cell lines and wild-type, naturally occurring HCV. Susceptibility of T cell lines to HCV infection and their ability to support HCV replication will be determined by molecular and immunological approaches, including identification of HCV expression, virus genome replicative intermediates and *de novo* synthesized of viral proteins. For comparison, equivalent studies will be carried out in primary T lymphocytes isolated from healthy donors exposed *ex vivo* to the same test HCV inocula.

2. To address the issue of why only some, but not other, human T cell lines can support HCV replication, the cell lines examined in this study will be evaluated for expression of CD3, CD4, CD8 and CD5 to confirm their T cell phenotype and they will be assessed for transcriptional activity of the genes encoding factors known to determine cell susceptibility or its lack to HCV infection or viral infections in general, such as tetraspanin CD81, IFN-α, IFN-γ and the interferon regulatory factors 3 and 7. Moreover, a role of CD81 in determining receptiveness to HCV infection will be examined in T cell lines found to be susceptible to HCV in the course of this study.

3. Following our finding made in the course of the preparation of this thesis dissertation that only T cell lines expressing CD5 appear to be prone to infection with naturally occurring, wild-type HCV, the aim will be to investigate a role of this molecule in mediating susceptibility of T cell lines and primary T lymphocytes to HCV invasion. Among others, the study will include examination of the relation between the level of CD5 display on T cells and their receptiveness

54

to HCV, the effect of CD5 upregulation on the susceptibility to HCV of the cells which display CD5 protein only after activation, and the effects of blocking of CD5 receptors with CD5-specific mAb and by knocking down CD5 expression in HCV-susceptible T cells using sequence-specific shRNA. To further confirm CD5 role in HCV infection, CD5-negative HEK-293 fibroblasts will be transfected with human CD5 cDNA and examined for evidence of HCV replication after exposure to naturally occurring HCV. In a supplementary study, expression of CD5 mRNA and protein will be examined in PHH and hepatoma-derived Huh7, Huh7.5 and HepG2 cells to recognize whether this molecule may also contribute to HCV towards hepatocytes.

4. To assess whether or not CD5 is functioning on T lymphocytes as a HCV receptor in conjunction with other HCV candidate receptors identified using the JFH-1-Huh7.5 cell system. For this purpose, T cell lines found to be susceptible or not to infection with wild-type HCV, total human PBMC and primary human T lymphocytes will be analyzed for expression of SR-B1, CLDN -1, CLDN-4, CLDN -6, and OCLN and the identified levels of both mRNA and protein expression will be compared to those in Huh7.5, HepG2 and control HEK-293 cells. Following our finding that OCLN, in addition to CD5 and CD81, may contribute to T cell receptiveness to HCV, infection experiments with T cell lines with knocked down expression of OCLN will be carried out. In a parallel study, the level of expression of HCV candidate receptors, such as CD5, CD81, OCLN and SR-B1, will be examined before and after exposure to HCV. The purpose of this study will be to assess whether exposure to HCV downregulates expression

of the molecules mentioned above, which may provide indirect evidence for their involvement in HCV entry to T cells.

5. To investigate whether the HCV JFH1$_T$ clone, that is a derivative of JFH-1 clone and carries adaptive mutations, and shows approximately 1000-fold greater infectivity towards Huh7.5 cells than the classical HCV JFH-1 strain can replicate in T cell lines found to be prone to wild-type HCV. This may eventually establish more simplified *in vitro* system of HCV propagation in T lymphocytes. In parallel, the susceptibility of hepatoma Huh7.5 cells to infection with plasma-derived wild-type HCV will be assessed.

The establishment of reproducible *in vitro* infection system utilizing readily available T cell lines and molecularly unmodified, naturally occurring HCV of different genotypes will be of significant importance not only in regard to the identification of factors determining host's susceptibility to HCV infection, particularly those mediating HCV lymphotropism, but also for evaluation of the effectiveness of the current and future antiviral agents against HCV propagating at this extrahaptocellular compartment. The system can be also applied in studies determining functional consequences of HCV replication in T cells and for recognizing molecular mechanisms underlying HCV-induced T cell functional hindrances.

CHAPTER THREE:

HEPATITIS C VIRUS INFECTION OF HUMAN T LYMPHOCYTES IS MEDIATED BY CD5

3.1   Introduction

HCV is one of the main causes of chronic liver disease. Although infection of hepatocytes is mostly responsible for manifestations of hepatitis C, the virus also invades the immune system by as yet an unidentified mechanism. Using human T cell lines and primary T lymphocytes as targets and patient-derived HCV, we aimed to identify how HCV gains entry to these cells. HCV replication was determined by detection of replicative (negative) HCV RNA strand and viral proteins, while specific antibody, knocking down gene expression and making otherwise resistant cells prone to HCV were employed to identify a receptor molecule determining permissiveness to HCV infection. The results revealed that only primary T cells and T cell lines which express CD5 protein are prone to HCV. This implied that T cell susceptibility to HCV requires CD5, a lymphocyte-specific glycoprotein belonging to the scavenger receptor cysteine-rich family, despite that the cells express CD81, the postulated customary HCV co-receptor. Blocking of T cell CD5 with antibody or silencing with specific shRNA decreased, while increasing CD5 expression by mitogen stimulation augmented susceptibility to HCV. Moreover, transfection of naturally CD5-nonreactive HEK-293 fibroblasts with CD5 facilitated infection of these otherwise HCV-resistant cells. In contrast to T cells, hepatocytes do not express CD5. The data revealed that CD5 is a

molecule essential for HCV entry to human T lymphocytes. This finding provides a direct insight into the mechanism of HCV lymphotropism and defines a target for potential interventions against HCV propagating at this extrahepatic compartment.

HCV infects over 170 million people globally and causes chronic hepatitis in up to 80% of patients - a condition that can progress to cirrhosis, liver cancer and is the leading reason for liver transplantation. Although HCV is conventionally known to infect hepatocytes, a significant body of molecular and clinical evidence indicates that HCV also invades and replicates in cells of the immune system (42, 86, 94, 151, 304, 436). These cells may in turn serve as a reservoir in which biologically competent virus persists.

HCV ability to infect human cells is currently interpreted in the context of the interactions identified between HCV JFH-1 strain or HCVpp and human hepatocarcinoma cell lines. Based on these data, tetraspanin CD81 (28), glycosaminoglycans (147), SR-B1 (28, 191), the tight junction protein CLDN-1 (102) and OCLN (32, 232, 323) have been proposed to being involved in HCV entry to human hepatocytes. On the other hand, factors determining HCV lymphotropism remain entirely unknown. Analysis of HCV compartmentalization in infected patients demonstrated virus replication in both T and B lymphocyte subsets (93, 214, 296, 312, 314). The susceptibility of normal human T lymphocytes *in vitro* to infection with patient-derived HCV and their ability to support the entire cycle of HCV replication have been shown (242, 243). HCV propensity to infect the immune system is consistent with a significantly greater

prevalence of lymphoproliferative disorders, such as non-Hodgkin's lymphoma and mixed cryoglobulinemia, and perhaps mucosa-associated lymphoid tissue lymphoma in patients infected with HCV (69, 118, 151, 436). It also is possible that HCV residing in immune cells, as in other persistent viral infections (72, 161, 272, 301), is an important contributor to long-term virus persistence and that the infected immune cells are reservoirs from which infection can spread, for example, in patients grafted with new livers due to HCV-related end-stage disease or in recipients of seemingly HCV-negative donor organs (260, 266, 394).

3.2   Materials and Methods

3.2.1. Cells

PBMC were isolated from two healthy donors who have no history and molecular evidence of HCV exposure, as confirmed by HCV RNA analysis of sera by RT-PCR/NAH assay with sensitivity <10 virus genome equivalents (vge) per ml and the absence of anti-HCV antibody by enzyme immunoassay (Abbott Molecular, Mississauga, Ontario, Canada) (242, 315). Primary T lymphocytes were affinity purified from monocyte-depleted PBMC by negative selection using MACS magnetic microbeads (Miltenyi Biotec, Auburn, CA), as reported (243, 319). T cells were 97-98% pure by flow cytometry. In some experiments, PBMC and primary T cells were stimulated with 5 µg/ml phytohemagglutinin (PHA; Sigma-Aldrich, Oakville, Ontario, Canada) for 72 h in the presence of 20 IU/ml human recombinant interleukin-2 (rIL-2; Roche Molecular Diagnostics,

Pleasanton, CA), as reported (242, 315). Molt4 (CRL-1582) and Jurkat (TIB-152) cells were acquired from the American Type Culture Collection (ATCC, Manassas, VA). PM1 cells were supplied by the National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, MD) and CCRF-CEM cells (CEM, ACC-240) by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Molt4, Jurkat and CEM cell lines were originally derived from patients with acute T lymphoblastic leukemia (125), while PM1 from a patient with acute cutaneous T cell lymphoma (241). The cells were cultured at 1 x 10^5 cells/well in 5 ml of culture medium containing RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine and 0.1 mM nonessential amino acids, all from Invitrogen Life Technologies (Burlington, Ontario, Canada). In some experiments, the cells were stimulated for 72 h with phorbol myristate acetate (PMA; Sigma-Aldrich) at 50 ng/ml in the presence of 500 ng/ml ionomycin (Sigma-Aldrich). In preliminary experiments, this treatment was found to be noncytopathic and augmenting efficiently CD5 expression on the T cell lines investigated (see Table 3.1). Cells not exposed to PMA/ionomycin, but cultured under the same conditions, served as controls.

Human hepatoma Huh7 cells, naive or carrying HCV AB12-A2 replicon, were kindly provided by Drs. Joyce Wilson and Christopher Richardson formerly from the Ontario Cancer Institute (Toronto, Ontario, Canada), while Huh7.5 cells infected with HCV JFH-AM2, a recombinant strain of HCV JFH-1 carrying a synonymous mutation at nucleotide 1681 of E2 and one nonsynonymous

Table 3.1. CD5 and CD81 protein expression following T cells stimulation

Cell Type	CD5		CD81	
	Untreated	Treated [b]	Untreated	Treated [b]
	% Positivity *(MFI)* [a]			
Molt4	99.8 *(84)*	100 *(104)*	99.8 *(106)*	96.4 *(54)*
Jurkat	97 *(77)*	100 *(103)*	98.8 *(171)*	66.3 *(38)*
PM1	10 *(18.8)*	53.6 *(30.1)*	95.4 *(107)*	80.0 *(57)*
CEM	6.3 *(6.4)*	20.6 *(14.5)*	97.7 *(166)*	55.7 *(79)*
PBMC	68 *(45)*	86 *(70)*	98.9 *(260)*	NT [c]
Primary T cells	86 *(56)*	97 *(75)*	99.8 *(213)*	NT

[a] MFI, mean fluorescence intensity.

[b] T cell lines were treated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 72 h, whereas monocyte-depleted PBMC and T cells affinity-purified from those PBMC were cultured in the presence of PHA (5 µg/ml) and IL-2 (20 IU/ml) for 72 hr. Percent of CD5 or CD81 positive cells was determined in untreated and treated cells by flow cytometry using respective specific antibodies and appropriate isotype antibody as controls. MFI values were read from flow cytometry plots.

[c] NT, not tested.

mutation in each of E2, p7, NS2 and NS5A sequences, were provided by Dr. Rodney Russell from Memorial University (St. John's, Newfoundland, Canada) (352). These cells served as positive HCV detection controls in selected experiments. Human hepatoma HepG2 (HB-8065) and HEK-293 (CRL-1573) cell lines were supplied by ATCC. The hepatoma cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FCS. PHH isolated by microperfusion of a healthy portion of the liver of a 58-year old male donor were supplied by Dominion Pharmakine (Derio-Bizkaia, Spain).

3.2.2. HCV Inocula

Plasma from 5 patients (3 males and 2 females; aged between 44 and 54 years) with progressing CHC, carrying HCV genotype 1a or 1b at levels 1.1×10^5 – 4.3×10^7 vge/ml, served as HCV infectious inocula (Table 3.2). PBMC of these individuals contained HCV at 2×10^3 to 2.8×10^5 vge/µg total RNA (Table 3.2). In some experiments, HCV JFH-1 strain derived from hepatoma Huh7 cells was used as inocula (403).

3.2.3. HCV Infection Assay

Cultured T cell lines, either intact or stimulated with PMA/ionomycin for 72 h at 1×10^5 cells/well, PHA-stimulated monocyte-depleted PBMC and primary T lymphocytes at 1×10^6 cells/well were supplemented with 5 ml of culture medium in 6-well plates and exposed for 24 h to HCV-positive plasma at ~1×10^5 HCV vge or to an equivalent volume of normal human plasma as a control or, in some experiments, to ~1×10^4 vge of JFH-1 virus. Then, the cells were extensively

Table 3.2. Virological characteristics of patients with chronic hepatitis C whose plasma served as HCV inocula [a]

Case Age (yrs)/ Sex (M/F)	HCV genotype	Plasma HCV RNA load (vge/ml) [b]	PBMC HCV RNA load (vge/µg total RNA) [b]
47/F	1a	1.1×10^5	1.8×10^4
52/M	1a	8.6×10^5	2×10^3
51/M-a [c]	1a	2.9×10^7	2.8×10^4
51/M-b [c]	1a	7.3×10^5	NA [d]
44/M	1a	5×10^6	1.6×10^3
54/F	1b	8.3×10^4	1.1×10^5

[a] M, male; F, female; vge, virus genome equivalents; n.a., not available.

[b] HCV RNA positive strand measured by real-time RT-PCR using RNA extracted from 250 µl of plasma or 1 µg of total RNA from PBMC.

[c] Samples a and b collected one year apart.

[d] NA, not available.

washed, suspended in fresh culture medium and cultured for 4-5 days post-infection (d.p.i.). In the case of PBMC and primary T cells exposed to HCV, culture medium was alternatively supplemented with PHA (5 μg/ml; Sigma-Aldrich) or PHA and rIL-2 (20 IU/ml; Roche) and cells maintained in culture for 14 days, as reported in our previous studies (242, 243).

3.2.4. Inhibition of HCV Infection in T cells by Treatment with Telaprevir

TLPV or VX-950, a HCV-specific protease inhibitor was purchased from Vertex Pharmaceuticals (Cambridge, MA) and used to treat Molt4 T cells infected with wild-type HCV (227). In preliminary experiments, naïve Molt4 cells were exposed to increasing concentrations of TLPV ranging from 0.8 to 80 μM in the presence of 0.5% DMSO for 7 days to determine cell nontoxic concentrations. Cell number and viability were determined by trypan blue exclusion using Countess Cell counter (Invitrogen). TLPV concentrations equal to and below 4 μM were found to be nontoxic. Accordingly, 2×10^5 Molt4 cells were incubated in duplicate with plasma-derived HCV in the presence or absence of 1 μM and 4 μM of TLPV in 0.5% DMSO. Then, cells were harvested after 7 d.p.i. for evaluation of HCV RNA positive and negative strand expression. Molt4 cells exposed to HCV and incubated in RPMI medium supplemented with 0.5% DMSO in the absence of TLPV were used as infection control.

3.2.5. Inhibition of HCV Infectivity with Anti-CD5 and Anti-CD81 Antibody

In order to ascertain the role of CD5 and CD81 in HCV entry to T cells, Molt4 at 1×10^5 cells/reaction were incubated for 30 min at 4°C and then for 30 min at 37°C with either anti-CD5 mAb (clone CD5-5D7; Invitrogen), anti-CD81

mAb (clone JS-81; BD Biosciences Pharminogen, Mississauga, Canada) or appropriate isotype antibody control at 2.5 μg in 50 μl of culture medium. The treated cells were transferred to a 6-well plate, supplemented with 5 ml culture medium, and inoculated with a patient's plasma containing approximately 1 x 10^5 HCV genome copies or, in some experiments, with ~1 x 10^4 vge JFH-1. After 24 h, cells were extensively washed, supplemented with fresh culture medium, and after 4 to 5-day culture, harvested for analysis.

3.2.6. Silencing of CD5 by RNA Interference

Lentiviral transduction particles encoding 5 different human CD5 shRNA (clones TRCN0000057653 to TRCN0000057657; designated as M-3 to M-7) with a titre between 1.5 x 10^7 and 2.2 x 10^7 infectious virus together with a non-target control transduction particles (#SHC001V) were prepared by Sigma-Aldrich. The particles were used to transduce 1 x 10^3 Molt4 cells in 100 μl RPMI 1640 medium at 1, 3 and 5 multiplicity of infection in 96-well plates in the presence of 2.5 μg/ml polybrene (Sigma-Aldrich). Cells were incubated for 24 h at 37°C and then allowed to revive in fresh medium for 24 h. Successfully transduced cells were selected in the presence of 1.7 μg/ml puromycin (Sigma-Aldrich) for 7 days. The level of CD5 knockout was determined by flow cytometry with anti-CD5 mAb. Clones M-5 and M-6 showed the greatest inhibition of CD5 protein expression (see Figure 3.9) and were used as targets in HCV infection experiments following the procedure described above.

3.2.7. Cloning of CD5 and Transfection of HEK-293 Cells

Complete CD5 cDNA sequence was amplified using total RNA from normal human PBMC and primers 5'-GCAGATCTATGCCCATGGGGTCTCTGC-3' (sense) and 5'-GCGAATTCTTAGGAGGAGCGATGCAGAACC-3' (antisense) containing *Bgl*II and *Eco*R1 restriction sites (underlined), respectively, at 95°C for 30 sec (denaturation), 59°C for 30 sec (annealing), and 72°C for 90 sec (extension) for 40 cycles. The 1444-base pair amplicon was cloned using PCRII TOPO TA system (Invitrogen) and the fragment excised was sequenced in both directions. The resulting sequence was identical to that of human CD5 deposited in GenBank under accession number NM-14207. To create a transcription vector, the CD5 DNA was excised from the TOPO TA by digestion with *Eco*RI and *Bgl*II restriction enzymes and ligated into the pIRES2-EGFP vector encoding the GFP (BD Biosciences, Clontech, Mountain View, CA). DH5α *E. coli* cells were transformed with the pIRES2-EGFPCD5 construct, cultured and enriched in LB kanamycin agar and then in broth. Plasmid DNA was purified using Qiaquik kit (Invitrogen). HEK-293 cells, which naturally do not express CD5 and are not susceptible to HCV, were grown to 70% confluence in a 6-well plate and transfected with 4 μg of pIRES2-EGFPCD5 vector or with empty vector in the presence of 10 μl Lipofectamine in 250 μl Opti-Mem medium (both Invitrogen). After 24 h, cells were supplemented with fresh medium and left to revive for 6 h. Transcription efficiency was evaluated by GFP positivity via fluorescent microscopy. CD5 gene expression was ascertained in transfected 293 cells by RT-quantitative-PCR, while CD5 protein by flow cytometry and confocal microscopy analyses. For HCV infection, the cells were exposed to plasma

containing ~1 x 10⁶ HCV genome copies or to ~1 x 10⁴ vge JFH-1. Inocula were removed after 48 h, cells extensively washed, supplemented with fresh medium, and cultured for 4 to 8 d.p.i. prior to analysis.

3.2.8. RNA Extraction and cDNA Synthesis

Total RNA was extracted from 5×10^6 to 1×10^7 cells or 300 µl cell culture supernatant with Trizol or Trizol LS, respectively (Invitrogen). Mock extractions were performed in parallel as contamination controls (315). RNA predestined for quantification of cellular gene expression was treated with DNAse and transcribed to cDNA with Maloney murine leukemia virus reverse transcriptase (Invitrogen) as reported (319, 315).

3.2.9. Detection of HCV Genome by RT-PCR/Nucleic Acid Hybridization

HCV genome was detected by PCR using complementary DNA derived from 1 or 2 µg of total RNA and primers against HCV 5'UTR and virus E2 region as described previously (315). PCR cycling parameters were as detailed before (315). Contamination controls routinely typically included a water sample instead of test cDNA, a mock extraction, and cDNA derived from PBMC of a healthy donor or from Molt4 or Jurkat cells exposed to normal human plasma (NHP; mock infection). As positive controls, cDNA from PBMC of patients with CHC providing for this study WHV inocula and the cloned HCV UTR-E2 (rHCV UTR-E2) fragment were used (315). The specificity of the signal detection and validity of controls were always confirmed by amplicon hybridization with ³²P-labeled recombinant HCV UTR-E2 (rHCV UTR-E2) (315). The sensitivity of this assay was generally <10 vge/ml (<2 IU/ml) or <2.5 vge/µg of total RNA. In addition,

when feasible, HCV genome was also evaluated by a real-time PCR using LightCycler 480 (Roche Diagnostics, Mannheim, Germany) under previously established conditions (315).

3.2.10. Detection of HCV Replication by Tth-Based RT-PCR/NAH Assay

HCV RNA negative (replicative) strand was determined by a strand-specific RT-PCR/NAH assay using r*Th* DNA polymerase (315). This enzyme catalyzes the polymerization of nucleotides into DNA using an RNA template in the 5´→3´ direction in the presence of manganese and the polymerization of nucleotides into duplex DNA in the 5´→3´ direction in the presence of magnesium. Details of this assay were described previously (315). Contamination controls were the same as those described above for the standard HCV genome detection assay. PCR cycling parameters were as reported previously (315). Suffice to mention here that specificity of this assay comes from the RT step in which a single HCV genome-specific sense primer (denoted as UTR1 in reference 315) was used to prime the synthesis of HCV cDNA from the viral RNA template. Subsequently, the first-round (direct) PCR was initiated by the addition of the HCV-specific antisense primer (denoted as RTU1 in reference 315). In this negative strand detection assay, ten-fold serial dilutions of HCV synthetic RNA (sRNA) negative strand were used as semi-quantitative standards, while those of HCV sRNA positive strand as the specificity control. As in the case of the standard HCV genome detection assay outlined above, the specificity of PCR signals and validity of controls were confirmed by nucleic acid hybridization using the ^{32}P-labeled rHCV UTR-E2 fragment (315). In this end,

this assay detects ~100 vge per reaction of the correct (negative) strand, while nonspecifically identifying $\geq 10^6$ vge per reaction of the positive strand (Figure 3.1) (315, 316). Given that total RNA templates used for this assay were usually between 2 to 4 µg, the overall sensitivity of this negative strand detection assay was about 25-50 vge/µg of total RNA (315).

3.2.11. RT-PCR Quantification of Cellular Gene Transcription

The level of expression of CD5 and CD81 in naïve and PMA/ionomycin- or PHA-stimulated T cell lines, PBMC and primary T cells, human hepatoma cell lines and isolated PHH were quantified by real-time RT-PCR. As well, expression of cytokines potentially influencing the susceptibility of T cells to HCV infection, such as IFN-α, IFN-γ, as well as IFN regulatory factor 3 (IRF-3) and IRF7 were identified by real-time RT-PCR. Beta-actin (ß-actin) and hypoxanthine phosphoribosyltransferase (HPRT) were measured as loading and expression controls. Primer sequences are shown in Table 3.3. All amplifications were carried out using cDNA derived from 50 ng of DNAse-treated RNA in a LightCycler 480 (Roche Diagnostics) for 40 cycles under the following conditions: denaturation at 95°C for 30 sec, annealing for 30 sec at 59°C for CD5, at 58°C for IFN-α, IFN-γ, IRF3, and IRF7, at 57 °C for ß-actin, at 56 °C for CD81 and at 55 °C for HPRT, and extension at 72°C for 20 sec.

3.2.12. Flow Cytometry

To determine or confirm phenotypic characteristics of the T cell lines used as HCV targets, the cells were stained with alexafluor488-conjugated anti-CD3 mAb (BD Pharminogen), phycoerythrin (PE)-conjugated anti-CD4 mAb

Table 3.3. Primer sequences used for real-time RT-PCR analysis

Gene	Forward primer	Reverse primer
CD5	5'-TCAAGCGTCAAAAGTCTGCC-3'	5'-AGCCACACTGGAGGTTGTTG-3'
CD81	5'-ACAAGGACCAGATCGCCAAG-3'	5'-AGTCAAGCGTCTCGTGGAAG-3'
IFN-α	5'-CAGCCTGAGTAACAGGAGGA-3'	5'-GCAGATGAGTCCTTTGTGGT-3'
IFN-γ	5'-TCAGCTCTGGATCGTTTTGG-3'	5'-TGTTTTAGCTGCTGGGGACA-3'
IRF3	5'-TACGTGAGGCATGTGCTGA-3'	5'-AGTGGGTGGCTGTTGGAAAT-3'
IRF7	5'- GCCCTTGCCTCCCCTGTTAT-3'	5'-CCACTGCAGCCCCTCATAG-3'

A

HCV RNA
negative strand

sHCV RNA negative strand

DW   NW   Mock 10^2 10^3 10^4 10^5 10^6

◄ 244 bp

B

HCV RNA
positive strand

sHCV RNA positive strand

DW   NW   Mock 10^2 10^3 10^4 10^5 10^6

◄ 244 bp
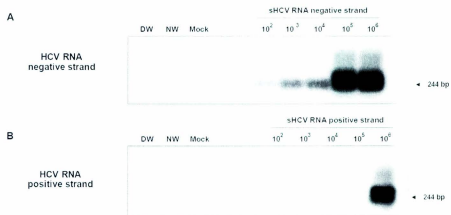
Figure 3.1

Figure 3.1. Verification of the specificity and the sensitivity of HCV RNA negative (replicative) strand detection by the strand-specific RT-PCR/NAH. (A) Ten-fold serial dilutions of synthetic HCV RNA negative strand or (B) synthetic HCV RNA positive strand were amplified using HCV-specific antisense primer, r*Tth* DNA polymerase and conditions described in Materials and Methods. Contamination controls included water added instead of synthetic RNA and amplified by direct (DW) and nested (NW) reactions and mock (M) treated as test RNA. Specificity of RT-PCR products was further verified by NAH using ^{32}P-labelled rHCV 5'-UTR-E2 fragment as a probe. Positive signals showed the expected 244-bp 5'-UTR sequence-specific fragments. As shown, the assay detects 100 copies of the correct (negative) strand, while nonspecifically identifying $\geq 10^6$ copies of the positive strand.

(Chemicon International, Temecula, CA) and PE-conjugated anti-CD8 mAb (Chemicon). Detection of CD5 was done with allophycocyanin (APC)-conjugated anti-CD5 mAb (Invitrogen), while that of CD81 with fluorescein isothiocyanate (FITC)-labeled CD81 mAb (Invitrogen). Briefly, approximately 1×10^6 cells per reaction were washed twice with phosphate-buffered saline and exposed to mAb or isotype antibody control on ice for 45 min. Cells were washed and then examined with a FACSCalibur cytometer (Becton Dickinson Biosciences, Mountain View, CA). The results from 10^4 gated cells were analyzed with CellQuest Pro software (Becton Dickinson Biosciences).

3.2.13.   *Confocal Microscopy*

To detect HCV NS5A and core proteins as well as CD5 protein, cells were fixed with 2% paraformaldehyde and permeabilized with 0.25% saponin. Double staining with rat anti-tubulin (Chemicon) and with either mouse anti-HCV NS5A mAb (Chemicon) or appropriate isotype antibody control (BD Biosciences) was done as previously reported (314). For staining of HCV core protein, cells were exposed to anti-HCV core mAb (Dainippon Sumitomo Pharma, Osaka, Japan) for 20 min on ice, washed and then incubated with alexaFluor488-labeled anti-mouse IgG mAb (Molecular Probes, Eugene, OR) for 20 min at ambient temperature. After washing, the cells were treated with 4',6-diamidino-2-phenylindole (DAPI; 0.1 µg/ml) (Vector Laboratories, Inc., Ontario, Canada) and examined. Huh7 cells naïve or carrying HCV AB12-A2 replicon and HuH7.5 cells infected with JFH-AM2 were used as controls. For double staining with anti-CD5 and anti-NS5a, cells were first exposed to anti-NS5a mAb overnight at 4 ºC,

washed and then exposed to Cy2-labeled donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) on ice for 60 min. Subsequently, the cells were treated with APC-conjugated anti-CD5 mAb (Invitrogen) for 30 min on ice, washed and counterstained with DAPI. Slides were examined under an Olympus BX50WI microscope with a FlouView FV300 confocal system (Olympus America Inc., Melville, NY). Approximately 1000 cells per preparation were examined, positive cells were counted, and the percentage of the positive cells versus the total cell number calculated.

3.2.14.   *Western Blotting*

To further ascertain that productive HCV infection was established in T cells, the presence of virus E2 protein was examined by immunoblotting in Molt4 cells infected with plasma-derived HCV. Thus, the infected cells collected at 5 d.p.i. were dissolved in ice-cold RIPA buffer (1% NP-40, 0.5% DOC, 0.1% SDS, 150 mmol/L NaCl in 50 mmol/L Tris, pH 8.0 (Sigma-Aldrich) and proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 20 µg protein/lane. After blotting onto a nitrocellulose membrane by wet transfer using the Bio-Rad SD cell system (Bio-Rad Laboratories, Mississauga, Ontario, Canada) (243, 274), the blots were exposed to 5% skimmed milk in Tris-buffered saline (pH 7.4) for 1 hour at room temperature and then overnight at 4°C to anti-E2 mouse mAb diluted 1:500 (provided by Dr. Arvind Patel, University of Glasgow, Glasgow, United Kingdom). Reactions were developed with horseradish peroxidase-conjugated goat anti-mouse IgG F(ab')$_2$

antibodies (Jackson ImmunoResearch) and signals visualized using an ECL detection kit (Sigma-Aldrich).

3.2.15. *Statistical Analyses.*

Data were analyzed using Prism 4 software (GraphPad Software, Inc., San Diego, CA). Statistical significance was determined by two-tailed Mann-Whitney or unpaired Student's-t test and P values less than 0.05 were considered significant.

3.3   Results And Discussion

To identify factors facilitating T cell susceptibility to infection with wild-type HCV, we explored a previously established T lymphocyte-based HCV infection system in which plasma from patients with CHC serves as infectious inocula (242, 243). In search of the most suitable and readily accessible human T cell targets, other than mitogen-induced primary T lymphocytes (242, 243), the susceptibility of Molt4, Jurkat, CEM and PM1 human lymphoblastic T cell lines to infection with genotype 1 HCV was investigated. This initial study showed, as previously reported (243, 373), that Molt4 and Jurkat cells as well as human primary T lymphocytes are susceptible to HCV and can support HCV replication, as identified by detection of virus RNA negative (replicative) strand (Table 3.4 and Figure 3.2). Susceptibility of Molt4 and Jurkat cells to HCV infection was also confirmed by cytoplasmic detection of HCV NS5a and core proteins (Figure 3.3).

Table 3.4. HCV infection of human T cell lines, PBMC and primary T lymphocytes with wild-type virus carried in plasma of CHC patients

HCV inoculum: Patient	HCV genotype	T cell lines				PBMC	Primary T cells
		Molt4	Jurkat	PM1	CEM		
		HCV RNA positive strand / HCV RNA negative strand[a]					
47/F	1a	$10^4/10^2$	$10^3/50$	ND[b]	ND	NT[c]	NT
52/M	1a	$10^4/10^2$	$10^4/10^2$	NT	NT	NT	NT
51/M-a	1a	$1x10^3/5x10^2$	NT	ND	NT	NT	NT
51/M-b	1a	$1.3x10^3/5x10^2$	$5.4x10^4/5x10^2$	34/ND	ND	$6.7x10^3/5x10^2$	$5.6x10^3/5x10^2$
44/M	1a	$1.5x10^7/10^3$	$2x10^6/10^3$	25/ND	ND	$1x10^5/10^3$	$1x10^5/10^3$
54/F	1b	$1.3x10^3/10^2$	NT	NT	NT	$5.5x10^4/10^3$	$1x10^4/10^3$

[a] HCV RNA-positive strand was determined by real-time RT-PCR or nested RT-PCR/NAH, whereas HCV RNA-negative strand by the strand-specific RT-PCR/NAH, as described in Materials and Methods. HCV load was expressed in vge/µg total RNA.

[b] ND, not detected; either both HCV RNA positive and negative strands or virus RNA negative strand only.

[c] NT, not tested.

Figure 3.2

Figure 3.2. Infection of T cell lines with HCV. (A) Molt4, Jurkat, PM1 and CEM T cells were exposed to HCV 44/M. HCV RNA positive and replicative strands were detected by RT-PCR/NAH assays as described in Materials and Methods. Synthetic HCV RNA positive strand (sHCV RNA pos) at 10^5 copies/reaction and rHCV UTR-E2 fragment were used as positive and specificity controls for the HCV RNA positive strand detection. Synthetic HCV RNA positive (pos) and negative (neg) strands at 10^5 copies/reaction confirmed the assay specificity for detection of the virus RNA negative strand. Water amplified in direct (DW) and nested (NW) reactions and a mock extraction served as contamination controls. The positive signals showed the expected 244-bp oligonucleotide fragments.

A

	Anti-NS5A / DAPI	Anti-NS5A / DAPI / TL	Anti-NS5A
Uninfected			

B

Huh7 / rHCV AB12-A2FL   Huh7   Molt4/HCV   Molt4/NHP

HCV E2 ◄ 70kDa

β-actin ◄ 45kDa
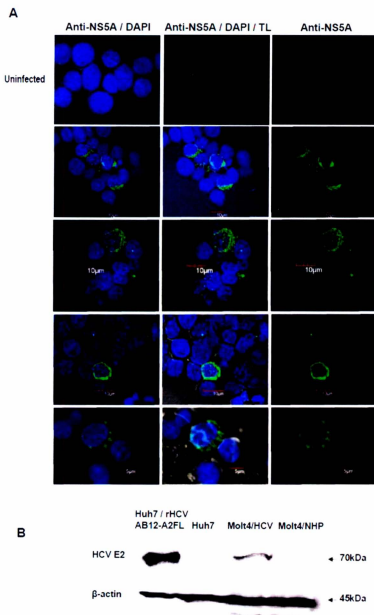
Figure 3.3

C
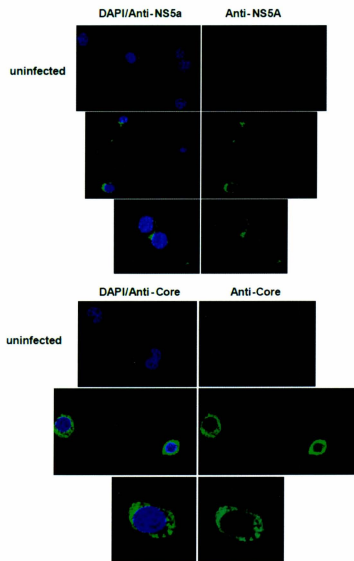
Figure 3.3

Figure 3.3. Evidence of infection of Molt4 and Jurkat T cell lines with wild-type HCV. (A) Detection of HCV NS5A in infected Molt4 by confocal microscopy. Cells nuclei were counterstained with DAPI to identify nuclei and examined under transmitted light (TL; central panel) to visualize the cytoplasm. Images captured at X60. (B) Identification of HCV E2 protein in Molt4 cells infected with wild-type HCV by Western blotting using anti-E2 mAb. Huh7 cells carrying rHCV AB12-A2FL replicon were used as a positive control, while naïve Huh7 cells and Molt4 cells exposed to normal human plasma (NHP) served as negative controls. Detection of β-actin protein served as a loading control. (C) Detection of HCV NS5A and core protein in Jurkat cells infected with patient-derived HCV inocula. Uninfected cells were used as controls. Nuclei were counterstained with DAPI. Images captured at X60.

