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Cell-Mediated Immune Responses Observed After the Adoptive Transfer of HCV-Primed Splenocytes in an HCV Transgenic Mouse Model

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TABLE OF CONTENTS

Abstract

HCV is a positive stranded RNA virus that infects up to 170 million people worldwide. Chronic infection is established in 80% of the cases and may lead to cirrhosis and hepatocellular carcinomas. A major debate regarding chronic HCV pathogenesis is whether liver damage is host or virus mediated. CD4+ and CD8+ immune responses are needed to control infection but these are often inefficient, resulting in chronic infection. The presence of these responses in chronic infection may contribute to liver pathogenesis. To study the relevance of immune-mediated liver damage, we created an immunization regimen which elicited humoral and cellular immune responses in B6C3F1 mice. HCV-primed donor splenocytes were stained with CFSE and transferred to a CE1E2 HCV Tg mouse model. Donor cells were recovered in various organs eight days following the transfer. HCV-primed CD4+ and CD8+ T cells selectively accumulated in the liver of the Tg mice where the CE1E2 transgene is predominantly expressed and caused liver pathology under the form of lymphocyte infiltration. The accumulation of these cells indicates a homing mechanism to this organ and a role in the development of liver pathologies. Further studies are required to unearth the mechanisms behind cellular trafficking and cell-mediated immune damage.

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LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa	Amino acids
ALT	Alanine aminotransferase
APC	Antigen presenting cell
AST	Aspartate aminotransferase
AxCANCre	Adenovirus expressing Cre recombinase
BSA	Bovine serum albumin
CBS	Canadian Blood Services
CCR	Chemokine receptor
cDNA	Complementary DNA
CE1E2	Core E1 E2 polyprotein
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DC-SIGN	DC-specific intracellular adhesion molecule 3 grabbing nonintegrin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ds-RNA	Double-stranded RNA
E1	Envelope 1
E2	Envelope 2
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
ER	Endoplasmic reticulum
Fas-L	Fas-ligand
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
H&E	Hematoxylin and eosin
His	Histidine
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HVR	Hypervariable region
ICAM	Intracellular adhesion molecule
IDU	Injection drug use
IFN	Interferon
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IL	Interleukin
IM	Intramuscular
IMDM	Iscove's Modified Dulbecco's Medium
IP	Intraperitoneally

IRES	Internal ribosomal entry site
IRF	IFN regulatory factor
ISGs	Interferon stimulated genes
JNKs	c-Jun amino-terminal kinases
kb	Kilobases
kDa	Kilodaltons
LSEC	Liver sinusoidal endothelial cell
MAPK	Mitogen activated protein kinase
MC	Mixed cryoglobulinemia
MCP	Monocyte chemoattractant protein
MHC-I	Major histocompatability complex I
MHC-II	Major histocompatability complex II
ml	Milliliter
NK	Natural killer cell
NS	Non structural
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PKR	Protein kinase
PMA	Photbol myristate acetate
pp	Pseudo particles
RNA	Ribonucleic acid
RBC	Red blood cells
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SFC	Spot forming cell
STAT	Signal transducer and activator of transcription
SVR	Sustained virological response
Tg	Transgenic mouse
Th	T helper
TNF- α	Tumor necrosis factor-alpha
T Regs	Regulatory T cells
UTR	Untranslated region
VLDL	Very low density lipoproteins
VLP	Viral-like particles
Wt	Wild type mouse

CHAPTER 1:
INTRODUCTION

BACKGROUND

Hepatitis C is a positive stranded enveloped RNA virus that infects up to 170 million people worldwide: the estimated global prevalence of infection is of 3% with 250 000 (0.8%) being Canadians (1). However, these statistics are estimated since many new hepatitis C virus (HCV) infections are asymptomatic and remain largely undiagnosed. HCV was initially named non-A or non-B hepatitis (there are six knows types of hepatitis, namely A, B, C, D, E and G) due to cases of post-transfusion hepatitis. The virus was first identified through molecular cloning as an unknown organism in 1987 and in 1989 the virus was named (2). The enveloped virus has a diameter of around 55-65 nm (3). The HCV genome consists of a single-stranded positive sense RNA genome of 9.6 kb in length (approximately 10 000 nucleotides in length or 3 010 amino acids). It is a member of the *Flavivirus* family (with other viruses such as dengue and yellow fever) and is more specifically from the *Hepacivirus* genus: hepatitis means inflammation of the liver (4). The virus has tropism for hepatocytes, more commonly known as liver cells, but has also been shown to infect extrahepatic immune cells such as peripheral blood mononuclear cells (PBMCs), dendritic cells, B cells and macrophages (5; 6; 7). This suggests that immune dysfunction might be caused by the capacity of the virus to infect immune cells.

Numerous studies have demonstrated that host immune responses are significantly impaired in HCV chronically infected individuals. The virus has been shown to persist despite the presence of HCV-specific circulating antibodies and the production of cytokines such as IFN-γ and IL-2 were shown to be suppressed in T cells. These immune deficiencies indicate that HCV might have an immunosuppressive

mechanism by which persistent chronic infection is established. However, further studies have been hampered by the lack of an appropriate small animal model or highly developed cell culture methods.

One of the modes of viral evasion for HCV may also be contributed to the high rate of replication and the mutation of epitopes. HCV which replicates at around 10^{12} virions per day is one of the fastest replicating viruses known to mankind (8). The sheer quantity of virus produced makes immunological control difficult to achieve due to the volume of emerging mutated virus.

This virus causes long-term complications to the liver, and in the case of chronic infection, a patient may develop steatosis, cirrhosis or hepatocellular carcinoma. No prophylactic or therapeutic vaccine exists to treat the virus and only a subset of patients (around 40%) respond to existing therapies. Consequently, this viral pandemic is a very important disease which causes a high burden on today's society. It is important to elucidate the mechanisms behind HCV pathogenesis in order to obtain a better understanding of the virus to tailor more effective therapies or vaccines. The various facets of HCV and the impact on humans will be discussed below.

MOLECULAR BIOLOGY OF HCV

HCV Viral Structure

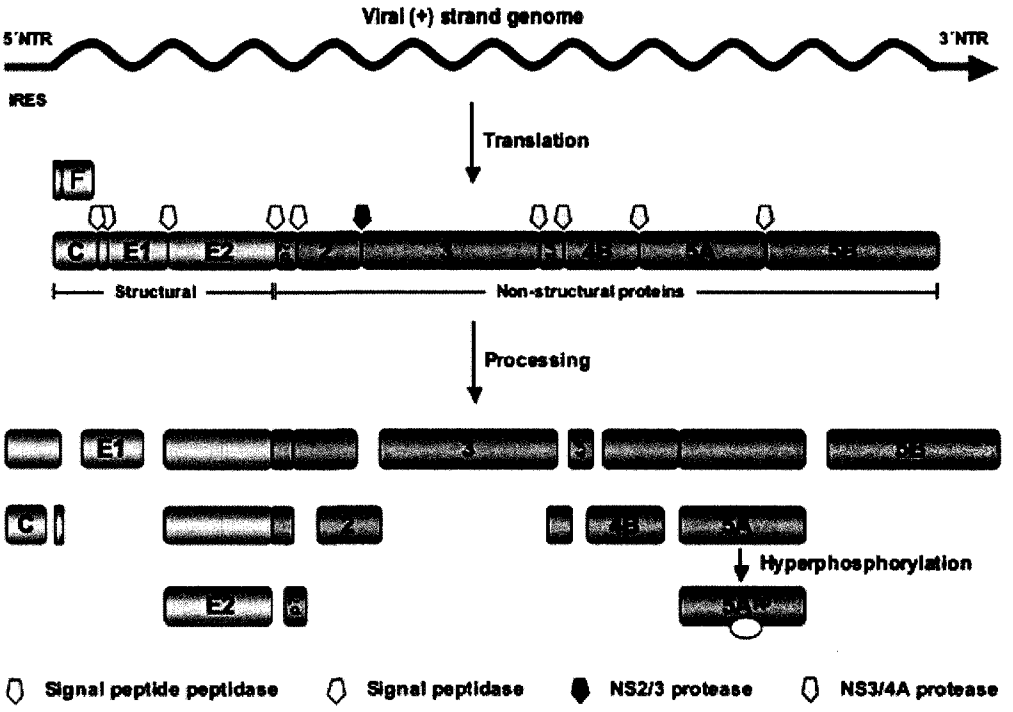
The hepatitis C virus genome consists of a single-stranded positive sense RNA genome of 9.6 kb in length (approximately 10 000 nucleotides in length) (4; 9). This hepatitis-causing virus is enveloped and has a diameter of around 55-65 nm: the envelope surrounds the nucleocapsid and the HCV RNA (3).

The HCV genome contains a long open reading frame (ORF) encoding a polyprotein of 3010 amino acids (aa). The ORF of the virus is flanked by two untranslated regions (UTRs) containing information which is required for viral protein and RNA processes such as initiation of translation and replication (10). The 5' UTR is especially important since it contains an internal ribosomal entry site (IRES) where protein translation begins (11). The polyprotein is processed by cellular (signal peptidase) and viral proteases in order to yield 10 products. These consist of 3 structural proteins at the 5' end core, envelope 1 and 2 and 7 non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (12). Cleavages by the host signal peptidase occur at C/E1, E2/E2, E2/p7 and p7/NS2 for the structural region (**Figure 1.1**).

HCV Structural Proteins

The hepatitis C virus contains three structural proteins, namely core, E1 and E2 which are the building blocks for the viral packaging processes and form a protective viral coat. Of the structural proteins, the highly basic 21 kDa core capsid (p22, which is non glycosylated) is required for nucleocapsid assembly (with the genomic RNA) to allow budding of the packaged virus through intracellular membranes: this protein associates with HCV RNA to form the nucleocapsid (10). The core protein is highly conserved and contains several B-cell epitopes in the N-terminus (13).

Figure 1.1. Schematic representation of HCV genome organization and processing pathways of the polyprotein. The HCV genome consists of a positive stranded enveloped RNA virus around 9.6 kb in size. The genome carries a long open frame encoding a polyprotein precursor of 3010 amino acids which is flanked by 5' and 3' non terminal regions (NTR). The 5' NTR of the genome contains an internal ribosome entry site. The HCV polyprotein is cleaved co- and post-translationally by cellular and viral proteases (as indicated by the arrows) in the cytoplasm of the host in order to yield 10 products: 3 structural proteins (core, E1 and E2) and 7 non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). Alleged functions of certain proteins are as follows: core (nucleocapsid), E1 and E2 (envelope glycoproteins), NS2 (transmembrane protein), NS3 (serine protease), NS4A and NS4B (cofactors), NS5A (IFN-resistance protein) and NS5B (RNA polymerase) (14).


Viral (+) strand genome
5'NTR
3'NTR
IRES
Translation
C
E1
E2
Structural
Non-structural proteins
Processing
Hyperphosphorylation
Signal peptide peptidase
Signal peptidase
NS2/3 protease
NS3/4A protease

Downstream of the core protein, HCV envelope glycoproteins, namely E1 and E2, interact to form a non-covalent heterodimer complex which is present at the surface of HCV particles: these heavily N-glycosylated proteins are needed for viral assembly at the cell membrane (15; 16). These proteins are of 33 and 70 kDa for E1 and E2 respectively when glycosylated. A hypervariable region (HVR) is found within the E2 protein at aa 1-27. It is likely that this highly variable region is subject to immune pressure (17). E2 may thus escape immune surveillance since the N-terminus of the protein has a surface loop which contains a B-cell epitope that undergoes antigenic evolution over time (18). When HCV proteins mutate, neutralizing antibodies directed to these epitopes no longer recognize the antigenic site. For our purposes, a neutralizing antibody can be defined as an antibody which reacts with a virus and inhibits or destroys its infectivity. When B cell and T cell epitopes change, existing immune responses are no longer effective. This has been suggested as one of the mechanisms by which HCV evades the immune response.

E1 and E2 proteins undergo folding by chaperones located in the endoplasmic reticulum (ER) (19). It is thought that these proteins are then transported to host cell membranes, where they eventually assemble around HCV viral RNA: the packaged virus then buds through the membrane containing the glycoproteins, consequently collecting pieces of host membrane. Although the roles of these proteins have not been completely elucidated, it is thought that E2 is involved in the binding of HCV to a cell receptor (CD81 has been proposed) and that E1 functions as a fusion protein (20; 21).

HCV Non-Structural Proteins

The non-structural proteins (NS) of HCV contribute at many different steps in the HCV viral life cycle: p7 (viroporin), NS2 (protease), NS3 (serine protease and helicase),

NS4A (protease co-factor), NS4B (formation of replication complexes), NS5A (RNA binding) and NS5B (RNA-dependant-RNA polymerase) (12; 22). As mentioned above, the NS2 protein is cleaved by host signal peptidase at the p7/NS2 junction. This is then autocleaved by a viral protease consisting of NS2 and the N-terminus of NS3 to release NS2 from the NS2/NS3 junction (22).

In addition to having protease activity at its N-terminus, NS3 is also known to encompass a RNA-helicase domain at the C-terminus, which is important during replication for strand separation (23). The remaining cleavages of the polyprotein to yield individual products are completed by NS3 or viral serine protease. NS3 has been found to interact with NS4A, being the protease co-factor, by forming a heterodimeric complex for the cleavage of NS3/4A, NS4A/4B, NS4B/5A, and 5NSA/5B) (24).

The non-structural proteins of HCV play important roles in RNA replication. Following polyprotein processing, the non-structural proteins of the virus form replication complexes with the viral RNA to start negative strand RNA transcription: HCV proteins and RNA form membrane-associated replication complexes in a perinuclear membranous web (10; 25). The RNA dependent RNA polymerase of HCV, namely NS5B, makes multiple copies of the original positive stranded HCV genome; these negative RNA strands act as intermediates in order to generate more positive RNA strands which will be encapsulated in later steps. Although the role of p7, also know as viroporin, is still controversial, it is thought to be an ion channel mediating membrane permeability and secretion. This would be important to protect glycoproteins during the late stages of release and maturation of the virus (26).

Viral Life Cycle

New *in vitro* cell culture methods have only recently become available for certain genotypes, but many steps in the HCV life cycle are still debated. Our understanding of HCV has mainly been derived from similar viruses (such as the bovine viral diarrhea virus) and the molecular analysis of HCV proteins. Briefly, HCV life cycle can be explained as follows: **1**) binding of HCV virion to hepatocyte surface receptor followed by receptor mediated endocytosis, **2**) uncoating of HCV virion to release RNA into the cell cytoplasm, **3**) translation of viral RNA directly in the cytoplasm using host's ribosomes to generate the polyprotein, **4**) processing of polyprotein and formation of replication complexes, **5**) transcription of negative sense complementary HCV RNA to be used as a template for the synthesis of progeny positive stranded RNA, **6**) packaging of newly produced positive stranded RNA with envelope proteins into virions, **7**) release of the progeny virions, and **8**) infection of new hepatocytes (or transmission to new host through contaminated blood products).

Controversy still surrounds the mechanism(s) and receptor(s) by which HCV gains access to the cell cytoplasm. However, it is likely that HCV E2 mediates binding to a receptor followed by the endocytosis of the virus (20). Possible receptors for viral entry will be discussed in the subsequent paragraph. Recent studies have indicated that the endocytosis of HCV is done in clathrin-coated pits and it is thought that a drop of pH within the phagosomal vesicle permits changes in the envelope proteins which lead to the fusion of viral and cellular membranes (27). As a result of this uncoating, the HCV RNA is delivered to the cytoplasm for translation where it works directly as an mRNA. As mentioned above, after the translation of the polyprotein, replication occurs and the

formation of more positive sense RNA genomes is done by the RNA dependent RNA polymerase (NS5B).

The processing of budding and release from the cell continues until the cell dies from exhaustion: the viral life cycle of HCV is around 3 hours, and this virus replicates at a rate of around 10^{12} virions per day (8). HCV RNA genomes released into the blood from infected hepatocytes reach a peak number 6 to 10 weeks into the acute phase of infection: this is the case regardless of future chronic state (28). Additional studies are required to elucidate all the mechanisms and processes involved in the HCV viral life cycle.

Receptors for HCV Viral Entry

Several molecules have been proposed as potential receptors or capture molecules for HCV absorption and penetration. However, due to difficulties associated with HCV *in vitro* propagation, the interaction of the whole virus with different cell receptors has yet to be studied. Advances in studying these receptors have been achieved through truncation of HCV E2 protein (truncated E2 protein is not secreted, retained in the ER), the creation of viral-like particles (VLPs), and HCV pseudoparticles (HCV pp) among others (19; 29; 30). As HCV is hepatotrophic, the required receptor should be predominant on hepatocytes.

CD81, a cell surface protein expressed on most human tissues, is usually involved in cellular activities such as cell adhesion, cell activation and signal transduction (31). The binding site for E2 on CD81 has been mapped to the large extracellular loop of this molecule (32). Interestingly, the use of anti-CD81 mAb inhibits the entry of HCV pp into hepatoma cell lines (33).

Another potential receptor involved in HCV entry would be the human scavenger receptor class B type I (SR-BI), a cell surface glycoprotein which interacts with low density lipoproteins (34; 35). SR-BI which has a high expression particularly in the liver, has been shown to interact with soluble E2: the blocking of SR-BI on Huh-7 cells reduced the infectivity of HCV pp (34; 36). Additionally, heparin sulfate, a carbohydrate which inhibits blood coagulation, has been shown to interact with E2 (37).

Taking into account the interaction of CD81 or SR-BI with HCV, it is attractive to state these receptors are required for HCV entry. However, their ectopic expression on non-liver cells does not lead to HCV pp entry, suggesting that additional molecules are also required (30).

Regardless of having tropism for hepatocytes, HCV may also be capable of binding receptors on different cell types. Soluble E2 can bind to DC-SIGN, a receptor on DC which interacts with T cells (38). This can act as a double edged sword since DC-SIGN binds HCV with high affinity, hence DC may increase infectivity of the virus by transmitting it to neighboring cells (38; 39).

These potential receptors, may act alone or in synergy for the endocytosis and entry of HCV in the cell. Further studies are required in improved cell culture systems to determine the exact viral receptors and the mechanisms behind viral entry.

HCV Genotypes (Genetic heterogeneity and quasispecies)

A genotype is defined as the genetic constitution of an organism. Currently, there exist 6 distinct yet related HCV genotypes and 50 plus subtypes (40). HCV subtypes are based on variations in the nucleotide sequence of the core and E1 genes, though the genotypes still have around 70% similarity (41). Viral genotypes differ according to

geographic region: 1, 2 and 3 are distributed worldwide while genotype 4 is found in the Middle East and Africa, genotype 5 in South Africa and genotype 6 in Southeast Asia (42; 43). Interestingly, higher clearance rates have been reported for genotype 2 in Africa (44). However, genotype 2 is far less common than genotype 1. People infected with HCV genotype 3 are most prone to develop steatosis. In terms of treatment, genotype 1 is the most resistant and frequently establishes chronic infection (45; 46).

Quasispecies are defined as non-identical but closely related RNA genomes or viral variants that develop within a host through evolutionary selection resulting in viral heterogeneity (47). Due to the high mutation rate (1 in 1 000 bases per year) of HCV within a host owing to the lack of proofreading function in the RNA dependent RNA-polymerase (NS5B), variants emerge under the selective pressure of host immunity creating quasispecies which are no longer recognized by the existing immune response (48; 49). These quasispecies are able to evade the host's immune responses which can explain the ability of the virus to establish chronic infection. The alterations in the antigenic epitopes allow these quasispecies to escape immune surveillance since they are no longer recognized by the existing T cells or neutralizing antibodies.

LIVING WITH HCV

Transmission in Humans

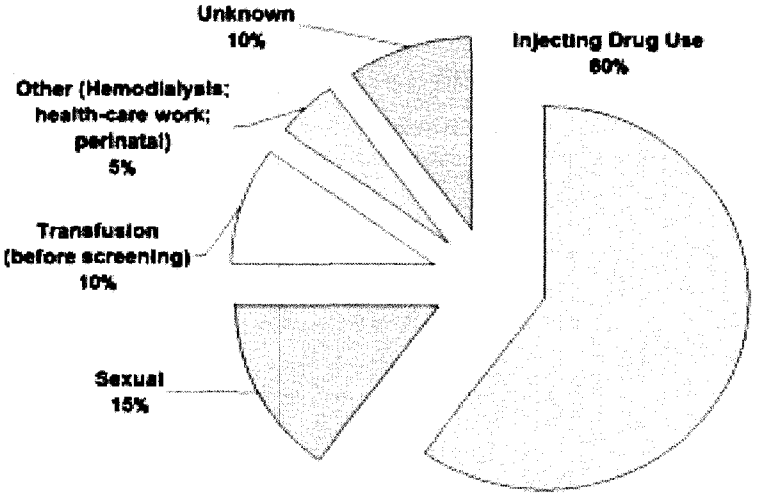
HCV is a blood borne pathogen which is transmitted through blood-to-blood contact. Worldwide distribution of HCV has been facilitated by its efficient transmission through blood products. The virus is frequently transmitted through blood transfusions from unscreened donors, by sharing infected needles or other drug related paraphernalia, through tattoos or medical procedures using poorly sterilized/ unsterilized equipment, by

having sexual relations with an infected individual, being born to an HCV-infected mother or by sharing household items such as a razor, toothbrush or nail file (**Figure 1.2**).

Individuals who received a blood transfusion or an organ transplant in Canada prior to 1990 (before routine Canadian Blood Services (CBS) screening) are at a higher risk for having contracted HCV. In recent years, the incidence of HCV infection has increased in developed countries due to needle sharing by intravenous drug users (50). It is estimated that injection drug use (IDU) could be the route of transmission responsible for approximately 70% to 80% of new HCV cases in Canada (51; 52).

Figure 1.2. Sources of infection for people living with HCV as defined by the Center for Disease Control. It is interesting to note that although this chart indicates that 15% of HCV transmission is sexual, this virus as of yet is not classified as a sexually transmitted infection. Additionally, it is evident that in the 21st century, the main source of infection is attributed to injection drug use (60%). The unknown route of transmission (10%) could be attributed to sharing household items such as razors and toothbrushes with an HCV infected individual. Health care workers may also be at a higher risk of contracting the virus as well as hemodialysis patients. Please note that this data was compiled in 2001 thus sources of infection might currently differ. (Source of chart: Center for Disease Control)

Sources of Infection for Persons with Hepatitis C



Many needle exchange programs and safe injection sites have been established in North America with the goal of reducing HCV transmission. However, in developing countries, blood transfusions remain a major cause of HCV transmission. In these countries, due to financial constraints, many injection devices are re-used (53).

Vertical transmission is infrequent and occurs in 6.7% of children who are born from women who are HCV RNA positive at the time of delivery (54). While HCV is not classified as a sexually transmissible infection, it is believed that 0-3% of straight people who have unprotected sex with a steady infected partner may contract HCV (though HCV is usually not found in semen or vaginal fluids) (55). Finally, in 20-40% of the cases, the route of transmission of the virus is unknown (56).

While HCV transmission occurs through blood to blood contact, an interesting study found that chimpanzee plasma containing HCV was still infectious for up to 16 hours after environmental exposures such as drying and storage at room temperature (57). This suggests that HCV may be able to survive outside the body, and that it may be more infectious than previously thought.

HCV Diagnosis

HCV is difficult to diagnose during the early acute stage due to a paucity of symptoms hence new infections are generally not diagnosed. Symptoms may include jaundice (caused by bilirubin accumulation in blood due to liver malfunction), abdominal pain, nausea, vomiting, fatigue and loss of appetite. Because these non-specific symptoms are manifested during acute infection, the diagnosis of infection is usually not done until the chronic phase. It is difficult to attribute which immune responses are first associated with the onset of infection and what triggers the infection to become chronic.

Early diagnosis is essential since it 1) increases the chances of successful therapy, 2) reduces possible transmission to others and 3) permits appropriate lifestyle changes before additional damage may occur (specifically drinking habits). An infection with HCV may be suspected on the basis of a patient's medical history (blood transfusions), if the patient has undergone unsafe tattooing or piercing or if abnormal or elevated liver enzymes are discovered during routine blood testing. However, during the time where this slow developing disease is left undiagnosed, it may cause irreversible liver damage.

Diagnosis of infection is usually made by identifying antiviral antibodies in the blood by using an enzyme linked immunoabsorbent assay (ELISA). 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 (58). Some individuals however do not develop antibodies until 12-27 weeks after initial exposure to the virus (59). Furthermore, in some immunosuppressed patients, despite infection, anti-HCV antibodies are not detected by ELISA. For example patients co-infected with HIV/HCV may become negative for anti-HCV antibodies despite chronic HCV infection (60). This means that some patients lose the capacity to produce HCV antibodies despite the presence of viremia, and progressively deplete existing antibodies. Further testing is essential to determine whether the infection is resolved or whether it is a chronic infection.

Polymerase chain reaction (PCR) is used on serum samples to detect the presence of HCV RNA. If the HCV RNA is negative, this suggests that the infection is resolved. However, if the HCV RNA is positive, it is evidence that the infection is chronic and further testing is required. The elevation of liver enzymes in serum and the presence of

HCV RNA usually require a liver biopsy to determine whether there has been continuous liver inflammation that requires antiviral treatment.

The PCR to detect HCV RNA is often used to determine the virus load in the blood of the infected patients. The PCR targets non-coding regions at the 5' and 3' ends since these regions are more conserved throughout genotypes (61). As discussed previously, the identification of specific viral genotypes is used for clinical prognostic significance. To determine genotype, reverse hybridization after PCR with genotype-specific probes can be used (62). In addition, CBS screens for potential HCV RNA contamination before using donor blood.

HCV is often first suspected in a patient due to elevation of liver enzymes found during routine blood testing. Alanine aminotransferase (ALT) is an intracellular enzyme that transfers amino groups from L-alanine to 2- ketoglutarate or from L-glutamic to pyruvate which is released into the blood stream: this enzyme is used as an indicator of hepatocyte damage. Many studies have shown that in HCV infection, the levels of ALT increase, indicating liver damage. In a study where chimpanzees were infected with HCV genotype 3, ALT levels peaked at 113 IU/liter on week 12 while viremia peaked at week 10, representing what would happen during an acute human infection (63). However, many HCV chronically infected individuals maintain normal levels of liver enzymes. A study examining accidental needle stick exposure of health care workers to HCV found that 4 out of 5 developed chronic infection, all had varied ALT levels, however, only 2 subjects with the highest levels of ALT progressed to jaundice (58). This indicates that serum ALT might not be the best indicator of liver damage. A liver biopsy

is usually done in patients who have elevated levels of liver enzymes to assess the grade of liver. This study is particularly important because it identifies the course of therapy.

Treatment or Therapy for HCV Infection

As of yet, there is no prophylactic or therapeutic vaccine to treat HCV infection: antivirals are used to treat chronic active infections. The goal of therapy is to prevent the advancement of liver disease by reducing inflammation in the liver and by attempting to control viral replication. It is known that in HCV infection, the core protein skews Th1 responses to Th2: recovered patients have predominant Th1 responses hence these are needed for successful viral clearance (64). Currently, the most common treatment for chronic HCV is combination therapy using pegylated IFN-α and ribavirin, which has a clearance rate of 45-80% in chronically infected individuals (10). The sooner a treatment is started after initial HCV infection, the greater the chance of a positive response to therapy.

IFN-α is a cytokine which is naturally secreted by cells in response to viral infection: it binds to the receptor (on a cell membrane) and initiates intracellular signaling to enhance the expression of certain genes. Some of these activities may include increasing the amount of CTL, normalizing ALT levels and inhibiting viral replication within an infected cell. IFN-α also induces the expression of IFN-γ, a Th1 cytokine, hence having a role in inhibiting viral replication (65). The pegylation of the IFN-α greatly improves the half life of the drug, permitting it to be retained for longer periods of time in the body at stable levels. It has been shown that IFN has a half life of 8 hours, but with pegylation, this can be increased to 45 hours, therefore allowing a patient to require fewer injections (66; 67).

Ribavirin, an antiviral drug, is a synthetic nucleoside that is used to block the replication of DNA or RNA viruses. Concerns surround the use of ribavirin, which causes serious side effects. It is taken by as an oral tablet, with a half life of 12 days, thus persisting in the body for long periods of time (68). Multiple studies have shown that combination therapy of ribavirin with pegylated IFN-α offers a more sustained response than IFN-α monotherapy since IFN-α has direct antiviral activity and enhances antigen-specific T cell responses to HCV while ribavirin reduces hepatic inflammation and further skews the host to a Th1 response (69; 70).

When deciding on the course of therapy, it is necessary to consider viral genotype since this is a good predictor of the effectiveness of treatment to attain a sustained virological response (SVR). It is estimated that 76 - 88% of people with genotype 2 or 3 will achieve a SVR as opposed to 42-48% of people with genotype 1a or 1b (71). Additionally, the duration of treatment for genotype 1 is 48 weeks as opposed to genotype 2 and 3 for which treatment is recommended for 24 weeks: these treatments aim to eliminate HCV RNA from the blood (72). Other factors, namely age, sex (women respond better to therapy maybe due to the positive effects of estrogen), lower viral load (patients with a viral load of less than 800 000 IU/ml are more likely to achieve a SVR), ethnicity (Caucasians and Asians respond better to current HCV therapy) and alcohol abuse can be affect treatment response (73; 74; 75; 76). Progression of HCV disease is especially dependent on the age of the patient at primary infection and incidence of alcohol abuse.

Nevertheless, many concerns are associated with the existing therapies for instance the high cost of the drugs, the relatively low success rate of therapy and the

many adverse side effects of the drugs (flu-like symptoms, depression, abnormal fetal development in child bearing women) (71; 77). In cases where cirrhosis and hepatocellular carcinoma are life threatening, a liver transplant may be indicated. However, donor livers almost always become reinfected: around one month after the transplant HCV RNA levels increase (these may progressively become higher than pre-transplant values) (78).

Co-infection with HIV

Due to the route of transmission of the virus and its association with high-risk behaviour, many patients with HCV are also infected with human immunodeficiency virus (HIV). HIV is a retrovirus which infects around 30 million people worldwide. Several clinical studies have indicated that people who are co-infected with HIV and HCV progress more rapidly to liver failure and cirrhosis. This could be due to the fact that certain protease inhibitors (such as Ritonavir which blocks a liver enzyme) used for HIV therapy can be toxic to the liver, having a synergistic damaging effect on the HCV infected liver (79). Thus, co-infection with HIV makes HCV progress more rapidly: however, the opposite is not true since HCV does not make HIV progress more rapidly.

HOST IMMUNE RESPONSES TO HCV INFECTION

Acute Infection

During the first weeks or up to 6 months of HCV infection, known as the acute stage, 60% - 75% of infected people are usually asymptomatic, and might experience mild flu like symptoms (80). It is thus difficult to diagnose the virus and start treatment before the infection becomes chronic. Host immune responses involve both the innate and adaptive arms of the immune response. In HCV infection, once the physical barrier

of the skin has been breached, the *innate* immune responses emerge first consisting of natural killer cells (NK), granulocytes and macrophages which secrete cytokines in response to the infection.

Acute HCV has a mean incubation period of 6 to 8 weeks in humans (81). In accordance with this, chimpanzee studies have found that virus-specific CD4+ and CD8+ T cells accumulated in the liver 8-14 weeks after infection: this overlaps with the viral clearance in this model (82; 83). During this incubation period, certain individuals manage to mount vigorous multi-specific and strong $CD4^+$ (T helper) and $CD8^+$ T cell (cytotoxic T lymphocytes) responses and manage to clear the virus (84). Clearance is representative of an acute infection.

