

Characterization of Liver Damage Mechanisms Induced by Hepatitis C Virus

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ABSTRACT

Hepatitis C Virus (HCV) is one of the most important causes of chronic liver disease, affecting more than 170 million people worldwide. The mechanisms of hepatitis C pathogenesis are unknown. Viral cytotoxicity and immune mediated mechanisms might play an important role in its pathogenesis. HCV infection and alcohol abuse frequently coexist and together lead to more rapid progression of liver disease, increasing the incidence and prevalence of cirrhosis and hepatocellular carcinoma. The cytopathic effect of HCV proteins, especially the core, E1 and E2 structural proteins, which induce liver steatosis, oxidative stress and cell transformation may be amplified by alcohol abuse. The purpose of this study was to characterize the liver damage mechanisms induced by HCV structural proteins and alcohol and to determine the potential molecular mechanism(s) that may promote chronic, progressive liver damage. A transgenic mouse model expressing HCV core, E1 and E2 was used to investigate whether alcohol increased HCV RNA expression. Real-time RT-PCR analysis of genes involved in lipid metabolism and transport confirmed their abnormal expression in the alcohol-fed transgenic mice. In addition, light and electron microscopy analysis were performed on liver tissues of transgenic mice on an alcoholic diet versus those on a normal diet, in order to identify histological changes. The severe hepatopathy in HCV transgenic mice was exacerbated by alcohol. Mitochondria and endoplasmic reticulum had severe abnormalities in the electron microscopy analysis. The second part of this study focused on adaptive immune responses, which may also play an important role in HCV pathogenesis. I focused my analysis on dendritic cells (DC), which have been the main suspects to explain immune impairment in HCV infection. Their powerful antigen-presenting function allows them to stimulate the antiviral response of CD4⁺ and CD8⁺ T cells, the

effector cells of the immune system. This unique function of the DC makes them possible targets for immune evasion by the Hepatitis C virus. In this study, DCs were generated from mouse bone marrow cells. I investigated their maturation capacity in the presence of structural proteins of HCV. The impact of HCV core/E1/E2 polyprotein on DCs cytokine expression and ability to activate T-cell lymphocytes was also analyzed. A dysfunctional CD4 T cell response was observed after exposure of DCs to core/E1/E2 polyprotein, indicating inefficient CD4 priming, which might lead to chronic HCV infection in humans. The presence of the core/E1/E2 polyprotein reduced the DC maturation capacity and the expression of certain cytokines (IL-12, IFN γ , IL-6, MCP-1) important for stimulation and chemotaxis of T cells and other immune cells. My studies contribute to the understanding of HCV pathogenesis and may have implications to the development of better therapies for HCV infection.

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LIST OF ABBREVIATIONS

Acaa	Acetyl-Coenzyme A acyltransferase
Acsvl3	Acyl-CoA synthetase long chain family member 3
ADH	Ethanol dehydrogenase
ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
AOX	Acyl-Coenzyme A oxidase
AP-1	Activating factor -1
Apo A IV	Apolipoprotein A IV
ApocII	Apolipoprotein C-II
AST	Aspartate aminotransferase
Bid	BH3 interacting domain death agonist
BM-derived DCs	Bone-marrow derived dendritic cells
BSA	Bovine serum albumin
C1q	Complement 1q
Cdk	Cyclin-dependent kinases
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CHO	Chinese hamster ovary
CLDN1	Claudin-1
CYP2E1	Cytochrome P450 2E1
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
E1	Envelope 1
E2	Envelope 2
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAEEs	Fatty acid ethyl esters
FCS	Fetal calf serum
FitC	Fluorescein isothiocyanate-labeled
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
JNK	c-Jun N-terminal Kinases
GPx	Glutathione Peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
H&E	Hematoxylin and Eosin
H ₂ O ₂	Hydrogen peroxide
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HCVpp	HCV pseudo-particles
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HVR 1	Hyper variable region (HVR) 1

IL-1 β	Interleukin 1 β
IL-12	Interleukin 12
IL-6	Interleukin 6
IFN γ	Interferon gamma
IFN α	Interferon alpha
IRES	Internal ribosome entry site
JNKs	c-Jun N-terminal kinases
LDL	Low density lipoprotein
LPO	Lipid peroxidation products
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocytes chemotactic protein 1
mDCs	Myeloid DCs
MEK	Mitogen-activated ERK kinase
MHCII	Major histocompatibility complex Class II
MIP-1	Macrophage inflammatory protein 1
NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
<i>NF-κB</i>	Nuclear Factor-KappaB
NS5	Non-structural protein 5
PBMCs	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PPAR α	Peroxisome proliferator-activated receptor - alpha
Rb	Retinoblastoma
RdRp	RNA dependent-RNA polymerase
ROS	Reactive oxygen species
RT-PCR	Reverse-transcription polymerase chain reaction
RXR- α	Retinoid X receptor alpha
<i>SCP-2</i>	Sterol carrier <i>protein 2</i>
SR-BI/Cla1	Scavenger receptor BI/Cla1
STAT1	Signal transducer and activator of transcription-1
TARC	Thymus and activation-regulated chemokine
TC	Tri-color
TGF α	Transforming growth factor alpha
TGF β	Transforming growth factor beta
TNF α	Tumor necrosis factor alpha
VLDL	Very low density lipoprotein

Chapter 1:

Introduction

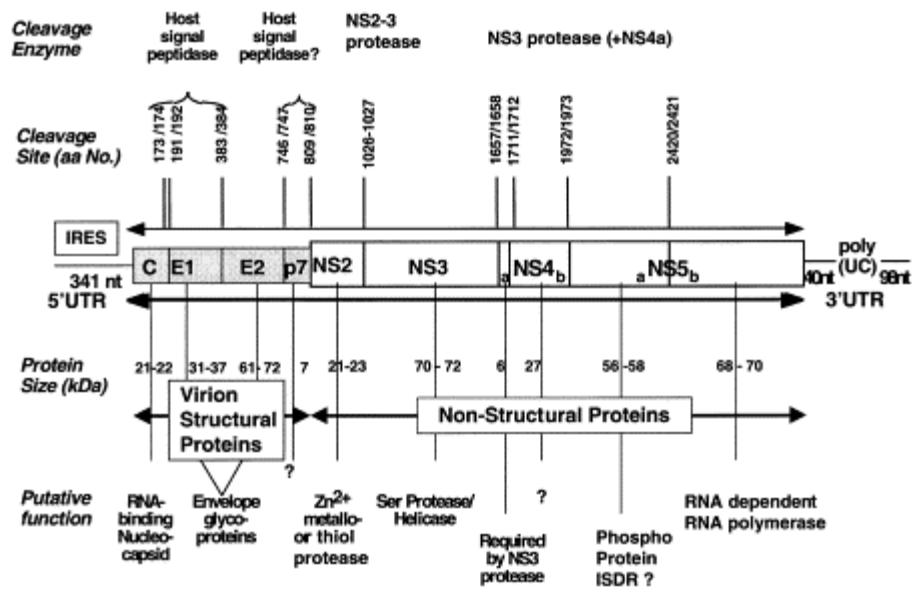
1.1 HCV infection

Hepatitis C Virus is one of the most important infectious causes of chronic liver disease. Hepatitis C Virus and alcohol frequently coexist and together lead to more rapid progression of liver disease, and an increased incidence and prevalence of cirrhosis and hepatocellular carcinoma. There are more than 170 million chronic HCV carriers worldwide (52), including 4 million within the United States and 240,000 individuals within Canada. Exposure to HCV may result in: acute infection with recovery, development of persistent infection without apparent disease, or persistent infection with chronic hepatitis. Eighty percent of infected individuals end up with persistent infections; therefore, it is very important to develop reliable antiviral treatments. The present treatments, combinations of α -interferon and ribavirin are only partially effective (less than 50%), depending on the specific HCV genotype (6 genotypes based on the NS5 region) (284). Using IFN α as a treatment for HCV infection has brought to light a number of host factors that influence clinical outcome. Response to treatment with interferon-based regimens is decreased in those who drink alcohol (245). Ethnicity, with African Americans having an attenuated response to IFN α (165), and gender, represented by female patients who respond significantly better to IFN α therapy than male patients due to a potentiating effect of estrogen on IFN α (126), are two other important factors to influence the HCV viral clearance. Also, genetic polymorphisms (126) (189) (282) affect IFN α therapy response via modulating host immune response. Age, with older individuals having a lower response to IFN α treatment than younger individuals (39) (189) (282), as well as obesity (39), and cirrhosis (24) reduce responsiveness to IFN α therapy (165).

1.2 Hepatitis C Virus Structure

Hepatitis C virus is a positive sense 9.6-kb ssRNA virus, a member of the Family *Flaviviridae*, Genus *Hepacivirus*. It only infects humans and chimpanzees (176). The genome is made up of 5' and 3' non-translated regions and an open reading frame that encodes a 3000 amino acid residue polyprotein, which is processed by viral and host proteinases to produce 10 viral structural and non-structural proteins. The N terminal part of the polyprotein is represented by the structural proteins: core, Envelope 1, and Envelope 2 proteins - part of the viral particle. The non-structural proteins are involved in viral replication (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) and are located at the C terminus (Figure 1.1). The 5' untranslated region (UTR) contains a transcriptional initiation site for positive-stranded viral RNA synthesis and an internal ribosome entry site (IRES) for viral polyprotein translational initiation (264). The 3' untranslated region is divided into three regions: a variable sequence of 40 bases, a variable length poly-UC rich tract, and a highly conserved 98 base region, with the 3'-terminal 46 bases believed to be mandatory for virus replication (157).

Figure 1.1 Genomic and proteomic map of the Hepatitis C virus. The functional and structural units of the HCV genome are schematically depicted. The 5' UTR, comprising the IRES, and the tripartite 3'UTR, flank the viral polyprotein coding sequence which occupies the vast majority of the viral genome. The N-terminal structural proteins are shaded and the non-structural proteins are white. The ten known polyprotein cleavage products are shown with their approximate sizes (in kDa) and putative functions. Proteins generally accepted to have unknown functions at this time are p7, NS4b, and NS5a. Proteins whose activities have been shown to be required for *in vivo* infectivity are NS2–3, NS3 (protease and helicase), and NS5b, as well as the 3'UTR. In addition, the proposed cleavage enzymes which generate these molecules are indicated along with the cleavage sites (254).



1.2.1 The HCV structural proteins

The structural proteins are the core, and the two envelope proteins, E1 and E2.

The HCV core is involved in nucleocapsid formation, and viral replication (145). The core protein functions as an RNA chaperone protein and drives profound structural modifications of HCV RNA *in vitro* (131). HCV core protein induces oxidative stress through increased reactive oxygen species (ROS) production in the absence of inflammation (213) which also contributes to the development of hepatocellular carcinomas (HCC). The core-induced oxidative stress was shown to be a result of direct effects on mitochondria followed by increased mitochondrial ROS production, greater sensitivity to Ca (2+)-induced cytochrome c release and oxidant-induced loss of mitochondrial membrane potential (323). The HCV core protein also induces alteration of the intracellular signalling cascade of mitogen-activated protein kinase (309) and activating factor (AP)-1 (125), leading to inappropriate function of the cell cycle control, resulting in HCC production (7). Studies suggest that activation of the MAPK/ERK pathway by core protein is done through upregulation of TGF α transcription via activation of NF-kB (270). The HCV core region also has impact on lipid metabolism, inducing hepatic steatosis, abnormal retention of lipids within hepatocytes, in patients with chronic hepatitis C (294) and transgenic mice expressing HCV core protein (215,216).

The E1 and E2 envelope proteins, cut from the polyprotein by host peptidases are involved in receptor binding and cell fusion. The functional form of the E1 and E2 proteins is a non-covalent heterodimer important in virus entry (70). E2 plays an important role in the binding of the virus to its major cellular receptors CD81 and SR-BI/Cla1 (234,271). There are also low density lipoprotein (LDL) receptors involved in HCV entry, which bind to unknown components on the viral envelope through a mechanism involving LDLs and

VLDLs. Both HCV envelope glycoproteins are highly glycosylated, which is also important in virus infectivity (112). It was suggested that both E1 and E2 proteins are involved in virus-cell fusion by either direct interaction of specific regions of the envelope proteins with lipid membranes or by involvement in the conformational changes of the E1/E2 heterodimer at low pH (164).

1.2.2 The HCV non-structural proteins

The p7 protein is a viroporin. It is thought to be an ion channel mediating membrane permeability and secretion. It is important during the late stages of release and maturation of the virus (287), and critical for releasing infectious viral particles (290).

Non-structural protein 2 (NS2) is a Zn^{2+} metallo- or thiol protease. It cleaves the NS2-3 junction having an important role in polyprotein processing, replication, and also in infectious virus production through the C-terminal region (72).

The NS3 serine protease is a 631 amino acid bi-functional protein, and has protease activity localized to the N-terminal portion (180 amino acids). The C-terminal portion has helicase (451 amino acids) and nucleoside triphosphatase activities required for viral replication. NS3 cleaves the non-structural region of the polyprotein at different sites: NS3-4a, NS4a-4b, NS4b-5a, NS5a-5b (341). This protease activity requires NS4a protein, a 54 amino acid hydrophobic protein whose function is to modulate the conformation of the N-terminal domain of NS3 and to dock the NS3 protein to the ER membrane. Moreover, the NS4a protein may have a role in temporal regulation of viral replication, inhibiting the NS2-3 autoproteolytic cleavage event and the helicase activity of NS3 (6,152).

Non-structural protein 4B (NS4B) is a hydrophobic protein of approximately 27-kDa in size, absolutely required for viral propagation. It forms the membranous web, a scaffold for

the replication process where all the non-structural proteins and the viral RNA colocalize following translation (6,81). NS4B possesses GTPase and ATPase activities (303). GTPase activity of NS4B protein might play a role in NS4B-induced cellular transformation and tumor formation (83). NS4B proved to bind to the 3' end of negative strand viral RNA suggesting that NS4B might link the HCV RNA to the membranous web and facilitate positive sense RNA synthesis (82). NS4B not only functions in RNA replication but also contributes to virus assembly and release (138).

Non-structural protein 5A (NS5A) plays an important role in HCV virion production and assembly of infectious viral particles, and might function as a molecular switch between replication and assembly, being recruited by core-associated lipid droplets (31,279). It was suggested that NS5a has a role in interferon response, through PKR inhibition (354).

NS5B is the RNA dependent-RNA polymerase (RdRp), the key component of the HCV replication. It has the ability to initiate replication from an RNA template without the need of a primer (de novo initiation). NS5B contains classic structural domains, fingers, palm, and thumb, with conserved aspartic acids that chelate the catalytic Mg^{2+} ions found in the palm. The finger and thumb domains are connected to each other, which confers rigidity to HCV RdRp enzyme, and does not allow a conformational change of the two domains independently of each other. This is also the case for other RdRps, as suggested by studies of double-stranded RNA bacteriophage $\Phi 6$ polymerase structure, providing an evolutionary link between double-stranded RNA viruses and flaviviruses (42).

1.3 HCV Life Cycle

The HCV virion comprises viral RNA and a core-nucleocapsid surrounded by a cell-derived lipid bilayer envelope containing embedded E1 and E2 proteins. The circulating HCV is represented by free virions or can be low-density lipoproteins (LDL), very-low-density lipoproteins (VLDL) and immunoglobulin-associated virions. The target cells for HCV infection are mainly hepatocytes and immune system cells represented by dendritic cells, and B-cells (210). The virus can enter the cells through different receptors, tetraspanin protein CD81, the LDL receptor, glycosaminoglycans, scavenger receptor class B type I (SR-BI), occludin and claudin-1 (a tight junction protein). The latter protein has been shown to induce non-hepatic cell-susceptibility to HCV and was identified as an essential factor in the late steps of virus entry, after the HCV-CD81 co-receptor binding (89). Occludin, a transmembrane component of the tight junctions interacts directly with E2 and facilitates viral entry (30,178). The virion is then internalized through clathrin-mediated endocytosis. Core protein is required for virus transport in cells, by high affinity binding to tubulin via amino acids 2-117 (253). Uncoating of the HCV virion, which releases HCV RNA into the cell cytoplasm, is still not well understood, and is followed by translation of viral RNA directly in the cell cytoplasm using host cell ribosomes to generate the polyprotein. HCV translation initiation occurs through the formation of a binary complex between the IRES and the 40S ribosomal subunit. Replication occurs after translation and cleavage, with replication complexes being identified close to specific perinuclear membrane alteration sites named the membranous web, derived from ER membranes. Formation of the membranous web is induced by NS4B (114). The latest studies suggest a very tight link between Hepatitis C virus replication and lipid metabolism (247). Synthesis of a complementary negative-strand RNA

using the genome as a template and the subsequent synthesis of genomic positive-strand RNA from this negative-strand RNA template is controlled by NS5B RdRp. NS2 and other non-structural proteins, as well as yet-to-be defined RNA structures, are involved in these processes. Packaging of newly produced positive stranded RNA with envelope proteins into virions is still not completely understood. Virions are believed to bud into the ER, or an ER-derived compartment, and exit the cell through the secretory pathway. Release of the progeny virions is thought to be closely related with lipoprotein metabolism (11).

1.4 HCV Genotypes

A genotype is defined as the genetic constitution of an organism. Currently, there are 6 distinct yet related HCV genotypes and 50 plus subtypes (284). Genotypes differ from each other by 31% to 33% at the nucleotide level depending on the differences in a 222 base fragment of the NS-5 gene (285) compared to 20% to 25% nucleotide difference between HCV subtypes, which are based on the nucleotide sequence of the core and E1 genes. Viral genotypes differ according to geographic region: Genotypes 1, 2 and 3 are distributed worldwide, with subtype 1a more prevalent in North and South America, Europe, and Australia, and subtype 1b more common in North America and Europe, and in some parts of Asia. Genotype 2 is present in most developed countries, but is less common than genotype 1. Genotype 4 is mostly found in the Middle East and Africa, genotype 5 in South Africa and genotype 6 in Southeast Asia (196). Patients infected with HCV genotype 3 are more prone to steatosis linked with high viral load (64). Genotype identification is clinically important because genotypes 1 and 4 are more resistant than genotypes 2 and 3 to the current standard of care, pegylated IFN α and ribavirin combination therapy, and are more often linked to chronic infection (128).

Studies showed that IFN α treatment was able to accelerate the evolution of HCV quasispecies in non-responder patients. Quasispecies are non-identical but closely related RNA genomes or viral variants that develop within a host through evolutionary selection resulting in viral heterogeneity (40, 62). These quasispecies are able to evade the host's immune responses which can explain the ability of the virus to establish chronic infection, since the virus can escape the CD8⁺ T cell response. Rapid evolution of the hypervariable region (HVR1) within the E2 envelope protein of HCV allows these quasispecies to escape immune surveillance since they are no longer recognized by the existing T cells and neutralizing antibodies.

1.5 Epidemiology of HCV Infection

The main route of HCV transmission is infected blood. The blood-borne virus is frequently transmitted through needle sharing by intravenous drug users, blood transfusion, solid organ transplantation from infected donors, unsafe medical practices using poorly sterilized or unsterilized equipment, occupational exposure to infected blood, transmission from HCV RNA positive mother to child, sex with an infected person, and high-risk sexual practices. In the United States and other developed countries, highly sensitive diagnostic tests developed after 1992 almost eliminated the transmission from HCV infected blood products and organ transplants. However, in developing countries, blood transfusions remain a major cause of HCV transmission (200). The highest HCV seroprevalence rates (up to 90%) have occurred in subpopulations like the homeless, incarcerated persons, injection drug users, and persons with hemophilia who were treated with clotting factors before 1992 (219).

The worldwide seroprevalence of Hepatitis C Virus (HCV) infection is about 3%, ranging from 1% in North America to 10% in North Africa, with higher prevalence in males

than in females. Three distinct types of transmission were identified. Injection drug use has been the predominant risk factor for HCV infection of 30-49 year old subjects (in countries like the United States and Australia). For countries like Japan and Italy, the highest frequency of HCV infections is found in older subjects, related to unsafe injections and contaminated equipment used in healthcare-related transmission occurring after the Second World War. In countries like Egypt, there is a spread of HCV infection through all age groups, indicating a continuous high risk for acquiring HCV infection by healthcare-related transmission (21) (329).

Prevalence of HCV infections in the United States is about 1.8% with the highest prevalence found among young adults, and 50% of which are the result of infections acquired from injecting illegal drugs (8) (274). There are three characteristics found among 85.1% of 20 to 59 year-old HCV RNA-positive cases. These are represented by abnormal serum ALT level, history of injection drug use, and history of blood transfusion before 1992 (14) (336).

There are 240,000 individuals infected with HCV in Canada. The incidence of newly acquired HCV infection declined from 3.3 to 2.1 cases per 100,000 from 1998 to 2004, with most of the new cases represented by 15 to 39 year old subjects, with unsafe drug injection as the most frequently reported route of transmission (336).

1.6 Epidemiology of HCV Infection and alcoholic liver disease (ALD)

More than 60% of patients with liver disease in the Western world are represented by hepatitis C virus (HCV) infection and ALD alone or in combination, and the outcomes are liver cirrhosis and hepatocellular carcinoma (HCC). It is known that 80 g of daily alcohol consumption increases the risk for HCC five times and the presence of HCV increases the risk 20 times, while the combination of both risk factors leads to a more than 100-fold risk

for HCC development. In the United States, 50% of liver related deaths are induced by alcohol consumption, with liver cirrhosis development in 10%-20% of heavy drinkers (more than 80 g of ethanol daily) (185) (186) (274). There is a 2-3 fold increased risk of severe liver disease in the presence of the two conditions compared with HCV patients without a history of drinking (69) (208). In recent years, the highest increase in alcohol-related disorders was found in China. In some parts of the country, the increase in alcohol consumption was approximately 400% (55).

1.7 Diagnosis of HCV Infections

Diagnosis of HCV infection is usually done in the chronic phase, because of the unspecific, low intensity symptoms present in the early acute stage of the disease. The identification of antiviral antibodies in the blood by an enzyme linked immunosorbent assay (ELISA) is a hallmark of HCV exposure. This method is suitable for screening high risk populations, and is recommended as the initial test for patients with clinical liver disease. HCV is usually detectable in the blood within 1-3 weeks following the initial infection, and antibodies to the virus are usually detectable within 3-12 weeks following the initial infection (301), with some exceptions for late sero-converters, which become positive for anti-HCV antibodies in the 12-27 week interval after exposure to the virus (314), or immunosuppressed patients, co-infected with HIV/HCV, which may become negative for anti-HCV antibodies but still have chronic HCV infection (47).

Due to the active replication of the virus present in acute or chronic infections, the presence of HCV RNA in serum can be detected by reverse transcriptase polymerase chain reaction (RT-PCR). The PCR targets are the non-coding regions at the 5' and 3' ends of the RNA since these regions are more conserved throughout genotypes (283). The identification

of specific viral genotypes is used for clinical prognostic significance. To determine HCV genotype, reverse hybridization after PCR with genotype-specific probes can be used (291).

1.8 Treatment of HCV Infections

The standard treatment of chronic hepatitis C is the combination of pegylated IFN α and ribavirin (4) (219). The pegylation of the IFN α molecule results from the attachment of one or more polyethylene glycols to the IFN α molecule, which drastically modifies the immunological, pharmacokinetic and pharmacodynamic properties of the drug. The efficacy of the treatment depends on genotype: 20% of subjects infected with HCV genotypes 2 and 3 are resistant to treatment (67, 317), while 50% – 60% of patients infected with HCV genotype 1 fail to eradicate the infection (101, 120). For genotype 4, the reported rate of failure was 30–45% (67, 87, 143). Genotypes 5 and 6 are less studied because of their minor distribution, but the rate of failure was found to be intermediate between genotype 1 and 2 (224). IFN α administration induces rapid decrease in HCV viremia (222), through induction of a cellular antiviral state, associated with the upregulation of numerous IFN α -stimulated genes (263). The response to IFN α -treatment correlates strongly with host factors like age, race, extent of fibrosis, obesity, insulin resistance, and alcohol consumption. Resistance to treatment among different HCV genotypes suggests true virus resistance to IFN α . Specific sequence variation in some viral proteins, such as the ‘interferon sensitivity determining region’ (ISDR) of the non-structural NS5A protein (49), the analysis of amino acid sequence covariance networks within the polyprotein (17), and also high levels of IFN α -stimulated genes before the treatment (51,162) can predict the outcome of the treatment.

