University of Alberta

MODULATION OF IMMUNE RESPONSE BY HCV-DERIVED F, CORE AND NS3 PROTEINS

By

Subodh Kumar Samrat

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ABSTRACT

The hepatitis C virus leads to chronic infection in the majority of infected individuals; however, in a minority of patients, acute infection is followed by viral clearance. The immune correlates of viral clearance are not yet clear, but have been extensively investigated, suggesting the role of multispecific and multifunctional cellular immunity.

In the current studies, we demonstrated that endogenous expression of the F protein in human DCs leads to contrasting effects on activation and apoptosis of DCs, allowing the activated DCs to efficiently internalize apoptotic DCs. These in turn result in the efficient ability of DCs to process and present antigen, and prime and stimulate antigen-specific T cells from HCV-naive individuals.

Our *in vivo* studies show that mice immunized with F- or core-containing adenovector induce dysfunctional T cells with reduced granzyme B (GrB) expression which are unable to kill peptide-loaded target cells. Exogenous addition of IL-2 in *in vitro* cultures, as well as immunization with the toll-like receptor (TLR) agonist poly I:C restores the GrB expression in T cells. Thus, we have discovered a new mechanism of T cell modulation by HCV-derived antigens and we have also delineated strategies to overcome this dysfunction.

Further, we have analyzed the early immune events in the mice immunized with recombinant adenovector containing core and NS3 in a time-course manner. Our results demonstrate that despite efficient expression of both antigens at the site of immunization, T cell proliferation and IL-2, IL-6 and IL-12 production were

significantly higher in NS3-immunized mice at 12-48 hours after immunization compared to the core-immunized mice. These studies have implied that early events in antigen encounters imprint the subsequent immunity and the final outcome; two distinct patterns of early events in immunity can be demonstrated for the antigens core and NS3.

Over all, my studies have discovered a new mechanism of immune modulation by HCV-derived antigens that could help in designing both prophylactic and therapeutic vaccine candidates against the HCV.

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LIST OF ABBREVIATION

AA	amino acid
Ag	antigen
ALT	Alanine transaminase
ANOVA	analysis of variance
APC	allophycocyanin
APCs	antigen presenting cells
ATCC	American type culture collection
BSA	bovine serum albumin
CCAC	Canadian council of animal care
CCR	chemokine receptor
CD	cluster of differentiation
CD-40L	CD-40 ligand
cDNA	complementary DNA
CFA	complete Freund's adjuvant
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CLRs	c-type lectin receptors
ConA	concanavalin A
CpG	cytosine-phosphate-guanine
CTL	cytotoxic T lymphocyte
CV	control vector
CTLA-4	cytotoxic t-lymphocyte antigen 4
CXCR6	chemokine (C-X-C motif) receptor 6
DAPI	4',6-diamidino-2-phenylindole
GrB	granzyme B
DC	dendritic cells
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DDA	direct acting antiviral
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-Linked ImmunoSorbent Assay
ER	endoplasmic Reticulum

ER	endoplasmic reticulum
FACS	fluorescent-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FoxP3	forkhead box P3
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hour
HCC	hepato cellular carcinoma
HCV	hepatitis c virus
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HSLAS	health sciences laboratory animals services
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HVR	hyper variable region
ICAM-I	Intercellular adhesion molecule-1
IDU	Intravenous drug use
IFN-γ	interferon-gamma
IL	interleukin
IM	intramuscular
ISG	Interferon stimulatory gene
JAK	Janus kinase
kDa	kilo Dalton
LDL	low density lipoprotein
LPS	lipopolysaccharide
mAb	monoclonal antibody
mDCs	myeloid dendritic cells
MFI	mean florescence intensity
mg	milligram
MHC	major histocompatibility complex
MHC	major histocompatibility complex
min	minute
mL	milliliter
NANBH	Non A Non B Hepatitis
NK cells	natural killer cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NS	non structural
OD	optical density
ORF	open reading frame
PAMPS	pathogen-associated molecular patterns
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PBMCs	peripheral blood mononuclear cells
PD-1	programmed cell death protein 1
PCR	polymerase chain reaction