Cellular Immune Responses

Cellular and humoral immune responses are *adaptive* and sequence specific to the HCV antigens. They are comprised of primarily humoral (B cells, antibodies) and cellular (T cells). If the virus is eradicated at this level, the adaptive immune response may remain for many years in the form of memory cells. However, an initial clearance and recovery from HCV infection may not prevent reinfection, putting into question whether protective immunity is efficient. (85) It may be that the virus is too heterogeneous or that the strength of the immune memory is not sufficient to avoid reinfection. However, the adaptive immune responses associated with HCV are required, but may not be sufficient for achieving viral clearance, resulting in liver injury in many infected individuals.

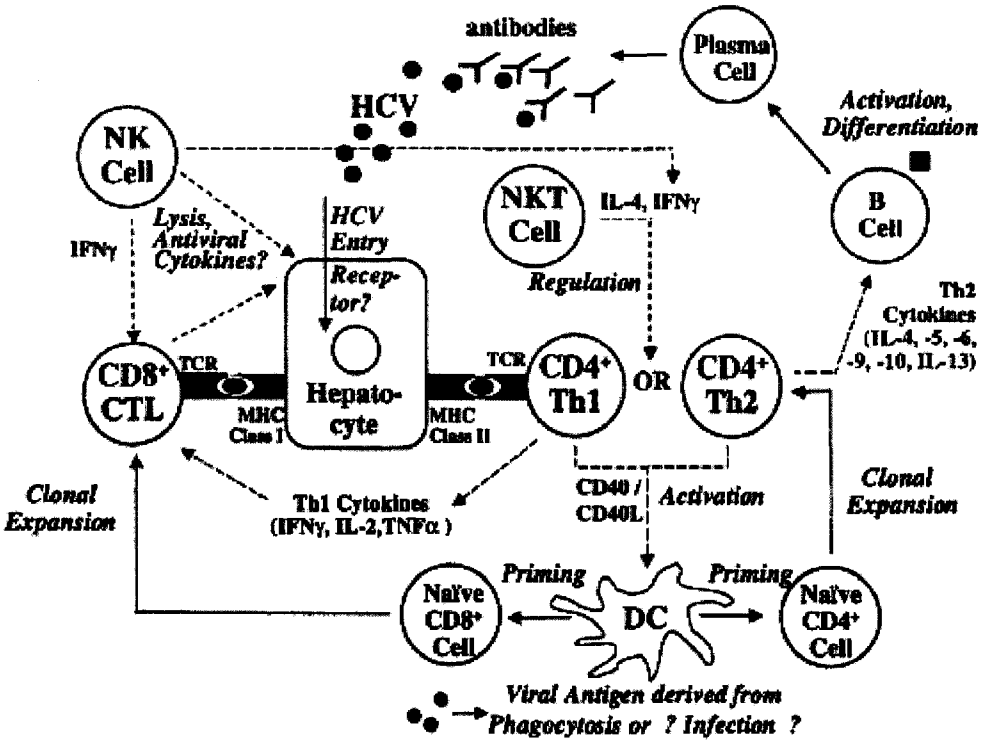
The immune responses needed to clear HCV are called cellular immune responses. These immune responses are *adaptive* and sequence specific to the HCV

antigens: they are primarily comprised of humoral (B cells, antibodies) and cellular (T cells) responses (**Figure 1.3**). If the virus is eradicated at this level, the adaptive immune response can remain in the form of memory in case of reinfection. However, an initial clearance and recovery from HCV does not prevent reinfection with the same viral strain, putting into question whether protective immunity is efficient in HCV infection (86). The adaptive immune responses associated with HCV play a role in both viral clearance and liver injury.

As a general rule, CD4+ T cells interact with major histocompatability complex II (MHC-II) on antigen presenting cells, which present exogenous antigen: CD4+ T cells activate macrophages, B cells and CD8+ T cells (through a cytokine network). Studies have demonstrated that strong multispecific and long lasting CD4+ T cell responses are associated with HCV clearance (87). In accordance with this, the depletion of CD4+ T cells in a chimpanzee experiment resulted in the emergence of viral escape mutations in MHC-I indicating that CD4 help is needed for both the expansion and maintenance of CD8+ T cells (88). These CD4+ T cells responses are often directed against highly conserved regions of the virus, namely the structural protein core and the non-structural proteins NS3/4 and NS5 (89; 90). Core and NS5 are the most conserved regions of HCV.

CD8+ T cells, also known as cytotoxic T lymphocytes (CTL), recognize what is presented by the MHC I of infected cells: these cells may directly kill the infected cell or can secrete cytokines such as IFN-γ and TNF-α which can inhibit viral replication without killing the cell (91). As mentioned above, CD4+ T cell responses are required for proper CD8+ T cell responses. However, these cells alone are also very important in controlling HCV infection.

Figure 1.3. Components of HCV-specific immune responses. Dashed lines represent regulatory effects while solid lines represent differentiation. HCV infection leads to the activation of many host immune responses such as the production of antibodies, IFN-γ production and the clonal expansion of CD4+ and CD8+ T cells. HCV has tropism for hepatocytes and gains access to these cells via as of yet, unknown receptors. Innate immune responses such as NK cells may be activated. Once inside the host, the virus can also be presented to CD4+ and CD8+ T cells via antigen presenting cells or hepatocytes to initiate antigen-specific immune responses. Antibody production may also be induced (194).


antibodies
Plasma Cell
HCV
Activation, Differentiation
NK Cell
NKT Cell
IL-4, IFNγ
B Cell
IFNγ
Lysis, Antiviral Cytokines?
HCV Entry
Recep-tor?
Regulation
Th2 Cytokines (IL-4, -5, -6, -9, -10, IL-13)
CD8+ CTL
TCR
MHC Class I
Hepato-cyte
MHC Class II
TCR
CD4+ Th1
OR
CD4+ Th2
Clonal Expansion
Th1 Cytokines (IFNγ, IL-2,TNFα)
CD40 / CD40L
Activation
Clonal Expansion
Priming
Priming
Naïve CD8+ Cell
DC
Naïve CD4+ Cell
Viral Antigen derived from Phagocytosis or ? Infection ?

Antibody-mediated depletion of CD8+ T cells in chimpanzees before reexposure to HCV resulted in prolonged viral infection indicating a critical role for these cells (92). In acute infection, strong broad CD8+ responses are required to control viremia but this only occurs in 20% of individuals (1; 93). Therefore, when HCV-specific CD8+ T cells are present in the blood, clearance of viremia and infection resolution are more probable.

Host Innate Immune Response: Interferon

Before the appearance of adaptive immune responses, innate responses such as NK cells play roles in controlling the infection. An innate immune response which is potentially important in the natural course of HCV infection is the host interferon responses. There exist different types of interferon: I and II which can be induced through HCV infection and act to inhibit viral replication and even apoptosis through a variety of mechanisms. Type II interferons, such as IFN-γ, unlike type I, are produced by specific T lymphocytes and are part of the adaptive immune response (antigen specific). Type I interferons on the other hand such as IFN-α and IFN-β are produced in most cells, perhaps in response to double-stranded RNA (dsRNA) intermediates which are seen during HCV replication (94). Once these type I interferons are produced, they can activate the Janus kinase-signal transducer and activator of transcription (Jak-STAT) pathway and activate interferon stimulated genes (ISGs) involved in the antiviral response such as 2',5' oligoadenylate synthetase (OAS) or the IFN-induced dsRNA-activated protein kinase (PKR) (95). The IFN will bind to its receptor and activates JAK proteins and subsequent STAT phosphorylation ensues: this STAT dissociates from the receptor and migrates to the nucleus where it can modulate gene transcriptional activity. dsRNA provides an immediate signal for the activation of NFkB, IFN regulatory factor

(IRF)-1 and 3 which stimulate the expression of host antiviral and antiproliferative gene products (96).

Following this, the ISG PKR can phosphorylate eukaryotic initiation factor 2 (eIF-2) which results in inhibition of cellular protein synthesis and viral replication (95). These processes are part of the host's defenses to protect itself against invading organisms. IFN-α and IFN-γ have been shown to have the potential to inhibit HCV replication in chimpanzees (97).

It is interesting to note that HCV expression in cells alters the expression of many intrahepatic genes involved in innate immune responses, specifically those involved in type I IFN responses (83; 98; 99). These alterations therefore cause type-I interferon dysregulation. NS5a has been shown to be capable of binding PKR and therefore suppressing IRF-1 dependent gene expression: other HCV proteins that are known to interfere with the interferon pathway are core, NS3/4A and NS5A (100). Studies in chimpanzees have shown that a strong IFN response does not necessarily correlate with viral clearance (58). Additionally, many individuals who are infected with HCV become resistant to type I IFN hence developing chronic infection.

Chronic HCV Infection

If an individual continues to live with the virus with no improvement for more than 6 months, the patient is usually considered to be chronically infected with HCV. In most cases, despite the presence of immune responses, viral persistence establishes itself in around 80% of patients (44; 101). The difference between a resolved infection and a persistent one has been linked to a vigorous, multi-specific and strong $CD4^+$ and $CD8^+$ T cell immune response: the mechanisms by which HCV evades these responses to

establish chronic infection have yet to be established. Chronically infected individuals have weaker and restricted CD4+ and CD8+ T cell responses compared to people who resolve the infection. In persistent infection, losses in CD4+ T cell functions, such as IL-2 secretion, have been demonstrated (102). As mentioned above, CD4+ T cell responses were required for the maintenance of CD8+ T cells. The dysfunction of early CD4+ T cells might then be responsible for viral persistence in chronic infection due to lack of CD8+ responses (88).

In chronic HCV infection, the host can only partially control the virus which can ultimately lead to forms of liver damage such as fibrosis, cirrhosis and eventually hepatocellular carcinoma. Evidence suggests that HCV-specific CD4+ and CD8+ T cells play roles in both viral control (T cells being protective) and injury (T cells contributing to immuopathology) in chronic infections. When chronic hepatitis leads to cirrhosis, liver function may decrease or the patient may experience portal hypertension, or other symptoms such as ascites (accumulation of fluid in the abdomen), varices (enlarged veins especially in the stomach and esophagus = caput medusa) and jaundice (103; 104). The only effective mechanism as of yet to help end-stage liver disease is a liver transplant. Nevertheless, there is usually reinfection of the new graft and recurrence of the disease usually within 5 years of the transplant, since HCV RNA is still in the blood of the patient (105; 106).

DISEASES ASSOCIATED WITH CHRONIC HCV INFECTION

Usual Manifestations of HCV

Although HCV currently infects 3% of the worldwide population, many HCV positive patients don't know they are infected. Despite having a high replication and

mutation rate, the HCV-related burden of disease is still quite low, considering only 1/3 of patients have symptoms and morbidity and mortality rates are of less than 30%. However, recent studies on the burden of disease have predicted that in England, the number of HCV infected people with cirrhosis will increase from 3705 in 2005 to 7550 in 2015 (107).

During the course of infection, if symptoms are observed, they will generally be non-specific and may include jaundice, abdominal pain, nausea, vomiting, fatigue and loss of appetite. In addition to these symptoms, the chronic effect of long-term HCV infection may induce steatosis, cirrhosis and hepatocellular carcinoma: these manifestations are to be expected since HCV has tropism for hepatocytes. Currently, HCV is the leading cause of liver disease worldwide and chronic infection resulting in cirrhosis and hepatocellular carcinomas are the highest indicators for liver transplant in the United States (108).

Fibrosis and Cirrhosis

Fibrosis, the main injury in HCV infection, is defined as the development of excess fibrous connective tissue, such as collagen, extracellular matrix and glycoproteins, in an organ. This is due to chronic liver inflammation such as hepatocyte apoptosis and cytokine production which lead to changes in hepatic architecture (109). Fibrosis is also the consequence of the body's sustained wound healing in response to HCV. Critical host factors which most affect disease progression and accelerate the rate of fibrosis are co-infection with HIV, aging (accelerates after 45) and alcohol consumption (110). 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), this accelerates

progression to cirrhosis by 3 fold (76). Other factors include sex, duration of infection and coinfection with HBV. In HCV infection, fibrosis usually first emerges around the portal areas of the liver, progressively extending to inside lobules and finally once the fibrosis connects the portal to central areas of the hepatic parenchyma, we see the emergence of cirrhosis (111).

Cirrhosis, which is the replacement of liver tissue by scar tissue in response to inflammation, is estimated to occur in 20 to 25% of HCV infected patients over their lifetime, usually after 20 to 30 years of infection (44; 112). Under the pressure of constant liver injury, hepatic stellate cells (main source of collagen and extracellular matrix) are activated and transdifferentiate into myofibroblasts which produce excessive amounts of collagen and large amounts of cytokines and chemokines (113; 114). This disease is the main cause of liver transplant worldwide. Currently, it is still unknown whether viral factors contribute directly to the progression of these diseases. However, recent studies have shown that rat hepatic stellate cells expressing an HCV core adenovirus showed accelerated cell activation, suggesting a direct role for core in regulating these cells (115). Additionally, HCV core has demonstrated oncogenic potential by transforming rat embryo fibroblasts and causing rapid proliferation with or without the presence of Ras-oncogene (116; 117). Further studies are needed to elucidate the exact mechanisms behind the development of fibrosis and cirrhosis.

Lipid Metabolism and HCV Proteins

Steatosis, the excessive accumulation of lipids within the hepatocyte cytoplasm, is a condition commonly observed in HCV chronically infected individuals at a mean prevalence of 55% (118). HCV can interfere with lipid metabolism through impairment

of lipid secretion thus causing accumulation of lipids in hepatocytes. The mechanisms behind the occurrence of steatosis in HCV infection are still unclear. However, it is thought that the structural protein core plays a big role in interfering with the lipid metabolism. To support this statement, studies using transgenic mice expressing core developed progressive steatosis in their livers (119). This could be related to the fact that HCV core (and NS5a) is localized to and interacts with the surface lipid droplets (organelles which store lipids and transport these to other organelles) (120). Individuals infected with genotype 3 have been linked to more severe steatosis. This could be attributed to differences in the core protein between genotypes: phenylalanine found in genotype 3 may have a higher affinity for lipid droplets than tyrosine (121). Interestingly, steatosis is resolved after a sustained virological response.

A study demonstrated that the expression of core in three different cell lines caused mitochondrial injury and increased reactive oxygen species (ROS) as well as lipid peroxidation products (122). The presence of ROS disrupts lipid metabolism consequently leading to steatosis. HCV core overexpression in a murine model has also been shown to interfere with hepatic assembly and secretion of very low density lipoproteins (VLDL): core seems to target microsomal triglyceride transfer protein (MTP) activity thus affecting VLDL secretion resulting in lipid accumulation (123). Altogether, these studies all suggest a role for core in the disruption of lipid metabolism.

Hepatocellular Carcinoma

Once cirrhosis has developed, the annual incidence of hepatocellular carcinoma (HCC), cancer of the liver, is of 0.5% to 7.9%, depending on the degree of liver fibrosis (124). According to these statistics, we can assert that the morbidity of HCV is relatively

low. The mechanism by which HCC occurs in HCV infection is still controversial since it is unknown if HCV plays a direct role in the pathogenesis or if the effect is indirect. Studies have indicated that at least 4 HCV proteins, namely core, NS3, NS4b and NS5a have the ability or potential to transform cells in tissue culture. Interestingly, a microarray study profiling the effect of the HCV proteins mentioned above and HBV found that these proteins affected different genes indicating that HBV and HCV induce HCC through different mechanisms (125). These viruses belong to different families, and HCV diverges from other hepatitis viruses since it does not integrate into the hepatocyte genome to become oncogenic.

HCV might induce cancer through repetitive viral replication in hepatocytes, thereby inducing death and regeneration of hepatocytes which could lead to the creation of carcinomas. Transformed hepatocytes found in cirrhotic nodules might grow to become adenomatous hyperplasia and ultimately, HCC (126). Further studies have demonstrated that gene abnormalities in p53 (G to T change in codon 249) have been reported in patients with HCV related HCC (127).

Cellular Transformation and HCV Core

Accumulating evidence indicates that many HCV proteins facilitate cell transformation in tissue culture. The structural protein core has especially been shown to have a role in hepatocarcinogenesis. In cells stably expressing core protein, a role in carcinogenesis was observed through the decrease of pRB (involved in tumor suppression pathway): this stimulates rapid cell proliferation and increased sensitivity to apoptosis, factors that could both lead to HCC (128). To support these results of core having a role in the formation of HCC, many mouse studies have shown that the expression of core

protein alone, or in combination with other proteins cause liver abnormalities (129; 130). The question remains if these animal studies are relevant to humans. Additional studies have shown that HCV core directly interacts with and activates STAT3 (implicated in tumorigenesis) through phosphorylation of a tyrosine residue: this resulted in rapid transformation and proliferation of the NIH-3T3 cell line (131).

Extrahepatic Manifestations: Chronic HCV Infection Related to B cell Dysfunction

One of the main difficulties associated with studying HCV infection is that the occurrence of the immune dysfunction observed, is also seen in people who are not HCV positive. This complicates specific diagnosis of HCV and makes it complex to confirm the relationship between HCV and a given disease or extrahepatic manifestations.

In chronic HCV infection, although the main site of infection is the liver, there are many extrahepatic manifestations that may emerge in the natural history of the virus (132). Expression of HCV proteins in mice has been confirmed in tissues such as salivary glands, kidney, testes, thymus and spleen (130; 133). It is estimated that 40-74% of infected patients will eventually develop an extra-hepatic manifestation (134).

Mixed cryoglobulinemia (MC), a condition in which abnormal blood proteins form and deposit in blood vessels, is the most common syndrome associated with HCV infection (135; 136). HCV stimulates the polyclonal proliferation of B cells in chronic infection thus creating abnormal immunoglobulins (IgG and IgM) (137).

Another extrahepatic manifestation of HCV which may emerge as a result of MC is non-Hodgkin lymphoma, a cancer of the lymphatic system. A lymphoma may occur after the proliferation of B lymphocytes, if there is translocation of bcl-2 (an oncogene) to the immunoglobulin heavy chain of IgG (136; 138).

Additionally, 13% of patients infected with chronic HCV will develop hypothyroidism, a condition in which the thyroid is underactive (139). Sjogren syndrome, an autoimmune disorder causing dry eyes and mouth, has also been associated with HCV infection (140). A mouse model expressing core, E1 and E2 polyprotein found HCV RNA in the salivary glands of fetal mice (130). Amongst others extra-hepatic manifestations of HCV we find porphyria cutanea tarda (a disorder in heme biosynthesis due a defective liver enzyme), glomurulonephritis (inflammation of the kidney) and HCV-related arthritis.

PATHOGENESIS OF HCV INFECTION

Immunopathogenesis of HCV infection

A recurring topic throughout this thesis is whether the diseases observed in HCV infection are virus or host-related. HCV proteins have been shown to have effects on apoptosis, signal transduction and cell transformation amongst others, which may suggest their involvement in immunopathogenesis. Immunopathogenesis of HCV pertains to the mechanism by which a disease develops in a host in response to the virus. Controversy surrounds the debate of whether liver damage is a direct cytopathic effect of HCV or if the damage is mediated through host immune responses: the damage might also be a combination of both factors. These mechanisms are still widely debated.

The injuries that are inflicted on the liver during infection are often attributed to vigorous host immune responses: previous studies have indicated that these are needed to clear the infection. Host immune responses consist of innate and adaptive immune responses. The goal of host immune responses is to eradicate the virus from the infected cells. However, with HCV infection, these responses are often insufficient and chronic

infection is established. Cell-mediated immune responses, mostly CTL, are thought to play a critical role in the development of HCV pathogenesis. The role of different cells and mechanisms in the development of chronic liver disease will be discussed.

Cell-Mediated Immune Responses and HCV Immunopathogenesis

It is important to note that it is difficult to study the acute phase of HCV since it is usually asymptomatic and the time of infection can rarely be identified. Consequently the majority of our knowledge on HCV immunopathogenesis and cellular immunity is derived from chimpanzee studies.

The adaptive immune response of the host is thought to play an important role in the development of chronic infection. As we know, strong, broad and sustained HCV-specific CD4+ and CD8+ T cell immune responses are required for viral clearance (87). CD4+ T cells are required for the priming of CD8+ T cells. It was demonstrated that individuals who managed to control HCV viremia had broad HCV T helper cells in their PBMCs (targeting up to 14 HCV epitopes): for the case of persistent viremia responses to a single epitope were detected (141). This indicates that when CD4+ T cell responses are inadequate chronic infection is established. However, no studies have yet demonstrated that CD4+ T cells mediate a direct cytopathic effect on the liver. CD4+ T cells act more like a helper for subsequent immune responses such as IL-2 production which induces the proliferation of activated CD8+ T cells. However, cytokines produced by CD4+ T cells may have a direct cytopathic effect if they are continually stimulated.

Much debate surrounds the question of whether CTL responses are responsible for the liver damage seen in HCV patients. CTL, like their name suggests, are cells which are capable of killing cells infected with virus. This can be done by cytolytic

mechanisms when a CD8+ T cell binds to its target and releases perforin and granzymes thus killing the infected cell. A CTL expressing Fas-L may also bind to Fas on the surface of a target cell thus inducing apoptosis of the cell. CTL may also act through a non-cytolytic mechanism by producing IFN-γ or TNF-α to suppress viral replication without killing the cell (91).

HCV-specific T cells have been located in the liver and blood of infected humans and chimpanzees. However, in HCV infection, according to chimpanzee studies, it was found that intrahepatic CD8+ T cells were detected only 4 weeks after the onset of viremia: these cells however, were not detected in the peripheral blood regardless of the outcome of infection (acute or chronic) (83). This delay in the appearance of these cells might be one of the factors associated with the occurrence of a persistent infection. During the acute phase of infection in humans, a high number of HCV specific CD8+ T cells could account for 3.5% of the CD8+ T cell pool: yet this number decreases when the infection becomes chronic (93). In chimpanzees, it was found that 1-3% of intrahepatic CD8+ T cells responded to HCV by producing IFN-γ(83). It has been shown that HCV-specific CTL may persist up to two decades after recovery from infection: interestingly, HCV-specific antibodies were not detected (142).

Studies using transgenic mice have demonstrated that CD4+ T cells expressing core which migrated to the liver of recipient mice had high Fas expression: these activated cells caused inflammation of the liver, maybe through interaction with hepatocytes expressing Fas (143). Considering that a broad and vigorous CTL response is required for viral clearance early in infection, the continual presence of anti-HCV CTL could be responsible for the accumulation of cell-mediated liver damage through

hepatocyte apoptosis (144). Additionally, since granzymes and perforin are released in the direction of the target cells, these may cause damage to neighboring hepatocytes.

However, certain studies have indicated the presence of protective immunity in HCV infection. Challenge experiments were conducted in previously infected HCV-recovered chimpanzees. In these subjects, proliferative responses were detected 2 weeks after the reinfection in the PBMC of the animals that previously cleared the infection indicating the presence of protective immunity and thus memory cells. HCV-specific T cells to NS3 and NS4 non-structural proteins underwent greater proliferation suggesting that immune responses to these proteins might be associated with viral clearance (145). A similar rechallenge study determined that the rapid viral clearance must be due to memory cells since antibodies to HCV glycoproteins were not detected, hence no defense was conferred through neutralizing antibodies (146).

Chimpanzee studies have also demonstrated that both CD4+ and CD8+ T cells are essential in chimpanzee rechallenge experiments. Antibody-mediated depletion of CD8+ T cells before rechallenge to HCV resulted in prolonged viral infection, indicating a critical role for CD8+ T cells (92). On the other hand, the depletion of CD4+ T cells in another model resulted in the emergence of viral escape mutations in MHC-I indicating that CD4 help is needed as cellular support for CD8+ T cells (88). Therefore, both CD4+ and CD8+ T cells play crucial roles in preventing the establishment of chronic infection.

Humoral Immune Responses and Immunopathogenesis

Protective immune responses to HCV are required to fight off the virus in the case of reexposure. Antibodies to HCV antigens have been detected 7 to 8 weeks after initial infection. In the case of HBV, it is known that neutralizing antibodies against surface

antigens protect the host. Nevertheless, the role of humoral immunity in HCV infection is controversial. Neutralizing antibodies, which are part of the humoral immune response and produced by B cells, can destroy or inhibit a virus. The capacity of a human host to produce neutralizing antibodies was previously tested. Plasma from an HCV chronically infected host was exposed to HCV *in vitro* to analyze if the antibodies present had neutralizing capacity: seronegative chimpanzees were afterwards inoculated with this mix. This study found that HCV did elicit neutralizing antibodies in humans that can prevent infection in chimpanzees (147). However, only the plasma taken 2 years after initial infection conferred protection and not plasma taken 11 years post-infection. This could be attributed to the frequency of viral mutations which might permit the virus to escape neutralizing antibodies and render the antibodies useless against quasispecies (148). Another study supported the ability of antibodies specific to the HVR1 of E2 to induce protection against homologous HCV infection in chimpanzees: this however did not protect against escape mutants (149). Additionally, some studies in humans have shown that the presence of antibodies against E2 is not associated with viral clearance but rather with chronic infection (150).

Nonetheless, while neutralizing antibodies might confer some protection to chimpanzees or an attenuated course of infection, HCV antibodies are often involved in the extra-hepatic manifestations of HCV. MC in which abnormal cryoglobulin proteins are formed causes complications such thickening of the wall of blood vessels which can increase the risk of blood clots (136). However, a good humoral immune response generated by the activation of B cells by CD4+ T cells does not necessarily correlate with a good outcome (148).

In summary, humoral immune responses may or may not protect the subject in case of reinfection with HCV, and although these antibodies may be present, they do not always provide protective immunity or correlate with viral clearance.

IMPAIRMENT OF IMMUNE FUNCTION: EVASION OF HOST IMMUNE RESPONSES

Due to the chronic nature of HCV, and the emergence of different diseases, it is recognized that HCV interferes with the host through different mechanisms. Whether the infection is acute of chronic, the immune system will be affected. These impairments will be discussed below.

Impairment of Natural Killer Cell Functions

Natural killer cells (NK), which are a form of cytotoxic lymphocyte, play an important role in innate immunity. These cells, which require no priming, release granules of perforin and granzyme which cause the target cell to die by apoptosis, therefore retaining the virus within the cell. This is an advantage compared to cell lysis since the virus remains contained. Studies have shown that in early infection, the amount of NK cells increases in the liver of infected individuals. NK cells secrete cytokines such as INF-γ and TNF-α. INF- γ, a type II INF is important in HCV clearance since it activates the Jak/STAT pathway which is important in the host's antiviral response (151).

Certain *in vitro* studies have shown that the function of NK cells from healthy individuals was inhibited by high concentrations of HCV E2 protein. In HCV infection, the E2 protein may bind to CD81 on the cell surface. In the case of NK cells, the binding of immobilized E2 inhibited cell function by limiting INF- γ production, cell cytotoxicity and cytokine production: NK function was not restored after exposure to a variety of

cytokines such as IL-2 (an important activator of NK cells) and IL-12 (152; 153). Studies analyzing HCV patient samples found that spontaneous NK cytotoxicity was 4 times lower in these patients than in normal donors (154). This supports the hypothesis that the establishment of a chronic infection is facilitated through NK cell dysfunction.

Meanwhile, contrasting studies have indicated that NK cells are important in viral clearance of HCV. A study also found that NK cell receptor KIR2DL3 (killer cell immunoglobulin-like receptors) and human leukocyte antigen C group (HLA-C1) ligand are important for the resolution of HCV infection (155). Individuals having previously resolved an HCV infection were more likely (37.5%) to have two copies of HLA-C1 than those whose infection became persistent (29.9%). This indicates an association between genotype, role of NK cells and viral clearance. However, complications arise in the study of how an infection becomes chronic since it is difficult to diagnose and follow the immune responses of an individual when they are initially infected with HCV.

Impairment of Dendritic Cell Functions

Dendritic cells (DC) are the most potent antigen presenting cells of the immune system which initiate immunological cascades and activate T cells, B cells and NK cells by the release of several cytokines (156). DC, which capture and process antigen, are necessary in order to induce antigen-specific immune responses by activating T cells (157). Once DC encounter antigen, they migrate to lymph organs to present this antigen to other immune cells: with their maturation, co-stimulatory molecules such as CD80 and CD86 are upregulated and DC secrete high levels of IL-12, a cytokine crucial for the maturation of CTL necessary for an effective Th1 response (158). Reduced IL-12 expression may inhibit the induction of a Th1 CD4+ T cell response which might be

critical in HCV clearance. Additionally, DC expressing core and E1 adenoviral construct had reduced ability to activate CD4+ T cells due to maturation defects caused by the presence of these structural proteins (159). These impairments (such as loss of IL-12 secretion and impaired antigen presentation) in persistent HCV infection lead to DC dysfunction and deficient humoral and cell-mediated immune responses (160). Thus, impairment of antigen presentation at the DC level may cause improper function of T cells. However, a lot of controversy surrounds the role of DC in chronic HCV infection. Many studies indicate normal DC function in HCV-infected patients while others demonstrate abnormal DC activity (161).

Impairment of T-Cell Functions and HCV Proteins

It is interesting to note that HCV core protein, which is 21 kDa in size and the first protein to be expressed in the natural course of infection, has been found in the bloodstream of infected patients (162). Since HCV only infects a small number of PBMC within a host, the finding that core protein is in the bloodstream indicates that the effect of core may not merely be limited to HCV infected cells.

T cell responses to HCV are very important in order to determine the outcome of the course of infection. It is accepted that strong sustained CD4+ and CD8+ T cell responses are required for viral clearance or elimination (82; 163). It is important to note that a sustained CD4+ T cell response is critical since certain patients initially have strong T cell responses but will progress to chronic infection once these responses disappear (87). Therefore, sustained responses are essential for the long term control of the virus. Since strong T cell responses are needed to clear the infection, it is possible that there is a problem in the primary activation of T cells. As indicated earlier, antigen presentation by

macrophages and DC is impaired in HCV infection as well as DC having reduced secretion of IL-12: both result in the ineffective priming of antigen-specific T cells (159; 164).

One of the factors contributing to the persistence of HCV in the human host would be the high mutation rate of the virus. When the virus mutates, the existing immune response no longer recognizes the new sequence: persistence of HCV is facilitated by the mutation of epitopes targeted by virus-specific host immune responses. A sequence within the HCV E2 protein, which has been identified as the hypervariable region, is associated with chronic viral infection (165).

It has been found that early in acute and chronic infection, CD8+ T cells can be impaired in their cytotoxicity and proliferative capacity: this occurred in both types of infection regardless of viral clearance (166; 167). This indicates that there is an occurrence of T cell dysfunction early in the infection. Constant exposure to high levels of virus may lead to T cell unresponsiveness: a mouse study discovered the inability of an HCV specific CD8+ T cell to produce IFN-γ in response to an HCV antigen (163). However, chronically infected human patients on therapy were shown to have enhanced CD4+ T cell proliferation indicating that viral load does affect the number of T cells (70; 168).

Previous studies have also found that core can inhibit T cell responses, and suppress the transcription of certain genes, therefore indicating a role for this protein in the establishment of chronic infection (169). When comparing chronic HCV patients to long-term recovered patients, CD4+/CD8+ T cell effector functions were diminished, as well as the production of Th1 cytokines which could contribute to viral persistence (167).

Core can also bind to a cytoplasmic domain of tumor necrosis factor receptor (TNFR) family members such as Fas and in T cells, to increase Fas-mediated apoptosis (170).

Another mechanism by which T cell dysfunction could occur is through impaired maturation of CD8+ T cells due to the presence of HCV (171). Lesser differentiated phenotypes such as CD28+CD27+ are more impaired in their usual effector functions (167). Additionally, other viral factors such as the presence of HCV core protein can inhibit CD8+ T cell function by impairing IFN-γ secretion (172).

Different mouse models using core from different HCV genotypes, either 1a or 1b found differential results on apoptosis, T cell dysregulation and lymphocyte infiltration (173; 174). Keeping in mind that the core protein is one of the most conserved HCV proteins, these differential responses to the core protein suggests that immunological effects might be genotype or subtype specific. Additionally, this suggests that the core protein alone cannot be designated as the mechanism by which HCV establishes chronic infection.