Ribavirin is a synthetic nucleoside which blocks the replication of DNA or RNA viruses, and is known to reduce hepatic inflammation (28). Still, the mechanism of action is

unknown. Ribavirin monotherapy has little antiviral effect, but in combination with pegylated IFN α increases substantially the number of cases with sustained virologic response (which is undetectable virus in the blood six months after the end of the treatment) (193). To increase the efficacy of the anti-HCV treatment, especially against genotype 1, a number of novel antiviral molecules are currently under development. The new therapeutic agents are represented by inhibitors of the NS3/4A serine protease and inhibitors of HCV replication like nucleoside/nucleotide analogues and non-nucleoside inhibitors of the HCV RNA-dependent RNA polymerase, NS5A inhibitors and cyclophilin inhibitors (235). The best results were reported for the combination of NS3/4A inhibitor, pegylated IFN α and ribavirin, with 70% HCV clearance (192).

1.9 Outcomes of HCV Infection

1.9.1 Hepatic Manifestations

Acute HCV infection, following a 6-8 week incubation period, is asymptomatic or paucisymptomatic in 90% of cases. The symptoms are usually nonspecific, represented by fatigue, anorexia, abdominal pain, mild hepatosplenomegaly, arthralgia, jaundice and scleral icterus. Acute HCV infection is frequently discovered on the basis of high serum levels of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) enzymes in asymptomatic patients. 60-85% of the acute HCV infections will progress to chronic infection. Symptomatic patients generally have a higher probability of eliminating the virus than asymptomatic patients, which are more prone to become chronically infected (273).

The symptomatology of chronic infection is usually not seen in immunocompetent subjects for 15-20 years, except for fluctuating levels of ALT (335). The virus causes

steatosis, inflammation, necrosis and apoptosis leading to gradual hepatic fibrosis, and 20–50% of chronically infected persons will eventually develop cirrhosis (severe fibrosis and nodular regeneration) or hepatocellular carcinoma (HCC). Chronic hepatitis C disease is the first cause of liver transplantation in developed countries. Death can occur from decompensated liver disease represented by esophageal varices, coagulopathy, and thrombocytopenia, as consequence of portal hypertension, HCC, or a combination of these conditions.

Steatosis (lipid accumulation) is the most common hepatic manifestation found in subjects with chronic HCV, occurring in 30% to 70% of liver biopsy samples (183). The development of steatosis is influenced by host and viral factors. Many studies showed a direct steatogenic effect of the virus, while some of the host factors are represented by increased BMI (body mass index) and insulin resistance (248). The presence of steatosis is a major determinant of the progression of fibrosis (43, 93), with correlation between the grade of steatosis and fibrosis (3). Some of the mechanisms by which steatosis can induce fibrosis are enhanced oxidative stress (183), activation of subsinusoidal stellate cells (43, 54), and increased susceptibility to apoptosis (95). Infection with HCV-3a genotypes was recognized as an independent risk factor associated with the development of steatosis in HCV-infected patients (133) (207).

The degree of fibrosis in liver biopsies can be used to grade the severity of the liver disease in chronic HCV infections. An early stage of disease is characterized by the presence of fibrosis in portal tracts, an intermediate stage consists of an expansion of the portal tracts and bridging between portal areas and central areas, and the late stage is represented by cirrhosis characterized by architectural disruption of the liver with fibrosis and regeneration (56).

Factors that can affect disease progression and accelerate the rate of fibrosis are co-infection with hepatitis B virus (32), immunosuppression (in HIV–HCV co-infection) (58) (302), and alcohol consumption (249) (334). When a chronically infected patient persistently consumes alcohol (> 40 g of alcohol /day for women and > 60 g of alcohol /day for men for more than 5 years), progression to cirrhosis accelerates by 3 fold (334).

Other factors which accelerate the progression of fibrosis include gender (men > women), older age at infection, longer duration of infection, and obesity (56). Cirrhosis, the final stage of fibrosis, precedes the onset of liver failure and development of primary liver cancer, which consists predominantly of hepatocellular carcinomas. Progression to cirrhosis is long, with an average of 30 years. Once the diagnosis of cirrhosis is established, the rate of developing decompensated liver disease is about 4% per year (190,277).

HCC is the fifth most common cancer worldwide and the third most common cause of cancer mortality (86, 155). The annual incidence rate of hepatocellular carcinoma among patients with HCV-related cirrhosis is 1% to 4%, with the highest, 7%, in Japan (155, 335). Additional risk factors for developing HCV-related HCC are HBV-HCV co-infection, with a relative risk of 82.5 for developing HCC, compared to HCV infection alone, which was associated with a relative risk of 6.6 (151). Alcohol consumption also significantly exacerbates the course of chronic hepatitis C, through development of cirrhosis (337).

1.9.2 Extrahepatic manifestations

HCV is also involved in the pathogenesis of autoimmune and rheumatic disorders like arthritis, vasculitis, sicca syndrome or Sjogren syndrome (an inflammatory disease of glands and other tissues of the body leading to decreased tears and dry eyes) (119), lichen planus,

nephropathies (202), thyroid diseases, and lung fibrosis, as well as in the development of B-cell lymphoproliferative diseases.

There are studies (5, 96, 261) suggesting a strong link between HCV infection and essential mixed cryoglobulinemia, a multisystem disorder characterized by deposition of circulating immune complexes in small and medium-sized blood vessels which results in arthralgias, Raynaud's syndrome (vasospastic disorder causing discoloration of the fingers, toes, and occasionally other extremities) and purpura. Furthermore, antiviral therapy with IFN α is an effective treatment for this disease.

An association between HCV infection and porphyria cutanea tarda (characterized by low levels of uroporphyrinogen decarboxylase, the enzyme responsible for the fifth step in heme production) was also reported (68, 91). The possible mechanism of porphyria cutanea tarda HCV-induction might be increased intracellular levels of iron leading to formation of active free radicals and oxidation of uroporphyrinogens (36).

Membranoproliferative glomerulonephritis was also shown to be associated with chronic HCV infection. This lymphoproliferative disease is characterized by the deposition of IgG, IgM, and C3 in cryoglobulin-like structures (36), which may contain HCV RNA and HCV antibodies (124, 137).

1.10 Models for studying HCV

The development of new anti-HCV therapeutics was slowed down by the unknown pathogenesis of HCV, an important impediment being the lack of a viral culture system or an inbred animal model (the only validated animal model is the chimpanzee). Because of the lack of efficient cell culture systems that can allow studies of HCV replication and pathogenesis, subgenomic selectable replicons were established by transfection of cloned

viral consensus genome sequences from an infected human liver into human hepatoma cell lines (33, 34, 182). The subgenomic replicons could be composed of part of the HCV genome that encodes for non-structural proteins or structural proteins. cDNA fragments containing core, E1, E2, p7 or non structural proteins from NS2/NS3 up to NS5b were amplified and cloned in different vectors. The expression of the proteins in these plasmids is under the control of the tetracycline responsive promoter, or the IRES of encephalomyocarditis virus. The plasmids contain a selectable marker too: neomycin phosphotransferase, which confers G418-resistance, so that the replicon containing cells can be identified. The HCV replicon system was used to study HCV RNA replication. Cells transfected with adaptive mutation-bearing subgenomic replicons were able to replicate HCV RNA at high levels, but the same replicons were incapable of producing infectious virus *in vivo* (chimpanzees), because of possible adaptive mutations which interfered with virus packaging and secretion (41). To explain the initial steps of the virus life cycle, like binding and entry, HCV pseudo-particles (HCVpp) were developed (98).

The other model used to study HCV pathogenic studies is the transgenic mouse (144, 167, 321). It has facilitated different studies like normal gene function, altered gene expression and production of animal models for human diseases. These transgenic mice are constructed by injecting cloned DNA (in this case, HCV genes) into fertilized mouse eggs. The surviving eggs are cultivated and transferred into the uterus of foster mouse females, which will deliver the pups in 19-21 days. The offspring will be analysed by PCR or Southern blot to detect the transgene carriers (the transgenic founder mice).

A core/E1/E2-expression vector was previously constructed in Dr. Diaz-Mitoma's lab to be used in the production of the transgenic model (216). Total RNA was extracted from a patient infected with HCV genotype 1a, one of the most predominant genotypes found in

North America. pVAX-HCV plasmid DNA was microinjected into the pronuclei of fertilized eggs from a B6C3F1 murine strain (Charles River Laboratories, Wilmington, MA, USA). After the injected eggs were implanted into the oviducts of pseudopregnant foster mothers, 5 founder mice carrying HCV fragments were identified as transgenic via PCR using tail DNA. Isolation of DNA from tail biopsies was followed by PCR amplification and identification of the transgene by gel electrophoresis to confirm successful transmission of the transgene (core, E1 and E2 fragment) to offspring. The transgenic colony was maintained by breeding transgenic mice with wildtype B6C3F1 mice thus creating a heterozygous mouse colony. This transgenic mouse model, expressing HCV core, E1 and E2, has been developed to investigate the oxidative stress and perturbation of lipid metabolism associated with hepatitis C infection. The expression of hepatitis C virus structural proteins in the liver of these transgenic mice resulted in an extensive hepatopathy (216).

Complete cell-culture systems with production of recombinant infectious HCV were developed in the last few years (256, 319) and have now made it possible to study the life cycle of HCV, including very early and late steps like viral entry, genome packaging, virion assembly, maturation, and release.

1.11 HCV Pathogenesis

Little is known about the mechanisms of HCV pathogenesis. Both immune-mediated mechanisms and direct viral toxicity have been suggested as factors in the pathological changes associated with hepatitis C. One of the major issues is whether the HCV proteins have a direct role in the pathogenesis of HCV-associated diseases. There have been numerous studies of various HCV proteins overexpressed in cultured cells and transgenic

mice, which showed multiple effects of these proteins on cellular signal transduction, growth regulation, apoptosis, and responses to cytokines (166, 169, 197, 213, 214, 238, 239, 310).

Many studies provide strong evidence that HCV proteins, particularly the viral core protein, play a direct role in the development of liver steatosis - the accumulation of lipid droplets in hepatocytes. Clinical studies showed the presence of steatosis and a synergistic effect of alcohol and HCV in liver injury, reflecting metabolic abnormalities. *In vitro* studies, using replicon expressing cell lines, revealed alterations in lipid metabolism and transportation, cell-cycle and apoptosis dysregulation, and cellular transformation (191, 204, 278, 306, 352). To explain these observations, studies have been done using transgenic mice expressing different HCV proteins. The advantage of these models is that the mice are immunologically tolerant to transgenes, so the findings are the result of viral protein cytotoxicity and not due to immunologic responses. Steatosis - lipid accumulation in hepatocytes - has been induced by HCV structural proteins, while the expression of the full-length genome increases lipid accumulation and carcinogenesis (166).

A study of steatosis in a core-transgenic model demonstrated reduced microsomal triglyceride transfer protein activity and modified hepatic very low density lipoprotein (VLDL) assembly and secretion by core protein binding to apolipoproteins A1 and A2 (239), which are involved in triglyceride accumulation and storage in hepatocytes. Liver lipid composition in HCV core transgenic mice and in chronic hepatitis C patients showed a high concentration of carbon 18 monosaturated (C18:1) fatty acids, such as oleic and vaccenic acids, known to increase membrane fluidity leading to higher cell division rates (214). Hepatocytes from HCV infected patients show glutathione depletion, which is a pivotal antioxidant, mitochondrial abnormalities, and high lipid peroxidation (26), which can be reversed by interferon therapy (140). The impairment of oxidative phosphorylation and

increased production of free radicals may explain in part the role of HCV core protein in lipid metabolism and in steatosis in the liver of HCV-positive patients. Moreover, alcohol and hepatitis C virus core protein have been proven to additively increase lipid peroxidation and synergistically trigger hepatic cytokine expression (TNF α and TGF β) in a transgenic mouse model (238).

Core protein has similar actions as complement 1q (C1q) in inhibition of T-cell proliferative responses. It binds to the globular domain of the C1q receptor (gC1qR), but at different sites than C1q. This effect can be counteracted by addition of anti-gC1qR antibody in a T-cell proliferation assay (15, 154). The interaction between core protein and gC1qR induces phosphorylation of extracellular signal-regulated kinase (ERK) and mitogen-activated ERK kinase (MEK), which inhibits IL-2 production and the expression of high-affinity IL-2 receptors. These results were demonstrated by the ability of anti-gC1qR antibody treatment to reverse HCV core-induced inhibition of ERK/MEK phosphorylation (344). Furthermore, it has been shown that core protein inhibits degradation of p27 (Kip1), a negative regulator of cyclin-dependent kinases (Cdk) 2/4 and cyclin E/D. Then, these cyclin-dependent kinases induce phosphorylation of retinoblastoma (pRb), and block cell cycle progression from G1 to S phase, keeping activated T cells at the G1 phase (342). There is a differential regulation of B and T lymphocytes by the HCV core-gC1qR interaction, with down-regulation of CD69 activation in T cells (343), but upregulation of CD69 activation and cell proliferation in B cells through down-regulation of suppressor of cytokine signalling-1 (SOCS-1) and upregulation of signal transducer and activator of transcription-1 (STAT1) phosphorylation (169, 213, 345).

One mechanism suggested to be involved in the development of hepatocellular carcinoma is oxidative stress. Accumulation of reactive oxygen species (ROS) can induce

genetic mutations and chromosomal alterations, which can lead to cancer development. Studies of transgenic mice expressing the core protein showed modifications of the oxidant/antioxidant state. The expression of core protein results in intracellular oxidative stress, which will be counteracted by the up-regulation of cellular antioxidant mechanisms in young mice. In older mice, this defence mechanism is overwhelmed, resulting in hepatocellular carcinoma (169, 213). To demonstrate how the core protein can induce ROS accumulation, studies of core expressing cell lines have been done. They start from the idea that the mitochondria are responsible for production of most of the ROS, and use an inhibitor of the flavoenzymes that blocks electron transport through mitochondrial complex 1. After the treatment, the high level of ROS, resulting from core protein expression, was decreased (228).

Another mechanism for explaining carcinogenesis was suggested to be through STAT3. STAT3 is one of the signal transducer and activator of transcription (STAT) proteins (310), and its phosphorylated form is involved in tumorigenesis. The result was that HCV core protein determines activation of STAT3, through phosphorylation of a critical tyrosine residue. The same study showed upregulation of effectors downstream of STAT3: Bcl-xl and Cyclin D1, which are related to cell-cycle progression and anti-apoptosis (350). NS3 non-structural protein was also shown to induce STAT3 activation in response to oxidative stress. Disturbance of intracellular calcium by the presence of this HCV non-structural protein triggers high levels of reactive oxygen species in mitochondria, followed by activation of NF- κ B and STAT-3 and its translocation into the nucleus (113, 327, 328).

It has been suggested that cytokines like TNF α and IL-1 β may be involved in hepatocarcinogenesis, because of their role in cell proliferation. The HCV core protein expressed in transgenic mice upregulates TNF α , IL-1 β expression, and activates JNK and

transcription factor AP-1, which are the downstream effectors of these cytokines (310). As antioxidants activate AP-1, it can also be activated in this transgenic mouse model by high levels of antioxidants, like catalase and glutathione, which have important roles in cellular defense against oxidative stress (197) (213).

On the other hand, HCV core has been shown to induce apoptosis and necrosis of cells, which might play an important role in the pathogenesis of HCV persistent infection and hepatocellular carcinoma, and different core domains of different HCV quasi-species might have some differences in their pathogenic effects (338). There are studies suggesting that viral evasion of cell-mediated immune responses leading to apoptotic death of hepatocytes may contribute to viral persistence in transgenic mice expressing the hepatitis C virus polyprotein. This deficit was associated with resistance of transgenic hepatocytes to apoptosis induced by Fas/APO1/CD95 death receptor stimulation, a major pathway of cell killing by cytotoxic T lymphocytes, by having a reduced abundance of Bid, a BH3-only member of the Bcl-2 family of apoptosis regulators (75). Such a mechanism might also contribute to the development of liver cancer in HCV.

The combination of HCV persistent infection with alcohol consumption was shown to also induce hepatocyte apoptosis, and this effect is determined by increased plasma levels of TNF α due to HCV presence and increased expression of TNF α receptor after alcohol ingestion (163) (257).

1.12 Ethanol influence on HCV-induced liver disease pathogenesis

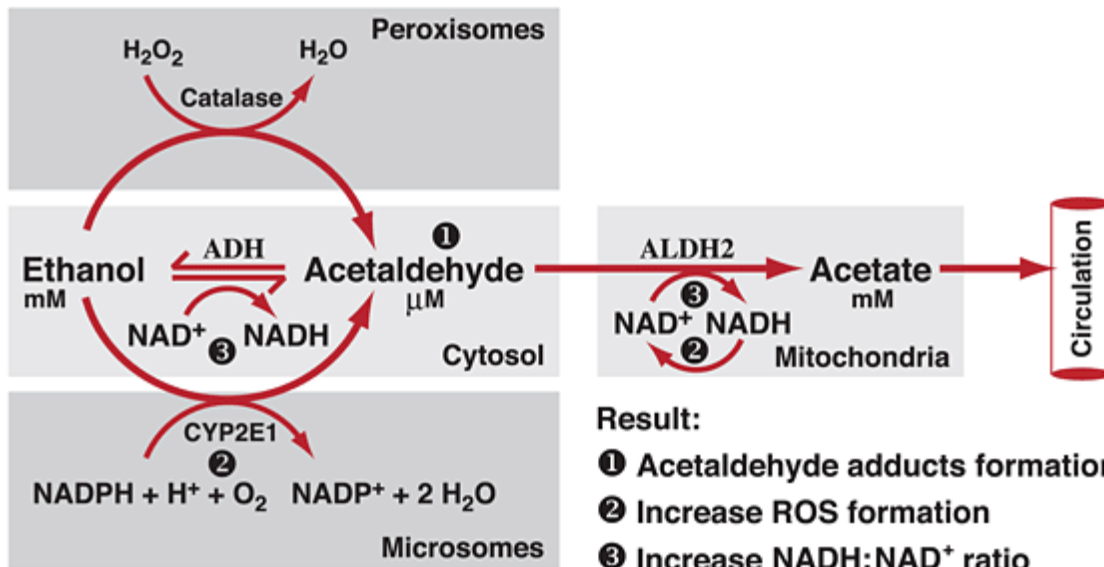
Most of the effects of ethanol on liver disease pathogenesis are due to its metabolism (Figure 1.2). There are three oxidative pathways of hepatic ethanol metabolism. The main one is the alcohol dehydrogenase catalysis of ethanol to acetaldehyde, with production of ROS which can indirectly interfere with the electron transfer system in the mitochondria and is associated with changes in the NADH levels and NADH/NAD⁺ redox ratios (66) (160) (188).

The second pathway by which ethanol can be converted to acetaldehyde is located in the endoplasmic reticulum and is represented by the microsomal ethanol-oxidizing system. The main enzyme responsible for ethanol catalysis at this location is cytochrome P450 2E1 (CYP2E1) (173). There are other isoforms of cytochrome P450, 1A2 and 3A4, with variable capacity of oxidizing ethanol (260).

The last oxidative pathway of ethanol metabolism is by catalase, and is located in the peroxisomes. Peroxidation via catalase supported by H₂O₂, formed by the peroxisomal beta-oxidation of fatty acids is the predominant pathway of alcohol oxidation in the fasting state (122).

There are also two non-oxidative pathways for ethanol metabolism. One results in the formation of molecules called fatty acid ethyl esters (FAEEs) from the reaction of alcohol with fatty acids. The other pathway results in the formation of phospholipids known as phosphatidyl ethanol. FAEEs' effect on liver damage has not yet been evaluated.

Figure 1.2 Oxidative pathways of alcohol metabolism. The enzymes alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1), and catalase contribute to oxidative metabolism of alcohol. Cytosolic ADH converts alcohol to acetaldehyde. This reaction involves an intermediate carrier of electrons, nicotinamide adenine dinucleotide (NAD^+), which is reduced by two electrons to form NADH. Peroxisomal catalase requires hydrogen peroxide (H_2O_2) to oxidize alcohol. CYP2E1, present predominantly in the cell's microsomes, assumes an important role in metabolizing ethanol to acetaldehyde at elevated ethanol concentrations. Acetaldehyde is metabolized mainly by aldehyde dehydrogenase 2 (ALDH2) in the mitochondria to form acetate and NADH. ROS, reactive oxygen species. (http://www.niaaa.nih.gov/Resources/GraphicsGallery/Metabolism/pathways_alcohol.htm).



1.12.1 Role of reactive oxygen species (ROS) in ethanol-induced liver disease pathogenesis

Enhanced oxidative stress may be a primary mechanism of synergistic liver injury found in chronic HCV infection and alcohol abuse. Alcohol-induced ROS such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are produced by the cytochrome P450-dependent microsomal mono-oxygenase system. During ethanol metabolism to acetaldehyde, increased electron leakage from the mitochondrial respiratory chain is associated with high levels of NADH entering mitochondria (18), hepatic iron overload (276), and activation of nitric oxide synthase, leading to the formation of the highly reactive peroxynitrite ($ONOO^-$) (48). Ethanol consumption is known to induce the expression of CYP2E1 (172) which can produce ROS in the presence or absence of ethanol. When CYP2E1 and core protein were expressed together in hepatoma cells, they enhanced reduced glutathione (GSH) depletion in mitochondria, and increased ROS production, mitochondrial depolarization, and alcohol-induced cytotoxicity (232) (324) (332). This suggests that induction of CYP2E1 may play a critical role in the exacerbation of chronic hepatitis C by alcohol.

1.12.2 Effect of ethanol on intrahepatic lipid accumulation

Both alcohol and HCV induce liver damage by increased lipid accumulation (steatosis), but the mechanisms are different. HCV mainly damages mitochondria, with decreased fatty acid metabolism, while ethanol-induced steatosis happens via increased lipogenesis. The mechanisms by which ethanol induces lipogenesis are through high production of ROS resulting from metabolism of alcohol to acetaldehyde, and release of $TNF\alpha$ cytokine via activated Kupffer cells, with subsequent activation of fatty acid release from adipocytes, increased hepatocyte lipogenesis, and inhibition of fatty acid β -oxidation (94) (123) (217).

Ethanol also impairs the transport and secretion of very low density lipoproteins (VLDL) (220).

1.12.3 Effect of ethanol on induction of liver fibrosis

A high number of studies have shown increased progression of fibrosis in HCV infections complicated with alcohol consumption (134). The degree of fibrosis is dependent on the dose of alcohol, and starts at less than 30 g ethanol/day (208) (240). The mechanism by which ethanol induces liver fibrosis is believed to be through high expression of type II TGF β receptor, resulting in activation of TGF β 1, a potent inducer of alpha 1-collagen. This effect is due to high levels of its metabolite – acetaldehyde (50). Moreover, mRNA levels of TGF β were synergistically increased by ethanol treatment of core-transgenic mice (238). Other studies showed a TGF β 1-induced activation of alpha 2 collagen promoter (10, 272).

1.12.4 Effect of ethanol on induction of HCC

Oxidative stress and cirrhosis are probably the most important factors in the pathogenesis of HCC. ROS accumulation in the liver is due to ethanol metabolism. Increased oxidative stress in the liver of core-transgenic and non-transgenic mice with short-term ethanol feeding activates p38 MAPK and ERK, and accumulation of reactive oxygen species closely associated with mitogen-activated protein kinases (MAPK) (309, 326). High levels of ROS induced by alcohol and HCV generate lipid peroxidation products like malondialdehyde and 4-hydroxynonenal (4-HNE), which can further react with deoxyadenosine and deoxycytidine DNA bases, to form highly mutagenic products (etheno-bridged nucleic acid bases) (85, 100, 326).

Chronic ethanol consumption is one of the major causes of liver cirrhosis, a well-recognized pre-neoplastic lesion evolving to HCC. In the alcohol-induced cirrhotic livers,

hepatic progenitor cells, termed 'oval cells', have been observed (286). These hepatic stem cells harbouring pluripotency have been shown to precede the development of some HCCs (255). Proliferation of these oval cells is activated by TNF α resulting from stimulation of Kupffer cells by bacterial endotoxins (304). Increased levels of TNF α activate nuclear factor κ B (NF- κ B), which will further activate cell-survival machinery involving anti-apoptotic mitochondrial proteins such as Bcl-2 and manganese superoxide dismutase (111). Proinflammatory NF- κ B signalling is also induced by both HCV core protein (153) and ethanol via acetaldehyde (352).

Both HCV and ethanol influence hepatocyte apoptosis (243). Chronic ethanol administration induces lower levels of retinoic acid, associated with inhibition of mitogen-activated protein kinase (MAPK) and increased levels of phosphorylated JNK. This was further associated with a functional downregulation of retinoic acid receptors and upregulation of AP1 (JUN and FOS) transcriptional complex expression, resulting in hepatic cell hyperproliferation and inhibition of apoptosis (325).