pDCs	plasmacytoid dendritic cells
PE	phycoerythrin
PEG-IFN	pegylated interferon
pg	picogram
PI	polydispersity index
PIAS	protein inhibitor of activated STAT
Poly (I:C)	Polyinosinic-polycytidylic acid
RBCs	red blood cells
PP2A	serine/threonine protein phosphatase 2A
PRP	Pattern recognition receptor
PRRs	pattern recognition receptors
rAd-F	Recombinant adenovector containing F protein
RBV	ribavirin
RIG-1	Retinoic acid inducible gene I
RMPI	roswell park memorial institute
RNA	ribonucleic acid
rpm	rounds per minute
Ac-IETD-pNA	acetyl-Ile-Glu-Thr-Asp-paranitroanilide
ARFP	alternate reading frame protein
CFSE	5(6)-Carboxyfluorescein N-hydroxysuccinimidyl ester
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription 3
SVR	sustained virologic response
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th	T helper
TLR	toll like receptor
TRAIL	(TNF)-related apoptosis-inducing ligand
UTR	untranslated region
VLDL	very low density lipoprotein
µg	micro gram
µl	micro liter

CHAPTER-1

GENERAL INTRODUCTION

1.1 VIRAL HEPATITIS

The word hepatitis describes a liver inflammation and originates from a combination of the ancient Greek words *"hepar"* (root word *hepat*), meaning 'liver,' and the '*itis,'* meaning inflammation. Hepatitis viruses such as hepatitis A, B, C, D and E, are known to result in liver damage (1). However, hepatitis can also be caused by alcohol consumption, toxins, infection, and by our own autoimmune processes. Hepatitis viruses infect and inflame the liver, and show similar symptoms. They differ mainly in the duration and magnitude of the infection, i.e. their structure, genetic makeup and the mechanism by which they are transmitted. In addition to these hepatitis viruses, other viruses that can also cause hepatitis include Cytomegalovirus, Epstein-Barr virus, and Yellow fever. Other than above mentioned hepatitis viruses, two other hepatitis viruses called hepatitis F and G have also been reported (2, 3). However, none of these are true hepatitis viruses as they have not shown any association with any liver disease and are not known to exacerbate liver disease caused by other hepatitis viruses.

1.2 Hepatitis C Virus (HCV)

Initially, the hepatitis A and hepatitis B viruses were thought to be agents of hepatitis, including the cases of blood transfusion associated hepatitis. The blood tests for hepatitis B and for hepatitis A were developed in 1963 and 1973 respectively. However, many of the blood samples tested negative for hepatitis A and hepatitis B, transfusion-associated hepatitis continued to occur. Cases of

transfusion-associated hepatitis were thought to be caused by some other unknown agents and were classified as non-A, non-B hepatitis (NANBH). Later on those cases were defined as Hepatitis C. Hepatitis C came into the picture a little more than three decades ago, when researchers transmitted NANBH from patients with transfusion-associated hepatitis to chimpanzees, demonstrating that the disease resulted from a transmissible agent (4). A major breakthrough came in 1989 with the cloning of the hepatitis C virus (HCV) genome by Choo *et al*., (5). Later, Kuo *et al*., developed an anti-HCV antibody detection system to diagnose HCV infection (6). Given the nature of the evolution of all viruses, hepatitis C is believed to have been around for thousands of years or more before its current strains evolved. Some experts speculate that since HGV/GBV-C, a close relative of HCV, originated in Old and New World primates, HCV might be traced back to 35 million years ago (7)**.** However, this is just a speculation and it is impossible to corroborate these theories because there is no blood sample available from that era.

1.3 MODE OF HCV TRANSMISSION

HCV is generally transmitted parenterally. Other common modes of transmission involve transfusions and/or parenteral contact with blood products. However, unlike most other blood-borne viruses (e.g. HIV), virtually any source of blood or blood products seems to be capable of carrying the HCV, even if the source is indirect, such as used razor. This makes hepatitis C far more transmissible than HIV; however HBV is the most transmissible virus by blood (8). Tattooing, as

well as many body piercing practices, such as acupuncture and ear-piercing, have contributed significantly to the spread of HCV, even in industrialised nations. Needle-stick injuries, contaminated medical equipment, and blood spills in health care settings are also responsible for many cases of HCV. In the 1970s and 1980s, many hepatitis C victims contracted the disease through blood transfusions. Rates of post-transfusion hepatitis during this period were determined to have been between 8% and 10% (9) since then; improved testing has led to drastic reductions in risk, down to less than 1% after 1993.