Liver Damage in Chronic HCV Infection

The mechanisms by which HCV infection becomes chronic are still highly debated: immune-mediated cell damage and direct viral effect have been proposed. Regardless, it is apparent that with time, chronic HCV can cause significant liver damage in the form of steatosis, fibrosis, cirrhosis and HCC. Little is know about the early acute stages of hepatitis since infections are usually asymptomatic and remain undiagnosed (80).

The occurrence of steatosis in core expressing cell lines or mouse models may suggest that HCV exerts a direct cytopathic effect. However, we must remember that

these cells do not necessarily represent real conditions and over-expression of this protein alone might lead to liver injury. In certain cases, individuals may be carriers of HCV but still express normal liver histology (175). Additionally, viral RNA levels are not predictive and do not correlate with disease severity (176). High levels of the liver enzyme ALT do not inevitably represent risk of liver damage or vice versa. These arguments therefore go against HCV being directly cytopathic.

On the other hand, in the attempt to clear the virus from the liver, T cell immune responses generated by the infected host seem to play a role in hepatocellular damage. Liver damage is seen in the form of lesions (portal lymphoid infiltration, necrosis and degenerative lobular lesions) which appear to be caused by the immune responses which are continuously amplified by the accumulation of T cells. The intrahepatic lymphocyte infiltration observed is composed of Th1 CD4+ T cells (in periportal space) secreting IFN-γ and unable to secrete IL-4 or IL-5 and CD8+ T cells (in periportal and lobular regions) (177; 178). These results suggest that host immune responses are very important in progression of HCV disease. Additional studies are hence needed to clarify the mechanisms surrounding HCV infection and subsequent liver damage.

HCV Persistence and Ability to Subvert Host Immune Responses

Since HCV establishes chronic infected in 80% of patients, it is widely accepted that this virus has yet unknown mechanisms by which it evades host immune responses. One of the primary mechanisms by which HCV escapes immune recognition is through constant mutations. When the virus mutates, thus generating quasispecies, existing immune responses no longer recognize their respective epitopes, therefore rendering neutralizing antibodies and T cells ineffective to fight off infection (179).

Another mechanism by which HCV subverts the host immune response is through DC dysfunction. Studies have indicated that HCV is capable of replicating in peripheral blood DC although at lower levels than other cell types: these cells could also be considered as a reservoir for HCV (6). During HCV infection, impaired DC function, maturation and antigen presentation leads to defects in Th1 expansion (and alterations in cytokine secretion) and therefore deficiencies in humoral and cell-mediated immune responses. This could be attributed to reduced expression of IL-12 and IFN-γ in chronic HCV patients, which may inhibit the induction of appropriate Th1-type of CD4+ T cells (160; 180; 181). Additionally, elevated levels of IL-10, an immunosuppressive molecule, and decreased levels of IL-12 decrease chances of achieving a CTL response (160). It was also shown that core/E1 proteins had an immunosuppressive effect on the stimulation of CD4+ T cell responses induced by DC: these T cells were unable to proliferate or to produce IL-2 (182).

Since HCV induces problems in the allostimulatory capacity of DC, this results in T cell dysfunction. The function of T cells may also be suppressed by HCV structural and non-structural proteins. Studies have indicated that HCV core can interact with the C1q receptor on the surface of T cells and inhibit T cell proliferation *in vivo* (169). T cell exhaustion is also quite likely to play a role in the persistence of HCV infection. CTL, which are generated in the lymphoid tissue, may die rapidly in the periphery when exposed to high levels of HCV antigen: additionally, HCV responses induced during acute infection are not sustained, which could mean that T cells are exhausted by the constant presence of virus (93).

Finally, HCV has tropism for the liver which is a very tolerogenic organ, which by itself could be a mechanism to escape immune response. In HCV studies, differences are always found between T lymphocytes from peripheral blood versus those found in the liver. Through these mechanisms, HCV manages to trick the immune response and to ultimately establish chronic infection.

HCV Vaccine Development

Currently, there exists no prophylactic or therapeutic vaccine to treat HCV infection. Many difficulties are associated with the creation of an effective vaccine due to the existence of multiple genotypes, the speed of viral replication and the emergence of subtypes. It is difficult to design a global vaccine since genetic variation exists between genotypes and even within the same strain. Studies done on chimpanzees have indicated that after primary infection, reinfection with an identical viral strain does not result in clearance of the virus (183). This may also be due to the generation of quasispecies which are no longer recognized by the host immune response.

Due to the asymptomatic nature of HCV, acute infection is rarely detected in the early stages, and the infection becomes chronic. To develop an effective vaccine, early immune responses in individuals who manage to clear the infection must be extensively studied. In terms of treatment, a prophylactic vaccine is needed in order to prevent all cases of infection especially in individuals at high risk of acquiring the virus. If this is impossible, the second best option would be a vaccine which could be used to treat chronic HCV infection: various vaccine options are currently being studied.

MODELS TO STUDY HCV PATHOGENESIS:

In Vitro Based Systems for HCV Cell Culture

Studies on HCV have long been hampered by the lack of a robust cell culture system capable of supporting HCV replication. Ideally, an *in vitro* cell culture system would incorporate hepatocytes since HCV has viral tropism for these cells. Certain groups have demonstrated that specific cell types can sustain HCV replication. *In vitro* systems have shown that serum from HCV positive patients can infect and replicate in primary human hepatocytes: despite the presence of the replication intermediate negative stranded RNA there was only a low yield of replication and RNA genomes (184; 185).

Other *In Vitro* Systems

In 2005, it was reported that the transfection of human hepatoma cell lines (Huh 7 or Huh 7.5) with a genomic RNA from the HCV strain JFH-1 resulted in the production of infectious particles (16). JFH-1 is a highly replicative strain of HCV from genotype 2a. The advantages associated with cell culture models are the control over cell type and genotype, quicker replication rate of cells (as compared to breeding animals) and additional control of culture conditions. However, some of the drawbacks associated with the 2005 model are that as of yet, it is restricted to genotype 2a and Huh 7 transformed cells.

In order for transfection to occur at a high rate, cells must be permissive to the virus: cells which are deficient for innate immune responses (such as Huh 7.5 cells which are mutated in the RIG protein) have higher replication rates. However, these studies are still restricted to certain cell types and do not represent actual hepatocytes and human conditions since they are mutated. Nevertheless, these cell culture systems are powerful

tools for studying HCV viral entry, life cycle and replication at a molecular level in a more controlled environment. Additionally, models are required to elucidate the role of individual HCV proteins in different steps of infection.

Hepatitis C Virus and Chimpanzees

Other than humans, chimpanzees are currently the only other known host which HCV naturally infects (98). Studies performed using chimpanzees have shed new light on our understanding of the virus regarding genetic drift, mode of transmission and pathogenesis. However, the clinical course of infection differs in these two species: 1) mother to infant transmission has not been reported in chimpanzees, 2) only about 30-50% of chimpanzees will progress to chronic infection while this occurs in 80% of humans and 3) chimpanzees do not progress to cirrhosis (186; 187). Additionally, chimpanzees have a higher clearance rate and a milder course of infection. The interspecies variation which exists brings into question whether chimpanzees are suitable models. Chimpanzees are expensive, endangered and need more care than a small animal model. Moreover, treatments which are effective in chimpanzees may not be relevant in humans. It is important to understand that many assumptions can be made while extrapolating data from one species to another. For instance, for HIV entry into cells in chimpanzees, only CCR5 is required, contrastingly in humans, CD4 and the chemokine receptor CCR5 are needed to gain access to a cell (188).

Furthermore, by using human strains to infect chimpanzees and then handling the blood taken from these same animals, new strains of HCV could emerge considering the RNA virus is highly mutable: this could result in a zoonosis (a new strain of virus that could be more infectious in humans). However, an advantage associated with using these

creatures is that you can infect the animal with a specific strain and follow the course of infection. In human studies, we are restricted to workplace injuries in which a worker is accidentally exposed, and most of the time the person will follow a treatment, so we do not see the natural course of HCV infection. Overall, even if humans and chimpanzees share 98-99% genetic similarity, we need to be careful in applying data obtained in chimpanzees to humans.

HCV Transgenic Mouse Models

Progress in research regarding HCV has been hampered by the lack of *in vitro* cell culture systems for this pathogen. Since the host range of HCV is restricted to humans and chimpanzees, throughout the years, many transgenic mouse models have been created to aid in furthering our knowledge on the mechanisms behind cell entry, pathogenesis, the development of cirrhosis and HCC *in vivo*. The general aim is to mimic what occurs in a human HCV infection in order to tailor improved therapies.

Mice are easy to handle, breed quickly, can be housed together and require a minimal amount of maintenance as compared to chimpanzees. However, HCV does not naturally replicate in mice, so many limits are associated with the development of a model in which to study HCV pathogenesis. Despite these complications, many research groups have attempted to create models using parts of the HCV genome: existing models have concentrated thus far on the HCV structural proteins expressed individually or together under different promoters, and their effect on the liver. The following section will review several mouse models and the important conclusions drawn from these studies.

Model Expressing HCV Core Protein

A mouse model expressing the HCV structural protein core from genotype 1b was created. This study found that the intrahepatic levels of core protein in transgenic mice were similar to those found in humans, therefore mimicking natural infection. Hepatic steatosis was detected in mice as early as 3 months of age, though, mice analyzed for pathological changes at 3, 6, 9 and 12 months of age had no inflammatory or neoplastic changes (abnormal proliferation of cells). Nonetheless, 16 month old mice formed nodules having the characteristics of hepatocellular adenoma. The fatty change and HCC occurring in these mice are characteristic of human HCV infection. The study also found that there was a higher expression of protein in the tumors. Additionally, male mice were more prone to develop HCV (as is consistent with human data): for the C21 line, 25.9% of mice developed HCC. However, no lymphocyte infiltration or changes in ALT and AST levels were observed. This study may conclude that the expression of core is oncogenic and causes cellular changes which ultimately result in the development of HCC, although the mechanism has to be elucidated (129).

Another mouse model which expressed HCV core specifically in T cells since the transgene was under the control of a CD2 promoter, found that transgenic mice developed histological changes which were accompanied with elevations in ALT levels (173).

Model Expressing HCV Envelope Proteins

A transgenic mouse model expressing E1 and E2 proteins was created by Koike *et al.* in which HCV genes were placed downstream of HBV regulatory elements. A previous study had demonstrated that high expression of HBx gene results in the development of HCC in transgenic mice which led the researchers to use the same

enhancer and promoter in order to induce HCC in mice expressing HCV proteins (189). They found that the glycosylated E1 and E2 proteins had highest overall expression in the mouse livers. However, extrahepatic manifestations of both E1 and E2 proteins were located in salivary glands while E1 was also detected in lung, intestine and kidney. In liver sections, the distribution of E1 and E2 was typically found in the cytoplasm of hepatocytes surrounding the central vein. However, this mouse model observed no lymphocyte infiltration, hepatocyte necrosis or neoplasia in mice up to 16 months in age. This study therefore suggests that HCV envelope proteins might not have the potential to cause direct liver damage (133).

Model Expressing All Structural Proteins

One group created a mouse model using genotype 1b which encompassed the entire structural region of HCV. Different transgenic lines were created using major urinary protein (MUP) and albumin promoters since these promoters are highly active in the liver (190). The authors were hoping to direct the HCV expression to the mouse liver since HCV is naturally hepatotrophic. In the selected transgenic lines, liver sections were positive for anti-core and anti-E2 antibodies. Regardless of core and E2 expression, no long term histological abnormalities were seen in these transgenic mice up to 6 months of age. According to these results, the authors concluded that a direct cytopathic effect of HCV structural proteins is doubtful. However, the main transgenic line studied was lost so no further studies were accomplished after 6 months of age: it is quite probable, when comparing time points for other transgenic models that changes could still have occurred after this time point (191).

A certain transgenic mouse model carrying HCV complementary DNA (C980) encoding HCV core and envelope proteins under the control of the promoter of the class I MHC gene was used to study lymphocyte infiltration (192). In this model, HCV RNA and core protein were detected in the livers of the mice. After 10 months of age, these mice developed focal infiltration of lymphocytes as well as other histological changes such as hepatocyte necrosis and degeneration. In 80% of mice aged 10 to 15 months, lymphocyte infiltration was mainly observed in necrotic hepatocytes surrounding the central veins: hepatocytes situated around these infiltrations appeared to be normal. In addition to lymphocyte infiltration, fatty change was present in 50% of the studied mice. Despite all these histological changes, these mice did not develop carcinomas within their 4 year observation period. It is interesting to note that alpha fetoprotein, which is used as a tumor marker in humans (usually at low levels in human adults) was detected in liver sections of the transgenic mice.

Summary of Mouse Models

In two of the mouse models discussed above expressing core, E1 and E2 different conclusions were reached. Contrastingly to the study by Kawamura *et al.*, the study by Honda *et al.* observed histological changes (191; 192). This brings into question whether the lesions observed in the mice were caused by core alone or in combination with the envelope proteins. Additionally the study by Moriya *et al.* demonstrated the emergence of HCC in the mice expressing only core. In view of the current mouse models, it is probable that core is the agent which causes changes within the cells since models studying only the envelope proteins demonstrated no pathologies (133).

Taken together, these studies suggest a role for HCV core alone or in combination with other proteins, in hepatocarcinogenesis. Future studies will need to consider the role of the host immune response in the development of HCV complications and to elucidate the molecular mechanisms in the formation of HCC.

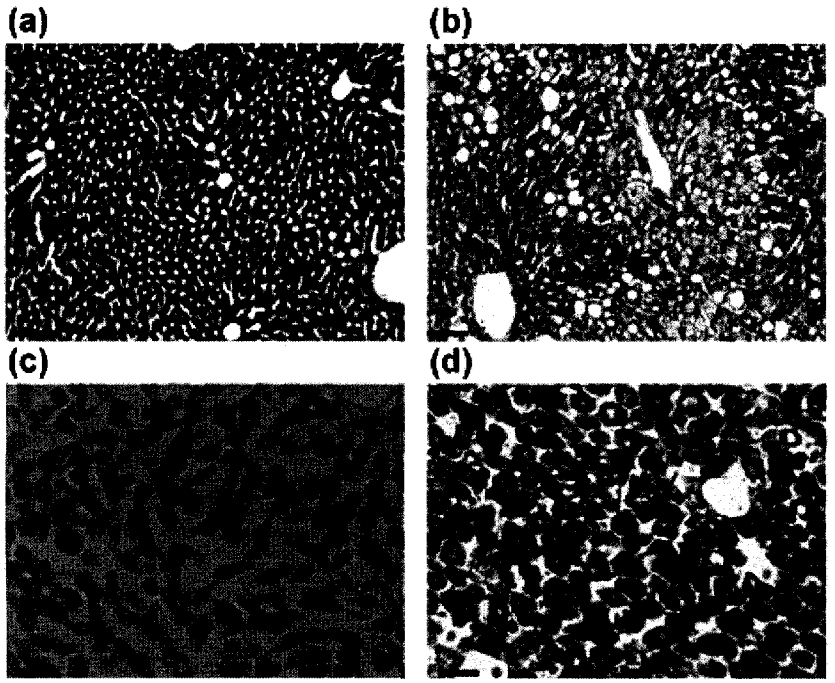
Our HCV Transgenic Mouse Model

Due to the lack of good existing models, for our purpose, a more appropriate transgenic mouse model was created to study the mechanism of HCV pathogenesis. In order to further investigate the effect of HCV core, E1 and E2 (CE1E2) proteins, our research group has successfully created a transgenic mouse model that mimics several features of human HCV infection (130). These transgenic mice express three HCV structural proteins (a polyprotein form of core, E1 and truncated E2) under the control of a universal cytomegalovirus (CMV) promoter. Although this transgenic model was found to express CE1E2 transcript in all tissues, the viral proteins were predominantly expressed in hepatocytes. Additionally, viral RNA, as well as viral proteins increased with age in the transgenic mice, and the mice developed detectable liver disease and liver abnormalities such as steatosis (**Figure 1.4**) and hepatocellular carcinomas (**Figure 1.5**). Since these transgenic mice have symptoms characteristic to the ones observed in human infections, we can determine that this is a good model in which to study chronic HCV infection. Additionally, the combination of 3 different structural proteins makes this model more credible than others expressing only one gene.

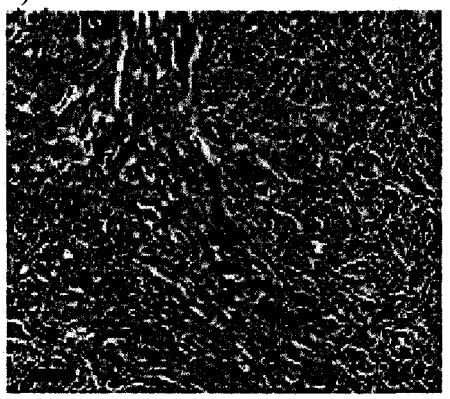
It is important to note that the protein encoded by our HCV transgene represents an endogenous antigen while it is in the intracellular environment.

Figure 1.4. Hepatic steatosis as observed in HCV transgenic mouse livers. Mouse livers were fixed in 4% paraformaldehyde and embedded in paraffin. Liver sections (5 μm thick) were stained with heamatoxylin and eosin (a and b) or Sudan Black B (c and d). Sections from a one year old wild type mouse (**a and c**) indicate absence of steatosis. Sections from a one year old transgenic mouse (**b and d**) demonstrate ballooning of hepatocytes indicating liver steatosis as well as the Sudan Black B stain showing lipids stained in black. These fatty change conditions are similar to those observed in HCV chronically infected humans (130).

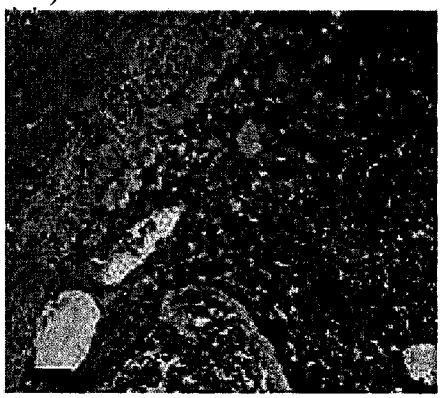
Figure 1.5. Liver tumors observed in HCV transgenic mice. Mouse livers were fixed in 4% paraformaldehyde, embedded in paraffin and liver sections (5 μm thick) were Trichrome stained. The development of hepatocellular carcinoma (**A**) and fibrosis (**B**) are shown in an 18 month old HCV transgenic mouse. The occurrence of these changes is similar to those observe in HCV chronically infected humans (130).


(a)
(b)
(c)
(d)

A)



B)



Prior studies have demonstrated that truncated HCV E2 protein is not secreted into the extracellular domain, but is retained in the endoplasmic reticulum (19). Since the sequence of the E2 protein of our HCV transgenic mouse model is prematurely terminated at amino acid 683, the products of the expressed HCV transgene remain in the cell. Yet, it is safe to assume that any liver cells that have died will release their contents into the extracellular space, thereby providing exogenous viral antigens which can be taken up by antigen presenting cells. However, since these mice express CE1E2 as part of their genome, it is assumed that these mice are tolerant to the transgene and do not mount immune responses to these antigens. Therefore, our HCV transgenic mouse model is an ideal small animal model with which to study the effects of immune damage through the adoptive transfer of HCV-specific immune cells.

CHAPTER 2:
HYPOTHESIS AND AIMS OF PROJECT

Rationale Behind Project

One of the major issues regarding HCV pathogenesis is the conundrum of whether the liver damage observed in chronic HCV infection is host or virus mediated. In the introduction, we learnt that individual HCV proteins have the ability to cause apoptosis, cell transformation and dysfunction amongst important immune cells such as DC and PBMC resulting in improper antigen presentation and suppression of T cell responses. Additionally, there is a consensus that CD4+ and CD8+ adaptive immune responses are very important to control acute infection but these are often inefficient thus resulting in chronic infection. Although these responses may be weak in chronic infection, their constant presence may contribute to liver pathogenesis.

The liver is a very tolerant organ which may be one of the mechanisms by which infection becomes chronic since HCV has tropism for the liver. Previous studies have investigated the effect of breaking tolerance to HCV antigens in transgenic mouse models where the transgene is activated later in life. However, with these models, it is difficult to study immune-mediated cell damage since these models do not represent the long-term effect of the immune system in chronic infection. Additionally, the mechanisms through which liver damage occurs, either by host factors or viral cytopathic effects are still unclear.

The mouse model used in this study expresses the HCV proteins core, E1 and E2 predominantly in the liver and the histological changes observed, such as hepatic steatosis and liver tumors, mimic several features of chronic human infection. What is interesting about this model is that because core, E1 and E2 are expressed as part of the mouse genome, mice do not naturally mount immune responses against these antigens.

These transgenic mice are immunotolerant to the transgene and thus eliminate T cells which would otherwise be reactive towards these antigens. Conversely, this creates an obstacle in studying host immune responses and resulting liver damage. For this reason, an experimental model in which an adoptive transfer of HCV-primed splenocytes into the HCV transgenic mouse model was created to investigate the role of immune-mediated liver injury in HCV pathogenesis. Adoptive immunotherapy might therefore be a useful tool to treat HCV infection.

Elucidating these mechanisms is important because it may not only provide a better understanding of the pathogenesis of HCV infection, but might ultimately contribute to the development of an effective vaccine and improved therapies for HCV infection.

Aims

1) To establish an efficient novel immunization method to induce strong HCV-specific immune responses in a murine model.
2) To create an adoptive transfer model to define the relevance of immune-mediated liver cell damage in HCV infection using transgenic mice.

Hypothesis

The adoptive cell transfer of HCV-primed splenocytes into an HCV transgenic mouse model will initiate potent immune-mediated cell damage.

CHAPTER 3:
MATERIALS AND METHODS

MATERIALS AND METHODS

Construction of Vector

Total RNA was extracted from a patient infected with HCV genotype 1a which is the most prominent genotype found in North America. The core, E1 and E2 section of the HCV genome was amplified using extracted RNA as a template. The fragment consisting of isolated core, E1 and truncated E2 genes (encoding for the amino acid residues 1-683) was constructed by real time-polymerase chain reaction (RT-PCR) using forward primer 5' ACC ATG AGC ACG AAT CCT AAA CCTC 3' and reverse primer 5' TGG TAG GGT TGT GAA GGA ACACG 3'. This fragment was then cloned into the EcoR1 sites of pCR 2.1 vectors using the TOPO-TA cloning kit (Invitrogen, Burlington, Ontario). The fragment was then sub-cloned into a pVax vector (Invitrogen, Burlington, Ontario), downstream of a cytomegalovirus (CMV) promoter. The pVax-HCV (core, E1 and E2) fragment was linearized by Mlu1 restriction enzyme digestion and used for the creation of the transgenic mice. The construction of the transgenic mice was done in association with Dr. Kothary at the Animal Care Facility at the University of Ottawa and Dr. Francisco Diaz-Mitoma's laboratory.

Transgenic Mice

The transgenic mice were generated by microinjecting the linearized plasmid containing the core, E1 and E2 fragment of the HCV genome into the pronuclei of fertilized eggs isolated from the B6C3F1 mouse strain from Charles River Laboratories (Wilmington, MA, USA). These eggs were implanted into the oviducts of pseudopregnant foster mothers. Transgenic founder mice were identified by isolation of DNA from tail biopsies, followed by PCR amplification and identification of transgene

by gel electrophoresis. Originally, five founder mice expressing the CE1E2 fragment were identified: the transgenic colony is maintained by breeding transgenic mice with B6C3F1 mice thus creating a heterozygous mouse colony. With each new generation, pups are screened for the presence of the transgene.

Genotyping of Mice

Total DNA was extracted from the tail biopsies of the pups using the DNeasy Tissue Kit (Qiagen, Mississauga, Ontario) and following the manufacturer's protocol. Briefly, mouse tail biopsies are performed by cutting a segment of 0.4 cm from the tail tip of each earmarked mouse. The end of the tail is cauterized with a silver nitrate stick in order to stop bleeding if it occurs. Proteinase K is added to the microcentrifuge tubes containing the tail samples in order to lyse the tissue and obtain the DNA. The DNA binds to the membrane of DNeasy mini spin column and is then 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 presence of DNA following the extraction.

PCR was performed in a final volume of 50 µl. Reagents were purchased from Invitrogen (Ontario, Canada) and primers were purchased from the University of Ottawa Sequencing Facility (Ontario, Canada) or Operon Biotechnologies Inc. (Alabama, USA). The amounts of the reagents used in the final master mix were as follows: 5µl 10X PCR buffer, 5 µl $MgCl_2$, 5 µl dNTP (mix of A, C, T and G nucleotides), 1 µl forward primer (CE1E2F1, 5' ACC ATG AGC ACG AAT CCT AAA CCT C 3'), 1 µl reverse primer (CE1E2R2, 5' AGT GAG CAC CAG CGT TCA TGT CCA AG 3'), 0.5 µl of Taq DNA polymerase, 22.5 µl of ion free water and 10 µl of the quantified DNA sample. The

amount of master mix prepared varied according to the number of samples, including a positive and negative control.

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, primers annealing at 55°C for 45 s, and elongation at 72°C for 1 min: one step of final elongation was done at 72°C for 10 minutes with a final 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. It is important to note that there is a 1/10 dilution of the primers. A mouse genotype was confirmed as positive if there was a 1.2 kb band present representing the CE1E2 transgene.

Donor and Recipient Mice for Adoptive Transfer Experiment

All mice used in these studies were purchased from Charles River Laboratories (Wilmington, MA, USA) and were from the B6C3F1 wild type background. Mice were bred and maintained in a pathogen-free state, were fed a diet of ordinary chows and kept in the Animal Care housing facility of the Faculty of Medicine at the University of Ottawa. All procedures were approved and in accordance to the guidelines of the Animal Care Committee of the University of Ottawa. Donor female mice for the adoptive transfer experiment were purchased at 6 to 8 weeks old and sacrificed at 24 weeks of age. Additionally, all recipient mice were males of around 12 weeks of age whether wild type or HCV transgenic.

Protein Purification

Chinese hamster ovaries cell lines (CHO-K1) were transfected with each pEF6 coreE1E2, pEF6 core, pEF6 E1 and pEF6 E2 inserts in order to develop stable cell lines expressing these inserts with histidine tags under the selective pressure of blasticidin. TOPO-TA HCV CE1E2 construct was subcloned into a pEF6/Myc-His vector: this was constructed by Masoud Ghorbani. This vector contains six histidine residues which permit purification of the His tagged proteins by immobilized metal affinity chromatography (Clontech Talon Metal Affinity Resin Kit, Palo Alto, CA). Transfection was performed by 2 electroporation shocks at 1.4–1.6 KV using an electroporation apparatus (BTX Inc., San Diego, CA). The transfected cells were incubated in IMDM (Sigma-Aldrich, St. Louis, MO) containing 10% FCS (Life Technologies Laboratories, Grand Island, NY) and 50 μg/mL of penicillin-gentamicin. The other pEF6/Myc-His vectors containing either core, E1 or E2 separate inserts were constructed by Xin Min Dong in our laboratory. Cells were cultured and maintained at 37°C until they were confluent (usually 3 days) and ready to be harvested using Ham's F-12 media and 1X trypsin (Sigma-Aldrich, St Missouri, USA) and then subjected to protein purification as described below. If cell pellets were not immediately used, these were frozen at -80°C.

The Talon Resin Protein Purification manufacturer's protocol was followed in order to obtain 4 purified proteins, namely CE1E2, core, E1 and E2. Briefly, the respective CHO cell pellet is lysed with 10 ml of Lysis buffer (50 mM sodium phosphate, 30 mM sodium chloride and 1% Triton-X) with 3.49 μl of 2-mercaptoethanol and 1 EDTA free protease inhibitor tablet (Roche Diagnostics, Indianapolis, USA). The pellet with this solution is mixed with the vortex and incubated for 30 minutes at room temperature. The cellular suspension is then sonicated 3 times for 10 second bursts with

a 1 minute break on ice in between each sonication. The sonicated cellular suspension is centrifuged at 10, 000 rpm for 30 minutes at 4°C in a 50 ml polycarbonate tube in order to create a visible pellet. The supernatant is then transferred to a 15 ml falcon tube containing washed Talon Superflow Metal Affinity Resin (BD Biosciences Clontech, California, USA). This mixture is left to rotate in the cold room for 1 hour to allow the protein to bind to the resin. After this incubation, the tube is centrifuged at 2400 rpm for 5 minutes and subsequently washed twice with 10 ml of 1X Equilibration /Wash buffer (5X Equilibration/Wash buffer: 250 mM sodium phosphate and 1.5 M sodium chloride). The resin is then resuspended in 5-6 ml of 1X Equilibration /Wash buffer and transferred to a Talon Disposable Gravity Column (Clontech, California, USA). The resin containing the protein is allowed to settle in the column until there is a pink layer above the filter. The cap is then removed, and the column is rinsed twice with 5 ml of 1X Equilibration /Wash buffer. The purified protein is collected in five fractions of 300 µl by adding 1X Elution buffer (150 mM Imidizole, 50 mM sodium phosphate and 300 mM sodium chloride) to the column each time. The protein concentrations of the fractions are verified with the Biorad Protein Assay protocol described below.

Determination of Protein Concentration

Protein concentration was determined using a Bio-Rad protein essay, more specifically the microtitre method. Standards were prepared by diluting bovine serum albumin (BSA) to 100 µg/ml in PBS to provide standards of 100, 50, 25, 12.5, 6.25, 2.125 and 1.56 µg/ml. Both 100 and 50 µg/ml standards were discarded. An amount of 0.4 ml of each standard is combined with 0.1 ml of the Coomassie Brilliant Blue G-250 dye. These standards are the point of comparison for the protein concentration of the

samples. This protocol is a dye-binding assay in which a differential colour change of dye occurs in the presence of various concentrations of protein.

Next, 20 µl of each sample (fractions 1-5 obtained from the protein purification) is mixed with 380 µl of PBS and 100 µl of the dye. These mixtures are incubated at room temperature for 5 minutes and then 200 µl of each standard and sample are transferred in duplicates to a 96-well plate (Nunc, Roskilde, Denmark). The absorbance of the duplicates is 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 accuracy of the purified protein must be verified by Western Blot.

Immunoprecipitation

Immunoprecipitation was done on protein extracts from core, E1 and E2 individual constructs expressed in CHO cell line. Briefly, 250 µg of protein lysates from each specimen were incubated with 1 µg affinity-purified antibody for each specific protein for 4 hours at 4°C on the shaker. Subsequently, 20 µL of protein G Dynabeads (Invitrogen, cat #100.03D) were added to allow the formation of protein G-IgG-Protein complexes. The samples were then placed on a shaker at 4° overnight. The next day, the samples were centrifuged and the supernatant was discarded. The complexes are then washed three times in lysis buffer containing protease inhibitors. Following these washes, the samples are centrifuged one final time, the supernatant is removed and 25 µl of 2x loading buffer is added to the tubes. These samples are then boiled at 95°C for 5 minutes to allow the denaturation of the protein and to separate the protein from the protein G beads. The samples are then centrifuged one final time for one minute and the supernatant is loaded on a 10 % SDS-PAGE gel. The proteins are then identified by

Western blot as explained below. All the centrifugations were carried at a speed of 14000 rpm and a temperature of 4°.