The percentages of NS5a-positive Molt4 cells enumerated under a confocal microscope ranged between 2 and 5% and the higher number of positivity cells correlated with the greater copy number of intracellular HCV RNA (see Table 3.4). In agreement with these findings was the identification of virus E2 protein in HCV-infected Molt4 T cells by Western blotting at the level which was evidently, but not surprisingly, lower than that displayed by Huh7 cells carrying HCV AB12-A2FL replicon (Figure 3.3B). Furthermore, treatment of Molt4 cells with 1 μM or 4 μM TLPV inhibited replication of HCV, as was evidenced by the absence of detectable HCV RNA replicative strand in these cells (Figure 3.4). However, in one of two repeats treated with 1 μM of TLPV, HCV RNA positive strand was detected, suggesting not complete inhibition of virus propagation at this lower drug concentration in this particular culture. On the other hand, Molt4 cells exposed to HCV in the absence of TLPV readily demonstrated replication of the virus (Figure 3.4). In contrast to Molt4 and Jurkat cells, PM1 cells were infrequently reactive for HCV RNA positive strand and did not display detectable levels of NS5a or core protein, while CEM cells were consistently negative following exposure to virus (Figure 3.2 and Table 3.4). To identify reasons behind the discrepant susceptibility to HCV infection between the T cell lines, we re-evaluated their phenotypic characteristics and assessed expression of CD81 as well as that of selected innate immunity response genes which are known to limit viral infection.

A

Molt4

DW   NW   Mock   UT   TLPV (µM)   rHCV
                                                    UTR E2

HCV RNA
positive strand   ◄— 244 bp

B

Molt4   sHCV RNA strand

DW   NW   Mock   UT   TLPV (µM)   neg   pos
                                                   10^2 10^3 10^2 10^3

HCV RNA
negative strand   ◄ 244 bp
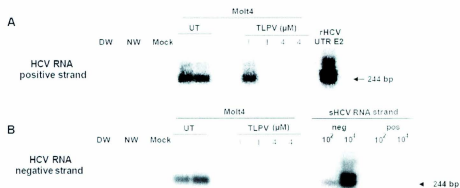
Figure 3.4

Figure 3.4. Inhibition of HCV infection in Molt4 T cells treated with the HCV-specific protease inhibitor TLPV. Molt4 cells infected with wild-type HCV were treated with 1 μM or 4 μM of TLPV or left untreated (UT) for 7 days post infection. HCV RNA positive strand was detected by RT-PCR with 5'-UTR-specific primers and amplicon specificity verified by NAH. Virus negative (replicative) strand was identified by the strand-specific RT-PCR/NAH assay in which synthetic HCV RNA positive (pos) and negative (neg) strands at 10^2 and 10^5 copies/reaction were used as specificity controls. As positive controls, total RNA from Molt4 cells infected with HCV but not treated with TLPV were used. Other controls were as described in the legend to Fig. 1.

In addition, we determined expression of CD5 which we had hypothesized to be involved in HCV entry based upon its essentially lymphocyte-restricted expression and, as is SR-B1, inclusion in the scavenger receptor cysteine-rich family (28, 361).

The flow cytometry data confirmed that Molt4 and Jurkat T cell lines were uniformly CD3 positive, essentially CD8 negative and variably CD4 reactive (Table 3.5). Interestingly, CD81 was displayed on comparable cell numbers (mean 98.2%, SEM 0.5) and with similar mean fluorescence intensity (MFI 106-171), as it was on fresh normal human PBMC and primary T cells (mean 99.1%, SEM 0.5; MFI 213-260).   Inversely, CD5 was displayed by a significantly ($P<0.0001$) greater number of Molt4 (mean 98%, SEM 0.6; MFI 84), Jurkat (mean 92%, SEM 2.7; MFI 77) and primary T cells (mean 80%, SEM 4.9; MFI 61) than on PM1 (mean 5.8%, SEM 1.6; MFI 34) or CEM cells (mean 5.6%, SEM 1.6; MFI 32). The expression of CD81 and CD5 proteins corresponded well to the transcriptional levels of the respective genes. Indeed, CD81 mRNA level was not significantly different in all T cell lines, while CD5 transcription was significantly ($P<0.0001$) greater in HCV-permissive Molt4, Jurkat, PBMC and primary T cells than in HCV-resistant PM1 and CEM cells (Figure 3.5). Therefore, the results suggested that T cell receptiveness to HCV infection requires CD5 and is unlikely mediated by CD81 alone. Furthermore, expression profiles of IFN-α, IFN-γ, IRF-3 and IRF-7 transcription revealed no apparent correlation with T cell susceptibility to infection with wild-type HCV (Figure 3.5).

Table 3.5. Surface markers expressed on T cell lines

Cell line	CD3 %[a]	CD4 %	CD8 %	CD81 %	CD5 %
Jurkat	99.8	5	0.28	99.5	97.12
Molt4	95	55	0.5	99.4	96.5
PM1	10	88	0.75	82.8	10
CEM	18	90	1.5	92.1	2

[a] percentage of positive cells by flow cytometry analysis.

Figure 3.5

87
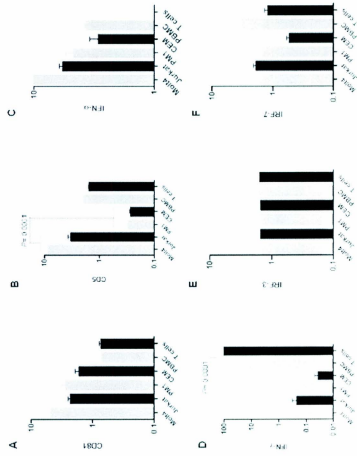
Figure 3.5. Expression of CD81, CD5, IFN-α, IFN-γ, IRF-3 and IRF-7 genes quantified in T cell lines, PBMC and primary T cells by real-time RT-PCR using an equivalent of 50 ng total RNA per reaction. Expression levels were normalized against that of ß-actin. Data bars show mean values $\pm$ SD of normalized copies of gene of interest.

Since our previous studies consistently demonstrated that ex vivo mitogen stimulation of normal human T lymphocytes predisposes them to HCV infection (242, 243) and, with some exceptions, augments HCV replication in PBMC from HCV-infected individuals (314, 315), we tested whether stimulation of PM1 or CEM cells may make them prone to HCV. In this regard, treatment with PHA or PMA had been shown to upregulate CD5 on T lymphocytes (237). Hence, we exposed T cell lines to nontoxic concentrations of PMA (50 ng/ml) with ionomycin (500 ng/ml), while normal human PBMC and purified primary T cells were treated with PHA prior to infection, as reported (243). As expected, the CD5 surface display was increased on all T cell lines examined (Table 3.1). However, while PM1 became prone to HCV infection, CEM remained virus resistant (Figure 3.6). This permissiveness appeared proportional to the cell positivity for CD5. In fact, while 10% (MFI 18.8) of naive PM1 were CD5 positive, 53.6% (MFI 30.1) of them became CD5 reactive after stimulation. Treatment of CEM augmented CD5 positivity from 6.3% (MFI 6.4) to 20.6% (MFI 14.5), but this appeared to be insufficient to facilitate HCV infection. Following the same treatment, CD5 expression on Molt4 and Jurkat cells was increased (Table 3.1), and the cell receptiveness to HCV infection augmented, albeit unremarkably (data not shown). Notably, stimulation of the T cell lines downregulated expression of cell surface CD81 (Table 3.1), making the postulated CD5 contribution to HCV T cell entry even more compelling.
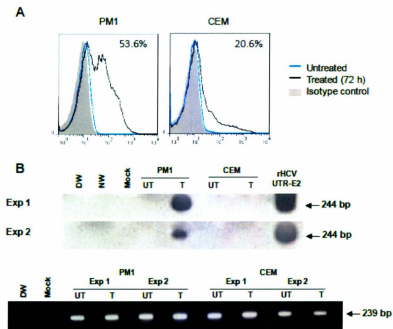
Figure 3.6

Figure 3.6. Upregulation of CD5 on nonpermissive PM1 cells confers their susceptibility to HCV infection. (A) HCV-resistant PM1 and CEM cells were treated with PMA/ionomycin for 72 h and examined for CD5 protein expression by flow cytometry. Stimulation significantly augmented CD5 positivity of PM1 but not CEM cells. The percentage of positive cells after stimulation is indicated in the upper right corner of each plot. (B) Untreated (UT) and treated/stimulated (T) PM1 and CEM cells were exposed to HCV 51/M-b for 24 h, extensively washed, supplemented with fresh medium, and after 5-day culture harvested for analysis. HCV RNA expression was detected in stimulated PM1 but not in stimulated CEM cells, while untreated cells were HCV nonreactive, indicating that an increase in CD5 expression on PM1 cells coincided with acquisition of their permissiveness to HCV infection. The results from two independent infection experiments are illustrated. (C) ß-actin expression in PM1 and CEM cells shown in (B).

In the context of the above findings and since no relevant information was available, we tested whether human hepatocytes are endowed in CD5. Flow cytometry revealed that hepatoma Huh7, Huh7.5 and HepG2 cells were essentially CD5 nonreactive, while they, with the exception of HepG2, were strongly CD81 positive (Figure 3.7). Quantification of CD5 mRNA by real-time RT-PCR revealed trace CD5 mRNA levels in the hepatoma cells and PHH, and comparably high CD81 mRNA levels (data not shown).

Taken together, it would be highly unlikely that CD5 can contribute to HCV infection of hepatocytes. To ascertain the role of CD5 in HCV entry to T cells, Molt4 cells and freshly purified human T lymphocytes were preincubated with anti-CD5 mAb and then exposed to HCV. Under the same conditions, interference of anti-CD81 mAb with HCV infectivity was examined. Blocking of either CD5 or CD81 abolished or significantly decreased susceptibility of Molt4 and primary T cells to HCV infection (Figure 3.8). Incubation with appropriate isotype controls (Figure 3.8) or unrelated mAb to CD6 (data not shown) was without effect.
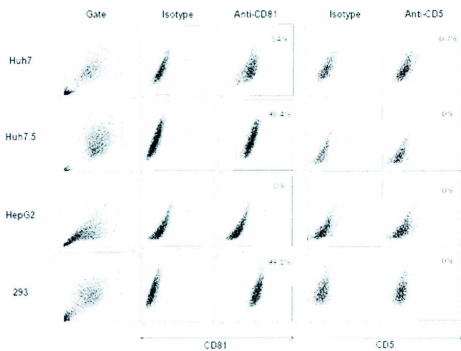
Figure 3.7

Figure 3.7. CD81 and CD5 protein expression by human hepatoma-derived cell lines and naïve HEK-293 cells. The proteins' display was determined by flow cytometry with anti-CD81 and anti-CD5 antibodies as well as appropriate isotype controls. The percentage of positive cells is indicated in the upper right corner of each plot.

Figure 3.8

95
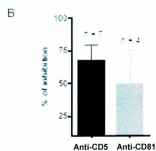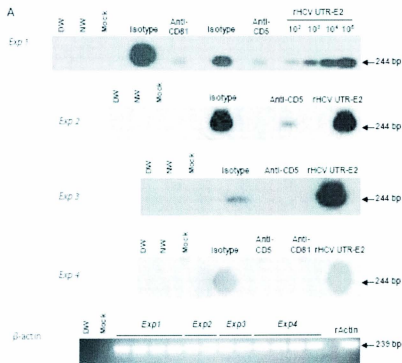
Figure 3.8. Inhibition of HCV infection of T cells with anti-CD5 and anti-CD81 antibody. (A) Molt4 cells (Experiments 1-3) or fresh primary T cells (experiment 4) were exposed to HCV 44/M (Experiments 1 and 4), HCV 51/M-b (Experiment 2) and HCV 51/M-a (Experiment 3) in the presence of anti-CD5, anti-CD81 or relevant isotype antibody control. Infection outcome was evaluated by RT-PCR/NAH. (B) Plot presentation of the data on inhibition of HCV RNA expression determined by RT-PCR/NAH assay in cells challenged with HCV which were obtained for all experiments performed including those shown in (A).

Overall, the data from 7 separate experiments with anti-CD5 mAb and 4 with anti-CD81 mAb using three different HCV inocula gave a mean inhibition of 67.2% (SEM 11.6) and 49.1% (SEM 25.6), respectively, in HCV RNA expression in Molt4 target cells (Figure 3.8B).

Furthermore, silencing of CD5 expression with lentiviruses encoding CD5-specific shRNAs significantly ($P<0.05$) inhibited HCV RNA expression which was accompanied by the loss of virus RNA replicative strand in Molt4 cells relative to those transduced with control shRNA or untreated cells (Figure 3.9A and B). This finding was supported by the significantly lower level ($P<0.05$) of HCV RNA released by CD5-silenced cells compared to those produced by untransduced Molt4 or by Molt4 transduced with scrambled shRNA (Figure 3.9D). It should be noted that selection of the cells in the presence of puromycin led to a partial decrease in their susceptibility to HCV infection as evidenced by the lower levels of HCV RNA in cells transduced with scrambled shRNA relative to those of untransduced cells (Figure 3.9E). The level of CD81 expression was not affected in these experiments (Figure 3.9C). The involvement of CD5 in the HCV entry was finally examined by transfection of CD5-negative, CD81-positive human embryonic kidney 293 fibroblasts with complete human CD5 cDNA and then exposing transfected cells to HCV. Up to 85% of HEK-293 cells became CD5 reactive (designated as 293-CD5), while those transfected with the empty vector (293-EV cells) remained negative (Figure 3.10 A). At 4 days post virus exposure, 293-CD5 cells, but not 293-EV cells, expressed virus positive and negative RNA strands (Figure 3.10 B) and displayed NS5a protein (Figure 3.10 C).

A

M3 MFI 93 / MFI 80 / MFI 97
M4 MFI 84 / MFI 80 / MFI 97
M5 MFI 11 / MFI 80 / MFI 97
M6 MFI 8.9 / MFI 80 / MFI 97
M7 MFI 54 / MFI 80 / MFI 97

% of Max

CD5

— clone M3-M7
— scrambled shRNA (MS)
— nontransduced cells (MN)
— isotype control

B CD5 copy number — $n \geq 3$ — MN MS M5 M6 — $P=0.01$

C CD81 copy number — $n \geq 3$ — MN MS M5 M6

D HCV copy number — $n = 2$ — MN MS M5 M6

E

	DW	NW	Mock	M5	M6	MN	MS	HCV sRNA Strand neg 10^3 pos 10^5
HCV RNA negative strand								← 244 bp

	DW	Mock	M5	M6	MN	MS	rActin
β-actin							← 239 bp

Figure 3.9

98

Figure 3.9. Inhibition of CD5 expression in Molt4 cells makes them resistant to HCV infection. (A) Clones M-5 and M-6 of lentiviral particles encoding CD5-shRNA were most effective in knocking down CD5 protein as revealed by flow cytometry with anti-CD5 antibody. (B) Inhibition of transcription of CD5 but not (C) CD81 with M-5 and M-6 clones as quantified by real-time RT-PCR. (D) Molt4 transduced with clone M-5 or M-6, scrambled shRNA or native cells were challenged with HCV 44/M and after 7-day culture HCV released to culture supernatant was quantified by real-time RT-PCR. (E) Replicating virus was identified by detection of HCV RNA negative strand. Specificity and contamination controls were the same as described in the legend to Fig. 3.1. ß-actin expression was used as a loading control, and recombinant human actin (rActin) as a positive control.

The association between cell positivity with CD5 and cytoplasmic expression of NS5a protein was clearly apparent. This demonstrated that the presence of CD5 facilitated infection of these otherwise CD5-negative and HCV-nonpermissive cells, albeit at a relatively low level ($\leq$ 1% of NS5A-positive cells).

It is of note that our attempts to infect T cell lines, primary T cells and PBMC or 293-CD5 cells with laboratory HCV JFH-1 strain were not successful. This may suggest that the ability to infect T lymphocytes is a characteristic of native, patient-derived HCV and, as we have found, does not depend on HCV genotype (G. Skardasi and T.I. Michalak, unpublished data) (242).

This study is the first to identify a molecule essential for the cell-specific HCV entry to human lymphocytes. In addition to primary T lymphocytes, only T cell lines displaying moderate to high levels of CD5 protein were found susceptible to HCV infection despite of comparable expression levels of CD81, the accepted customary HCV co-receptor. Since anti-CD5 and anti-CD81 mAb inhibited infection of permissive T cells, this may argue that both of these molecules are required for HCV entry to T cells. Notably, the ability of anti-CD81 to inhibit infection of human T cells with wild-type HCV was previously documented (242, 243). Considering the restricted expression of CD5, which in adults is essentially limited to T cells and a minor subset of B cells, it is likely that while CD81 may contribute to a broad recognition of cells by HCV, CD5 facilitates HCV tropism specifically towards lymphocytes. On this note, while we clearly documented in this study the involvement of CD5 in T cell susceptibility to HCV infection, it remains to be established whether this protein may also play a role in

HCV entry to B cells. Since the factors mediating HCV entry to hepatoma Huh7 cells reported thus far are not unique to hepatocytes, CD5 is the first molecule identified that governs cell permissiveness to HCV in a cell-type specific manner. Our findings provide not only a direct insight into the nature of HCV lymphotropism, but also open new options for the development of antiviral approaches targeting HCV that propagates in the lymphatic system. Although this is not yet fully appreciated site of extrahepatic HCV occurrence, it may in fact constitute under certain clinical conditions the largest reservoir of a potentially pathogenic virus.

A

293CD5
293EV
Isotype control

% of Max

CD5

B

HCV RNA positive strand

DW NW Mock 293-EV 293-CD5 rHCV UTR-E2

← 244 bp

HCV RNA negative strand

DW NW Mock 293-EV 293-CD5

HCV sRNA
Strand
pos 10⁵ neg 10⁵

← 244 bp

β-actin

DW Mock 293-EV 293-CD5 rActin

← 239 bp

C

Anti-CD5 Anti-NS5a DAPI/Anti-CD5/Anti-NS5a

293-EV

293-CD5/ uninfected

293-CD5/ HCV 44/M

293-CD5/ HCV 51/M-b
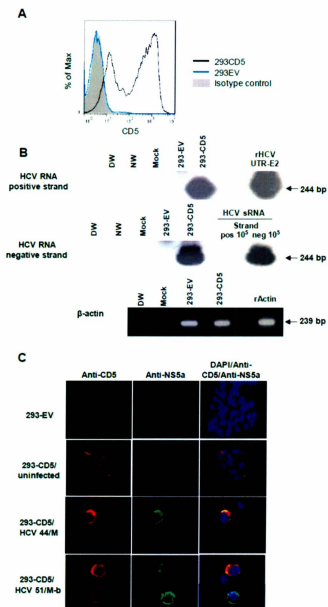
Figure 3.10

Figure 3.10. HEK-293 cells transfected with CD5 become susceptible to HCV. (A) HCV-resistant, CD5-negative 293 cells were transfected with plasmid encoding human CD5. (B) 293 cells transfected with CD5 (293-CD5) or empty vector (293-EV) were exposed to HCV 51/M-b inoculum and examined for HCV RNA positive and negative strands by RT-PCR/NAH as described in Materials and Methods. Specificity and contamination controls were outlined in the legend to Fig. 1. ß-actin expression was used as a loading control. (C) 293-EV, uninfected 293-CD5 and 293-CD5 challenged with HCV 44/M or 51/M-b inoculum were stained with anti-CD5 and anti-NS5a antibody, and examined by confocal microscopy. Nuclei were counterstained with DAPI. Images captured at X60.

CHAPTER FOUR:

DIFFERENTIAL EXPRESSION OF HEPATITIS C VIRUS CANDIDATE RECEPTORS IN HUMAN T LYMPHOCYTES PRONE OR RESISTANT TO WILD-TYPE VIRUS AND HEPATOMA CELLS SUSCEPTIBLE TO INFECTION WITH HCV JFH-1 CLONE.

4.1. Introduction

HCV is a hepatotropic virus that also propagates in the human immune system. Recently, a lymphocyte-specific CD5 glycoprotein has been identified as an essential factor for infection of normal human T lymphocytes with wild-type HCV. However, several other molecules have been proposed as receptors mediating HCV tropism towards human hepatocytes based on the studies applying laboratory adapted HCV strains or pseudoparticles and hepatoma cell lines. To assess whether these candidate receptors may play a role in infection of T lymphocytes, we characterized expression of SR-B1, CLDN-1, 4 and 6 and OCLN, as well as CD5 and CD81, in HCV-prone and resistant T cell lines and primary T lymphocytes in comparison to Huh7.5 and HepG2 hepatoma cells and, when feasible, PHH. The results showed that, although SR-B1 was transcribed in all cell types examined, only HCV-resistant PM1 and CEM T cells and hepatoma cell lines displayed SR-B1 protein. CLDN-1 protein was detected exclusively in HCV-resistant PM1 cells, while CLDN-4 and CLDN-6 were found at

exclusively in HCV-resistant PM1 cells, while CLDN-4 and CLDN-6 were found at comparably high levels in all cells tested, except HepG2 cells. Interestingly, OCLN was displayed by HCV-susceptible Molt4 and Jurkat T cell lines, but not by PBMC and occasionally only at low levels by primary T cells. Finally, CD5 was presented by HCV-susceptible Molt4 and Jurkat T cell lines, primary T cells and PBMC but not by HCV-resistant T cell lines and hepatoma cells, while CD81 was expressed at comparable levels in all cells, except HepG2. Since involvement of CD5 and CD81 was previously documented, a role of OCLN in infection of T lymphocytes by wild-type HCV was the focus of the subsequent investigation. In this regard, knocking down OCLN with specific shRNA in HCV-prone Jurkat T cells inhibited HCV infection, while de novo infection with HCV downregulated OCLN and CD81, upregulated CD5, but did not modify SR-B1 expression. It was also uncovered that suppression of OCLN in T cells inhibited the display of HCV envelope protein without modifying virus genome transcription. The data suggested that while OCLN may play a role in infection of virus-susceptible T cell lines, its contribution to infection of normal human primary T cells is less likely, pointing to a difference in factors determining permissiveness of cultured tumor-derived and primary cells to HCV. Overall, while no association between expression of SR-B1 or CLDN-1 and T cell susceptibility to HCV infection was found, CD5 was confirmed to be essential for HCV lymphotropism with CD81 contributing as a potential coreceptor. Our study narrowed the range of cellular factors utilized by wild-type HCV infecting human T lymphocytes amongst those previously identified as receptors using HCV preparations and Huh7.5 cells.

Their identification may affect future development of preventive and therapeutic strategies against HCV.

HCV is a positive single stranded RNA virus that chronically infects more than 170 million people. This infection represents a major health problem worldwide despite significant advancements in blood-screening techniques. Currently, there are no vaccines preventing HCV infection, however the new therapies show a significantly improved anti-viral potency and augmented rates of virus elimination as determined by measurements of plasma HCV RNA by the currently available clinical laboratory assays (57, 227, 262). The efforts to establish a robust HCV culture system have succeeded in recent years by transfecting human hepatoma Huh7 cells with a full-length HCV derived from a Japanese patient with fulminant hepatitis C (JFH-1), which resulted in secretion of *in vitro* infectious HCV particles (HCVcc) (231, 403, 430). This infection model and other HCV surrogate systems, such as HCVpp (28, 346), were utilized to identify receptors mediating HCV infection of Huh7 cells and related cell lines. As a result, tetraspanin CD81 (322), glycosaminoglycans (147), SR-B1 (28, 363), and the TJ proteins CLDN-1 (102) and OCLN (32, 323) have been proposed as factors determining HCV tropism to human hepatocytes. However, it remains uncertain to what degree these models reflect natural infection of hepatocytes with wild-type virus. An attempt to infect PHH with HCV JFH-1 clone using micropatterened co-cultures resulted in detection of virus trace signals that sustained for several weeks as revealed by highly sensitive reporter systems;

however neither HCV RNA positive and negative strands nor viral proteins were detected (324).

Accumulated experimental and clinical evidence indicate that HCV infects not only hepatocytes but also cells in extrahepatic compartments, particularly those of the immune system, as well as astrocytes and glial cells (127, 412). In regard to infection of circulating lymphoid cells, studies on HCV compartmentalization in patients with either symptomatic chronic or clinically silent infection documented HCV replication in both T and B lymphocyte subsets and in monocytes (93, 314). The susceptibility of human primary T lymphocytes *ex vitro* and particular T cell lines to infection with patient-derived HCV and their ability to support the entire cycle of HCV replication in culture have also been demonstrated (242, 243, 360, 373). The propensity of HCV to infect the host's immune system is consistent with a significantly greater prevalence of lymphoproliferative disorders, such as mixed cryoglobulinemia and non-Hodgkin's lymphoma, in patients infected with HCV (42, 69, 149, 192, 347). In contrast to the several cellular molecules proposed to be mediators of HCV tropism towards hepatocytes, factors determining HCV lymphotropism are just being recognized. In our recent study, a lymphocyte-specific glycoprotein CD5, belonging to the scavenger receptor cysteine-rich family, has been found to be essential for infection of human T lymphocytes with wild-type HCV (360). A contribution of CD81 to infection of T cells has also been demonstrated (242, 243, 360). In the current study, the expression of SR-B1, CLDN-1, CLDN-4, CLDN-6 and OCLN, as well as CD5 and CD81, in HCV-prone and HCV-resistant

107

human T cell lines and in human primary T lymphocytes was investigated to assess their potential contribution to HCV infection of lymphocytes and to compare their expression with that in hepatoma JFH-1-prone Huh7.5 and JFH-1-resistant HepG2 cell lines. Based on the results from this comparative analysis, we subsequently examined involvement of OCLN in HCV lymphotropism.

4.2. Materials and Methods

4.2.1. Cell lines, primary cells and culture conditions.

Molt4 (CRL-1582) and Jurkat (TIB-152) T cell lines were provided by the ATCC (Manassas, VA). PM1 cells were acquired from the National Institutes of Health AIDS Research and Reference Program (Rockville, MD) and CCRF-CEM cells (CEM, ACC-240) from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Molt4, Jurkat and CEM cell lines were originally derived from patients with acute T lymphoblastic leukemia (125), while PM1 from a patient with acute cutaneous T cell lymphoma (241). The cells were cultured at 1 x 10^5 cells/well in 5 ml of medium containing RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine and 0.1 mM nonessential amino acids (Invitrogen). In some experiments, the cells were stimulated for 72 h with PMA (Sigma-Aldrich) at 50 ng/ml in the presence of 500 ng/ml ionomycin (Sigma-Aldrich), as reported (360).

PBMC were isolated from two healthy donors by gradient centrifugation in Ficoll-HyPaque (Pharmacia Biotech, Quebec, Canada) (315). Primary T lymphocytes were affinity-purified from monocyte-depleted PBMC by negative

selection using MACS magnetic microbeads (Miltenyi Biotec), as reported (319, 360). CD4+ and CD8+ T cell subsets were isolated from PBMC by affinity chromatography using AutoMacs Pro-Separator system (Miltenyi Biotec) following the manufacture's instruction. T cells and their subsets were 97-98% pure by flow cytometry analysis. For some experiments, PBMC and affinity-purified primary T cells were stimulated for 72 h with 5 μg/ml PHA (Sigma-Aldrich) in the presence of 20 IU/ml human rIL-2 (Roche Molecular Diagnostics), as described (243, 315).

HCV replicon AB12-A2FL Huh7 cell line, containing full-length HCV genotype 1b was provided by Drs Christopher Richardson and Joyce Wilson formerly from Ontario Cancer Institute, University of Toronto, Canada. The human hepatoma Huh7.5 cells were provided by Dr. Rodney Russell from Memorial University of Newfoundland, St. John's, NL Canada. Human hepatoma HepG2 (HB-8065) and HEK-293 (CRL-1573) cell lines were supplied by ATCC. The cells were maintained in DMEM supplemented with 10% FCS. PHH isolated by microperfusion of a healthy portion of the liver of a 58-year old male donor were purchased from Dominion Pharmakine (Derio-Bizkaia, Spain) (163).

4.2.2. HCV Infection.

Cultured T cell lines, either intact or stimulated with PMA/ionomycin for 72 h at 1 x 10^5 cells/well, supplemented with 2 ml of culture medium in 6-well plates were exposed for 24 h to ~1 x 10^5 vge of HCV genotype 1a-positive plasma pool prepared from patients with progressing CHC who were antiviral treatment naive. Infectivity of the pool was established in preliminary experiments using Molt4 and

Jurkat T cell lines and PHA-stimulated primary T lymphocytes, as previously reported (243, 360). Cells exposed under identical conditions to an equivalent volume of NHP served as negative controls. After exposure to HCV or NHP, the cells were extensively washed, suspended in fresh culture medium and cultured for 4-7 d.p.i., as reported in detail previously (360).

4.2.3. Silencing of OCLN by RNA Interference.

Lentiviral transduction particles encoding different human OCLN-specific shRNA (clones: TRCN0000158463, TRCN0000158804, TRCN0000159413, TRCN0000159613 and TRCN0000159771; designated as C-1 to C-5) with a titer between 2.9×10^7 and 3.9×10^7 infectious virus and 4.6×10^7 non-target control transduction particles (#SHC001V; designated as irr) were prepared by Sigma-Aldrich. 1×10^3 Jurkat cells in 100 µl RPMI 1640 medium was transduced at 4 multiplicity of infection (MOI) in 96-well plates in the presence of 2.5 µg/ml polybrene (Sigma-Aldrich). Cells were spun down at 4 ^{o}C for 10 min at 2,000 rpm, incubated at 37 ^{o}C for 24 h, and allowed to revive in fresh medium for another 24 h. Transduced Jurkat cells were selected in the presence of 2 µg/ml puromycin (Sigma-Aldrich) for 7 days. The level of OCLN expression was determined by real-time RT-PCR and Western blotting (see below). Only C-1, C-2 and C-3 were used in subsequent experiments, since C-4 and C-5 were found to be toxic to Jurkat cells. Subsequently, Jurkat cells transduced with C-1, C-2 and C-3 or with the control scrambled shRNA (i.e., irr), and the nontransduced cells were used as targets in HCV infection following the procedure described above.

4.2.4. RNA and Transcription to cDNA.

Total RNA was extracted using Trizol (Invitrogen). RNA used for quantification of cellular genes was treated with DNAse, as reported (316). cDNA was transcribed with Moloney murine leukemia virus RT (Invitrogen), as previously described (315, 360, 319).

4.2.5. HCV Detection.

HCV RNA positive and negative (replicative) strands were detected using cDNA derived from 1 or 2 μg and 2 or 3 μg, respectively, of total RNA and the strand-specific amplification conditions reported in detail previously (315, 360). The specificity of the signal detection and validity of controls were normally confirmed by NAH with ^{32}P-labeled rHCV UTR-E2 as a probe (315). The sensitivity of RT-PCR/NAH assay for HCV RNA-positive strand identification was <10 vge/ml (<2 IU/ml) or <5 vge/μg of total RNA, while that for HCV RNA-negative strand was 25-50 vge/μg of total RNA (315, 360).

4.2.6. Cloning of SR-B1 and tight junction gene sequences.

To facilitate quantification of the levels of expression of SR-B1 and TJ genes in the cells examined, cDNA transcribed from human PBMC or liver was amplified by PCR with primers shown in Table 4.1. Amplified fragments were cloned into the dual promoter vector PCRII using the TOPO TA cloning system (Invitrogen) and then excised and sequenced in both directions to confirm the gene specificity. The cloned fragments were used as quantitative standards to establish the sensitivity of real-time RT-PCR assays and to determine the copy numbers of the amplified gene sequences. Human CD5 and CD81 gene fragments were previously generated (360).

Table 4.1. Primer sequences used for real-time RT-PCR analysis

Gene	Forward primer	Reverse primer
CD5	5'-TCAAGCGTCAAAAGTCTGCC-3'	5'-AGCCACACTGGAGGTTGTTG-3'
CD81	5'-ACAAGGACCAGATCGCCAAG-3'	5'-AGTCAAGCGTCTCGTGGAAG-3'
OCLN	5'-TGCATGTTCGACCAATGC-3'	5'-AAGCCACTTCCTCCATAAGG-3'
SR-B1	5'-TCGCAGGCATTGGACAAACT-3'	5'-CTCCTTATCCTTTGAGCCCTTTT-3'
CLDN-1	5-TACTCCTATGCCGGCGACA-3'	5'-GACATCCACAGCCCCTCGT-3'
CLDN-4	5'-CATCGGCAGCAACATTGTCA-3'	5'-GCACCTTACACGTAGTTGCT-3'
CLDN-6	5'-AGAAGGATTCCAAGGCCCG-3'	5'-GATGTTGAGTAGCGGGCCAT-3'
Actin	5'-CATCCTCACCCTGAAGTACC-3'	5'-CATACTCCTGCTTGCTGATCC-3'
HPRT	5'-TGACACTGGCAAAACAATGCA-3'	5'-GGTCCTTTTCACCAGCAAGCT-3'

4.2.7. Real-Time RT-PCR.

The expression of SR-B1, CLDN-1, CLDN-4, CLDN-6, OCLN, CD81 and CD5 was quantified by real-time RT-PCR during 40 cycles using the LightCycler 480 (Roche Molecular Diagnostics). Reactions were performed in 10 µL volumes, each containing 2 µL cDNA derived from 50 ng DNAse-treated RNA using primer pairs shown in Table 4.1. Annealing temperature of $56^{\circ}C$ were used for amplification of OCLN, CD81, β-actin and HPRT, while $60\ ^{\circ}C$ annealing temperature for SR-B1, CLDN-1, -4 and -6 and CD5. Expression of genes of interest was evaluated in at least 4 separate experiments. β-actin and HPRT were used as loading controls. The sensitivity of the real-time RT-PCR assays was determined using serial, 10-fold dilutions of the cloned fragments of the genes of interest and was found to be between 10 to 10^2 copies/reaction. In regard to HCV RNA positive strand quantification, the copy numbers were enumerated by real-time RT-PCR using 10-fold serial dilution of rHCV UTR-E2, as described previously (314).

4.2.8. Western Blotting.

Cells were treated with ice-cold RIPA buffer (1% NP-40, 0.5% DOC, 0.1% SDS, 150 mmol/L NaCl in 50 mmol/L Tris, pH 8.0; Sigma-Aldrich) and cellular debris removed by centrifugation. Proteins were separated by SDS-PAGE at 20 µg protein/lane or as indicated and then blotted onto a nitrocellulose membrane (Amersham Biosciences) by wet transfer using the Bio-Rad SD cell system (Bio-Rad) (274). The blots were incubated with 5% skimmed milk in Tris-buffered saline, pH 7.4, for 1 h at room temperature and exposed overnight at 4°C to

113

working dilutions of appropriate antibodies diluted in the same blocking buffer. Mouse mAb to SR-B1 (clone 25/CLA-1) and OCLN (clone 19) were purchased from B.D. Biosciences, rabbit antibodies to CLDN-1 and CLDN-2 were from Invitrogen, while rabbit antibodies to CLDN-6 (ab75055) and CD5 (ab52964) were acquired from Abcam Inc. (Cambridge, MA). In some instances, the presence of HCV envelope protein in infected lymphocytes and control AB12-A2FL Huh7 cells was detected with anti-E2 mouse mAb provided by Dr. Arvind Patel, University of Glasgow, Glasgow, United Kingdom), as reported (360). Detection of β-actin with specific rabbit mAb (Sigma-Aldrich) served as protein loading control. Reactions were developed with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG F(ab')$_2$ antibodies (Jackson ImmunoResearch) and visualized using an ECL detection kit (Sigma-Aldrich). Protein signals were quantified by densitometry using the computer-assisted image processing application ImageJ from the National Institute of Health (150). For comparative evaluations, the density of the protein signals detected in Huh7.5 cells were taken as 100%, except for CD5 protein for which the signal detected in affinity-purified primary T cells was used as 100% (see Figure 4.5).