1.12.5 Iron accumulation in hepatitis C infection and chronic ethanol consumption

Chronic HCV infection and chronic consumption of mild doses of ethanol were associated with accumulation of iron (92, 130) in hepatocytes or Kupffer cells. Iron is known to be one of the most powerful profibrogenic factors, and can induce liver fibrosis and HCC, possibly through inhibition of the anti-tumor activity of macrophages (116, 225). Oxidative stress, represented by sustained non toxic levels of hydrogen peroxide, induces upregulation of transferrin receptor 1 (TfR1), the receptor responsible for internalization of the trivalent iron-transferrin complex into hepatocytes, followed by increased transferrin-mediated iron uptake, and hepatocellular accumulation of iron (13). This mechanism may

explain the progression of alcohol liver diseases. Transferrin receptor 2 (TfR2) was also higher in the livers of patients with chronic hepatitis C, than in those with hepatitis B (296). Hepcidin, a liver-produced peptide hormone, that is a key regulator of iron metabolism in mammals, was also lower in chronic hepatitis C patients than in chronic hepatitis B patients (103). Hepcidin has an important role in the regulation of iron homeostasis, by binding to ferroportin 1(FP1), a protein responsible for the transfer of iron from intestinal cells to liver cells. By doing this, hepcidin contributes to the regulation of iron delivery to hepatocytes, from macrophage iron stores and intestinal mucosa, and plays an important role in the pathogenesis of hepatitis C. High iron diets were proven to induce mitochondrial alterations, hepatic steatosis, fatty acid oxidation, formation of 8-hydroxy- 2'-deoxyguanosine (8-OHdG), biomarker of oxidative DNA damage, and development of HCV-related HCC in transgenic mice expressing the HCV polyprotein (104). The standard treatment for chronic hepatitis C patients in Japan is iron reduction therapy by phlebotomy, coupled with low iron diets. This treatment was shown to reduce lipid peroxidation, oxidative stress (141), and hepatic content of 8-OHdG (149). The same therapy for compensated HCV-related liver cirrhosis reduced serum aminotransferase and α -fetoprotein levels, a major risk factor for the development of HCC (297).

1.12.6 Effects of ethanol on the immune system

Alcohol has an effect on the immune system, being associated with alteration of the cytokine profile, for example suppressed secretion of IL-2 and interferon γ (IFN γ), (88). Ethanol and HCV inhibit the cellular IFN γ response by interfering with the JAK–STAT signalling pathway (175) (245), and ethanol is also known to lower the efficacy of interferon therapy in chronic hepatitis C patients (74) (205). The anti-HCV action of IFN α proved to be

inhibited by ethanol metabolism by CYP2E1 through inhibition of the JAK–STAT signalling pathway (191). Oxidative stress, induced by both ethanol and HCV, decreases IFN γ expression through the JAK–STAT signalling pathway (74) (175). Moreover, ethanol has inhibitory effects on the antigen-presenting cell function of myeloid DCs (293). There are studies which suggest that ethanol treatment induces reduction of CD4+ inflammatory T cell and CD8+ cytotoxic T lymphocyte responses to HCV (106).

1.12.7 Inhibition of proteasome activity by ethanol and HCV

The inhibition of proteasome activity may be another mechanism of the synergistic liver injury found in HCV infection and chronic ethanol consumption. The HCV core induces high levels of ROS, which are further upregulated in CYP2E1-expressing cells and by alcohol exposure (232, 332). The proteasome, the major cellular protein-degrading complex, is sensitive to cellular ROS levels (27, 150, 229) and is inhibited by ethanol (78) (231). Osna et al. have reported ethanol-induced downregulation of proteasome activity previously upregulated by low levels of HCV core-induced oxidative stress (230).

1.12.8 Ethanol and HCV replication

Several clinical studies have found that moderate alcohol intake leads to increased viral load (240) (252). Zhang et al showed that alcohol enhances HCV RNA expression in replicon containing hepatic cells through activation of NF-kB promoter (352). Using hepatocyte-derived cell lines constitutively expressing CYP2E1, an alcohol-metabolizing enzyme, and harboring HCV replication, McCartney et al. showed that alcohol increases HCV-replication dependent on CYP2E1-mediated production of oxidative stress (191). Ethanol was also proven to enhance HCV genotypes 2a and 1b replication in Huh7 human

hepatoma cells naturally expressing CYP2E1 through lipid metabolism and alteration of the cellular NADH/NAD⁺ ratio (278). Furthermore, alcohol has been found to be associated with a significant increase in quasispecies complexity, determined by the high mutation rates of the E1 and E2 envelope regions, especially of the amino terminal hypervariable region 1 (HVR1) of E2 (295).

1.13 Immune response in HCV Infection

HCV reaches high serum titres within 1 week of infection, followed by 1 month-delayed adaptive cellular immune responses and 2 month-delayed humoral immune responses (300, 301). 8–12 weeks post-infection, HCV RNA titres decline, and more than 80% of patients develop chronic hepatitis with relatively stable viral titres.

1.13.1 Innate immune response to HCV Infection

There are studies suggesting low sensitivity of HCV to the antiviral effects of IFN α and IFN β . The mechanisms by which the HCV may inhibit innate immunity are suggested to be: (1) blocking of IRF3- mediated induction of type I IFN by serine protease NS3, (2) E2 and NS5A inhibition of PKR. E2 has a homologous sequence to the phosphorylation sites of both the enzyme and its substrate, the eukaryotic translation factor 2 α (EIF2 α), (3) NS5A forms heterodimers with PKR, and (4) disfunction of natural killer (NK) cells. The impairment in NK cell function may be due to (1) HCV E2 binding to tetraspanin CD81 at the surface of NK cells with inhibition of cytotoxicity and cytokine production, (2) lower NK capacity to activate dendritic cells, through high expression of the receptor CD94–NKG2A (NK group 2, member A) and (3) production of TGF β and interleukin-10 (IL-10) (63) (135) (307).

1.13.2. Adaptative immune responses in HCV Infection

Chronic HCV infection does not induce vigorous multi-epitope specific CD4⁺ and CD8⁺ T-cell responses. Induction of T-cell responses and IFN γ expression in the liver coincides with a decrease in HCV RNA titres (59, 187). HCV-specific T cell response in HCV infection are delayed and characterized by low levels of perforin and IFN γ with decreased proliferation capacity and cytotoxic activity, all indicators of lower probability of viral clearance. Humoral immune responses are represented by neutralizing antibodies specific for the HCV envelope glycoproteins (E1 and E2) (90). The highest antibody titres are found in patients with chronic hepatitis C, while recovered patients test negative, which is consistent with the emergence of HCV escape mutants (181).

1.13.3 HCV Evasion of immune responses

Viruses activate innate immunity through viral RNA recognition by RIG-I-like receptors (RLR). These receptors (349) contain two tandem caspase association and recruitment domains (CARD) in the N-terminus. RNA ligand activation of RIG-I is followed by binding to IFN β promoter stimulator-1 (IPS-1 or Cardif) which in turn activates transcription factors like interferon regulatory factor-3 (IRF-3) and NF- κ B, and triggers induction of IFN β (136). HCV evades the host innate immune response by disrupting the RIG-I signalling pathway through HCV NS3/4A protease-mediated cleavage of IPS-1 (99). NS3/4A also cleaves the TLR3 adaptor molecule TRIF, thus blocking TLR3-dependent signalling (168). NS5A HCV-protein also reduces type I IFN induction through inhibition of MyD88-dependent TLR signalling (1). HCV core protein modulates type I IFN signaling by inducing the expression of a negative regulator of the JAK/STAT signal pathway called suppressor of cytokine signalling (SOCS)-3 (35).

Besides evasion of the innate immune responses HCV was shown to interfere with the adaptive immune system. There are studies suggesting HCV inhibits early stages of adaptive immune activation by dendritic cell dysfunction resulting in a reduced ability to stimulate an antigen-specific response represented by functional helper ($CD4^+$) T cells and effector ($CD8^+$) T cells (71). Dendritic cells isolated from the blood of HCV-infected patients have an altered cytokine-secretion profile (308). During HCV infection, DCs induce defective expansion of $CD4^+CD25^+$ regulatory T cells (T_{REG}) which negatively affect activation of other T-cell populations (77).

B-cell-mediated humoral responses are also inhibited by HCV. Humoral immune responses through antibody production are important for virus clearance. Antibodies bind to virus particles inducing neutralization and opsonization, followed by degradation of viral antigens. HCV infection is associated with ineffective humoral responses. HCV envelope proteins, especially E2, have high rate of mutation within the HVRs (233). This results in production of HCV variants that impair the recognition and binding of neutralizing antibodies. Glycosylation on E2 impairs antibody-mediated inhibition of viral attachment to surface receptors and allows viral entry (127, 233).

A robust intrahepatic activation of $CD4^+$ and $CD8^+$ T cells is required to achieve effective control of the acute HCV infection (301). HCV core protein binds with the gC1q receptor on dendritic cells and results in low levels of IL-12 important for TH1 differentiation (301, 318). Mutations of NS3 epitope that promotes a TH1-like immune response may generate escape variants with important consequences for T cell recognition (322). HCV infection is also associated with dysfunctional $CD8^+$ T cells. $CD8^+$ T cells have diminished capacity for proliferation, poor peptide-specific cell-killing ability, decreased secretion of $IFN\gamma$ and tumor necrosis factor, reduced perforin and granzyme A production,

and low expression levels of Fas (CD95) (184, 237, 331) . Genetic mutation may result in ineffective HCV-specific CD8⁺ T cells (223, 275) through weak T-cell recognition of epitopes, altered binding of viral peptide to MHC molecules or dysfunction in antigen processing.

1.14 Hypothesis and Objectives

Hypothesis: The structural proteins of the Hepatitis C virus (core, E1 and E2) induce pathological changes in the liver due to alteration of hepatocyte metabolism. A direct virus-mediated cytopathic effect, through overproduction of reactive oxygen species, results in progression of hepatic injury in chronic hepatitis C infection. The liver damage is accelerated by alcohol intake. The ultrastructural alteration of mitochondria seen in the HCV genotype 1a transgenic mouse model is further augmented by ethanol treatment and represents the anatomical impact of a larger functional impairment of gene expression and oxidative phosphorylation, resulting in increased lipoperoxidation, fibrosis and tumor development. The structural proteins of HCV (core, E1 and E2) are important for establishing persistent infections by inducing deficient immune response through impaired antigen presentation and reduced T cell priming.

AIM 1: The purpose of this study was to determine how the presence of alcohol affects the dynamic structural and functional properties of HCV-core/E1/E2 transgenic hepatocytes.

Rationale: by analysing this HCV-transgenic mouse model which expresses the structural proteins core, E1 and E2 of HCV genotype 1a, I will learn more about the mechanisms of disease production of this important human pathogen. Electron microscopy analysis of HCV-transgenic hepatocytes revealed abnormalities of the structure and morphology of mitochondria (216). Alcohol treatment may induce additional changes in

mitochondria through several alternate mechanisms: 1) direct effects of viral protein on mitochondria through upregulation of HCV RNA/protein levels; 2) inducing intracellular oxidative stress causing secondary mitochondrial dysfunction, possibly by activating peroxisome-proliferator-activated receptor α ; 3) other unknown mechanisms. This analysis has the potential to assist in the design of novel therapeutic strategies to control this infection.

Specific objectives:

1. To identify histological liver changes in ethanol-fed HCV-transgenic mice
2. To investigate whether alcohol affects HCV RNA and protein expression in this mouse model
3. To determine molecular mechanism(s) by which alcohol and HCV act synergistically to promote chronic, progressive liver damage

AIM 2: To study the effects of HCV structural genes on dendritic cell functions, such as cytokine expression and antigen presentation, as potential mechanisms of HCV escape from immune surveillance.

Rationale: earlier studies on the adaptive cellular immune response to hepatitis C virus established the predominance of cytotoxic T-cell responses important for clearing infection (143). Recent studies (108, 218) re-emphasize the role of CD4⁺ T cells, showing recurring HCV viraemia after apparent viral clearance from the circulation, this being temporally related to a loss of detectable CD4⁺ T-cell responses. The reasons for the loss of CD4⁺ T-cell responsiveness need to be further analyzed, but one possible mechanism may be the deficit in T cell priming induced by potential impairment of dendritic cell function.

Specific objectives:

4. To investigate dendritic cell maturation in the presence of structural proteins of HCV
5. To investigate dendritic cell function by analyzing their cytokine expression and ability to activate T-cell lymphocytes.

Chapter 2:

Materials and methods:

2.1 Mice

The B6C3F1 mice used in this study were purchased from Charles River Laboratories (Wilmington MA, USA). The wild-type and the HCV transgenic mice generated in our laboratory (216) were bred and maintained in a pathogen-free state at the Animal Care housing facility of the Faculty of Medicine at the University of Ottawa. All procedures were approved and in accordance with the guidelines of the Animal Care Committee of the University of Ottawa.

HCV-transgenic and non-transgenic littermates were divided into 4 groups: alcohol-fed transgenic mice, transgenic mice and the control groups, alcohol-fed non-transgenic and non-transgenic littermates, each group being represented by 8 mice. For the ethanol loading, 6-month old mice were given water ad libitum containing 4% ethanol. After 5 weeks, the mice were sacrificed by cervical dislocation and the liver tissues were collected for histology, lipid peroxidation product quantification and RNA extraction.

Bone-marrow donor mice used for dendritic cell generation and dendritic cell immunizations were purchased at 6-8 weeks old from Charles River Laboratories.

Body weight measurements were taken pre (T_0)- and post ($T_{5\text{weeks-2days}}$)-alcohol treatment. Blood was also collected by saphenous vein puncture from alcohol-fed transgenic mice, transgenic mice and the control groups, alcohol-fed non-transgenic and non-transgenic littermates at $T_{5\text{weeks-2days}}$.

2.2 Mouse Genotyping

Total DNA was extracted from the tail biopsies of the pups using the DNeasy Tissue Kit (Qiagen, Mississauga, Ontario) following the manufacturer's protocol. Briefly, mouse tail biopsies were performed by cutting a segment of 0.4 cm from the tail tip of each 3-5 week-old earmarked mouse. The tail samples were lysed by Proteinase K to obtain the DNA. The DNA was isolated by binding to the membrane of DNeasy mini spin column and eluted as purified DNA. The absorbance of all samples was read at 260 nm with the Biorad Microplate Reader 3550-UV (Cortland, New-York, USA) to quantify the amount of DNA following the extraction.

The 50 µl PCR final volume contained 5µl 10X PCR buffer, 5 µl 25mM MgCl₂, 5 µl 10mM dNTP (Invitrogen, Ontario, Canada), 2 ul of 10mM forward primer (CE1E2F1, 5' ACC ATG AGC ACG AAT CCT AAA CCT C 3') and reverse primer (CE1E2R2, 5' AGT GAG CAC CAG CGT TCA TGT CCA AG 3') mix (University of Ottawa Sequencing Facility, Ontario, Canada or Operon Biotechnologies Inc., Alabama, USA), 2.5U of Taq DNA polymerase(Invitrogen (Ontario, Canada), 2-10 µl of the quantified DNA sample.

The PCR was carried out in the GeneAmp PCR System 2700 (Applied Biosystems, Singapore) using the following cycles: 1 cycle of DNA denaturation at 94°C for 5 min; 40 cycles consisting of DNA denaturation at 94°C for 45 s, primer annealing at 55°C for 45 s, and elongation at 72°C for 1 min; the final step of elongation was done at 72°C for 10 minutes followed by holding of the samples at 4°C. The PCR products were analyzed immediately on a 2% agarose gel containing ethidium bromide in 0.5X TBE or stored at -20°C until further use.

2.3 Detection of serum transaminases

Liver cytotoxicity induced by HCV and alcohol was detected by measuring the level of serum transaminases: alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The testing was done using VITROS ALT/AST slides (Ortho-Clinical Diagnostics, Rochester, NY, USA).

For detection of ALT enzyme activity, a drop of unhemolyzed serum was deposited on a slide containing ALT substrates L-alanine and sodium α -ketoglutarate. In the presence of the enzyme, the amino group of the alanine was transferred to α -ketoglutarate producing pyruvate and glutamate. Lactate dehydrogenase further catalyzed the conversion of pyruvate and NADH to lactate and NAD⁺. The rate of change in reflectance density given by oxidation of NADH was proportional with enzyme activity. For AST detection, the amino group of L-aspartate was transferred to α -ketoglutarate in the presence of pyridoxal-5-phosphate producing glutamate and oxaloacetate. The oxaloacetate decarboxylase catalyzed the conversion of oxaloacetate to pyruvate and carbon dioxide. Pyruvate was further oxidized to acetylphosphate and hydrogen peroxide by pyruvate oxidase. The final step of the reaction was represented by peroxidase-catalyzed oxidation of a leuco dye to produce a colored dye. The rate of change in reflectance density given by the oxidation of leuco dye was proportional with enzyme activity.

2.4 Protein Purification

Chinese hamster ovary cells (CHO-K1) were transfected by electroporation with pEF6/Myc-His vector containing core/E1/E2 inserts in order to develop stable cell lines expressing the insert. pEF6/Myc-His vector containing core/E1/E2 inserts, designed for over-

production of recombinant proteins in mammalian cells, was obtained by subcloning TOPO-TA HCV CE1E2 construct into a pEF6/Myc/His vector. The six histidine residues tagging the C terminal of encoded proteins make them suitable for purification by immobilized metal affinity chromatography (Clontech Talon Metal Affinity Resin Kit, Palo Alto, CA). Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (Life Technologies Laboratories, Grand Island, NY) and blasticidin (Invitrogen, Burlington, ON), and maintained at 37°C, 5%CO₂ until they were confluent (usually 3 days). For protein purification purposes, large quantities of core/E1/E2 expressing CHO cells were grown. The cells were harvested using 1X trypsin (Sigma-Aldrich, St. Missouri, USA) for expansion and cell scrapers for protein purification. The Talon Resin Protein Purification manufacturer's protocol was followed in order to obtain purified proteins, namely core/E1/E2 polyprotein. To summarize, each CHO cell pellet was lysed with 10 ml of lysis buffer (25mM Tris base, 1% Triton-X100, 2.5mmol/L 2-mercaptoethanol and 1 EDTA free protease inhibitor tablet (Roche Diagnostics, Indianapolis, USA), mixed by vortexing, and incubated for 30 minutes at room temperature. The cellular suspension was then sonicated 3 times for 10 second bursts with a 1 minute break on ice in between each sonication. The resulting cellular lysate was centrifuged at 10,000 rpm for 30 minutes at 4°C in a 50 ml polycarbonate tube in order to eliminate cellular debris. The supernatant containing the cell lysate was then transferred to a 15 ml Falcon tube containing washed Talon Superflow Metal Affinity Resin (BD Biosciences Clontech, California, USA). The His-tagged cellular proteins were absorbed to the resin by 1hour rotation at 4°C. After this incubation, the protein-resin mixture was centrifuged at 2400 rpm for 5 minutes, washed twice with 10 ml of 1X Equilibration /Wash buffer (5X Equilibration/Wash buffer: 250 mM sodium phosphate and 1.5 M sodium chloride), resuspended in 5-6 ml of 1X Equilibration

/Wash buffer, and transferred to a Talon Disposable Gravity Column (Clontech, California, USA). The protein-bound resin was allowed to settle in the column, and rinsed twice with 5 ml of 1X Equilibration /Wash buffer. The purified protein was collected in fractions of 300 μ l by adding 1X Elution buffer (150 mM Imidazole, 50 mM sodium phosphate and 300 mM sodium chloride). The protein quantification of each fraction was conducted using the Biorad Protein Assay protocol described below.

Other types of samples, namely tissue lysates, were also subject to protein quantification. Liver lysates were obtained by grinding liver tissue using mortar and pestle in the presence of RLT buffer (RNeasy Mini Kit, Qiagen). These samples were centrifuged for 14000 rpm for 10min at 4⁰C. The supernatants were analysed for total protein content, and equal amounts of total protein from each sample were further subject to immunoprecipitation followed by SDS-Page and Western blot analysis.

2.5 Determination of Protein Concentration

Protein concentration was determined using a Bio-Rad protein assay. Standards were used as point of comparison for the protein concentration of the samples. They were 2 fold-dilutions of 100 μ g/ml bovine serum albumin (BSA) in PBS. An amount of 0.4 ml of each standard was combined with 0.1 ml of the Coomassie Brilliant Blue G-250 dye. This protocol is a dye-binding assay in which a differential colour change occurs in the presence of various concentrations of protein. Next, 400 μ l of different dilutions of each sample (fractions obtained from the above protein purification) were mixed with 100 μ l dye. These mixtures were incubated at room temperature for 5 minutes and then 200 μ l of each standard and sample were transferred in duplicates to a 96-well plate (Nunc, Roskilde, Denmark). The absorbance of the duplicates was then read at 595 nm on the Bio-Rad Microplate Reader

3550-UV (Cortland, New-York, USA) to indicate the concentration of protein contained in each fraction. The identity of the purified protein was further verified by Western Blot analysis.

2.6 Immunoprecipitation

For immunoprecipitation, 250 ug protein lysate from core/E1/E2 expressing CHO cell lines or 150 ug liver lysate was incubated for 1hour at room temperature on a shaker in the presence of 1ug mouse monoclonal antibody to HCV-core protein (Biodesign, UK) and 1ug rabbit polyclonal antibody to β -actin (Abcam, Cambridge, US). 20 ul of Dynabeads ProteinG (Invitrogen, US) was further added to each lysate and samples were incubated over night at 4⁰C on a shaker to allow protein G-IgG-protein complex formation. The mixtures were washed 3 times with lysis buffer containing protease inhibitors. After the last centrifugation (8000 rpm at 4⁰C for 10 min) the supernatants were removed, and the proteins were separated from the protein-G beads by boiling for 5 min at 95-100⁰C in the presence of 15 ul protein loading buffer. After 30sec centrifugation at 12000 rpm the supernatants were analyzed by SDS-PAGE gel followed by Western blot analysis.

2.7 Western Blot Analysis

Briefly, proteins were previously purified using the Clontech Protein Purification Protocol and then subjected to immunoprecipitation. Protein samples were denatured by heating for 5 minutes at 100⁰C in SDS sample loading buffer, separated by electrophoresis on 10% SDS-PAGE gels and transferred to PVDF membranes (BioRad, US). The membranes were incubated overnight on a shaker at 4⁰C in blocking buffer (5% BSA in TBST) with the respective primary antibodies. The primary antibodies used were as follows: mouse

monoclonal anti-HCV core (1:1000) (Biodesign, UK) for core protein detection in liver lysates, and human patient heat inactivated serum (1:1000) for CE1E2 polyprotein detection in CHO cell lysates. After washing 3 times with TBST for 10 minutes on a shaker, the secondary antibodies were added for 1 hour at room temperature in the blocking buffer. The secondary antibodies used were as follows: goat anti-mouse (1:5000) for core detection (BioRad), and horseradish peroxidase conjugated goat anti-human (1:5000) for CE1E2 detection (BioRad). To control for protein loading, the membranes were stripped and reprobed with antibodies directed against beta actin. The immunoblots were visualized by ECL (Amersham Biosciences). The ladder used was precision plus protein dual color standards (BioRad, Mississauga, ON). The computer software used for analysis was GeneSnap version 6.05.

2.8 Immunohistochemistry using Immunoperoxidase staining

To observe the effect of alcohol on HCV core protein levels in transgenic livers, 5 μ m liver sections fixed with 4% paraformaldehyde and embedded in paraffin were deparaffinated using toluene and absolute alcohol. After an extensive wash, the endogenous peroxidase activity was blocked by treatment with 3% H_2O_2 , followed by blocking of unspecific binding with BSA and normal swine serum. For HCV core protein detection, primary antibodies represented by mouse monoclonal anti-core (1:500) (Biodesign, UK) were used in combination with universal biotinylated anti-mouse secondary antibodies and avidin D, followed by diaminobenzidine (DAB) development and hematoxylin and eosin (H&E) counterstaining.