Western Blot

Briefly, proteins were previously purified and quantified using the Clontech Protein Purification Protocol. An amount of 10 μg of respective protein was denatured by heating for 5 minutes at 100°C in SDS sample loading buffer. The protein samples were then separated by electrophoresis on 10% SDS-PAGE gels and transferred to PVDF membranes (BioRad, Cat # 162-0177). The membranes were incubated overnight on the shaker at 4°C in blocking buffer (5% BSA in TBST) with their respective antibodies. The primary antibodies used were as follows: mouse monoclonal core (1:1000) for core protein, goat polyclonal to E2 (1:1000) for E2 protein, human heat inactivated patient serum (1:1000) for E1 protein and for CE1E2 polyprotein. The core and E2 proteins were purchased from Biodesign International (Maine, USA) and the rabbit polyclonal to CE1E2 was prepared in the laboratory by Masoud Ghorbani. After washing 3 times with TBST for 10 minutes on the 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, rabbit anti-goat (1:5000) for E2, goat anti-human horseradish peroxidase (1:5000) for E1 and CE1E2. The secondary antibodies were purchased from BioRad. The ladder used for these Westerns was precision plus protein dual color standards (Cat #161-0374) from Biorad. The computer software used for analysis of the membranes is GeneSnap version 6.05.

pVax Plasmid Purification

DNA plasmids were constructed for all the proteins mentioned above, namely CE1E2, core, E1 and E2. The different sequences were sub-cloned into pVAX (Invitrogen, Burlington, ON). DH5α cells were transfected with recombinant plasmid containing the correctly oriented inserts, amplified, and purified using the Endofree plasmid purification kit (Qiagen). Plasmid DNA was obtained as described in the EndoFree plasmid purification handbook (Qiagen, Crawly, UK).

Transient Transfection to Verify Protein Expression

CHO-K1 cells were transiently transfected with pVax core, pVax E1 and pVax E2 constructs to verify expression of proteins within the cells. Briefly, CHO-K1 cells are plated at $2*10^5$ cells/ml in a 24 well plate in complete Ham's F-12 media. After a 24 hour incubation period at 37°C cells should be 95% confluent and ready for transfection. Next, 2 µl of Lipofectamine 2000 Reagent (Invitrogen, Burlington, Ontario) and 50 µl of OPTI-MEM (Invitrogen, Burlington, Ontario) are combined and incubated at room temperature for 5 minutes. Following this, 1 µg of each respective DNA is mixed with 50 µl of OPTI-MEM. The lipofectamine and DNA mixes are combined and incubated at room temperature for 30 minutes. The lipofectamine/ DNA mixes are then added to the respective wells in the plate. After a 24 hour incubation period, the supernatant is taken out of the wells: the wells are washed with PBS and then trypsinized with 150 µl of 1X Trypsin. An amount of 1 ml of complete Ham's F-12 is added to each well and the cells are transferred to a small Petri dish containing an autoclaved glass cover slip at the bottom. Another 2 ml of complete Ham's F-12 is added to the Petri dish to ensure the cover slip is immersed in media. The cells are incubated 24-48 hours or until confluent.

Once cells are confluent, they are fixed on the cover slips with acetone: first for 1 minute with 30% acetone, followed by 1 minute at each 50% and 80% acetone. The cells are then washed 3 times with PBS. A primary antibody against either core, CE1E2, E1 or E2 is prepared at a 1/100 dilution and the slips are incubated for 2 hours with 200 µl of the diluted antibody. The primary antibody is washed off 6 times with PBS. The secondary antibody conjugated to Fit-C is added at the same dilution and the slides are incubated for 1 hour at 4°C in the dark. Excess secondary antibody is washed off 6 times with PBS. The slides are then mounted with mounting media and analyzed by fluorescent microscopy.

Immunization of Donor Mice

Donor B6C3F1 mice were immunized four times with different HCV vaccine components, with a 3-5 week wait interval period between each immunization. The donor mice were sacrificed 10 days after the final immunization. Donor group 1 contained 10 mice which were used as negative control donors and donor group 2 contained 11 mice which received HCV antigens. The first immunization was done intra-peritoneally where group 1 received $2 * 10^7$ pfu of rVV1170 (empty vaccinia) in PBS and group 2 received $2 * 10^6$ pfu of rVVE12C/B, a modified vaccinia virus containing HCV-1 truncated core, E1, E2, p and truncated NS2 (aa 134-966) inserted into the Eco R1 site of the vector. This reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: rVV E12C/B from Chiron Corporation. (Germantown, USA). In the second immunization, each mouse from group 1 received the adjuvants CpG (30 µl) and montanide (60 µl, ISA-51, Seppic Inc., Fairfield, NJ) in a PBS solution and group 2 received CE1E2 polyprotein (25 µg per

mouse), CpG (30 μl) and montanide (60 μl) in a PBS solution. For the third immunization, group 1 received empty pVax, montanide (45 μl) and CpG (30 μl) in a PBS solution and group 2 received pVax Core DNA (75 μg), pVax E1 DNA (75 μg), pVax E2 DNA (75 μg), CE1E2 polyprotein (25 μg), montanide (45 μl) and CpG (30 μl) in a PBS solution. For the fourth immunization, group 1 and 2 received the same content as the third immunization, except the mice from group 2 received 25 μg of each pVax DNA. The last three boosting immunizations were done intramuscularly in the quadriceps major. The amount of vaccine injected varied from 100 μl to 190 μl per mouse.

ELISA: Humoral Immune Response of Donor Mice

Antibody titers against HCV antigens found in the vaccine were assessed in the donor mice. Mice were bled after each immunization and the humoral immune response was assessed by ELISA using mouse sera. Blood was collected in a capillary type tube the Microvette (Sarstedt, Numbrecht, Germany) by puncturing the saphenous vein with a needle. After the blood was collected, samples were centrifuged at 3000 rpm for 15 minutes to obtain serum. Serum levels of HCV-specific antibodies were measured using the HCV recombinant core, E1 or E2 individual proteins as a capture molecules and a mouse-specific monoclonal antibody–horseradish peroxidase (HRP) conjugate detection system. EIA/RIA Strip Well TM plates (Corning CoStar Inc., New York, NY) were coated with 20 μg/ml of respective recombinant protein dissolved in sterile distilled/ deionized water (coat at 100 μl per well), sealed with film and incubated overnight at 4°C. After this incubation, these plates were washed 6 times with 300 μl/well of PBS/0.05% Tween20 (Sigma-Aldrich). After washing, the plates were blocked with 1%

BSA (Sigma-Aldrich, St. Louis, MO) in PBS for 1 hour at 37°C. The plates were then washed and dilutions of sera in 1% BSA/PBS were incubated for 2 hours at 37°C. After this incubation, the plates were washed again. Antibodies were detected with a 1/1000 dilution in 1% BSA/PBS of the required goat anti-mouse IgG (H+L)-HRP conjugate (Biorad Laboratories, Hercules, CA). O-phenylenediamine dihydrochloride (OPD, Sigma-Aldrich) and hydrogen peroxide in a citrate/phosphate buffer were used to develop the colour reaction. The reaction was stopped after about 30 minutes of colour development by the addition of 50 µl of 1 N HCl to each well. The optical density was read at 490 nm using the Biorad Microplate Reader 3550-UV (New-York, USA).

Lymphocyte Proliferation Assay

For the CD4+ T cell proliferation assay, the CFSE stained cells derived from the spleen were split according to the following conditions: unstimulated or CE1E2 stimulated (10 µg/ml). After the addition of the appropriate stimulants, cells were incubated in the dark at 37°C in 5% CO_2 for 4 days. It is important to remember that cell manipulations should be done in the dark due to the presence of the CFSE dye.

Cell Surface Staining of Donor Cells for Flow Cytometry

Following the 4 day incubation period, cells are washed with 3 ml of PBS/0.1% azide/5% FCS and centrifuged at 1600 rpm at 4°C for 5 minutes. The supernatant is then dumped and the cells are stained with the appropriate surface antibodies. The cells are stained with 2 µl of each CD3 PE, CD4 PE-Cy7 and CD8 PerCP (antibodies from BD Pharmingen), the tubes are vortexed and then incubated for 20 minutes at room temperature in the dark. The cells are then washed with 3 ml of PBS/0.1 azide/5% FCS and centrifuged at 1600 rpm at 4°C for 5 minutes. Following this, the supernatant is

dumped and the cells are analyzed on BD FACS Canto Flow Cytometer (BD Biosciences, California, USA). We gated on the living cells and the number of events obtained for each sample was at least 10 000 events from the gated cells. Surface expression of the molecules was analyzed with FCS Express V3. Marker expression was evaluated as the percentage of positive cells in the gated population as defined by forward and side scatter characteristics. When cells are first stained with CFSE, they have a very bright fluorescence. As the cells proliferate, daughter cells have only half the fluorescence of the parent cells and the fluorescence of the cell population decreases from bright to dim.

ELISPOT INF-γ Assay

The ELISPOT for INF-γ was done according to the protocol established by Mabtech (Mariemont, OH, USA). Recombinant core protein, HCV peptide mix or CE1E2 polyprotein were incubated with donor splenocytes to determine HCV-specific CD8+ T cell responses.

Briefly, 96-well filtration plates (Millipore, Beford, MA, USA) were pre-wet with 15 µl of 70% ethanol for 1 minute at room temperature, washed 5 times with 200 µl of sterile PBS per well and mouse anti-IFN-γ monoclonal coating antibody (mAb AN18, Mabtech, Mariemont, OH, USA) was diluted to 15 µg/ml in sterile PBS and added at 100 µl per well and the plates incubated overnight at 4°C. Following the incubation period, excess antibody was removed by washing the plate 5 times with 200 µl/well of sterile PBS. Medium containing 10% of the same serum used for the cell suspensions was added at 200 µl/well and incubated at room temperature for ≥ 30 minutes. After the

incubation, this medium was removed and the cell suspensions containing the possible stimulatory agents were added to make a final volume of 100-150 µl/well.

Cells were suspended at 200 000 cells/well and stimulated with CE1E2 polyprotein, peptide mix, and core protein, all used at 10 µg/ml. The CE1E2 polyprotein and core protein were obtained in the laboratory as described above. The peptides, namely core 1 (35-44, YLLPRRGPRL), core 2 (132-140, DLMGYIPLV) and E1 (220-229, ILHTPGCV) were prepared by a peptide chemist, Dr. Andrei Ogrel from Variation Biotechnologies Inc. (Gatineau, Quebec). Unstimulated cells were used as a negative control and PMA/ION stimulated cells were used as a positive control. All conditions were tested in duplicate. The plates were placed in a humidified incubator with 5% CO_2 and incubated for 48 hours. Following the incubation, the cells were removed by emptying the plate and washing 5 times with 200 µl/well of sterile PBS/0.05% Tween20.

The secondary antibody, anti-mouse IFN-γ (mAb R4-6A2-biotin, Mabtech, Mariemont, OH, USA) was diluted to 1 µg/ml in PBS/0.5% FCS, and added at 100 µl/well for an incubation of 2 hour at room temperature. The plates were then washed and streptavidin conjugated with ALP (Streptavidin-ALP-PQ, 1:1000) in PBS/0.5% FCS was added at 100 µl/well and incubated for 1 hour at room temperature. The plates were washed to remove excess antibody and 100 µl/well of substrate solution, BCIP/NBT alkaline phosphatase substrate (Moss, Inc.) was added and the plates were incubated until spots emerged at the site of responding cells (around 20 to 30 minutes). Colour development was halted by extensive washing of the plates under tap water. The underdrain (plastic under plate) was removed in order to rinse the back of the membranes. The plates were left to dry, images were taken with the KS Elispot-Axioplan

21 (Zeis) and spot-forming cells (SFC) were counted and analyzed using the Immunospot Software version 3.2. The mean number of SFC was calculated in the following manner: mean SFC of stimulated condition - mean SFC from unstimulated condition, thus data is shown after the background subtraction of spots.

Preparation of Lymphocytes from Donor Mice Spleens

Donor mice were anesthetized by injecting 0.2 ml of Somnotal (MTC Pharmaceuticals, Cambridge, ON, Canada) into the intra-peritoneal cavity. Blood was taken from the heart by cardiac puncture (for sera analysis) and spleens were surgically removed and placed in eppendorf tubes containing sterile PBS.

Spleens were then processed into cellular suspensions by gently pressing the organ with a plastic pipette through a 100 μ nylon mesh (BD Biosciences) into a 50 ml sterile falcon tube (BD Biosciences): 20 ml of PBS (Wisent Bioproducts, St-Bruno, QC, Canada) was added to aid the passing of the cells through the mesh. The tubes were then centrifuged at 1600 rpm at 4°C for 5 minutes. The supernatant was dumped and the cellular suspensions were depleted of red blood cells (RBC) with the addition of 10 ml of RBC lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) for 5 minutes at room temperature. The cells were recentrifuged at 1600 rpm for 5 minutes at 4°C after the addition of 10 ml of warmed RPMI 1640 (Wisent Bioproducts, St-Bruno, QC, Canada). The supernatant was then dumped and the cells were washed another 2 times with 10 ml RPMI. The cells were then counted using a hematocytometer and divided into several fractions for later experiments: 1) cells labeled with CFSE and used for injections and CFSE proliferation, and 2) cells for CTL/ELISPOT assays. Remaining cells were frozen at -80°C in freezing media containing 90% dimethyl sulphoxide

(DMSO, Sigma-Aldrich, Oakville, ON, Canada) and 10% fetal calf serum (FCS, Wisent Bioproducts, St-Bruno, QC, Canada).

Labeling cells with CFSE

To prepare 10 mM of CFSE stock solution, 90 µl of dimethyl sulfoxide (DMSO, company) was added to 500 µg lyophilized powder of CFSE dye (Molecular Probes, Oregon USA, V-12883 Component A: 5(6)-CFDA, SE, mixed isomers, 500 µg, Lot 02D1-1, MW 557.47). The stock solution was then diluted in sterile PBS/0.1% BSA to obtain a working concentration of 10 µM.

An equal volume of 10 µM of CFSE dye solution was added to 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 cRPMI (complete RPMI consists of RPMI 1640 (Gibco, Invitrogen Corporation, Grand Island NY, USA), supplemented with 5% fetal calf serum from Wisent and 1% penicillin/gentamicin from Sigma) followed by a 5 minute incubation of the cellular suspensions on ice. It is important to use ice-cold RPMI. Cells were then centrifuged at 1600 rpm for 5 minutes at 4°C and the supernatant was removed. Following this, the cells were washed 2 times with warmed complete RPMI. The cells were then resuspended in cRPMI and some cells were removed for the proliferation assay. The remaining cells were then pooled, centrifuged and resuspended at 50 * 10^6 cells per 100 µl sterile PBS. The CFSE staining was verified before injection to recipient mice by looking at CFSE labeled cells by flow cytometry.

Adoptive Transfer of Donor Cells to Recipient Mice

CFSE labeled cells from the donor mice were injected through tail veins to the recipient mice (n = 8 per group, 4 groups). 50 * 10^6 cells suspended in 100 µl of sterile PBS was injected per mouse. One mouse was bled 24 hours after the injection to verify the presence of CFSE labeled cells in the blood, in order to confirm a successful tail vein injection.

Sacrificing Recipient Mice

Recipient mice were sacrificed 8 days post adoptive transfer. The following organs or liquids were collected: blood, lymph nodes, spleen and liver. The blood, lymph nodes and spleen were solely collected for cellular analysis on flow cytometry. These were processed into individual cellular suspensions as described for the donor mice. However, the liver was split into 3 sections: 1) for cellular analysis on flow cytometry, 2) for histology analysis and 3) for fluorescent microscopy (frozen sections). The various organs were immediately analyzed on the flow cytometry.

Cell Surface Staining of Recipient Cells for Flow Cytometry

Cells were immediately harvested from the recipient mice and prepared for flow cytometry. Cells were then stained in the same manner as mentioned above for the proliferation assay of the donor mice. The cells are stained with 2 µl of CD3 PE, CD4 PE-Cy7 and CD8 PerCP (all antibodies from BD Pharmingen). The cells are analyzed on BD FACS Canto Flow Cytometer (BD Biosciences, California, USA). We gated on the living cells and the number of events obtained for each sample was at least 10 000 events from the gated cells. Surface expression of the molecules was analyzed with FCS Express V3. Marker expression was evaluated as the percentage of positive cells in the gated population as defined by forward and side scatter characteristics.

Histological Analysis

Liver tissue samples from donor mice were fixed in 4% formalin, embedded in paraffin, sectioned (5 μm thick) and stained with hematoxylin and eosin: this staining was done according to standard methods used in the Department of Pathology and Laboratory Medicine at the Faculty of Medicine, University of Ottawa. The sections were then analyzed for the presence of lymphocyte infiltration and liver damage.

Fluorescence Microscopy

Fluorescent microscopy was used to attempt to locate CFSE positive lymphocytes in the livers of the recipient mice. Liver cryosections, 5 μm thick, were placed on polylysine-coated microscope slides and examined under fluorescence microscopy.

Statistical Analysis

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

CHAPTER 4:
RESULTS

TRANSGENIC MICE AND MOLECULAR ANALYSIS

Genotyping and Generation of Transgenic Mice

Mice were constructed with the goal of characterizing the immunonomodulatory effects of HCV structural proteins *in vivo*. Mice were bred in the Animal Care Facility at the University of Ottawa to maintain the CE1E2 transgenic line. Previously determined transgenic females were mated with one B6C3F1 wild type male, while transgenic males were mated with two B6C3F1 females at a time: this was done to maintain a heterozygous population. With each new generation, pups are screened for the presence of the transgene. HCV transgene expression was determined 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 4.1**). We observe a predominance of transgene expression in the male gender. Transgenic males seemed to transmit the transgene more efficiently to male offspring. Once mice were genotyped, transgenic mice were separated from non-transgenic littermates. Alopecia (a form of hair loss) was also identified in many of the transgenic mice. This could be due to a secondary effect of the transgene expression or could be a result of aging.

Protein Purification Concentration

The concentration of extracted proteins was always verified after purification. As a general trend, the concentration of the proteins was higher in the 2^{nd} and 3^{rd} elutions and progressively decreased. However, despite the use of the same cell line and growth conditions and confluence for all cell lines, the protein extractions did not all yield the same concentration of protein. (**Table 4.1**). This could be due to the fact that core alone can be toxic to the cells.

Figure 4.1. Transmission of HCV transgene in the mouse colony. The genotype of the mice was confirmed by gel electrophoresis. These mice were generated by breeding founder CE1E2 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 mice that carry the transgene have a 1.2 kb band (represented by the arrow) in their genomic DNA (lanes 2-5), indicating that they have successfully integrated the transgene. This band is absent in mice that are non-transgenic for this transgene (lanes 1 and 6). pVax CE1E2 plasmid was used as a positive control (Plasmid). Mice that are previously designated as transgenic can also be used as positive controls. L represents the 1 kb DNA ladder used for this experiment.

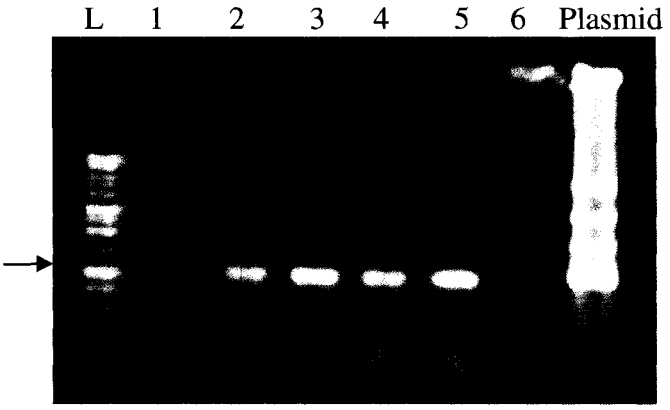

L
1
2
3
4
5
6
Plasmid

Table 4.1. Concentration of proteins obtained after protein purification. Concentration of proteins (μg/ml) as determined by the Bio-Rad Microtitre Method following protein purification of CE1E2, core, E1 and E2 proteins from stably transfected CHO-K1 cells. For each respective CHO-K1 cell pellet, protein was extracted using the Talon Resin Protein Purification Method and five elutions were collected. As a general trend, fractions 2 and 3 usually had the highest concentration of protein. The concentrations obtained are based on a linear curve fit. Although the confluence of cells was equivalent, the yield of protein for each cell line different with core having the lowest yield. This could be attributed to the fact that core can be toxic to cells.

	Concentration (µg/ml)			
Fraction	**CE1E2**	**Core**	**E1**	**E2**
1	270.4	82	50.9	95.3
2	632.7	466.6	700.9	848.4
3	249.2	328.4	510.4	608.2
4	186.8	157.4	246.3	234.4
5	121.8	67.5	112.9	99.6

Protein Detection by Western Blot

Following the protein assay, a Western blot was done to confirm that the isolated samples were indeed the CE1E2 polyprotein, core, E1 or E2 proteins. CE1E2 polyprotein is approximately 110 kDa in size (20). However, in certain lanes, additional bands were detected indicating the presence of cleaved polyprotein in the form of partial E1 or E2 fragments (**Figure 4.2**). Western blots were also done for individual core (**Figure 4.3**), E1 (**Figure 4.4**) and E2 (**Figure 4.5**) protein constructs. Western blots indicate the proteins at their respective size: core at 21 kDa, E1 at 33 kDa and E2 at 70 kDa (193).

Plasmid Purification

Many plasmids were used over the course of this experiment. DH5- α cells were transfected with the different hepatitis C constructs. Plasmid purification was then done to obtain specific DNA segments. To certify that the purified DNA segments were the correct inserts, plasmid digestion was done. pVax core, pVax E1 and pVax E2 constructs were digested with *Eco*R1 and *Xba*I enzymes to separate the pVax from the insert. These digests were verified by gel electrophoresis. Bands corresponding to the weight of the constructs were confirmed. Each lane had a band representing the pVax (at 3kb) and core had a band at 573 bp, E1 at 576 bp and E2 at 1089 bp certifying that each pVax construct contained the proper DNA insert (**Figure 4.6**).

Figure 4.2. Western blot detecting the presence of core, E1 and E2 polyprotein. The proteins were purified from CHO pEF6 CE1E2 transfected cells using Talon Resin protein purification method. Cells were grown in the presence of blasticidin, as a selective antibody. The primary antibody used was heat inactivated patient serum at 1:1000 (obtained from the Clinical Laboratory at the Children's Hospital of Eastern Ontario) and the secondary antibody was goat anti-human horseradish peroxidase at 1:5000. **A)** ladder, **B)** untransfected CHO cell lysates, **C)** polyprotein and cleaved E1E2 protein and **D-G)** CE1E2 polyprotein. The polyprotein may yield different protein products due to processing by cellular proteases.

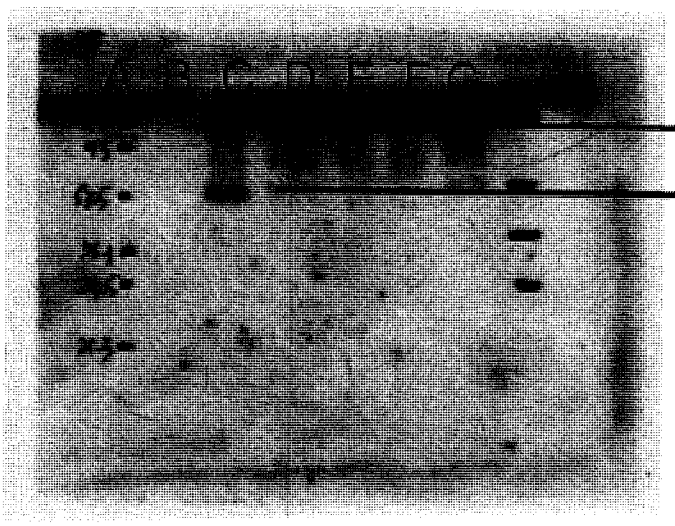

Glycosylated polyprotein (110 kd)
Partial E1, E2 proteins

Figure 4.3. Western blot detecting the presence of core protein. The proteins were purified from CHO pEF6 core transfected cells using Talon Resin protein purification method. Cells were grown in the presence of blasticidin, as a selective antibody. The primary antibody used was mouse monoclonal core at 1:1000 from Biodesign International (Maine, USA) and the secondary antibody was goat anti-mouse at 1:5000. Bio-Rad Dual Colour ladder (**L**), samples from transgenic mouse livers indicating the presence of core (**A-D**), sample from wild type mouse liver lacking core expression (**E**), protein purified from CHO pEF6 core transfected cells (**F**). The core protein is indicated by the circles.

L A B C D E F

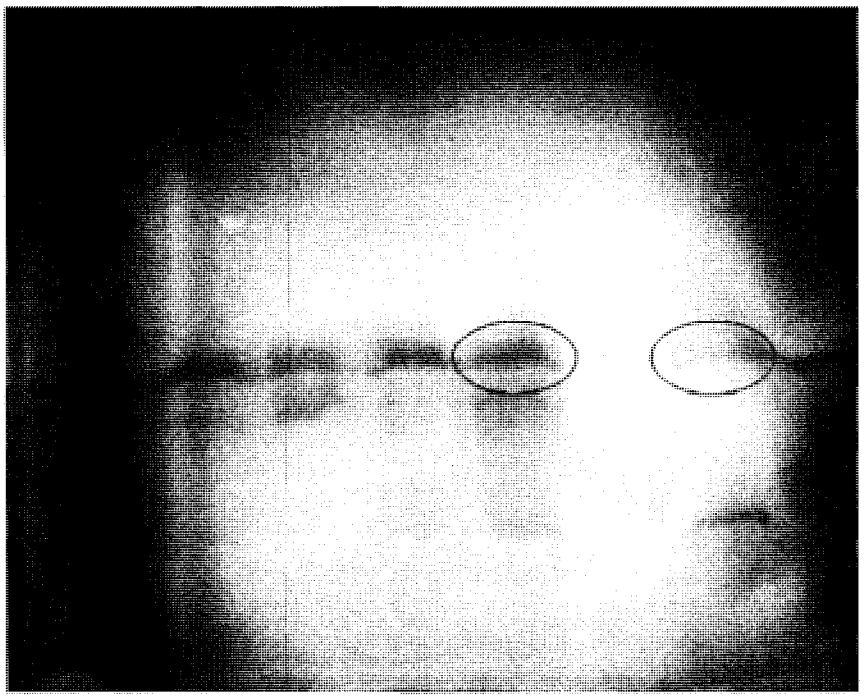


Figure 4.4. Western blot detecting the presence of E1 protein. The proteins were purified from CHO pEF6 E1 transfected cells using Talon Resin protein purification method. Cells were grown in the presence of blasticidin, as a selective antibody. The primary antibody used was heat inactivated patient serum at 1:1000 (obtained from the Clinical Laboratory at the Children's Hospital of Eastern Ontario) and the secondary antibody was goat anti-human horseradish peroxidase at1:5000. The presence of E1 protein is indicated by the circle (**E1**).

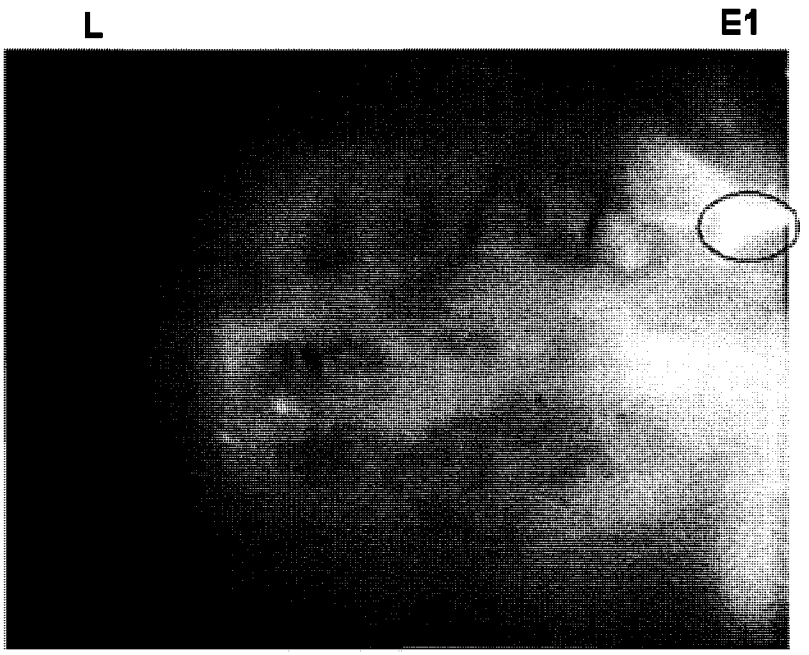

L
E1

Figure 4.5. Western blot detecting the presence of E2 protein. The proteins were purified from CHO pEF6 E2 transfected cells using Talon Resin protein purification method. Cells were grown in the presence of blasticidin, as a selective antibody. The primary antibody used was goat polyclonal to E2 at 1:1000 from Biodesign International (Maine, USA) and the secondary antibody was rabbit anti-goat at1:5000. The presence of E2 protein is indicated by the circle (**E2**).

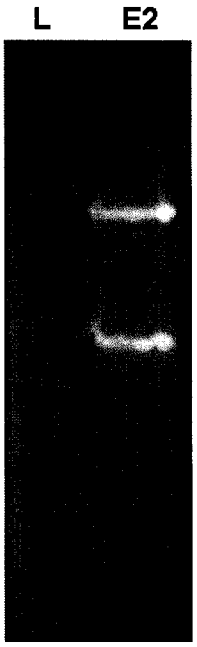
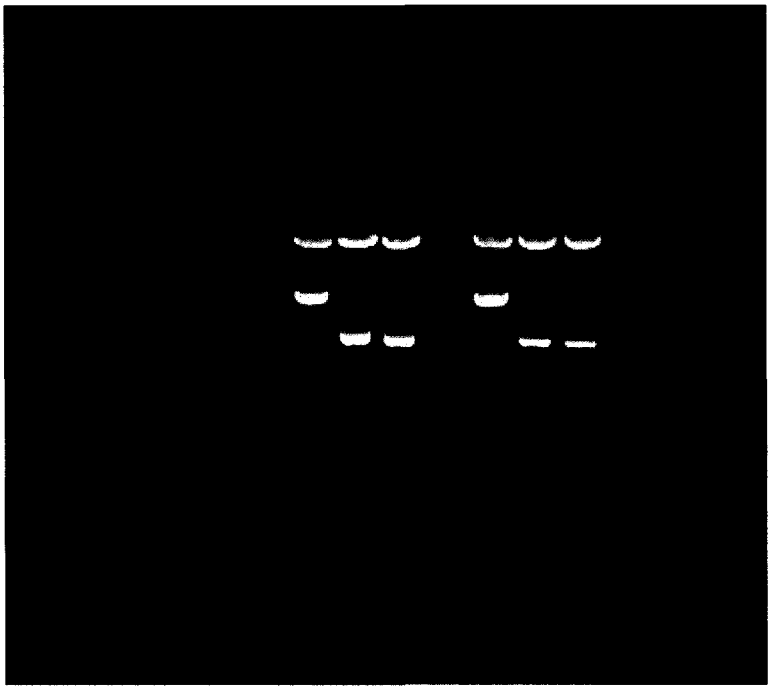

L
E2

Figure 4.6. Plasmid purification from DH5-α cells individually transfected with pVax core, E1 and E2 constructs. Plasmid DNA was digested with *Eco*R1 and *Xba*I enzymes. The bands, compared with the 1KB ladder (**L**) represent the corresponding weight of each construct, **A)** E2 at 1089 bp **B)** E1 at 576 bp and **C)** core at 573 bp (pVax remains at 3kb in each instance).



In vitro Expression of Plasmids

The ability of pVax expression vectors to produce proteins in cell culture was tested in mammalian cells. Chinese hamster ovaries (CHO) cells were transiently transfected with pVax core, pVax E1 and pVax E2 individual constructs. These cells were cultured in normal conditions for 3 days, following a 24 hour incubation on glass slides. After this, the cells were fixed and stained. Rabbit polyclonal antibody to CE1E2 was used for immunolabeling. Anti-rabbit FitC-conjugated antibody was used as a secondary antibody. This process indicated that these proteins were expressed mainly in the cytoplasm of these cells (**Figure 4.7**).