The main cause of HCV infection is intravenous drug use (IDU), which is responsible for about 30-40% of all identified cases of hepatitis C (10). As with HIV, sharing contaminated needles and other drug paraphernalia dramatically increases the chance of infection. The incidence of infection among intravenous drug users has surpassed 50 percent in many studies, and reached 100 percent in others (11). Cocaine users have also been shown to transmit the virus by sharing snorting straws. Heterosexual or homosexual activity with multiple sexual partners has been clearly identified as another mode of transmission; however, the exact risk is unknown (12). Moreover, in more than 10% of all cases, no risk factor can be identified, indicating the possibility of yet-unidentified modes of transmission.

1.4 EPIDEMIOLOGY

HCV continues to be a major disease burden in various countries throughout the world. Countries with high rates of chronic infection are Egypt (15%), Pakistan

(4.8%) and China (3.2%) (13). The main mode of transmission in these countries is thought to be unsafe injections (i.e., those using contaminated equipment). In 2012, the WHO estimated that every year, 3–4 million people are infected with the HCV. About 150 million people are chronically infected and are at risk of developing liver cirrhosis and/or liver cancer. More than 350,000 people die from hepatitis C-related liver diseases every year (14).

As of December 2007, it was estimated that 242,500 people in Canada (approximately 0.7% of the Canadian population) were infected with HCV, with an estimated 7,900 individuals newly infected in 2007, mostly through IDU (Public Health Agency of Canada). In Canada, approximately 20% of reported HCV infections occur in the immigrant community. HCV infections from transfusion of blood products accounts for approximately 13% of all cases (15). The genotyping of HCV isolates showed that subtype 1a (48%) was predominant in Canada. The other subtypes detected were 1b (19%), 2a (6%), 2b (3%), 3a (22%) and 4a (1%). In Winnipeg, Manitoba, subtype 3a (47%) was more prevalent than subtype 1a (37%), and, in Guelph, Ontario, both subtypes 1a and 3a had equal (40%) distribution. The prevalence of subtype 3a was significantly higher in injection drug users (27%) than in nonusers (10%) (16). Figure 1-1 shows the distribution of HCV around the world.

1.5 GENOMIC STRUCTURE AND VARIABILITY

HCV belongs to the Flaviviridae family, which includes yellow fever virus, dengue fever virus, Japanese encephalitis virus, and Tick-borne encephalitis virus.

HCV, with 7 genotypes and numerous subtypes, is a member of the hepacivirus genus, which includes tamarin virus and GB virus B (GBV-B), and is closely related to human GB virus C (GBV-C) (17). The HCV is a small (50 nm in size), enveloped, single-stranded, positive-sense RNA virus. The HCV envelope is a lipid bilayer in which two envelope proteins (E) are anchored. The envelope surrounds the nucleocapsid, which is composed of multiple copies of a small basic protein called core protein. The nucleocapsid contains the RNA genome of 9600 bps (18). Within an HCV genotype, several subtypes (designated a, b, c and so on) can be defined that differ in their nucleotide sequence by 20–25%. The genetic heterogeneity of the population of HCV genomes that coexist in an infected individual is called a quasispecies (19).

More than 40,000 HCV sequences have been identified and reported in databases. Three complementary databases specifically dedicated to HCV are the European HCV Database, the Los Alamos National Laboratory HCV Database and the Japanese Hepatitis Virus Database (18, 20). These offer several specialized features, including useful tools for HCV sequence comparisons, structural analyses of viral proteins and $CD4^+$ and $CD8^+$ T-cell epitope information.

1.5 VIRAL LIFE CYCLE

1.5.1 Entry of virus into host cell

In the blood of infected patients, HCV is physically associated with very low density lipids (VLDL), low density lipids (LDL), and high density lipids (HDL).

Lipo viral particles bind to a variety of receptors on the surface, including the tetraspanin CD81 (21, 22) and the scavenger receptor class B type I (23). This receptor complex then translocate to the tight junction where co-receptors claudin (24) and occludin (25, 26) mediate Clatharin-dependent endocytosis. HCV also binds to other molecules, such as glycosaminoglycans, the LDL receptor, and the lectins DC-SIGN and L-SIGN (27, 28), but these are not essential entry factors and do not confer tissue specificity. After clathrin-mediated endocytosis, virus particles are released from endosome due to the pH gradient. HCV translation and replication start in the cytosol, where HCV protein translation initiates through an internal ribosomal entry site in the 5' untranslated region (UTR). As a result of the translation, HCV generates a single open reading frame encoding polyprotein of about 3000 amino acids (29).