2.9 Real-time RT-PCR

To elucidate the effect of alcohol on HCV RNA levels in transgenic livers, total RNA was extracted from the liver of each mouse using RNeasy Mini Kit (Qiagen, Mississauga, ON). Briefly, 20mg frozen liver was disrupted using mortar and pestle in ice cold RLT buffer, followed by homogenization with a QIAshredder homogenizer. After centrifugation, the flow-through of the resulting clear lysate was ethanol treated and transferred to the RNeasy spin column. The total RNA bound to the column was washed a few times using RW1 and RDE buffers, and eluted using RNase-free water. The resulting total RNA was the template for reverse transcription with Murine Leukemia virus (Mul.V) reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and random hexamers (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Briefly, reverse transcription was performed for 60 minutes at 37⁰C using 1µg total RNA in a 20µl reaction, followed by 15 min at 72⁰C to stop the reaction. HCV-specific sequences in the reverse-transcription product, cDNA, was used as template for Real time PCR amplification, performed in 36-well microtiter plates (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA) with an iCycler (Bio-Rad, Mississauga, ON). The primers used to amplify the HCV-core gene were 5'ACC ATG AGC AAT CCT AAA CCT 3' (forward) and 5' GCA ACA AGT AAA CTC CAC CAA CGA 3' (reverse), and the fluorescent signals were generated using the Quantitec SYBR Green PCR kit (Qiagen, Mississauga, ON). The internal control used was Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with the following sense and antisense primers: 5'ATG TGT CCG TCG TGG ATC TGA 3' and 5' TTG AAG TCG CAG GAG ACA ACT 3'. The amplification protocol included 15 min at 95⁰C, and 40 cycles of 15 sec at 94⁰C, 30 sec at 66⁰C, and 30sec at 72⁰C. All experiments were run in triplicate,

and negative controls (no cDNA added, and non-reverse-transcribed RNA) were used each time. The iCycler software detected the threshold cycle (CT) for each amplicon. Normalization was performed using $2^{-\Delta\Delta CT}$ method (31).

Microarray technology was used previously in the lab (Naas et al., manuscript in preparation) to determine which transcripts were differentially expressed in the transgenic mice compared with the normal littermates. From a total number of 15,297 genes analysed, six were found to be up/downregulated. Some of these genes are involved in β -oxidation and lipid biosynthesis (Apolipoprotein A IV (Apo A IV), Acetyl-Coenzyme A acyltransferase (Acaa), Acyl-Coenzyme A oxidase (AOX)) (previous work). I analysed the expression of these genes in the ethanol-fed transgenic mice. RNA extraction and real-time RT-PCR techniques were previously described. The following differentially expressed genes were analyzed using oligonucleotide primers (300 nM): AOX (Acyl-CoA oxidase) sense 5' GAA CTC CAG ATA ATT GGC ACC TA 3' and antisense 5' AGT GGT TTC CAA GCC TCG AA 3', and Apo A-4 (apolipoprotein A-IV) sense 5' CTG GTG GCC ATC ACC GGC AC 3' and antisense 5' AGT CCT GGA AGA GGG TAC TGA GC 3'.

2.10 Histological staining

Liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin. H&E and osmium stainings were performed on 5 μ m paraffin-embedded sections, according to standard methods used by the Faculty of Medicine, University of Ottawa. Histopathological morphology of the liver tissues was performed and interpreted in collaboration with Dr. Rudy Muller at the Department of Pathology and Laboratory Medicine, University of Ottawa. A semi-quantitative method was used to quantify steatosis in analysed tissue samples. 1+ was assigned to samples with lipid droplets present in sporadic hepatocytes, 2+

for moderate steatosis represented by hepatocytes containing lipid droplets present in 2 or more of 5 fields analysed, 3+ for moderate to severe steatosis represented by lipid droplets present in more than 50% of the analysed fields, and 4+ for very severe steatosis, in which lipid accumulation was observed everywhere in the analysed liver tissue.

2.11 Electron Microscopy

The electron microscopy study was done according to the standard procedures at the electron microscopy facility at the Children's Hospital of Eastern Ontario in Ottawa. Approximately 0.25 mm thick liver tissue slices were briefly post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer dehydrated in graded ethanol and embedded in Spur epoxy resin. Ultrathin 60-70 nm sections were cut using a Leica Ultracut R ultra-microtome, mounted on 200 mesh copper grids and stained with 10% uranyl acetate in 50% methanol, followed by Reynold's lead citrate. Grids were examined and micro-graphed using a JEOL 1010 transmission electron microscope at 60 KV. Liver tissues of alcohol-treated core/E1/E2 transgenic mice and transgenic mice on a normal diet were analysed.

2.12 Measurement of lipid peroxidation (LPO) products

LPO products were measured in livers from core/E1/E2 transgenic and B6C3F1, wild type (WT) alcohol-fed mice with the LPO-586 assay kit (Oxis International, Portland, OR). Livers were isolated, disrupted using mortar and pestle with ice cold buffer containing PBS and 5 mM butylated hydroxytoluene, and centrifuged at 5,000g for 20 min at 4°C. Supernatants were collected and the malonaldehyde content, which is the main product of lipid peroxidation, was measured colorimetrically following the manufacturer's procedures.

2.13 Measurement of anti-oxidant reserve

The GPx (Glutathione Peroxidase) activity is a useful method to monitor the effectiveness of antioxidant intervention strategies, and indirectly to measure oxidative stress. Oxidative stress in mouse livers was determined using a GPx 340™ assay kit (Oxis International, Portland, OR). Livers were isolated, homogenized using Tris HCl buffer, 5mM EDTA, and 1 mM 2-mercaptoethanol, and centrifuged at 5,000g for 20min at 4°C. Supernatants were collected and the GPx activity was detected as a change in absorbance at 340 nm as NADPH is oxidized to NADP⁺ providing a spectrophotometric means for monitoring GPx enzyme activity.

2.14 Generation of bone marrow-derived dendritic cells

B6C3F1 mice were anesthetised by injecting 0.2 ml of Somnotal (MTC Pharmaceuticals, Cambridge, ON, Canada) into the intra-peritoneal cavity. Fresh murine wild type bone-marrow cells were isolated from femurs and tibias of donor mice. The bones were removed from each mouse using dissection scissors. The muscles were cut away to expose the bones. The bones were removed by cutting above and below the joints to leave as much of the epiphysis intact as possible. The femurs and tibias were submerged for 5 minutes in a dish containing 70% ethanol for sterilization. After sterilization the bones were transferred to a culture dish containing PBS. Using sterile tweezers and scissors, each epiphysis (ends of the bone) were cut off to expose the bone marrow, which is the bright red center of the bone. Needles (30 gauge) attached to a sterile syringe were inserted into one side of the bone to wash bone marrow with 0.5-1 mL of sterile PBS. The bone marrow cells were washed and the red blood cells were lysed using NH₄Cl-containing buffer. After

centrifugation at 1600 rpm for 5 minutes at 4⁰C, the cells were resuspended at 2x10⁶/ml in RPMI 1640 medium (Wisent Bioproducts, St-Bruno, QC, Canada) supplemented with 10% fetal bovine serum and 100U/ml penicillin and 100ug/ml gentamicin (complete RPMI) and cultured in tissue culture grade sterile 6-well plates. After 18-24 hours of culture at 37⁰C, in an atmosphere containing 5% CO₂, the non-adherent cells were transferred to fresh complete RPMI containing 10ng/ml GM-CSF (Biosource International, Camarillo, CA, USA) and 10ng/ml IL4 (Biosource International, Camarillo, CA, USA) and cultured for another 96 hours at 37⁰C, 5%CO₂ (half the media was changed at 48 hours). On the 5th day of culture the immature dendritic cells were loaded with Core/E1/E2 purified polyprotein and/or HIV-Gp120 (addition of 10ug/ml total protein in the culture media). Dendritic cell maturation was induced by adding 200 ng/ml TNF α (Biosource International, Camarillo, CA, USA) on the 6th day of culture. The bone marrow derived mature dendritic cells were harvested on the 7th day of culture and used (after Flow Cytometry characterization) in CD4+ T cell proliferation assays.

To determine whether loading of BM-derived DCs with core/E1/E2 diminishes responses of dendritic cells to lipopolysaccharide (LPS) activation compared to other antigens (for example HIV-Gp120), BM-derived DCs were cultured on the 6th day in the presence of 0.1ug/ml LPS. On the 7th day DC's culture supernatants were tested using the RayBio mouse cytokine antibody array to identify the cytokine expression. The viability of the dendritic cells was also determined by Trypan Blue staining.

2.15 Mouse Cytokine Antibody Assay

The RayBio mouse cytokine antibody array (RayBiotech, Norcross, GA, USA) was used for each culture supernatant of BM-derived DC culture in the presence or absence of core/E1/E2 polyprotein, HIV-Gp120 and LPS according to the manufacturer's instructions. Briefly, cytokine array membranes were blocked in 2 ml of 1x blocking buffer for 30 min and then incubated with 1 ml of samples overnight at 4⁰C on a shaker. Samples were then decanted from each container, and the membranes were washed three times with 2 ml of 1x wash buffer I, followed by two washes with 2 ml of 1x wash buffer II at room temperature with shaking. Membranes were then incubated in 1:250-diluted biotin-conjugated primary antibodies at room temperature for 2 hours and washed as described above before incubation in 1:1000-diluted horseradish peroxidase-conjugated streptavidin. After incubation in horseradish peroxidase-conjugated streptavidin for 2 hours, membranes were washed thoroughly and exposed to a peroxidase substrate (detection buffers C and D; RayBiotech, Inc.) for 5 min in the dark before imaging. The signal intensities were quantified by densitometry using a Bio-Rad VersaDoc Imaging System 3000 and analyzed with Quantity One software (Bio-Rad). Biotin-conjugated immunoglobulin G served as a positive control at six spots, where it was used to identify membrane orientation and to normalize the results across different membranes. For each spot, the net optical density level was determined by subtracting the background optical level from the total raw optical density level.

2.16 Flow Cytometry

For characterization of dendritic cell culture, flow cytometry analysis was conducted for each 7 day-BM-derived DC culture. In summary, 200 μ l aliquots of DC cultures, containing $0.3\text{--}0.5 \times 10^6$ cells, were washed with 3 ml of PBS/0.1% azide/5% FCS and centrifuged at 1600 rpm at 4°C for 5 minutes. The cell pellets were stained with fluorescein isothiocyanate-labeled (FitC) mouse monoclonal anti-MHC class II antibody and phycoerythrin-labeled (PE) monoclonal anti-mouse B7-2/CD86 (BD Biosciences, Mississauga, ON) at 2 μ l per $0.3\text{--}0.5 \times 10^6$ cells. The tubes were vortexed to ensure proper mixing of the antibodies with the cells, and incubated for 20 minutes at room temperature in the dark. Following washing in 3 ml of PBS/0.1% azide/5% FCS the antibody-cell complexes were fixed with 2% paraformaldehyde, and analyzed using a BD FACS Canto Flow Cytometer (BD Biosciences, California, USA). The gating strategy was to first gate on the dendritic cell population as defined by forward and side scatter characteristics, followed by quantification of the double-positive MHCII⁺/CD86⁺ cells. Surface expression of the molecules was analyzed with FCS Express V3. Marker expression was evaluated as the percentage of double-positive cells in the DC gated population.

2.17 Allogeneic Lymphocyte Proliferation Assay

250-300 μ l of blood was collected in ammonium heparinized capillary tubes from the saphenous vein of BMdDC immunized B6C3F1 mice. The blood was incubated for 5 minutes at room temperature in the presence of lysis buffer (155 mM NH_4Cl , 10 mM KHCO_3 and 0.1 mM EDTA) for red blood cell depletion. The cellular suspension was washed twice with PBS/0.1% BSA and viable cells were counted using Trypan Blue staining.

HCV-induced CD4⁺ T cell proliferation was assessed by labeling PBMCs with carboxyfluorescein diacetate succinimidyl ester (CFSE) dye. CFSE is known to passively diffuse into cells where the succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are well retained. The dye-protein complexes formed in the labeled cells are retained by the cells throughout development and meiosis, and is inherited by daughter cells (half the fluorescence of the parent cells) after each cell division and not transferred to adjacent cells in a population. For CFSE labelling, a 10 mM CFSE stock solution was prepared by adding 90 μ l of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Oakville, ON, Canada) to 500 μ g lyophilized CFSE (Molecular Probes, Oregon USA). The stock solution was then diluted in sterile PBS/0.1% BSA to obtain a working concentration of 10 μ M.

An equal volume of 10 μ M CFSE dye solution was added to $2-3 \times 10^6$ lymphocytes suspended in PBS/0.1% BSA. These cells were then vortexed and incubated at 37°C for 15 minutes. The staining was quenched by the addition of 5 volumes of ice-cold RPMI 1640 (Gibco, Invitrogen Corporation, Grand Island NY, USA), supplemented with 5% fetal calf serum (FCS) (Wisent Bioproducts, St-Bruno, QC, Canada) and 1% penicillin/gentamicin (Sigma-Aldrich, Oakville, ON) followed by a 5 minute incubation of the cellular suspensions on ice. Cells were then centrifuged at 1600 rpm for 5 minutes at 4°C with the removal of the supernatant, washed 2 more times with warm medium and resuspended at 2×10^6 cells/ml.

For the CD4⁺ T cell proliferation assay, the CFSE-stained PBMCs were derived from B6C3F1 mice injected with unloaded BMdDC. 2×10^5 irradiated (30Rad) BMdDCs were adoptively transferred subcutaneously to each mouse, four times at one month intervals, for breaking tolerance. PBMCs from injected mice were isolated 10 days post last injection and cultured in the presence of unloaded or HCV core/E1/E2 or HIV Gp120 loaded BMdDCs, at

a ratio of 2 PBMCs to 1 BMdDC. Each CFSE-labelled cell suspension was split into two 4 ml round bottom flow tubes according to the following conditions: the first one contained unloaded BMdDCs and the second one contained HCV core/E1/E2 loaded DC (dendritic cells were previously loaded with 10 µg/ml core/E1/E2 polyprotein) or HIV Gp120 loaded DCs (loaded with the same concentration of HIV protein - 10 µg/ml). The cell suspensions were mixed and incubated in the dark (due to the presence of the CFSE dye) at 37°C in 5% CO₂ for 5 days.

2.18 Cell Surface Staining of CD4⁺ T cells for Flow Cytometry detection

Following the 5 day incubation period, each cell culture tube was washed with 3 ml of PBS/0.1% azide/5% FCS and centrifuged at 1600 rpm at 4°C for 5 minutes. The supernatant was removed and the cells were surface stained with the appropriate surface antibodies. Antibodies used were Tri-color (TC)-labeled rat monoclonal anti-mouse CD3 and phycoerythrin-labeled (PE) rat monoclonal antimouse CD4 (Caltag Laboratories, Burlingame, CA) 1 µl per 0.3-0.5x10⁶ cells. The tubes were mixed by vortexing and incubated for 20 minutes at room temperature in the dark. After surface staining, the cells were washed again with 3 ml of PBS/0.1% azide/5% FCS to remove excess fluorescence-labelled antibodies, centrifuged at 1600 rpm at 4°C for 5 minutes to remove the supernatant, fixed with 2% paraformaldehyde, and analyzed on a BD FACS Canto Flow Cytometer (BD Biosciences, California, USA). The gating strategy was to first gate on the lymphocyte population as defined by forward and side scatter characteristics, and the number of events obtained for each sample was at least 10 000 events, then to gate on CD3⁺ cells, and lastly on CD4⁺/CFSE⁺ cells. Surface expression of the molecules was analyzed with FCS Express

V3. Marker expression was evaluated as the percentage of double-positive cells in the gated population.

2.19 Immunofluorescence analysis (IFA)

For IFA of cells in suspension, the cells were pelleted and resuspended in 75ul PBS. Each 75ul cell pellet was added to one polylysine-coated microscope slide. Cells were immediately fixed by adding cold methanol for 5 minutes followed by 5 minutes incubation with cold acetone. Following fixation, the slides were washed 3 times for 10 minutes each in PBS, and then incubated overnight at 4⁰C. To perform IFA, the fixed loaded and unloaded BM-derived DCs were incubated for 1 hour at room temperature on a shaker with blocking buffer containing 5% normal goat serum, and 0.1% Triton x-100 in PBS. Rabbit anti-core/E1/E2 polyclonal antibody (prepared in Dr. Diaz-Mitoma's laboratory according to the University of Ottawa Animal Care Facility protocols for antibody production) was added to the slides as primary antibody, and incubated with the cells at a dilution of 1:50 for an hour at room temperature, followed by a one-hour incubation with FitC-labelled anti-rabbit IgG secondary antibodies (Sigma, Oakville, ON) at 1:200 dilution. After mounting the slides using Vectashild mounting media (Vector, Burlingame, CA), the cells were examined with a fluorescence microscope.

2.20 Statistical Analysis

All statistical analyses were performed using Student's t test. A value of $p < 0.05$ was regarded as statistically significant.

Chapter 3:

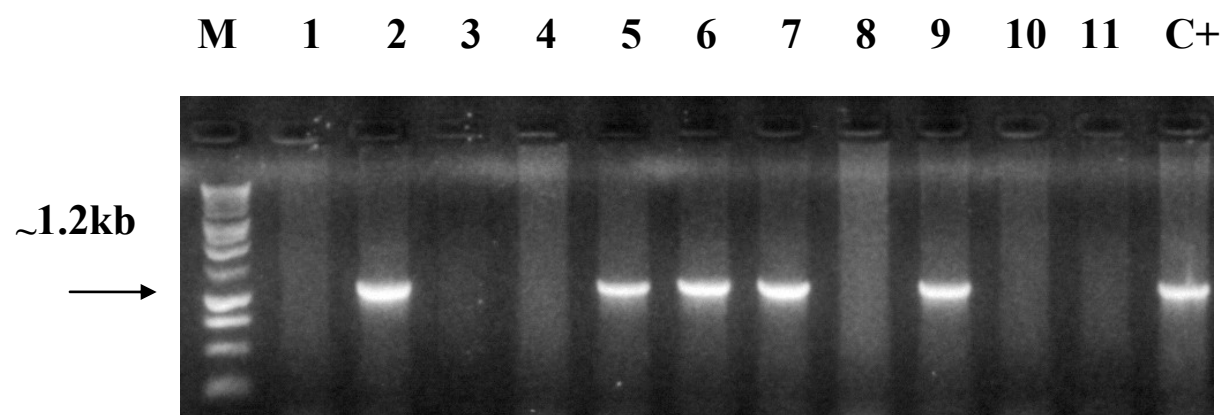
**Characterization of liver damage mechanisms induced by alcohol and
Hepatitis C virus**

3.1 Establishment of the ethanol-treated HCV-transgenic mouse model

The HCV transgenic mice were bred in the Animal Care Facility at the University of Ottawa to maintain the core/E1/E2 transgenic colony. Males known to be positive for the transgene were mated with B6C3F1 wild type females in order to maintain a heterozygous population. With each new generation, pups were screened for the presence of the transgene by PCR using genomic DNA extracted from the tails of the mice: a band present at 1.2 kb indicated the presence of the transgene (Figure 3.1). During breeding, a predominance of transgene expression and liver histological abnormalities in male mice was observed. Interestingly, this is representative of human HCV infection since males are more frequently infected than females.

The presence of HCV RNA and proteins were detected in transgenic mouse model tissues, such as liver, kidney, and spleen. The highest HCV RNA and viral protein expression was found in the liver, followed by kidney and spleen, and the levels increased in the liver with age. Histological analysis of liver cells demonstrated steatosis in transgenic mice older than 3 months, which progressed with age. To determine the effects of alcohol on liver disease, HCV-transgenic and non-transgenic littermates were divided into 4 groups (Table 3.1): ethanol-fed transgenic mice, transgenic mice on a normal diet, ethanol-fed non-transgenic and non-transgenic littermates on normal diet, each group being represented by 8 mice. For the ethanol treatment, 6-month old mice were given water ad libitum containing 4% ethanol. Serum transaminase measurements were used to evaluate disease progression 2 days prior to euthanasia. After 5 weeks, the mice were sacrificed by cervical dislocation and the liver tissues were collected for histopathology, measurement of HCV RNA and viral protein expression and detection of lipid peroxidation products.

Figure 3.1 Confirmation of the presence of HCV core/E1/E2 transgene in the mouse colony. HCV transgenic mice were heterogenic for the transgene and were generated by breeding of founder core/E1/E2 positive mice with B6C3F1 mice. DNA was extracted from mouse tail biopsies and a polymerase chain reaction was carried out using transgene-specific primers. The presence of the core/E1/E2 transgene fragment in the PCR product was confirmed by gel electrophoresis. The mice that carry the transgene have a 1.2 kb band in their genomic DNA (lanes 2, 5, 6, 7, 9), indicating that they have successfully integrated the transgene. This band is absent in mice that are negative for this transgene (lanes 1, 3, 4, 8, 10, 11). The positive control(C+) used in the assay was a core/E1/E2 transgenic positive mouse, which was previously confirmed as transgenic by PCR-Gel electrophoresis. M represents the 1 kb DNA ladder used for this experiment.



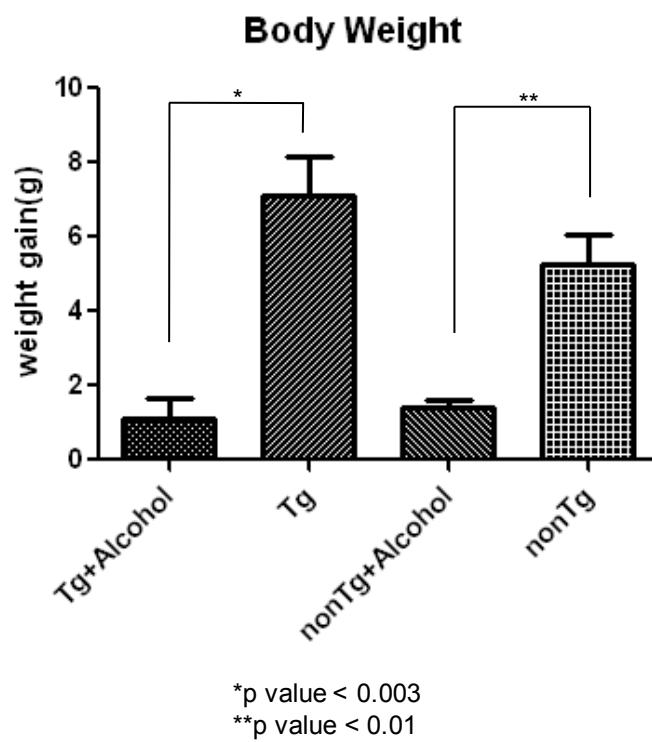
	Mouse Groups			
	1	2	3	4
Presence of Core/E1/E2 Transgene	Transgenic+	Transgenic+	Transgenic-	Transgenic-
Ethanol Treatment	+ ethanol	-	+ ethanol	-
Body weight measurements (at T ₀ and T _{5weeks-2days})	✓	✓	✓	✓
Blood Collection for liver enzyme measurements (at T _{5weeks-2days})	✓	✓	✓	✓
Liver tissue collection (at T _{5weeks})	✓	✓	✓	✓

Table 3.1 Experimental design for the ethanol treatment (n=8). 6-month old HCV-transgenic and non-transgenic littermates were divided into 4 groups: ethanol-fed transgenic mice, transgenic mice and the control groups, ethanol-fed non-transgenic and non-transgenic littermates. For the ethanol treatment, water containing 4% ethanol was given ad libitum. After 5 weeks, the mice were sacrificed by cervical dislocation and blood was collected by saphenous vein puncture. The liver tissues were collected for histology, lipid peroxidation product quantification and RNA extraction.

The weight of the animals was recorded before starting the ethanol treatment at T_0 , and 2 days before the end of the 5-week ethanol treatment ($T_{5\text{week}-2\text{days}}$). During the 5 week experiment, the body weight of the mice is expected to increase slowly. In contrast, the ethanol treatment induced a significant decrease of body weight gain (percentage weight gain) in both transgenic and non-transgenic littermates (Figure 3.2).