IMMUNE RESPONSES IN HCV-PRIMED OR CONTROL DONOR MICE

Humoral Immune Responses, ELISA

Many ELISA were done over the course of the experiment to examine if there was progress in the immune responses mounted by the donor mice. Control mice, or mice receiving HCV antigens were injected 4 times with different combinations of empty vectors, or vectors expressing HCV antigens as explained in **Table 4.2**. IgG antibodies, the most abundant form of antibody in human blood, were measured. This antibody can bind virus and cause classic complement activation with the goal of eliminating the virus (for example through phagocytosis).

ELISA results indicated significant increases of IgG antibodies against HCV core, E1 and E2 proteins. The measurement of IgG antibodies in the serum of control and HCV immunized mice two weeks after the first immunization indicated only slight but not statistically significant differences between the control and immunized group (**Figure 4.8**).

Figure 4.7. In vitro expression of individual hepatitis C proteins. Immunofluorescent staining of CHO K1 cells transiently transfected with **A)** pVax E2 DNA showing expression of E2 in the cytoplasm, or **B)** empty pVax. Cells express E2 proteins in the cytoplasm of the cells. The cells were cultured in normal conditions for 3 days and transferred to glass slips for 24 hours. Rabbit polyclonal antibody to CE1E2 was used as a primary antibody on the transfected cells. Anti-rabbit FitC-conjugated antibody was used as a secondary antibody. (Magnification power 20x)

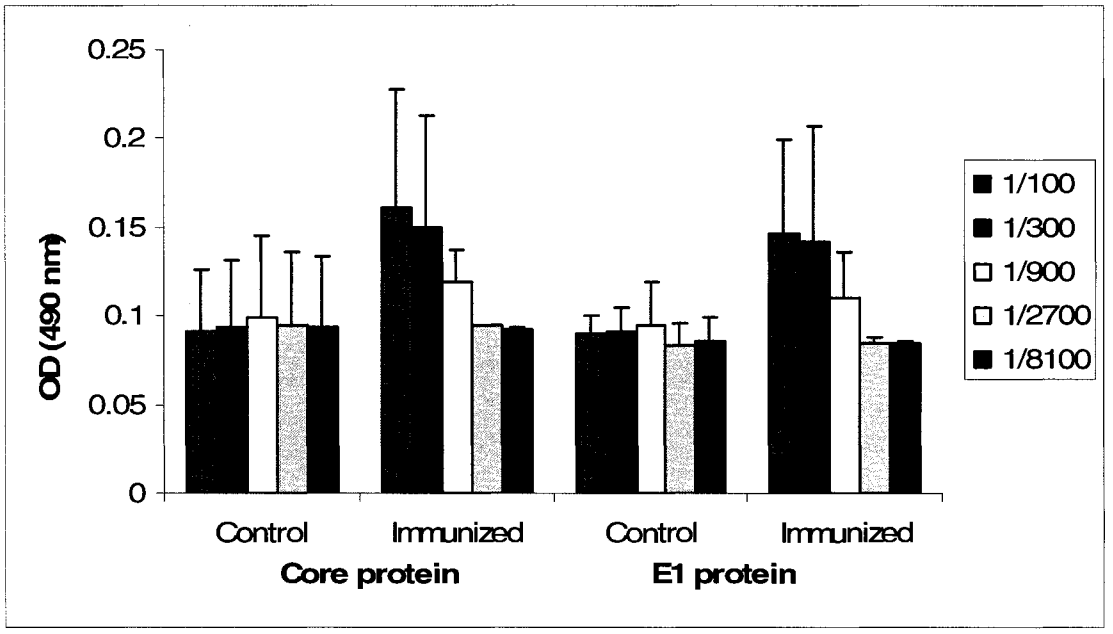
A)

B)

Table 4.2. Summary of vaccination regimen used in B6C3F1 donor mice. Group 1 was the control group which received empty expression vectors while group 2 was the group which received HCV antigens as described below. It is important to note that the route of immunization for the first vaccination was intra-peritoneal (IP) and the three following boosters were done intramuscularly (IM) in the quadriceps major. rVVCE12 B/C represents poly vaccinia, an MVA expressing HCV core, E1, E2, p7 (NS1) and NS2 (aa 1-966) from the National Institute of Health (NIH). Empty vaccinia, VV1170, was also obtained from the NIH. Vaccinia virus vectors were administered at a dose of at $2*10^7$ pfu/mouse. Mice were first vaccinated at 6 to 8 weeks of age whereas there was a 4 to 5 week wait interval between each subsequent immunization (4 weeks between first and second immunization and 5 weeks between subsequent immunizations). Control and HCV immunized mice always received immunizations on the same day. Mice were sacrificed 2 weeks after the final immunization.

Immunization	Group 1	Group 2	Route	Volume
First	Empty vaccinia virus (VV1170) and PBS	rVVCE12 B/C and PBS	IP	200 µl
Second	Montanide, CpG and PBS	CoreE1E2 polyprotein (25 µg/mouse), Montanide, CpG and PBS	IM	150 µl
Third	Empty pVax, Montanide, CpG and PBS	pVax Core, E1 and E2 DNA (each at 75 µg/mouse), CoreE1E2 polyprotein (25 µg/mouse), Montanide, CpG and PBS	IM	190 µl
Fourth	Empty pVax, Montanide, CpG and PBS	pVax Core, E1 and E2 DNA (each at 25 µg/mouse), CoreE1E2 polyprotein (25 µg/mouse), Montanide, CpG and PBS	IM	150 µl

Figure 4.8. Measurement of anti-core or anti-E1 IgG antibodies in serum of control and HCV immunized mice after the first immunization. In the first immunization, B6C3F1 control mice (n=10) were immunized with empty vaccinia plus the adjuvants montanide and CpG. B6C3F1 mice receiving HCV antigens (n=11) were immunized with HCV poly vaccinia plus montanide and CpG. Two weeks after the first immunization, sera was obtained and analyzed for the presence of anti-core or anti-E1 IgG antibodies. For the control group, average results are obtained from mouse number 4 and 6 (n=2). For the immunized group, average results are obtained from mouse number 15 and 19 (n=2). Plates were coated with 20 μg/ml of core or E1 protein which were extracted from CHO stable cell lines and purified by Talon Resin Protein Purification method. Values for the mean antibody levels of pooled mouse sera with standard deviations are shown above. Leftover sera were aliquoted and frozen at -20°C. Antibody levels in both groups did not differ significantly.


0.25
0.2
0.15
0.1
0.05
0
OD (490 nm)
1/100
1/300
1/900
1/2700
1/8100
Control
Immunized
Control
Immunized
Core protein
E1 protein

It is important to remember that after the first immunization, the mice receiving HCV antigens were only exposed to the poly vaccinia (rVVCE12 B/C) indicating this might not be the most efficient way to induce strong humoral immune responses.

After the third immunization, the mice receiving HCV antigens had been exposed to antigens in the form of poly vaccinia, CE1E2 polyprotein and individual pVax DNA constructs for core, E1 and E2. Significant increases in total IgG for pooled mouse sera were seen for all 3 antigens studied when comparing the control and immunized groups: **1**) anti-core IgG antibodies (**Figure 4.9**) at a 1:100 antibody titer (P value < 0.05) and at 1:900 (P value < 0.01), **2**) anti-E1 IgG antibodies (**Figure 4.10**) at 1:100 antibody titer (P value < 0.05) and at 1:300 (P value < 0.05) and **3**) anti-E2 IgG antibodies (**Figure 4.11**) at 1:300 antibody titer (P value < 0.05), at 1:900 (P value < 0.01) and at 1:2700 (P value < 0.05).

A comparative analysis of the humoral immune responses of donor mice to HCV antigens was done (**Figure 4.12**) for core, E1 and E2 proteins in control and immunized mice. The amount of IgG antibodies to the various proteins differed, with immunized mice having a higher response to E1 protein followed by core while the response to E2 protein was weaker. Control mice had similar IgG responses, or lack thereof, to all three HCV proteins. After the fourth immunization individual mouse humoral responses were analyzed for anti-core IgG antibodies (**Figure 4.13**). Statistically significant differences (P value < 0.01) were observed for each antibody titer studied when comparing the control group to the immunized group. Additionally, the amount of IgG antibodies to core protein increased after the fourth immunization compared to the amount seen after the third immunization.

Figure 4.9. Measurement of anti-core IgG antibodies in serum of control and HCV immunized mice after the third immunization. B6C3F1 control mice (n=10) were immunized with empty vaccinia, empty pVax plasmid plus the adjuvants montanide and CpG. B6C3F1 mice receiving HCV antigens (n=11) were immunized with HCV poly vaccinia, CE1E2 polyprotein, pVax core, E1 and E2 individual DNA constructs plus montanide and CpG. Two weeks after the third immunization, sera was obtained and analyzed for the presence of anti-core IgG antibodies. For the control group, sera from mouse 1 to 5 and 6 to 10 were pooled and an average was obtained. The same was done for the immunized group with pooled sera from mouse 11 to 15 and 16 to 21. Plates were coated with 20 μg/ml of core protein extracted from CHO stable cell line expressing core and purified by Talon Resin Protein Purification method. Values for the mean antibody levels of pooled mouse sera with standard deviations are shown above. Leftover sera were aliquoted and frozen at -20°C. Antibody levels in both groups differed significantly. There was a significant difference in total IgG between the control and immunized groups with a 1:100 antibody titer (* P value < 0.05) and at 1:900 (* P value < 0.01).

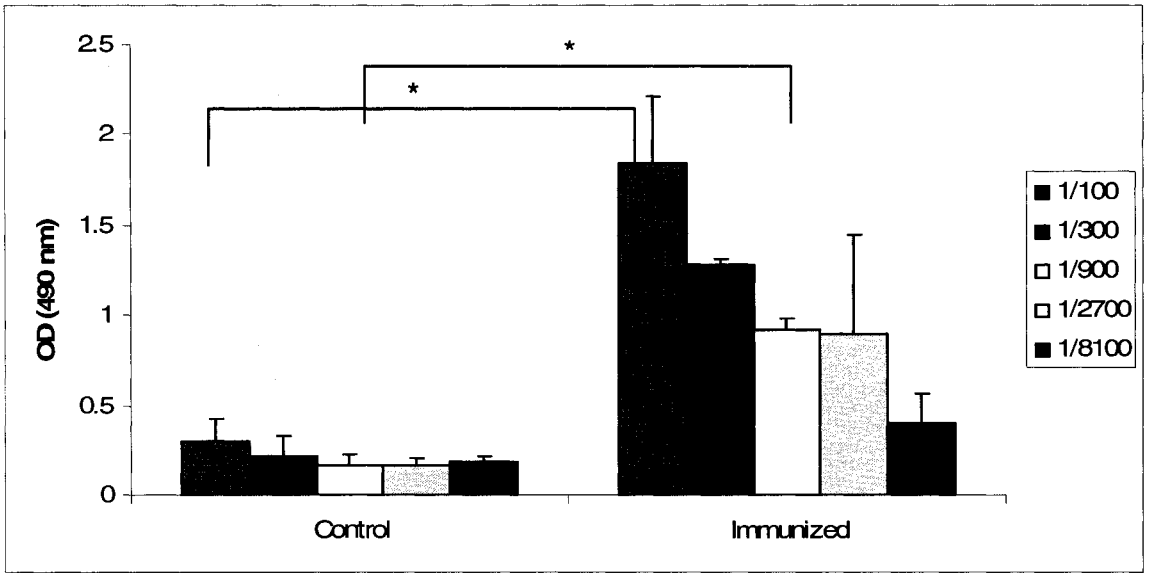

2.5
2
1.5
1
0.5
0
OD (490 nm)
*
*
Control
Immunized
1/100
1/300
1/900
1/2700
1/8100

Figure 4.10. Measurement of anti-E1 IgG antibodies in serum of control and HCV immunized mice after the third immunization. B6C3F1 control mice (n=10) were immunized with empty vaccinia, empty pVax plasmid plus the adjuvants montanide and CpG. B6C3F1 mice receiving HCV antigens (n=11) were immunized with HCV poly vaccinia, CE1E2 polyprotein, pVax core, E1 and E2 individual DNA constructs plus montanide and CpG. Two weeks after the third immunization, sera was obtained and analyzed for the presence of anti-E1 IgG antibodies. From the control group, sera from mouse 1 to 5 were pooled and an average was obtained. The same was done for the immunized group with pooled sera from mouse 11 to 15 and 16 to 21. Plates were coated with 20 μg/ml of E1 protein extracted from CHO stable cell line expressing core and purified by Talon Resin Protein Purification method. Values for the mean antibody levels of pooled mouse sera with standard deviations are shown above. Please note, standard deviations are not shown for the control group since there was only one group of pooled sera. Leftover sera were aliquoted and frozen at -20°C. Antibody levels in both groups differed significantly. There was a significant difference in total IgG between the control and immunized groups with a 1:100 antibody titer (* P value < 0.05) and at 1:300 (* P value < 0.05).

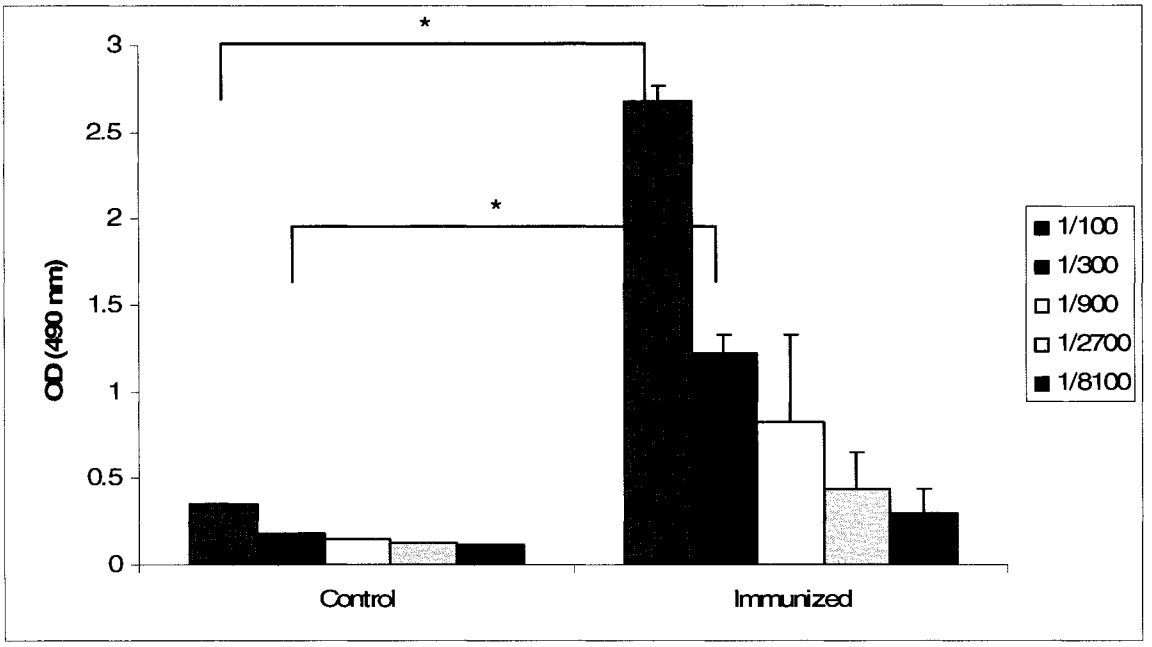

*
*
3
2.5
2
1.5
1
0.5
0
OD (490 nm)
Control
Immunized
1/100
1/300
1/900
1/2700
1/8100

Figure 4.11. Measurement of anti-E2 IgG antibodies in serum of control and HCV immunized mice after the third immunization. B6C3F1 control mice (n=10) were immunized with empty vaccinia, empty pVax plasmid plus the adjuvants montanide and CpG. B6C3F1 mice receiving HCV antigens (n=11) were immunized with HCV poly vaccinia, CE1E2 polyprotein, pVax core, E1 and E2 individual DNA constructs plus montanide and CpG. Two weeks after the third immunization, sera was obtained and analyzed for the presence of anti-E2 IgG antibodies. From the control group, sera from mouse 1 to 5 and 6 to 10 were pooled and an average was obtained. The same was done for the immunized group with pooled sera from mouse 16 to 21. Plates were coated with 20 μg/ml of E2 protein extracted from CHO stable cell line expressing core and purified by Talon Resin Protein Purification method. Values for the mean antibody levels of pooled mouse sera with standard deviations are shown above. Please note, standard deviations are not shown for the immunized group since there was only one group of pooled sera. Leftover sera were aliquoted and frozen at -20°C. Antibody levels in both groups differed significantly. There was a significant difference in total IgG between the control and immunized groups with a 1:300 antibody titer (* P value < 0.05), at 1:900 (* P value < 0.01) and at 1:2700 (* P value < 0.05).

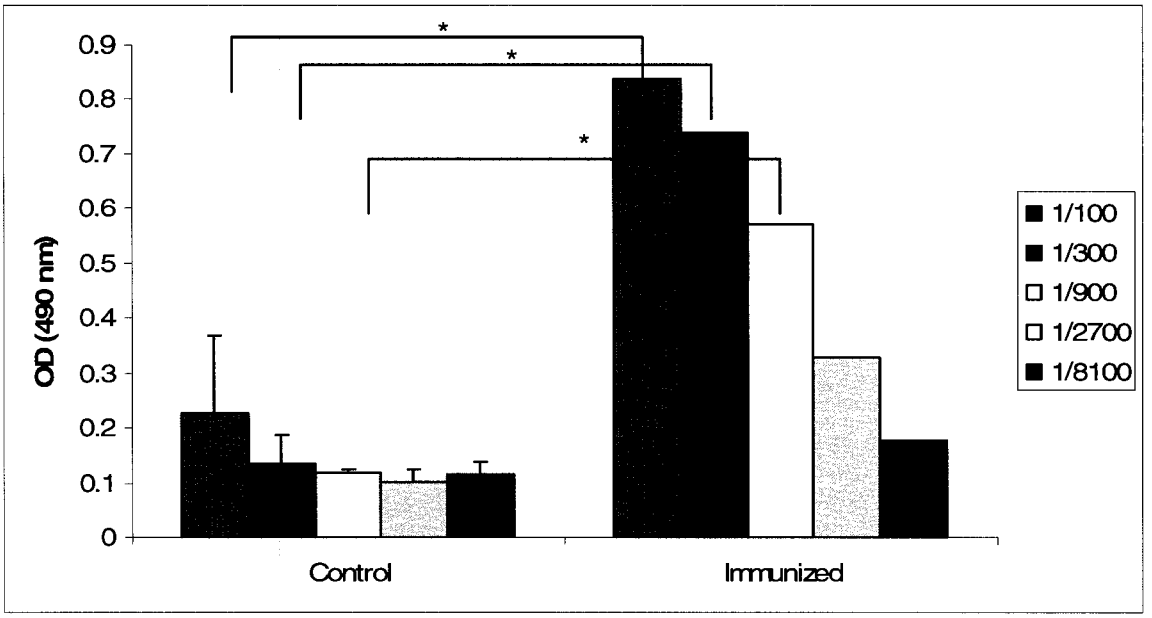

OD (490 nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Immunized
1/100
1/300
1/900
1/2700
1/8100
*
*
*

Figure 4.12. Comparative analysis of humoral immune responses of donor mice to various HCV antigens after the third immunization. B6C3F1 control mice (n=10) were immunized with empty vaccinia, empty pVax plasmid plus the adjuvants montanide and CpG. B6C3F1 mice receiving HCV antigens (n=11) were immunized with HCV poly vaccinia, CE1E2 polyprotein, pVax core, E1 and E2 individual DNA constructs plus montanide and CpG. Two weeks after the third immunization, sera was obtained and analyzed for the presence of anti-core, anti-E1 and anti-E2 antibodies. From the control group, sera from mouse 1 to 5 and 6 to 10 were pooled and an average was obtained. The same was done for the immunized group with pooled sera from mouse 16 to 21. Plates were coated with 20 μg/ml of each respective protein extracted from CHO stable cell lines and purified by Talon Resin Protein Purification method. Mean values of anti-core, anti-E1 and anti-E2 IgG are shown above for various dilutions of pooled mouse sera from control and immunized groups of mice. Protein specific antibodies differed in number, with immunized mice having a higher response to E1 protein followed by core. The response to E2 protein was not strong. The control mice had similar responses to all three HCV proteins.

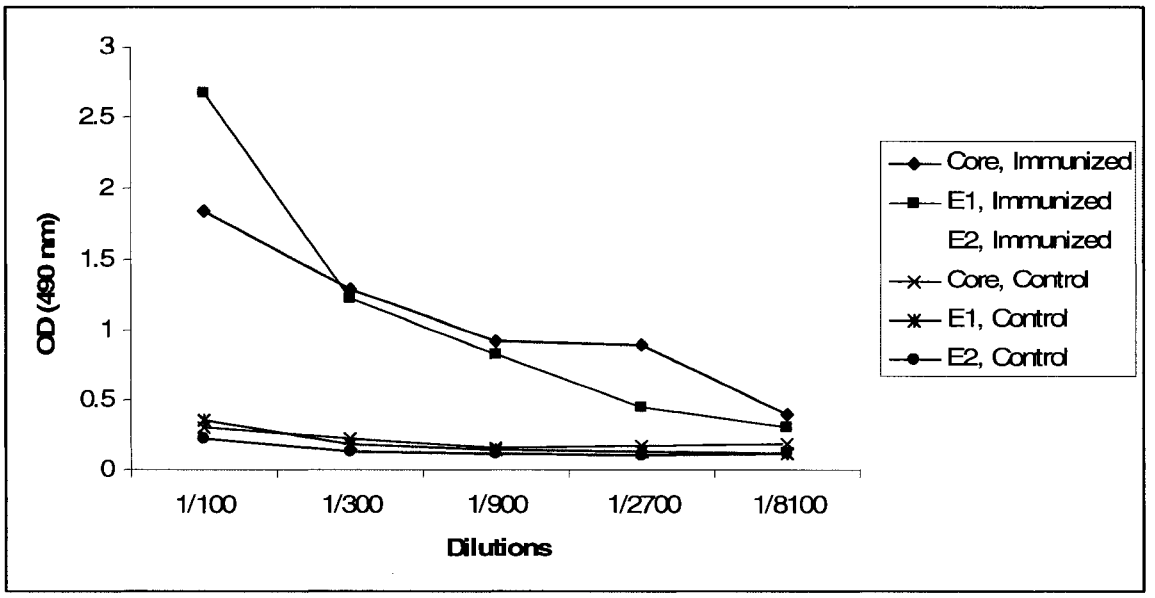
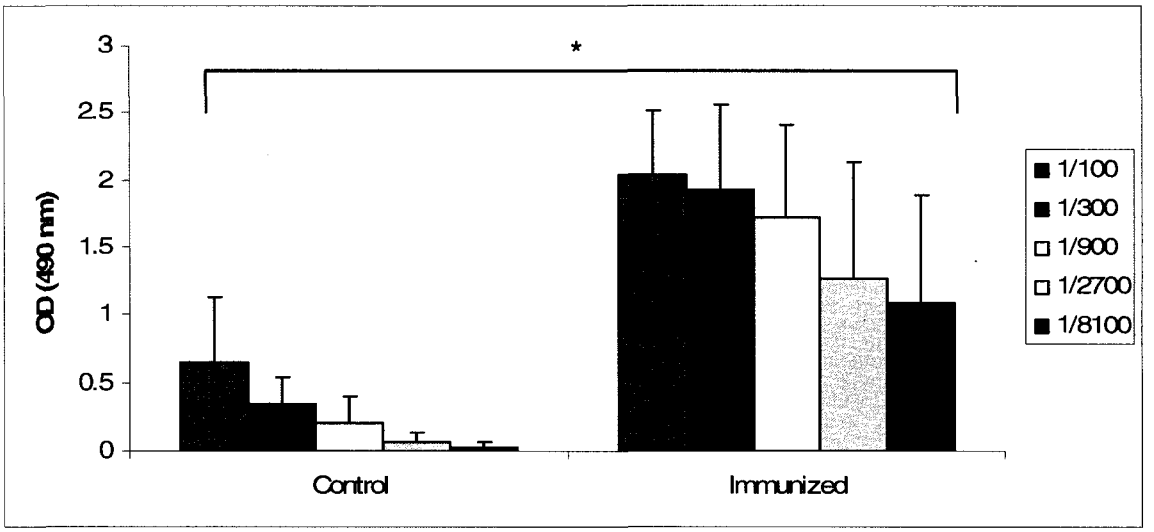

3
2.5
2
1.5
1
0.5
0
OD (490 nm)
1/100
1/300
1/900
1/2700
1/8100
Dilutions
Core, Immunized
E1, Immunized
E2, Immunized
Core, Control
E1, Control
E2, Control

Figure 4.13. Measurement of anti-core IgG antibodies in serum of control and HCV immunized mice after the fourth immunization. B6C3F1 control mice (n=8, mouse number 1 to 8) were immunized with empty vaccinia, empty pVax plasmid plus the adjuvants montanide and CpG. B6C3F1 mice receiving HCV antigens (n=11, mouse number 11 to 21) were immunized with HCV poly vaccinia, CE1E2 polyprotein, pVax core, E1 and E2 individual DNA constructs plus montanide and CpG. Two weeks after the final immunization, sera was obtained by cardiac puncture and analyzed for the presence of anti-core antibodies. Plates were coated with 20 μg/ml of core protein extracted from a CHO stable cell line and purified by Talon Resin Protein Purification method. Results for means with standard deviation are shown above. Leftover sera were aliquoted and frozen at -20°C. There was a significant difference in total IgG between the control and immunized groups for all the dilutions (* P value < 0.01).


*
3
2.5
2
1.5
1
0.5
0
OD (490 nm)
Control
Immunized
1/100
1/300
1/900
1/2700
1/8100

Overall, mice receiving HCV antigens demonstrated higher titers of IgG antibodies to various HCV antigens, indicating the presence of effective humoral immune responses. Moreover, these responses increased over time with subsequent boosters.

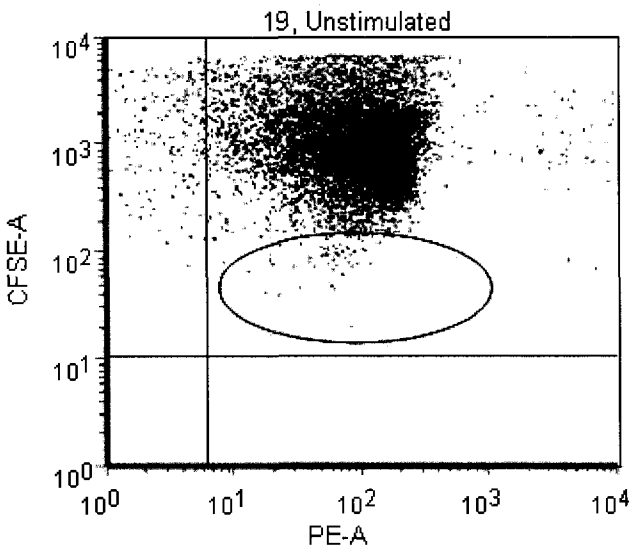
Proliferation of Donor CD4+ T cells

Two weeks after the final immunization, donor mice were sacrificed and their splenocytes were processed into individual cell suspensions. These cells were tested for their ability to proliferate in the presence of HCV antigens. This was done by staining the cells with CFSE membrane dye. This dye permits the user to track proliferating cell populations since when cells proliferate, the dye splits between daughter cells: on the flow cytometry, the intensity of the CFSE dye diminishes and the population shifts to a lower intensity. Cells were left unstimulated or cultured in the presence of CE1E2 polyprotein for 4 days at 37°C, 5% CO_2. Cells were stained with CFSE dye, CD3-PE and CD4-PercP. Cells were gated primarily on lymphocytes, and then on CD3+ T cell populations. The CD4+CFSE positive population was then selected.

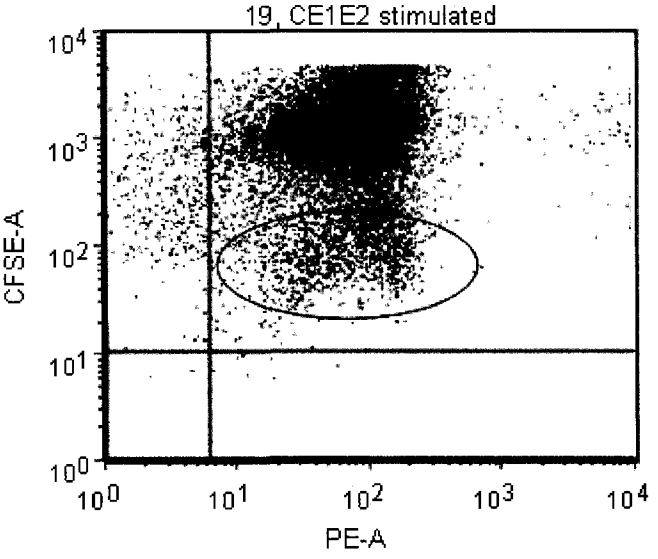
The splenocytes from mouse 19, an immunized donor, were cultured in unstimulated conditions and indicated no evident proliferation (**Figure 4.14 A**). However these splenocytes proliferated in response to the CE1E2 polyprotein antigens (**Figure 4.14 B**). The proliferation is indicated by the shift of cells towards a lower intensity of CFSE since this dye is split amongst daughter cells when cells proliferate. These figures are representative of the CD4+ proliferation for all the immunized donor mice.

Figure 4.14. CD4+ T cell proliferation of immunized donor splenocytes in response to HCV antigens. Splenocytes from immunized donors were stained with CFSE and incubated for 4 days at 37°C, 5% CO_2. **A**) Immunized mouse 19 splenocytes in unstimulated conditions showing no proliferation. **B**) Immunized mouse 19 splenocytes stimulated with CE1E2 polyprotein showing proliferation of cells. The proliferation is indicated by the shift of cells towards a lower intensity of CFSE (dye is split amongst daughter cells when cells proliferate). Cells were stained with CFSE dye, CD3-PE and CD4-PerCP. Cells were gated on lymphocytes, and then on CD3+ T cell populations. The CD4+CFSE positive population was then selected to be represented as shown above on the CFSE and CD3 figure. The upper right quadrants of the above figures represent cells which are CD3+CD4+CFSE positive T cells. These figures are representative of the CD4+ proliferation for all the immunized donor mice.