1.5.2 Replication of HCV

After the HCV infects the host cell, its genomic material is released into the cytoplasm and is translated. The viral and host protease then process the polyprotein (30). A negative strand of RNA is synthesized by viral replicase composed of NS3-5B and serves as a template to produce a positive strand. By interacting with structural proteins, the positive strand of RNA is encapsidated. Processed viral proteins remain associated with the endoplasmic reticulum (ER) membrane. The virus particles are enveloped by budding into the lumen of the ER and are exported through the Golgi complex (31). Various stages of the HCV's life cycle are shown in Figure 1-2.

1.5.3 HCV polyprotein processing

The polyprotein is cleaved by cellular and viral proteases into 4 structural and 6 non-structural proteins as shown in (Figure 1-3). The structural proteins and the p7 polypeptide are processed by the ER signal peptidase whereas the non-structural proteins are processed by two viral proteases, the NS2–3 protease and the NS3–4A serine protease (32). Apart from these proteins, within the core encoding region, a protein called the F protein or alternate reading frame protein (ARFP) has been reported to be expressed from an alternative +1 reading frame (33-35).

1.5.4 HCV assembly and release

The packaging and release of the HCV is not very well known. However, it is proposed that it is closely linked to lipid metabolism (36), during which the virus infection changes the cellular distribution of lipid droplets (LDs). The nucleocapsid assembly in the HCV generally involves oligomerization of the capsid protein and encapsidation of genomic RNA. This process is thought to occur when the core protein interacts with viral RNA. The core-RNA interaction may be critical for switching from RNA replication to packaging (37, 38). Once an HCV nucleocapsid is formed in the cytoplasm, it acquires an envelope as it buds through an intracellular membrane (39). Further, it is believed that HCV particles are released from the cell through the secretory pathway. HCV structural proteins have been observed both in the ER and Golgi apparatus (40). In addition,

complex N-linked glycans, which transit through the Golgi apparatus, have been detected on the surface of HCV particles isolated from patient sera (41).

1.5.5 Function of HCV viral proteins and their interaction with host proteins

Core protein: The first structural protein from the N-terminus of the polyprotein is the core protein, which is 191 aa in length. It constitutes the virion nucleocapsid and most likely interacts with the viral RNA (39). The full-length core protein has been shown to localize in the cytoplasm on the external membrane of the ER, but some of its truncated forms have been found in the nucleus (42, 43). The core protein has been extensively studied and appears to play multiple roles in various cellular signalling pathways, and potentially in oncogenesis (44, 45). It induces hepatocellular carcinoma when expressed in transgenic mice (46, 47). It could also induce the formation of lipid droplets and may play a direct role in steatosis formation (46-48). The core protein modulates the immune response by interacting with T cells and DCs. Mice immunized with an HCV-derived core protein containing adenovector show an expression of Tregs and a reduction in GrB expression by T cells (49).

F protein: The F protein is encoded by a reading frame that overlaps the core protein coding sequence (Figure 1-4). The length of the F protein varies depending on the genotypes. For genotype 1a such as the HCV-1 isolate, the F protein is 161 amino acids long (50). Previous work suggests that the F protein is expressed in natural HCV infections (51) and exhibits B- and T-cell mediated immune responses specific to ARFP (52). It has also been reported that an anti-F

antibody is significantly increased in patients with HCC, suggesting a possible role and/or presence of the F protein during carcinogenesis (51). Although the F protein is not essential for viral replication in cell culture or *in vivo* (53), its role in disease progression and in the development of HCC has not been ruled out.

E1 and E2: E1 and E2 have molecular weights of 33-35 and 70-72 kDa, respectively, and assemble as noncovalent heterodimers (54). These are essential components of the HCV virion envelope and are necessary for viral entry and fusion (55, 56). Cell surface expression of E1 and E2 is very limited, which may explain why the infected cells can escape from immune recognition. Their ER localization strongly suggests that, for other *Flaviviridae* members, the HCV assembles at the ER membrane. Localization of the core protein near this structure is consistent with this hypothesis. The E1 and core proteins can interact with each other, suggesting that the viral nucleocapsid is enveloped through this interaction. Besides its structural role, E2 has been shown to modulate the IFN-α response (57).

p7: p7 is a small, 63 aa polypeptide, which is a membrane-associated protein. Their precise roles in viral structure or replication are not yet clear (58). *In vitro* studies suggest that p7 belongs to the viroporin family and could act as a calcium ion channel (59). However, these results have not yet been confirmed *in vivo*.