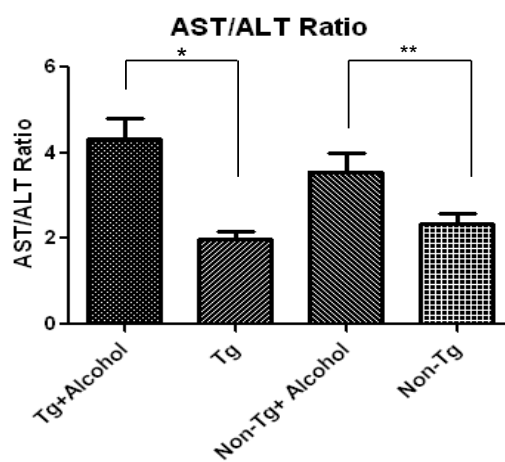
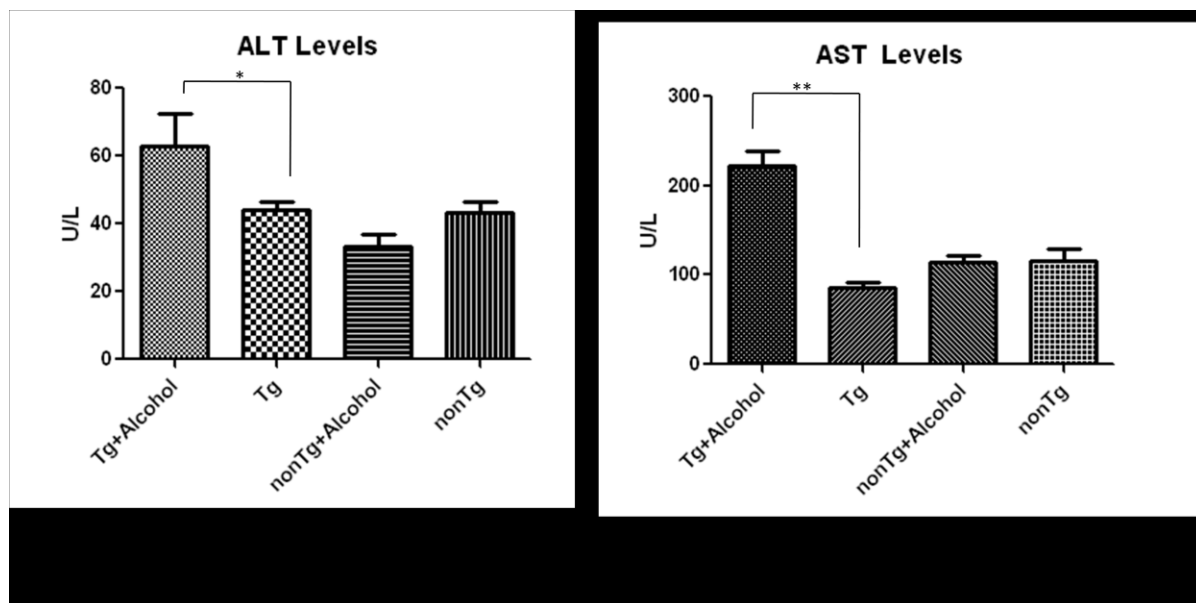
The enzymes ALT and AST are present in high levels in the liver, but their concentrations in serum increase when there is destruction or cytotoxicity of liver cells (hepatocytes). Serum ALT increases rapidly in liver cell necrosis, hepatitis, hepatic cirrhosis, liver tumors and bile duct obstruction, resulting in jaundice. AST serum levels are especially increased in ethanol-induced cirrhosis as well as in viral hepatitis (118,199). In order to examine the cytotoxic effects of ethanol and HCV structural proteins on the transgenic murine liver, the levels of ALT and AST were determined in mouse sera at the end of the ethanol treatment, 2 days prior to euthenisia. There was a statistically significant increase in the levels of serum ALT and AST between the ethanol treated HCV core/E1/E2 transgenic mice and the transgenic mice on the normal diet (* $p < 0.05$ for the ALT values, and $p < 0.0001$ for the AST values). In contrast, neither the ethanol fed nor untreated wild type mice demonstrated increases in liver enzymes (Figure 3.3). The ratios between the AST and ALT values were also calculated, to differentiate nonalcoholic steatohepatitis from alcoholic liver disease. When the ratios are greater than 2.0, it is more likely to be associated with alcoholic hepatitis. There was a significant difference of AST/ALT ratios between the ethanol treated groups and the groups on normal diet (* $p < 0.004$ for the transgenic groups with or without ethanol treatment, and $p < 0.05$ for the non-transgenic groups with or without ethanol treatment). These results demonstrate that expression of the core/E1/E2 viral proteins plus ethanol ingestion has a synergistic cytotoxic effect on the murine liver.

Figure 3.2 Body weight after 5 weeks of ethanol treatment (n=8). The weight of the animals was recorded at T_0 (before starting the ethanol treatment) and 2 days before the end of the 5-week ethanol treatment ($T_{5\text{week-2days}}$). The recorded values are the averages of the differences between weights at T_0 and weights at $T_{5\text{week-2days}}$ plus/minus the standard deviation. The ethanol treatment induced a significant decrease in the amount of weight gained in both transgenic and non-transgenic littermates. The P values between the groups of transgenic animals with or without ethanol were statistically significant (* $P < 0.003$). The age of the mice at $T_0 = 6$ months



	Mouse Groups			
	Transgenic+ + ethanol	Transgenic+	Transgenic- + ethanol	Transgenic-
Body Weight Gain (g)	1.1	7.1	1.4	5.25
Standard Deviation Error	+/- 0.56	+/- 1.06	+/- 0.18	+/- 0.77

Figure 3.3 Liver cytotoxicity induced by ethanol ingestion in mice expressing HCV proteins in the liver as shown by the level of serum transaminases ALT (n=6) and AST (n=6). These enzymes were measured using a Vitros 250 analyzer that employs reflectance spectrophotometry to estimate the level of liver enzymes. The ALT and AST levels are expressed as units per litre (U/L), and the measurements were taken from blood collected 2 days before the end of the 5-week ethanol treatment. Values are presented as the mean with the standard deviation.



*p value < 0.004
**p value < 0.05

3.2 Increased expression level of HCV-core RNA in the ethanol-fed transgenic mice:

Ethanol consumption is known to increase HCV viral loads in patients with chronic hepatitis C (240, 252, 268) and ethanol exposure activates virus replication in HCV replicon systems (191, 278, 306). The effect of ethanol on HCV-core RNA levels in transgenic livers was determined by comparing the HCV-core RNA levels in the ethanol-fed transgenic mice and the transgenic littermates on normal diet (aged 7 months). Real-time RT-PCR was performed using total RNA extracted from mouse livers. The results showed a 14 – fold increase in the expression level of HCV-core RNA in the ethanol-fed transgenic mice compared to the control transgenic group that did not receive ethanol (Table 3.2).

3.3 Changes in Expression of HCV Proteins in Livers of Transgenic Mice after Ethanol Ingestion.

To confirm the higher levels of HCV-core RNA in the ethanol-fed transgenic mice compared with the transgenic littermates on normal diet, the HCV-core protein expression levels were also analysed. The liver lysates obtained by grinding liver tissue using mortar and pestle in the presence of RLT buffer were initially centrifuged and the supernatants were analysed for total protein content by Bradford assay. Equal amounts of total protein (150 µg) from each sample were further subjected to immunoprecipitation (IP) followed by Western blot. Western blot analysis demonstrated that transgenic mice treated with ethanol had a higher liver HCV core protein expression than untreated transgenic littermates (Figure 3.4). Also immunohistochemistry results, using an indirect labelling antibody assay and anti-core monoclonal antibodies, showed higher HCV-core protein expression levels in the ethanol-

	Ethanol-fed Transgenic Mice	Transgenic Mice
Avg. HCV-Core CT	24.67	28.27
Avg. GAPDH CT	13.32	13.07
Δ CT (Avg. HCV Core CT - Avg. GAPDH CT)	11.35	15.2
$\Delta \Delta$ CT (Avg. Δ CT Transgenic+Ethanol - Avg. Δ CT Transgenic)	-3.85	0
Normalized Core Transgenic+Ethanol relative to Transgenic ($2^{-\Delta\Delta CT}$)	14.42	1

Table 3.2 Relative expression of HCV-core RNA in the livers of transgenic mice fed with ethanol, as determined by real-time RT-PCR (n=6). There was a 14-fold increase in the expression of core protein after 5-week ethanol treatment of HCV core/E1/E2 transgenic mice compared with the transgenic mice on normal diet. The first row represents the threshold cycle average where the HCV-core RNA signal was detected. The second row represents the threshold cycle average where the internal gene, GAPDH, RNA signal was detected. The third row is the Δ CT, which is the difference between the threshold cycle average of the HCV-core RNA and the threshold cycle average of the GAPDH RNA. The fourth row is the $\Delta \Delta$ CT, which is the difference between the Δ CT of the HCV core/E1/E2 transgenic group on ethanol treatment and the HCV core/E1/E2 transgenic group on normal diet. The last row ($2^{-\Delta\Delta CT}$) is the normalization of the HCV core/E1/E2 transgenic group on ethanol treatment relative to the HCV core/E1/E2 transgenic group on normal diet.

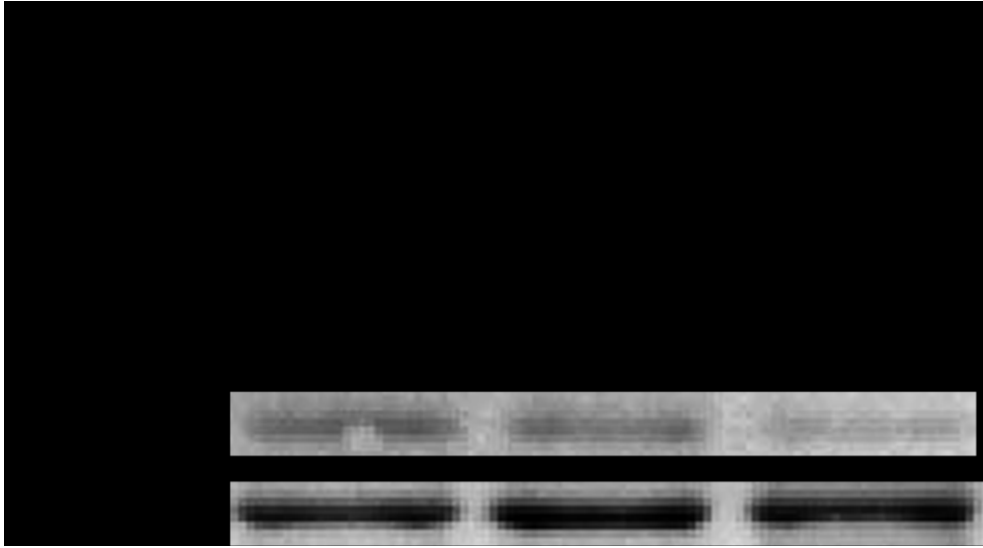
treated transgenic mice than the untreated transgenic littermates. This high level of HCV protein expression was localized around the lipid droplets in the hepatocytes (Figure 3.5).

Core protein was previously shown to co-localize with lipid droplets by studies on different cell lines expressing hepatitis C virus core protein, liver samples from HCV-infected patients or cultured cells infected with HCV (25, 195, 209, 256, 259, 279, 280, 346). Immunohistochemistry analysis also confirmed the Core protein localization around the lipid droplets (Figure 3.5).

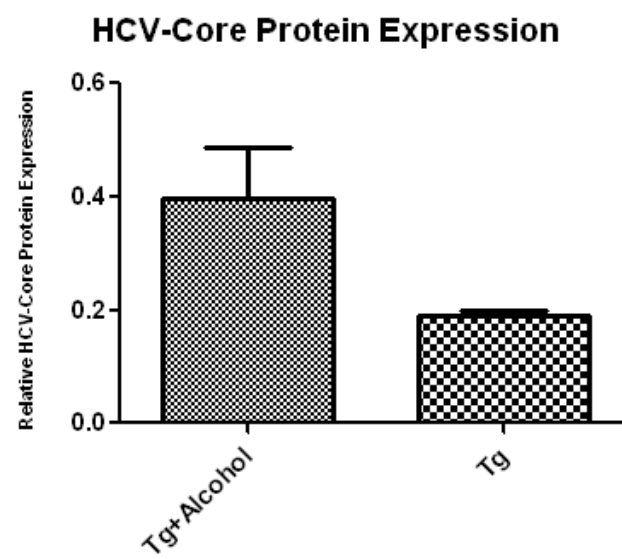
3.4 Histopathological abnormalities of the ethanol-fed HCV-transgenic mouse livers:

Clinical studies suggested a synergistic effect of alcohol and HCV in liver injury represented by accelerated steatosis, reflecting metabolic abnormalities. The transgenic mouse model expressing HCV core, E1 and E2 structural proteins was used to investigate the oxidative stress and perturbation of lipid metabolism associated with hepatitis C infection (216). In order to characterize steatosis in the ethanol-fed HCV-transgenic mice, 5 μ m sections of paraffin-embedded liver tissue were examined after H&E staining. Hematoxylin stains cell nuclei blue, while eosin stains cytoplasm, connective tissue and other extracellular substances pink or red. The histopathological analysis of the H&E stained liver sections showed a severe micro- and macrovacuolar degeneration characteristic of steatosis in both groups of transgenic mice. There was a centrilobular distribution of macrovesicular steatosis, with microvesicular pattern more generally distributed in the liver of the transgenic mice on a normal diet, while in the livers of ethanol-fed transgenic mice, steatosis had a portal distribution (Figure 3.6). On the other hand in the ethanol-fed non-transgenic mice a diffuse

Figure 3.4 Increase in HCV-core protein expression in the livers of HCV-transgenic mice fed with ethanol. A. Equal amounts of liver lysates (150 μ g of total protein) were subjected to immunoprecipitation using anti-HCV core mouse monoclonal and β -actin polyclonal rabbit antibodies followed by SDS-PAGE and Western blot analysis. To control for loading of equal amounts of proteins, the membranes were stripped for removal of primary and secondary antibodies, and reprobed with anti- β -actin polyclonal rabbit sera. The results shown are from one of three independent experiments with similar results. B. Relative HCV-core expression (ratio of HCV-core/ β -actin) for HCV-transgenic mice on normal or ethanol diets determined by densitometry (n=5). The results shown are the means + SD.



A.



B.

Figure 3.5 Increased HCV-core protein expression in ethanol treated HCV-core/E1/E2 transgenic mice as shown by immunohistochemistry. Paraformaldehyde fixed and paraffin embedded 5µm liver sections were stained for HCV-core protein using a mouse monoclonal to HCV core and a secondary anti-mouse immunoperoxidase-labeled antibody (brown-orange colour), followed by H&E counterstaining A. HCV-core protein in the ethanol-fed HCV-core/E1/E2 transgenic mouse with the HCV-core protein concentrated around lipid droplets indicated by arrows B. HCV-core protein in the HCV transgenic mouse on the normal diet. C. Non-transgenic negative control mouse. (magnification in all cases 10X)

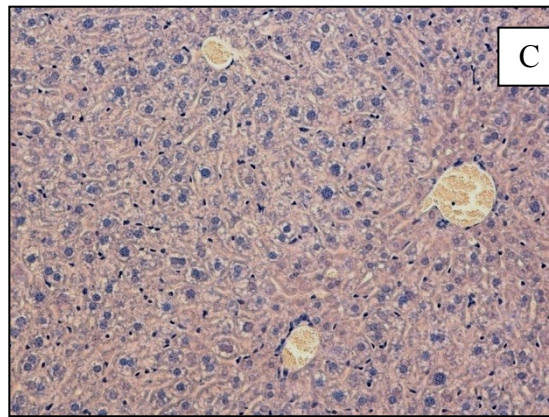
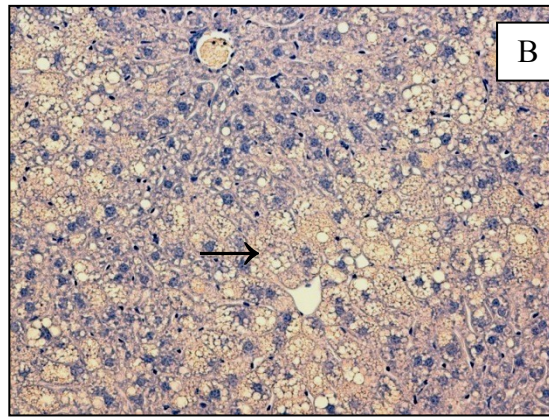
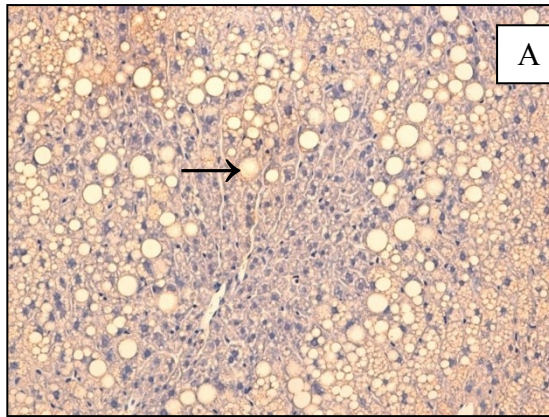
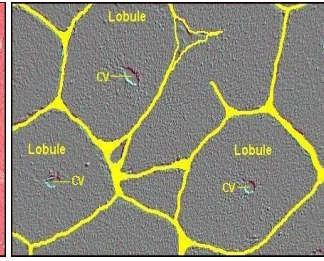


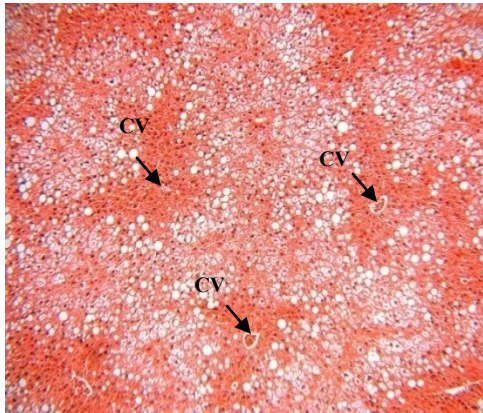
Figure 3.6 Steatosis in livers of ethanol fed transgenic mice. Paraformaldehyde fixed and paraffin embedded 5µm liver sections were stained with H&E. A. H&E staining of normal mouse liver. B. Schematic representation of the normal histology of the liver (CV represents centrilobular vein). The structural unit of the liver is the hepatic lobule. (<http://arbl.cvmbs.colostate.edu/hbooks.html>). At the vertices of the lobules are regularly distributed portal triads, containing a bile duct and a terminal branch of the hepatic artery and portal vein. C. Periportal distribution of macro- and microvesicular steatosis, visible as large droplets respective small droplets of accumulated triglycerides in livers from ethanol-fed six-month-old HCV core/E1/E2 transgenic mice. D. Centrilobular distribution of macro- and microvesicular steatosis in livers from 6-month-old HCV core/E1/E2 transgenic mice fed the normal diet. The nucleus on the cells is stained blue, while the cytoplasm is red.



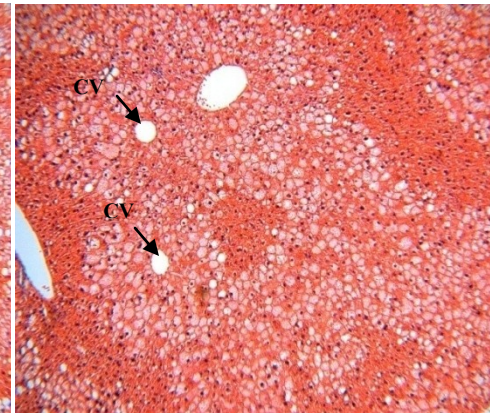
A.



B.



C.



D.

panlobular distribution of lipid droplets was observed. The same patterns of distribution of steatosis were confirmed by Osmium staining of liver sections (Figure 3.7, Table 3.3). Osmium tetroxide is used in optical microscopy to stain lipids. It dissolves in fats, and is reduced by organic materials to elemental osmium, an easily visible black substance.

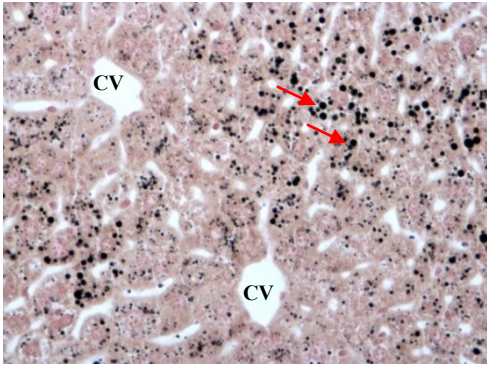
3.5 Subcellular abnormalities in HCV-core/E1/E2 transgenic hepatocytes:

Electron microscopy revealed several abnormalities of liver ultrastructure in both transgenic and ethanol-fed transgenic mice, but not the wild type mice. The mitochondria structure was disturbed (swelling and loss of cristae), with aberrant shapes, elongated or oblong; the rough endoplasmic reticulum was proliferated and dilated, with an increase dilatation in the ethanol-treated transgenic mice compared with the ones on normal diet (Figure 3.8). There was also an increase in the number and volume of peroxisomes in the ethanol-treated transgenic mice compared with the transgenic littermate mice on normal diet.

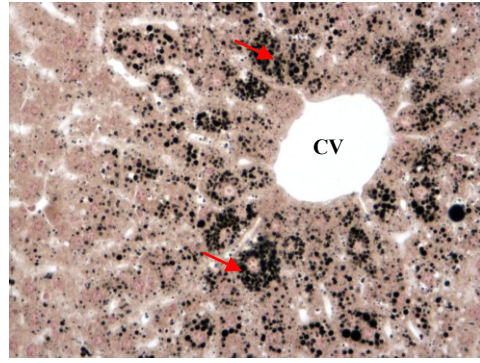
3.6 Effect of ethanol and HCV on hepatic gene expression:

Microarray analysis of 15,000 genes in the livers of transgenic and normal mice (Naas et al., manuscript in preparation) indicated that 6 genes consistently had differential expression, with the upregulation of three genes that are directly involved in lipid metabolism: apolipoprotein A IV (Apo A IV), acyl-Coenzyme A oxidase (AOX) and acyltransferase (Acaa). Both AOX and Acaa are also involved in the classical peroxisomal β -oxidation pathway. This pathway is important in the initial oxidation of very long chain fatty acids in peroxisomes. The final products, shorter chain fatty acids, will be further degraded in

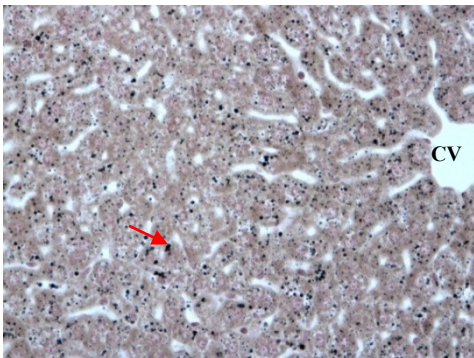
Figure 3.7 Distribution of steatosis in livers of ethanol fed transgenic mice. Osmium staining of liver sections, showing different distribution of hepatic steatosis (marked by red arrows) (40X magnification) Microvesicular hepatic steatosis is represented by accumulation of triglycerides in cytoplasm of hepatocytes observed as black droplets (stained by osmium tetroxide). A. Periportal distribution of microvesicular steatosis in ethanol-fed HCV transgenic mouse (six-month-old, 5% ethanol treatment for 5 weeks). B. Centrilobular distribution of microvesicular steatosis in HCV transgenic mouse on normal diet (six-month-old, sibling). C. Panlobular distribution of microvesicular steatosis in ethanol-fed non-transgenic mouse (six-month-old, 5% ethanol treatment for 5 weeks). CV represents centrilobular vein



A.



B.



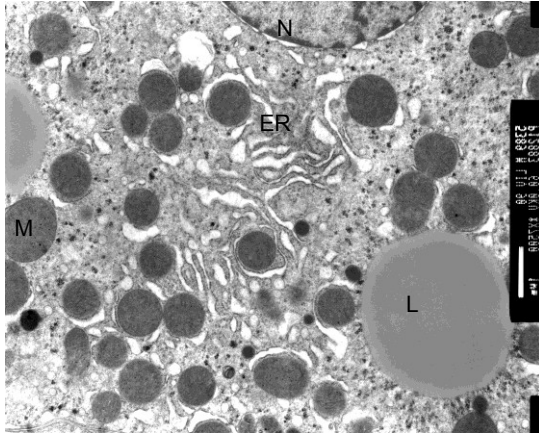
C.

Table 3.3 Relative quantification of steatosis in livers of ethanol-fed HCV core/E1/E2 transgenic mice, as detected by osmium tetroxide staining of triglycerides. The extent of liver steatosis (macro- or microvesicular) was analyzed using a semi-quantitative method. 1+ was assigned to samples with lipid droplets present in sporadic hepatocytes, 2+ for moderate steatosis represented by hepatocytes containing lipid droplets present in 2 or more of 5 fields analysed, 3+ for moderate to severe steatosis represented by lipid droplets present in more than 50% of the analysed fields, and 4+ for very severe steatosis, in which lipid accumulation was observed everywhere in the analysed liver tissue. The pattern of distribution was also analysed for each condition. Liver sections were stained using osmium tetroxide (n=8).