A)


19, Unstimulated
CFSE-A
PE-A
10^0
10^1
10^2
10^3
10^4

B)

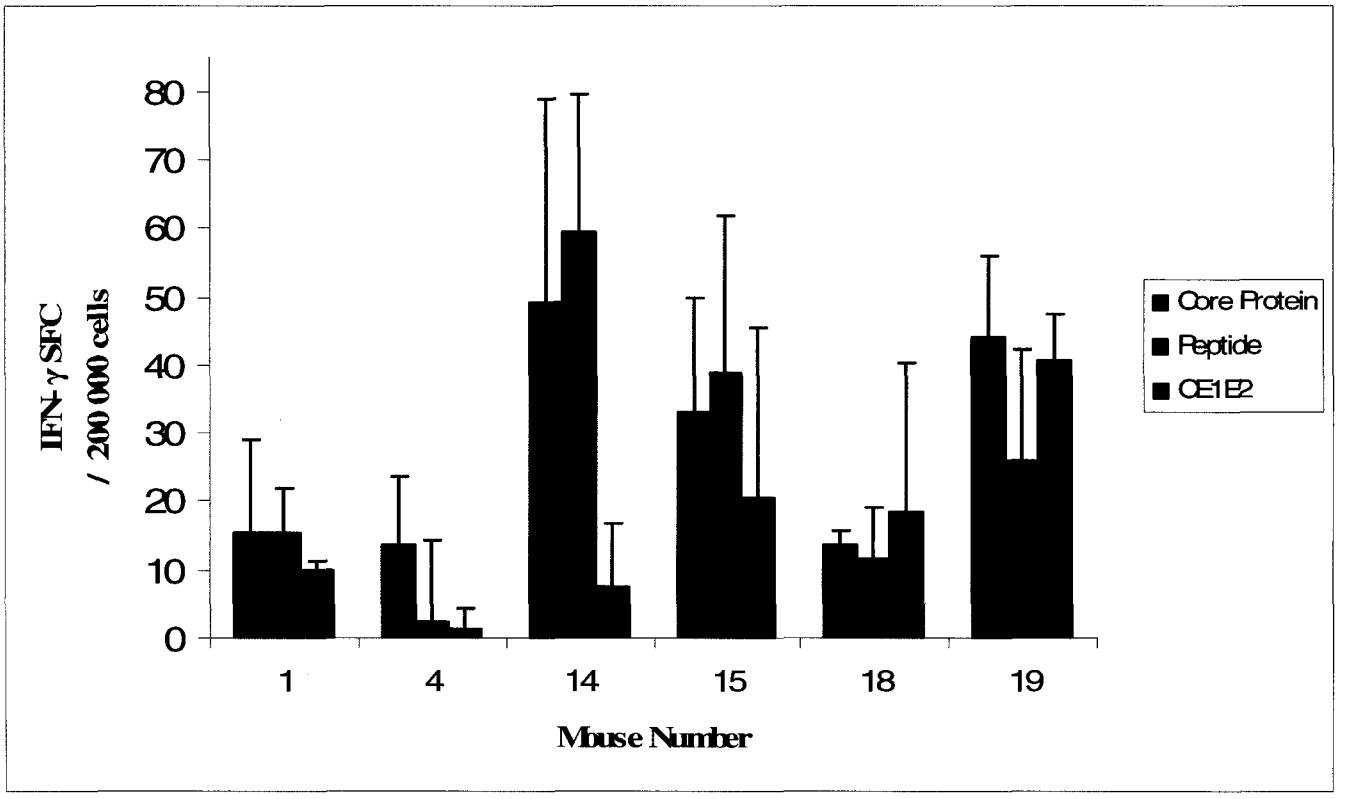

19, CE1E2 stimulated
CFSE-A
PE-A
10^0
10^1
10^2
10^3
10^4

Interferon-γ ELISPOT: CTL Response

ELISPOT was done to observe the IFN-γ response of donor splenocytes to a variety of HCV antigens. This method is highly sensitive and may be used as a surrogate to measure T cell responses. The antigens used were core and CE1E2 proteins extracted from CHO cells prepared in the laboratory and an HCV peptide mix. The peptides used are as follows: core 1 (35-44, YLLPRRGPRL), core 2 (132-140, DLMGYIPLV) and E1 (220-229, ILHTPGCV). We aimed to determine if the immunization strategy used induced a higher frequency of HCV-specific IFN-γ secreting splenocytes. Responses here are either CD4+ or CD8+ since neither T cells were depleted: overall, we are measuring T cell responsiveness.

All cells had a higher than expected amount of SFC in the unstimulated conditions and seemed to possess a generalized state of activation (control and HCV immunized donor mice both received montanide and CpG as adjuvants). However, the background (considered as the mean SFC in the unstimulated condition) has been subtracted from each stimulated condition (**Figure 4.15**). The assay was done using 2 control donor mice (number 1 and 4) and 4 HCV-immunized donor mice (number 14, 15, 18 and 19). From the immunized donors, 3 out of the 4 mice (14, 15 and 19) had statistically significant increases in the mean number of SFC as compared to the control mice. Mouse number 14 and 15 had the highest mean number of SFC for the HCV peptide mix followed by core protein and finally CE1E2 polyprotein. In contrast, mouse 19 had the highest mean number of SFC for the core protein followed by the CE1E2 polyprotein and finally the HCV peptide mix.

Figure 4.15. Frequency of HCV-specific interferon-γ producing splenocytes in donor mice. The ELISPOT assay was done to assess interferon-γ T-cell responses. Briefly, splenocytes at 200 000 cells/well were incubated in the presence of core protein (10 μg/ml), HCV peptide mix (10 μg/ml) or CE1E2 polyprotein (10 μg/ml). Each sample was tested in duplicate and PMA/ionomycin was used as a positive control (data not shown). Mice 1 and 4 were from donor Group 1 and did not receive any HCV antigens during immunizations. Mice 14, 15, 18 and 19 were from donor Group 2 and received HCV antigens during immunizations. These cells were incubated for 48 hours in the presence of different antigens and analyzed for the emergence spot forming cells (SFC) representing HCV-specific interferon-γ producing cells. SFC were counted using the KS Elispot-Axioplan 21 and analyzed with the Immunospot Software version 3.2. Each spot counted represents a single activated cell. The background number of spots (unstimulated condition) was subtracted from the stimulated conditions. The results are shown as the mean with the standard deviation of the duplicates. Differences between reactivity to different HCV antigens were statistically significant for mouse 14, 15 and 19 concerning core protein and HCV peptide mix ($P<0.05$) and for the CE1E2 polyprotein for mouse 19 only ($P<0.01$). These were compared to mouse 1 which was more reactive to the antigens than mouse 4. No differences between groups were observed concerning initial spleen size.


IFN-γ SFC
/ 200 000 cells
80
70
60
50
40
30
20
10
0
1
4
14
15
18
19
Mouse Number
Core Protein
Peptide
CE1E2

Overall, interferon-γ production was significantly higher in response to HCV antigens in the mice which received immunizations with a variety of HCV antigens as opposed to mice who did not receive any. These results suggest that the vaccination with HCV-antigens elicited strong interferon-γ responses in the donor mice, indicating that the immunization regimen induced good cellular immune responses.

IMMUNE RESPONSES IN RECIPIENT MICE

Donor mice were sacrificed and HCV-primed and control donor splenocytes were stained with CFSE before the adoptive transfer of cells to the recipient mice. Previous to the injection 50 x 10^6 cells/mouse, the labeling of splenocytes with CFSE was confirmed by flow cytometry (**Figure 4.16**). Cells were then transferred to the recipient mice by intravenous injection. One mouse was bled 24 hours after the intravenous infection to confirm the presence of CFSE positive cells in the blood of the recipient mice (**Figure 4.17**). The goal was to observe the effect of HCV-primed splenocytes in the HCV transgenic mouse model: splenocytes kinetics could shed insight into cell-mediated immune responses.

Tracking of CFSE Positive Cells: Recipient Mice

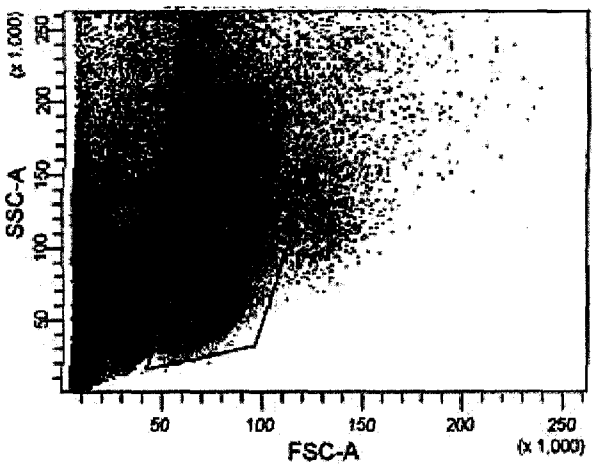
Eight days after the adoptive transfer of CFSE positive HCV-primed or control splenocytes, recipient mice (4 groups of n=8) were euthanized and various lymphoid organs were analyzed by flow cytometry. We attempted to locate CFSE positive cells to reveal which organ cells are homed to by looking at the blood, the spleen, the liver and the lymph nodes. The spleen and the lymph nodes are important secondary lymphoid organs and are popular sites for the induction of effector functions in T cells.

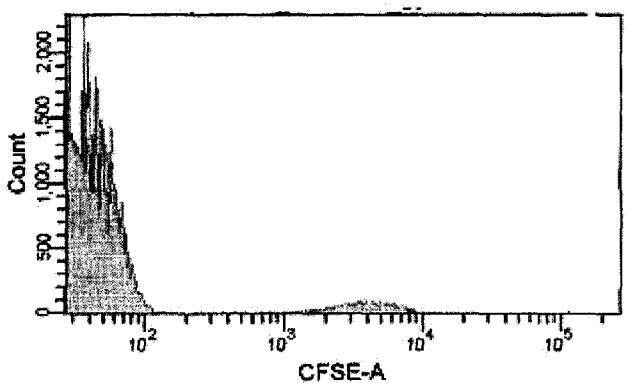
Figure 4.16. Verification of CFSE staining on donor splenocytes. Prior to intravenous injection of the donor cells to the recipient mice, donor cells were observed by flow cytometry to confirm the presence of CFSE positive cells. Two cell populations are visible on the left hand side of the figure representing a cell population and the other representing the CFSE positive stained lymphocytes (P1), our population of interest. The population of interest is visible on the right hand side of the figure where the peak (P2) confirms the presence of CFSE positive cells. This figure confirms the successful staining of splenocytes with CFSE. Whole donor splenocytes were used to avoid excessive cell manipulations (preserve integrity of cells) and to transfer other immune cells (B cells, macrophages, monocytes, etc.) along with CD4+ and CD8+ T cells.

FSC-A
(x 1,000)
50
100
150
200
250
SSC-A (x 1,000)
50
100
150
200
250
P1

CFSE
10^2
10^3
10^4
10^5
Count
0
5
10
15
P2

Figure 4.17. Verification of successful intravenous injection of CFSE positive cells into recipient mice. An individual recipient mouse (# 7) was bled 24 hours post adoptive transfer to certify that CFSE stained cells were present in the blood of the recipient mice. Briefly, blood was depleted of red blood cells and unstained lymphocytes were analyzed on flow cytometry. A small peak of cells to the right hand side of the figure represents the CFSE positive cells which were adoptively transferred to the recipients indicating successful intravenous injection of the mice.


SSC-A
(x 1,000)
250
200
150
100
50
50
100
150
200
250
(x 1,000)
FSC-A


Count
2,000
1,500
1,000
500
0
10^2
10^3
10^4
10^5
CFSE-A

To simplify the explanation of the results, the terms Wt (wild-type) and Tg (transgenic) will be used along with immunized representing the HCV-primed splenocytes and control representing donor splenocytes from mice which did not receive HCV antigens.

When CFSE positive cells were detected in the respective tissue, the number of CD4+ or CD8+ T cells was always similar within the same group and were not statistically significant in any case. A higher percentage of CFSE positive CD4+ or CD8+ T cells were located in the spleens of the Wt immunized followed by Wt control, Tg control and finally the least amount of cells detected in the spleen was in the Tg immunized (**Figure 4.18**). The highest average percentage was of 0.72% for CD4+CFSE+ and of 0.53% for CD8+CFSE+ both from the Wt immunized group. The differences were statistically significant ($P < 0.05$) between Wt immunized and the three other groups. Differences were also significant between Wt control and Tg immunized and Wt control and Tg control but only for CD4+CFSE+ cells. The Tg immunized group had the lowest average percentages of CFSE positive cells 0.15% and 0.11% for CD4+ and CD8+ respectively.

For the blood, the percentage of CFSE positive CD4+ or CD8+ T cells was highest in the Wt immunized followed by Wt control, Tg immunized and finally the least amount of cells detected in the blood was in the Tg control (**Figure 4.19**). The highest average percentage was of 0.76% for CD4+CFSE+ and of 0.44% for CD8+CFSE+ both from the Wt immunized group. The differences were statistically significant ($P < 0.05$) between Wt immunized and the three other groups. Differences were also significant between Wt control and Tg immunized and Wt control and Tg control.

Figure 4.18. Flow cytometry analysis of recipient mice spleens. Wild type (Wt) or HCV transgenic (Tg) mice were sacrificed 8 days after injection with CFSE stained HCV-primed splenocytes (immunized) or control splenocytes (control). The percentage of CFSE positive CD4+ or CD8+ T cells in the spleen of the different recipient mice groups (n=8 per group) is shown. Cells were surface stained with anti-CD3, anti-CD4 and anti-CD8 then analyzed by flow cytometry. FCS Express Version 3 was used to analyze the data. (* P value <0.05, the + symbol represents that the differences in mean values were only significant for CD4+CFSE+ cells between Wt Control and Tg Control).

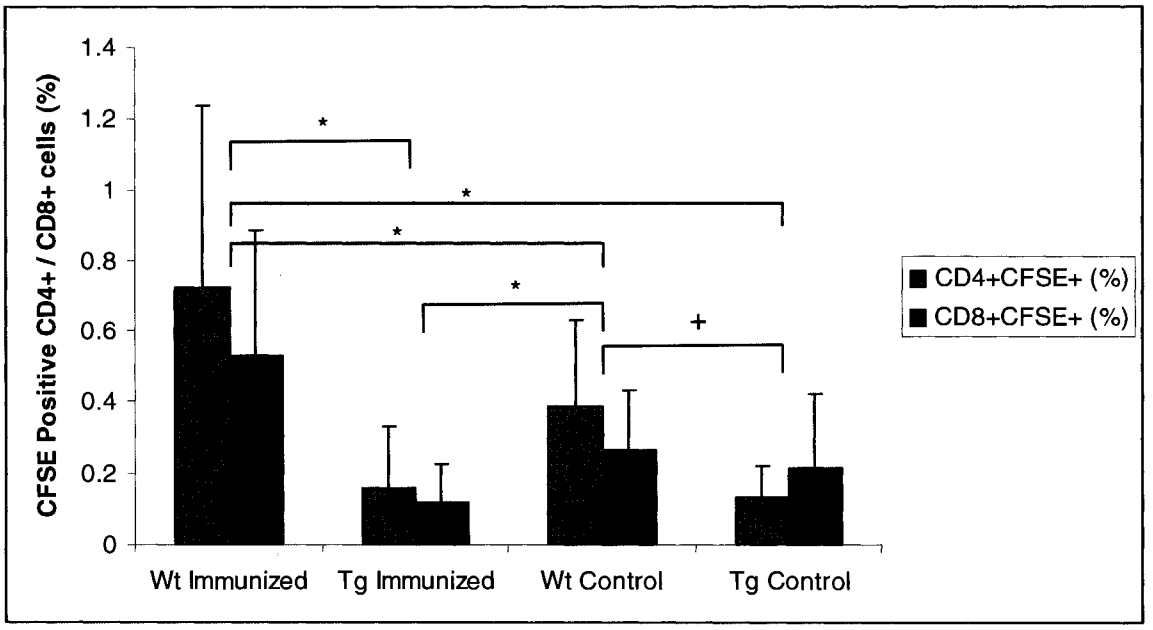
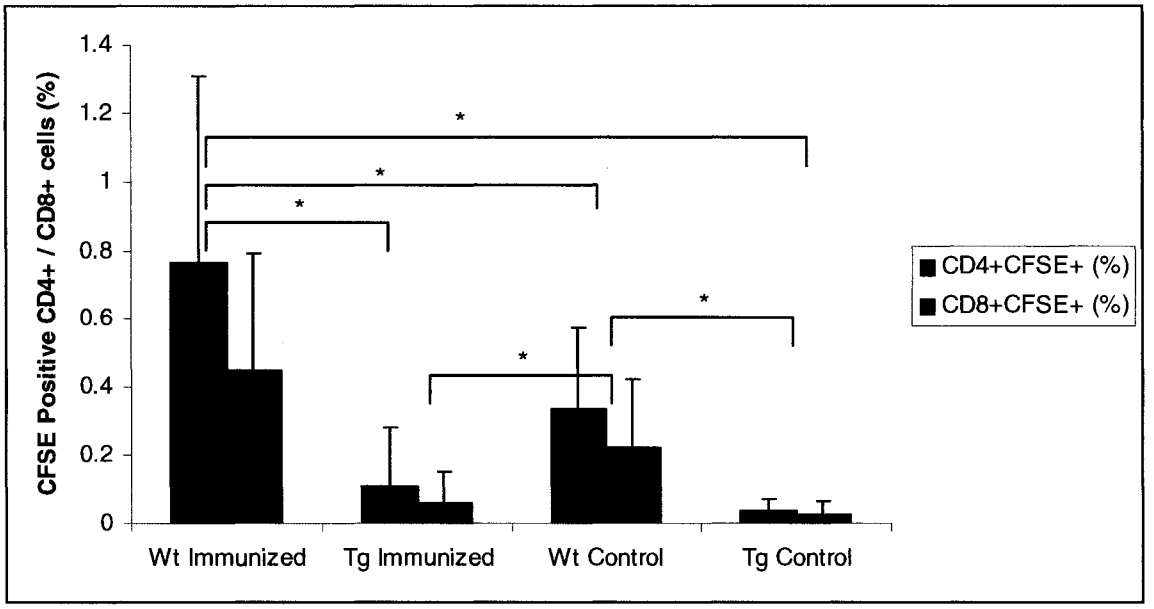

CFSE Positive CD4+ / CD8+ cells (%)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
*
+
Wt Immunized
Tg Immunized
Wt Control
Tg Control
CD4+CFSE+ (%)
CD8+CFSE+ (%)

Figure 4.19. Flow cytometry analysis of recipient mice blood. Mice were sacrificed 8 days after injection with CFSE stained HCV-primed splenocytes (immunized) or control splenocytes (control). The percentage of CFSE positive CD4+ or CD8+ T cells in the blood of the different recipient mice groups (n=8 per group) is shown. Cells were surface stained with anti-CD3, anti-CD4 and anti-CD8 then analyzed by flow cytometry. FCS Express Version 3 was used to analyze the data. (* P value <0.05)


CFSE Positive CD4+ / CD8+ cells (%)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
*
*
Wt Immunized
Tg Immunized
Wt Control
Tg Control
CD4+CFSE+ (%)
CD8+CFSE+ (%)

The Tg control group had the lowest average percentages of CFSE positive cells 0.03% and 0.02% for CD4+ and CD8+ respectively.

In the case of the livers, the percentage of CFSE positive CD4+ or CD8+ T cells was highest in the Tg immunized followed by Wt immunized, Tg control and finally the least amount of cells detected in the blood was in the Wt control (**Figure 4.20**). HCV-primed donor splenocytes were two-fold higher in both CD4+ and CD8+ T cells compared to all other groups of recipient mice. The highest average percentage was of 1.6% for CD4+CFSE+ and of 2.02% for CD8+CFSE+ both from the Tg immunized group. The differences were statistically significant ($P < 0.05$) between Tg immunized while the differences between the other groups were negligible. The Tg immunized group had the lowest average percentages of CFSE positive cells 0.55% and 0.69% for CD4+ and CD8+ respectively.

Last of all, in the case of the lymph nodes the percentage of CFSE positive CD4+ or CD8+ T cells was highest in the Wt immunized followed by Wt control, Tg immunized and finally the least amount of cells detected in the blood was in the Tg control (**Figure 4.21**). The highest average percentage was of 0.77% for CD4+CFSE+ and of 0.59% for CD8+CFSE+ both from the Wt immunized group. The differences were statistically significant ($P < 0.05$) between Wt immunized and the three other groups. Differences were also significant between Wt control and Tg immunized and Wt control and Tg control but only for CD8+CFSE+ cells. The Tg control group had the lowest average percentages of CFSE positive cells 0.01% and 0.03% for CD4+ and CD8+ respectively.

Figure 4.20. Flow cytometry analysis of recipient mice livers. Mice were sacrificed 8 days after injection with CFSE stained HCV-primed splenocytes (immunized) or control splenocytes (control). The percentage of CFSE positive CD4+ or CD8+ T cells in the l of the different recipient mice groups (n=8 per group) is shown. Cells were surface stained with anti-CD3, anti-CD4 and anti-CD8 then analyzed by flow cytometry. FCS Express Version 3 was used to analyze the data. (* P value <0.05)

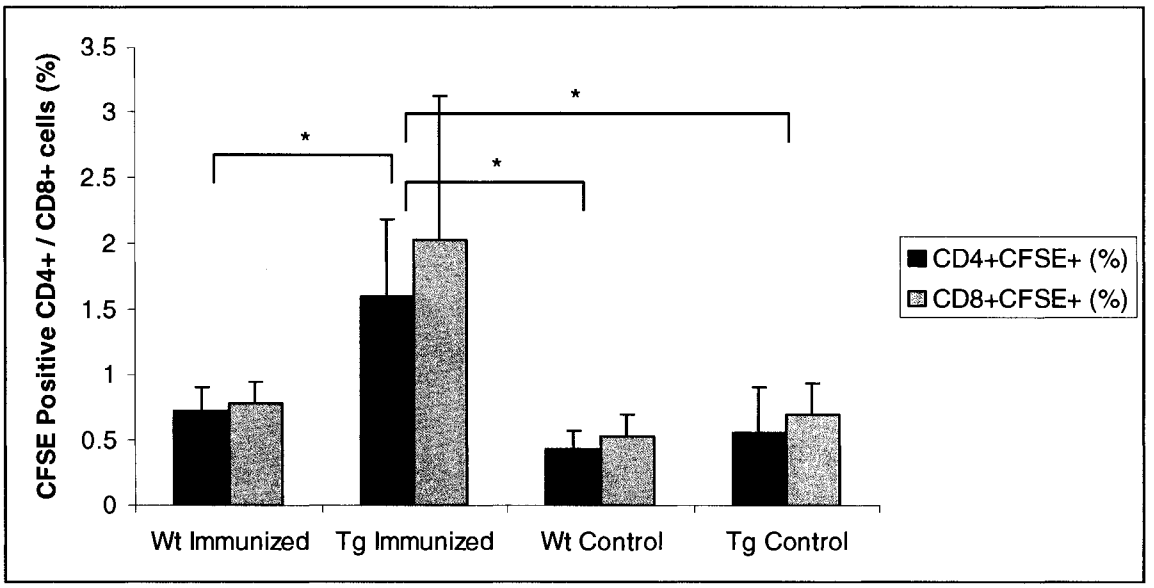
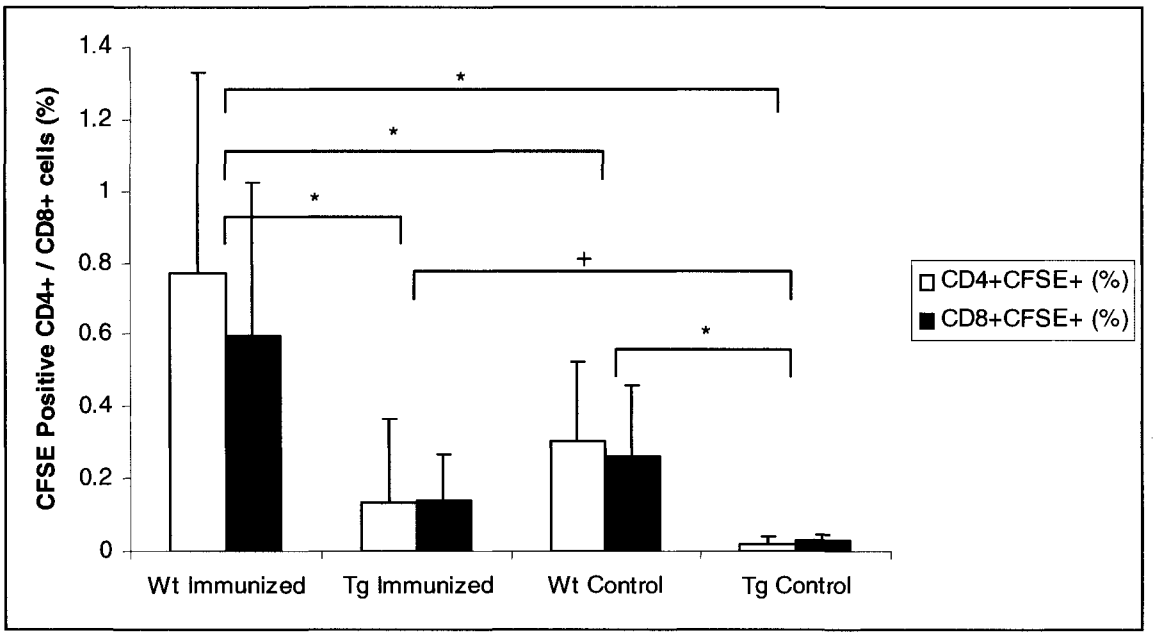

CFSE Positive CD4+ / CD8+ cells (%)
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
Wt Immunized
Tg Immunized
Wt Control
Tg Control
CD4+CFSE+ (%)
CD8+CFSE+ (%)

Figure 4.21. Flow cytometry analysis of recipient mice lymph nodes. Mice were sacrificed 8 days after injection with CFSE stained HCV-primed splenocytes (immunized) or control splenocytes (control). The percentage of CFSE positive CD4+ or CD8+ T cells in the lymph nodes of the different recipient mice groups (n=8 per group) is shown. Cells were surface stained with anti-CD3, anti-CD4 and anti-CD8 then analyzed by flow cytometry. FCS Express Version 3 was used to analyze the data. (* P value <0.05, the + symbol represents that the differences in mean values were only significant for CD8+CFSE+ cells between Wt Control and Tg Control)


CFSE Positive CD4+ / CD8+ cells (%)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
+
*
Wt Immunized
Tg Immunized
Wt Control
Tg Control
CD4+CFSE+ (%)
CD8+CFSE+ (%)

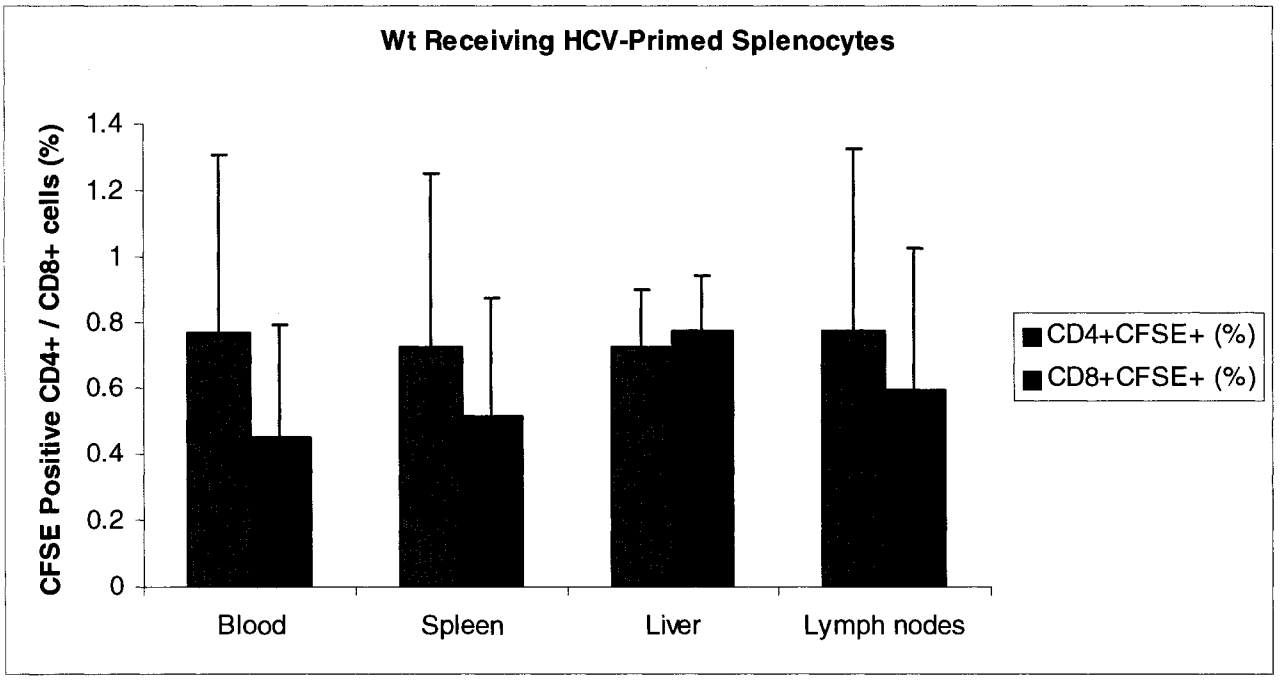
The comparative analysis of differences of averages of CFSE positive CD4+ or CD8+ T cells in different organs was done for Wt immunized and Tg immunized groups. In the case of Wt immunized (**Figure 4.22 A**) the amount of CFSE positive CD4+ and CD8+ T cells were similar in all four organs. However, if we compare organs for the Tg group that received HCV-primed splenocytes, we observe that the amount of CFSE positive CD4+ and CD8+ T cells is significantly higher in the liver (**Figure 4.22 B**). These results indicate that HCV-primed T cells are attracted to the transgene in the transgenic mouse liver potentially through a homing mechanism.

Measurement of ALT and AST values

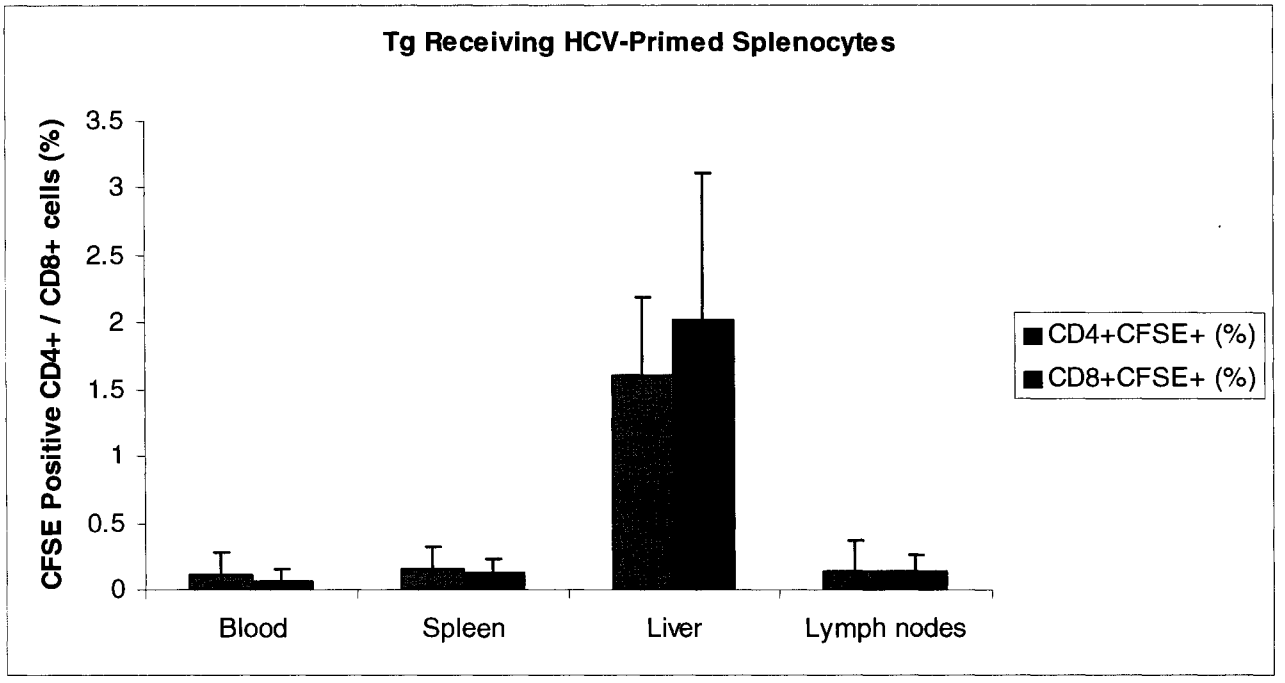
Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are both part of the transaminase enzyme family. These can be used as indicators of liver damage, and elevations in these enzymes are often seen with the occurrence of hepatitis and other acute liver diseases. When cells are damaged, they may leak these enzymes into the bloodstream. ALT is considered as an indicator of liver damage and is more specific to this organ while AST can also be found in the heart. If a blood sample was hemolyzed, the ALT or AST value was removed from the mean since red blood cells carry high levels of these enzymes and could skew the results.