4.2.9. Statistical Analyses.

Results were analyzed by a one way analysis of variance or unpaired Student t test with Welch's correction using GraphPad Prism software (GraphPad Software Inc.). Differences between experimental conditions were considered to be significant when two-sided P values were below or equal to 0.05.

4.3. Results

4.3.1. Molt4 and Jurkat T Cells and Primary T Lymphocytes Demonstrate Comparable Susceptibility to Infection with Wild-Type HCV.

Primary human T cells, PBMC from which the T cells were derived, and T cell lines, including HCV-prone Molt4 and Jurkat cells and HCV-resistant PM1 and CEM cells (360), were exposed to patient-derived HCV and subsequently examined for expression of HCV RNA, as described in Materials and Methods. As the results showed (Figure 4.1), Molt4 and Jurkat T cells displayed HCV RNA positive strand at levels comparable to those in primary T lymphocytes and PBMC (P=0.6), while CEM cells where entirely nonreactive and PM1 cells only occasionally demonstrated a low level, *i.e.,* in one of 3 experiments (Figure 4.1A). These results were compatible to those previously reported, including the observation that PM1 cells have potential to become prone to HCV infection when activated (360).

Similarly as HCV RNA positive strand, the negative strand was detected at similar levels in Molt4 and Jurkat T cells and in primary T cells and PBMC after exposure to the same HCV inocula, as illustrated for one of the inoculum in Figure 4.1B. In contrast, PM1 and CEM cells, which have been found in the previous study to be resistant to HCV infection (360), were HCV RNA replicative strand nonreactive following exposure to the same infectious virus.

A
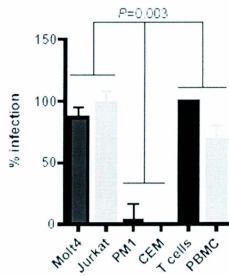
B

Figure 4.1

Figure 4.1. Identification of HCV in T cell lines and primary T lymphocytes investigated. Molt4, Jurkat, PM1 and CEM T cell lines, affinity-purified primary T cells and PBMC were exposed to plasma-derived, wild-type HCV and subsequently evaluated for the expression of HCV RNA positive strand by real-time RT-PCR and HCV RNA negative strand by RT-PCR/NAH. T cell lines were analyzed after 7 d.p.i. Primary human T cells and PBMC isolated from healthy donors were exposed to the same virus as T cell lines and analyzed for HCV after 14 d.p.i., as described in Materials and Methods. (A) Quantification of HCV RNA positive strand. Data represent means from 3 separate experiments and are presented as percentage of the expression found in primary T cells that was taken as 100%. (B) Representative example of the detection of HCV RNA negative strand by RT-PCR/NAH in the cells from one of 3 experiments shown in A. Synthetic HCV RNA positive (pos) and negative (neg) strands at 10^5 copies/reaction confirmed the assay specificity for detection of the virus RNA replicative (negative) strand. Water amplified in direct (DW) and nested (NW) reactions and a mock extraction served as contamination controls. The positive signals showed the expected 244-bp oligonucleotide fragments.

4.3.2. Uniformly High Expression of CD81 and Aberrant Transcription of SR-B1 Characterize T Cells Either Prone or Resistant to HCV Infection.

Comparative analysis revealed that CD81 was ubiquitously expressed at similarly high levels in primary T cells, their CD4+ and CD8+ subsets and in all T cell lines investigated, independent of whether they were or were not susceptible to HCV infection, as well as in Huh7.5, PHH, and control HEK-293 cells (Figure 4.2A). The exception was HepG2 cells. Thus, CD81 mRNA levels typically ranged between 10^6 and 10^7 copies per μg of total RNA, but HepG2 cells transcribed the gene at about 10^3-fold lower levels (Figure 4.2A).

Both HCV-susceptible and resistant T cell lines expressed SR-B1 at significantly higher ($P=0.007$) levels than primary T cells, purified CD4+ or CD8+ T lymphocyte subsets or total PBMC. In general, primary T cells, their subsets and PBMC transcribed SR-B1 at the mean level of 3.9 x 10^3 $\pm$ SEM 628 copies/μg RNA, which was 2 to 3-fold lower ($P=0.007$) than that detected in the T cell lines (Figure 4.2B). Moreover, HCV-resistant PM1 and CEM cells transcribed significantly more ($P=0.001$) SR-B1 compared to virus-prone Molt4 and Jurkat T cells. In regard to Huh7.5 and HepG2 hepatoma cells, no significant difference in SR-B1 expression was found (Figure 4.2B). Further, PHH expressed about 10-fold less SR-B1 mRNA than Huh7.5 cells ($P=0.001$) (Figure 4.3B). There also was significantly lower transcription of SR-B1 in HCV-prone Molt4 and Jurkat cells ($P=0.0001$) and in primary T cells, their subsets and PBMC ($P=0.0001$) than in Huh7.5 cells.

A
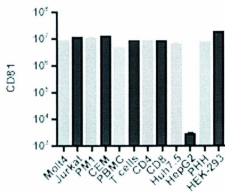
B
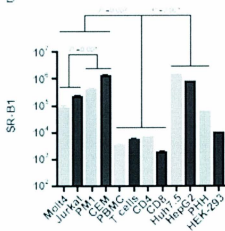
Figure 4.2

Figure 4.2. Quantification of CD81 and SR-B1 transcription in T cell lines, primary T cells, their CD4+ and CD8+ subsets, total PBMC, hepatoma-derived cell lines, and human primary hepatocytes. (A) Expression levels of CD81. (B) Expression levels of SR-B1. An equivalent of 50 ng DNAse-treated RNA was used as template for real-time RT-PCR quantifications. The results represent means $\pm$ SEM from 4 or more independent experiments and are presented as copies/μg total RNA. For other details see Materials and Methods.

It is of note that the expression levels of house-keeping ß-actin and HPRT genes were closely comparable in all cell types tested, with one minor exception. Thus, the ß-actin mRNA levels ranged between 10^7 to 10^8 copies/µg total RNA with no statistically significant variations except PHH, in which approximately 10-fold lower transcription level was detected (data not shown). For HPRT, highly comparable mRNA levels were detected in all cells examined, including PHH.

4.3.3. Expression of CLDN-1, -4 and -6 Does Not Coincide with T Cell Susceptibility to HCV Infection.

CLDN-1 mRNA level was found to be high in Huh7.5 cells ($3 \times 10^5 \pm$ SEM 2×10^4 copies/µg total RNA), while HepG2 and PHH showed 10 to 100-fold lower copy numbers (P=0.002 and P=0.003, respectively) (Figure 4.3A). In contrast, HCV-susceptible Molt4 and Jurkat T cells essentially did not express CLDN-1, while PM1, but not CEM, transcribed CLDN-1 at the level greater than 10^4 copies/µg RNA. Primary T cells, their CD4+ and CD8+ subtypes and PBMC demonstrated CLDN-1 mRNA levels between 1×10^2 and 1.5×10^2 copies/µg RNA, which were significantly lower (P=0.0001) than that detected in Huh7.5 cells (Figure 4.3A).

The level of CLDN-4 expression was closely comparable in both HCV-prone and HCV-resistant T cell lines, as well as in primary T cells or PBMC, and the differences in their transcription levels were not greater than 10-fold (Figure 4.3B). CD4+ and CD8+ T cell subtypes transcribed approximately 10-fold more CLDN-4 than the total primary T cells and these levels were comparable to that found in Huh7.5 cells.
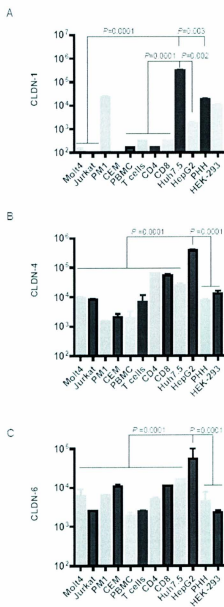
Figure 4.3

Figure 4.3. Quantification of CLDN-1, CLDN-4 and CLDN-6 expression in T cell lines and primary T lymphocytes, their subsets, PBMC, hepatoma cells and primary hepatocytes. (A) Expression levels of CLDN-1; (B) Expression levels of CLDN-4, and (C) Expression levels of CLDN-6. The results are presented as mean copies/µg total RNA from at least 4 independent experiments.

HepG2 transcribed CLDN-4 most efficiently and at levels which were significantly higher (P=0.0001) than those detected in other cell types tested (Figure 4.3B). PHH showed CLDN-4 mRNA at the level comparable (P=0.7) to that identified in primary T cells and HCV-susceptible Molt4 and Jurkat T cells, which were approximately 100-fold lower than the level found in HepG2 cells (P=0.0001).

In regard to CLDN-6, no significant difference (P=0.14) was found in this gene expression between HCV-prone and resistant T cell lines and Huh7.5 cells, while primary T cells, PBMC and PHH tended to transcribe approximately 10-fold lower levels than Huh7.5 cells (P=0.01) (Figure 4.3C). As in the case of CLDN-4, HepG2 cells expressed the highest levels of CLDN-6 among the cells tested (P=0.0001).

4.3.4. High Expression of CD5 in Primary and Cultured T Cells and OCLN in T Cell Lines Coincides with Susceptibility to HCV Infection.

In regard to CD5, which transcription is lymphocyte restricted and the presence of CD5 protein has been identified to be essential for infection of T cells with HCV (360), it was confirmed that the gene expression was significantly greater (P=0.0001) in HCV-prone primary T lymphocytes, their CD4+ and CD8+ subsets, PBMC and in Molt4 and Jurkat T cell lines, as well as in HEK-293 cells transfected with human CD5 serving as a control, than in virus-resistant PM1 and CEM T cells (Figure 4.4A). Furthermore, PHH and HepG2 cells did not transcribe this gene, while hepatoma Huh7.5 cells displayed CD5 mRNA at the mean level of $9.6 \times 10^3 \pm$ SEM 1.6×10^3 copies/µg RNA (Figure 4.4A).

A
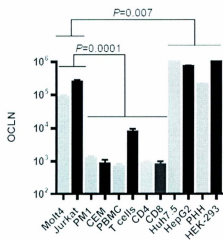
B
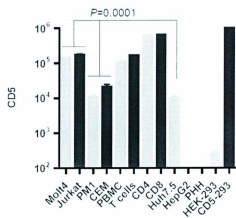
Figure 4.4

Figure 4.4. Quantification of OCLN and CD5 transcription in T lymphocyte lines and other cell types investigated in this study. (A) OCLN expression levels. (B) Expression levels of CD5. The results represent mean copies/µg total RNA from at least 4 independent experiments.

OCLN mRNA was expressed at high levels (10^5-10^6 copies/µg RNA) in Huh7.5, HepG2, PHH and HEK-293 cells, while it was transcribed at approximately 10-fold lower ($P=0.007$) levels in HCV-susceptible Molt4 and Jurkat cells (Figure 4.4B). However, HCV-resistant PM1 and CEM cells, as well as PBMC and CD4+ and CD8+ T cell subsets transcribed OCLN at 10^2-10^3-fold lower ($P=0.0001$) levels than the susceptible T cell lines (Figure 4.4B). The total primary T cells demonstrated approximately 10-fold greater expression than their subsets or total PBMC and the reason behind that is not yet clear.

4.3.5. Display of CD5 and OCLN Proteins is Most Closely Associated with T Cells Susceptibility to HCV Infection.

T cell lines, primary T cells, total PBMC, as well as Huh7.5 and HepG2 cells were examined for the display of the HCV candidate receptor proteins by probing with appropriate antibodies. In regard to SR-B1, this protein was displayed in HCV-resistant CEM (~65% of the signal detected in Huh7.5 cells) and PM1 (~40%), and at evidently lower levels in HCV-prone Molt4 (11%) and Jurkat (20%) T cells (Figure 4.5A). In contrast, HepG2 displayed approximately 30% more of SR-B1 protein and control HEK-293 cells transfected with human CD5 about twice less than Huh7.5 cells. These results overall well corresponded to SR-B1 mRNA levels detected in the same cell types (Figure 4.2B). However, primary T cells, their CD4+ and CD8+ subsets and total PBMC were found to be SR-B1 protein negative (Figure 4.5A and B). This indicated that the SR-B1 mRNA detected in these cells by real-time RT-PCR, ranging between 10^3 and 10^4

copies/µg RNA (Figure 4.2B), were not translated to the protein amounts detectable by Western blotting (Figure 4.5).

In regard to the tight junction proteins, CLDN-1 protein was detected only in HCV-resistant PM1 T cells (~30% of the signal exhibited by Huh7.5 cells) and in HepG2 hepatoma cells (~170% of the signal identified in Huh7.5 hepatoma cells), and at a lower level (~25%) by control HEK-293 cells transfected with CD5 (Figure 4.5A and B). Notably, primary T cells, their CD4+ and CD8+ subsets and total PBMC, as well as HCV-prone Molt4 and Jurkat cells were entirely CLDN-1 protein nonreactive (Figure 4.5A and B). This was consistent with CLDN-1 mRNA data which showed the levels below 10^3 copies/µg RNA in primary T cells and undetectable levels in HCV-prone Molt4 and Jurkat T cells (Figure 4.3A). On the other hand, CLDN-4 and CLDN-6 proteins were detected in all cell types at the levels which were essentially similar to that detected in Huh7.5 cells (Figure 4.5A). Again, CLDN-4 and CLDN-6 mRNA levels overall well-corresponded to the protein signals identified in individual cell types (Figure 4.3A and B).

In regard to OCLN protein, HCV-susceptible Molt4 and Jurkat cells displayed approximately 90% and 210% of the protein signal detected in Huh7.5 cells, respectively (Figure 4.5A). However, primary T lymphocytes presented the protein at about 10-fold lower level than Huh7.5 cells, while PBMC were nonreactive. Furthermore, PM1 and CEM T cells exhibited trace levels of OCLN comparing to Huh7.5, i.e., approximately 5% of the signal detected in Huh7.5 cells (Figure 4.5A).
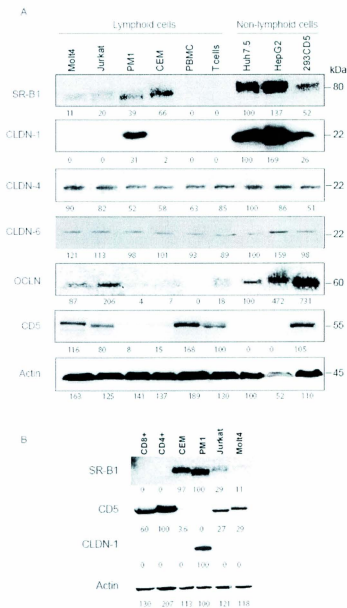
Figure 4.5

Figure 4.5. Differential display of HCV candidate receptor proteins in HCV-prone and resistant T cell lines, primary T cells and their subsets, PBMC and hepatoma cell lines. Proteins were separated at 25 μg/well by SDS-PAGE and after blotting probed with appropriate antibodies. β–actin served as a loading control. (A) Identification of SR-B1, CLDN-1, CLDN-4, CLDN-6, OCLN, CD5 and actin in T cell lines, primary T cells, PBMC, Huh7.5, HepG2 and in control HEK-293 cells transfected with human CD5. The level of a given protein in a particular cell type is presented as a relative percentage of this protein signal detected in Huh7.5 that was taken as 100%, except CD5 protein for which display in primary T cells was taken as 100%. (B) Detection of SR-B1, CD5, CLDN-1 and actin in CD4+ and CD8+ subsets of primary T cell and in T cell lines investigated. The level of each protein in a particular cell type was measured at least twice and was presented as a relative percentage of the protein density signal detected in PM1 cells that was taken as 100%.

HepG2 and control CD5-transfected HEK-293 demonstrated the protein at levels more than 4-fold and 7-fold greater than Huh7.5 cells, respectively (Figure 4.5A). The density of OCLN protein signals appeared to well coincide with the gene expression levels detected in the same cells (Figure 4.4A).

CD5 protein was identified in HCV-susceptible Molt4 and Jurkat T cell lines, primary total T cells and PBMC (Figure 4.5A) and in T cell subsets (data not shown) and at trace levels in PM1 but not in CEM cell lines. Huh7.5 and HepG2 cells were CD5 protein nonreactive, while control CD5-transfected HEK-293 cells showed the protein signal at a level comparable to that detected in primary T cells and T cell lines prone to HCV (Figure 4.5A). In general, there was a good agreement between CD5 protein display and the CD5 gene transcriptional activity identified in the cells investigated.

4.3.6. HCV Infection Downregulates CD81 and OCLN but Augments CD5 Expression in Virus-Prone T Cell Line.

Jurkat and Molt4 T cells exposed to wild-type HCV or NHP were cultured for 5 days and examined for expression of CD81, SR-B1, OCLN and CD5 by the appropriate real-time RT-PCR assays. The result showed a significant decrease in the level of transcription of CD81 (P=0.001) and OCLN (P=0.0008) in HCV-infected cells comparing to those uninfected, as was shown for Jurkat cells (Figure 4.6). In contrast, CD5 mRNA levels were significantly (P=0.01) augmented in infected cells, while that of SR-B1 remained unchanged after infection (P=0.54) (Figure 4.6).
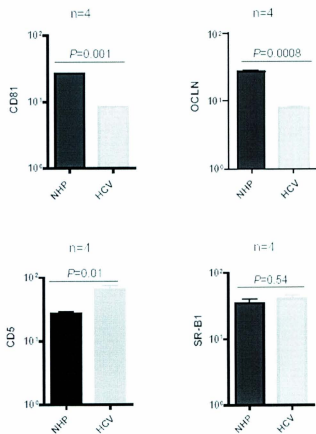
Figure 4.6

Figure 4.6. Transcription of, OCLN, CD81, CD5 and SR-B1 in Jurkat cells infected with wild-type HCV. Jurkat T cells infected with plasma-derived HCV or exposed to the equivalent amount of normal human plasma (NHP) were cultured for 5 days and then evaluated for the expression of (A) CD81, (B) OCLN, (C) CD5 and (D) SR-B1 by appropriate real-time RT-PCR assays. The results are presented as ratios of mean copy numbers/μg total RNA from 4 independent experiments of a given gene to actin.

This differential effect of HCV infection on the expression of CD81, OCLN and CD5, but not SR-B1 gene, provided supporting evidence that the respective proteins might be engaged in the infection process induced by HCV in T cells.

4.3.7. Knockdown of OCLN Inhibits HCV Replication in HCV-Prone T Cell Line.

Since the data on the gene and the protein expression implied that OCLN may contribute to the susceptibility of Molt4 and Jurkat T cells to HCV infection, although rather unlikely to the receptiveness of primary T lymphocytes to this virus, the effect of the suppression of OCLN transcription on the infectivity of Jurkat cells with wild-type HCV was investigated. In this regard, Jurkat cells were transduced with OCLN-shRNA C-1, C-2 or C-3 or with the control irrelevant shRNA, and then exposed to HCV. The results showed that the level of OCLN expression was significantly (P=0.0001) downregulated in OCLN-shRNA-transduced cells compared to control cells treated with irrelevant shRNA (Figure 4.7A). It also is of note that mRNA levels of CD81 (Figure 4.7B), SR-B1 (Figure 4.7C) and CD5 (Figure 4.7D) were not affected by treatment with either OCLN-shRNA or irrelevant shRNA. Further, the display of OCLN protein was inhibited by ~50% after transduction with OCLN-shRNA comparing to the control cells (Figure 4.7E). Most interestingly, although the knocking down of the OCLN expression had no effect on the level of HCV RNA positive and negative (replicative) strands detected after exposure to virus, comparing to the control cells transduced with irrelevant shRNA (Figure 4.7F and G, respectively), the display of HCV E2 protein was totally inhibited in cells treated with OCLN-shRNA

134

but not in those with irrelevant shRNA (Figure 4.7H). This suggested that in fact downregulation of OCLN severely affected HCV replication, most likely on the posttranscriptional level.

4.4. Discussion

Despite that HCV is a hepatotropic virus and infection of hepatocytes is chiefly responsible for manifestations of hepatitis C, substantial experimental and clinical evidence indicate that HCV replication can also be supported by cells of the immune system, including T lymphocytes. The HCV RNA negative strand, representing virus genome replicative intermediate, viral proteins and virus sequences distinct from those occurring in the liver have been identified in total PBMC, T and B lymphocytes, CD4+ and CD8+ T cell subsets, and in monocytes from patients with CHC (93, 203, 216, 299, 314, 339, 413) and from individuals with clinically apparent sustained virological response to FNα or IFNα-RBV therapy for CHC (312, 314-316, 319, 339). HCV infection resulting in production of infectious virus has also been documented in de novo infected mitogen-stimulated normal human T lymphocytes (242, 243, 437), cultured B cells (23, 287) and monocytes (93, 214). A recent study from this laboratory has shown that naturally occurring HCV utilizes CD5, a lymphocyte unique glycoprotein, to infect T cells (360). This protein is not displayed by PHH or hepatoma-derived cell lines, indicating that HCV uses a distinct molecular strategy to enter into hepatocytes and lymphocytes.

Figure 4.7

136
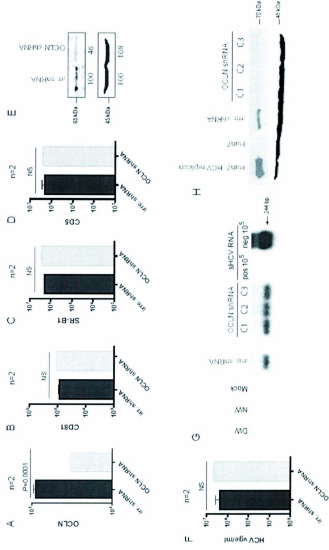
Figure 4.7. Effect of OCLN knockout on infection of Jurkat T cells with wild-type HCV. Jurkat cells transduced with either OCLN-specific shRNA or control irrelevant (irr) shRNA were infected with wild-type virus as described in Material and Methods. The levels of transcription of (A) OCLN, (B) CD81, (C) SR-B1 and (D) CD5 were evaluated at 7 d.p.i. using appropriate real-time RT-PCR assays. The data are presented as mean copy numbers/µg total RNA from 2 independent experiments. (E) Western blot identification of OCLN protein in Jurkat cells transduced with either irr-shRNA or OCLN shRNA. β–actin served as a loading control. The level of OCLN protein in the cells transduced with OCLN shRNA is presented as a relative percentage of the OCLN protein signal detected in control that was taken as 100%. (F) Quantification of HCV RNA positive strand in Jurkat cells transduced with either control irr-shRNA or three separate clones of OCLN shRNA and subsequently infected with wild-type HCV. The data represent the mean copy (vge) numbers $\pm$ SEM from 2 independent experiments. (G) Detection of HCV RNA negative (replicative) strand in Jurkat cells transduced with either irr-shRNA or three different clones (C1-C3) of OCLN shRNA and infected with HCV, as indicated in (F). Intact Jurkat cells not transduded or infected with HCV were used as a negative control. DW, NW and mock as described in the legend to Fig 4.1. (H) HCV E2 protein detection in Jurkat cells transduced with irr-shRNA or with three OCLN shRNA clones (C1-C3) and then infected with HCV by Western blotting. Huh7 replicon expressing and naïve Huh7 cells served as HCV E2 positive and negative controls, respectively. β-actin served as a loading control.

Subsequently, this may contribute to different kinetics of virus replication and persistence in these two very distinct cell types. As in other chronic viral infections (72, 302), HCV-infected T cells constitute a reservoir in which virus can persist independently from its progeny multiplying in hepatocytes. In this regard, distinct HCV variants have been identified in the different compartments of virus occurrence, *i.e.*, liver and circulating lymphomononuclear cells (93, 94, 296, 296, 299, 312), and HCV has been detected in PBMC but not in the livers of individuals with persistent low-level (occult) infection (299, 312, 312, 339). It has also been suggested that HCV variants inhabiting lymphatic cells can reinfect newly transplanted livers (82, 216, 340). Furthermore, HCV immune evasion and drug resistance may result, at least in part, from the virus' ability to persistently replicate in the lymphoid cell compartment, as in the case of hepatitis B virus infection in patients on suppressive antiviral therapy (73).

The host's factors facilitating HCV infection of human cells were mainly recognized using HCV JFH-1 clone or HCVpp and human hepatoma cell lines. The JFH-1-Huh7.5 model provides a robust system for HCV replication. In this system, JFH-1 HCVcc infect hepatoma Huh7.5 cells which are characterized by a deficient innate immune response due to the lack of RIG-I expression, perturbed signalling and endocytic pathways, and dysfunctional mitochondria (51, 52, 83, 188, 205, 207). On the other hand, the genetic variance between JFH-1 or related HCV clones and the wild-type HCV may additionally affect the nature and the strength of interactions with molecules mediating HCV cell attachment and entry, increasing the uncertainty to what degree the initial stages of infection of

primary hepatocytes with wild-type virus are mirrored in the JFH-1-Huh7.5 system (403). In this regard, JFH-1 produced in Huh7.5 cells have failed to infect human PBMC or primary T cells in culture (255), which are prone to wild-type HCV in both *in vivo* and *in vitro* conditions (243, 314). Also, JFH-1 HCVcc were not able to efficiently infect chimpanzees (193) or polarized PHH in MPCCs despite that these cells display all proposed HCV receptors (324).

Previously, we have found that some T cell lines, such as CEM and unstimulated PM1, are resistant to infection with wild-type HCV, in contrast to Molt4 and Jurkat cells which can support propagation of wild-type virus (360). In the current study, we have also established that Molt4 and Jurkat cells are susceptible to infection and maintain HCV replication at approximately the same levels as primary T lymphocytes after exposure to the same HCV inocula. In order to identify cellular factors, other than CD5 and CD81 (360), which determine T cell susceptibility to HCV, we analyzed expression of the proposed HCV candidate receptors in HCV-prone and resistant T cells, and compared them to JFH-1-susceptible Huh7.5 and JFH-1-resistant HepG2 hepatoma cells. This comparative analysis revealed that some of the postulated HCV receptors are not involved in determining HCV lymphotropism, while others, such OCLN, may contribute to infectivity of T cell lines but seemingly unlikely primary T cells.

Considering distribution of the individual HCV receptors proposed in the cells tested, the expression of tetraspanin CD81, which is known to require other cellular factors to mediate HCV binding and entry into hepatocytes (77), was detected at comparable levels in all lymphocytic and non-lymphocytic cells

examined, except HepG2 (see Fig. 2A). Although CD81 was found to contribute to infection of T cells, based on the fact that antibodies against CD81 inhibited infection with wild-type virus of both primary T cells and Molt4 and Jurkat cells (243, 360), CD81 is not the limiting factor of the infection since HCV-resistant PM1 and CEM cells express CD81 at similar levels as those prone to this virus.

Another HCV candidate receptor is SR-B1, expressed at high levels in liver tissue and is considered an important HCV entry factor. Studies using soluble HCV E2 protein and HepG2 cells lacking CD81 but expressing SR-B1 showed infection of these cells. This interaction was found to be highly selective since neither mouse SR-BI nor the closely related human scavenger receptor CD36 was able to bind E2 (363). However, although antibodies to SR-B1 markedly inhibited the binding of E2 or HCV-like particles to primary tupaia hepatocytes, they were not able to block infection with wild-type, plasma-derived HCV (24), implying an important limitation of the surrogate soluble E2 or HCV-like particles when applied for recognition of the actual wild-type virus-host cell interactions. Considering lymphocytes, a significantly (P=0.007) lower expression of SR-B1 was detected in T cell lines, primary T cells and PBMC, which was comparable to that identified in Huh7.5, HepG2 and PHH. Moreover, HCV-resistant PM1 and CEM cells transcribed significantly (P=0.001) more SR-B1 than HCV-susceptible Molt4 and Jurkat T cells. Also, despite being susceptible to HCV, primary T cells, their subsets and PBMC did not display SR-B1 protein. This conclusively excluded the contribution of this protein to HCV lymphotropism. This finding appears to be consistent with that reported by others where soluble

140

truncated HCV E2 protein and PBMC, B cells, monocytes and dendritic cells as targets were used (420). Taken together, the data from this and previous studies imply that while CD81 is involved, likely as a co-receptor, SR-B1 is not required for recognition of T lymphocytes by HCV.

Although a pivotal contribution of CD5 to HCV T cell tropism has been previously delineated (360), we re-analyzed expression of CD5 in the context of other HCV candidate receptors in the current study. In general, the data obtained remained in agreement with those previously reported (360) and showed CD5 gene transcription at significantly (P=0.0001) greater levels in HCV-susceptible versus resistant T cells. This difference coincided with the detection of CD5 protein in HCV-susceptible but not in HCV-resistant T cells, providing direct evidence for the association between T cell CD5-positivity and their susceptibility to infection. This was verified in our previous study by inhibition of HCV infection with CD5-specific antibody and shRNA, and by making CD5-deficient HEK-293 fibroblast permissive to wild-type HCV infection after their transfection with human CD5 cDNA (360). In contrast to T cells, Huh7.5 and HepG2 cells did not display CD5 protein, as previously reported (360).

Recent studies have demonstrated the role for TJ proteins in the susceptibility of Huh7.5 cells to infection with JFH-1 clone (170, 232). However, the expression of these proteins in lymphoid cells and their potential role in determining permissiveness of these cells to HCV remained unknown. We compared the levels of CLDN-1, CLDN-4, CLDN-6 and OCLN mRNAs and proteins in the cells examined. CLDN-1, a protein previously reported to be an

HCV entry factor mediating infection of hepatic cells, as determined by examining HCVpp and HCVcc preparations and Huh7.5 cells (102), was expressed only by hepatoma cells and PM1 T cells, which are resistant to HCV infection unless activated (360). Thus, the results clearly showed that CLDN-1 is not involved in determining susceptibility of primary T lymphocyte to HCV. Further, CLDN-4 and CLDN-6 mRNA and proteins were identified at comparable levels in both HCV-susceptible and resistant cells, making their potential contribution to HCV infectivity towards T lymphocytes highly unlikely. In this regard, others have also shown that human PBMC transcribe CLDN-6 but not CLDN-1 (429), which is consistent with our findings. The study mentioned also reported that human hepatoma cell line Bel7402 was recognized by HCVpp, although the cells did not express CLDN1 (429). This indicates that CLDN-1 is dispensable not only for infection of lymphocytes but also for infection of non-lymphoid cells.

OCLN, which is another member of the TJ protein family, was shown to mediate infection of Huh7.5 cells by HCVcc and to be likely involved at the late stage of HCV entry (323). Although expressed at significantly higher levels in hepatic cells, OCLN was both transcribed and translated in HCV-prone Molt4 and Jurkat T cells at much greater levels than in HCV-resistant PM1 and CEM cells and, importantly, primary T cells and PBMC. To confirm potential relevance of OCLN to T cell susceptibility to HCV infection, the effect of OCLN-knockout on the level of HCV replication in Jurkat T cells was investigated. While both HCV RNA positive and negative strands were detected at comparable levels in the cells transduced with OCLN-shRNA or irrelevant-shRNA, virus E2 protein was

detected only in the control cells treated with irr-shRNA. This observation appears to be consistent with the previous report which showed no effect of OCLN silencing on the HCV RNA level in Huh7 cells carrying genomic or subgenomic HCV replicons (32). The reason why HCV E2 protein was absent in the cells with silenced OCLN is not clear. It might be due to the interaction between E2 glycoprotein and OCLN and to the possibility that OCLN facilitates translocation of the viral protein to the appropriate intracellular compartment and/or prevents its premature release from the infected cell. In this regard, HCV E2 and OCLN have been found to partially co-localize in hepatocyte endoplasmic reticulum and co-immunoprecipitate (31). Overall, the data obtained imply that OCLN can posttranscriptionally affect the HCV E2 protein display without influencing the virus genome transcription in infected T cells.

In agreement with the previous observations, where down regulation of OCLN and CLDN-1 was identified in Huh7 cells after infection with HCVcc (232), the level of OCLN and CD81 expression significantly decreased in Jurkat T cells infected with wild-type HCV. Meanwhile, the transcription of CD5 became augmented and that of SR-B1 unchanged. These findings can be interpreted as an indication that HCV downregulates OCLN and CD81 molecules to prevent superinfection, as it has been postulated before (232), providing also evidence for the importance of these proteins in the HCV infection process in lymphocytes. On the other hand, upregulation of CD5 expression may protect cells from apoptosis and allow for more prolonged and robust HCV replication in the cells

infected (81, 134, 398, 439). Certainly, further investigations are necessary to assess validity of these hypotheses.

The data obtained in this study contribute to our understanding of the nature of HCV lymphotropism, which remains a vaguely recognized property of naturally propagating virus. Overall, they imply that the virus utilizes a distinct set of cellular factors to gain entry into lymphocytes in comparison to that expected to mediate HCV tropism towards human hepatocytes. Among the molecules analyzed, only expression of CD5 is uniquely restricted to lymphocytes, implying a central role of this glycoprotein in HCV lymphotropism. No association between expression of SR-B1 or CLDN-1 and the susceptibility of T cells to HCV infection was found, while contribution of CD81 as a potential co-receptor was confirmed. The role of OCLN as an HCV entry factor to T cells remains ambiguous since this protein is essentially absent or displayed at trace quantities in primary T cells but is robustly presented by T cell lines known to be susceptible to HCV. Considering a surprisingly large number of cellular receptors implicated in HCV entry to hepatocytes, it cannot be excluded that other, not yet identified molecules are involved in the HCV tropism towards both T cells and other cell subsets in the human lymphatic system.

CHAPTER FIVE:

WILD-TYPE HEPATITIS C VIRUS AND HCV JFH-1 CLONES CONTRASTINGLY DIFFER IN THEIR ABILITY TO INFECT HUMAN LYMPHOCYTES AND HEPATOMA CELLS.

5.1. Introduction:

Hepatitis C virus (HCV) is a hepatotropic virus that also infects cells of the immune system. HCV clones cultivated in human hepatoma Huh-7.5 cells have significantly advanced our understanding of HCV replication and candidate hepatocyte receptors. However, naturally occurring wild-type HCV, in contrast to the HCV JFH-1 clone, is unable to infect Huh-7.5 cells, while it can replicate in human primary T cells and selected T cell lines as recently documented (360). To better understand this incongruity, we examined the susceptibility of primary T cells, PBMC and T cell lines to infection with wild-type HCV, the classical HCV JFH-1 and a cell culture-adapted JFH1$_T$ known to be highly infectious to Huh-7.5 cells. We also tested whether Huh-7.5 cells are prone to virus readily infecting T lymphocytes. The results revealed that while primary T cells and Molt4 and Jurkat T cell lines were susceptible to wild-type HCV, they were resistant to infection with either JFH1$_T$ or JFH-1. However, JFH1$_T$ clone interacted more firmly, although non-productively, with the cells than JFH-1. Further, Huh-7.5 cells robustly supported replication of JFH1$_T$ but not wild-type virus despite using highly sensitive detection assays. In conclusion, JFH-1 and JFH1$_T$ clones were unable to establish productive infection in human primary T cells, PBMC and T cell lines known to be prone to wild-type virus, while Huh-7.5 cells were totally

totally resistant to infection with patient-derived HCV. The data showed that the ability to infect lymphocytes is a distinctive characteristic of native virus but not laboratory adapted HCV clones.