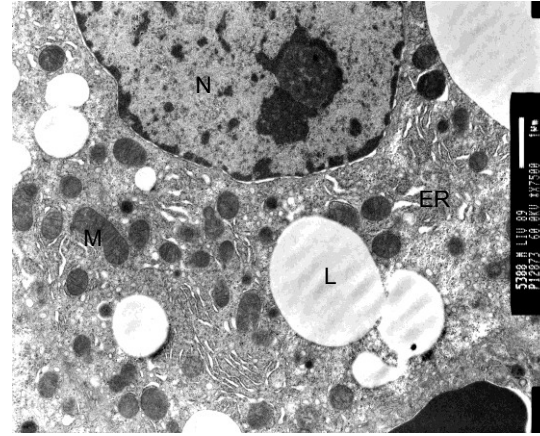
Distribution of Steatosis

			<i>Distribution of Steatosis</i>	
<i>Conditions</i>		<i>Macrosteatosis</i>	<i>Microsteatosis</i>	
	<i>HCV-Core/E1/E2 transgenic + Ethanol</i>	4	4	Microvesicular around the centrolobular vein, macrosteatosis interlobular space
		2	2	Microvesicular around the centrolobular vein, macrosteatosis interlobular space
		2	3	Macro- and microvezicular steatosis around the centrolobular vein
		1	2	Macro- and microvesicular around the interlobular space
		2	3	Microvesicular around the centrolobular vein, macrosteatosis interlobular space
		1	2	Macro- and microvesicular steatosis evenly distributed
		2	3	Microvesicular around the centrolobular vein, macrosteatosis interlobular space
		2	3	Microvesicular around the centrolobular vein, macrosteatosis interlobular space
	<i>HCV-Core/E1/E2 transgenic</i>	2	3	Macro- and microvesicular around the centrolobular vein
		1	2	Macro- and microvesicular around the centrolobular vein
		2	3	Macro- and microvesicular around the centrolobular vein
		1	3	Macro- and microvesicular around the centrolobular vein
		3	4	Macro- and microvesicular around the centrolobular vein
		0	2	Microvesicular steatosis evenly distributed
		2	3	Macro- and microvesicular around the centrolobular vein
		0	2	Microvesicular steatosis evenly distributed
	<i>Non transgenic + Ethanol</i>	0	2	Microvesicular steatosis evenly distributed
		0	1	Microvesicular steatosis evenly distributed
0		2	Microvesicular steatosis evenly distributed	
0		2	Microvesicular steatosis evenly distributed	
0		1	Microvesicular steatosis evenly distributed	
0		2	Microvesicular steatosis evenly distributed	
0		2	Microvesicular steatosis evenly distributed	
0		2	Microvesicular steatosis evenly distributed	
<i>Non transgenic</i>	0	0	N/A	
	0	1	Microvesicular steatosis evenly distributed	
	0	1	Microvesicular steatosis evenly distributed	
	0	1	Microvesicular around the centrolobular vein	
	0	0	N/A	
	0	2	Microvesicular around the centrolobular vein	
	0	0	N/A	

Figure 3.8 Subcellular abnormalities of hepatocytes from ethanol-fed HCV core/E1/E2 transgenic mice (25000X magnification) Electron micrographs show the subcellular organization of the HCV transgenic hepatocytes +/- ethanol treatment. The mitochondrial ultrastructure is disturbed with swelling and loss of cristae. Also mitochondria with aberrant shapes, elongated or oblong can be observed. A. Ethanol-fed HCV transgenic mouse (six-month-old, 5% ethanol treatment for 5 weeks) shows proliferated and dilated endoplasmic reticulum and an increase in the number of the peroxisomes. B. HCV transgenic mouse (six-month-old sibling) M – mitochondria; ER – endoplasmic reticulum; N – nucleus; L – lipids.



A.



B.

Table 3.4 Relative expression of Acyl-Coenzyme A oxidase (AOX) RNA in the liver of HCV core/E1/E2 transgenic mice with or without ethanol by Real-time RT-PCR. Acyl-Coenzyme A oxidase, a gene involved in lipid metabolism peroxisomal β oxidation, was shown to be upregulated in ethanol treated transgenic mice, compared with the transgenic mouse on a normal diet and the non-transgenic mice. The first row represents the threshold cycle average where the AOX RNA signal was detected. The second row represents the threshold cycle average where the internal gene, GAPDH, RNA signal was detected. The third row is the Δ CT is the difference between the threshold cycle average of the AOX RNA and the threshold cycle average of the GAPDH RNA. The fourth row is the $\Delta \Delta$ CT, which is the difference between the Δ CT of the HCV core/E1/E2 transgenic group on ethanol treatment, or the HCV core/E1/E2 transgenic group on normal diet compared to the non-transgenic group. The last row ($2^{-\Delta\Delta CT}$) is the normalization of the AOX RNA for HCV core/E1/E2 transgenic group on ethanol treatment or on normal diet relative to the AOX RNA for the non-transgenic group on a normal diet. CT is the threshold cycle where the specific RNA signal was detected. The samples were assayed in triplicate using the Quantitec SYBR Green PCR kit and iCycler software for CT detection of each amplicon (n=4).

	Ethanol-fed Transgenic Mice	Transgenic Mice	Non- Transgenic mice
Avg. AOX CT	19.77	20.05	21.85
Avg. GAPDH CT	14.17	13.75	14.95
Δ CT (Avg. AOX CT - Avg. GAPDH CT)	5.6	6.3	8.05
$\Delta \Delta$ CT (Avg. Δ CT _X - Avg. Δ CT _{0X})	-2.45	-1.75	0
Normalized AOX Transgenic+Ethanol/Transgenic relative to nonTransgenic ($2^{-\Delta\Delta CT}$)	5.46	3.36	1

the mitochondria. Apo AIV is a transporter of dietary triglycerides, phospholipids, cholesterol and cholesterol esters from enterocytes to hepatocytes and other tissues. To determine if ethanol would further increase the expression of these genes in the transgenic mice..... An increased expression of AOX and Apo A IV RNA was observed by real-time RT-PCR analysis in the ethanol-fed transgenic mice compared with transgenic mice on normal diet. There was a 5.46 fold increase in AOX transcript (Table 3.4), and a 5.27 fold increase of the Apo A IV transcript (Table 3.5).

3.7 Measurement of lipid peroxidation products (LPO):

To provide direct evidence of the consequences of oxidative stress induced by HCV proteins alone or in the presence of ethanol, LPO were measured in liver from core/E1/E2 transgenic and ethanol-fed non-transgenic littermate mice. Supernatants from liver homogenates were collected, and the content of malonaldehyde, which is the main product of lipid peroxidation, was quantified. The results showed an increase in the lipid peroxidation of transgenic versus non-transgenic mice, while in the ethanol-treated HCV-transgenic mice lower amounts of LPO were observed (Figure 3.9). The increased production of free radicals may lead to mitochondrial dysfunction, perhaps associated with mitochondrial DNA depletion.

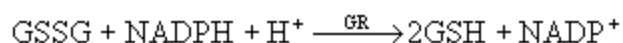
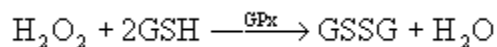
3.8 Measurement of anti-oxidant reservoir (Glutathione Peroxidase):

Under normal circumstances, intracellular oxidative stress should be counteracted by the up-regulation of cellular antioxidant mechanisms. Glutathione (GSH) in its reduced form is a major antioxidant in human tissues that provides reducing equivalents for the glutathione peroxidase (GPx) catalyzed reduction of hydrogen peroxide and lipid hydroperoxides to

Table 3.5 Relative expression of Apolipoprotein A IV(ApoAIV) RNA in the liver of HCV transgenic mice with/or without ethanol by real-time RT-PCR Apolipoprotein A IV, a gene involved in lipid metabolism, was shown to be upregulated in the ethanol treated transgenic mice, compared with the transgenic mice on a normal diet and the non-transgenic mice. The first row represents the threshold cycle average where the ApoAIV RNA signal was detected. The second row represents the threshold cycle average where the internal gene, GAPDH, RNA signal was detected. The third row is the Δ CT is the difference between the threshold cycle average of the ApoAIV RNA and the threshold cycle average of the GAPDH RNA. The forth row is the $\Delta \Delta$ CT, which is the difference between the Δ CT of the HCV core/E1/E2 transgenic group on ethanol treatment, on the HCV core/E1/E2 transgenic group on normal diet compared to the non-transgenic group. The last row ($2^{-\Delta\Delta CT}$) is the normalization of the APO AIV RNA of HCV core/E1/E2 transgenic group on ethanol treatment or on a normal diet relative to the APO AIV RNA of non-transgenic group on a normal diet. CT is the threshold cycle where the specific RNA signal was detected. The samples were assayed in triplicates using the Quantitec SYBR Green PCR kit and iCycler software for CT detection of each amplicon (n=4).

	Ethanol-fed Transgenic Mice	Transgenic Mice	Non- Transgenic mice
Avg. ApoAIV CT	17.9	18.52	21.15
Avg. GAPDH CT	16.65	16.1	17.5
Δ CT (Avg. ApoAIV CT - Avg. GAPDH CT)	1.25	2.42	3.65
$\Delta \Delta$ CT (Avg. Δ CTransgenic - Avg. Δ CTransgenic+Ethanol)	-2.4	-1.22	0
Normalized ApoAIV Transgenic+Ethanol relative to nonTransgenic ($2^{-\Delta\Delta CT}$)	5.27	2.33	1

water and the respective alcohol. During this process, GSH becomes oxidized glutathione (GSSG). The GSSG is then recycled to GSH by the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), catalyzed by glutathione reductase (GR). When mammalian cells are exposed to increased oxidative stress, the ratio of GSH/GSSG will decrease as a consequence of GSSG accumulation.



There are studies proving the association of Hepatitis C and lower levels of reduced glutathione in plasma and lymphocytes, and this depletion is associated with liver disease. The HCV-core/E1/E2 transgenic animal model has similar features to HCV infection in humans, demonstrating decreased glutathione peroxidase activity. Also, as expected, the ethanol treatment of the non-transgenic mice showed a decrease in the enzyme activity.

During alcohol consumption combined with HCV infection, high and prolonged levels of oxidative stress induces up-regulation of cellular antioxidant mechanisms resulting in higher activity of Glutathione Peroxidase, which may not be sufficient to eliminate the oxidative stress. As expected, the Glutathione peroxidase activity in the ethanol-treated HCV-transgenic mice was increased (Figure 3.10), but unable to counteract the high levels of lipid peroxidation (Figure 3.9).

Figure 3.9 Increased lipid peroxidation in mice fed ethanol for 5 weeks. Lipid peroxidation products were measured in liver from ethanol fed HCV core/E1/E2 transgenic and ethanol-fed non-transgenic littermates by measuring malonaldehyde, the main product of lipid peroxidation. The amount of malonaldehyde is expressed as micromoles. There is a significant increase in the levels of malonaldehyde in the transgenic mice compared with the non-transgenic ones. The results shown are means + SD of livers from 4 mice in each group.

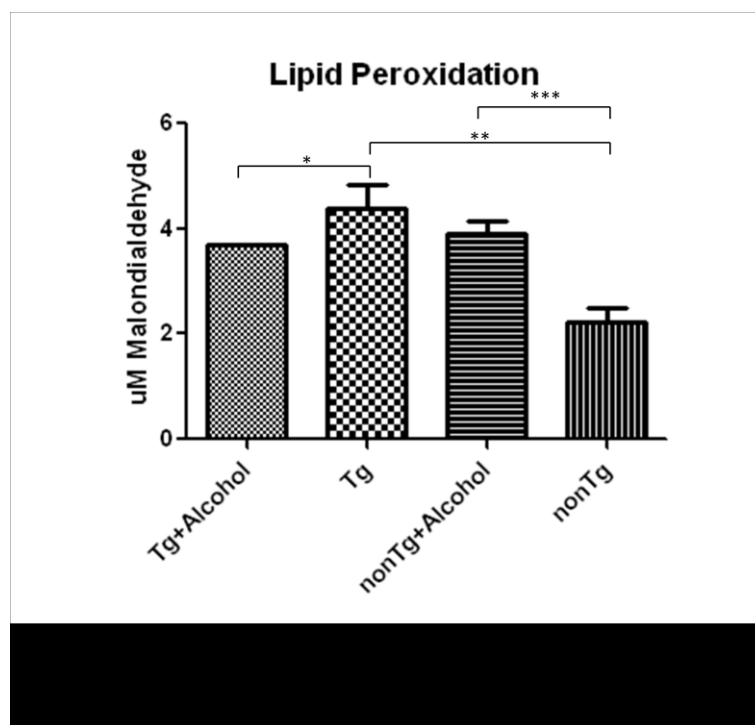
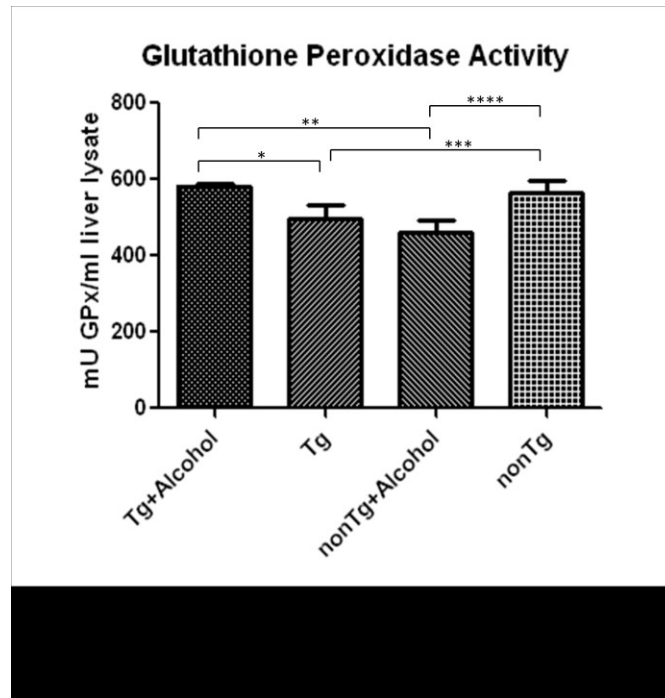


Figure 3.10 Antioxidant activity induced by ethanol diet in the HCV core/E1/E3 Transgenic mice. In order to monitor the antioxidant activity, Glutathione Peroxidase (GPx) activity was measured with a GPx 340™ assay kit (Oxis International, Portland, OR). Mouse liver homogenates were analysed for the oxidation of NADPH to NADP⁺, which is accompanied by a decrease in absorbance at 340nm, providing a spectrophotometric way of monitoring GPx enzyme activity. The glutathione peroxidase activity in ethanol-treated HCV-transgenic mice was higher than the lower than normal levels induced by either HCV-transgene or ethanol alone. The GPx enzyme activity is expressed as mU/ml of liver lysate. The results shown are mean + SD (n=6).



Chapter 4:

The effects of HCV structural genes on cytokine expression and dendritic cell function as a potential mechanism of HCV escape from immune surveillance

Only 20% of people who become infected by HCV are able to spontaneously clear the virus. Previous studies have suggested that strong CD4⁺ helper T cell and CD8⁺ cytotoxic T cell immune responses are critical for viral clearance while insufficient immune activation during acute infection is associated with viral persistence. Acute HCV infection associated with viral clearance is characterized by a vigorous CD4⁺ and CD8⁺ T cell response with predominant Th1-type immune activation (143). However, in the patients who develop chronic HCV infection, T cell activity specific to HCV antigens is drastically weaker and insufficient for viral elimination (281). These observations suggest that a robust T cell immune response following HCV infection is the decisive factor where viral clearance is concerned. Recurring HCV viraemia after apparent viral clearance from the circulation has been associated to a loss of detectable CD4⁺ T-cell responses (115). The reasons for the loss of CD4⁺ T-cell responsiveness need to be further analyzed, but one possible mechanism may be the deficit in T cell priming induced by potential impairment of dendritic cell function (108, 218). Several studies suggested impaired myeloid DC (mDC) function in chronic HCV infection (16, 20, 146) and decreased DC maturation capacity (265, 292), induced by different HCV proteins. Core structural protein was shown to inhibit the MHC class I antigen-processing pathway and was associated with impairment in the ability of mDCs to stimulate CD8⁺ T cells (227) and decreased secretion of IL-12 with subsequent inhibition of Th1 responses (318). HCV core protein was also found to be partially responsible for the inhibition of TLR7-induced plasmacytoid DC CD40 expression, followed by low CD4 T cell priming (170).

Although impaired antigen presentation induced by HCV proteins has been proposed as a possible mechanism used by the virus to cause T cell dysfunction (76, 292), the exact details of this inhibition have yet to be defined. In the second part of my study I investigated

dendritic cell maturation in the presence of structural proteins of HCV, core, E1 and E2, and also their effects on DC function by analyzing DC cytokine expression and the ability of DCs to activate T-cell lymphocytes (143).

4.1 Inhibition of bone marrow-derived dendritic cell maturation by HCV

Core/E1/E2 polyprotein

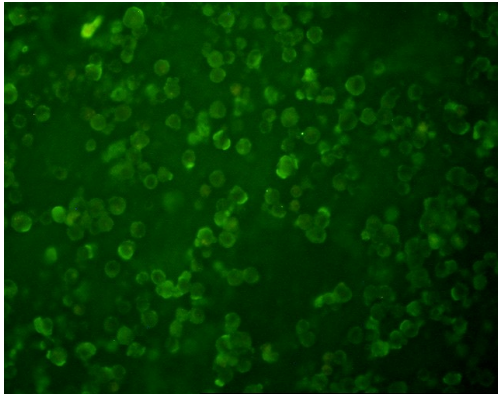
To analyse the DC maturation induced in the presence of HCV-core/E1/E2 polyprotein, bone-marrow derived DC from wild-type mice (B6C3F1) were loaded with 10 µg/ml of purified HCV-core/E1/E2 or HIV-Gp120 proteins, in the presence or absence of 0.1µg/ml LPS on the 5th day of DC culture.

The cells were analysed by fluorescent immunolabelling with anti-core/E1/E2 rabbit polyclonal antibodies, and observed by fluorescence microscopy on the 7th day of culture. Core/E1/E2 proteins were detected in the loaded DCs, while the unloaded DCs demonstrated no fluorescence (Figure 4.1).

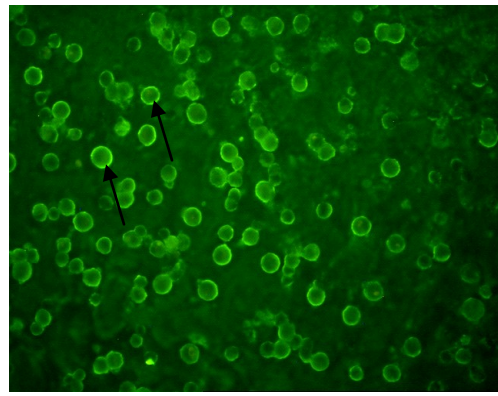
The HIV-Gp120-loaded DC showed changes in surface marker expression characteristic of DC maturation with up-regulation of MHC class II and CD86 (enhanced numbers of double positive MHCI/CD86). In contrast, the core/E1/E2-loaded DCs showed reduced numbers of double positive cells for these markers compared to unloaded DCs (Figure 4.2, Table 4.1).

To eliminate the possibility of cell death as the cause of low percent of double positive cells (and maturation of DCs) in the presence of core/E1/E2, the viability of dendritic cells was also tested. A higher percentage of cell death were seen at 1µg/ml concentration of LPS.

Figure 4.1 Detection of core/E1/E2 in passively loaded bone-marrow-derived DCs. DC were prepared from BM cells isolated from B6C3F1 mice by seven days of culture in the presence of GM-CSF, IL4 and TNF α . At day 5 of culture, purified core E1/E2 was added to the media at a concentration of 10ug/ml. Control cultures received no protein. Cells were fixed at day 7, and the presence of HCV core protein was detected using rabbit anti-core/E1/E2 polyclonal antibodies (primary) and FitC-labelled anti-rabbit IgG antibodies (secondary antibodies). A. Control dendritic cells that were not treated with recombinant protein B. core/E1/E2-loaded bone marrow derived DCs ($\rightarrow$).



A.



B.

Figure 4.2 HCV-C/E1/E2 induced inhibition of maturation of bone-marrow derived DCs The source of DCs was bone-marrow cells from wild-type mice (B6C3F1). The cells were cultivated for 7 days in the presence of 10ng/ml GM-CSF, 10ng/ml IL4, 0.1µg/ml LPS, and then loaded with 10 µg/ml HCV core/E1/E2 or HIV-Gp120 proteins. Different surface markers were analysed by flow cytometry using anti-MHC Class II-FitC conjugated and anti-CD86-phycoerythrin (PE) conjugated antibodies. A. HCV core/E1/E2 loaded bone marrow-derived DCs showed lower percentage of double positive MHCII+CD86+ cells (27.79%) in the presence of 0.1µg/ml LPS. B. HIV Gp120 loaded DCs showed higher percentage of double positive MHCII+CD86+ cells (50.14%) in the presence of 0.1µg/ml LPS.

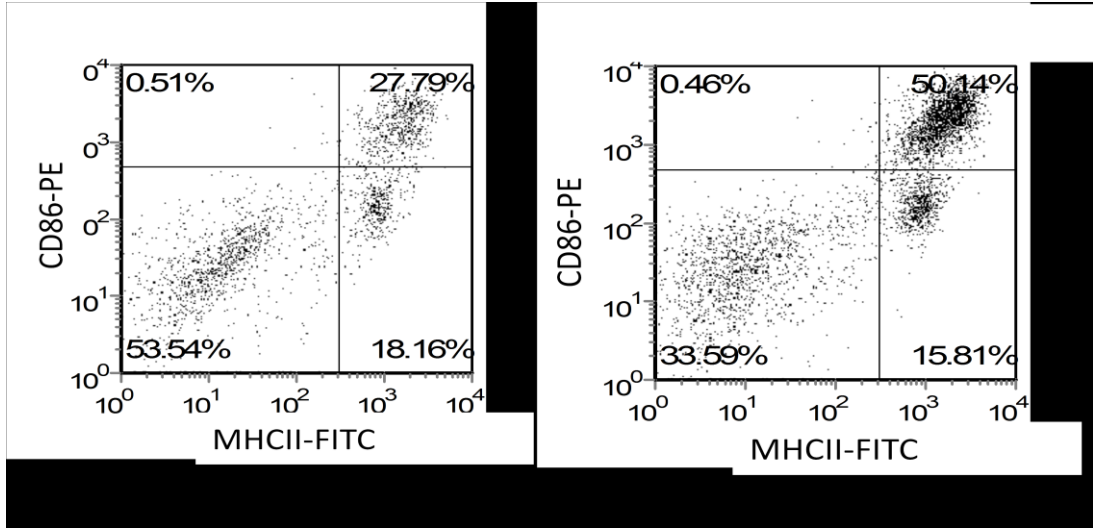


Table 4.1 HCV core/E1/E2 inhibits cytokine induced maturation of DC derived from bone marrow. Antibodies used to label the cells were anti-MHC Class II- FitC conjugated and anti-CD86-PE conjugated. Dendritic cells were isolated from bone marrow-derived cultures from B6C3F1 mice (Wt mice). Bone-marrow cells from wild-type mice (B6C3F1) were cultivated for 7 days in the presence of 10ng/ml GM-CSF and 10ng/ml IL4, and in the absence(A) or presence(B) of 0.1µg/ml LPS. DCs were loaded with 10 µg/ml HCV core/E1/E2 or HIV Gp120 proteins. Student's t-test was used for data analysis and values of $P \leq 0.05$ were considered statistically significant.

	Wt	Wt + HCV-Core/E1/E2	Wt + HIV-Gp120	[Wt + HCV-Core/E1/E2] - [Wt]	[Wt + HIV-Gp120] - [Wt]	
Percentage of MHC II+ CD86+ DCs						
Experiments	#1	77.89	74.93	-2.96		
	#2	41.83	35.63	-6.2		
	#3	38.73	30.28	-8.45		
	#4	82.07	64.05	-18.02		
	#5	31.7	25.25	36.22	-6.45	4.52
	#6	47.17	37.45	68.56	-9.72	21.39
	#7	61.03	53.89	68.05	-7.14	7.02
	#8	42.4	30.25	58.63	-12.15	16.23
	#9	62.61	54.4	64.97	-8.21	2.36
	Average				-8.81	10.30
StDevErr				1.42	3.64	
p value					0.004	

A.

		Wt + LPS	Wt + HCV-Core/E1/E2 + LPS	Wt + HIV-Gp120 + LPS	[Wt + HCV-Core/E1/E2 + LPS] – [Wt + LPS]	[Wt + HIV-Gp120 + LPS] – [Wt + LPS]
<i>Percentage of MHC II+ CD86+ DCs</i>						
Experiments	#1	82	71	86.51	-11	4.51
	#2	30.1	27.79	50.14	-2.31	20.04
	#3	78.89	67	85.45	-11.89	6.56
	Average				-8.4	10.37
	StDevErr				3.05	4.87
	p value					0.039
B.						

However, no correlation between the addition of core/E1/E2 to the cell cultures and cell death was observed (Table 4.2).