ALT and AST levels of the recipient mice were measured prior to and 8 days after the transfer. The amount of ALT significantly decreased in each group 8 days post adoptive transfer (P value < 0.05) (**Figure 4.23**). However the results did not differ between the 4 groups of recipient mice. The differences between the pre and post values for AST for each group of recipient mice were not statistically significant (**Figure 4.24**).

Figure 4.22. Comparative analysis of averages of CFSE positive CD4+ or CD8+ cells (%) in organs of wild type mice (**A**) or transgenic mice (**B**) who received HCV-primed donor splenocytes. Mice were sacrificed 8 days after injection of CFSE positive donor cells. Flow cytometry analysis of recipient mice (n=8) was done on cells from blood, spleen, liver and lymph nodes. Cells were surface stained with anti-CD3, anti-CD4 and anti-CD8 then analyzed by flow cytometry. FCS Express Version 3 was used to analyze the data.


Wt Receiving HCV-Primed Splenocytes
CFSE Positive CD4+ / CD8+ cells (%)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Blood
Spleen
Liver
Lymph nodes
CD4+CFSE+ (%)
CD8+CFSE+ (%)

A)


Tg Receiving HCV-Primed Splenocytes
CFSE Positive CD4+ / CD8+ cells (%)
3.5
3
2.5
2
1.5
1
0.5
0
Blood
Spleen
Liver
Lymph nodes
CD4+CFSE+ (%)
CD8+CFSE+ (%)

B)

Figure 4.23. Analysis of plasma alanine aminotransferase (ALT) levels in recipient mice. These were calculated by the Vitros 250 using reflectance spectrophotometry. The ALT levels are expressed as units per liter (U/L). Recipient mice were bled the day before (pre) they received the splenocytes from the donor mice, and 8 days after the adoptive transfer (post). The first two groups are B6C3F1 mouse strain and they received 1) splenocytes from immunized donors and 2) splenocytes from the control donors. The last two groups are HCV transgenic mice and they received 3) splenocytes from immunized donors and 4) splenocytes from the control donors. Each group included n=8 mice: however, 3 results were not considered since the specimens were hemolyzed. Values are presented as the mean with the standard deviation. Differences between the pre and post ALT values within each group were statistically significant (* $P < 0.05$), however no differences were observed between groups.

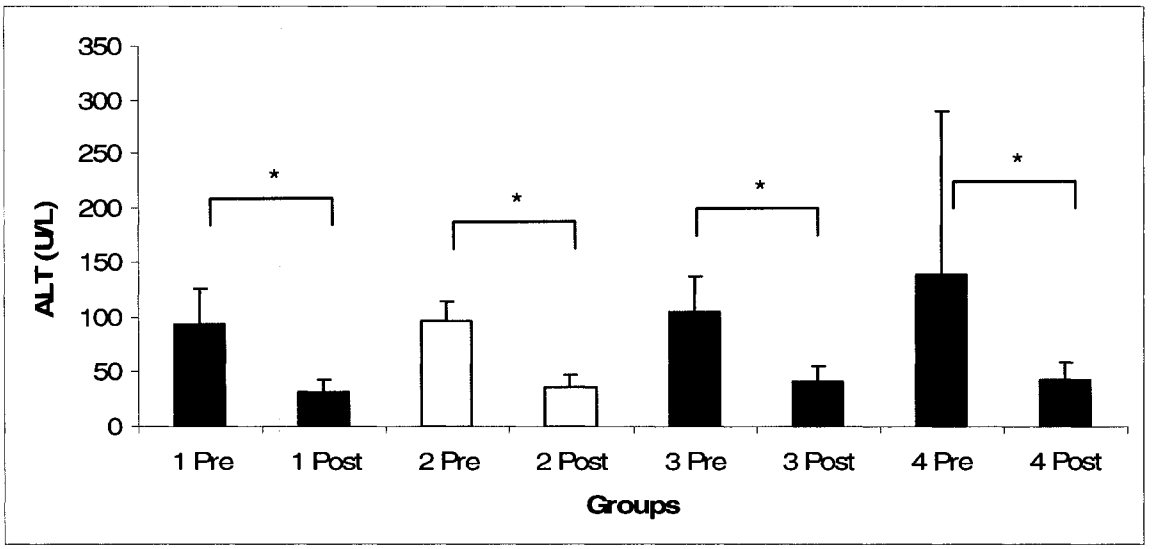
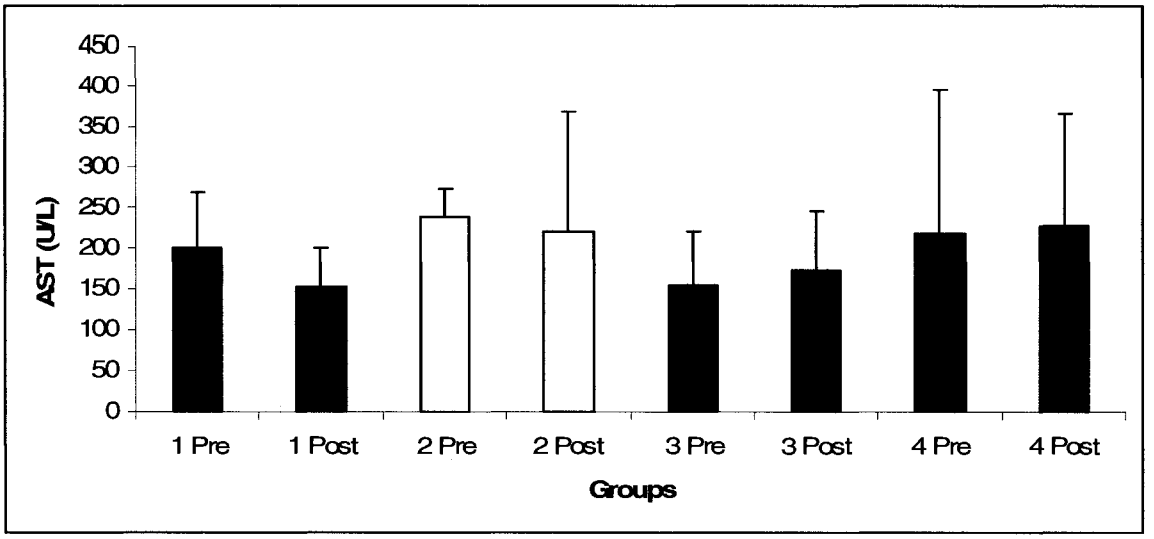

350
300
250
200
150
100
50
0
ALT (U/L)
*
*
*
*
1 Pre
1 Post
2 Pre
2 Post
3 Pre
3 Post
4 Pre
4 Post
Groups

Figure 4.24. Analysis of plasma aspartate aminotransferase (AST) levels in recipient mice. These were calculated by the Vitros 250 using reflectance spectrophotometry. The AST levels are expressed as units per liter (U/L). Recipient mice were bled the day before (pre) they received the splenocytes from the donor mice, and 8 days after the adoptive transfer (post). The first two groups are B6C3F1 mouse strain and they received 1) splenocytes from immunized donors and 2) splenocytes from the control donors. The last two groups are HCV transgenic mice and they received 3) splenocytes from immunized donors and 4) splenocytes from the control donors. Due to the small availability of mouse plasma, AST values are not available for all mice. Additionally, hemolyzed specimens were not included. Groups 1 is representative of n=4 for the pre condition and n=8 for post, groups 2 and 3 have n=5 for each condition and group 4 has n=7 for the pre condition and n=8 for the post condition. Values are presented as the mean with the standard deviation. Differences between the pre and post AST values for all groups were not statistically significant.


450
400
350
300
250
200
150
100
50
0
AST (U/L)
1 Pre
1 Post
2 Pre
2 Post
3 Pre
3 Post
4 Pre
4 Post
Groups

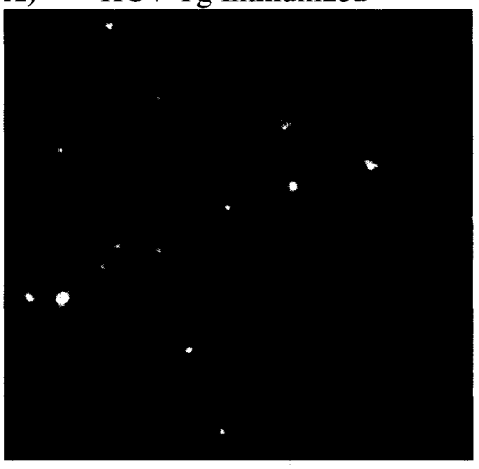
Immunofluorescent and Histological Analysis of Recipient Livers

Livers were harvested from HCV transgenic or wild type mice receiving either control splenocytes of HCV-primed splenocytes. Visual analysis of immunofluorescense in the livers of recipient mice showed a higher number of CFSE positive cells in the livers of the transgenic mice that received the HCV-primed splenocytes (**Figure 4.25 A**) while few cells were present in the transgenic group which received control cells (**Figure 4.25 B**). Results for wild type groups, regardless if they received HCV-primed or control splenocytes are similar to the transgenic group which received control cells (data not shown).

Hematoxylin and eosin staining was done on paraffin-embedded liver sections in order to detect the presence of lymphocyte infiltration in the livers of the recipient mice. Marked lymphocyte infiltration was detected in transgenic mice receiving the HCV-primed splenocytes (**Figure 4.26 B**) while this was absent in all other groups (**Figure 4.26 A, C and D**). Steatosis (which can be observed as the ballooning on hepatocytes) was observed in both groups of transgenic mice, due to the expression of the transgene. Liver pathology observed in the transgenic group (**Figure 4.27 A and B**) which received HCV-primed splenocytes resembles that of chronically infected individuals.

Figure 4.25. Tracking of CFSE positive lymphocytes in frozen liver sections of recipient transgenic mice receiving HCV-primed or control CFSE labeled splenocytes. **A**) Immunofluorescent analysis of frozen liver section of transgenic mouse 1915 (5 μm thickness) showing distribution of CFSE positive lymphocytes in the cryosection (objective 20x). **B**) Immunofluorescent analysis of frozen liver section of transgenic mouse 1932 (5 μm thickness) showing few CFSE positive lymphocytes in the cryosection (objective 20x). Results are representative for n=8 mice and each respective group.

A) HCV Tg Immunized



B) HCV Tg Control

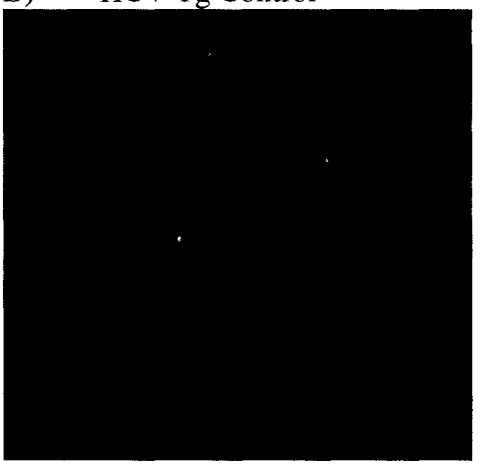
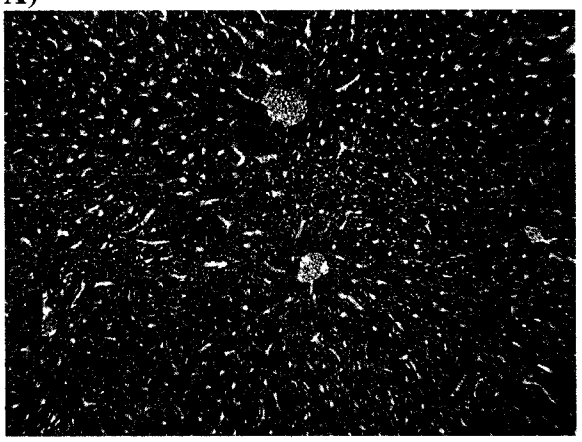
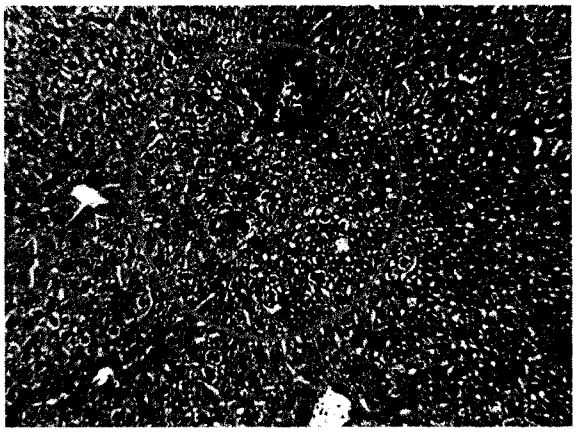


Figure 4.26. Lymphocyte infiltration in transgenic recipient mouse livers. Livers were harvested from HCV transgenic or wild type mice 8 days after the adoptive transfer of HCV-primed or control splenocytes. Hematoxylin and eosin stained paraffin-embedded liver sections showing **A**) normal liver histology in wild type mouse (#15) that received HCV-primed splenocytes, **B**) lymphocyte infiltration (as indicated by the circle) in transgenic mouse liver (#1927) that received HCV-primed splenocytes along with steatosis, **C**) normal liver histology in wild type mouse (#15) that received normal splenocytes and **D**) the presence of steatosis in transgenic mouse (#1932) that received normal splenocytes. The presence of steatosis in both transgenic mice is due to the expression of the transgene, and is manifested by the ballooning of hepatocytes. Marked lymphocyte infiltration was only observed in the transgenic mice that received HCV-primed splenocytes indicating liver infiltration of HCV-specific cells while this liver pathology was absent in all other groups of mice (n=8 for each group). Liver pathology observed resembles that of chronically infected individuals (objective 20x).

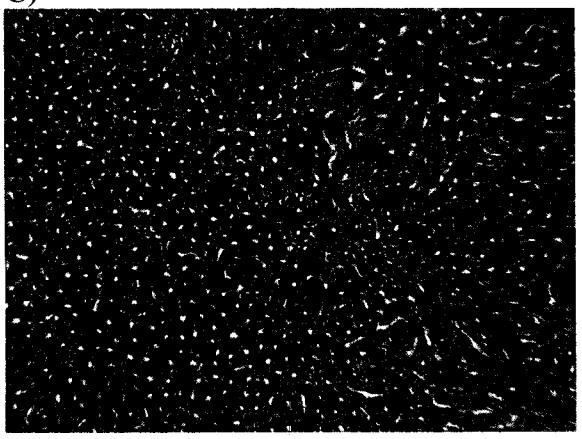
A)



B)



C)



D)

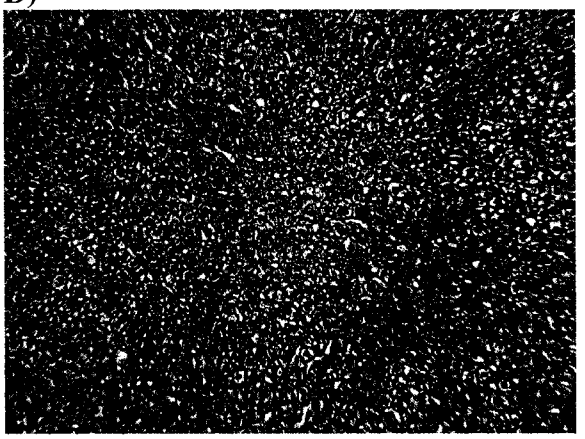
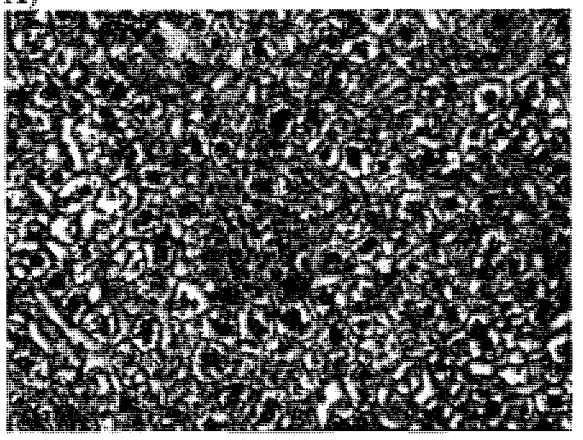
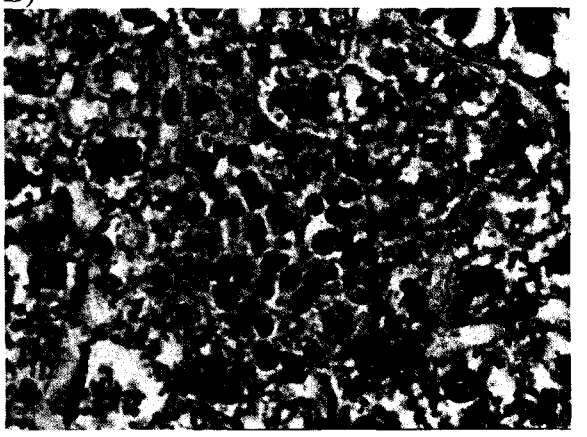


Figure 4.27. Magnification of lymphocyte infiltration in transgenic mouse liver receiving HCV primed splenocytes. Livers were harvested from HCV transgenic mice 8 days after the adoptive transfer of CFSE stained HCV-primed splenocytes. Hematoxylin and eosin stained paraffin-embedded liver section from mouse # 1919 (representing n=8) showing marked lymphocyte infiltration and steatosis at a magnification power of 40X (**A**) and 100X (**B**). Liver pathology observed resembles that of chronically infected individuals. These images represent the effect of the HCV-specific immune responses on hepatic injury *in vivo* and are representative for all transgenic mice which received HCV-primed cells.

A)



B)



CHAPTER 5:
DISCUSSION

DISCUSSION

The purpose of this study was to investigate the role of cell-mediated immune damage in HCV pathogenesis. This was done through an adoptive transfer model in which CFSE stained HCV-primed splenocytes were transferred and tracked in an HCV transgenic mouse model. Since the transgenic mice express a CE1E2 transgene from HCV genotype 1a, it is safe to assume the mice are immunotolerant to the transgene and do not mount immune responses to these antigens (self reactive immune responses would have been eliminated at a young age to avoid auto-immunity). Hence the CD4+ and CD8+ T cells found within the transgenic mice would not cause direct immunopathology to the cells expressing the transgene. Previous studies on this model have demonstrated that mice develop steatosis and hepatocellular carcinomas, clinical manifestations seen in HCV chronically infected humans (130). This model is therefore useful to try to elucidate the immunopathogenesis of HCV infection. This study had two objectives: **1**) to establish an efficient novel immunization regimen to induce strong HCV-specific immune responses in a murine model and **2**) to create an adoptive transfer model to define the relevance of immune-mediated liver cell damage in HCV infection using transgenic mice.

Since there is a lack of commercially available HCV proteins, many of the antigens used in this experiment were prepared in the laboratory. The western blot of the purified CE1E2 polyprotein confirmed the predicted protein at 110 kDa (**Figure 4.2**). However, some of the protein fraction samples had another band visible at 53.5 kDa. This second band was not as intense as the 110 kDa band. This band represents a cleaved section of the polyprotein. Since viral and cellular proteases play roles in processing the

HCV polyprotein, it is likely that a cellular protease present in the CHO-K1 cells was able to cleave the polyprotein into different fragments of partial E1/E2 (10). Additionally, it is important to note that the E2 segment of the polyprotein was truncated thus the protein remained in the cytoplasm of the cell until extraction (19). This polyprotein is useful because it is present in different forms, therefore mimicking epitopes which would be available in human HCV infection. If the polyprotein was not cleaved, certain epitopes would otherwise remain unavailable, thus reducing potential immune responses.

Western blots were also done to identify the HCV individual proteins for CHO purified core (**Figure 4.3**), E1 (**Figure 4.5**) and E2 (**Figure 4.5**) which were used as primary antibodies for the ELISA and ELISPOT assays. The hierarchy in immune responses detected in the ELISA assays indicates that protein constructs evidently presented different epitopes since differential levels of humoral immune responses were observed in response to the different proteins.

For the first part of this study, we aimed to establish an efficient novel immunization regimen to induce strong HCV-specific immune responses in a murine model. It is possible to initiate potent immune responses against specific antigens through immunizations: various vaccine approaches in mice have been tested such as vaccinations with DNA, recombinant proteins, peptides and VLPs amongst others. For our purposes, wild type B6C3F1 strain donor mice were immunized with HCV antigens and the cellular and humoral immune responses were assessed.

Previous studies observing immunizations with modified vaccinia Ankara vectors expressing several different HCV antigens found high increases in humoral immune responses and slight increases in cellular immune responses. (195) In our study, the first

immunization with HCV poly vaccinia or empty vaccinia was injected intraperitoneally since this technique is a good way to induce systemic immunity (antigen passes by lymphatic uptake, to the thoracic duct and reaches the bloodstream). Subsequent immunizations were all completed through the intramuscular route (get rapid spread, pushes towards Th1 response). Our immunization regimen contained many different sources of antigen (**Table 4.2**). DNA vaccines have been shown to express intracellular antigens and induce effective cellular immune responses (196). DNA plasmid vector vaccines allow the plasmid to gain access to a cell, be translated into a protein in the cytoplasm and finally to be degraded by proteosomes into intracellular peptides which are presented by the MHC-I on the cell surface: this can activate cell-mediated immune responses when effector cells recognize these endogenously processed virus-derived peptides (197). Immunizations with vaccinia virus constructs were administered first since these induce strong humoral immune responses and CD4+ T cells have been shown to be required to induce effective CD8+ T cell responses. Subsequent DNA immunizations were done to induce strong CD8+ T cell responses and to push towards a Th1 profile.

Wild type mice immunized with HCV antigens showed significant immune responses to a variety of HCV antigens compared to control mice, in which no antigen specific responses were detected. IgG antibody titers to the different HCV proteins increased in the HCV-immunized mice with the number of vaccinations and diversity of antigens presented. Humoral immune responses to the individual protein constructs under the form of anti-HCV core, E1 and E2 IgG antibodies in the serum of HCV-immunized mice were detected by ELISA. After the third immunization, these responses

were found to be statistically significant in the HCV-immunized group when compared to the control group. Serum IgG antibodies to specific proteins were highest for E1 > core > E2 (**Figure 4.12**). After the fourth immunization, humoral responses to core protein were assessed individually in the mice: IgG anti-core antibodies were higher after the fourth immunization compared to the third and statistically significant compared to the control group (**Figure 4.13**). However, not every immunized mouse exhibited strong responses to the protein: mice number 11 and 21 had weak titers. This is comparable to human HCV infection, since not all individuals develop antibodies to HCV. Nonetheless, the majority of immunized mice exhibited high IgG antibodies to the various HCV antigens indicating the presence of effective humoral immune responses. The hierarchy of humoral antibody responses was not due to the amount of antigen administered since all mice received CE1E2 polyprotein from the same fraction, and more than one injection. However, the E2 segment of our polyprotein is truncated so the C-terminal hydrophobic region from aa 706 to 809 is omitted: certain strong B cell epitopes could potentially be missing.

Donor splenocytes proliferated in response to HCV antigens. CFSE stained immunized donor splenocytes, which were incubated for 4 days in the presence of CE1E2 polyprotein, demonstrated robust proliferation of CD3+CD4+ T cell populations while the unstimulated condition indicated no proliferation (**Figure 4.14**). Due to the lack of commercially available HCV antigens, donor splenocytes were restimulated with the CE1E2 polyprotein which was part of the immunization regimen. However, the ELISA assays confirmed the presence of antibodies to core, E1 and E2 individual proteins which would indicate that if these splenocytes were stimulated with either of these proteins,

CD4+ T cell proliferation would probably ensue. Competent CD4+ T cells are required for the T-cell dependent activation of B cells: the cytokines secreted are required for B cells to differentiate into antibody producing plasma cells, therefore cellular proliferation concords with humoral immune responses (148). Hence, there exists a strong correlation linking the proliferation of helper T cells in the presence of HCV antigens. As scientific literature indicates, in the case of HCV infection, CD4+ T cell responses are required since strong multispecific and long lasting CD4+ T cell responses are associated with HCV clearance (87). Additionally the depletion of CD4+ T cells in chimpanzees results in the emergence of viral escape mutations in MHCI indicating that T helper cells are required to establish efficient CD8+ T cell responses (88).

All mice exhibited a general state of activation throughout the ELISPOT assay (raw data not shown). This is probably due to the fact that each immunization contained a combination of the adjuvants montanide and CpG. Montanide is a mineral oil-based adjuvant which stimulates the immune system, while CpG is a DNA motif which can stimulate the immune system through TLR9 expressed on B cells and DC in humans and mice (198). Therefore, montanide is a good inducer of cellular immune responses while CpG is a good inducer of humoral immune responses (199). Immunized donor and control mice splenocytes were incubated in the presence of various HCV antigens and assessed for their HCV-specific IFN-γ immune responses. In the mice immunized with HCV antigens, the mean SFC for 2 out of the 3 mice which differed significantly from the control group (P value < 0.05) had the highest response to the HCV peptide mix, followed by the core protein, indicating a hierarchy of cellular immune responses (**Figure 4.15**). HCV peptides have previously been mapped for the presence of CTL epitopes.

However, it is important to remember that peptides from different genotypes contain different epitopes thus might not be recognized. The core peptide used for stimulation in this ELISPOT contained part of the epitope (LMGYUPLVGA) which is recognized by CTL in both mice and humans (200). Although the E1 peptide sequence we used differs, previous immunizations of Balb-C mice with HCV E1 peptide (SGHRMAWDM) indicated that this peptide induced strong CD8+ T cell responses (201). Additionally, the peptide SGPSQKIQLV from the E2 protein has been shown to be an immunodominant CTL epitope (202). Although we did not stimulate our cells with all these peptides, our vaccine incorporated all HCV structural proteins and specific responses to these could have been induced. Our results indicate that the cells which reacted in the ELISPOT are CTLs since they responded strongly to the antigens. Mouse 18 which received HCV antigens did not differ from the control group for mean number of IFN-γ producing cells with any of the three tested antigens. Similarly, not all humans develop strong CD8+ T cell responses since chronic infection is established in 80% of the cases, indicating that immune response are not always mounted when an individual, be it mouse or human, is presented with an antigen. Overall, the frequency of IFN-γ producing cells in the HCV-immunized mice confirms that immunizations were effective in inducing strong cellular immune response under the form of CTL.

The second aim of this project was to create an adoptive transfer model to define the relevance of immune-mediated liver cell damage in HCV infection using HCV transgenic mice. This was done through the adoptive cell transfer of CFSE stained HCV-primed splenocytes from the wild type donor mice into an HCV transgenic mouse model.

In chronic HCV infection, the mechanisms of liver cell damage are still poorly understood. HCV enters the host through an extralymphatic route (blood borne virus), gains access to the liver via the hepatic artery or portal vein and replicates mainly in hepatocytes. For this reason, our adoptive transfer was done intravenously hence initially the donor splenocytes would be located in the circulatory system (**Figure 4.17**).

The liver, which can be designated as the body's "filter", is an important organ that is required for the induction and maintenance of peripheral tolerance and appears to favor the induction of immune tolerance rather than immunity: this could be due to the presence of liver sinusoidal endothelial cells (LSEC) which may present exogenous antigens to CD8+ T cells therefore inducing tolerance to these antigens rather than immune responses (203). Debates exist over whether T cell responses are primed in the liver in HCV infection or if APC migrate to lymphatic organs to prime naïve cells. Studies have indicated that it is possible for infected hepatocytes to present antigen to T cells. LSEC can express CD80, CD86 and MHCII and uptake and process antigens similarly to DCs: however, the expression of these costimulatory molecules is low and when a naïve CD8+ T cell come into direct contact with a LSEC, this suboptimal antigen presentation often leads to ineffective T cell activation (204). When activated T cells came into contact with LSEC, around 30% underwent apoptosis (205; 206). This could be one of the mechanisms by which peripheral tolerance is maintained in the liver and explains why although HCV is hepatotropic, this organ is not a main priming site for HCV-specific CD8+ T cells. Additional models are required to reveal where HCV specific immune responses are initiated.

It is commonly accepted that most HCV responses are triggered by APC (which most probably came into contact with HCV in the liver due to high levels of antigen expressed) in extrahepatic locations and not by resident liver cells. A resting naïve T cell in a secondary lymphoid organ could become activated after the binding of the T-cell receptor with a specific peptide-MHCI complex on an APC expressing CD80 and CD86. This would lead to clonal expansion of CD8+ T cells and differentiation into effector cells, which are part of the adaptive immune responses. HCV-specific CD8+ T cells may home to the liver and detect viral antigens presented on the MHCI of the infected hepatocyte (since HCV replicates within the cytoplasm). Reports confirm that during infection, HCV-specific T cells possess a Th1 profile and are shown to be present at higher amounts in the liver than in the blood (207). Additionally, studies show that HCV-specific CTL (for envelope and non-structural protein regions home to the hepatic parenchyma of HCV infected individuals (208).

We attempted to track donor CFSE positive splenocytes, whether CD4+ T helpers or CD8+ CTL, by locating these cells within different lymphoid organs 8 days after the adoptive transfer. These CFSE positive HCV-primed donor splenocytes were injected intravenously in the recipient mice at an amount of 50 x 10^6 cells per mouse. We were interested in discovering how these cells would react to the transgene expressed in our HCV transgenic mouse model, and to track these cells *in vivo*. As mentioned earlier, the transgenic mice do not naturally mount an immune response to the CE1E2 transgene. Essentially, we are transferring an HCV-sensitized immune system or immunity to the transgenic mice. Flow cytometry was done to relocate these donor cells within the blood, spleen, liver and lymph nodes. These organs were chosen for various reasons.

The *spleen* acts like the immunological filter of the blood (also a center of destruction for red blood cells) where B and T cells, macrophages, NK cells and DC, are present: these cells capture antigen on location. Additionally, DCs have been shown to migrate to the spleen to present antigen to a corresponding B or T cell. The *lymph nodes*, similarly to the spleen, are the immunological filters for the lymphatic fluids and are numerous in mammalians. These centers are composed of B cells, T cells, DCs and macrophages. The lymph nodes filter the lymph fluid arriving from respective tissues and immune cells will capture antigen for presentation. The *blood* of the recipient mice was also analyzed since the donor cells were injected intravenously. We wanted to observe, if cells left the bloodstream to migrate to specific lymphoid organs or to the liver. Finally, we selected to analyze the *liver* of the recipient mice since HCV is hepatotrophic and our mice express the HCV transgene predominantly in their liver.

Throughout this study, we wanted to assess hepatocellular injury caused by the adoptive transfer of HCV-primed splenocytes in HCV transgenic mice. Briefly, recipient mice were analyzed for changes in the ALT enzyme, cells were tracked by flow cytometry in different organs and liver sections were analyzed for evidence of hepatocellular injury. We speculated that HCV-primed donor cells would react in one of numerous ways in the recipient transgenic mice: 1) donor cells would not recognize the transgene and not react, 2) donor cells would recognize the transgene and home to the liver. For option 2, we thought the cells would home to the liver, directly kill cells presenting HCV antigens and also proliferate in response to these cells. Throughout the experiment, due to reactivity of the cells (as demonstrated by experiment with donor cells), we risked possibility of cells proliferating beyond the CFSE detection point. This

means that the percentage of CFSE positive cells recovered cells in recipient mice might not actually represent the number of cells that would have homed to a certain organ – additional time course experiments are required to determine the progression of cellular homing.

The presence of HCV in a host is often revealed by the appearance of abnormal ALT and AST enzymes during routine blood testing: these can be indicative of continuous liver damage in chronic infection. For our purposes, the elevation of these enzymes would indicate that HCV-specific splenocytes are recognizing the transgene in the Tg mice and lysing the hepatocytes in which it is expressed. These values were recorded before (pre) and 8 days after (post) the adoptive transfer for individual mice. We found that ALT values decreased in every mouse group after the adoptive transfer although these decreases were not statistically significant (**Figure 4.23**).