HCV is a hepatotropic virus that also infects cells of the immune system. Studies applying HCV clones cultivated in human hepatoma Huh7.5 cells have significantly advanced our understanding of the HCV replication strategy and candidate receptors potentially mediating infection of human hepatocytes. However, naturally occurring wild-type HCV, in contrast to JFH-1 clone, is unable to infect Huh7.5 cells, while it can enter and replicate in human primary T cells, PBMC, and selected human T cell lines. In order to better recognize this incongruity, we examined the susceptibility of affinity-purified primary T cells, total PBMC and T cell lines to infection with wild-type HCV, the classical HCV JFH-1 strain and its triple mutant $JFH1_T$ known to be highly infectious to Huh7.5 cells. We also tested whether Huh7.5 cells are prone to patient-derived virus readily infecting T cells when measured by highly sensitive HCV detection assays. The results revealed that while primary T cells and Molt4 and Jurkat T cell lines were susceptible to wild-type virus, as previously reported, they were resistant to infection with either $JFH1_T$ or JFH-1. Nonetheless, $JFH1_T$ clone more firmly, although non-productively, interacted with the lymphoid cells than JFH-1, as quantification of the cell-associated HCV RNA positive strand revealed. On the other hand, Huh7.5 cells robustly supported propagation of $JFH1_T$ but not at all wild-type virus despite employing highly sensitive detection assays. In conclusion, JFH-1 and $JFH1_T$ clones were unable to establish productive

146

infection in human primary T cells, PBMC and T cell lines known to be prone to wild-type virus, while Huh7.5 cells were totally resistant to infection with patient-derived HCV. The data showed that the ability to infect lymphocytes is a distinctive characteristic of native virus but not laboratory adapted HCV clones.

HCV is a small enveloped RNA virus belonging to the *Flaviviridae* family that affects as a symptomatic chronic infection over 170 million people worldwide (2). This form of infection frequently progresses to cirrhosis and hepatocellular carcinoma (396).

In addition to this clinically apparent chronic infection, HCV can persist as a silent, essentially asymptomatic infection in the liver and the immune system which is usually detectable by thorough testing for HCV genome expression using nucleic acid amplification assays of enhanced sensitivity (64, 312-315, 339)]. Overall, the evidence accumulated in the last decade indicates that HCV not only infects hepatocytes but also enters and replicates in immune cells, including T lymphocytes (42, 86, 94, 151, 222, 304).

Since the discovery of HCV (67), there have been significant efforts to establish a robust *in vitro* cell culture system and a small animal model supporting the entire replication cycle of native, naturally occurring virus (269, 403). With the isolation and cloning of an HCV strain from a Japanese patient with fulminant hepatitis (JFH-1) (403) this goal was partially achieved and a clone propagating to very high levels in human hepatoma Huh7.5 cell line and producing viral particles infectious to these cells has been established. This robust system and its subsequent modifications provided an important tool for

molecular studies of the HCV life cycle and preclinical testing of novel antiviral strategies. However, the degree to which this system mimics the actual events occurring during *in vivo* infection of human hepatocytes with wild-type HCV remains unsettled. In this regard, Kato *et al.* (2001) demonstrated a significant genetic distance between JFH-1 and the wild-type HCV derived from patients with CHC (194). The phylogenetic analysis showed that although JFH-1 clone clusters with genotype 2a viruses from patients with CHC, there are numerous nucleotide deviations from the wild-type virus sequences which are most prominent in the 5'-UTR and in the core, NS3 and NS5A genomic regions with a mean genetic distance of 0.1136 ± 0.0073 (194). These differences, in addition to the unique immune-deficient milieu existing within Huh7.5 cells and abnormalities in their signalling and endocytic pathways and function of mitochondria, may account for the clone's ability to replicate in Huh7 (51, 83, 188, 205, 207). Although JFH-1 clone is highly infectious to Huh7.5 cells, it displays a very low replication capacity and liver pathogenic potency with short course of infection in experimentally infected chimpanzees, which represent the closest immunopathogenic model of human HCV infection and hepatitis C (193, 231). Further, it has been shown that administration of plasma-derived HCV to chimpanzees induces infection of PBMC (371, 372), whereas a similar situation has not yet been reported for JFH-1. Considering findings in HCV-infected patients, HCV genome, its negative (replicative) strand, unique variants distinct from these occurring in the liver or plasma, and intracellularly expressed viral proteins have been identified in different subsets of circulating immune cells (127,

148

222, 314). The ability of patient-derived HCV to infect *in vitro* PBMC, T and B lymphocytes, and monocytes/ macrophages has also been shown by several groups (42, 86, 94, 151, 222, 304). In contrast, JFH-1 was found to be non-infectious to human lymphoid cells (255, 292).

In the current study, we utilized an adapted JFH-1 virus, designated as JFH1$_T$ clone, which has been found to be highly infectious to Huh7.5 cells with 100-1000-fold greater production of viral particles than the classical JFH-1 strain (187, 352). We aimed to identify if such highly infectious, readily available virus can infect human primary T cells and/or T cell lines previously identified to be prone to infection with wild-type HCV (242, 243, 360) and, if so, whether the clone can be utilized in studies on HCV lymphotropism. For comparison, the same target cells exposed to the classical JFH-1 clone or patient-derived HCV were analyzed. Further, to conclusively determine whether hepatoma Huh7.5 cells are truly resistant to infection with wild-type HCV, we investigated the cells exposed to infectious HCV derived from patients with progressing CHC for the expression of HCV genome and evidence of virus replication using highly sensitive HCV RNA detection assays.

5.2. Material and Methods

5.2.1. Cell lines, Primary Cells and Culture Conditions.

Molt4 (CRL-1582) and Jurkat (TIB-152) T cell lines were provided by the ATCC (Manassas). These cells were found to be susceptible to wild-type, patient-derived HCV and be able to support its replication, as documented in our

previous studies (360). PM1 cells were acquired from the National Institutes of Health AIDS Research and Reference Program (Rockville, MD) and CCRF-CEM cells (CEM, ACC-240) from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig). These two T cell lines were not permissive to infection with naturally occurring HCV; however PM1 cells could be infected with wild-type HCV after simulation with PMA and inomycin, as previously reported (360). The cells were cultured at 1×10^5 cells/well in 5 ml of medium containing RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine and 0.1 mM nonessential amino acids (Invitrogen). Total PBMC were isolated from healthy donors by gradient centrifugation in Ficoll-HyPaque (Pharmacia), as described elsewhere (315). Primary T lymphocytes were affinity-purified from monocyte-depleted PBMC of a healthy human donor by negative selection using MACS magnetic microbeads (Miltenyi Biotec), as reported (319, 360). T cells were 98% pure by flow cytometry evaluations. PBMC and affinity-purified primary T cells were stimulated with 5 µg/ml PHA (Sigma-Aldrich) for 24 h before exposure to virus (243). The human hepatoma-derived Huh7.5 cells were propagated in DMEM (Invitrogen) supplemented with 10% FCS and 1% penicillin-streptomycin.

5.2.2. HCV JFH-1 and JFH1$_T$ Infectious Strains.

The classical HCV JFH-1 strain and its derivative JFH1$_T$, provided by Dr. Rodney Russell, Memorial University, St. John's, NL, Canada, were used for a comparative infection study of T cells and Hu7.5 cells. The JFH1$_T$ strain carries a

synonymous point mutation in each of E2, p7, NS2 and NS5A coding sequences and has demonstrated 100 to 1000-fold greater ability to infect Huh7.5 cells than the classical JFH-1 virus (352).

JFH-1 or its triple mutant JFH1$_T$ was propagated in Huh7.5 cells following the protocol reported elsewhere (187, 352). Briefly, cells were transfected with RNA transcribed from linearized DNA plasmids using lipofectamine transfection reagent (Invitrogen). Then, the cells were washed and cultured in DMEM for 72 h. The resulting culture supernatants were assayed for the virus infectious titre by limiting dilutions in a plaque formation assay and titres were expressed as the number of focus-forming units (FFU) per ml of culture supernatant as reported (187, 352). In addition, JFH-1 and JFH1$_T$ genome copy numbers were quantified by real-time RT-PCR as described below and reported in detail before.

5.2.3.   Wild-type, Patient-Derived HCV

Plasma from 2 male patients age 44 and 52 years with progressing antiviral therapy-naïve CHC carrying HCV genotype 1a at levels 5×10^4 and 7×10^5 vge/ml, respectively, served as HCV infectious inocula. PBMC of these individuals contained HCV RNA positive strand at 1.6×10^2 and 2×10^3 vge/µg total RNA and were HCV RNA replicative strand reactive. In preliminary experiments, the inocula demonstrated comparable infectivity towards HCV-naïve Molt4 and Jurkat cell lines as well as total PBMC and primary T cells.

5.2.4.   Infection Assays with Wild-Type HCV and JFH Strains.

T cell lines, primary T cells and PBMC were cultured in 6-well plates supplemented with 2 ml of culture medium. For primary T cells and PBMC, the cells were pretreated with PHA 5 µg/ml for 24 h before exposure to inocula. Then, cells were exposed to ~1 x 10^5 vge of wild-type, patient-derived HCV or 1 x 10^5 vge of JFH-1 or the triple JFH1$_T$ mutant. In addition, cells treated under identical conditions and exposed to an equivalent volume of NHP served as negative controls. After exposure to either HCV viruses or NHP, the cells were extensively washed and cultured for 7-10 d.p.i. in the presence of either PHA alone or PHA and human recombinant interleukin-2 (IL-2; 20 IU/ml; Roche Molecular Diagnostics), as reported (243). For T cell lines, the cells were extensively washed after exposure to inoculum, supplemented with fresh medium and cultured for 4-7 d.p.i., as reported in detail previously (360).

For infection of Huh-7.5 cells, the cells were seeded at 5 x10^5 cells/well in 8-well chamber slides and after 24-h incubation exposed to identical virus copy numbers (10^4 vge/ml) of HCVcc-containing filtered culture supernatant or wild-type HCV. Cells incubated with NHP or culture supernatant from noninfected Huh7.5 cells were used as controls. After 4-h exposure, the inocula were removed, and cells were washed and cultured for 3 days.

5.2.5. RNA Extraction and cDNA Transcription.

Total RNA was extracted from 1 x 10^6 to 1 x 10^7 cells using Trizol or from 250 µl patient plasma or 300 µl cell culture supernatant with Trizol S reagent

(both Invitrogen). Mock extractions were performed in parallel as contamination controls (315). cDNA was transcribed with Moloney murine leukemia virus RT (Invitrogen), as previously described (315, 360, 319).

5.2.6. Detection of HCV by RT-PCR/NAH Assays.

HCV RNA positive and negative (replicative) strands were detected using cDNA derived from 2 μg and 4 μg of total RNA, respectively, and the strand-specific amplification conditions were reported in detail previously (315, 360). The specificity of the signal detection and validity of controls were routinely confirmed by NAH with ^{32}P-labeled rHCV UTR-E2 as a probe (315). The sensitivity of RT-PCR/NAH assay for HCV RNA-positive strand identification was <10 vge/ml (<2 IU/ml) or <5 vge/μg of total RNA, while that for HCV RNA-negative strand was 25-50 vge/μg of total RNA, as reported (315, 360). The relative density of the RT-PCR/NAH signals was quantified by densitometry computer-assisted analysis using ImageJ software from the National Institutes of Health (Washington, DC) and presented in relative density units.

5.2.7. Quantification of HCV by Real-Time RT-PCR.

Enumeration of HCV RNA copy numbers was done by real-time RT-PCR during 45 cycles using the LightCycler 480 (Roche Diagnostics) and conditions as previously reported (360). Briefly, reactions were performed in 10 μL volumes, each containing 2 μL cDNA derived from 50 ng of total RNA using primers described before (315, 360). Ten-fold serial dilutions of rHCV UTR-E2 were used

as standards to determine the HCV genome copy numbers. The sensitivity of the assay was between 10 and 10^2 vge/reaction.

5.2.8. *Detection of HCV Proteins by Confocal Microscopy.*

Huh7.5 cells exposed to wild-type HCV or infected with JFH1$_T$ as a positive control were grown on chamber slides and then fixed in cold acetone for 2 min, washed with phosphate-buffered saline, pH 7.4 (PBS), and incubated with anti-HCV core mouse mAb (B2; Anogen-YES Biotech Laboratories, Ontario, Canada) at 1:1000 for 20 min at ambient temperature. Subsequently, cells were washed with PBS and incubated with Alexa Fluor 488 conjugated anti-mouse antibody for 20 min.

Finally, cells were washed again and mounted using Vectashield hard set mounting medium with DAPI (Vector Laboratories). To detect HCV NS5A protein in Molt4 and Jurkat cells infected with wHCV, cells were fixed with 2% paraformaldehyde and permeabilized with 0.25% saponin. Staining with mouse anti-HCV NS5A mAb (Chemicon) or appropriate isotype antibody control (BD Pharminogen) was done as previously reported (360). Cells were examined under a confocal microscopy and images recorded at x10 and/or x40 magnifications.

5.2.9. *STATISTICAL ANALYSES.*

Results were analyzed by a one way analysis of variance or unpaired Student *t* test with Welch's correction using GraphPad Prism software (GraphPad

Software, Inc.). Differences between experimental conditions were considered to be significant when two-sided P values were below or equal to 0.05.

5.3. Results

5.3.1. *Susceptibility of Primary T Lymphocytes to Infection with JFH-1 and JFH1$_T$.*

Primary human T cells from healthy donors were exposed to either classical JFH-1 strain or the triple mutant JFH1$_T$ and after 7 or 10-day culture evaluated for the expression of HCV RNA.   As shown in Figure 5.1A, the HCV RNA positive strand was detected in T cells exposed to JFH1$_T$ virus but not to JFH-1 virus. In agreement with this observation, HCV RNA was also detected in the supernatant of T cells exposed to JFH1$_T$ but not in the supernatant from the cells incubated with JFH-1 (Figure 5.1A).   Comparable results were obtained from two experiments in which T cells obtained from a healthy donor were tested (Figure 5.1A). To assess whether the detection of HCV RNA positive strand in the cells exposed to JFH1$_T$ reflected active virus replication or virus potentially adhered to the cells, the expression of HCV RNA negative (replicative) strand was evaluated. The results showed that the detection of the HCV RNA positive strand did not coincide with the presence of the virus replicative intermediate in primary T cells exposed to JFH1$_T$ (Figure 5.1B).

A

			T cells		supernatant		sHCV RNA
DW	NW	Mock	JFH-1	JFH1₁	JFH-1	JFH1₁	10⁵

Exp 1 ← 244 bp

			supernatant		T cells		sHCV RNA
DW	NW	Mock	JFH1₁	JFH-1	JFH1₁	JFH-1	10⁵

Exp 2 ← 244 bp

B

					sHCV RNA	
DW	NW	Mock	JFH1₁	JFH-1	pos 10⁴	neg 10⁴

← 244 bp
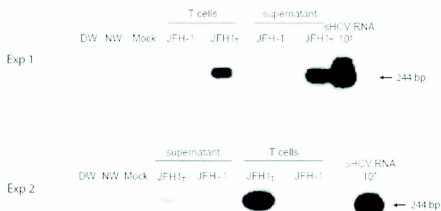
Figure 5.1

Figure 5.1. Determination of the presence of HCV genome positive and negative (replicative) strands in human primary T cells exposed to HCV JFH-1 or JFH1$_T$. Primary T cells affinity purified from a healthy donor were exposed to the same copy numbers of JFH-1 or JFH1$_T$ clones in two parallel experiments (Exp 1 and Exp 2), as described in Materials and Methods. (A) Identification of HCV RNA positive strand. Synthetic HCV RNA positive strand (sHCV RNA) at 10^5 copies/reaction was used as positive and specificity control. (B) Testing for HCV RNA negative strand. Synthetic HCV RNA positive (pos) and negative (neg) strands at 10^5 copies/reaction confirmed the assay specificity for detection of the virus RNA negative strand. Water amplified in direct (DW) and nested (NW) reactions and a mock extraction served as contamination controls. The positive signals showed the expected 244-bp 5'-UTR sequence-specific fragments.

Therefore, the data demonstrated that neither JFH-1 nor JFH1$_T$ was able to establish replication in primary T lymphocytes, although JFH1$_T$ tended to adhere more firmly than JFH-1to these cells.

5.3.2. Infectivity of Wild-Type HCV Versus JFH1$_T$ Towards T cell Lines and Huh7.5 Cells

Molt4, Jurkat, PM1 and CEM T cells, as well as Huh7.5 cells, were exposed to the same copy numbers of patient-derived (wild-type) HCV or JFH1$_T$ virus. After culture the cells were evaluated for expression of HCV by real-time RT-PCR. As shown for wild-type virus in Figure 5.2A, HCV RNA positive strand was detected in Molt4 and Jurkat T cell lines but not in PM1 or CEM cells, while Huh7.5 cells showed trace signals not exceeding 10 vge/µg total RNA. On the other hand, T cell lines exposed to JFH1$_T$ showed variable but overall very low levels of the HCV RNA positive strand ranging between 10 and 10^3 vge/µg total RNA, while Huh7.5 cells, as expected, displayed very high levels exceeding 10^7 vge/µg total RNA (Figure 5.2A). Further, Molt4 and Jurkat T cells after exposure to wild-type virus showed 10^3-10^5-fold greater levels (P=0.001) of HCV RNA positive strand expression comparing to those exposed to the same copy numbers of JFH1$_T$ (Figure 5.2A and B). In order to determine which cells were in fact infected, the expression of HCV RNA negative strand was examined. In agreement with the previous findings (360), only Molt4 and Jurkat T cells lines exposed to patient-derived HCV expressed HCV genome replicative intermediate (Figure 5.2C). In contrast, PM1 and CEM T cells exposed to wild-type virus, as

well as all cell types exposed to JFH1$_T$ virus, except Huh7.5 cells (Figure 5.2C), were HCV RNA negative strand nonreactive. Thus, these results confirmed that, on one hand, wild-type HCV recognizes and infects cultured T cells which have been previously identified to be susceptible to this type of virus (360) but it does not infect Huh7.5 cells and, on the other hand, they showed that JFH1T clone, similarly as JFH-1, does not infect wild-type virus-susceptible T cell lines. However, in contrast to JFH-1, JFH1$_T$, showed a greater ability to interact with the cells, likely with their plasma membranes, as the results on HCV RNA positive strand detection indicated (Figure 5.1A).

5.3.3. Wild-Type HCV but not JFH1$_T$ Virus Infects Circulating Lymphoid Cells

To further test whether human PBMC are susceptible to laboratory adapted JFH1$_T$, PHA-stimulated PBMC obtained from a healthy donor were exposed to 1 x 10^5 vge of JFH1$_T$ or to naturally circulating HCV from 3 different patients with progressing CHC. After culture, as described in Materials and Methods, cells were evaluated for the expression of HCV RNA positive and negative strands. The results showed that circulating lymphoid cells exposed to patient-derived HCV became HCV RNA both positive and negative strand reactive, while those exposed to JFH1$_T$ displayed weak positive strand signals and were negative strand nonreactive (Figure 5.3). The data demonstrated that PBMC were prone to infection with wild-type virus, which is consistent with the previous reports (243, 360), but not to infection with JFH1$_T$.

A

B

Figure 5.2

C

			wHCV				JFH1_T				sHCV RNA	
DW	NW	Mock	Molt4	Jurkat	PM1	CEM	Huh7.5	Molt4	Jurkat	PM1	CEM	Huh7.5	pos 10^4 neg 10^4

← 244 bp

D

	Anti-NS5a/DAPI	Anti-NS5a/DAPI/TL	Anti-NS5a
NHP			
Molt4/HCVcc			
Jurkat/HCVcc			
Molt4/wHCV			
Jurkat/wHCV			
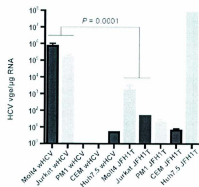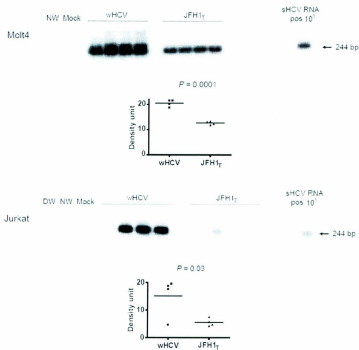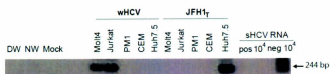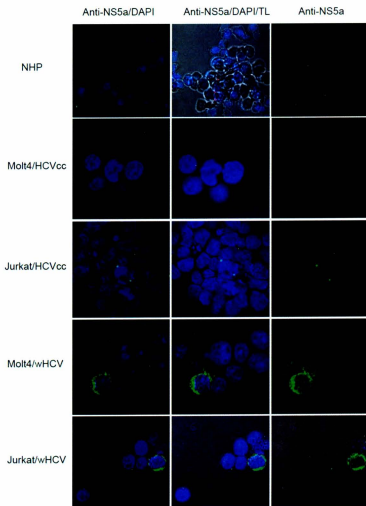
Figure 5.2

Figure 5.2. Infectivity of wild-type HCV and JFH1$_T$ clone towards different human T cell lines and Huh7.5 cells. Molt4 and Jurkat T cell lines susceptible to wild-type HCV (wHCV), PM1 and CEM T cells resistant to infection with that virus, and hepatoma Huh7.5 cells were exposed to comparable numbers of wHCV or JFH1$_T$, as outlined as in Materials and Methods. (A) Quantification of HCV RNA positive strand by real-time RT-PCR. (B) Molt4 and Jurkat cells exposed to wHCV or JFH1$_T$ were evaluated for the presence of HCV RNA positive strand by RT-PCR/NAH. The relative density of the RT-PCR/NAH signals was quantified by densitometry as described by Materials and Methods. The data represent mean $\pm$ SD from 4 experiments. (C) Identification of HCV RNA negative strand in T cell lines and Huh7.5 cells exposed to wHCV or JFH1$_T$ clone. Specificity and contamination controls for B and C were as those described in the legend to Figure 5.1. (D) Identification of HCV NS5a protein in Molt4 and Jurkat cells exposed to JFH1$_T$ clone (HCVcc) or wild-type HCV (wHCV) by confocal microscopy. Green fluorescence represents HCV NS5a protein in HCV-infected cells. The cells were counterstained with DAPI to identify nuclei and examined under transmitted light (TL) to visualize the cytoplasm. The images were captured at X60 magnification.

A

DW NW Mock wHCV #1 JFH1$_T$ wHCV #2 JFH1$_T$ wHCV #3 JFH1$_T$ sHCV RNA pos 10^5

← 244 bp

B

DW NW Mock JFH1$_T$ wHCV sHCV RNA

#1 #2 #3 pos 10^2 neg 10^4

← 244 bp
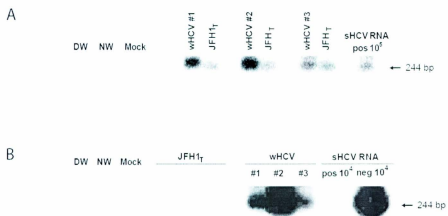
Figure 5.3

Figure 5.3. Wild-type HCV but not JFH1$_T$ virus infects human lymphomononuclear cells. PHA-stimulated PBMC isolated from a healthy donor were exposed to 1 x 10^5 copies of JFH1$_T$ virus or wHCV from 3 different patients with progressing untreated CHC. (A) Identification of HCV RNA positive strand. (B) Detection of HCV RNA negative (replicative) strand. Specificity and contamination controls were as those described in the legend to Figure 5.1.

5.3.4. Huh7.5 Cells are not Susceptible to Infection with Wild-Type HCV

Finally, to further our understanding of the differences in infectivity between wild-type HCV and the JFH1$_T$ virus, Huh7.5 cells were exposed in parallel to ~10^4 vge/ml of either JFH1$_T$ or wild-type virus. Following 3-d.p.i. culture, the cells were examined for the expression of HCV core protein. The data showed that while Huh7.5 cells robustly supported replication of JFH1$_T$ virus, they were not susceptible to infection with the wild-type HCV, as illustrated in Figure 5.4.

5.4. Discussion

Although HCV is a highly hepatotropic virus, the data accumulated in recent years have convincingly documented that the virus also invades and replicates in cells of the immune system lymphocytes (42, 86, 94, 151, 222, 304). The lack of small animal models and a robust tissue culture system capable of supporting infection with wild-type HCV hindered the studies on the virus-host cell interactions naturally occurring *in vivo*, particularly those mediating the initial host's cell target recognition and associations with functionally important intracellular factors or pathways operating within naturally infected cells. Since hepatoma Huh7 cells significantly differ from normal human hepatocytes due to their deficient innate immune response, perturbed physiological functions and functionally altered mitochondria (51, 83, 188, 205, 207) and since the JFH-1 and related clones are highly adapted to propagate in these cells and they are
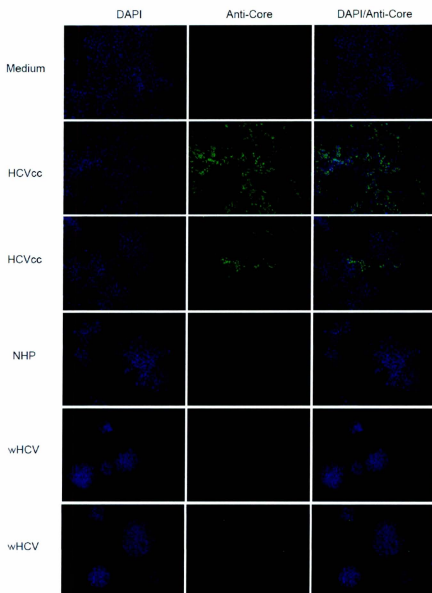
Figure 5.4

Figure 5.4. Huh7.5 cells are susceptible to infection with JFH1$_T$ but not to wild-type HCV. Equal numbers of Huh7.5 cells were exposed to comparable copy numbers of either JFH1$_T$ virus or wHCV. Cells exposed to NHP or DMEM served as negative controls. Green fluorescence represents HCV core protein in HCV-infected cells. Nuclei were counterstained with DAPI. The images were captured at X40 magnification.

molecularly distinct from wild-type virus, the virus-cell interactions observed in this system should be interpreted with caution.

The finding that molecularly intact, wild-type HCV can productively infect normal human primary T cells (242, 243) (Skardasi, G. and Michalak, T.I. – in preparation) provides the means to investigate naturally occurring events, although in the context of lymphoid cells only, and to assess, for example, the effectiveness and the mode of action of agents interfering with replication of fully infectious and pathogenic HCV. The application of the HCV-T cell *in vitro* infection model already led to the finding that unique HCV variants emerge during replication in primary T cells (243), that the lymphocyte-specific CD5 molecule is essential for HCV infection of T lymphocytes (360), and that a number of molecules proposed to mediate HCV tropism towards hepatocytes unlikely play a role in infection of primary lymphocytes indicating that the virus may utilize different receptors to enter its natural cell targets (Sarhan *et al.* - submitted). However, in an attempt to simplify investigations on HCV lymphotropism, a search for a readily available and well characterized virus was undertaken. Cell culture-derived JFH1$_T$ clone showing greatly enhanced infectivity and robust replication in Huh7.5 cells, compared to the classical JFH-1 virus (187, 352), appeared to be the best candidate. Therefore, in the current work, we wanted to delineate, via comparative analysis of the infectivity towards different T lymphocyte targets, whether JFH1$_T$ virus would be a suitable analogue of wHCV in studies on HCV lymphotropism.

Analysis of the HCV RNA positive strand revealed a strong signal in primary T cells exposed to JFH1$_T$ and non in the cells exposed to JFH-1, suggesting that JFH1$_T$ clone tended to bind to the T cells more tightly than JFH-1 despite routinely performed extensive washing of the cells after inoculation with virus (see Figure 5.1). However, examination for HCV RNA negative (replicative) stand revealed the lack of detectable signal, indicating that JFH1$_T$ did not replicate in primary T cells or that its replication progressed at a very low level not identifiable by otherwise highly sensitive RT-PCR/NAH assay (sensitivity ~100 copies/reaction). Detection of HCV RNA positive strand in culture supernatants of T cells exposed to JFH1$_T$ could be interpreted in support of this possibility. JFH1$_T$ clone also interacted to a variable degree with all other types of T cells examined, including T cell lines either susceptible or not to infection with wHCV, as the analysis of HCV RNA positive strand showed (see Figure 5.2A, B and 5.3A). However, HCV RNA replicative strand was not detected in any of the cells except Huh7.5 cells, as expected (see Figure 5.2C). In future, it would be interesting to explore whether this non-productive interaction between JFH1$_T$ and T cells reflects naturally occurring wHCV-cell binding or is a phenomenon unique to this clone since JFH-1 virus did not display such binding reactivity.

In contrast to cells exposed to JFH1$_T$ virus, PBMC as well as Molt4 and Jurkat T cell lines exposed to wHCV demonstrated readily detectable HCV RNA replicative strand (see Figure 5.2C and 5.3B), while PM1 and CEM cells, known to be resistant to infection with wHCV (360) (Sarhan *et al.* - submitted), were negative strand nonreactive (see Figure 5.2C). Taken together, a clear picture

169

emerged indicating that while molecularly intact, patient-derived wHCV was infectious and capable of replication in primary lymphoid cells and T cell lines previously identified to be prone to this type of virus (242, 243, 360) (Sarhan *et al.* - submitted), the highly infectious to Huh7.5 cells JFH1$_T$ clone was unable to establish replication in either primary lymphocytes or wHCV-prone T cell lines. Similarly, it has been shown that JFH-1 HCVcc does not infect B or T lymphocytes, monocytes, macrophages, or dendritic cells from healthy donors (255). Also, T cells and PBMC were not recognized by HCV pseudoparticles, however they were found to enter hepatoma Huh7 and HepG2 cells transfected with CD81, and to human primary hepatocytes (77).

On the other hand, Huh7.5 cells were highly susceptible to infection with JFH1$_T$ clone, as documented before (187, 352), while being entirely resistant to infection with wHCV despite the fact that highly sensitive assays for detection of HCV RNA positive and negative strands were used throughout the study. This was consistent with the results on HCV core protein staining in Huh7.5 cells exposed to JFH1$_T$ or wHCV. Thus, cells exposed to JFH1$_T$ but not those incubated with wHCV were reactive for this protein.

The results from this study document a contrasting difference between wild-type, patient-derived HCV and JFH1$_T$ or JFH-1 clones in infectivity of human primary and cultured lymphocytes, and they showed that the mutations introduced to JFH1$_T$ greatly enhancing its infectivity of Huh7.5 cells were without effect on infection of lymphocytes. This divergence could be attributed, as already mentioned, to the significant genetic differences between the naturally

occurring HCV and the cloned and cell culture-adapted JFH viruses. However, at this stage, it cannot be completely excluded that the plasma-derived HCV may contain a minor subpopulation of lymphotropic variants or carry host factors predisposing virus to attach, enter and/or preferentially replicate in lymphoid cells, which are absent in the preparations of the cell culture-derived JFH-related viruses. In conclusion, our study clearly shows that primary lymphocytes and selected T cell lines can support replication of wild-type HCV but not the otherwise highly infectious and replication efficient JFH-1-related strain. Since JFH-1-derived viruses are unlikely suitable for investigations on HCV lymphotropism, there is a need for generation of HCV clones displaying properties more closely resembling those of the wild-type virus which can be applied for the type of investigations mentioned. Nonetheless, this and our previous studies (19, 20, 33) (Sarhan *et al.* - submitted) showed that, although using wild-type virus for *in vitro* experimentations is highly challenging, it is also fully feasible and can generate valuable data which cannot be obtained otherwise.

CHAPTER SIX: GENERAL DISCUSSION

Human hepatocytes are considered the primary and the main site of HCV replication. However, as summarized before (Section 1.7), there is also substantial molecular and clinical evidence implying that HCV invades and propagates in extrahepatic sites, including cells of the immune system (243, 273, 319, 360). Cellular factors and molecules that mediate the susceptibility of T cells to HCV infection were not investigated to any significant degree until the studies presented in this thesis. HCV candidate receptors potentially mediating virus tropism toward hepatocytes, such as CD81, SR-B1 and the TJ proteins CLDN-1 and OCLN, have been identified as being involved in HCV infection of human hepatoma Huh7 cells, mainly by using nonpathogenic HCV pseudoparticles and other HCV surrogate models. However, overexpression of these molecules does not allow many HCV resistant cells to become HCV susceptible, suggesting that other factors necessary for HCV cell entry and infection of are yet to be identified.

The main purpose of our studies was to identify a mechanism by which wild-type, patient-derived HCV recognizes and enters human primary T lymphocytes, which are one of the sites of HCV replication in the host's immune system. In this regard, the susceptibility of freshly isolated human T cells and different human T cell lines to infection with wild-type HCV was examined to uncover the nature of cellular factors mediating the T cell permissiveness to HCV and to identify which of the HCV hepatocyte candidate receptors may contribute

to infection of human primary T cells, their CD4+ and CD8+ subsets, total PBMC and T cell lines, which were found to be prone to HCV infection in the course of my study, in comparison to hepatoma-derived Huh7.5 and in HepG2 cell lines, PHH and control HEK-293 cells. Furthermore, we compared the infectivity of naturally occurring, plasma-derived HCV and Huh7.5 cells highly infectious HCV JFH-1 clone to human T lymphocytes and Huh7.5 cells.

For this purpose, we established an *in vitro* T cell culture system using readily available T cell lines which have been isolated from patients with acute lymphoblastic leukemia or acute T cell lymphoma and employed a HCV-primary T lymphocyte infection system previously developed in this laboratory (243, 319). *De novo* infection with HCV was identified by detection of HCV RNA positive and negative (replicative) strands and expression of virus NS5A, E2 or core proteins. Specificity of HCV binding to cellular molecules of interest was confirmed by blocking experiments with specific antibodies or shRNAs and, in some investigations, by transfection of the naturally HCV resistant cells with cDNA encoding a molecule identified as HCV T cell receptor.

The advantages of our T cell culture system rely on the fact that we used easy to handle target human cells and molecularly intact wild-type HCV virus as inocula. Moreover, the cells can be exposed to and support infection of naturally occurring HCV of different genotypes. In contrast, this is not feasible in the current JFH-1-Huh7.5 culture model in which a clone, adapted in culture HCV genotype 2b displaying multiple mutations when compared to wild-type virus, is used. On the other hand, hepatoma-derived Huh7 cells, as indicated before

(Sections 1.10.2.3), were shown to have a perturbed intracellular innate immune response, dysfunctional mitochondria and aberrant signalling and endocytosis pathway (51, 52, 83, 188, 207, 83). Although the JFH-1-Huh7.5 culture system supports robust HCV production that allow the study on JFH-1 entry and its interactions with host factors at different stages of virus life cycle, the genetic distance between JFH-1 (and related clones) and the wild-type HCV (403) and distinctive characteristics of Huh7 cells likely affects, although to an unknown degree, the properties of the interactions observed. Among others, JFH-1 produced in Huh7.5 cells failed to infect human PBMC (255), which were found to be susceptible to wild-type virus both *in vivo* and *in vitro* (Section 1.7.2). This finding is not surprising, as JFH-1 HCVcc also failed to efficiently infect polarized PHH and chimpanzees (324).

Our HCV-T cell culture model is certainly less robust than the JFH-1-Huh7 system and requires highly sensitive methods to detect and quantify replicating virus and virions released by infected cells. However, the highly sensitive and well controlled assays available in our laboratory allow the identification of specific HCV RNA positive strand at levels below 5 vge/µg of total RNA and virus RNA negative strand at 25-50 vge/µg of total RNA (360). For comparison, the infection of PHH with wild-type HCV *in vitro* has been shown to have a very low efficiency which further declined after 8 days with no evidence of the production of virions (131, 280, 281). Furthermore, less than 15% of the sera from CHC patients were found to be infectious to PHH in other studies (131). Therefore, it is more appropriate to compare our wild-type HCV-T cell culture system with the

174

PHH cultures exposed to patient derived virus but not to the artificial JFH-1 system. In this case, the HCV-T cell culture system appears to be more efficient, reproducible, and convenient.