4.2 Inhibition of allogeneic CD4⁺ T cell proliferation in the presence of Core/E1/E2 loaded bone marrow-derived DCs

Impaired antigen presentation induced by HCV proteins was previously suggested as possible mechanism used by the virus to cause T cell dysfunction. The capacity of the HCV core/E1/E2-loaded bone marrow-derived DC populations to stimulate proliferation of CD4⁺ T cells was evaluated in this study. A commonly used technique to investigate the immunological response of lymphocytes is the mixed lymphocyte reaction. In this assay, two different types of white blood cells are mixed together. The first population serves as the antigen and these cells are named stimulator cells, while the second population, so-called responder cells, is largely composed of the CD4⁺ lymphocytes. In this case, irradiated stimulator cells represented by DCs loaded with HCV core/E1/E2 or HIV gp120 were mixed with responder cells, PBMCs, which proliferation was measured after 5-7 days. In order for the responder cell to proliferate after stimulation with syngeneic DCs, the PBMCs were isolated from mice injected with mature BMdDC for breaking their tolerance against self antigens. T-cell proliferative responses of the responder cells were measured by CFSE decay. The CFSE dye is an intracellular dye which is diluted 50% everytime there is cell division and is easily measured by flow cytometry. Following exposure to HIV Gp120 protein, TNF α -induced mature bone marrow-derived DCs showed high T-cell stimulatory capacity, while HCV core/E1/E2-loaded DC showed low or no T cell stimulatory capacity (Figure 4.3).

4.3 Treatment of bone marrow-derived DCs with core/E1/E2, but not HIV-Gp120, diminishes responses of DCs to LPS

To determine if HCV-core/E1E2 loaded DC have impaired function, HCV core/E1/E2 loaded and unloaded DC cells were tested for their capacity to produce cytokines in response to LPS stimulation. The presence of the core/E1/E2 polyprotein reduced the secretion of several cytokines (IL-12, $\text{INF}\gamma$, MCP-1 and MCP-5) important for stimulation and chemotaxis of T cells, as compared to DCs treated with LPS only. In contrast HIV Gp120 did not result in lower secretion of these cytokines (Figure 4.4).

Table 4.2 Viability of bone marrow-derived dendritic cells in the presence of 10µg/ml HCV core/E1/E2 polyprotein and different concentrations of LPS. Trypan blue stain is a vital stain used for testing cell viability. Live (viable) cells do not take up the dye and appear bright yellowish in color upon microscopic analysis, while dead (non-viable) cells take up the dye and become blue in color. The source of counted cells was bone marrow-derived DC culture from B6C3F1 mice. Bone-marrow cells from wild-type mice (B6C3F1) were cultivated for 7 days in the presence of 10ng/ml GM-CSF, 10ng/ml IL4, with or without 0.1µg/ml LPS, and loaded with 10 µg/ml HCV core/E1/E2 proteins or not. The data are representative of three different experiments with similar results. Cells were counted on a hemocytometer. The counts represent the total number of cells in four squares. Cell viability was estimated for each experiment.

Conditions		# of viable cells	# of dead cells	Total # of cells	% of dead cells from the total # cells
	1µg/ml LPS + 10µg/ml Core/E1/E2	28	3	31	9.7
	1µg/ml LPS	25	5	30	16.7
	0.1µg/ml LPS + 10µg/ml Core/E1/E2	32	2	34	5.9
	0.1µg/ml LPS	30	2	32	6.3
	0.01µg/ml LPS + 10µg/ml Core/E1/E2	35	0	35	0
	0.01µg/ml LPS	26	1	27	3.7
	10µg/ml Core/E1/E2 + no LPS	38	0	38	0
	no Core/E1/E2 + no LPS	45	0	45	0

Figure 4.3 Mixed Lymphocyte Reaction - CD4⁺ T cell proliferation is inhibited when stimulator cells express HCV core/E1/E2 polyprotein. PBMCs were isolated from B6C3F1 wild-type mice. The mice were vaccinated subcutaneously four times with 2×10^5 irradiated (30Rad) BMdDCs per mouse, at one month interval. PBMCs from vaccinated mice were isolated 10 days post last vaccination, labelled with CFSE dye, and cultured for 5 days in the presence of untreated or HCV core/E1/E2 treated bone marrow-derived DCs. Following a 5 day incubation, T-lymphocyte proliferation was measured by CFSE decay. The CFSE-labelled mouse peripheral blood mononuclear cells were harvested and surface stained with antimouse-CD3-PE-Cy5 and antimouse-CD4-PE conjugated antibodies. A. Co-culture of PBMCs from bone marrow-derived DC-vaccinated mice with untreated DCs resulted in CD3⁺CD4⁺ T cell proliferation. Dot plot analyses were performed in cells stained with CFSE only. These cells were analyzed by flow cytometry and were gated on both CD3⁺ and CD4⁺ markers. B. Co-culture of PBMCs from vaccinated animals and core/E1/E2-loaded bone marrow derived DCs resulted in inhibition of CD3⁺CD4⁺ T cell proliferation. C. Most cells had arrested proliferation at the parental generation (black peak) when co-cultured with HCV core/E1/E2 loaded bone marrow-derived DCs. In contrast, co-culture of untreated bone marrow-derived DCs and mouse PBMCs resulted in T-cell proliferation. The red peaks represent successive generations of peripheral blood T-cells. (n=6).

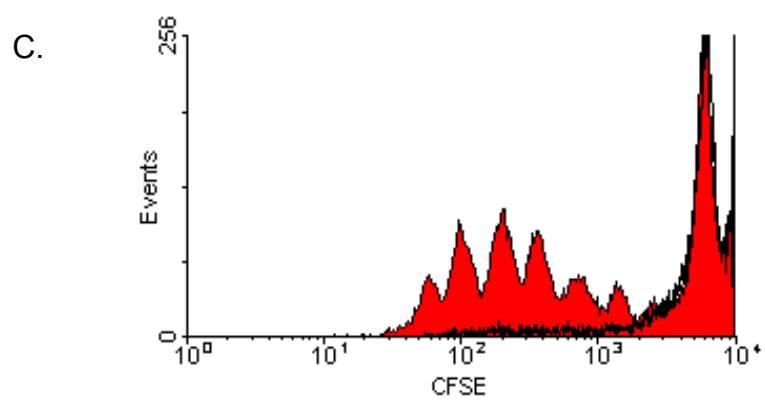
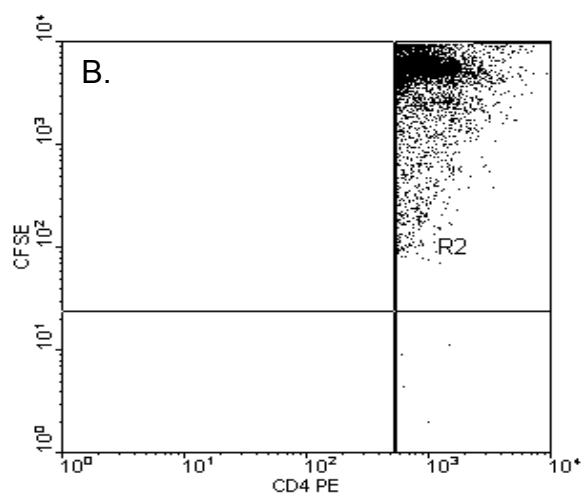
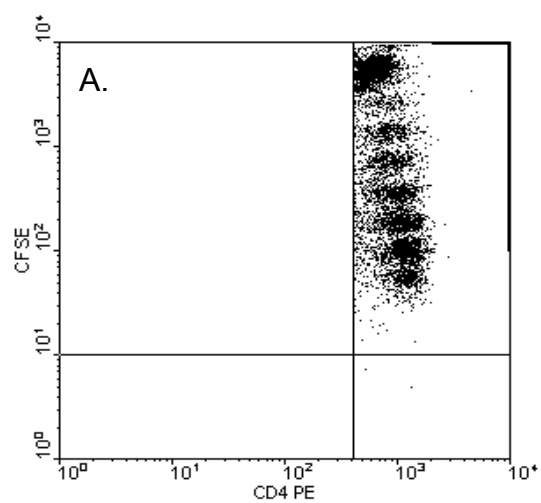
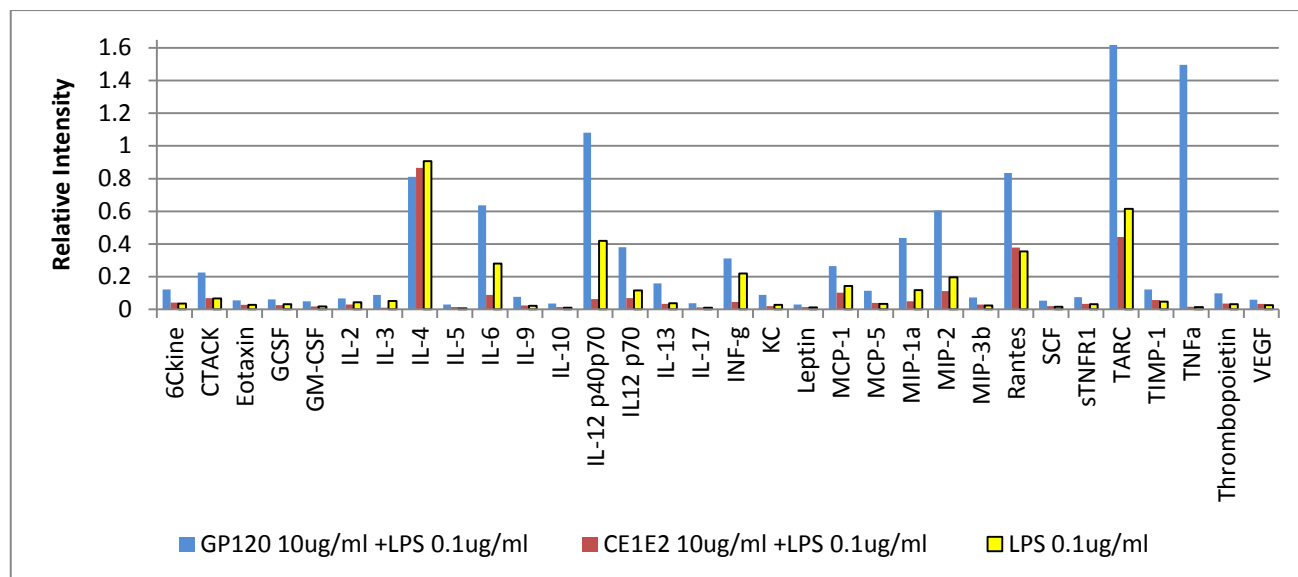


Figure 4.4 Cytokine pattern induced by LPS stimulation of HCV core/E1/E2 loaded DCs. A mouse cytokine antibody array was used to detect cytokines produced in supernatants of 7 day-cultured B6C3F1 bone marrow-derived DCs after stimulation with 0.1µg/ml LPS +/- purified 10µg/ml HCV core/E1/E2 polyprotein or HIV Gp120. The samples were supernatants of bone marrow-derived dendritic cell cultures from B6C3F1 mice (Wt mice). The bone-marrow cells from wild-type mice (B6C3F1) were cultivated for 7 days in the presence of 10ng/ml GM-CSF, 10ng/ml IL4, with addition of 0.1µg/ml LPS the 6th day of culture, and 10 µg/ml HCV-core/E1/E2 or HIV-Gp120 proteins on the 5th day of culture. The presence of the core/E1/E2 polyprotein reduced the LPS-stimulated bone marrow-derived DC's secretion of certain cytokines and chemokine (IL-12, INF γ , MCP-1, MIP-1a, MIP-2, TARC), and had no effect on others (IL4, Rantes). The data are representative of two different experiments with similar results.



Chapter 5: Discussion

One goal of this study was to determine the effect of ethanol on the disease induced by expression of the HCV core protein, how the consumption of ethanol affects the structural dynamics and functional properties of transgenic HCV-core/E1/E2 hepatocytes. Analysis of the HCV-transgenic mouse model allowed me to learn more about the mechanisms of disease production of both ethanol consumption and hepatitis C virus.

Together, alcoholic liver disease (ALD) and chronic HCV infection are the most frequent chronic liver diseases in the Western world, and the present study tried to mimic the coexistence of these two conditions, which happens frequently in the human population. The five weeks of ethanol treatment in mice induced a significant decrease of body weight gain compared with the control mice, both transgenic and non-transgenic littermates on normal diets (Figure 3.2). This finding is supported by other studies which suggest that ethanol consumption does not result in increased body weight (177) even though ethanol has a relatively high caloric value (7.1 Cal/gram in comparison with 1 gram of carbohydrate, which contains 4.5 Cal/gram). The first and second National Health and Nutrition Examination Surveys (NHANES I and II) found that higher caloric intake among drinkers did not result in higher body weight compared with nondrinkers. Moreover, women drinkers had significantly lower body weight than female nondrinkers. Also, as the ethanol intake among men increased, their body weight decreased (57) (117). A study by Liu et al reported a lower weight gain during a 10-year follow-up period for both men and women drinkers. Increased levels of ethanol intake were strongly related to decreased body weight among women (177). Energy wastage during ethanol metabolism by the microsomal ethanol oxidizing system (MEOS) may be one reason for relatively low body weight (171) (244). Chronic ethanol consumption induces MEOS activation, with ethanol oxidation without phosphorylation, in which a high energy compound, NADPH, is utilized, but no high-energy

compound is formed, and the reaction only generates heat. If this calorigenesis exceeds the needs for thermoregulation, it can be considered as energy wastage. By contrast ethanol oxidation to acetaldehyde via the ethanol dehydrogenase (ADH) pathway is associated with the generation of the high energy compound NADH (from NAD), which leads to ATP synthesis (174). Another possible reason for ethanol-induced low body weight is mitochondrial inefficiency in fatty acid oxidation secondary to chronic ethanol consumption and acetaldehyde toxicity (171).

The levels of serum ALT and AST in the ethanol treated core/E1/E2-transgenic mice, compared with the other groups were substantially increased (Figure 3.4). Elevations in ALT and AST reflect hepatic injury and are commonly used to monitor liver damage. Chronic hepatitis due to HCV infection is frequently diagnosed after detection of persistently high serum aminotransferase levels (9). There is no correlation between serum aminotransferase levels and histological disease in chronic HCV-infected individuals (97), but high levels of ALT/AST are indicative of continuing infection and liver damage (118, 199).

Ethanol ingestion induced significantly higher AST/ALT ratios in both HCV-transgenic and non-transgenic mice (Figure 3.3). Increasing ethanol intake is known to be associated with higher AST/ALT ratios and higher levels of AST (305). Thus, AST/ALT ratios are used as a conventional biomarker to differentiate ethanol liver diseases from fatty liver diseases not related to alcohol consumption (80),

The effect of ethanol on HCV-core RNA levels in transgenic livers was a 14-fold increase in expression compared to transgenic mice on a normal diet (Table 3.2). Also HCV-core protein expression was higher in ethanol-treated transgenic mice than in untreated transgenic littermates (Figure 3.4 and Figure 3.5). These findings are consistent with the known clinical effects of ethanol consumption in patients with chronic hepatitis C,

represented by high viral load (240, 252, 268) and activation of virus replication in HCV replicon systems (191, 278, 306). Moreover, this is the first time that ethanol has been demonstrated to specifically induce an increase in the expression of viral RNA as well as protein in a transgenic mouse model (Table 3.2, Figure 3.4 and Figure 3.5).

One possible mechanism responsible for increase in HCV replication may be ethanol-induced oxidative stress following Cyp2e1-mediated metabolism of ethanol. The increase in HCV replication could be blocked in the presence of the antioxidant N-acetyl cysteine (191). Oxidative stress is known to activate different cellular transcription factors such as activating protein-1 and NF- κ B (246). Activation of NF- κ B promoter activity was also suggested as an additional mechanism responsible for ethanol action on HCV replication by Zhang et al (352). To support this data, caffeic acid phenethyl ester, a specific inhibitor of the activation of NF-kappaB, abolished ethanol-induced HCV RNA expression.

Ethanol metabolism generates high levels of reactive oxygen species, which has been recognized as a key mechanism of ethanol hepatotoxicity (15, 45, 180, 315). There are three mechanisms of metabolizing ethanol, each one of them resulting in increased hepatic oxidative stress: ethanol can be metabolized by cytosolic alcohol dehydrogenase to acetaldehyde, followed by production of ROS and accumulation of NADH which further interferes with the electron transfer system in the mitochondria, generating more ROS (194) (66). Microsomal ethanol-oxidizing system, located on the endoplasmic reticulum, and represented by ethanol-inducible cytochrome P450 2E1 (CYP2E1), converts ethanol to acetaldehyde and also generates ROS (46). On the other hand, chronic ethanol consumption significantly decreases cytosolic and mitochondrial glutathione peroxidase activities (19). Acetaldehyde interaction with mitochondrial glutathione may cause a decrease in the antioxidant defence of the mitochondria with increased susceptibility to oxidative stress. My

results are in agreement with these observations, and show increased lipid peroxidation products in the ethanol-treated non-transgenic mice, as proof of increased oxidative stress, and decreased glutathione peroxidase activity as marker of lower antioxidative reserve (Figure 3.9, 3.10).

Moreover, HCV core protein has been shown to increase the levels of reactive oxygen species and lipid peroxidation products (159). Hepatitis C virus was shown to increase production of oxidative stress in the liver in the absence of inflammation (158). This overproduction of reactive oxygen species results in increased lipid peroxidation, as is also seen in our transgenic mouse model (Figure 3.9). An analysis of the antioxidant profile revealed that antioxidative molecules are decreased despite the overproduction of reactive oxygen species in the liver of core/E1/E2 transgenic mice: glutathione peroxidase levels were lower in the transgenic mice than in the nontransgenic littermates (Figure 3.10).

There are studies suggesting an inhibition of HCV replication induced by oxidative stress (53) through involvement of the MEK-ERK1/2 signaling pathway (340). This inhibition can be prevented by treatment with vitamin E, a lipid-soluble antioxidant (129) (339) and others antioxidants such as vitamin C, and N-acetyl cysteine (161). This suggests that the HCV RNA replication can be inhibited through modulation of the glutathione redox system and oxidative stress. My results are in agreement with these studies, and suggest an increase in glutathione peroxidase activity in the ethanol treated HCV-transgenic mice (Figure 3.10) but lower levels of lipid peroxidation products (Figure 3.9) despite the presence of HCV-core protein and alcohol which individually increase oxidative stress.

Another possible mechanism by which ethanol exposure can increase HCV replication may be by alteration of the cellular NADH/NAD⁺ ratio induced by downstream metabolites

of ethanol as suggested by Seronello et al., with further modulation of lipid metabolism through increased fatty acid synthesis (278).

Also ethanol-induced oxidative stress may stimulate HCV replication through upregulation of COX-2, a key cellular regulator of the oxidative stress pathway (179, 226, 306). Ethanol and HCV have additive effects on increased COX-2 expression and activity, and through its product, prostaglandin E2, activate viral promoters (79, 156, 211), followed by increased viral expression.

HCV replicates in cholesterol-rich compartments in the cell, and the ethanol and its metabolites acetaldehyde, acetone, and acetate are known to increase the total intracellular cholesterol content which may facilitate the virus replication (278). Moreover, cholesterol and fatty acid metabolism have been shown to play an important role in HCV replication (147, 148, 258, 347, 348). This core/E1/E2 transgenic mouse model shows co-localization of HCV-core protein and lipid droplets (Figure 3.5) with an increase in core expression induced by ethanol treatment. The core protein co-localization with lipid droplets was also shown by other studies in different cell lines expressing HCV core protein, liver samples from HCV-infected patients or cultured cells infected with HCV (25, 195, 209, 256, 269, 279, 280, 346). In cultured cells infected with HCV, the core protein was initially detected at single punctuate sites on each lipid droplet, and later the entire organelle was progressively surrounded by core protein thought to be transferred from the ER membrane (37).

Chronic HCV infection is known to induce steatosis, associated with disease progression (221, 251). HCV replication in cultured cells leads to lipid accumulation, with core protein being the main inducer, since the in vitro expression of this protein alone resulted in lipid accumulation (2) while the expression of HCV replicons lacking the protein did not (204). In the transgenic mouse model used in this study, as for two other lines of

transgenic mice (212, 215) expressing HCV core protein in the liver developed hepatic steatosis, which is exacerbated by ethanol treatment (Figure 3.8, Table 3.3). The mechanisms underlying lipid accumulation in the liver are not completely understood and were investigated using this mouse model. Acyl-CoA synthetase long chain family member 3, *Acsvl3*, is an important activator of long and very long chain fatty acids through thioesterification to CoA, by which these lipids are metabolized in the mitochondria (236) (330). *Acsvl3* expression in the core/E1/E2 transgenic mouse model was down-regulated (Naas et al, manuscript in preparation) which may cause fatty acid accumulation due to lack of fatty acid activation. The microarray analysis of the HCV transgenic mice compared with their nontransgenic littermates also demonstrated down regulation of lipid transporters: *Sep-2* protein (important in transport, hepatic metabolism of cholesterol and its intracellular distribution) (206, 316) and *Apoa1* (important in reverse cholesterol transport) (201) were down regulated. Low expression of these two genes can result in decreased cholesterol transport and consequently, accumulation inside hepatocytes. Accumulating cholesterol levels in hepatocytes may facilitate HCV replication in cholesterol-rich compartments in the cell.

On the other hand, *Apo A-IV* (important in transport of dietary triglycerides, phospholipids and cholesterol esters from enterocytes to liver and an activator of lecithin:cholesterol acyltransferase (288, 289) was up-regulated in our core/E1/E2 transgenic model. The ethanol treatment of these transgenic mice further increased the expression of *Apo A-IV* (Table 3.5). This effect probably happens indirectly, through up-regulation of core protein expression.

Furthermore *Apo A-IV* was proven to have a dose-dependent anorectic effect, through suppression of food intake by decreasing meal size (65, 102). *ApoA-IV*'s role in food intake

was also demonstrated in studies using cortical injection of Apo A-IV and its antiserum at the level of the fenestrated part of the hypothalamus and the arcuate nucleus (250). The up-regulation of the ApoA-IV gene may be the explanation behind the decreased body weight in the ethanol-fed transgenic mice.

Also, Apoc2, an inhibitor of lipoprotein lipase (139), was up-regulated with possible consequent accumulation of triglycerides due to low metabolism of triglyceride-rich lipoproteins. Taken together, these results imply a direct effect of the HCV proteins on lipid metabolism and transport with consequent liver steatosis.

In relation to lipid metabolism, the HCV core protein has also been found to interact with retinoid X receptor (RXR)- α (311). This nuclear receptor RXR- α forms homodimers or heterodimers with other nuclear receptors, including PPAR (peroxisome proliferator-activated receptor) - α , and has an important role in regulating gene expression related to lipid metabolism, cell differentiation, and proliferation. The HCV core protein was found to induce spontaneous, persistent, age-dependent activation of PPAR α in transgenic mice, which may also contribute to human core-induced hepatocarcinogenesis (241, 299). The transgenic mouse model used in this study showed increasing liver cancer in older mice (216). Activation of PPAR α induces transcriptional activation of genes which are part of classical peroxisomal β -oxidation pathway for very long chain fatty acids (320). This pathway (Figure 5.1), metabolizes very long chain fatty acid ($>C_{20}$), long chain dicarboxylic acids, eicosanoids, bile acid precursors, and side chains of some xenobiotics. The same pathway is responsible for generating H_2O_2 , known to be involved in oxidative stress related to HCV pathogenesis.