Takaku *et al.* created a transgenic mouse model in which there was conditional transgene activation of HCV core, E1, E2 and NS2 cDNA through a Cre/loxP system using an adenovirus expressing Cre recombinase (AxCanCre): this means that a few days after inoculation with AxCanCre, the expression of HCV proteins will be activated. These proteins were detected mainly in the liver of the mice and were accompanied by massive elevations of ALT a week after the activation of the HCV cDNA. This indicates that cells which were expressing the antigens were likely being eliminated since ALT elevation correlates with hepatic injury and viral elimination. This theory can be confirmed since following the ALT peak, the mice presented very little HCV in their livers (209). The difference between this model and ours is that these transgenic mice are not tolerant to the transgene, and thus may mount immune responses to the HCV antigens

when the transgene is activated. It is common knowledge that strong multispecific CD4+ and CD8+ T cell responses are required on location, to clear an acute infection (84). While our transgenic model is more representative of a chronic infection where the transgene is constantly present but not necessarily eliminated, this Cre/loxP system is more representative of an acute infection since the mice mounted immediate immune responses and cleared the transgene.

In their follow up study, Takaku *et al.* created CD8+ T cell deficient mice (IRF-deficient) expressing HCV proteins. Thus this is similar to our transgenic model since these mice lack HCV-specific CTL responses. The group isolated CTL from the transgenic mice described above and discovered that the CTL responses which cleared the transgene were specific to only E1 and neither core nor E2 (209). Notably, the hierarchy of humoral immune responses seen in our study indicated strongest responses to the E1 protein: this could indicate that E1 contains strong epitopes for both humoral and cellular CTL responses. The effect of the E1-specific CTL in the CD8+ T cell deficient HCV transgenic mice was tested through an adoptive transfer of cells. In order for ALT enzymes to become elevated at least 5 x 10^6 cells had to be inoculated (2.5 * 10^6 cells had no effect). The ALT levels were found to be significantly elevated 24 hours after the transfer of cells, however, these returned to normal 48 hours later.

In our study, blood was collected before the adoptive transfer and 8 days after the transfer due to restrictions in handling of the animals and the presence of ALT or AST enzymes was tested. According to the results from the previous study, the 8 day gap period might have permitted ALT levels to peak and to stabilize without our knowledge.

Further studies are needed to reveal how the ALT levels fluctuate in response to our adoptive cell transfer.

Another feature of HCV infection is that histological changes such as lymphocyte infiltration are observed in chronically infected livers. Individuals usually display massive amounts of lymphocyte infiltration, however not all the cells found in the liver in these infiltrations are HCV specific. This infiltration is often associated with elevated ALT. A study examining the function of core protein expressed specifically in T lymphocytes observed increased inflammation, accumulation of lymphocytes in the liver and statistically significant increases in ALT in the Tg mice although T lymphocyte responses and IL-2 production were also suppressed. Of these core Tg mice, 8 out of 21 had an increased number of intrahepatic lymphocytes which induced spotty necrosis and inflammation of the liver, suggesting that these T cells localized here and induced hepatocyte death and hepatitis (173). It is interesting to note that many chronically infected HCV patients have no correlation between the grade of liver inflammation and serum ALT levels (210). This means that immune-mediated cell damage can play a role in HCV pathogenesis and cause liver damage despite the absence of obvious biochemical changes such as ALT increases.

The immunofluorescent analysis of frozen liver sections indicated that CFSE positive cells accumulated in the livers of the transgenic mice that received HCV-primed splenocytes (**Figure 4.25**). Additionally, lymphocyte infiltration was observed in our transgenic recipient mice which received the HCV-primed splenocytes while this was absent in the transgenic mice which received control splenocytes (**Figure 4.26- 4.27**). These changes were absent in the wild type mice. The *Takaku* adoptive transfer

experiment using HCV transgenic CD8+ T cell deficient mice demonstrated histopathological injury indicating that there was specific lysis of HCV-infected hepatocytes. However, 1 to 2 months after the adoptive transfer, core protein was still detected in the liver indicating that the adoptive transfer was insufficient to eliminate the presence of HCV thus suggesting a role for HCV specific immune cells in the progression of liver damage (209).

Satoi *et al.* created a transgenic mouse lineage which expresses HCV core, E1 and E2 under the control of a liver specific albumin promoter. In this study, wild type and transgenic mice were immunized with a plasmid vector expressing HCV core or a vector expressing HCV core, E1 and E2 (poly). It was observed that the wild type or transgenic mice had little or no humoral immune responses and that with poly vector immunization, wild type mice responded to core and E1/E2 for T cell proliferation while the transgenic mice were unresponsive (211). Additionally, wild type mice exhibited CTL responses against E2 antigens, while the transgenic mice still had no detectable CD8+ T cell response. According to these results, it appears that the DNA-immunization was incapable of breaking the tolerance in the transgenic mice.

In the *Satoi* experiment, the adoptive transfer of cells from wild type mice immunized with poly vector (induction of CTL response) to transgenic mice did not result in increases of ALT or any sign of hepatitis despite the presence of E2-specific CTL. Thus, regardless of the presence of cellular immune responses from the donor mice, no ALT elevations or histopathological changes occurred in these transgenic livers. These results are similar to our study in the perspective that we did not observe ALT elevations 8 days after the adoptive transfer, though we did observe lymphocyte

infiltration. This suggests that the E2-specific CTL of the Satoi *et al.* experiment might not be properly homing to the liver which is problematic since it is essential that virus-specific cells must locate to the liver in order to clear the infection. The absence of homing to the liver might explained by the lack of variety in CTL responses, since only E2 responses were detected despite the presence of core and E1 in the DNA vaccine. This suggests the presence of a hierarchy of immune responses to the HCV proteins. However, amid the differential cellular and humoral immune responses observed in the HCV transgenic mouse models, we have to remember that diverse vaccine combinations produce discrepancies and differences will be observed in terms of tolerance induction and other immune-mediated damages. Although the Satoi *et al.* study was only able to detect E2-specific CTL responses, human studies have also detected CTL responses against core and E1 (200; 201). Core is the most conserved of the HCV structural proteins while the envelope proteins are more subject to mutations, thus even if E2 responses are formed, due to the hypervariable nature of this protein, these responses might be inefficient. Another explanation for the lack of homing of these lymphocytes to the liver could be insufficient levels of antigen expression or the absence of essential homing factors.

Cytokines and chemokines are essential in the regulation of cellular activation and homing of lymphocytes for any immune response. Chemokines are very important since they have chemotactic and immunoregulatory functions (attract lymphocytes to the site of inflammation). Due to the sheer amount of cytokines and chemokines, there exists a great deal of redundancy and pleiotropy since most receptors can recognize more than one chemokine and most chemokines may also bind more than one receptor. In HCV

infection, it is necessary for HCV-specific cellular immune responses to home and accumulate in the infected liver where the antigen is predominantly expressed.

As a general rule, naïve T cells circulate from the blood to secondary lymphoid organs (lymph nodes and spleens) to detect antigen and relocate to the blood: once these cells encounter a specific antigen, presented on an APC, these naïve cells become activated and can proliferate and become effector cells. The splenocytes for our adoptive transfer experiment were injected intravenously at 50 x 10^6 cells per mouse because of their ability of to recirculate to the blood and lymphoid organs in search of antigen.

Eight days after the adoptive transfer, we attempted to locate CFSE positive cells in the liver and different lymphoid organs. CFSE positive CD4+ and CD8+ T cells were located at high percentages in the blood of wild type mice which received immunized cells while there was a significant decrease in the percentage of these donor cells in the transgenic group. Since the transgenic mice received the same HCV-immunized cells, this indicates that in this group, cells migrated from the blood to different lymphoid organs or the liver. It was found that in immunized Tg mice, the highest percentage of CFSE positive cells was located in the liver: in all other groups, cells did not accumulate in this organ. This indicates that the HCV-primed lymphocytes were attracted to the CE1E2 transgene and migrated to this organ where the transgene is preferentially expressed in transgenic livers (**Figure 4.20**). In contrast, donor HCV-primed splenocytes seemed to have no preferential migration pattern and accumulated in all organs studied at similar percentages (**Figure 4.22 A**). Additionally, control donor splenocytes and HCV-primed donor splenocytes also migrated to the spleen and lymph nodes, in other groups

indicating that these cells express different homing receptors and react differently to the chemokines produced.

A study observing the cellular profile of lymphocytes purified from the tissue of HCV infected livers determined that NK cells (CD56+) were the predominant cell type followed by T cells (CD3+), B cells (CD19+) and NK-T cells (CD3+CD56+) (212). In accordance with this, despite there being a 30 fold increase of HCV-specific T cells in the liver as compared to the lymph nodes and blood, the majority of liver infiltrating intrahepatic CD8+ T cells are not HCV specific thus, liver damage is caused by bystander cells (213). Following a liver transplant, if a sustained virological response is not achieved, there is often reinfection of hepatocytes with HCV and lymphocyte infiltration is observed (105; 106). Thus, we know that lymphocytes are homed to the HCV infected liver when HCV RNA is still present within the host: the cellular trafficking occurs through association with adhesion molecules and the recognition of chemokines at the homing site. Additionally, both CD4+ and CD8+ T cell responses (T helper and CTL) are required and dependent on each other for HCV viral clearance and these cells must have the ability to migrate to the site of infection to locate and kill the infected hepatocytes. Thus, we suggest that our HCV-primed lymphocytes recognized the viral antigens in our transgene.

We assume that our donor splenocytes contained memory T cells since immunizations were done over a 4 month period thus providing enough time for memory responses to appear. Additionally, we presume that effector T cells were also present in since mice were sacrificed 2 weeks after the last immunization allowing time for the reactivation or formation of fresh immune responses. Our donor splenocytes probably

contained both short-lived effector and long-lived memory CD4+ and CD8+ T cells responses although an in depth phenotypic analysis of donor cells (apart from CD3, CD4 and CD8) was not performed in this study. However, considering the mice were sacrificed two weeks after the last immunization, if most of the HCV primed lymphocytes were reactive with the last antigen stimulation, a large portion of the cells may still be in the effector stage. For the purpose of this experiment, it might have been useful to wait 4-6 weeks after the last immunization to perform the adoptive transfer.

When effector cells are no longer exposed to antigen, many of these cells die but some remain in the form of memory cells (CD44 in mice and CD45RO in humans) which are present in the blood and lymphoid organs: these cells remain in case of antigen reappearance (214). CD45RA is a marker which can be found on naïve T cells before they become effector memory cells. In our adoptive transfer experiment, high percentages of CFSE positive cells in both wild type groups of mice which received either HCV-primed splenocytes or control splenocytes were detected in the lymph nodes. One possible explanation for this could be the lack of detection of antigen, hence cells homed to lymphoid organs as memory cells to await antigen reexposure. Studies have also shown that viral infection resulted in the accumulation of CD8+ memory cells in non-lymphoid tissues such as lung, kidney, fat pad and liver amongst others: thus, these cells may recirculate through peripheral tissues or may permanently reside in them to be immediately available in case of reexposure to the antigen (215). Our study did not take into account these non-lymphoid tissues, thus donor cells may have migrated to these. Further studies are required for a more in depth analysis of these cells. These studies are

needed since naïve or memory CD8+ T cells have different homing potentials and migrate to different organs (216)

Nonetheless, amid the uncertainties associated with the mechanisms of cellular homing, it is accepted that strong CD4+ and CD8+ T cell responses in the liver are required to clear infection, although these responses are also involved in the progression of liver damage during chronic infection. CTL are known to mediate cell injury and specific cell lysis through the production of IFN-γ and/or perforin and granzymes in addition to Fas-mediated apoptosis. According to recent literature, there is minimal evidence that CD4+ T cells mediate direct liver cell injury in chronic HCV infection though, the presence of these cells is likely to play a role in facilitating other immune-mediated cell damage such as CTL (84). In chronic infection, liver infiltrating CD8+ T cells often express FasL which has the ability to bind to Fas expressed on hepatocytes and induce apoptosis (217). The presence of a larger number of CD8+ effector T cells will frequently correlate with a higher degree of liver damage.

In a transgenic mouse model expressing core and envelope proteins, mice were injected with a sufficient amount of anti-Fas antibody to cause moderate liver cell injury (at high levels, anti-Fas antibody can induce fatal damage in normal mice 8 hours following injection) (218). This study found that ALT levels dramatically increased in the serum of transgenic mice 24 hours after the injection but only slightly in control littermates (192). Therefore, this study can conclude that the hepatocytes in HCV transgenic mice are more sensitive to Fas-mediated apoptosis. In accordance with this, one study indicated a higher sensitivity to Fas-mediated apoptosis in HepG2 cells transfected with HCV structural protein core while another showed higher levels of FasL

on the lymphocytes which migrated to the liver and induced inflammation and apoptosis in a mouse model where core was specifically expressed in CD4+ T cells (143; 219). These results indicate that core is an agent which sensitizes cells to Fas-mediated apoptosis and thus liver injury: due to high levels of FasL, this may also induce bystander apoptosis of uninfected cells furthering liver damage. Since our mouse model expresses core among E1 and E2, hepatocytes may express high levels of Fas receptor and may be sensitive to the adoptive transfer of HCV-primed splenocytes which potentially express high levels of FasL. Further studies are required to determine the levels of Fas and FasL in our adoptive transfer model.

The majority of HCV-specific CD4+ T cells found in the peripheral blood of human patients who spontaneously resolved the infection were CCR7+CD45RA-CD27+, which corresponds to a central memory phenotype: these cells proliferate rapidly in the presence of antigen and produce IL-2 (141). Conversely, Th1 helper cells secrete IFN-γ and IL-2 and are found at high levels in the liver of HCV chronically infected individuals: these cytokines can be involved in the progression of tissue damage. Chemokines (expressed in the infected livers) that attract these cells are important in understanding of chronic disease progression. Chronically infected individuals who responded to combination therapy had increases in CD8+CD45RA+CCR7+ cells in their livers. These results indicate that CCR7 is important in directing cells to the HCV infected liver for a favorable outcome.

Additional chemokine receptors such as CXCR3 (predominantly expressed on activated T cells) and CCR5 (predominantly expressed on activated and memory T cells) are usually found on Th1 inflammatory cells and in HCV infected patients, these

receptors are expressed on higher proportion of intrahepatic T cells compared to the peripheral blood of HCV-infected individuals and finally compared to normal controls (220; 221; 222; 223). In normal livers, levels of CXCR3 and CCR5 are low, yet a study by Larrubia *et al.* found that most liver-infiltrating lymphocytes in HCV chronically infected individuals possessed a CD8+CCR5^{high}/CXCR3 ^{high} phenotype and correlated with liver inflammation (224). This indicates a function for these receptors in the migration of CD4+ and CD8+ cells to the liver and suggests a role of these cells in the progression of chronic liver damage.

In accordance with the differential expression of these chemokine receptors, studies have shown that the following chemokines, which interact with CXCR3 are differentially expressed in HCV infection. Intrahepatic IFN-γ inducible protein 10 (IP-10, produced by hepatocytes) mRNA levels are correlated with liver inflammation in HCV infection since this chemokine attracts inflammatory T cells through CXCR3 (225). Additionally in chronic infection, peripheral and intrahepatic levels of monokine induced by IFN-γ (Mig) and intrahepatic levels of interferon-inducible T-cell-α (I-TAC) are increased in HCV infection and correlate with the grade of liver inflammation (222; 226). Chimpanzee studies have confirmed that IP-10 and Mig are important in acute infection since transcripts of both these chemokines were increased (227). The following chemokines, which interact with CCR5 receptor on T cells, are also differentially expressed in HCV infection. Elevated intrahepatic levels of RANTES (CCL5) have been found in HCV chronically infected patients as well as increased levels of macrophage inflammatory protein 1-α (MIP-1 α or CCL3) and MIP-1β (CCL4) (222; 228).

Most of the chemokines mentioned above specifically attract T cells to the site of infection, in addition to elevated levels being correlated with a higher grade of liver inflammation: accordingly the interaction between a chemokine and its receptor is important for activating and recruiting Th1 helper cells and CTL to the site of infection (229). It is important to mention that many of the samples analyzed were obtained from HCV chronically infected individuals that comprise a variety of other diseases, hence results detected might be influenced by other manifestations.

Additional chemokines which have been detected in inflamed livers, as well as HCV infected livers are CCL2, CCL3, CCL4, CXCL9 and CXCL10 (221; 230). Due to all the extrahepatic manifestations of HCV and its association with a variety of diseases, we must question if the recruitment of the cells to the liver is the direct result of HCV infection or if this occurrence might be associated with general liver inflammation (other liver pathologies such as hepatitis B or alcoholic liver disease). Additionally, the progression of liver disease may be due to the effect of the host immune mechanisms in chronic infection since even if T cell responses are ineffective (loss of IL-2 and IFN- γ production or cellular proliferation), these cells still produce cytokines and chemokines which attract other immune cells furthering liver damage. Thus, we must consider viral versus host factors: although the development of cirrhosis may take up to three decades, immune-mediated cell damage may be continuous but at a low frequency (231).

The use of chemokines should be strongly considered in HCV therapy. Monocyte chemoattractant proteins (MCP) are potent attractants of T cells, NK cells and DC (232). In chronic HCV infection, hepatic expression of MCP-1 is found to be upregulated, indicating recruitment of cells to the liver (233). Thus, future therapies should consider

using chemokine antagonists since these could be useful in HCV infection to interfere with chemokine systems and reduce inflammation (234). We recognize that lymphocytes are homed to the liver through a yet unknown mechanism/s using chemokines. By revealing a chemokine which induces HCV-specific CTL to migrate specifically to the liver and modulating the expression of this chemokine, we could reduce inflammation and eventually possibly delay disease progression.

It is commonly accepted that chemokine receptors are upregulated on T cells depending on their homing location and that chemokines are expressed at high levels in certain tissues to attract specific T lymphocytes to the area of inflammation (235). In the case of hepatitis C virus, since core protein has been shown to be found in the bloodstream, naïve T lymphocytes could encounter core in an extrahepatic site, and then migrate to the liver where there is a high level of antigen expression. Once in the liver, these T cells can undergo clonal expansion to eventually display effector functions which can lead to liver injury, primarily by cytotoxic T lymphocytes (236; 237). CD8+ T cells may act as a double edged sword regarding HCV infections. These cells may either clear an infection (which is rare since 80% of individuals progress to chronic infection), or these cells can cause additional liver damage through repetitive cycles of hepatocyte lysis in attempts to clear the virus. Further studies should compare the differential chemokine/ receptor expression during acute versus chronic infection. Nevertheless, our knowledge on lymphocyte recruitment is still limited and more studies are required to understand the selective recruitment of lymphocytes to the HCV chronically infected liver.

CD4+ T cells are often neglected in HCV studies since it is known that CD8+ T cells cause liver damage progress. However, regulatory T cells (T Regs) are a subset of

CD4+ T cells which express CD25 and which act as immunosuppressive cells in order to protect a host against autoimmunity (238). Subsets of T Regs that are CD4+CD25+ comprise about 10% of the CD4+ pool in mice and around 2-5% of the CD4+ pool in humans (239; 240). In humans, HCV acute infection is associated with strong Th1 responses and multispecific CTLs (64). However, it is interesting to note that HCV chronically infected patients have a higher proportion of IL-10 (anti-inflammatory cytokine, downregulates Th1 cytokines) producing HCV-specific T cells in the peripheral blood than patients who managed to clear the infection. (MacDonald, 2002) As a result of this, a HCV infection may become chronic due to the immunosuppressive effect of this subset of cells on CTL thus preventing viral clearance (167). However, chronic HCV infection is also associated with liver pathologies such as steatosis, cirrhosis and HCC and theoretically, T Regs should protect the host from immunopathology due to virus specific cells. An explanation for these pathologies is that the frequency of T Regs may decrease over time and the host would lose ability to control CD8+ T cells. Therapies using T Regs might be valuable in treating chronic HCV infection in order to suppress host immune responses.

In HBV infection, protection against the virus is conferred by antibodies to hepatitis B surface antigen. DNA immunization can result in the production of new proteins within the host which can mimic the presence of virus and induce immune responses such neutralizing antibodies, T helper cells and CTL similarly to a natural infection (241; 242). Studies using wild type mice have shown that injection of a plasmid DNA vector with this antigen resulted in antibody production (243). This is similar to our wild type mice which demonstrated humoral immune responses against HCV proteins

after immunizations (**Figure 4.8 – 4.13**). Additional studies with HBV have indicated that DNA immunization with a HBV construct in transgenic mice (HBV carrier state) broke the tolerance, induced hepatitis and resulted in clearance of antigen by T cells (244).

For our purposes, our goal was to induce strong humoral and cellular immune responses to HCV in order to eliminate the virus or observe the effects of cell-mediated immune damage. However, the question remains if protective immunity is useful for HCV infection due to the high mutation rate of the virus. Additionally, due to the association of HCV immune responses with the progression of immune damage, the selection of which proteins should be incorporated into a vaccine is important since certain proteins are involved in the pathology of the virus. In HCV infection, core protein has independently been located in the bloodstream and numerous studies suggest that core is related to HCV pathogenesis. Another study using BALB/c mice found that CTL responses were *only* induced if core protein was part of the plasmid DNA (245). We must therefore consider this double edged sword when designing therapies.

CONCLUSIONS

The novelty of this study lies in the tracking of HCV-primed non-tolerant T cells to various lymphoid organs and the liver *in vivo* in a HCV transgenic mouse model to unearth the mechanisms behind cellular trafficking and cell-mediated immune damage. Core, E1 and E2 structural proteins were selected as antigens against which to induce strong humoral and cellular immune responses since these proteins are important for viral entry and capsid formation. Additionally, certain CTL epitopes are located on the core and E2 proteins, and since strong CD8+ T cell responses are required to clear the virus in

human infection, we wanted to induce strong responses against these epitopes. While core may be hidden in the three dimensional virion form of HCV, this protein has been detected under free form in the blood of infected patients and is very immunogenic and conserved across genotypes: this protein has well-defined human B cell, T helper and CTL epitopes (162). Additionally, E1 and E2 protein are often linked with extrahepatic manifestations. Therefore, our goal was to mimic real infection as closely as possible.

In HCV chronic infection, CTL activity is always a double edged sword since antigen specific cells are required to clear the infection, however, these must be maintained at a certain level as to not cause cell-mediated immune damage. The accumulation of HCV-specific intrahepatic CTL and T helper cells in the livers of HCV chronically infected individuals indicates a homing mechanism to this organ and a role in the establishment of chronic infection and development of liver pathologies. These T cells secrete type 1 cytokines and destroy HCV infected hepatocytes thus suggesting a role for these cells in viral control or tissue damage and the progression of immunopathogenesis. The molecular mechanisms surrounding HCV pathogenesis and the progression of liver damage in chronic infection are still poorly understood.

The expression of the CE1E2 transgene in the transgenic mice induced histological abnormalities such as steatosis and hepatocellular carcinomas in the transgenic mice, which are similar to the clinical manifestations seen in chronically infected humans. Additionally, although the transgene was mainly expressed in the liver, there were extrahepatic manifestations which also concord with human studies. Although chronically infected individuals still exhibit weak immune responses, our model is a surrogate model for chronic infection. Due to the tolerance of the transgenic mice to the

transgene, the adoptive transfer of cells from HCV-immunized donor mice could shed light upon the immunopathogenesis of HCV infection.

Our study showed that we could elicit strong humoral and cellular CD4+ and CD8+ T cell HCV-specific immune responses to a variety of HCV antigens in wild type B6C3F1 mice indicating a successful immunization regimen. In response to immunizations, a hierarchy of the humoral antibody response against the 3 structural proteins of HCV was observed with E1 having the highest antibody production followed by core and finally E2 in the mice that received HCV antigens. CD4+ donor T cells proliferated in response to HCV antigens and the CD8+ T cells demonstrated strong HCV-specific IFN-γ CTL responses in the ELISPOT. This is important since strong multispecific T helper and CTL responses are often associated with viral clearance in acute infection, therefore our donor cells represent robust cellular immune responses with cells already primed for future antigen encounters. Further experiments should evaluate if this immunization strategy is effective at protecting against future HCV infection in donor mice. If these cells can effectively be transferred to another mouse, these strong responses may have the ability to clear the infection, thus having an antiviral effect.

Eight days after the adoptive transfer of CFSE positive splenocytes, cells were recovered *in vivo* in the blood, spleen, liver and lymph nodes. It was found that HCV-primed CD4+ and CD8+ T cells selectively accumulated in the liver of the transgenic mice while these responses were absent in all other groups. We suggest that through chemokine networks, donor cells are recognizing the transgene expressed in the liver and these lymphocytes are homing to and attacking transgene expressing cells. The pathology in the liver indicates a link between HCV-primed splenocytes and the presence

of the transgene since this group exhibited the highest percentage of CD4+ and CD8+ T cells in the livers: this assumption is supported by histological analysis confirming evident lymphocyte infiltration. In human chronic HCV infections, cirrhosis will precede hepatocellular carcinoma. It is possible that lymphocytes may infiltrate area of infection after the injury has occurred. Moreover, if donor cells are killing transgene expressing hepatocytes, bystander killing may induce death in non-infected cells due to proximity causing greater liver damage. We must also consider the scenario where T cells are proliferating and therefore, these might not have been considered in our model due to proliferation going beyond our level of detection.

These results may suggest either further hepatic injury mediated by cell-mediated immune responses in chronic infection or another school of thought could propose that these cells possess an antiviral effect to clear the transgene expressing hepatocytes which could be useful in HCV therapies. Long term studies are required to track levels of mRNA CE1E2 transcripts in the transgenic mouse livers to observe if the cell transfer is successful at reducing transgene expression, thus suggesting the use of HCV-specific T cells in immunotherapy. Additionally, in depth cellular phenotype analysis of the chemokines produced in transgenic livers and the receptors expressed on donor T lymphocytes are required: modulation of these chemokines or receptors may be promising for therapy. Additional studies and mouse models are required to identify the mechanisms leading to HCV immunopathogenesis.

The progression of host immune responses to HCV and pathogenesis of this virus depend on many factors: viral versus host factors, acute versus chronic infection,

deficiencies in cellular immune responses or ability to control the virus, natural susceptibility of host and maybe even to some degree, protective immunity.

LIMITATIONS AND DIFFICULTIES ASSOCIATED WITH THE STUDY

It is important to critically evaluate the results obtained throughout this study and the experimental design. This study has certain limitations which need to be considered when looking at the applicability of the study.

Although transgenic mouse models may be extremely powerful tools for studying immune mechanisms, many limitations are always associated with these designs. In nature, HCV only infects humans and chimpanzees. HCV does not naturally infect mice, thus results obtained using the transgenic mouse model may or may not be representative of the natural course of infection in humans. Furthermore, our transgene only comprised of three structural proteins, thus the effects of the seven non-structural proteins have been ignored. Additional research is required to observe at the effect of non-structural proteins or the entire HCV genome in a similar tolerogenic mouse model. Interestingly, although higher percentages of CFSE positive CD4+ and CD8+ T cells were located in the liver of transgenic mice receiving HCV-primed cells, human studies have found that these responses are particularly primed towards the HCV non-structural proteins, especially NS3 (246). This is interesting, since our immunization protocol only took into account HCV structural proteins but strong proliferative and CTL responses were still detected. Additionally, our model was based on genotype 1a therefore results from this experiment might not be representative for other genotypes or even subtypes. Many studies have shown that genotype is very important in deciding the course of treatment and even that

steatosis is more highly associated with genotype 3 (247). Additionally, we must consider the physiological integrity of the cells after *in vitro* handling.

A vital part of this experiment was recovering the CFSE-labeled CD4+ and CD8+ T cells in the recipient mice. This was done after an 8 day incubation in which the cells may have proliferated in response to HCV antigen. It is possible that the CFSE dye faded during the incubation and proliferation process which could explain why we did not recover a large quantity of CFSE positive cells in the liver of transgenic mice receiving immunized cells. The difficulties associated with using the impermanent CFSE dye were not only linked to the flow experiments but also to the immunohistochemistry attempted on frozen liver sections. The CFSE fluorescence is rapidly quenched when tissues are viewed under the fluorescent microscope which can be problematic.

Another important question that we must ask is if the expression of the transgene and the resulting steatosis in the transgenic mouse model is as strong as the first generations. When analyzing liver samples from the first generation of mice compared to the ones used in the current study, the severity of steatosis seems to be reduced. Long term studies are required to ensure that transgene expression is strongly maintained.

Another possible limitation of the study is the use of pVax as a vector for the DNA vaccination. It is important to note that different constructs might not have the same integration site in cells which could result in differential humoral immune responses to the various antigens.

A further limitation of working with HCV is that since the virus is highly mutable, there are few commercially available products, especially for genotype 1a which is the genotype of our transgenic mouse model. Even if a product is commercially

available, due to the high level of mutations, these products will not necessarily be compatible with our strain. Complexities arose for the Western analysis of the lab-grown proteins.

In the future, a more appropriate mouse model could be designed for this study or we could consider using HCV viable cell culture which might be more representative of a real infection.

FUTURE DIRECTIONS

Future experiments should consider DNA immunizations of the HCV transgenic mouse model to attempt to break immunological tolerance to the transgene. DNA immunization of the HBV transgenic mouse model with a HBV expression construct resulted in induction of hepatitis (244). Breaking the tolerance to antigen in our HCV mouse model could give insight into the induction of autoimmunity and cellular-mediated liver damage.

More in depth studies of donor splenocytes are required to assess the proportions of the following cell markers: CD44 (CD45RA in humans), CD45RO, FasL, CCR5, CCR7, CD25 and intracellular IFN-γ production. In terms of recipient hepatocytes, details on the secretion of chemokines and the percentage of cells expressing the Fas receptor (and the amount of cellular apoptosis) after the adoptive transfer are essential. Time courses should be completed to track if changes in chemokine secretion occur. Additionally, chemokine secretion profiles in secondary lymphoid organs should be defined to uncover how differential expression could affect cellular trafficking.

In addition, future studies should also consider targeting HCV-specific immune responses to hepatocytes by using liver specific promoters or it may be promising to

administer the vaccine at the portal vein to ensure the maximal amount of cells attain the liver. Furthermore, due to hierarchies observed in humoral and cellular immune responses in reaction to different antigens, it may be valuable to test different vaccine combinations to avoid using antigens which are futile in inducing effective immune responses. Concentrating on a few select proteins may generate more efficient treatments.

Furthermore, studies employing dendritic cells seem to be promising since these are the most potent antigen presenting cell of our immune system. Results from studies pulsing dendritic cells with different HCV proteins have been optimistic (248). A dendritic cell treatment would be ideal since these can be pulsed with important cytotoxic T lymphocyte epitopes *in vitro* and these cells can be transferred to HCV infected hosts in the hopes of inducing host immune responses to eliminate infected hepatocytes. This therapy is promising since we know strong CTL responses are needed to clear an infection and the host would be mounting its own immune responses and would retain memory cells in case of reinfection.

In the grand scheme of things, what we propose is to pulse wild type dendritic cells with select singular epitopes *in vitro* and to adoptively transfer these cells *in vivo* to our HCV transgenic mouse model to see if this may have an antiviral effect. Different factors will need to be tracked such as the amount of HCV mRNA transcripts in the mouse liver, IFN-γ production and finally a cytokine antibody array should be done on the supernatant from hepatocyte cultures from mice sacrificed at different time points to define the timely chemokine secretion pattern in response to these DC. Additionally, it will be necessary to perform histological analyses to track changes in the liver. This may

be a promising new therapy that could lead to clearance of the virus and could potentially be applied to chronically infected humans.

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