Moreover, it has been proposed that infection of immune cells with HCV contributes to HCV persistence by acting as a reservoir of the pathogenic virus and by likely altering the ability of the infected immune cells to mount an effective anti-viral response. Persistence of HCV in this compartment is also implicated in the pathogenesis of extrahepatic disorders and can be responsible for reinfection of the grafts after liver transplantation (290, 328, 366, 434). The persistence of HCV in immune cells may also contribute to virologic relapse after successful antiviral treatment (312, 315) and influence the response to IFN-α treatment (154). For these reasons, as well as to advance our understanding of the principal biological properties of HCV, it was important to uncover cellular molecules that mediate the permissiveness of immune cells, specifically T lymphocytes, to HCV infection. We examined the receptiveness of four human T cell lines (Molt4, Jurkat, PM1 and CEM) to infection with wild-type HCV. The differential analysis of their susceptibility to HCV led to the discovery that lymphocyte unique CD5 molecule, belonging to the scavenger receptor cysteine-rich domain superfamily (342), is essential for recognition of T lymphocytes by this virus. This finding not only defines the molecule involved in determination of HCV lymphotropism but also a new target for potential interventions against HCV propagating at this extrahepatic compartment.

Subsequently, to further characterize the differences between HCV-susceptible and nonsusceptible T cells, we assessed the cells for the expression of receptors such as CD3, CD4, CD8 and CD81 in addition to the pan-T marker CD5. All cells were CD3 positive, CD81 positive, CD8 negative with variable expression of CD4. Interestingly, only T cells susceptible to HCV expressed CD5 molecules. The level of infection of these susceptible cells with HCV was inhibited by CD5-specific antibodies and shRNAs and was comparable to that of CD81 blocking. We also found that the differential susceptibility of the examined T cell lines was not correlated to the transcription levels of IFN-α and IFN-γ, IRF3 and IRF7 molecules. On the other hand, upregulation of CD5 in PM1 cells confered susceptibility to HCV infection. Moreover, the naturally resistant HEK-293 cells become susceptible to HCV infection, although at low levels, when transfected with a plasmid containing CD5. The results revealed that only primary T cells and T cell lines which express CD5 protein are prone to HCV. This implied that T cell susceptibility to HCV requires CD5, a lymphocyte-specific glycoprotein that belongs to the scavenger receptor cysteine-rich domain superfamily (342). Considering the restricted expression of CD5 in T cells, it is likely that CD5 specifically facilitates HCV lymphotropism. This finding defines a new target for potential interventions against HCV propagation at this extrahepatic compartment.

HCV cell entry to hepatocytes appears to be a multi-step process that involves a number of different host cell factors. Following initial engagement with GAGs and the LDL receptor, HCV entry proceeds via interactions with the CD81,

SR-BI, and the tight-junction proteins CLDN1 and OCLN (103). These receptors have been previously shown to be necessary for HCV infection of hepatoma Huh7 cells, but whether or not they are necessary for infection of human T lymphocytes by wild-type virus was not investigated. To assess the role of HCV hepatocyte candidate receptors in infection of T cells, we examined primary T cells, CD4+ and CD8+ T lymphocyte subsets, total PBMC and T cell lines for the level of expression of these postulated receptors in comparison to hepatoma-derived cells, PHH and HEK-293 cells. The level of mRNA transcription and protein expression were identified using quantitative real-time RT-PCR and Western blotting, respectively. Our findings showed that CD81 is unlikely the limiting factor for HCV infection of T cells since both nonsusceptible (PM1 and CEM) and susceptible (Molt4 and Jurkat) T cell lines, as well as primary T cells, expressed similar levels of CD81. Further, we found that SR-B1, a molecule highly expressed in liver tissue and which is considered to be an important HCV hepatocyte entry factor (28, 65) seems not to play a role in T cell susceptibility to HCV infection. Thus, primary T cells, CD4+ and CD8+ T cell subsets and PBMC, despite being susceptible to wild-type HCV (203, 314), were found to express significantly lower levels of SR-B1 mRNA comparing to Huh7.5 and HepG2 cells and no SR-B1 protein. Our results in regard to SR-B1 expression in lymphoid cells appears to be compatible with other studies (255, 420).

The resistance of PM1 T cells to HCV infection despite the expression of CLDN-1 and the susceptibility of Molt4 and Jurkat T cells to HCV which are CLDN-1 negative, indicates that CLDN-1 is also likely irrelevant to

permissiveness of normal human T cells as well as Molt4 and Jurkat T cell lines to infection with naturally occurring HCV. Our findings were consistent with what has been shown before that CLDN-1 and SR-BI were not detectable in lymphocytes (255, 429) and that CLDN-1 could be absent in cells which are HCV-susceptible, such as the human hepatocellular carcinoma cell line Bel7402 (429). In regard to CLDN-4 and CLDN-6, no correlation between the level of expression and susceptibility of T cells to HCV infection was identified.

OCLN expression was significantly higher in hepatoma cells and PHH, and in HCV-susceptible lymphoid cells Molt4 and Jurkat T cell lines than in primary T cells, PBMC and HCV-resistant PM1 and CEM T cells. In fact, we were not able to detect OCLN protein in PBMC, however traces were identified in primary T cells. It has been reported that OCLN is not expressed by peripheral lymphoid cells, but it has been shown to be expressed in activated T cells under certain conditions (13). In regard to the role of OCLN in HCV infection of T cells, we found that OCLN-knockdown did not interfere with virus attachment and replication but it interfered with HCV E2 protein expression. This may suggest that OCLN may act at later, intarcellular stages in HCV infection of T cells. Further, the downregulation of OCLN after infection of Jurkat cells with HCV again implied its role in the infection process.

Taken together, our data showed that the lack of association between T cell expression of SR-B1 or CLDNs and the susceptibility to HCV infection, while CD5 and CD81 were identified to be essential to HCV lymphotropism. In addition, OCLN appears to be likely involved in infection of the susceptible T cell

lines but its precise role is not clear. A study by Marukian et al. (2008) reported that JFH-1 HCVcc does not infect PBMC and that SR-B1 and CLDN-1-transfected B cells did not become infectable with HCVpp. The authors suggested that OCLN could be the missing receptor for infection of lymphoid cells. However, they also showed that lymphocytes do not support replication of transfected HCV RNA, implying that multiple blocks in HCV infection of lymphocytes may exist, at last when a laboratory virus is used for this type of experimentation. The study by Marukian et al. (2008) was built on the use of modified JFH-1 and HCVpp, the authors did not test the effect of OCLN or other TJ proteins on the infection of PBMC or T cells with wild-type HCV.

The recent identification of the TJ proteins as candidate hepatocyte receptors for HCV was considered to be an important step in the development of a small animal model for HCV infection. However, investigators were unable to induce replication of HCV in mouse cells, suggesting that other host-specific factors than CD81 and OCLN might be required to support of the complete life cycle of HCV in mice.

In the third project completed in the course of my thesis work, we wanted to delineate via comparative analysis of HCV cell susceptibility, whether JFH1$_T$ virus, known to be highly infectious to Huh7.5 cells, would be a suitable alternative to wHCV in studies of HCV lymphotropism. In this regard, the infectivity of HCV JFH-1 and its culture adapted clone JFH1$_T$ (352) towards T cells were tested in comparison to the plasma-derived wild-type HCV. In parallel, the susceptibility of hepatoma Huh7.5 cells to infection with wild-type HCV was

assessed. We found that primary T cells, PBMC and T cell lines known to be prone to plasma-derived HCV (360), were not susceptible to either JFH-1 or JFH1$_T$ clones. On the other hand, Huh7.5 cells were only susceptible to the JFH-1 clones but not to patient-derived HCV which was infectious to T cells. The preferential replication of plasma-derived HCV but not JFH clones in lymphoid cells document a contrasting difference between the wild-type, patient-derived HCV and JFH1$_T$ or JFH-1 clones, and show that the mutations introduced to JFH1$_T$ greatly enhanced the clone infectivity towards Huh7.5 cells but not to lymphocytes. This divergence could be attributed, as already mentioned, to the significant genetic differences between the naturally occurring HCV and the cloned and cell culture-adapted JFH viruses. Thus, our study clearly showed that human primary lymphocytes and selected, readily available T cell lines can support replication of wild-type HCV but not the otherwise highly infectious and replication efficient JFH-1-related strain. Since JFH-1-derived clones are unlikely suitable for investigations of HCV lymphotropism, there is a need for generation of HCV clones with properties more closely resembling those of the wild-type virus that can be utilized in the study of HCV lymphotropism. Nonetheless, we demonstrated that although using wild-type, patient-derived virus for *in vitro* experimentations is highly challenging it is also fully feasible and can generate valuable data which cannot be obtained otherwise.

Overall, our studies applied a physiologically relevant infection system reflecting natural behaviour of wild-HCV and its interactions with human cells. Applying this system, we discovered that CD5, a lymphocyte-specific glycoprotein

belonging to the scavenger receptor cysteine-rich family, is an essential factor for infection of T cells by molecularly unmodified, wild-type virus. In consequence, we showed that HCV utilizes different cellular molecules to infect different cell types naturally prone to infection with this virus. We also narrowed the range of cellular molecules utilized by a pathogenic HCV in infection of T lymphocytes among those previously proposed based on studies applying laboratory adapted HCV strains or HCVpp and Huh7.5 cells. Further, we demonstrated that the current JFH-1-based infectious HCV clones are not suitable for studies on HCV lymphotropism. While identification of CD5 and other cellular factors mediating susceptibility of human T cells may affect development of future preventive and therapeutic strategies against HCV, further studies are required to delineate nature of receptors determining permissiveness of other types of immune cells to infection with pathogenic HCV.

CHAPTER SEVEN: CONCLUSIONS AND FUTURE DIRECTIONS

7.1. Conclusions

The findings presented in this dissertation can be summarized and concluded as follows:

1. We have established reproducible *in vitro* HCV infection system using readily available human Molt4 and Jurkat T cell lines and wild-type HCV derived from patients actually infected with HCV as inocula. Using T cell lines as infection targets, we had overcome a drawback of our previous HCV-T cell infection model in which freshly isolated human PBMC were used as a source of HCV target T cells. The current system is as efficient as the previous one, considering the rate of HCV replication, but certainly is more straightforward and less work demanding. Although our HCV-T cell infection system is significantly less robust than the culture adapted JFH-1 strains propagating in hepatoma Huh7.5 cells, it offers important advantages for investigations of interactions between naturally occurring virus and host cells, and as a tool to further study cellular and viral factors promoting HCV lymphotropism and affecting HCV replication in immune cells, which cannot be investigated otherwise.

2. Differential analysis of HCV infection in permissive and resistant T cell lines and in primary T lymphocytes led us to discover that CD5 is a T cell-specific molecule that is important for HCV infection of human lymphocytes. We also

found that CD81 contributes to HCV infection, but it is not the limiting factor for HCV infection of resistant T cell lines.

3. Analysis of the expression of the proposed HCV hepatocyte candidate receptors previously identified using JFH-1 and related clones or HCVpp and Huh7.5 cells revealed that, in addition to CD5 and CD81, OCLN is involved in HCV infection of permissive human T cells lines, but rather unlikely in infection of primary T lymphocytes. We also uncovered that SR-B1 and tight junction protein CLDN-1 do not play a role in HCV lymphotropism.

4. Comparing HCV cell tropism of the wild-type, patient-derived HCV and the JFH clones towards lymphoid cells and hepatoma-derived cells, we demonstrated that JFH-1 or the adapted JFH1$_T$ strain, which is known to be highly infectious to Huh7.5 cells, can efficiently infect Huh7 cells but not human primary T lymphocytes or T cell lines known to be susceptible to wild-type HCV. In contrast, wild-type HCV derived from infected patients was only infectious to the lymphoid cells but not to Huh7.5 cells.

7.2. Future Directions

1. Further efforts should be made to improve the efficiency of the HCV-T lymphocyte infection system. Serial passage of virus in T lymphocytes and subculture of HCV-infected T cells may produce virions that may more efficiently infect virus-naive T cells and allow for more robust HCV propagation in these cells. It is not excluded that HCV variants preferentially infecting and replicating in T cell cultures could be created. Their sequence analysis and examinations of

biophysical properties may potentially explain the difference between hepatotropic and lymphotropic HCV strains.

2. The exact molecular mechanism of CD5 interaction with HCV virions is yet unknown. Therefore, creating CD5 clones with site mutations may help identify certain CD5 regions that interact with the virus. Also, transfection of these mutated or truncated clones of CD5 into T cells with knockout CD5 may enhance our knowledge about its function during the HCV attachment and potentially in the subsequent stages of the virus intracellular life cycle. On the other hand, although HCV E2 protein most likely mediates binding to CD5, it would be to appropriate to formally document this expectation.

3. The mechanism of HCV infection of other immune cells expressing CD5, such as a minor subpopulation of B cells, or not expressing CD5, such as monocytes, macrophages and the majority of mature circulating B cells, should be investigated to explain in full the nature of HCV lymphotropism.

4. It has been postulated that the occurrence of lymphoproliferative disorders in HCV-infected patients, such as B cell non-Hodgkin's lymphoma and mixed cryoglobulinemia, could be related to the expansion of CD5-positive B cells. The mechanism of this expansion is unknown and examination of this issue would be a clinically important area of further investigations. In addition, recognition of the possible differential susceptibility of CD5-positive and CD5-negative B cells to HCV infection may advance our understanding of the contribution of CD5 to HCV lymphotropism.

5. Whether or not soluble CD5 can be used as a component of anti-HCV therapy to limit virus spread and to prevent the establishment of HCV reservoir in the lymphatic system will require separate studies.

In the studies presented in this dissertation, we uncovered important, previously unknown aspects of HCV biology that improved our understanding of the nature of HCV lymphotropism. These findings may shape the future strategies of anti-HCV treatment and the ways how the progression of HCV infection and its therapy are monitored. We believe that HCV residing in immune cells may contribute to the failure of antiviral treatment and infection relapse. We also think that future therapies should consider combating HCV in this extrahepatic reservoir.

CHAPTER EIGHT: REFERENCES

1. **Anonymous** 1998. Recommendations for prevention and control of hepatitis C virus (HCV) infection and HCV-related chronic disease. Centers for Disease Control and Prevention. MMWR Recomm Rep. **47:**1-39.

2. **Anonymous** 1999. Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. J. Viral Hepat. **6:**35-47.

3. **Anonymous** 2011. Telaprevir (Incivek) and boceprevir (Victrelis) for chronic hepatitis C. Med. Lett. Drugs Ther. **53:**57-59.

4. **Abad, F. X., R. M. Pinto, and A. Bosch.** 1994. Survival of enteric viruses on environmental fomites. Appl. Environ. Microbiol. **60:**3704-3710.

5. **Abe K, Inchauspe G, Shikata T, and Prince AM.** 1992. Three different patterns of hepatitis C virus infection in chimpanzees. Hepatology. **15:**690-5.

6. **Acosta-Rivero N, Rodriguez A, Musacchio A, Falcón V, Suarez VM, Martinez G, Guerra I, Paz-Lago D, Morera Y, de la Rosa MC, Morales-Grillo J, and Dueñas-Carrera S.** 2004. In vitro assembly into virus-like particles is an intrinsic quality of Pichia pastoris derived HCV core protein. Biochem. Biophys. Res. Commun. **325:**68-74.

7. **Acosta-Rivero, N., J. C. Aguilar, A. Musacchio, V. Falcon, A. Vina, M. C. de la Rosa, and J. Morales.** 2001. Characterization of the HCV core virus-like particles produced in the methylotrophic yeast Pichia pastoris. Biochem. Biophys. Res. Commun. **287:**122-125. doi: 10.1006/bbrc.2001.5561.

8. **Adinolfi, L. E., A. Andreana, R. Utili, R. Zampino, E. Ragone, and G. Ruggiero.** 1998. HCV RNA levels in serum, liver, and peripheral blood mononuclear cells of chronic hepatitis C patients and their relationship to liver injury. Am. J. Gastroenterol. **93:**2162-2166. doi: 10.1111/j.1572-0241.1998.00613.x.

9. **Aggarwal, R., and S. Jameel.** 2011. Hepatitis E. Hepatology. . doi: 10.1002/hep.24674; 10.1002/hep.24674.

10. **Agnello, V., G. Abel, M. Elfahal, G. B. Knight, and Q. X. Zhang.** 1999. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc. Natl. Acad. Sci. U. S. A. **96:**12766-12771.

11. **Agnello, V., R. T. Chung, and L. M. Kaplan.** 1992. A role for hepatitis C virus infection in type II cryoglobulinemia. N. Engl. J. Med. **327:**1490-1495. doi: 10.1056/NEJM199211193272104.

12. **Agnello, V., and F. G. De Rosa.** 2004. Extrahepatic disease manifestations of HCV infection: some current issues. J. Hepatol. **40:**341-352.

187

13. **Alexander, J. S., T. Dayton, C. Davis, S. Hill, T. H. Jackson, O. Blaschuk, M. Symonds, N. Okayama, C. G. Kevil, F. S. Laroux, S. M. Berney, and D. Kimpel.** 1998. Activated T-lymphocytes express occludin, a component of tight junctions. Inflammation. **22:**573-582.

14. **Alter, M. J.** 2007. Epidemiology of hepatitis C virus infection. World J. Gastroenterol. **13:**2436-2441.

15. **Alter, M. J., D. Kruszon-Moran, O. V. Nainan, G. M. McQuillan, F. Gao, L. A. Moyer, R. A. Kaslow, and H. S. Margolis.** 1999. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. N. Engl. J. Med. **341:**556-562. doi: 10.1056/NEJM199908193410802.

16. **Alter, M. J., H. S. Margolis, K. Krawczynski, F. N. Judson, A. Mares, W. J. Alexander, Pin Ya Hu, J. K. Miller, M. A. Gerber, R. E. Sampliner, E. L. Meeks, and M. J. Beach.** 1992. The natural history of community-acquired hepatitis C in the United States. N. Engl. J. Med. **327:**1899-1905.

17. **Antonelli, A., C. Ferri, P. Fallahi, A. Pampana, S. M. Ferrari, F. Goglia, and E. Ferrannini.** 2005. Hepatitis C virus infection: Evidence for an association with type 2 diabetes. Diabetes Care. **28:**2548-2550.

18. **Arema A. Pereira, and Ira M. Jacobson.** New and experimental therapies for HCV. .

19. **Armstrong, G. L., A. Wasley, E. P. Simard, G. M. McQuillan, W. L. Kuhnert, and M. J. Alter.** 2006. The prevalence of hepatitis C virus infection in the United States, 1999 through 2002. Ann. Intern. Med. **144:**705-714.

20. **Babcock GJ, Decker LL, Volk M, and Thorley-Lawson DA.** 1998. EBV persistence in memory B cells in vivo. Immunity. **9:**395-404.

21. **Babitt, J., B. Trigatti, A. Rigotti, E. J. Smart, R. G. Anderson, S. Xu, and M. Krieger.** 1997. Murine SR-BI, a high density lipoprotein receptor that mediates selective lipid uptake, is N-glycosylated and fatty acylated and colocalizes with plasma membrane caveolae. J. Biol. Chem. **272:**13242-13249.

22. **Bantel, H., A. Lugering, J. Heidemann, X. Volkmann, C. Poremba, C. P. Strassburg, M. P. Manns, and K. Schulze-Osthoff.** 2004. Detection of apoptotic caspase activation in sera from patients with chronic HCV infection is associated with fibrotic liver injury. Hepatology. **40:**1078-1087. doi: 10.1002/hep.20411.

23. **Bare, P., I. Massud, C. Parodi, L. Belmonte, G. Garcia, M. C. Nebel, M. Corti, M. T. Pinto, R. P. Bianco, M. M. Bracco, R. Campos, and B. R. Ares.** 2005. Continuous release of hepatitis C virus (HCV) by peripheral blood mononuclear cells and B-lymphoblastoid cell-line cultures derived from HCV-infected patients. J. Gen. Virol. **86:**1717-1727. doi: 10.1099/vir.0.80882-0.

24. **Barth, H., R. Cerino, M. Arcuri, M. Hoffmann, P. Schurmann, M. I. Adah, B. Gissler, X. Zhao, V. Ghisetti, B. Lavezzo, H. E. Blum, F. von Weizsacker, A. Vitelli, E. Scarselli, and T. F. Baumert.** 2005. Scavenger receptor class B type I and hepatitis C virus infection of primary tupaia hepatocytes. J. Virol. **79:**5774-5785. doi: 10.1128/JVI.79.9.5774-5785.2005.

25. **Barth, H., C. Schafer, M. I. Adah, F. Zhang, R. J. Linhardt, H. Toyoda, A. Kinoshita-Toyoda, T. Toida, T. H. Van Kuppevelt, E. Depla, F. Von Weizsacker, H. E. Blum, and T. F. Baumert.** 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J. Biol. Chem. **278:**41003-41012. doi: 10.1074/jbc.M302267200.

26. **Barth, H., E. K. Schnober, C. Neumann-Haefelin, C. Thumann, M. B. Zeisel, H. M. Diepolder, Z. Hu, T. J. Liang, H. E. Blum, R. Thimme, M. Lambotin, and T. F. Baumert.** 2008. Scavenger receptor class B is required for hepatitis C virus uptake and cross-presentation by human dendritic cells. J. Virol. **82:**3466-3479. doi: 10.1128/JVI.02478-07.

27. **Bartosch, B., G. Verney, M. Dreux, P. Donot, Y. Morice, F. Penin, J. M. Pawlotsky, D. Lavillette, and F. L. Cosset.** 2005. An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. J. Virol. **79:**8217-8229. doi: 10.1128/JVI.79.13.8217-8229.2005.

28. **Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, and F. L. Cosset.** 2003. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J. Biol. Chem. **278:**41624-41630. doi: 10.1074/jbc.M305289200.

29. **Bassett SE, Brasky KM, and Lanford RE.** 1998. Analysis of hepatitis C virus-inoculated chimpanzees reveals unexpected clinical profiles. J. Virol. **72:**2589-99.

30. **Behrens, S. E., L. Tomei, and R. De Francesco.** 1996. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J. **15:**12-22.

31. **Benedicto, I., F. Molina-Jimenez, O. Barreiro, A. Maldonado-Rodriguez, J. Prieto, R. Moreno-Otero, R. Aldabe, M. Lopez-Cabrera, and P. L. Majano.** 2008. Hepatitis C virus envelope components alter localization of hepatocyte tight junction-associated proteins and promote occludin retention in the endoplasmic reticulum. Hepatology. **48:**1044-1053. doi: 10.1002/hep.22465.

32. **Benedicto, I., F. Molina-Jimenez, B. Bartosch, F. L. Cosset, D. Lavillette, J. Prieto, R. Moreno-Otero, A. Valenzuela-Fernandez, R. Aldabe, M. Lopez-Cabrera, and P. L. Majano.** 2009. The tight junction-associated protein occludin is required for a postbinding step in hepatitis C virus entry and infection. J. Virol. **83:**8012-8020. doi: 10.1128/JVI.00038-09.

33. **Benhamou, Y., M. Bochet, V. Di Martino, F. Charlotte, F. Azria, A. Coutellier, M. Vidaud, F. Bricaire, P. Opolon, C. Katlama, and T. Poynard.** 1999. Liver fibrosis progression in human immunodeficiency virus and hepatitis C virus coinfected patients. The Multivirc Group. Hepatology. **30:**1054-1058. doi: 10.1002/hep.510300409.

34. **Benhamou, Y., V. Di Martino, M. Bochet, G. Colombet, V. Thibault, A. Liou, C. Katlama, T. Poynard, and MultivirC Group.** 2001. Factors affecting liver fibrosis in human immunodeficiency virus-and hepatitis C virus-coinfected patients: impact of protease inhibitor therapy. Hepatology. **34:**283-287. doi: 10.1053/jhep.2001.26517.

35. **Berenguer, M., F. X. López-Labrador, and T. L. Wright.** 2001. Hepatitis C and liver transplantation. J. Hepatol. **35:**666-678.

36. **Berenguer, M., M. Prieto, F. San Juan, J. M. Rayon, F. Martinez, D. Carrasco, A. Moya, F. Orbis, J. Mir, and J. Berenguer.** 2002. Contribution of donor age to the recent decrease in patient survival among HCV-infected liver transplant recipients. Hepatology. **36:**202-210. doi: 10.1053/jhep.2002.33993.

37. **Bertaux, C., and T. Dragic.** 2006. Different domains of CD81 mediate distinct stages of hepatitis C virus pseudoparticle entry. J. Virol. **80:**4940-4948. doi: 10.1128/JVI.80.10.4940-4948.2006.

38. **Berzsenyi, M. D., D. S. Bowden, and S. K. Roberts.** 2005. GB virus C: Insights into co-infection. Journal of Clinical Virology. **33:**257-266. doi: DOI: 10.1016/j.jcv.2005.04.002.

39. **Bialek, S. R., and N. A. Terrault.** 2006. The changing epidemiology and natural history of hepatitis C virus infection. Clin. Liver Dis. **10:**697-715. doi: 10.1016/j.cld.2006.08.003.

40. **Bienz, K., D. Egger, T. Pfister, and M. Troxler.** 1992. Structural and functional characterization of the poliovirus replication complex. J. Virol. **66:**2740-2747.

41. **Biron CA, and Brossay L.** 2001. NK cells and NKT cells in innate defense against viral infections. Curr. Opin. Immunol. **13:**458-64.

42. **Blackard, J. T., N. Kemmer, and K. E. Sherman.** 2006. Extrahepatic replication of HCV: insights into clinical manifestations and biological consequences. Hepatology. **44:**15-22. doi: 10.1002/hep.21283.

43. **Blank, C., T. F. Gajewski, and A. Mackensen.** 2005. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. Cancer Immunol. Immunother. **54:**307-314. doi: 10.1007/s00262-004-0593-x.

44. **Blight, K. J., and C. M. Rice.** 1997. Secondary structure determination of the conserved 98-base sequence at the 3' terminus of hepatitis C virus genome RNA. J. Virol. **71:**7345-7352.

45. **Boddi, M., R. Abbate, B. Chellini, B. Giusti, V. Solazzo, F. Soft, G. Pratesi, C. Pratesi, G. Gensini, and A. L. Zignego.** 2007. HCV infection facilitates asymptomatic carotid atherosclerosis: preliminary report of HCV RNA localization in human carotid plaques. Digestive and Liver Disease. **39:**S55-S60.

46. **Bradley, D. W.** 2000. Studies of non-A, non-B hepatitis and characterization of the hepatitis C virus in chimpanzees. Curr. Top. Microbiol. Immunol. **242:**1-23.

47. **Branch, A. D., D. D. Stump, J. A. Gutierrez, F. Eng, and J. L. Walewski.** 2005. The hepatitis C virus alternate reading frame (ARF) and its family of novel products: the alternate reading frame protein/F-protein, the double-frameshift protein, and others. Semin. Liver Dis. **25:**105-117. doi: 10.1055/s-2005-864786.

48. **Brinton, M. A., and J. H. Dispoto.** 1988. Sequence and secondary structure analysis of the 5'-terminal region of flavivirus genome RNA. Virology. **162:**290-299.

49. **Broide, E., S. Reif, E. Brazovski, R. Shapira, B. Weiss, Y. Bujanover, H. Hager, and N. Amir.** 2004. Chronic hepatitis C in Israeli children. Fetal. Pediatr. Pathol. **23:**231-239.

50. **Brown, E. A., H. Zhang, L. H. Ping, and S. M. Lemon.** 1992. Secondary structure of the 5' nontranslated regions of hepatitis C virus and pestivirus genomic RNAs. Nucleic Acids Res. **20:**5041-5045.

51. **Buck, M., and M. Chojkier.** 1996. Muscle wasting and dedifferentiation induced by oxidative stress in a murine model of cachexia is prevented by inhibitors of nitric oxide synthesis and antioxidants. EMBO J. **15:**1753-1765.

52. **Buck, M., L. Zhang, N. A. Halasz, T. Hunter, and M. Chojkier.** 2001. Nuclear export of phosphorylated C/EBPbeta mediates the inhibition of albumin expression by TNF-alpha. EMBO J. **20:**6712-6723. doi: 10.1093/emboj/20.23.6712.

53. **Bukh, J.** 2004. A critical role for the chimpanzee model in the study of hepatitis C. Hepatology. **39:**1469-1475. doi: 10.1002/hep.20268.

54. **Bukh, J., R. H. Miller, and R. H. Purcell.** 1995. Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. Semin. Liver Dis. **15:**41-63. doi: 10.1055/s-2007-1007262.

55. **Bukh, J., R. H. Purcell, and R. H. Miller.** 1993. At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. Proc. Natl. Acad. Sci. U. S. A. **90:**8234-8238.

56. **Burlone, M. E., and A. Budkowska.** 2009. Hepatitis C virus cell entry: role of lipoproteins and cellular receptors. J. Gen. Virol. **90:**1055-1070. doi: 10.1099/vir.0.008300-0.

57. **Butt, A. A., and F. Kanwal.** 2012. Boceprevir and telaprevir in the management of hepatitis C virus-infected patients. Clin. Infect. Dis. **54:**96-104. doi: 10.1093/cid/cir774.

58. **Cacoub, P., C. Renou, E. Rosenthal, P. Cohen, I. Loury, V. Loustaud-Ratti, A. M. Yamamoto, A. C. Camproux, P. Hausfater, L. Musset, P. Veyssier, G. Raguin, and J. C. Piette.** 2000. Extrahepatic manifestations associated with hepatitis C virus infection. A prospective multicenter study of 321 patients. The GERMIVIC. Groupe d'Etude et de Recherche en Medecine Interne et Maladies Infectieuses sur le Virus de l'Hepatite C. Medicine (Baltimore). **79:**47-56.

59. **Cai, Z., C. Zhang, K. S. Chang, J. Jiang, B. C. Ahn, T. Wakita, T. J. Liang, and G. Luo.** 2005. Robust production of infectious hepatitis C virus (HCV) from stably HCV cDNA-transfected human hepatoma cells. J. Virol. **79:**13963-13973. doi: 10.1128/JVI.79.22.13963-13973.2005.

60. **Cao, J., E. B. Yang, J. J. Su, Y. Li, and P. Chow.** 2003. The tree shrews: adjuncts and alternatives to primates as models for biomedical research. J. Med. Primatol. **32:**123-130.

61. **Castet, V., C. Fournier, A. Soulier, R. Brillet, J. Coste, D. Larrey, D. Dhumeaux, P. Maurel, and J. M. Pawlotsky.** 2002. Alpha interferon inhibits hepatitis C virus replication in primary human hepatocytes infected in vitro. J. Virol. **76:**8189-8199.

62. **Castillo I., Bartolome J., Quiroga J.A., Barril G., and Carreno V.** 2010. Diagnosis of occult hepatitis C without the need for a liver biopsy. J.Med.Virol.Journal of Medical Virology. **82:**1554-1559.

63. **Castillo I, Pardo M, Bartolomé J, Ortiz-Movilla N, Rodríguez-Iñigo E, de Lucas S, Salas C, Jiménez-Heffernan JA, Pérez-Mota A, Graus J, López-Alcorocho JM, and Carreño V.** 2004. Occult hepatitis C virus infection in patients in whom the etiology of persistently abnormal results of liver-function tests is unknown. J. Infect. Dis. **189:**7-14.

64. **Castillo, I., E. Rodriguez-Inigo, J. M. Lopez-Alcorocho, M. Pardo, J. Bartolome, and V. Carreno.** 2006. Hepatitis C virus replicates in the liver of patients who have a sustained response to antiviral treatment. Clin. Infect. Dis. **43:**1277-1283. doi: 10.1086/508198.

65. **Catanese, M. T., R. Graziani, T. von Hahn, M. Moreau, T. Huby, G. Paonessa, C. Santini, A. Luzzago, C. M. Rice, R. Cortese, A. Vitelli, and A. Nicosia.** 2007. High-avidity monoclonal antibodies against the human scavenger class B type I receptor efficiently block hepatitis C virus infection in the presence of high-density lipoprotein. J. Virol. **81:**8063-8071. doi: 10.1128/JVI.00193-07.

66. **Cheng, J. C., M. F. Chang, and S. C. Chang.** 1999. Specific interaction between the hepatitis C virus NS5B RNA polymerase and the 3' end of the viral RNA. J. Virol. **73**:7044-7049.

67. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science. **244**:359-362.

68. **Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina-Selby, and P. J. Barr.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. U. S. A. **88**:2451-2455.

69. **Chuang, S. S., Y. L. Liao, S. T. Chang, Y. C. Hsieh, S. Y. Kuo, C. L. Lu, W. S. Hwang, I. H. Lin, C. J. Tsao, and W. T. Huang.** 2010. Hepatitis C virus infection is significantly associated with malignant lymphoma in Taiwan, particularly with nodal and splenic marginal zone lymphomas. J. Clin. Pathol. **63**:595-598. doi: 10.1136/jcp.2010.076810.

70. **Chung, N. S., and K. M. Wasan.** 2004. Potential role of the low-density lipoprotein receptor family as mediators of cellular drug uptake. Adv. Drug Deliv. Rev. **56**:1315-1334. doi: 10.1016/j.addr.2003.12.003.

71. **Ciancio, A., A. Smedile, C. Giordanino, C. Colletta, G. Croce, M. Pozzi, G. Cariti, A. Macor, A. Biglino, A. Di Napoli, G. F. Tappero, M. Andreoni, A.**

198

Manca, G. Prandi, G. Calleri, P. G. Orsi, G. Ciccone, M. Rizzetto, and G. Saracco. 2006. Long-Term Follow-Up of Previous Hepatitis C Virus Positive Nonresponders to Interferon Monotherapy Successfully Retreated with Combination Therapy: Are They Really Cured? Am. J. Gastroenterol. **101:**1811-1816.

72. **Ciurea, A., P. Klenerman, L. Hunziker, E. Horvath, B. Odermatt, A. F. Ochsenbein, H. Hengartner, and R. M. Zinkernagel.** 1999. Persistence of lymphocytic choriomeningitis virus at very low levels in immune mice. Proc. Natl. Acad. Sci. U. S. A. **96:**11964-11969.

73. **Coffin, C. S., P. M. Mulrooney-Cousins, G. van Marle, J. P. Roberts, T. I. Michalak, and N. A. Terrault.** 2011. Hepatitis B virus quasispecies in hepatic and extrahepatic viral reservoirs in liver transplant recipients on prophylactic therapy. Liver Transpl. **17:**955-962. doi: 10.1002/lt.22312; 10.1002/lt.22312.

74. **Collett, M. S., D. K. Anderson, and E. Retzel.** 1988. Comparisons of the pestivirus bovine viral diarrhoea virus with members of the flaviviridae. J. Gen. Virol. **69 (Pt 10):**2637-2643.

75. **Conry-Cantilena, C., M. VanRaden, J. Gibble, J. Melpolder, A. O. Shakil, L. Viladomiu, L. Cheung, A. DiBisceglie, J. Hoofnagle, and J. W. Shih.** 1996. Routes of infection, viremia, and liver disease in blood donors found to have hepatitis C virus infection. N. Engl. J. Med. **334:**1691-1696. doi: 10.1056/NEJM199606273342602.