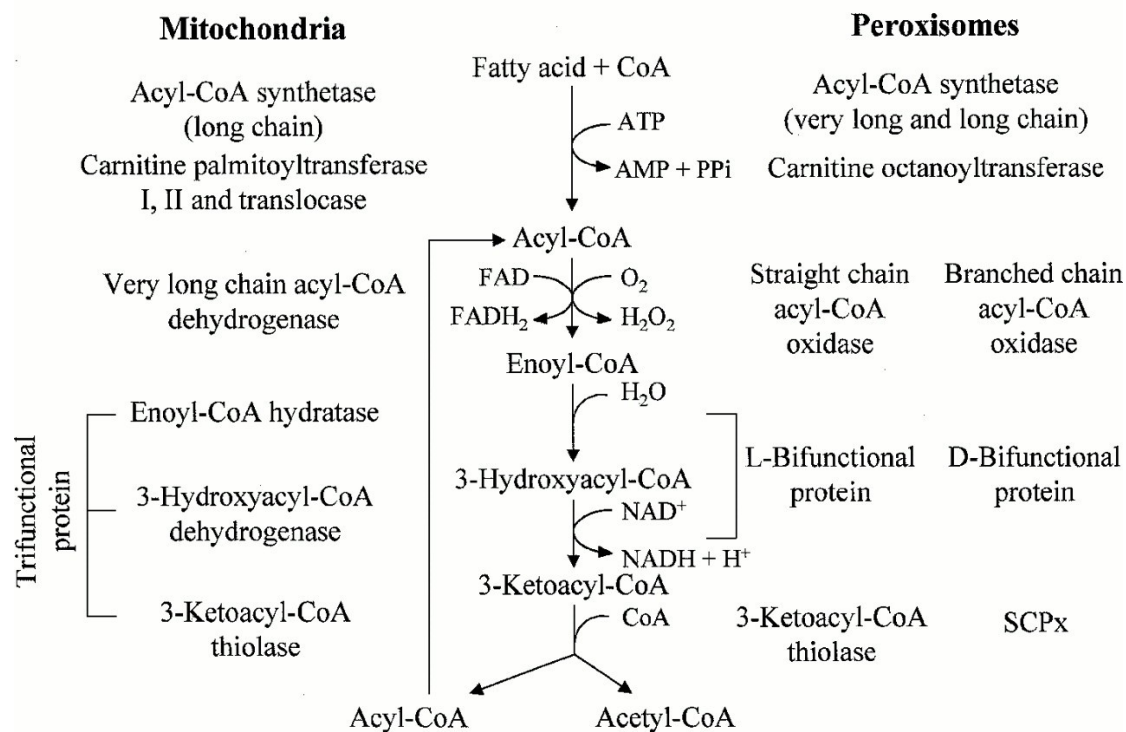
Acyl CoA-oxidase 1(AOX) and Acetyl-CoA acyltransferase 1(Acaa1) are both part of this pathway, and were up-regulated in the HCV-transgenic model. The addition of ethanol,

probably through its effect on HCV-core expression, further increases the AOX expression (Table 3.4). AOX is the rate-limiting enzyme of the peroxisomal fatty acid β -oxidation pathway, and catalyzes the oxidation of the very long chain acyl-CoA thioesters to their corresponding trans-2-enoyl-CoAs ,

Activation of PPAR α should lead to improvement rather than aggravation of steatosis, which is not the case. Tanaka et al (298) showed that steatosis was absent in PPAR α -null or-heterozygous HCV core gene transgenic mice, but present in PPAR α -intact HCV core gene transgenic mice at the age of 9 or 24 months, probably due to mitochondrial dysfunction in the core gene transgenic mice. It is known that the function of the peroxisomal fatty acid oxidation system is to shorten the fatty acid chains and not to degrade fatty acids to completion. The medium-chain acyl-CoA esters resulting from peroxisomal fatty acid oxidation are shuttled to the HCV core-damaged mitochondria for full oxidation to CO₂ and H₂O, resulting in increased steatosis. There was an increase in the number of peroxisomes in the ethanol-treated transgenic mice compared with the transgenic littermate mice on normal diet (Figure 3.8), which might be explained by the presence of higher levels of HCV-core induced by ethanol, which further activates PPAR α receptors. In the absence of HCV, ethanol consumption impairs PPAR α function (60, 105). Ethanol ingestion is known to induce high levels of hepatic fatty acids which should serve as ligands for PPAR α . In reality, ethanol decreases the protein level of retinoid X receptor alpha (RXR α) (29), probably through increased plasma lipopolysaccharide levels (29, 312).

Figure 5.1 Fatty acid β -oxidation Fatty acid β -oxidation occurs in mitochondria and peroxisomes. The very long chain (greater than C-20) fatty acids undergo initial oxidation in peroxisomes which is followed by mitochondrial oxidation. Very long chain fatty acid β -oxidation is not coupled to ATP synthesis, with the transfer of the high-potential electrons to O_2 , which yields H_2O_2 . Hydrogen peroxide is converted to water and oxygen by catalase, an enzyme found exclusively in peroxisomes and which also metabolizes alcohol.

FATTY ACID β -OXIDATION



Ethanol decreases as well the ability of PPAR α /RXR in liver nuclear extracts to bind its consensus sequence, giving further inhibition of fatty acid-metabolizing enzyme.

The antagonistic effects of HCV and ethanol on RXR α receptors may explain the different distribution of steatosis found in our model. Centrolobular distribution of fat globules found in the HCV transgenic mice on a normal diet (Figure 3.6, 3.7, Table 3.3) may be related to the constant activation of PPAR α due to core/RXR α interaction. The alcohol treatment of non-transgenic mice should result in pan-distribution of lipid droplets due to inhibition of PPAR α due to ethanol-reduced levels of RXR α . In the presence of both the HCV transgene and ethanol the distribution of steatosis is changed from centrolobular pattern to periportal, due to opposite effects of HCV-core protein and ethanol on the centrolobular RXR α . Similar patterns of lipid droplet distribution were seen in patients with chronic hepatitis, where centrolobular distribution is more frequently seen in comparison with pan-steatosis in alcohol liver disease (351). Also, microvesicular steatosis was more frequent in patients with chronic hepatitis C, in contrast with macrovesicular steatosis of patients with alcohol liver diseases. My results correlate with this study, suggesting a higher frequency of macrosteatosis in the ethanol-treated transgenic mice, compared with microsteatosis in the HCV-transgenic mice on a normal diet (Table 3.3). Ultrastructural changes were represented by disturbed mitochondrial structure likely due to increased production of free radicals. Also the endoplasmic reticulum was proliferated and dilated, with an increase dilatation in the ethanol-treated transgenic mice compared with the ones on a normal diet a result of HCV-proteins accumulation following higher protein expression induced by ethanol (Figure 3.8). Normally, newly synthesized proteins with proper folded structure are transported from the ER to other intracellular destinations, while the incompletely folded or misfolded proteins are retained and eventually degraded (121). There are studies (198) suggesting that the carboxy-

terminal domain of E2 (presumably its transmembrane domain, and missing in the transgenic model studied here because the E2 protein is prematurely terminated at amino acid 683) is important for E1/E2 heterodimer formation and stabilization and also assists E1 in its folding. The lack of this carboxy-terminal domain of E2 in this transgenic model may result in accumulation of these proteins in the ER.

The purpose of the second part of my thesis was to investigate the effects of HCV structural proteins, core, E1 and E2, on cytokine expression and other DC functions, as potential mechanisms of HCV escape from the immune system. To accomplish this goal, DCs were obtained by culturing bone marrow cells for seven days in the presence of GM-CSF, IL-4 and TNF α or LPS with or without HCV core/E1/E2 polyprotein. Bone-marrow cells were isolated from the femur and tibia of B6C3F1 mice. After addition of GM-CSF and IL-4, most cells differentiate into immature DCs which are able to take up antigens by endocytosis (23). There are two major DC subsets, which are differentiated by their surface markers: CD11C⁺ myeloid DCs (mDCs) and CD123⁺ plasmacytoid DCs (pDCs) (22). GM-CSF and IL-4 induce mainly mDCs. These cells prime Th1 T-cell responses and are known to produce large amounts of interleukin-12 (IL-12) after bacterial or viral infection (22). In my case, I examined immature DC differentiation into mature DCs after addition of HCV core/E1/E2 polyprotein or HIV Gp120 protein. This was accompanied by upregulation of major histocompatibility complex (MHC) and costimulatory molecules like CD86 (B7-2), which play an important role in effective induction of antigen-specific immune responses. DCs are the professional cells capable of presenting processed antigens to naïve T cells in order to generate effector T cells (44). The flow cytometry analysis of cell subpopulations in the bone-marrow derived DC culture showed increased expression of CD11c, CD86, and MHC class II.

There are conflicting results regarding DC dysfunction during HCV infection. Several studies suggested impaired mDC function in chronic HCV infection (16, 20, 146), with different HCV proteins having various effects on DCs. Core protein inhibits the MHC class I antigen-processing pathway and was associated with impairment in the ability of mDCs to stimulate CD8⁺ T cells (227) and decreased secretion of IL-12 with subsequent inhibition of Th1 responses (318). HCV also reduces circulating DCs in chronically infected HCV patients (333). Core, E1, and NS3 proteins were also associated with decreased DC maturation capacity (265, 292). Increased IL-10 and reduced IL-12 production induced by core and NS3, but not E2, were associated with DC dysfunction (76). In addition, HCV core protein was found to be partially responsible for the inhibition of TLR7-induced plasmacytoid DC CD40 expression, followed by low CD4 T cell priming (170).

One objective of my project was to investigate DC maturation in the presence of the HCV structural proteins. My results showed that DCs loaded with core/E1/E2 were limited in maturation, as measured by expression of MHCII and CD86, compared to a control antigen, HIV-gp120. (Figure 4.1, Table 4.1). These data suggest that HCV-core/E1/E2 polyprotein is not an efficient inducer of DC maturation. Other studies suggest an inhibitory effect of these viral proteins on DC maturation. Chronic HCV infection affects the maturation and allostimulatory function of DCs (16, 107, 146). Core protein and NS3 were shown to inhibit DC differentiation (76) while recently, HCV replication was found to inhibit in vitro DC maturation (84).

Several studies highlight the importance of cell-mediated immune responses in the spontaneous clearance of acute hepatitis C. Strong and multi-specific CD4⁺ and CD8⁺ T cell antiviral responses in the early stages of HCV infection are critical for disease resolution and virus clearance (203, 313). The mechanisms by which the T-cell mediated responses

contribute to viral clearance in acutely HCV-infected patients is not fully understood. However, failure to generate or sustain an antiviral CD4⁺ T cell response is a likely mechanism of viral persistence (115). If there is no appropriate DC maturation, CD4⁺ T cells may fail to get activated, which could also interfere with the emergence of protective CD8⁺ T cell responses that destroy infected cells. Rebounds in HCV viral load are frequently associated with low CD4⁺ T cell responses accompanied by persistent, but defective CD8⁺ T cell responses (38, 108).

Another objective of my project was to analyze the capacity of the HCV core/E1/E2-loaded mDCs to activate CD4⁺ T-cell lymphocytes. These loaded mDCs inhibited the polyclonal activation of CD4⁺ T-cell lymphocytes (Figure 4.2). To further investigate mDCs dysfunction, I analyzed the expression of cytokines following LPS treatment. Core/E1/E2 polyprotein reduced the LPS-elicited secretion of cytokines (IL-12, IFN γ , IL-6, MCP-1 and MCP-5) that mediate the stimulation and chemotaxis of T cells (Figure 4.3).

Viral antigens stimulate up-regulation of costimulatory receptor expression on DCs and the secretion of numerous proinflammatory cytokines, including IL-12. These inflammatory signals are important for CD4⁺ T cell differentiation, especially the Th1 cytokine IFN γ . IL-12 is known to stimulate the growth and function of T cells, and further induces production of IFN γ . My results agree with other studies (76, 142, 146, 318), and suggest HCV-core/E1/E2 inhibition of IL-12/ IFN γ production by mDCs. Another potential HCV mechanism of inhibition of Th1 differentiation of CD4⁺ T cells is the interaction of HCV core protein with the gC1q receptor followed by suppression of DC IL-12 production (318).

IL-6 induces intracellular signaling cascades followed by inflammatory cytokine production. My data suggest an inhibition of IL-6 production induced by the structural proteins of HCV. Monocyte-derived DCs from chronic HCV patients have been shown to

exhibit aberrant cytokine profile, with decreased IL-6 and TNF α production (107); also lower levels of IL-6 were detected in the serum of HCV-infected patients (61).

Under normal conditions, DC maturation is associated with a different pattern of chemokine production. After stimulation with LPS, TNF α or CD40 ligand, the inflammatory chemokines MIP-1 α , MIP-1 β are produced rapidly at high levels, but for a short period of time. In contrast, RANTES and MCP-1 are produced in a sustained fashion. The TARC chemokine is constitutively expressed in immature DC and is up-regulated following maturation. Constitutive production of inflammatory chemokines provides DC with the capacity to self-regulate their traffic and also to recruit other immune cells (259).

To support my findings of reduced activation of DCs and the loss of CD4⁺ T-cell responsiveness in the transgenic mouse model expressing HCV core, E1, and E2 proteins, microarray analysis of hepatocytes from the transgenic mice (Naas et al, manuscript in preparation) revealed inhibition of MHC II genes expression, histocompatibility 2, class 2 antigen A, alpha (H2Aa), cathepsin L (CtSL) and MHC class II locus Mb1 (H2-DM b1). The microarray results were further confirmed by real time PCR analysis of MHC II gene expression, and indicate significantly more down-regulation of the MHC II genes in older mice than in younger transgenic mice.

Exogenously viral peptides are processed and presented to CD4⁺ T cells via the MHC II pathway (73). Under normal conditions, the $\alpha\beta$ chains assemble in the endoplasmic reticulum with the help of an Ii chaperone protein (262). The CLIP region of the Ii binds the peptide-binding groove of the $\alpha\beta$ chains, and blocks any premature peptide loading (110). The supercomplex formed by the MHC II molecules and Ii enter the MHC class II compartment (MIIC) (242). Here the Ii is degraded by cathepsins, until only the CLIP remains bound to the peptide binding groove and viral peptide loading can proceed. H2-DM

protein (or H-2M in mice) is required to stabilize the MHC class II molecule while it is in the peptide-free state (353). The MHC II molecule/peptide complex is then sent to the cell surface, where it presents the antigenic peptide to an immature CD4⁺ T cell. The primed CD4⁺ T cell migrates to the lymph node, where it can proliferate and activate an immune response against the specific antigen. Sarobe et al showed that expression of HCV core and E1 in DCs induced abnormal priming of CD4⁺ T cells, leading to inhibited CD4⁺ T cell proliferation both in vitro and in vivo (264, 266, 267).

A functional MHC II antigen presentation pathway is dependent on the intact expression of all the genes involved. Naas et al (manuscript in preparation) demonstrated that three of the genes involved in this pathway were down-regulated in the transgenic mice. The H2-Aa gene encodes for the α chain of the MHC II molecule, and is essential in the formation of the peptide-binding groove. Without this pocket, Ii would be unable to associate with the MHC II molecule, and the complex would not be trafficked to the MIIC. Cathepsin (Ctsl) cleaves Ii resulting in the CLIP fragment which will be exchanged with the antigenic peptide. Lastly, H2-Mb1 is essential for the stabilization of the MHC II molecule in the peptide-free state (110). The absence of this protein would prevent efficient loading of any antigenic peptides and presentation to CD4⁺ T cells.

A dysfunctional MHC II antigen presentation pathway compromises the breadth and strength of the CD4⁺ T cell response against HCV antigens. These findings are a potential mechanism that explains the deficit in T cell priming induced by impairment of dendritic cell function during persistent HCV infection.

General discussion: The mechanisms of hepatitis C pathogenesis are still unknown. An important impediment to advance this area was the lack of a viral culture system and the lack of appropriate animal models. Replicon-expressing cell lines and transgenic murine models

have been developed and many studies demonstrate multiple effects of HCV structural proteins on cytokine response, cellular signal transduction, growth regulation, cellular transformation, necrosis and apoptosis. Viral cytotoxicity and immunomediated mechanisms have been suggested to play important roles in the pathogenesis. However, few studies address these questions. The first part of my work focused on explaining the mechanisms underlying the cytopathic effect of viral structural proteins (core, E1 and E2) combined with ethanol treatment and induction of pathological changes in the liver of ethanol-fed HCV-transgenic mice. I tried to associate histological abnormalities with the alteration of the hepatocyte metabolism uncovered by my analysis. The histopathological changes observed in the liver of the HCV-transgenic mice were exacerbated by ethanol administration, an important exogenous factor also implicated in reducing responsiveness to IFN α therapy.

The five week ethanol treatment of the HCV-transgenic mice induced a significant decrease in body weight gain in both transgenic and non-transgenic littermates, induced probably by an altered systemic metabolism and an imbalance in thermoregulation. The co-existence of the structural proteins of HCV and ethanol administration increased liver histological damage, also reflected biochemically in high serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

This study demonstrated that ethanol specifically induces an increase in HCV-viral mRNA and viral protein expression *in vivo* in a transgenic mouse model. However, the higher expression of HCV-viral mRNA in this transgenic mouse model cannot be compared with HCV replication during viral infection, with HCV-mRNA expression being driven from a different promoter in the transgenic mouse model, the CMV promoter. On the other hand, the higher expression of HCV viral proteins after alcohol treatment, followed by liver

pathological changes observed in these transgenic mice are comparable with the outcomes of the HCV viral infection in alcoholic patients.

The levels of lipid peroxidation were elevated as a result of increased oxidative stress in both ethanol-treated nontransgenic mice, as well as in transgenic mice expressing HCV core/E1/E2 on a normal diet. An analysis of the antioxidant profile revealed that antioxidative molecules were decreased in both cases despite the overproduction of reactive oxygen species. In contrast, the presence of both HCV and ethanol induced higher glutathione peroxidase activity. Furthermore, the levels of lipid peroxidation in the presence of both conditions were still higher than normal, and suggest that the antioxidative system is not able to counteract the high levels of lipid peroxidation.

Acyl CoA-oxidase 1 mRNA was elevated in the HCV-core/E1/E2 transgenic mice (Naas T. et al, manuscript in preparation) and is the rate-limiting enzyme of the peroxisomal fatty acid β -oxidation pathway. This enzyme is activated by RXR α - PPAR α dimerisation. Ethanol treatment further upregulated the mRNA levels of Acyl CoA-oxidase 1 in the HCV-transgenic mice. Some of these results require further evaluation to better explain the new findings. For example, the activation of PPAR- α activates fatty acid β -oxidation and should lead to improvement rather than aggravation of steatosis. In contrast, I detected increased steatosis, probably due to mitochondrial dysfunction in the transgenic mice. The function of the peroxisomal fatty acid β -oxidation system is to shorten the fatty acid chains, but not to degrade fatty acids to completion. The medium-chain acyl-CoA esters resulting from the peroxisomal fatty acid oxidation are then shuttled to the HCV core/E1/E2-damaged mitochondria for full oxidation to CO₂ and H₂O, resulting in increased steatosis. Ethanol in the absence of HCV decreases the protein level of retinoid X receptor alpha (RXR α). Antagonistic effects of HCV structural proteins and ethanol on RXR α receptors may explain

the different distribution of steatosis found in the transgenic model. In the presence of both HCV transgene and ethanol treatment the distribution of steatosis was changed from a centrilobular pattern, observed in the HCV-transgenic mice on normal diet, to periportal, due to the opposite effects of HCV-core protein and ethanol on centrilobular RXR α .

The ultrastructural changes seen in mitochondria are likely due to increased production of free radicals with a severe impact on the morphology of these organelles. The proliferation of endoplasmic reticulum, seen in the transgenic mice is exacerbated by alcohol consumption, and is probably due to the accumulation of HCV structural proteins following the higher protein expression induced by ethanol. There was also an increase in the number of peroxisomes in the ethanol-treated transgenic mice compared with the transgenic littermate mice on a normal diet, which might be explained by the presence of higher levels of HCV-core which further activates PPAR α receptors.

The modulation of the immune response may be one more mechanism of HCV pathogenesis. Earlier studies on the adaptive cellular immune response to HCV established the predominance of the cytotoxic T-cell responses, but recently the role of CD4⁺ T cells in viral clearance was re-emphasized. In the second part of my work, the potential impairment of dendritic cell function was analyzed, as one possible mechanism to explain the loss of CD4⁺ T-cell responsiveness induced by HCV infection.

The viral structural proteins induced inhibition of DC maturation. The capacity of the HCV-core/E1/E2-loaded mDCs to activate and polarize CD4⁺ T-cell lymphocytes was analyzed. The loaded mDCs inhibited the polyclonal activation of CD4⁺ T-cell lymphocytes indicating a dysfunctional CD4 T cell response during core/E1/E2 polypeptide stimulation.

Inefficient CD4 priming suggested by my results might lead to chronic HCV infection in humans. This data is supported by the microarray analysis of the core/E1/E2 transgenic

mice, showing inhibition of MHC II gene expression, including histocompatibility 2, class 2 antigen A, alpha(H2Aa), cathepsin L (Ctsl) and MHC class II locus Mb1 (H2-DM b1). (Naas T. et al, manuscript in preparation). The presence of the core/E1/E2 polyprotein also reduced the DC expression of certain cytokines (IL-12, INF γ , MCP-1, IL-6) important for stimulation and chemotaxis of T cells and other immune cells.

Significance: future studies should address the molecular mechanism of pathogenesis and synergy between ethanol and HCV infection in chronic liver disease and how they involve the modulation of the host immune response. However, emphasis on novel prevention methods may have a larger impact than the study of these molecular mechanisms. The result of the more detailed molecular description and metabolic impact of viral infection and ethanol abuse could unveil new approaches that may be adjuncts to vaccination programs and educational programs to prevent ethanol abuse.

Final Conclusions and Thoughts: The diagram below represents a summary of what I believe are important elements that explain the pathogenesis of liver disease in HCV infection and ethanol abuse. The functions of antigen-presenting dendritic cells and other key immune cells are disrupted by both ethanol and HCV proteins (293). Geissler and colleagues noted that chronic alcohol feeding of mice inhibited T-helper cell and cytotoxic T-lymphocyte functions important in the clearance of HCV. In addition, alcohol consumption is known to significantly decrease the efficacy of interferon therapy in chronic hepatitis C patients (205). Oxidative stress, alcohol, and HCV core protein have each been proposed to inhibit the cellular interferon response by interfering with the JAK–STAT signaling pathway. This inhibition of the immune response would allow for viral persistence and exacerbated pathogenesis via oxidative stress.

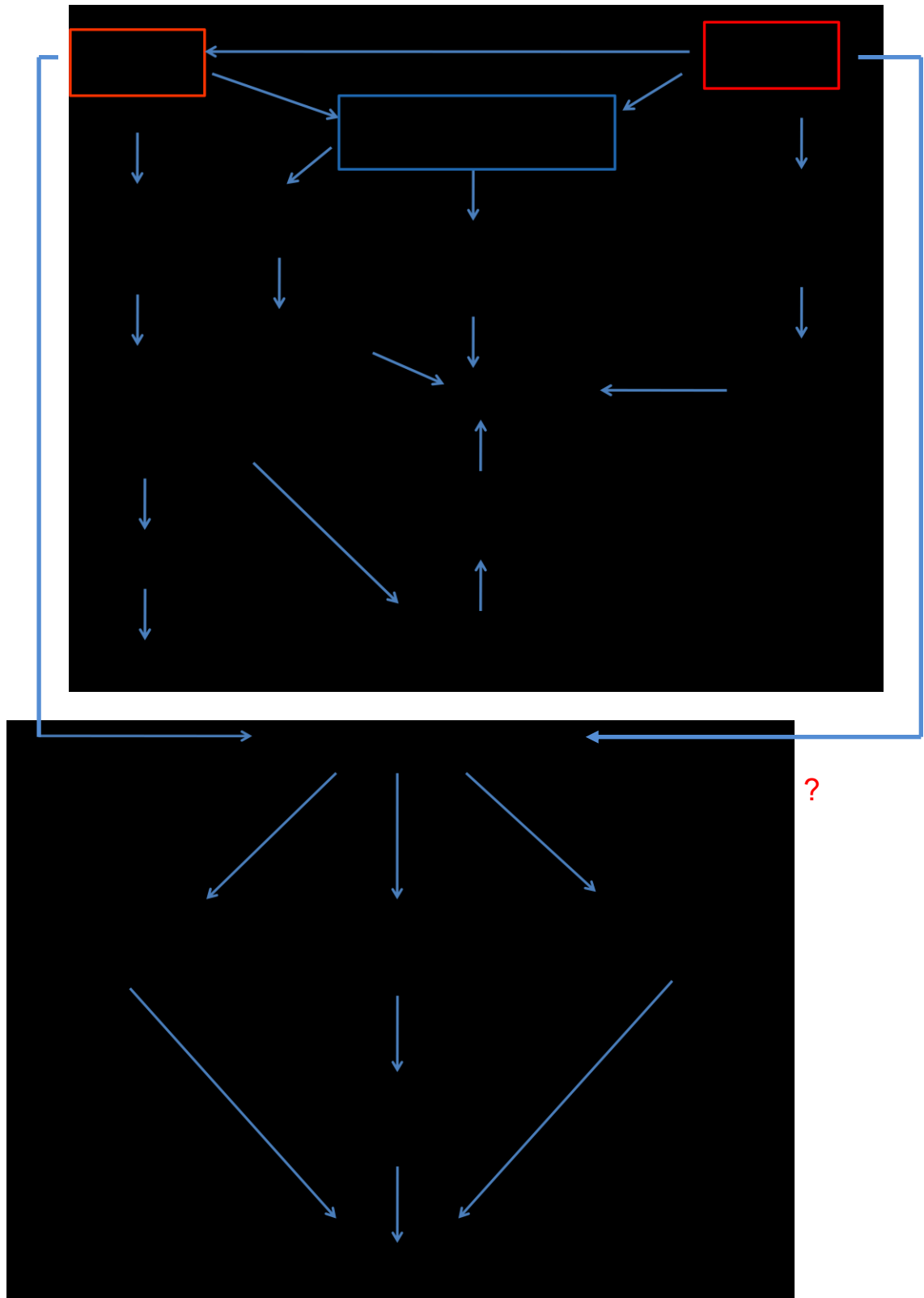


Figure 5.2 Model of HCV pathogenesis

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