76. **CORADO, J., F. TORO, H. RIVERA, N. E. BIANCO, L. DEIBIS, and J. B. DE SANCTIS.** 1997. Impairment of natural killer (NK) cytotoxic activity in hepatitis C virus (HCV) infection. Clinical & Experimental Immunology. **109:**451.

77. **Cormier, E. G., F. Tsamis, F. Kajumo, R. J. Durso, J. P. Gardner, and T. Dragic.** 2004. CD81 is an entry coreceptor for hepatitis C virus. Proc. Natl. Acad. Sci. U. S. A. **101:**7270-7274. doi: 10.1073/pnas.0402253101.

78. **Costafreda, M. I., A. Bosch, and R. M. Pinto.** 2006. Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. Appl. Environ. Microbiol. **72:**3846-3855. doi: 10.1128/AEM.02660-05.

79. **Cribier, B., C. Garnier, D. Laustriat, and E. Heid.** 1994. Lichen planus and hepatitis C virus infection: An epidemiologic study. J. Am. Acad. Dermatol. **31:**1070-1072.

80. **Crotta, S., A. Stilla, A. Wack, A. D'Andrea, S. Nuti, U. D'Oro, M. Mosca, F. Filliponi, R. M. Brunetto, F. Bonino, S. Abrignani, and N. M. Valiante.** 2002. Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. J. Exp. Med. **195:**35-41.

81. **Curry, M. P., L. Golden-Mason, D. G. Doherty, T. Deignan, S. Norris, M. Duffy, N. Nolan, W. Hall, J. E. Hegarty, and C. O'Farrelly.** 2003. Expansion of

innate CD5pos B cells expressing high levels of CD81 in hepatitis C virus infected liver. J. Hepatol. **38:**642-650.

82. **Dahari, H., A. Feliu, M. Garcia-Retortillo, X. Forns, and A. U. Neumann.** 2005. Second hepatitis C replication compartment indicated by viral dynamics during liver transplantation. J. Hepatol. **42:**491-498. doi: 10.1016/j.jhep.2004.12.017.

83. **Damm, E. M., L. Pelkmans, J. Kartenbeck, A. Mezzacasa, T. Kurzchalia, and A. Helenius.** 2005. Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. J. Cell Biol. **168:**477-488. doi: 10.1083/jcb.200407113.

84. **Dash, S., A. B. Halim, H. Tsuji, N. Hiramatsu, and M. A. Gerber.** 1997. Transfection of HepG2 cells with infectious hepatitis C virus genome. Am. J. Pathol. **151:**363-373.

85. **De Marco L, Gillio-Tos A, Fiano V, Ronco G, Krogh V, Palli D, Panico S, Tumino R, Vineis P, Merletti F, Richiardi L, and Sacerdote C.** 2009. Occult HCV infection: an unexpected finding in a population unselected for hepatic disease. PloS One. **4:**.

86. **Di Liberto, G., A. M. Roque-Afonso, R. Kara, D. Ducoulombier, G. Fallot, D. Samuel, and C. Feray.** 2006. Clinical and therapeutic implications of hepatitis

C virus compartmentalization. Gastroenterology. **131:**76-84. doi: 10.1053/j.gastro.2006.04.016.

87. **Di Martino, V., P. Lebray, R. P. Myers, E. Pannier, V. Paradis, F. Charlotte, J. Moussalli, D. Thabut, C. Buffet, and T. Poynard.** 2004. Progression of liver fibrosis in women infected with hepatitis C: long-term benefit of estrogen exposure. Hepatology. **40:**1426-1433. doi: 10.1002/hep.20463.

88. **Dolganiuc, A., S. Chang, K. Kodys, P. Mandrekar, G. Bakis, M. Cormier, and G. Szabo.** 2006. Hepatitis C virus (HCV) core protein-induced, monocyte-mediated mechanisms of reduced IFN-alpha and plasmacytoid dendritic cell loss in chronic HCV infection. J. Immunol. **177:**6758-6768.

89. **Dreux, M., T. Pietschmann, C. Granier, C. Voisset, S. Ricard-Blum, P. E. Mangeot, Z. Keck, S. Foung, N. Vu-Dac, J. Dubuisson, R. Bartenschlager, D. Lavillette, and F. L. Cosset.** 2006. High density lipoprotein inhibits hepatitis C virus-neutralizing antibodies by stimulating cell entry via activation of the scavenger receptor BI. J. Biol. Chem. **281:**18285-18295. doi: 10.1074/jbc.M602706200.

90. **D'Souza M, Fontenot AP, Mack DG, Lozupone C, Dillon S, Meditz A, Wilson CC, Connick E, and Palmer BE.** 2007. Programmed death 1 expression on HIV-specific CD4+ T cells is driven by viral replication and associated with T cell dysfunction. Journal of Immunology (Baltimore, Md.: 1950). **179:**1979-87.

91. **Dubuisson, J.** 2007. Hepatitis C virus proteins. World J. Gastroenterol. **13:**2406-2415.

92. **Dubuisson, J., H. H. Hsu, R. C. Cheung, H. B. Greenberg, D. G. Russell, and C. M. Rice.** 1994. Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. J. Virol. **68:**6147-6160.

93. **Ducoulombier, D., A. M. Roque-Afonso, G. Di Liberto, F. Penin, R. Kara, Y. Richard, E. Dussaix, and C. Feray.** 2004. Frequent compartmentalization of hepatitis C virus variants in circulating B cells and monocytes. Hepatology. **39:**817-825. doi: 10.1002/hep.20087.

94. **Durand, T., G. Di Liberto, H. Colman, A. Cammas, S. Boni, P. Marcellin, A. Cahour, S. Vagner, and C. Feray.** 2010. Occult infection of peripheral B cells by hepatitis C variants which have low translational efficiency in cultured hepatocytes. Gut. **59:**934-942. doi: 10.1136/gut.2009.192088.

95. **Duvet S, Cocquerel L, Pillez A, Cacan R, Verbert A, Moradpour D, Wychowski C, and Dubuisson J.** 1998. Hepatitis C virus glycoprotein complex localization in the endoplasmic reticulum involves a determinant for retention and not retrieval. The Journal of Biological Chemistry. **273:**32088-95.

96. **Dwight, M. M., K. V. Kowdley, J. E. Russo, P. S. Ciechanowski, A. M. Larson, and W. J. Katon.** 2000. Depression, fatigue, and functional disability in patients with chronic hepatitis C. J. Psychosom. Res. **49:**311-317.

97. **Elmowalid, G. A., M. Qiao, S. H. Jeong, B. B. Borg, T. F. Baumert, R. K. Sapp, Z. Hu, K. Murthy, and T. J. Liang.** 2007. Immunization with hepatitis C virus-like particles results in control of hepatitis C virus infection in chimpanzees. Proc. Natl. Acad. Sci. U. S. A. **104:**8427-8432. doi: 10.1073/pnas.0702162104.

98. **Eren, R., D. Landstein, D. Terkieltaub, O. Nussbaum, A. Zauberman, J. Ben-Porath, J. Gopher, R. Buchnick, R. Kovjazin, Z. Rosenthal-Galili, S. Aviel, E. Ilan, Y. Shoshany, L. Neville, T. Waisman, O. Ben-Moshe, A. Kischitsky, S. K. Foung, Z. Y. Keck, O. Pappo, A. Eid, O. Jurim, G. Zamir, E. Galun, and S. Dagan.** 2006. Preclinical evaluation of two neutralizing human monoclonal antibodies against hepatitis C virus (HCV): a potential treatment to prevent HCV reinfection in liver transplant patients. J. Virol. **80:**2654-2664. doi: 10.1128/JVI.80.6.2654-2664.2006.

99. **Erhardt, A., A. Maschner-Olberg, C. Mellenthin, G. Kappert, O. Adams, A. Donner, R. Willers, C. Niederau, and D. Haussinger.** 2003. HFE mutations and chronic hepatitis C: H63D and C282Y heterozygosity are independent risk factors for liver fibrosis and cirrhosis. J. Hepatol. **38:**335-342.

100. **Esumi, M., T. Rikihisa, S. Nishimura, J. Goto, K. Mizuno, Y. H. Zhou, and T. Shikata.** 1999. Experimental vaccine activities of recombinant E1 and E2

glycoproteins and hypervariable region 1 peptides of hepatitis C virus in chimpanzees. Arch. Virol. **144:**973-980.

101. **European Association for the Study of the Liver.** 2011. EASL Clinical Practice Guidelines: management of hepatitis C virus infection. J. Hepatol. **55:**245-264. doi: 10.1016/j.jhep.2011.02.023.

102. **Evans, M. J., T. von Hahn, D. M. Tscherne, A. J. Syder, M. Panis, B. Wolk, T. Hatziioannou, J. A. McKeating, P. D. Bieniasz, and C. M. Rice.** 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature. **446:**801-805. doi: 10.1038/nature05654.

103. **Eyre, N. S., H. E. Drummer, and M. R. Beard.** 2010. The SR-BI partner PDZK1 facilitates hepatitis C virus entry. PLoS Pathog. **6:**e1001130. doi: 10.1371/journal.ppat.1001130.

104. **Eyster, M. E., H. J. Alter, L. M. Aledort, S. Quan, A. Hatzakis, and J. J. Goedert.** 1991. Heterosexual co-transmission of hepatitis C virus (HCV) and human immunodeficiency virus (HIV). Ann. Intern. Med. **115:**764-768.

105. **Fabris, M., L. Quartuccio, S. Sacco, G. De Marchi, G. Pozzato, C. Mazzaro, G. Ferraccioli, T. S. Migone, and S. De Vita.** 2007. B-Lymphocyte stimulator (BLyS) up-regulation in mixed cryoglobulinaemia syndrome and hepatitis-C virus infection. Rheumatology (Oxford). **46:**37-43. doi: 10.1093/rheumatology/kel174.

106. **Fallahian, F., and A. Najafi.** 2011. Epidemiology of hepatitis C in the Middle East. Saudi J. Kidney Dis. Transpl. **22:**1-9.

107. **Farci, P.** 2003. Delta hepatitis: an update. J. Hepatol. **39 Suppl 1:**S212-9.

108. **Farci, P., J. Bukh, and R. H. Purcell.** 1997. The quasispecies of hepatitis C virus and the host immune response. Springer Semin. Immunopathol. **19:**5-26.

109. **Farci, P., W. T. London, D. C. Wong, G. J. Dawson, D. S. Vallari, R. Engle, and R. H. Purcell.** 1992. The natural history of infection with hepatitis C virus (HCV) in chimpanzees: comparison of serologic responses measured with first- and second-generation assays and relationship to HCV viremia. J. Infect. Dis. **165:**1006-1011.

110. **Farci, P., A. Shimoda, A. Coiana, G. Diaz, G. Peddis, J. C. Melpolder, A. Strazzera, D. Y. Chien, S. J. Munoz, A. Balestrieri, R. H. Purcell, and H. J. Alter.** 2000. The Outcome of Acute Hepatitis C Predicted by the Evolution of the Viral Quasispecies. Science. **288:**339-344. doi: 10.1126/science.288.5464.339.

111. **Farquhar, M. J., and J. A. McKeating.** 2008. Primary hepatocytes as targets for hepatitis C virus replication. J. Viral Hepat. **15:**849-854. doi: 10.1111/j.1365-2893.2008.01051.x.

112. **Fauquet, C. M., and D. Fargette.** 2005. International Committee on Taxonomy of Viruses and the 3,142 unassigned species. Virol. J. **2:**64. doi: 10.1186/1743-422X-2-64.

113. **Fearon, D. T., and M. C. Carroll.** 2000. Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. Annu. Rev. Immunol. **18:**393-422. doi: 10.1146/annurev.immunol.18.1.393.

114. **Feinstone, S. M., A. Z. Kapikian, and R. H. Purcell.** 1973. Hepatitis A: detection by immune electron microscopy of a viruslike antigen associated with acute illness. Science. **182:**1026-1028.

115. **Ferri C, Caracciolo F, Zignego AL, La Civita L, Monti M, Longombardo G, Lombardini F, Greco F, Capochiani E, Mazzoni A, and et al.** 1994. Hepatitis C virus infection in patients with non-Hodgkin's lymphoma. Br. J. Haematol. **88:**392-4.

116. **Ferri, C., M. A. Bertozzi, and A. L. Zignego.** 2002. Erectile dysfunction and hepatitis C virus infection [7]. J. Am. Med. Assoc. **288:**698-699.

117. **Ferri, C., L. La Civita, C. Cirafisi, G. Siciliano, G. Longombardo, S. Bombardieri, and B. Rossi.** 1992. Peripheral neuropathy in mixed cryoglobulinemia: Clinical and electrophysiologic investigations. J. Rheumatol. **19:**889-895.

118. **Ferri, C., M. Monti, L. La Civita, G. Longombardo, F. Greco, G. Pasero, P. Gentilini, S. Bombardieri, and A. L. Zignego.** 1993. Infection of peripheral blood mononuclear cells by hepatitis C virus in mixed cryoglobulinemia. Blood. **82:**3701-3704.

119. **Ferri, C., M. Sebastiani, D. Giuggioli, M. Cazzato, G. Longombardo, A. Antonelli, R. Puccini, C. Michelassi, and A. L. Zignego.** 2004. Mixed cryoglobulinemia: Demographic, clinical, and serologic features and survival in 231 patients. Semin. Arthritis Rheum. **33:**355-374.

120. **Firestone Cruz, M., B. Fischer, J. Patra, K. Kalousek, B. Newton-Taylor, J. Rehm, and M. Tyndall.** 2007. Prevalence and associated factors of hepatitis C infection (HCV) in a multi-site Canadian population of illicit opioid and other drug users (OPICAN). Can. J. Public Health. **98:**130-133.

121. **Fischer, B., E. Haydon, J. Rehm, M. Krajden, and J. Reimer.** 2004. Injection drug use and the hepatitis C virus: considerations for a targeted treatment approach--the case study of Canada. J. Urban Health. **81:**428-447. doi: 10.1093/jurban/jth128.

122. **Flint, M., and J. A. McKeating.** 2000. The role of the hepatitis C virus glycoproteins in infection. Rev. Med. Virol. **10:**101-117.

123. **Flint, M., J. M. Thomas, C. M. Maidens, C. Shotton, S. Levy, W. S. Barclay, and J. A. McKeating.** 1999. Functional analysis of cell surface-expressed hepatitis C virus E2 glycoprotein. J. Virol. **73:**6782-6790.

124. **Flint, M., T. von Hahn, J. Zhang, M. Farquhar, C. T. Jones, P. Balfe, C. M. Rice, and J. A. McKeating.** 2006. Diverse CD81 proteins support hepatitis C virus infection. J. Virol. **80:**11331-11342. doi: 10.1128/JVI.00104-06.

125. **FOLEY, G. E., H. LAZARUS, S. FARBER, B. G. UZMAN, B. A. BOONE, and R. E. MCCARTHY.** 1965. Continuous Culture of Human Lymphoblasts from Peripheral Blood of a Child with Acute Leukemia. Cancer. **18:**522-529.

126. **Folgori, A., S. Capone, L. Ruggeri, A. Meola, E. Sporeno, B. B. Ercole, M. Pezzanera, R. Tafi, M. Arcuri, E. Fattori, A. Lahm, A. Luzzago, A. Vitelli, S. Colloca, R. Cortese, and A. Nicosia.** 2006. A T-cell HCV vaccine eliciting effective immunity against heterologous virus challenge in chimpanzees. Nat. Med. **12:**190-197. doi: 10.1038/nm1353.

127. **Forton, D. M., P. Karayiannis, N. Mahmud, S. D. Taylor-Robinson, and H. C. Thomas.** 2004. Identification of unique hepatitis C virus quasispecies in the central nervous system and comparative analysis of internal translational efficiency of brain, liver, and serum variants. J. Virol. **78:**5170-5183.

128. **Forton, D. M., H. C. Thomas, C. A. Murphy, J. M. Allsop, G. R. Foster, J. Main, K. A. Wesnes, and S. D. Taylor-Robinson.** 2002. Hepatitis C and

cognitive impairment in a cohort of patients with mild liver disease. Hepatology. **35:**433-439.

129. **Foster, G. R., C. Hezode, J. P. Bronowicki, G. Carosi, O. Weiland, L. Verlinden, R. van Heeswijk, B. van Baelen, G. Picchio, and M. Beumont.** 2011. Telaprevir alone or with peginterferon and ribavirin reduces HCV RNA in patients with chronic genotype 2 but not genotype 3 infections. Gastroenterology. **141:**881-889.e1. doi: 10.1053/j.gastro.2011.05.046.

130. **Foster, G. R., C. Hezode, J. P. Bronowicki, G. Carosi, O. Weiland, L. Verlinden, R. van Heeswijk, T. Vangeneugden, G. Picchio, M. Beumont-Mauviel, and Abstracts of the International Liver Congress TM 2009. 44th Annual Meeting of the European Association for the Study of the Liver.** 2009. Activity Of Telaprevir Alone Or In Combination With Peginterferon Alfa-2a And Ribavirin In Treatment-Naive Genotype 2 And 3 Hepatitis-C Patients: Interim Results Of Study C209. J. Hepatol. **50:**S22.

131. **Fournier, C., C. Sureau, J. Coste, J. Ducos, G. Pageaux, D. Larrey, J. Domergue, and P. Maurel.** 1998. In vitro infection of adult normal human hepatocytes in primary culture by hepatitis C virus. J. Gen. Virol. **79 (Pt 10):**2367-2374.

132. **Frank, C., M. K. Mohamed, G. T. Strickland, D. Lavanchy, R. R. Arthur, L. S. Magder, T. El Khoby, Y. Abdel-Wahab, E. S. Aly Ohn, W. Anwar, and I.**

Sallam. 2000. The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. Lancet. **355:**887-891.

133. **Friebe, P., and R. Bartenschlager.** 2002. Genetic analysis of sequences in the 3' nontranslated region of hepatitis C virus that are important for RNA replication. J. Virol. **76:**5326-5338.

134. **Friedlein, G., F. El Hage, I. Vergnon, C. Richon, P. Saulnier, Y. Lecluse, A. Caignard, L. Boumsell, G. Bismuth, S. Chouaib, and F. Mami-Chouaib.** 2007. Human CD5 protects circulating tumor antigen-specific CTL from tumor-mediated activation-induced cell death. J. Immunol. **178:**6821-6827.

135. **Frolov, I., M. S. McBride, and C. M. Rice.** 1998. cis-acting RNA elements required for replication of bovine viral diarrhea virus-hepatitis C virus 5' nontranslated region chimeras. RNA. **4:**1418-1435.

136. **Fukuma, T., N. Enomoto, F. Marumo, and C. Sato.** 1998. Mutations in the interferon-sensitivity determining region of hepatitis C virus and transcriptional activity of the nonstructural region 5A protein. Hepatology. **28:**1147-1153. doi: 10.1002/hep.510280433.

137. **Furuse, M., M. Hata, K. Furuse, Y. Yoshida, A. Haratake, Y. Sugitani, T. Noda, A. Kubo, and S. Tsukita.** 2002. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J. Cell Biol. **156:**1099-1111. doi: 10.1083/jcb.200110122.

138. **Galossi, A., R. Guarisco, L. Bellis, and C. Puoti.** 2007. Extrahepatic manifestations of chronic HCV infection. J. Gastrointest Liver Dis. **16:**65-73.

139. **Galun, E., N. A. Terrault, R. Eren, A. Zauberman, O. Nussbaum, D. Terkieltaub, M. Zohar, R. Buchnik, Z. Ackerman, R. Safadi, Y. Ashur, S. Misrachi, Y. Liberman, L. Rivkin, and S. Dagan.** 2007. Clinical evaluation (Phase I) of a human monoclonal antibody against hepatitis C virus: safety and antiviral activity. J. Hepatol. **46:**37-44. doi: 10.1016/j.jhep.2006.08.019.

140. **Gane, E.** 2003. The natural history and outcome of liver transplantation in hepatitis C virus-infected recipients. Liver Transpl. **9:**S28-34. doi: 10.1053/jlts.2003.50248.

141. Gao M., Fridell R.A., Sun J.-H., O'Boyle Ii D.R., Lemm J.A., Wang C., Colonno R.J., Nettles R.E., Chien C., Grasela D.M., Belema M., Snyder L.B., Nguyen V.N., Serrano-Wu M.H., Meanwell N.A., Hamann L.G., Langley D.R., and Knipe J.O. 2010. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. Nature Nature. **465:**96-100.

142. **Garcia-Monzon, C., P. Jara, M. Fernandez-Bermejo, L. Hierro, E. Frauca, C. Camarena, C. Diaz, A. De la Vega, J. Larrauri, C. Garcia-Iglesias, M. J. Borque, P. Sanz, L. Garcia-Buey, J. A. Moreno-Monteagudo, and R. Moreno-Otero.** 1998. Chronic hepatitis C in children: a clinical and immunohistochemical comparative study with adult patients. Hepatology. **28:**1696-1701. doi: 10.1002/hep.510280633.

143. **Garcia-Retortillo, M., X. Forns, A. Feliu, E. Moitinho, J. Costa, M. Navasa, A. Rimola, and J. Rodes.** 2002. Hepatitis C virus kinetics during and immediately after liver transplantation. Hepatology. **35:**680-687.

144. **Gardner, J. P., R. J. Durso, R. R. Arrigale, G. P. Donovan, P. J. Maddon, T. Dragic, and W. C. Olson.** 2003. L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. Proc. Natl. Acad. Sci. U. S. A. **100:**4498-4503. doi: 10.1073/pnas.0831128100.

145. **Gastaminza, P., K. A. Dryden, B. Boyd, M. R. Wood, M. Law, M. Yeager, and F. V. Chisari.** 2010. Ultrastructural and biophysical characterization of hepatitis C virus particles produced in cell culture. J. Virol. **84:**10999-11009. doi: 10.1128/JVI.00526-10.

146. **Ge, D., J. Fellay, A. J. Thompson, J. S. Simon, K. V. Shianna, T. J. Urban, E. L. Heinzen, P. Qiu, A. H. Bertelsen, A. J. Muir, M. Sulkowski, J. G. McHutchison, and D. B. Goldstein.** 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. **461:**399-401. doi: 10.1038/nature08309.

147. **Germi, R., J. Crance, D. Garin, J. Guimet, H. Lortat-Jacob, R. W. H. Ruigrok, J. Zarski, and E. Drouet.** 2002. Cellular glycosaminoglycans and low density lipoprotein receptor are involved in hepatitis C virus adsorption. J. Med. Virol. **68:**206-215. doi: 10.1002/jmv.10196. http://dx.doi.org/10.1002/jmv.10196.

148. **Ghany, M. G., D. R. Nelson, D. B. Strader, D. L. Thomas, and L. B. Seeff.** 2011. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. Hepatology. **54:**1433-1444. doi: 10.1002/hep.24641; 10.1002/hep.24641.

149. **Giannini, C., F. Giannelli, and A. L. Zignego.** 2006. Association between mixed cryogtobulinemia, translocation (14;18), and persistence of occult HCV lymphoid infection after treatment [1]. Hepatology. **43:**1166-1167.

150. **Girish, V., and A. Vijayalakshmi.** 2004. Affordable image analysis using NIH Image/ImageJ. Indian J. Cancer. **41:**47.

151. **Gisbert, J. P., L. García-Buey, J. M. Pajares, and R. Moreno-Otero.** 2003. Prevalence of hepatitis C virus infection in B-cell non-Hodgkin's lymphoma: systematic review and meta-analysis. Gastroenterology. **125:**1723-1732. doi: DOI: 10.1053/j.gastro.2003.09.025.

152. **Glynn, S. A., D. J. Wright, S. H. Kleinman, D. Hirschkorn, Y. Tu, C. Heldebrant, R. Smith, C. Giachetti, J. Gallarda, and M. P. Busch.** 2005. Dynamics of viremia in early hepatitis C virus infection. Transfusion. **45:**994-1002. doi: 10.1111/j.1537-2995.2005.04390.x.

153. **Golden-Mason, L., B. E. Palmer, N. Kassam, L. Townshend-Bulson, S. Livingston, B. J. McMahon, N. Castelblanco, V. Kuchroo, D. R. Gretch, and**

H. R. Rosen. 2009. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. J. Virol. **83:**9122-9130. doi: 10.1128/JVI.00639-09.

154. **Gong, G. Z., L. Y. Lai, Y. F. Jiang, Y. He, and X. S. Su.** 2003. HCV replication in PBMC and its influence on interferon therapy. World J. Gastroenterol. **9:**291-294.

155. **Gonzalez, M. E., and L. Carrasco.** 2003. Viroporins. FEBS Lett. **552:**28-34.

156. **Gosert R, Egger D, Lohmann V, Bartenschlager R, Blum HE, Bienz K, and Moradpour D.** 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J. Virol. **77:**5487-92.

157. **Gosert, R., A. Kanjanahaluethai, D. Egger, K. Bienz, and S. C. Baker.** 2002. RNA replication of mouse hepatitis virus takes place at double-membrane vesicles. J. Virol. **76:**3697-3708.

158. **Goutagny, N., A. Fatmi, V. De Ledinghen, F. Penin, P. Couzigou, G. Inchauspe, and C. Bain.** 2003. Evidence of viral replication in circulating dendritic cells during hepatitis C virus infection. J. Infect. Dis. **187:**1951-1958. doi: 10.1086/375350.

159. **Grakoui, A., N. H. Shoukry, D. J. Woollard, J. H. Han, H. L. Hanson, J. Ghrayeb, K. K. Murthy, C. M. Rice, and C. M. Walker.** 2003. HCV persistence

and immune evasion in the absence of memory T cell help. Science. **302:**659-662. doi: 10.1126/science.1088774.

160. **Greene, W. K., M. K. Cheong, V. Ng, and K. W. Yap.** 1995. Prevalence of hepatitis C virus sequence variants in South-East Asia. J. Gen. Virol. **76 (Pt 1):**211-215.

161. **Griffin, D. E.** 2010. Measles virus-induced suppression of immune responses. Immunol. Rev. **236:**176-189. doi: 10.1111/j.1600-065X.2010.00925.x.

162. **Griffin, S. D. C., L. P. Beales, D. S. Clarke, O. Worsfold, S. D. Evans, J. Jaeger, M. P. G. Harris, and D. J. Rowlands.** 2003. The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. FEBS Lett. **535:**34-38. doi: DOI: 10.1016/S0014-5793(02)03851-6.

163. **Guy, C. S., S. L. Rankin, J. Wang, and T. I. Michalak.** 2008. Hepatocytes can induce death of contacted cells via perforin-dependent mechanism. Hepatology. **47:**1691-1701. doi: 10.1002/hep.22228.

164. **Haddad, J., P. Deny, C. Munz-Gotheil, J. -. Ambrosini, J. -. Trinchet, D. Pateron, F. Mal, P. Callard, and M. Beaugrand.** 1992. Lymphocytic sialadenitis of Sjogren's syndrome associated with chronic hepatitis C virus liver disease. Lancet. **339:**321-323.

165. **Han, J. H., V. Shyamala, K. H. Richman, M. J. Brauer, B. Irvine, M. S. Urdea, P. Tekamp-Olson, G. Kuo, Q. L. Choo, and M. Houghton.** 1991. Characterization of the terminal regions of hepatitis C viral RNA: identification of conserved sequences in the 5' untranslated region and poly(A) tails at the 3' end. Proc. Natl. Acad. Sci. U. S. A. **88:**1711-1715.

166. **Hass, M., U. Golnitz, S. Muller, B. Becker-Ziaja, and S. Gunther.** 2004. Replicon system for Lassa virus. J. Virol. **78:**13793-13803. doi: 10.1128/JVI.78.24.13793-13803.2004.

167. **Heathcote, E. J.** 2007. Antiviral therapy: chronic hepatitis C. J. Viral Hepat. **14 Suppl 1:**82-88. doi: 10.1111/j.1365-2893.2007.00921.x.

168. **Heathcote, E. J., M. L. Shiffman, W. G. Cooksley, G. M. Dusheiko, S. S. Lee, L. Balart, R. Reindollar, R. K. Reddy, T. L. Wright, A. Lin, J. Hoffman, and J. De Pamphilis.** 2000. Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. N. Engl. J. Med. **343:**1673-1680. doi: 10.1056/NEJM200012073432302.

169. **Heathcote, J.** 2000. Antiviral therapy for patients with chronic hepatitis C. Semin. Liver Dis. **20:**185-199. doi: 10.1055/s-2000-9941.

170. **Helle, F., and J. Dubuisson.** 2008. Hepatitis C virus entry into host cells. Cell Mol. Life Sci. **65:**100-112. doi: 10.1007/s00018-007-7291-8.

171. **Hijikata, M., Y. K. Shimizu, H. Kato, A. Iwamoto, J. W. Shih, H. J. Alter, R. H. Purcell, and H. Yoshikura.** 1993. Equilibrium centrifugation studies of hepatitis C virus: evidence for circulating immune complexes. J. Virol. **67:**1953-1958.

172. **Hikosaka, K., H. Noritake, W. Kimura, N. Sultana, M. T. Sharkar, Y. Tagawa, T. Uezato, Y. Kobayashi, T. Wakita, and N. Miura.** 2011. Expression of human factors CD81, claudin-1, scavenger receptor, and occludin in mouse hepatocytes does not confer susceptibility to HCV entry. Biomed. Res. **32:**143-150.

173. **Honda, M., E. A. Brown, and S. M. Lemon.** 1996. Stability of a stem-loop involving the initiator AUG controls the efficiency of internal initiation of translation on hepatitis C virus RNA. RNA. **2:**955-968.

174. **Hoofnagle JH, and di Bisceglie AM.** 1997. The treatment of chronic viral hepatitis. N. Engl. J. Med. **336:**347-56.

175. **Hoofnagle, J. H.** 2006. Hepatitis B--preventable and now treatable. N. Engl. J. Med. **354:**1074-1076. doi: 10.1056/NEJMe058309.

176. **Hoofnagle, J. H., K. D. Mullen, D. B. Jones, V. Rustgi, A. Di Bisceglie, M. Peters, J. G. Waggoner, Y. Park, and E. A. Jones.** 1986. Treatment of chronic non-A,non-B hepatitis with recombinant human alpha interferon. A

preliminary report. N. Engl. J. Med. **315:**1575-1578. doi: 10.1056/NEJM198612183152503.

177. **Hou, J., D. L. Paul, and D. A. Goodenough.** 2005. Paracellin-1 and the modulation of ion selectivity of tight junctions. J. Cell. Sci. **118:**5109-5118. doi: 10.1242/jcs.02631.

178. **Hsu, M., J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc. Natl. Acad. Sci. U. S. A. **100:**7271-7276. doi: 10.1073/pnas.0832180100.

179. **Ilan, E., J. Arazi, O. Nussbaum, A. Zauberman, R. Eren, I. Lubin, L. Neville, O. Ben-Moshe, A. Kischitzky, A. Litchi, I. Margalit, J. Gopher, S. Mounir, W. Cai, N. Daudi, A. Eid, O. Jurim, A. Czerniak, E. Galun, and S. Dagan.** 2002. The hepatitis C virus (HCV)-Trimera mouse: a model for evaluation of agents against HCV. J. Infect. Dis. **185:**153-161. doi: 10.1086/338266.

180. **Imhof, I., and P. Simmonds.** 2011. Genotype differences in susceptibility and resistance development of hepatitis C virus to protease inhibitors telaprevir (VX-950) and danoprevir (ITMN-191). Hepatology. **53:**1090-1099. doi: 10.1002/hep.24172; 10.1002/hep.24172.

181. **Ishizaka, N., Y. Ishizaka, E. Takahashi, E. -. Tooda, H. Hashimoto, R. Nagai, and M. Yamakado.** 2002. Association between hepatitis C virus

seropositivity, carotid-artery plaque, and intima-media thickening. Lancet. **359:**133-135.

182. **Ito, T., and M. M. Lai.** 1997. Determination of the secondary structure of and cellular protein binding to the 3'-untranslated region of the hepatitis C virus RNA genome. J. Virol. **71:**8698-8706.

183. **Itoh, M., M. Furuse, K. Morita, K. Kubota, M. Saitou, and S. Tsukita.** 1999. Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2 and ZO-3, with the COOH termini of claudins. J. Cell Biol. **147:**1351-1363. doi: 10.1083/jcb.147.6.1351.

184. **Jara, P., M. Resti, L. Hierro, R. Giacchino, C. Barbera, L. Zancan, C. Crivellaro, E. Sokal, C. Azzari, M. Guido, and F. Bortolotti.** 2003. Chronic hepatitis C virus infection in childhood: clinical patterns and evolution in 224 white children. Clin. Infect. Dis. **36:**275-280. doi: 10.1086/345908.

185. **Ji, H., C. S. Fraser, Y. Yu, J. Leary, and J. A. Doudna.** 2004. Coordinated assembly of human translation initiation complexes by the hepatitis C virus internal ribosome entry site RNA. Proc. Natl. Acad. Sci. U. S. A. **101:**16990-16995. doi: 10.1073/pnas.0407402101.

186. **Johnson, R. J., D. R. Gretch, H. Yamabe, J. Hart, C. E. Bacchi, P. Hartwell, W. G. Couser, L. Corey, M. H. Wener, C. E. Alpers, and R. Willson.**

1993. Membranoproliferative glomerulonephritis associated with hepatitis C virus infection. N. Engl. J. Med. **328:**465-470.

187. **Jones, D. M., A. M. Atoom, X. Zhang, S. Kottilil, and R. S. Russell.** 2011. A genetic interaction between the core and NS3 proteins of hepatitis C virus is essential for production of infectious virus. J. Virol. **85:**12351-12361. doi: 10.1128/JVI.05313-11.

188. **Jones, S. M., K. E. Howell, J. R. Henley, H. Cao, and M. A. McNiven.** 1998. Role of dynamin in the formation of transport vesicles from the trans-Golgi network. Science. **279:**573-577.

189. **Kaito, M., S. Watanabe, K. Tsukiyama-Kohara, K. Yamaguchi, Y. Kobayashi, M. Konishi, M. Yokoi, S. Ishida, S. Suzuki, and M. Kohara.** 1994. Hepatitis C virus particle detected by immunoelectron microscopic study. J. Gen. Virol. **75 (Pt 7):**1755-1760.

190. **Kanto, T., N. Hayashi, T. Takehara, H. Hagiwara, E. Mita, M. Naito, A. Kasahara, H. Fusamoto, and T. Kamada.** 1994. Buoyant density of hepatitis C virus recovered from infected hosts: two different features in sucrose equilibrium density-gradient centrifugation related to degree of liver inflammation. Hepatology. **19:**296-302.

191. **Kapadia, S. B., and F. V. Chisari.** 2005. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. Proc. Natl. Acad. Sci. U. S. A. **102:**2561-2566. doi: 10.1073/pnas.0409834102.

192. **Kasama, Y., S. Sekiguchi, M. Saito, K. Tanaka, M. Satoh, K. Kuwahara, N. Sakaguchi, M. Takeya, Y. Hiasa, M. Kohara, and K. Tsukiyama-Kohara.** 2010. Persistent expression of the full genome of hepatitis C virus in B cells induces spontaneous development of B-cell lymphomas in vivo. Blood. **116:**4926-4933. doi: 10.1182/blood-2010-05-283358.

193. **Kato, T., Y. Choi, G. Elmowalid, R. K. Sapp, H. Barth, A. Furusaka, S. Mishiro, T. Wakita, K. Krawczynski, and T. J. Liang.** 2008. Hepatitis C virus JFH-1 strain infection in chimpanzees is associated with low pathogenicity and emergence of an adaptive mutation. Hepatology. **48:**732-740. doi: 10.1002/hep.22422.

194. **Kato, T., A. Furusaka, M. Miyamoto, T. Date, K. Yasui, J. Hiramoto, K. Nagayama, T. Tanaka, and T. Wakita.** 2001. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. J. Med. Virol. **64:**334-339.

195. **Kawamura, T., A. Furusaka, M. J. Koziel, R. T. Chung, T. C. Wang, E. V. Schmidt, and T. J. Liang.** 1997. Transgenic expression of hepatitis C virus structural proteins in the mouse. Hepatology. **25:**1014-1021. doi: 10.1002/hep.510250437.

196. **Kayali, Z., V. E. Buckwold, B. Zimmerman, and W. N. Schmidt.** 2002. Hepatitis C, cryoglobulinemia, and cirrhosis: A meta-analysis. Hepatology. **36:**978-985.

197. **King, E., C. Trabue, D. Yin, Z. Q. Yao, and J. P. Moorman.** 2007. Hepatitis C: the complications of immune dysfunction. Expert Rev. Clin. Immunol. **3:**145-157. doi: 10.1586/1744666X.3.2.145.

198. **Kiyosawa, K., T. Sodeyama, E. Tanaka, Y. Nakano, S. Furuta, K. Nishioka, R. H. Purcell, and H. J. Alter.** 1991. Hepatitis C in hospital employees with needlestick injuries. Ann. Intern. Med. **115:**367-369.

199. **Kneteman, N. M., and C. Toso.** 2009. In vivo study of HCV in mice with chimeric human livers. Methods Mol. Biol. **510:**383-399. doi: 10.1007/978-1-59745-394-3_29.

200. **Kneteman, N. M., A. J. Weiner, J. O'Connell, M. Collett, T. Gao, L. Aukerman, R. Kovelsky, Z. J. Ni, Q. Zhu, A. Hashash, J. Kline, B. Hsi, D. Schiller, D. Douglas, D. L. Tyrrell, and D. F. Mercer.** 2006. Anti-HCV therapies in chimeric scid-Alb/uPA mice parallel outcomes in human clinical application. Hepatology. **43:**1346-1353. doi: 10.1002/hep.21209.

201. **Knobler, H., R. Schihmanter, A. Zifroni, G. Fenakel, and A. Schattner.** 2000. Increased risk of type 2 diabetes in noncirrhotic patients with chronic hepatitis C virus infection. Mayo Clin. Proc. **75:**355-359.

202. **Kolykhalov, A. A., S. M. Feinstone, and C. M. Rice.** 1996. Identification of a highly conserved sequence element at the 3' terminus of hepatitis C virus genome RNA. J. Virol. **70:**3363-3371.

203. **Kondo, Y., V. M. Sung, K. Machida, M. Liu, and M. M. Lai.** 2007. Hepatitis C virus infects T cells and affects interferon-gamma signaling in T cell lines. Virology. **361:**161-173. doi: 10.1016/j.virol.2006.11.009.

204. **Koppel, E. A., K. P. van Gisbergen, T. B. Geijtenbeek, and Y. van Kooyk.** 2005. Distinct functions of DC-SIGN and its homologues L-SIGN (DC-SIGNR) and mSIGNR1 in pathogen recognition and immune regulation. Cell. Microbiol. **7:**157-165. doi: 10.1111/j.1462-5822.2004.00480.x.

205. **Korenaga, M., M. Okuda, K. Otani, T. Wang, Y. Li, and S. A. Weinman.** 2005. Mitochondrial dysfunction in hepatitis C. J. Clin. Gastroenterol. **39:**S162-6.

206. **Krieger, M.** 2001. Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. J. Clin. Invest. **108:**793-797. doi: 10.1172/JCI14011.

207. **Kroemer, G., and M. Jaattela.** 2005. Lysosomes and autophagy in cell death control. Nat. Rev. Cancer. **5:**886-897. doi: 10.1038/nrc1738.

208. **Kunkel, M., M. Lorinczi, R. Rijnbrand, S. M. Lemon, and S. J. Watowich.** 2001. Self-assembly of nucleocapsid-like particles from recombinant hepatitis C virus core protein. J. Virol. **75:**2119-2129. doi: 10.1128/JVI.75.5.2119-2129.2001.

209. **Lamarre, D., P. C. Anderson, M. Bailey, P. Beaulieu, G. Bolger, P. Bonneau, M. Bos, D. R. Cameron, M. Cartier, M. G. Cordingley, A. M. Faucher, N. Goudreau, S. H. Kawai, G. Kukolj, L. Lagace, S. R. LaPlante, H. Narjes, M. A. Poupart, J. Rancourt, R. E. Sentjens, R. St George, B. Simoneau, G. Steinmann, D. Thibeault, Y. S. Tsantrizos, S. M. Weldon, C. L. Yong, and M. Llinas-Brunet.** 2003. An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. Nature. **426:**186-189. doi: 10.1038/nature02099.

210. **Lanford, R. E., B. Guerra, H. Lee, D. Chavez, K. M. Brasky, and C. B. Bigger.** 2006. Genomic response to interferon-alpha in chimpanzees: implications of rapid downregulation for hepatitis C kinetics. Hepatology. **43:**961-972. doi: 10.1002/hep.21167.

211. **Lanford, R. E., E. S. Hildebrandt-Eriksen, A. Petri, R. Persson, M. Lindow, M. E. Munk, S. Kauppinen, and H. Orum.** 2010. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science. **327:**198-201. doi: 10.1126/science.1178178.

212. **Laporte, J., C. Bain, P. Maurel, G. Inchauspe, H. Agut, and A. Cahour.** 2003. Differential distribution and internal translation efficiency of hepatitis C virus

quasispecies present in dendritic and liver cells. Blood. **101:**52-57. doi: 10.1182/blood-2002-03-0818.

213. **Laporte, J., I. Malet, T. Andrieu, V. Thibault, J. J. Toulme, C. Wychowski, J. M. Pawlotsky, J. M. Huraux, H. Agut, and A. Cahour.** 2000. Comparative analysis of translation efficiencies of hepatitis C virus 5' untranslated regions among intraindividual quasispecies present in chronic infection: opposite behaviors depending on cell type. J. Virol. **74:**10827-10833.

214. **Laskus, T., M. Radkowski, A. Piasek, M. Nowicki, A. Horban, J. Cianciara, and J. Rakela.** 2000. Hepatitis C virus in lymphoid cells of patients coinfected with human immunodeficiency virus type 1: evidence of active replication in monocytes/macrophages and lymphocytes. J. Infect. Dis. **181:**442-448. doi: 10.1086/315283.

215. **Laskus, T., M. Radkowski, L. F. Wang, M. Nowicki, and J. Rakela.** 2000. Uneven distribution of hepatitis C virus quasispecies in tissues from subjects with end-stage liver disease: confounding effect of viral adsorption and mounting evidence for the presence of low-level extrahepatic replication. J. Virol. **74:**1014-1017.

216. **Laskus, T., M. Radkowski, J. Wilkinson, H. Vargas, and J. Rakela.** 2002. The origin of hepatitis C virus reinfecting transplanted livers: serum-derived versus peripheral blood mononuclear cell-derived virus. J. Infect. Dis. **185:**417-421. doi: 10.1086/338635.

217. **Laskus, T., L. F. Wang, M. Radkowski, S. J. Jang, H. Vargas, F. Dodson, J. Fung, and J. Rakela.** 1997. Hepatitis G virus infection in American patients with cryptogenic cirrhosis: no evidence for liver replication. J. Infect. Dis. **176:**1491-1495.

218. **Law, M. G., G. J. Dore, N. Bath, S. Thompson, N. Crofts, K. Dolan, W. Giles, P. Gow, J. Kaldor, S. Loveday, E. Powell, J. Spencer, and A. Wodak.** 2003. Modelling hepatitis C virus incidence, prevalence and long-term sequelae in Australia, 2001. Int. J. Epidemiol. **32:**717-724.

219. **Lee WM, Polson JE, Carney DS, Sahin B, and Gale M Jr.** 2005. Reemergence of hepatitis C virus after 8.5 years in a patient with hypogammaglobulinemia: evidence for an occult viral reservoir. J. Infect. Dis. **192:**1088-92.

220. **Lee, H., H. Shin, E. Wimmer, and A. V. Paul.** 2004. cis-acting RNA signals in the NS5B C-terminal coding sequence of the hepatitis C virus genome. J. Virol. **78:**10865-10877. doi: 10.1128/JVI.78.20.10865-10877.2004.

221. **Lehmann, M., M. F. Meyer, M. Monazahian, H. L. Tillmann, M. P. Manns, and H. Wedemeyer.** 2004. High rate of spontaneous clearance of acute hepatitis C virus genotype 3 infection. J. Med. Virol. **73:**387-391. doi: 10.1002/jmv.20103.

222. **Lerat, H., F. Berby, M. A. Trabaud, O. Vidalin, M. Major, C. Trepo, and G. Inchauspe.** 1996. Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. J. Clin. Invest. **97:**845-851. doi: 10.1172/JCI118485.

223. **Lerat, H., Y. K. Shimizu, and S. M. Lemon.** 2000. Cell type-specific enhancement of hepatitis C virus internal ribosome entry site-directed translation due to 5' nontranslated region substitutions selected during passage of virus in lymphoblastoid cells. J. Virol. **74:**7024-7031.

224. **Lescar, J., A. Roussel, M. W. Wien, J. Navaza, S. D. Fuller, G. Wengler, G. Wengler, and F. A. Rey.** 2001. The Fusion glycoprotein shell of Semliki Forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH. Cell. **105:**137-148.

225. **Li, X., L. J. Jeffers, L. Shao, K. R. Reddy, M. de Medina, J. Scheffel, B. Moore, and E. R. Schiff.** 1995. Identification of hepatitis C virus by immunoelectron microscopy. J. Viral Hepat. **2:**227-234.

226. **Lidove, O., P. Cacoub, T. Maisonobe, J. Servan, V. Thibault, J. -. Piette, and J. -. Léger.** 2001. Hepatitis C virus infection with peripheral neuropathy is not always associated with cryoglobulinaemia. Ann. Rheum. Dis. **60:**290-292.

227. **Lin, K., R. B. Perni, A. D. Kwong, and C. Lin.** 2006. VX-950, a novel hepatitis C virus (HCV) NS3-4A protease inhibitor, exhibits potent antiviral

activities in HCv replicon cells. Antimicrob. Agents Chemother. **50:**1813-1822. doi: 10.1128/AAC.50.5.1813-1822.2006.

228. **Lindenbach B D, Thiel H-J and,Rice C.** 2007. Flaviviridae. Fields Virology. 1101. **http://mun-resolver.asin-risa.ca/?genre=article&spage=1101&date=2007-01-01&aulast=Lindenbach+B.+D.&aufirst=Thiel+H.-J.+and+Rice+C.+M.&title=Fields+Virology&atitle=Flaviviridae**.

229. **Lindenbach BD, and Rice CM.** 2005. Unravelling hepatitis C virus replication from genome to function. Nature. **436:**933-8.

230. **Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science. **309:**623-626. doi: 10.1126/science.1114016.

231. **Lindenbach, B. D., P. Meuleman, A. Ploss, T. Vanwolleghem, A. J. Syder, J. A. McKeating, R. E. Lanford, S. M. Feinstone, M. E. Major, G. Leroux-Roels, and C. M. Rice.** 2006. Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. Proc. Natl. Acad. Sci. U. S. A. **103:**3805-3809. doi: 10.1073/pnas.0511218103.

232. **Liu, S., W. Yang, L. Shen, J. R. Turner, C. B. Coyne, and T. Wang.** 2009. Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and

are downregulated during infection to prevent superinfection. J. Virol. **83:**2011-2014. doi: 10.1128/JVI.01888-08.

233. **Llovet, J. M.** 2005. Updated treatment approach to hepatocellular carcinoma. J. Gastroenterol. **40:**225-235. doi: 10.1007/s00535-005-1566-3.

234. **Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science. **285:**110-113.

235. **Lozach, P. Y., A. Amara, B. Bartosch, J. L. Virelizier, F. Arenzana-Seisdedos, F. L. Cosset, and R. Altmeyer.** 2004. C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles. J. Biol. Chem. **279:**32035-32045. doi: 10.1074/jbc.M402296200.

236. **Lozach, P. Y., H. Lortat-Jacob, A. de Lacroix de Lavalette, I. Staropoli, S. Foung, A. Amara, C. Houles, F. Fieschi, O. Schwartz, J. L. Virelizier, F. Arenzana-Seisdedos, and R. Altmeyer.** 2003. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. J. Biol. Chem. **278:**20358-20366. doi: 10.1074/jbc.M301284200.

237. **Lozano, F., J. Alberola-lla, L. Places, T. Gallart, and J. Vives.** 1990. Protein kinase C-dependent up-regulation of CD5 surface expression on normal and lymphoblastoid T cells. Immunology. **70:**434-439.

238. **Ludwig, I. S., A. N. Lekkerkerker, E. Depla, F. Bosman, R. J. Musters, S. Depraetere, Y. van Kooyk, and T. B. Geijtenbeek.** 2004. Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation. J. Virol. **78:**8322-8332. doi: 10.1128/JVI.78.15.8322-8332.2004.

239. **Luik, P., C. Chew, J. Aittoniemi, J. Chang, P. Wentworth Jr, R. A. Dwek, P. C. Biggin, C. Venien-Bryan, and N. Zitzmann.** 2009. The 3-dimensional structure of a hepatitis C virus p7 ion channel by electron microscopy. Proc. Natl. Acad. Sci. U. S. A. **106:**12712-12716. doi: 10.1073/pnas.0905966106.

240. **Lunel, F., L. Musset, P. Cacoub, L. Frangeul, P. Cresta, M. Perrin, P. Grippon, C. Hoang, J. C. Piette, J. -. Huraux, and P. Opolon.** 1994. Cryoglobulinemia in chronic liver diseases: Role of hepatitis C virus and liver damage. Gastroenterology. **106:**1291-1300.

241. **Lusso, P., F. Cocchi, C. Balotta, P. Markham, A. Louie, P. Farci, R. Pal, R. Gallo, and M. Reitz Jr.** 1995. Growth of macrophage-tropic and primary human immunodeficiency virus type 1 (HIV-1) isolates in a unique CD4+ T-cell clone (PM1): failure to downregulate CD4 and to interfere with cell-line-tropic HIV-1. J. Virol. **69:**3712-3720.

242. **MacParland, S. A., T. N. Pham, C. S. Guy, and T. I. Michalak.** 2009. Hepatitis C virus persisting after clinically apparent sustained virological response to antiviral therapy retains infectivity in vitro. Hepatology. **49:**1431-1441. doi: 10.1002/hep.22802.

243. **MacParland, S. A., T. N. Q. Pham, S. A. Gujar, and T. I. Michalak.** 2006. De novo infection and propagation of wild-type Hepatitis C virus in human T lymphocytes in vitro. J. Gen. Virol. **87:**3577-3586. doi: 10.1099/vir.0.81868-0.

244. **Maeda, N., S. Okamoto, H. Ishii, T. Yamada, J. Hata, N. Hozumi, A. Katsume, M. Kohara, T. Kanai, and M. Watanabe.** 1999. A Novel Hepatitis C Model By Reconstitution Of Normal And HCV-Infected Human Liver Tissues Into Nod/Scid Mouse. Gastroenterology. **116:**L0272.

245. **Maeda, N., M. Watanabe, S. Okamoto, T. Kanai, T. Yamada, J. Hata, N. Hozumi, A. Katsume, H. Nuriya, J. Sandhu, H. Ishii, M. Kohara, and T. Hibi.** 2004. Hepatitis C virus infection in human liver tissue engrafted in mice with an infectious molecular clone. Liver Int. **24:**259-267. doi: 10.1111/j.1478-3231.2004.0909.x.

246. **Maillard, P., K. Krawczynski, J. Nitkiewicz, C. Bronnert, M. Sidorkiewicz, P. Gounon, J. Dubuisson, G. Faure, R. Crainic, and A. Budkowska.** 2001. Nonenveloped nucleocapsids of hepatitis C virus in the serum of infected patients. J. Virol. **75:**8240-8250.

247. **Major ME, Dahari H, Mihalik K, Puig M, Rice CM, Neumann AU, and Feinstone SM.** 2004. Hepatitis C virus kinetics and host responses associated with disease and outcome of infection in chimpanzees. Hepatology. **39:**1709-20.

248. **Major, M. E., H. Dahari, K. Mihalik, M. Puig, C. M. Rice, A. U. Neumann, and S. M. Feinstone.** 2004. Hepatitis C virus kinetics and host responses associated with disease and outcome of infection in chimpanzees. Hepatology. **39:**1709-1720. doi: 10.1002/hep.20239.

249. **Majumder, M., A. K. Ghosh, R. Steele, X. Y. Zhou, N. J. Phillips, R. Ray, and R. B. Ray.** 2002. Hepatitis C virus NS5A protein impairs TNF-mediated hepatic apoptosis, but not by an anti-FAS antibody, in transgenic mice. Virology. **294:**94-105. doi: 10.1006/viro.2001.1309.

250. **Manns, M. P., H. Wedemeyer, and M. Cornberg.** 2006. Treating viral hepatitis C: efficacy, side effects, and complications. Gut : Journal of the British Society of Gastroenterology. **55:**1350-1359.

251. **Marcucci, F., and A. Mele.** 2011. Hepatitis viruses and non-Hodgkin lymphoma: epidemiology, mechanisms of tumorigenesis, and therapeutic opportunities. Blood. **117:**1792-1798. doi: 10.1182/blood-2010-06-275818.

252. **Martell, M., J. I. Esteban, J. Quer, J. Genesca, A. Weiner, R. Esteban, J. Guardia, and J. Gomez.** 1992. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. J. Virol. **66:**3225-3229.

253. **Martin-Blondel, G., A. Gales, J. Bernad, L. Cuzin, P. Delobel, K. Barange, J. Izopet, B. Pipy, and L. Alric.** 2009. Low interleukin-10 production

by monocytes of patients with a self-limiting hepatitis C virus infection. J. Viral Hepat. **16:**485-491. doi: 10.1111/j.1365-2893.2009.01094.x.

254. **Martins, T., J. L. Narciso-Schiavon, and L. Schiavon Lde.** 2011. Epidemiology of hepatitis C virus infection]. Rev. Assoc. Med. Bras. **57:**107-112.

255. **Marukian, S., C. T. Jones, L. Andrus, M. J. Evans, K. D. Ritola, E. D. Charles, C. M. Rice, and L. B. Dustin.** 2008. Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells. Hepatology. **48:**1843-1850. doi: 10.1002/hep.22550.

256. **Masciopinto, F., G. Freer, V. L. Burgio, S. Levy, L. Galli-Stampino, M. Bendinelli, M. Houghton, S. Abrignani, and Y. Uematsu.** 2002. Expression of human CD81 in transgenic mice does not confer susceptibility to hepatitis C virus infection. Virology. **304:**187-196.

257. **Matsuo, K., A. Kusano, A. Sugumar, S. Nakamura, K. Tajima, and N. E. Mueller.** 2004. Effect of hepatitis C virus infection on the risk of non-Hodgkin's lymphoma: A meta-analysis of epidemiological studies. Cancer Science. **95:**745-752.

258. **Mattsson, L., A. Sonnerborg, and O. Weiland.** 1993. Outcome of acute symptomatic non-A, non-B hepatitis: a 13-year follow-up study of hepatitis C virus markers. Liver. **13:**274-278.

259. **McCarthy, K. M., S. A. Francis, J. M. McCormack, J. Lai, R. A. Rogers, I. B. Skare, R. D. Lynch, and E. E. Schneeberger.** 2000. Inducible expression of claudin-1-myc but not occludin-VSVG results in aberrant tight junction strand formation in MDCK cells. Journal of Cell Science. **113:**3387-3398.

260. **McCaughan, G. W., N. A. Shackel, P. Bertolino, and D. G. Bowen.** 2009. Molecular and cellular aspects of hepatitis C virus reinfection after liver transplantation: how the early phase impacts on outcomes. Transplantation. **87:**1105-1111. doi: 10.1097/TP.0b013e31819dfa83.

261. **Mchutchison, J. G., S. C. Gordon, E. R. Schiff, M. L. Shiffman, W. M. Lee, V. K. Rustgi, Z. D. Goodman, M. Ling, S. Cort, and J. K. Albrecht.** 1998. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N. Engl. J. Med. **339:**1485-1492.

262. **McHutchison, J. G., M. P. Manns, A. J. Muir, N. A. Terrault, I. M. Jacobson, N. H. Afdhal, E. J. Heathcote, S. Zeuzem, H. W. Reesink, J. Garg, M. Bsharat, S. George, R. S. Kauffman, N. Adda, A. M. Di Bisceglie, and PROVE3 Study Team.** 2010. Telaprevir for previously treated chronic HCV infection. N. Engl. J. Med. **362:**1292-1303. doi: 10.1056/NEJMoa0908014.

263. **Mele, A., A. Pulsoni, E. Bianco, P. Musto, A. Szklo, M. Grazia Sanpaolo, E. Lannitto, A. De Renzo, B. Martino, V. Liso, C. Andrizzi, S. Pusterla, F. Dore, M. Maresca, M. Rapicetta, F. Marcucci, F. Mandelli, and S. Franceschi.**

2003. Hepatitis C virus and B-cell non-Hodgkin lymphomas: An Italian multicenter case-control study. Blood. **102:**996-999.

264. **Mellor, J., E. C. Holmes, L. M. Jarvis, P. L. Yap, and P. Simmonds.** 1995. Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: implications for virus classification. The International HCV Collaborative Study Group. J. Gen. Virol. **76:**2493-2507.

265. **Melnick, J. L.** 1995. History and epidemiology of hepatitis A virus. J. Infect. Dis. **171 Suppl 1:**S2-8.

266. **Melon, S., M. C. Galarraga, M. Villar, A. Laures, J. A. Boga, M. de Ona, and E. Gomez.** 2005. Hepatitis C virus reactivation in anti-hepatitic C virus-positive renal transplant recipients. Transplant. Proc. **37:**2083-2085. doi: 10.1016/j.transproceed.2005.03.045.

267. **Meltzer, M., E. C. Franklin, K. Elias, R. T. McCluskey, and N. Cooper.** 1966. Cryoglobulinemia-A clinical and laboratory study. II. Cryoglobulins with rheumatoid factor activity. Am. J. Med. **40:**837-856.

268. **Mercer, D. F., D. E. Schiller, J. F. Elliott, D. N. Douglas, C. Hao, A. Rinfret, W. R. Addison, K. P. Fischer, T. A. Churchill, J. R. Lakey, D. L. Tyrrell, and N. M. Kneteman.** 2001. Hepatitis C virus replication in mice with chimeric human livers. Nat. Med. **7:**927-933. doi: 10.1038/90968.

269. **Meuleman, P., and G. Leroux-Roels.** 2009. HCV Animal Models: A Journey of More than 30 Years. Viruses Viruses. **1:**222-240.

270. **Meunier, J. C., R. E. Engle, K. Faulk, M. Zhao, B. Bartosch, H. Alter, S. U. Emerson, F. L. Cosset, R. H. Purcell, and J. Bukh.** 2005. Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1. Proc. Natl. Acad. Sci. U. S. A. **102:**4560-4565. doi: 10.1073/pnas.0501275102.

271. **Micallef, J. M., J. M. Kaldor, and G. J. Dore.** 2006. Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. J. Viral Hepat. **13:**34-41. doi: 10.1111/j.1365-2893.2005.00651.x.

272. **Michalak, T. I.** 2000. Occult persistence and lymphotropism of hepadnaviral infection: insights from the woodchuck viral hepatitis model. Immunol. Rev. **174:**98-111.

273. **Michalak, T. I.** 2010. Immune cell reservoirs of persisting hepatitis C virus. Gut. **59:**867-868. doi: 10.1136/gut.2010.210054.

274. **Michalak, T. I., P. D. Hodgson, and N. D. Churchill.** 2000. Posttranscriptional inhibition of class I major histocompatibility complex presentation on hepatocytes and lymphoid cells in chronic woodchuck hepatitis virus infection. J. Virol. **74:**4483-4494.

275. **Michalak, T. I., I. U. Pardoe, C. S. Coffin, N. D. Churchill, D. S. Freake, P. Smith, and C. L. Trelegan.** 1999. Occult lifelong persistence of infectious hepadnavirus and residual liver inflammation in woodchucks convalescent from acute viral hepatitis. Hepatology. **29:**928-938. doi: 10.1002/hep.510290329.

276. **Michalak, T. I., C. Pasquinelli, S. Guilhot, and F. V. Chisari.** 1994. Hepatitis B virus persistence after recovery from acute viral hepatitis. J. Clin. Invest. **93:**230-239. doi: 10.1172/JCI116950.

277. **Miller, R. H., and R. H. Purcell.** 1990. Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. Proc. Natl. Acad. Sci. U. S. A. **87:**2057-2061.

278. **Misiani, R., P. Bellavita, D. Fenili, G. Borelli, D. Marchesi, M. Massazza, G. Vendramin, B. Comotti, E. Tanzi, and G. Scudeller.** 1992. Hepatitis C virus infection in patients with essential mixed cryoglobulinemia. Ann. Intern. Med. **117:**573-577.

279. **Mizuno, M., G. Yamada, T. Tanaka, K. Shimotohno, M. Takatani, and T. Tsuji.** 1995. Virion-like structures in HeLa G cells transfected with the full-length sequence of the hepatitis C virus genome. Gastroenterology. **109:**1933-1940.

280. **Molina, S., V. Castet, C. Fournier-Wirth, L. Pichard-Garcia, R. Avner, D. Harats, J. Roitelman, R. Barbaras, P. Graber, P. Ghersa, M. Smolarsky, A. Funaro, F. Malavasi, D. Larrey, J. Coste, J. M. Fabre, A. Sa-Cunha, and P.**

238

Maurel. 2007. The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. J. Hepatol. **46:**411-419. doi: 10.1016/j.jhep.2006.09.024.

281. **Molina, S., V. Castet, L. Pichard-Garcia, C. Wychowski, E. Meurs, J. M. Pascussi, C. Sureau, J. M. Fabre, A. Sacunha, D. Larrey, J. Dubuisson, J. Coste, J. McKeating, P. Maurel, and C. Fournier-Wirth.** 2008. Serum-derived hepatitis C virus infection of primary human hepatocytes is tetraspanin CD81 dependent. J. Virol. **82:**569-574. doi: 10.1128/JVI.01443-07.

282. **Monazahian, M., I. Bohme, S. Bonk, A. Koch, C. Scholz, S. Grethe, and R. Thomssen.** 1999. Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. J. Med. Virol. **57:**223-229.

283. **Mondelli, M. U., and E. Silini.** 1999. Clinical significance of hepatitis C virus genotypes. J. Hepatol. **31 Suppl 1:**65-70.

284. **Moorman, J., Z. P. Dong, L. Ni, C. Zhang, T. Borthwick, and Z. Q. Yao.** 2009. Abnormal B-cell activation associated with TALL-1 over-expression and SOCS-1 suppression during chronic hepatitis C virus infection. Immunology. **128:**227-235. doi: 10.1111/j.1365-2567.2009.03106.x.

285. **Morishima C, Paschal DM, Wang CC, Yoshihara CS, Wood BL, Yeo AE, Emerson SS, Shuhart MC, and Gretch DR.** 2006. Decreased NK cell frequency

in chronic hepatitis C does not affect ex vivo cytolytic killing. Hepatology. **43:**573-80.

286. **Morita, K., M. Furuse, K. Fujimoto, and S. Tsukita.** 1999. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc. Natl. Acad. Sci. U. S. A. **96:**511-516.

287. **Morsica, G., G. Tambussi, G. Sitia, R. Novati, A. Lazzarin, L. Lopalco, and S. Mukenge.** 1999. Replication of hepatitis C virus in B lymphocytes (CD19+). Blood. **94:**1138-1139.

288. **Mosley, J. W.** 1975. The epidemiology of viral hepatitis: an overview. Am. J. Med. Sci. **270:**253-270.

289. **Muchmore E, Popper H, Peterson DA, Miller MF, and Lieberman HM.** 1988. Non-A, non-B hepatitis-related hepatocellular carcinoma in a chimpanzee. J. Med. Primatol. **17:**235-46.

290. **Müller HM, Pfaff E, Goeser T, Kallinowski B, Solbach C, and Theilmann L.** 1993. Peripheral blood leukocytes serve as a possible extrahepatic site for hepatitis C virus replication. J. Gen. Virol. **74:**669-76.

291. **Mulrooney-Cousins, P. M., and T. I. Michalak.** 2007. Persistent occult hepatitis B virus infection: experimental findings and clinical implications. World J. Gastroenterol. **13:**5682-5686.

292. **Murakami, K., T. Kimura, M. Osaki, K. Ishii, T. Miyamura, T. Suzuki, T. Wakita, and I. Shoji.** 2008. Virological characterization of the hepatitis C virus JFH-1 strain in lymphocytic cell lines. J. Gen. Virol. **89:**1587-1592. doi: 10.1099/vir.0.83618-0.

293. **Mushahwar, I. K.** 2008. Hepatitis E virus: molecular virology, clinical features, diagnosis, transmission, epidemiology, and prevention. J. Med. Virol. **80:**646-658. doi: 10.1002/jmv.21116.

294. **Nakajima, N., M. Hijikata, H. Yoshikura, and Y. K. Shimizu.** 1996. Characterization of long-term cultures of hepatitis C virus. J. Virol. **70:**3325-3329.

295. **Nakamoto, N., D. E. Kaplan, J. Coleclough, Y. Li, M. E. Valiga, M. Kaminski, A. Shaked, K. Olthoff, E. Gostick, D. A. Price, G. J. Freeman, E. J. Wherry, and K. M. Chang.** 2008. Functional restoration of HCV-specific CD8 T cells by PD-1 blockade is defined by PD-1 expression and compartmentalization. Gastroenterology. **134:**1927-37, 1937.e1-2. doi: 10.1053/j.gastro.2008.02.033.

296. **Navas, S., J. Martin, J. A. Quiroga, I. Castillo, and V. Carreno.** 1998. Genetic diversity and tissue compartmentalization of the hepatitis C virus genome in blood mononuclear cells, liver, and serum from chronic hepatitis C patients. J. Virol. **72:**1640-1646.

297. **Nicolau, A., R. Tanasescu, E. Balanescu, P. Balanescu, R. Patrascu, and C. Tanasescu.** 2011. Hepatitis C virus--mixed cryoglobulinemia--lymphoma relationship. Rom. J. Intern. Med. **49:**3-10.

298. **Niu, M. T., P. J. Coleman, and M. J. Alter.** 1993. Multicenter study of hepatitis C virus infection in chronic hemodialysis patients and hemodialysis center staff members. Am. J. Kidney Dis. **22:**568-573.

299. **Okuda, M., K. Hino, M. Korenaga, Y. Yamaguchi, Y. Katoh, and K. Okita.** 1999. Differences in hypervariable region 1 quasispecies of hepatitis C virus in human serum, peripheral blood mononuclear cells, and liver. Hepatology. **29:**217-222. doi: 10.1002/hep.510290117.

300. **Okuda, M., K. Li, M. R. Beard, L. A. Showalter, F. Scholle, S. M. Lemon, and S. A. Weinman.** 2002. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. Gastroenterology. **122:**366-375.

301. **Oldstone, M. B.** 1996. Virus-lymphoid cell interactions. Proc. Natl. Acad. Sci. U. S. A. **93:**12756-12758.

302. **Oldstone, M. B.** 2009. Anatomy of viral persistence. PLoS Pathog. **5:**e1000523. doi: 10.1371/journal.ppat.1000523.

303. **Op De Beeck, A., C. Voisset, B. Bartosch, Y. Ciczora, L. Cocquerel, Z. Keck, S. Foung, F. L. Cosset, and J. Dubuisson.** 2004. Characterization of functional hepatitis C virus envelope glycoproteins. J. Virol. **78:**2994-3002.

304. **Pal, S., D. G. Sullivan, S. Kim, K. K. Lai, J. Kae, S. J. Cotler, R. L. Carithers Jr, B. L. Wood, J. D. Perkins, and D. R. Gretch.** 2006. Productive replication of hepatitis C virus in perihepatic lymph nodes in vivo: implications of HCV lymphotropism. Gastroenterology. **130:**1107-1116. doi: 10.1053/j.gastro.2005.12.039.

305. **Pascarella, S., and F. Negro.** 2011. Hepatitis D virus: an update. Liver Int. **31:**7-21. doi: 10.1111/j.1478-3231.2010.02320.x; 10.1111/j.1478-3231.2010.02320.x.

306. **Pascual, M., L. Perrin, E. Giostra, and J. A. Schifferli.** 1990. Hepatitis C virus in patients with cryoglobulinemia type II. J. Infect. Dis. **162:**569-570.

307. **Pasquariello, A., C. Ferri, L. Moriconi, L. La Civita, G. Longombardo, F. Lombardini, F. Greco, and A. L. Zignego.** 1993. Cryoglobulinemic membranoproliferative glomerulonephritis associated with hepatitis C virus. Am. J. Nephrol. **13:**300-304.

308. **Pasquinelli, C., J. M. Shoenberger, J. Chung, K. M. Chang, L. G. Guidotti, M. Selby, K. Berger, R. Lesniewski, M. Houghton, and F. V. Chisari.**

1997. Hepatitis C virus core and E2 protein expression in transgenic mice. Hepatology. **25:**719-727. doi: 10.1002/hep.510250338.

309. **Pavlovic, D., D. C. Neville, O. Argaud, B. Blumberg, R. A. Dwek, W. B. Fischer, and N. Zitzmann.** 2003. The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. Proc. Natl. Acad. Sci. U. S. A. **100:**6104-6108. doi: 10.1073/pnas.1031527100.

310. **Penin, F., J. Dubuisson, F. A. Rey, D. Moradpour, and J. M. Pawlotsky.** 2004. Structural biology of hepatitis C virus. Hepatology. **39:**5-19. doi: 10.1002/hep.20032.

311. **Pestova, T. V., I. N. Shatsky, S. P. Fletcher, R. J. Jackson, and C. U. Hellen.** 1998. A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. Genes Dev. **12:**67-83.

312. **Pham, T. N., C. S. Coffin, N. D. Churchill, S. J. Urbanski, S. S. Lee, and T. I. Michalak.** 2012. Hepatitis C virus persistence after sustained virological response to antiviral therapy in patients with or without past exposure to hepatitis B virus. J. Viral Hepat. **19:**103-111. doi: 10.1111/j.1365-2893.2011.01442.x; 10.1111/j.1365-2893.2011.01442.x.

313. **Pham, T. N., C. S. Coffin, and T. I. Michalak.** 2010. Occult hepatitis C virus infection: what does it mean? Liver Int. **30:**502-511. doi: 10.1111/j.1478-3231.2009.02193.x.

314. **Pham, T. N., D. King, S. A. Macparland, J. S. McGrath, S. B. Reddy, F. R. Bursey, and T. I. Michalak.** 2008. Hepatitis C virus replicates in the same immune cell subsets in chronic hepatitis C and occult infection. Gastroenterology. **134:**812-822. doi: 10.1053/j.gastro.2007.12.011.

315. **Pham, T. N., S. A. MacParland, P. M. Mulrooney, H. Cooksley, N. V. Naoumov, and T. I. Michalak.** 2004. Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C. J. Virol. **78:**5867-5874. doi: 10.1128/JVI.78.11.5867-5874.2004.

316. **Pham, T. N., S. E. Mercer, and T. I. Michalak.** 2009. Chronic hepatitis C and persistent occult hepatitis C virus infection are characterized by distinct immune cell cytokine expression profiles. J. Viral Hepat. **16:**547-556. doi: 10.1111/j.1365-2893.2009.01092.x.

317. **Pham, T. N., and T. I. Michalak.** 2006. Occult hepatits C virus persistence: identification and characteristics. MLO Med. Lab. Obs. **38:**20-22.

318. **Pham, T. N., and T. I. Michalak.** 2008. Hepatitis C virus in peripheral blood mononuclear cells of individuals with isolated anti-hepatitis C virus antibody reactivity. Hepatology. **48:**350-1; author reply 351-2. doi: 10.1002/hep.22384.

319. **Pham, T. N. Q., S. A. MacParland, C. S. Coffin, S. S. Lee, F. R. Bursey, and T. I. Michalak.** 2005. Mitogen-induced upregulation of hepatitis C virus expression in human lymphoid cells. J. Gen. Virol. **86:**657-666. doi: 10.1099/vir.0.80624-0.

320. **Pietschmann, T., A. Kaul, G. Koutsoudakis, A. Shavinskaya, S. Kallis, E. Steinmann, K. Abid, F. Negro, M. Dreux, F. L. Cosset, and R. Bartenschlager.** 2006. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc. Natl. Acad. Sci. U. S. A. **103:**7408-7413. doi: 10.1073/pnas.0504877103.

321. **Pietschmann, T., M. Zayas, P. Meuleman, G. Long, N. Appel, G. Koutsoudakis, S. Kallis, G. Leroux-Roels, V. Lohmann, and R. Bartenschlager.** 2009. Production of infectious genotype 1b virus particles in cell culture and impairment by replication enhancing mutations. PLoS Pathog. **5:**e1000475. doi: 10.1371/journal.ppat.1000475.

322. **Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani.** 1998. Binding of hepatitis C virus to CD81. Science. **282:**938-941.

323. **Ploss, A., M. J. Evans, V. A. Gaysinskaya, M. Panis, H. You, Y. P. de Jong, and C. M. Rice.** 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature. **457:**882-886. doi: 10.1038/nature07684.
246

324. **Ploss, A., S. R. Khetani, C. T. Jones, A. J. Syder, K. Trehan, V. A. Gaysinskaya, K. Mu, K. Ritola, C. M. Rice, and S. N. Bhatia.** 2010. Persistent hepatitis C virus infection in microscale primary human hepatocyte cultures. Proc. Natl. Acad. Sci. U. S. A. **107:**3141-3145. doi: 10.1073/pnas.0915130107.

325. **Pockros, P. J.** 2008. Emerging therapies for chronic hepatitis C virus. Gastroenterol. Hepatol. (N. Y). **4:**729-734.

326. **Pockros, P.** 2010. Review: New direct-acting antivirals in the development for hepatitis C virus infection. Therapeutic Advances in Gastroenterology. **3:**191-202.

327. **Pohlmann, S., J. Zhang, F. Baribaud, Z. Chen, G. J. Leslie, G. Lin, A. Granelli-Piperno, R. W. Doms, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. J. Virol. **77:**4070-4080.

328. **Powers, K. A., R. M. Ribeiro, K. Patel, S. Pianko, L. Nyberg, P. Pockros, A. J. Conrad, J. McHutchison, and A. S. Perelson.** 2006. Kinetics of hepatitis C virus reinfection after liver transplantation. Liver Transpl. **12:**207-216. doi: 10.1002/lt.20572.

329. **Poynard, T., P. Bedossa, and P. Opolon.** 1997. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Lancet. **349:**825-832.

247

330. **Poynard, T., M. Colombo, J. Bruix, E. Schiff, R. Terg, S. Flamm, R. Moreno-Otero, F. Carrilho, W. Schmidt, T. Berg, T. McGarrity, E. J. Heathcote, F. Goncales, M. Diago, A. Craxi, M. Silva, P. Bedossa, P. Mukhopadhyay, L. Griffel, M. Burroughs, C. Brass, J. Albrecht, and Epic Study Group.** 2009. Peginterferon alfa-2b and ribavirin: effective in patients with hepatitis C who failed interferon alfa/ribavirin therapy. Gastroenterology. **136:**1618-28.e2. doi: 10.1053/j.gastro.2009.01.039.

331. **Prati, D.** 2006. Transmission of hepatitis C virus by blood transfusions and other medical procedures: a global review. J. Hepatol. **45:**607-616. doi: 10.1016/j.jhep.2006.07.003.

332. **Prieto, M., M. Berenguer, A. Rimola, C. Loinaz, C. Barrios, G. Clemente, J. Figueras, V. Vargas, F. Casafont, J. A. Pons, and J. I. Herrero.** 1998. Liver transplantation in hepatitis C. A Spanish multi-centre experience. Eur. J. Gastroenterol. Hepatol. **10:**771-776.

333. **Puig, M., K. Mihalik, J. C. Tilton, O. Williams, M. Merchlinsky, M. Connors, S. M. Feinstone, and M. E. Major.** 2006. CD4+ immune escape and subsequent T-cell failure following chimpanzee immunization against hepatitis C virus. Hepatology. **44:**736-745. doi: 10.1002/hep.21319.

334. **Pumeechockchai, W., D. Bevitt, K. Agarwal, T. Petropoulou, B. C. Langer, B. Belohradsky, M. F. Bassendine, and G. L. Toms.** 2002. Hepatitis C virus particles of different density in the blood of chronically infected

immunocompetent and immunodeficient patients: Implications for virus clearance by antibody. J. Med. Virol. **68:**335-342. doi: 10.1002/jmv.10208.

335. **Puoti M, Zonaro A, Ravaggi A, Marin MG, Castelnuovo F, and Cariani E.** 1992. Hepatitis C virus RNA and antibody response in the clinical course of acute hepatitis C virus infection. Hepatology. **16:**877-81.

336. **Qin H, Shire NJ, Keenan ED, Rouster SD, Eyster ME, Goedert JJ, Koziel MJ, Sherman KE, and Multicenter Hemophilia Cohort Study Group.** 2005. HCV quasispecies evolution: association with progression to end-stage liver disease in hemophiliacs infected with HCV or HCV/HIV. Blood. **105:**533-41.

337. **Quiroga JA, Llorente S, Castillo I, Rodríguez-Iñigo E, Pardo M, and Carreño V.** 2006. Cellular immune responses associated with occult hepatitis C virus infection of the liver. J. Virol. **80:**10972-9.

338. **Racanelli, V., D. Sansonno, C. Piccoli, F. P. D'Amore, F. A. Tucci, and F. Dammacco.** 2001. Molecular characterization of B cell clonal expansions in the liver of chronically hepatitis C virus-infected patients. J. Immunol. **167:**21-29.

339. **Radkowski, M., J. F. Gallegos-Orozco, J. Jablonska, T. V. Colby, B. Walewska-Zielecka, J. Kubicka, J. Wilkinson, D. Adair, J. Rakela, and T. Laskus.** 2005. Persistence of hepatitis C virus in patients successfully treated for chronic hepatitis C. Hepatology. **41:**106-114.

340. **Radkowski, M., L. F. Wang, H. E. Vargas, J. Rakela, and T. Laskus.** 1998. Detection of hepatitis C virus replication in peripheral blood mononuclear cells after orthotopic liver transplantation. Transplantation. **66:**664-666.

341. **Raimondo, G., T. Pollicino, M. Levrero, and A. Craxi.** 2011. Occult hepatitis B virus infection and hepatocellular carcinoma development in patients with chronic hepatitis C. Hepatology. **54:**373-4; author reply 374. doi: 10.1002/hep.24379; 10.1002/hep.24379.

342. **Raman, C.** 2002. CD5, an important regulator of lymphocyte selection and immune tolerance. Immunol. Res. **26:**255-263. doi: 10.1385/IR:26:1-3:255.

343. **Ramia, S., and J. Eid-Fares.** 2006. Distribution of hepatitis C virus genotypes in the Middle East. Int. J. Infect. Dis. **10:**272-277. doi: 10.1016/j.ijid.2005.07.008.

344. **Raziorrouh, B., A. Ulsenheimer, W. Schraut, M. Heeg, P. Kurktschiev, R. Zachoval, M. C. Jung, R. Thimme, C. Neumann-Haefelin, S. Horster, M. Wachtler, M. Spannagl, J. Haas, H. M. Diepolder, and N. H. Gruner.** 2011. Inhibitory Molecules That Regulate Expansion and Restoration of HCV-Specific CD4(+) T Cells in Patients With Chronic Infection. Gastroenterology. **141:**1422-1431.e6. doi: 10.1053/j.gastro.2011.07.004.

345. **Raziorrouh, B., A. Ulsenheimer, W. Schraut, M. Heeg, P. Kurktschiev, R. Zachoval, M. Jung, R. Thimme, C. Neumann–Haefelin, S. Horster, M.**

Wächtler, M. Spannagl, J. Haas, H. M. Diepolder, and N. H. Grüner. Inhibitory Molecules That Regulate Expansion and Restoration of HCV-Specific CD4+ T Cells in Patients With Chronic Infection. Gastroenterology. . doi: 10.1053/j.gastro.2011.07.004.

346. **Regeard, M., M. Trotard, C. Lepere, P. Gripon, and J. Le Seyec.** 2008. Entry of pseudotyped hepatitis C virus into primary human hepatocytes depends on the scavenger class B type I receptor. J. Viral Hepat. **15:**865-870. doi: 10.1111/j.1365-2893.2008.01048.x.

347. **Richiardi, L., L. De Marco, A. Gillio-Tos, F. Merletti, V. Fiano, D. Palli, G. Masala, C. Agnoli, G. Tagliabue, S. Panico, A. Mattiello, R. Tumino, G. Frasca, P. Vineis, and C. Sacerdote.** 2010. Persistent infection by HCV and EBV in peripheral blood mononuclear cells and risk of non-Hodgkin's lymphoma. Cancer. Epidemiol. **34:**709-712. doi: 10.1016/j.canep.2010.07.014.

348. **Roccasecca, R., H. Ansuini, A. Vitelli, A. Meola, E. Scarselli, S. Acali, M. Pezzanera, B. B. Ercole, J. McKeating, A. Yagnik, A. Lahm, A. Tramontano, R. Cortese, and A. Nicosia.** 2003. Binding of the hepatitis C virus E2 glycoprotein to CD81 is strain specific and is modulated by a complex interplay between hypervariable regions 1 and 2. J. Virol. **77:**1856-1867.

349. **Rollier, C. S., G. Paranhos-Baccala, E. J. Verschoor, B. E. Verstrepen, J. A. Drexhage, Z. Fagrouch, J. L. Berland, F. Komurian-Pradel, B. Duverger, N. Himoudi, C. Staib, M. Meyr, M. Whelan, J. A. Whelan, V. C. Adams, E.**

Larrea, J. I. Riezu, J. J. Lasarte, B. Bartosch, F. L. Cosset, W. J. Spaan, H. M. Diepolder, G. R. Pape, G. Sutter, G. Inchauspe, and J. L. Heeney. 2007. Vaccine-induced early control of hepatitis C virus infection in chimpanzees fails to impact on hepatic PD-1 and chronicity. Hepatology. **45:**602-613. doi: 10.1002/hep.21573.

350. **Rosa, D., S. Campagnoli, C. Moretto, E. Guenzi, L. Cousens, M. Chin, C. Dong, A. J. Weiner, J. Y. Lau, Q. L. Choo, D. Chien, P. Pileri, M. Houghton, and S. Abrignani.** 1996. A quantitative test to estimate neutralizing antibodies to the hepatitis C virus: cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells. Proc. Natl. Acad. Sci. U. S. A. **93:**1759-1763.

351. **Rubin, A., V. Aguilera, and M. Berenguer.** 2011. Liver transplantation and hepatitis C. Clin. Res. Hepatol. Gastroenterol. . doi: 10.1016/j.clinre.2011.04.009.

352. **Russell, R. S., J. C. Meunier, S. Takikawa, K. Faulk, R. E. Engle, J. Bukh, R. H. Purcell, and S. U. Emerson.** 2008. Advantages of a single-cycle production assay to study cell culture-adaptive mutations of hepatitis C virus. Proc. Natl. Acad. Sci. U. S. A. **105:**4370-4375. doi: 10.1073/pnas.0800422105.

353. **Saadoun, D., T. Asselah, M. Resche-Rigon, F. Charlotte, P. Bedossa, D. Valla, J. -. Piette, P. Marcellin, and P. Cacoub.** 2006. Cryoglobulinemia is associated with steatosis and fibrosis in chronic hepatitis C. Hepatology. **43:**1337-1345.

354. **Sabatos, C. A., S. Chakravarti, E. Cha, A. Schubart, A. Sanchez-Fueyo, X. X. Zheng, A. J. Coyle, T. B. Strom, G. J. Freeman, and V. K. Kuchroo.** 2003. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. Nat. Immunol. **4:**1102-1110. doi: 10.1038/ni988.

355. **Saeed, M., M. Shiina, T. Date, D. Akazawa, N. Watanabe, A. Murayama, T. Suzuki, H. Watanabe, N. Hiraga, M. Imamura, K. Chayama, Y. Choi, K. Krawczynski, T. J. Liang, T. Wakita, and T. Kato.** 2011. In vivo adaptation of hepatitis C virus in chimpanzees for efficient virus production and evasion of apoptosis. Hepatology. **54:**425-433. doi: 10.1002/hep.24399; 10.1002/hep.24399.

356. **Sakai, A., M. S. Claire, K. Faulk, S. Govindarajan, S. U. Emerson, R. H. Purcell, and J. Bukh.** 2003. The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. Proc. Natl. Acad. Sci. U. S. A. **100:**11646-11651. doi: 10.1073/pnas.1834545100.

357. **Sansonno, D., V. De Re, G. Lauletta, F. A. Tucci, M. Boiocchi, and F. Dammacco.** 2003. Monoclonal antibody treatment of mixed cryoglobulinemia resistant to interferon α with an anti-CD20. Blood. **101:**3818-3826.

358. **Santantonio, T., E. Sinisi, A. Guastadisegni, C. Casalino, M. Mazzola, A. Gentile, G. Leandro, and G. Pastore.** 2003. Natural course of acute hepatitis C: a long-term prospective study. Dig. Liver Dis. **35:**104-113.

359. **Santolini, E., L. Pacini, C. Fipaldini, G. Migliaccio, and N. Monica.** 1995. The NS2 protein of hepatitis C virus is a transmembrane polypeptide. J. Virol. **69:**7461-7471.

360. **Sarhan, M. A., T. N. Q. Pham, A. Y. Chen, and T. I. Michalak.** 2012. Hepatitis C Virus Infection of Human T Lymphocytes is Mediated by CD5. J. Virol. 2012, **86**(7):3723. doi: 10.1128/JVI.06956-11.

361. **Sarrias, M. R., J. Gronlund, O. Padilla, J. Madsen, U. Holmskov, and F. Lozano.** 2004. The Scavenger Receptor Cysteine-Rich (SRCR) domain: an ancient and highly conserved protein module of the innate immune system. Crit. Rev. Immunol. **24:**1-37.

362. **Saunier, B., M. Triyatni, L. Ulianich, P. Maruvada, P. Yen, and L. D. Kohn.** 2003. Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes. J. Virol. **77:**546-559.

363. **Scarselli, E., H. Ansuini, R. Cerino, R. M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, and A. Vitelli.** 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. **21:**5017-5025.

364. **Schiff, E. R., and N. Ozden.** 2003. Hepatitis C and alcohol. Alcohol Res. Health. **27:**232-239.

365. **Schultheiss, T., Y. Y. Kusov, and V. Gauss-Muller.** 1994. Proteinase 3C of hepatitis A virus (HAV) cleaves the HAV polyprotein P2-P3 at all sites including VP1/2A and 2A/2B. Virology. **198:**275-281.

366. **Schvoerer, E., C. Thumann, E. Soulier, C. Royer, S. Fafi-Kremer, N. Brignon, B. Ellero, M. L. Woehl-Jaegle, C. Meyer, P. Wolf, D. Jaeck, and F. Stoll-Keller.** 2006. Recurrence of hepatitis C virus (HCV) infection after liver transplantation for HCV-related disease: host factors and viral factors implicated in the occurrence and the severity of HCV recurrence. Pathol. Biol. (Paris). **54:**556-560. doi: 10.1016/j.patbio.2006.07.027.

367. **Semmo, N., and P. Klenerman.** 2007. CD4+ T cell responses in hepatitis C virus infection. World J. Gastroenterol. **13:**4831-4838.

368. **Shepard, C. W., L. Finelli, and M. J. Alter.** 2005. Global epidemiology of hepatitis C virus infection. Lancet Infect. Dis. **5:**558-567. doi: 10.1016/S1473-3099(05)70216-4.

369. **Sherman, M., V. Bain, J. P. Villeneuve, R. P. Myers, C. Cooper, S. Martin, and C. Lowe.** 2004. The management of chronic viral hepatitis: A Canadian consensus conference 2004. Can. J. Infect. Dis. Med. Microbiol. **15:**313-326.

370. **Shiffman, M. L.** 2010. Treatment of hepatitis C in 2011: what can we expect? Curr. Gastroenterol. Rep. **12:**70-75. doi: 10.1007/s11894-009-0085-4.

371. **Shimizu, Y. K., H. Igarashi, T. Kanematu, K. Fujiwara, D. C. Wong, R. H. Purcell, and H. Yoshikura.** 1997. Sequence analysis of the hepatitis C virus genome recovered from serum, liver, and peripheral blood mononuclear cells of infected chimpanzees. J. Virol. **71:**5769-5773.

372. **Shimizu, Y. K., H. Igarashi, T. Kiyohara, M. Shapiro, D. C. Wong, R. H. Purcell, and H. Yoshikura.** 1998. Infection of a chimpanzee with hepatitis C virus grown in cell culture. J. Gen. Virol. **79 (Pt 6):**1383-1386.

373. **Shimizu, Y. K., A. Iwamoto, M. Hijikata, R. H. Purcell, and H. Yoshikura.** 1992. Evidence for in vitro replication of hepatitis C virus genome in a human T-cell line. Proc. Natl. Acad. Sci. U. S. A. **89:**5477-5481.

374. **Shimizu, Y. K., R. H. Purcell, and H. Yoshikura.** 1993. Correlation between the infectivity of hepatitis C virus in vivo and its infectivity in vitro. Proc. Natl. Acad. Sci. U. S. A. **90:**6037-6041.

375. **Shimoike, T., S. Mimori, H. Tani, Y. Matsuura, and T. Miyamura.** 1999. Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. J. Virol. **73:**9718-9725.

376. **Shoukry, N. H., A. Grakoui, M. Houghton, D. Y. Chien, J. Ghrayeb, K. A. Reimann, and C. M. Walker.** 2003. Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. J. Exp. Med. **197:**1645-1655. doi: 10.1084/jem.20030239.

377. **Silver, D. L., N. Wang, X. Xiao, and A. R. Tall.** 2001. High density lipoprotein (HDL) particle uptake mediated by scavenger receptor class B type 1 results in selective sorting of HDL cholesterol from protein and polarized cholesterol secretion. J. Biol. Chem. **276:**25287-25293. doi: 10.1074/jbc.M101726200.

378. **Silvestri F, Pipan C, Barillari G, Zaja F, Fanin R, Infanti L, Russo D, Falasca E, Botta GA, and Baccarani M.** 1996. Prevalence of hepatitis C virus infection in patients with lymphoproliferative disorders. Blood. **87:**4296-301.

379. **Simmonds, P.** 1999. Viral heterogeneity of the hepatitis C virus. J. Hepatol. **31 Suppl 1:**54-60.

380. **Simmonds, P.** 2004. Genetic diversity and evolution of hepatitis C virus--15 years on. J. Gen. Virol. **85:**3173-3188. doi: 10.1099/vir.0.80401-0.

381. **Simmonds, P., J. Bukh, C. Combet, G. Deleage, N. Enomoto, S. Feinstone, P. Halfon, G. Inchauspe, C. Kuiken, G. Maertens, M. Mizokami, D. G. Murphy, H. Okamoto, J. M. Pawlotsky, F. Penin, E. Sablon, T. Shin-I, L. J. Stuyver, H. J. Thiel, S. Viazov, A. J. Weiner, and A. Widell.** 2005. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology. **42:**962-973. doi: 10.1002/hep.20819.

382. **Simons, J. N., T. P. Leary, G. J. Dawson, T. J. Pilot-Matias, A. S. Muerhoff, G. G. Schlauder, S. M. Desai, and I. K. Mushahwar.** 1995. Isolation

257

of novel virus-like sequences associated with human hepatitis. Nat. Med. **1:**564-569.

383. **Spahn, C. M., J. S. Kieft, R. A. Grassucci, P. A. Penczek, K. Zhou, J. A. Doudna, and J. Frank.** 2001. Hepatitis C virus IRES RNA-induced changes in the conformation of the 40s ribosomal subunit. Science. **291:**1959-1962. doi: 10.1126/science.1058409.

384. **Steinmann, E., F. Penin, S. Kallis, A. H. Patel, R. Bartenschlager, and T. Pietschmann.** 2007. Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions. PLoS Pathog. **3:**e103. doi: 10.1371/journal.ppat.0030103.

385. **Tan, S.** 2006. Hepatitis C viruses : genomes and molecular biology. Horizon Bioscience, Wymondham, Norfolk, U.K.

386. **Tanaka, T., N. Kato, M. J. Cho, and K. Shimotohno.** 1995. A novel sequence found at the 3' terminus of hepatitis C virus genome. Biochem. Biophys. Res. Commun. **215:**744-749. doi: 10.1006/bbrc.1995.2526.

387. **Tanaka, T., N. Kato, M. J. Cho, K. Sugiyama, and K. Shimotohno.** 1996. Structure of the 3' terminus of the hepatitis C virus genome. J. Virol. **70:**3307-3312.

388. **Tanaka, Y., T. Shimoike, K. Ishii, R. Suzuki, T. Suzuki, H. Ushijima, Y. Matsuura, and T. Miyamura.** 2000. Selective binding of hepatitis C virus core protein to synthetic oligonucleotides corresponding to the 5' untranslated region of the viral genome. Virology. **270:**229-236. doi: 10.1006/viro.2000.0252.

389. **Terrault, N. A.** 2002. Sexual activity as a risk factor for hepatitis C. Hepatology. **36:**S99-105. doi: 10.1053/jhep.2002.36797.

390. **Thimme, R., D. Oldach, K. M. Chang, C. Steiger, S. C. Ray, and F. V. Chisari.** 2001. Determinants of viral clearance and persistence during acute hepatitis C virus infection. J. Exp. Med. **194:**1395-1406.

391. **Thio, C. L.** 2008. Host genetic factors and antiviral immune responses to hepatitis C virus. Clin. Liver Dis. **12:**713-26, xi. doi: 10.1016/j.cld.2008.03.002.

392. **Thomas, D. L., A. M. Di Bisceglie, H. J. Alter, and N. A. Terrault.** 2010. Understanding the natural history of chronic HBV and HCV infections. J. Fam. Pract. **59:**S17-22.

393. **Thomas, D. L., J. M. Zenilman, H. J. Alter, J. W. Shih, N. Galai, A. V. Carella, and T. C. Quinn.** 1995. Sexual transmission of hepatitis C virus among patients attending sexually transmitted diseases clinics in Baltimore--an analysis of 309 sex partnerships. J. Infect. Dis. **171:**768-775.

394. **Thomas, R. M., J. J. Brems, G. Guzman-Hartman, S. Yong, P. Cavaliere, and D. H. Van Thiel.** 2003. Infection with chronic hepatitis C virus and liver transplantation: a role for interferon therapy before transplantation. Liver Transpl. **9:**905-915. doi: 10.1053/jlts.2003.50166.

395. **Thurner, C., C. Witwer, I. L. Hofacker, and P. F. Stadler.** 2004. Conserved RNA secondary structures in Flaviviridae genomes. J. Gen. Virol. **85:**1113-1124.

396. **Tong, M. J., N. S. el-Farra, A. R. Reikes, and R. L. Co.** 1995. Clinical outcomes after transfusion-associated hepatitis C. N. Engl. J. Med. **332:**1463-1466. doi: 10.1056/NEJM199506013322202.

397. **Tong, Y., Y. Zhu, X. Xia, Y. Liu, Y. Feng, X. Hua, Z. Chen, H. Ding, L. Gao, Y. Wang, M. A. Feitelson, P. Zhao, and Z. T. Qi.** 2011. Tupaia CD81, SR-BI, claudin-1, and occludin support hepatitis C virus infection. J. Virol. **85:**2793-2802. doi: 10.1128/JVI.01818-10.

398. **Toubi, E., A. Kessel, R. Peri, Z. Shmuel, E. Bamberger, E. Sabo, and E. Zuckerman.** 2004. Enhanced apoptosis of peripheral CD5-negative B lymphocytes from chronically hepatitis C virus-infected patients: reversal after antiviral treatment. J. Virol. **78:**11379-11384. doi: 10.1128/JVI.78.20.11379-11384.2004.

399. **Tran, T. T.** 2009. Management of hepatitis B in pregnancy: weighing the options. Cleve. Clin. J. Med. **76 Suppl 3:**S25-9. doi: 10.3949/ccjm.76.s3.06.

400. **Trestard, A., Y. Bacq, L. Buzelay, F. Dubois, F. Barin, A. Goudeau, and P. Roingeard.** 1998. Ultrastructural and physicochemical characterization of the hepatitis C virus recovered from the serum of an agammaglobulinemic patient. Arch. Virol. **143:**2241-2245.

401. **Tseng, C. T., and G. R. Klimpel.** 2002. Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions. J. Exp. Med. **195:**43-49.

402. **Voisset, C., N. Callens, E. Blanchard, A. Op De Beeck, J. Dubuisson, and N. Vu-Dac.** 2005. High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I. J. Biol. Chem. **280:**7793-7799. doi: 10.1074/jbc.M411600200.

403. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11:**791-796. doi: 10.1038/nm1268.

404. **Walker, C. M.** 1997. Comparative features of hepatitis C virus infection in humans and chimpanzees. Springer Semin. Immunopathol. **19:**85-98.

405. **Walters, K. A., M. A. Joyce, J. C. Thompson, M. W. Smith, M. M. Yeh, S. Proll, L. F. Zhu, T. J. Gao, N. M. Kneteman, D. L. Tyrrell, and M. G. Katze.** 2006. Host-specific response to HCV infection in the chimeric SCID-beige/Alb-

uPA mouse model: role of the innate antiviral immune response. PLoS Pathog. **2:**e59. doi: 10.1371/journal.ppat.0020059.

406. **Wang, C., S. Y. Le, N. Ali, and A. Siddiqui.** 1995. An RNA pseudoknot is an essential structural element of the internal ribosome entry site located within the hepatitis C virus 5' noncoding region. RNA. **1:**526-537.

407. **Wasley, A., and M. J. Alter.** 2000. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin. Liver Dis. **20:**1-16.

408. **Weiner, A. J., C. Christopherson, J. E. Hall, F. Bonino, G. Saracco, M. R. Brunetto, K. Crawford, C. D. Marion, K. A. Crawford, and S. Venkatakrishna.** 1991. Sequence variation in hepatitis C viral isolates. J. Hepatol. **13 Suppl 4:**S6-14.

409. **Westaway, E. G., A. A. Khromykh, and J. M. Mackenzie.** 1999. Nascent flavivirus RNA colocalized in situ with double-stranded RNA in stable replication complexes. Virology. **258:**108-117. doi: 10.1006/viro.1999.9683.

410. **Wiedermann, G., M. Kundi, F. Ambrosch, A. Safary, E. D'Hondt, and A. Delem.** 1997. Inactivated hepatitis A vaccine: long-term antibody persistence. Vaccine. **15:**612-615.

411. **Wiesner, R. H., M. Sorrell, F. Villamil, and International Liver Transplantation Society Expert Panel.** 2003. Report of the first International

Liver Transplantation Society expert panel consensus conference on liver transplantation and hepatitis C. Liver Transpl. **9:**S1-9. doi: 10.1053/jlts.2003.50268.

412. **Wilkinson, J., M. Radkowski, and T. Laskus.** 2009. Hepatitis C virus neuroinvasion: identification of infected cells. J. Virol. **83:**1312-1319. doi: 10.1128/JVI.01890-08.

413. **Willems, M., K. Peerlinck, H. Moshage, I. Deleu, C. Van den Eynde, J. Vermylen, and S. H. Yap.** 1994. Hepatitis C virus-RNAs in plasma and in peripheral blood mononuclear cells of hemophiliacs with chronic hepatitis C: evidence for viral replication in peripheral blood mononuclear cells. J. Med. Virol. **42:**272-278.

414. **Wolk, B., D. Sansonno, H. G. Krausslich, F. Dammacco, C. M. Rice, H. E. Blum, and D. Moradpour.** 2000. Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. J. Virol. **74:**2293-2304.

415. **Workowski, K. A., S. Berman, and Centers for Disease Control and Prevention (CDC).** 2010. Sexually transmitted diseases treatment guidelines, 2010. MMWR Recomm Rep. **59:**1-110.

416. **Wu L, Gerard NP, Wyatt R, Choe H, Parolin C, Ruffing N, Borsetti A, Cardoso AA, Desjardin E, Newman W, Gerard C, and Sodroski J.** 1996. CD4-

induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. Nature. **384:**179-83.

417. **Wunschmann, S., J. D. Medh, D. Klinzmann, W. N. Schmidt, and J. T. Stapleton.** 2000. Characterization of hepatitis C virus (HCV) and HCV E2 interactions with CD81 and the low-density lipoprotein receptor. J. Virol. **74:**10055-10062.

418. **Xiang, J., S. Wunschmann, W. Schmidt, J. Shao, and J. T. Stapleton.** 2000. Full-length GB virus C (Hepatitis G virus) RNA transcripts are infectious in primary CD4-positive T cells. J. Virol. **74:**9125-9133.

419. **Yagnik, A. T., A. Lahm, A. Meola, R. M. Roccasecca, B. B. Ercole, A. Nicosia, and A. Tramontano.** 2000. A model for the hepatitis C virus envelope glycoprotein E2. Proteins. **40:**355-366.

420. **Yamada, E., M. Montoya, C. G. Schuettler, T. P. Hickling, A. W. Tarr, A. Vitelli, J. Dubuisson, A. H. Patel, J. K. Ball, and P. Borrow.** 2005. Analysis of the binding of hepatitis C virus genotype 1a and 1b E2 glycoproteins to peripheral blood mononuclear cell subsets. J. Gen. Virol. **86:**2507-2512. doi: 10.1099/vir.0.81169-0.

421. **Yi, M., and S. M. Lemon.** 2003. 3' nontranslated RNA signals required for replication of hepatitis C virus RNA. J. Virol. **77:**3557-3568.

422. **Yi, M., and S. M. Lemon.** 2003. Structure-function analysis of the 3' stem-loop of hepatitis C virus genomic RNA and its role in viral RNA replication. RNA. **9:**331-345.

423. **Yi, M., Y. Ma, J. Yates, and S. M. Lemon.** 2007. Compensatory mutations in E1, p7, NS2, and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus. J. Virol. **81:**629-638. doi: 10.1128/JVI.01890-06.

424. **Zamai L, Ponti C, Mirandola P, Gobbi G, Papa S, Galeotti L, Cocco L, and Vitale M.** 2007. NK cells and cancer. Journal of Immunology (Baltimore, Md.: 1950). **178:**4011-6.

425. **Zeisel, M. B., G. Koutsoudakis, E. K. Schnober, A. Haberstroh, H. E. Blum, F. L. Cosset, T. Wakita, D. Jaeck, M. Doffoel, C. Royer, E. Soulier, E. Schvoerer, C. Schuster, F. Stoll-Keller, R. Bartenschlager, T. Pietschmann, H. Barth, and T. F. Baumert.** 2007. Scavenger receptor class B type I is a key host factor for hepatitis C virus infection required for an entry step closely linked to CD81. Hepatology. **46:**1722-1731. doi: 10.1002/hep.21994.

426. **Zekri AR, Mohamed WS, Samra MA, Sherif GM, El-Shehaby AM, and El-Sayed MH.** 2004. Risk factors for cytomegalovirus, hepatitis B and C virus reactivation after bone marrow transplantation. Transpl. Immunol. **13:**305-11.

427. **Zeuzem, S., M. Buti, P. Ferenci, J. Sperl, Y. Horsmans, J. Cianciara, E. Ibranyi, O. Weiland, S. Noviello, C. Brass, and J. Albrecht.** 2006. Efficacy of

24 weeks treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C infected with genotype 1 and low pretreatment viremia. J. Hepatol. **44:**97-103. doi: 10.1016/j.jhep.2005.10.003.

428. **Zhang, J., G. Randall, A. Higginbottom, P. Monk, C. M. Rice, and J. A. McKeating.** 2004. CD81 Is Required for Hepatitis C Virus Glycoprotein-Mediated Viral Infection. J. Virol. **78:**1448-1455. doi: 10.1128/JVI.78.3.1448-1455.2004.

429. **Zheng A, Yuan F, Li Y, Zhu F, Hou P, Li J, Song X, Ding M, and Deng H.** 2007. Claudin-6 and claudin-9 function as additional coreceptors for hepatitis C virus. J. Virol. **81:**12465-71.

430. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. U. S. A. **102:**9294-9299. doi: 10.1073/pnas.0503596102.

431. **Zhu, C., A. C. Anderson, A. Schubart, H. Xiong, J. Imitola, S. J. Khoury, X. X. Zheng, T. B. Strom, and V. K. Kuchroo.** 2005. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat. Immunol. **6:**1245-1252. doi: 10.1038/ni1271.

432. **Zignego, A. L., and C. Brechot.** 1999. Extrahepatic manifestations of HCV infection: facts and controversies. J. Hepatol. **31:**369-376.

433. **Zignego, A. L., M. De Carli, M. Monti, G. Careccia, G. La Villa, C. Giannini, M. M. D'Elios, G. Del Prete, and P. Gentilini.** 1995. Hepatitis C virus infection of mononuclear cells from peripheral blood and liver infiltrates in chronically infected patients. J. Med. Virol. **47:**58-64.

434. **Zignego, A. L., C. Ferri, S. A. Pileri, P. Caini, and F. B. Bianchi.** 2007. Extrahepatic manifestations of Hepatitis C Virus infection: A general overview and guidelines for a clinical approach. Digestive and Liver Disease. **39:**2-17.

435. **Zignego, A. L., C. Giannini, and C. Ferri.** 2007. Hepatitis C virus-related lymphoproliferative disorders: an overview. World J. Gastroenterol. **13:**2467-2478.

436. **Zignego, A. L., C. Giannini, M. Monti, and L. Gragnani.** 2007. Hepatitis C virus lymphotropism: lessons from a decade of studies. Dig. Liver Dis. **39 Suppl 1:**S38-45.

437. **Zignego, A. L., D. Macchia, M. Monti, V. Thiers, M. Mazzetti, M. Foschi, E. Maggi, S. Romagnani, P. Gentilini, and C. Brechot.** 1992. Infection of peripheral mononuclear blood cells by hepatitis C virus. J. Hepatol. **15:**382-386.

438. **Zingoni A, Sornasse T, Cocks BG, Tanaka Y, Santoni A, and Lanier LL.** 2005. NK cell regulation of T cell-mediated responses. Mol. Immunol. **42:**451-4.

439. **Zuckerman, E., A. Kessel, G. Slobodin, E. Sabo, D. Yeshurun, and E. Toubi.** 2003. Antiviral treatment down-regulates peripheral B-cell CD81 expression and CD5 expansion in chronic hepatitis C virus infection. J. Virol. **77:**10432-10436.