NS2 and NS3: NS2 and NS3 are the two viral proteases responsible for the cleavage of all the NS proteins (61, 62). Furthermore, NS3 has a helicase and an NTPase activity, suggesting that it plays a role in RNA replication as well (62).

NS3 is also involved in modulating the T cell function. NS3 also has an effect on granzyme expression. Studies conducted on mice immunized with NS3 containing adenovector showed that the NS3 increased the GrB expression from T cells of those mice (49).

NS4A: This is a cofactor of NS3, with which it forms a heterodimer (63, 64). NS3-4A also gains additional properties through its interaction with host cell pathways and proteins. These properties may be important in the lifecycle and pathogenesis of infection. Not surprisingly, the NS3-NS4A protease is one of the most popular viral targets for anti-HCV therapeutics (65, 66).

NS4B: NS4B is an integral ER membrane protein. Its function is poorly characterized. One of its functions is to induce the formation of the membranous web, which serves as a scaffold for the HCV replication complex. (67-69). Additional putative properties include the inhibition of cellular protein synthesis (70, 71), modulation of HCV NS5B RNA-dependent RNA polymerase (RdRp) activity (64), transformation of NIH3T3 cell lines (73), and induction of IL-8 (74).

NS5A: NS5A is a 56-58 kDa phosphorylated zinc-metalloprotein that plays an important role in virus replication and cell regulation. This protein is phosphorylated (75) and has been shown to interfere with the IFN response (76). Recent studies on HCV-RNA replicon have shown that many adaptive mutations that enhance viral replication are localized in NS5A, suggesting that it play an important role in viral replication (69, 70). HCV replicon RNA replication was inhibited by mutations in the NS5A sequence (31, 79) and abolished by alterations

of the zinc-binding site (72). NS5A also bears transcriptional activation functions (81, 82) and appears to be involved in the regulation of cell growth and cellular signalling pathways (83, 84). However, these observations remain to be confirmed *in vivo*. NS5A, a component of the HCV polymerase complex, has diverse effects, including control of cellular proliferation and apoptosis, inhibition of protein kinase activity, and induction of IL-8 expression (85). These effects may inhibit the action of IFN-α (85).

NS5B: This is the viral RNA-dependent RNA polymerase (RdRp); it does not show rigid template specificity *in vitro* but can copy a full-length HCV genomic RNA (86). NS5B has a hydrophobic domain and its C-terminus allows it to be inserted into the membrane (87). Interactions between NS5B and cellular components have also been reported. The C-terminus of NS5B can interact with the N-terminus of hVAP-33, and the interaction may play an important role in the formation of the HCV replication complex (88, 89). More recently, NS5B was reported to bind cyclophilin B, a cellular peptidyl-prolyl *cis-trans* isomerase that apparently regulates HCV replication through modulation of the RNA binding capacity of NS5B (90).

1.6 TYPE OF INFECTION

1.6.1 Acute HCV infection

Acute hepatitis C infection is rarely diagnosed because in the majority of acutely infected individuals, it is asymptomatic. Approximately 70% to 80% of cases have been found asymptomatic in the transfusion setting, where acute onset of

HCV infection has been best recorded (91). About 20% to 30% of adults with acute HCV infection may develop clinical symptoms. In acute hepatitis C infection, the onset of symptoms occurs between 3 and 12 weeks after exposure (92, 93). Symptoms may include malaise, weakness, anorexia, and jaundice. Alanine aminotransferase (ALT) levels in serum, which indicate hepatocyte necrosis, begin rising 2 to 8 weeks after exposure, and often reach levels of greater than 10 times the upper limits of normal. HCV RNA, which can be detected in the serum within 1 to 2 weeks after exposure (93, 94), rises rapidly during the first few weeks, and then peaks between 10^5 to 10^7 IU/ml, shortly before the peak of serum aminotransferase levels and the onset of symptoms. Approximately one to three months after exposure, HCV antibodies develop and can be detected by enzyme immunoassay. However, up to 30% of patients test negative for the anti-HCV antibody at the onset of symptoms, making HCV testing unreliable for diagnosing acute infection (94). Almost all patients eventually develop the antibody to HCV; however, titers can be low or undetectable in immunodeficient patients. The anti-HCV assay detects greater than 90% of HCV infections after the initial 3 months (95, 96). Although acute HCV infection can be severe, fulminant liver failure is rare (97).

Recently, nucleotide polymorphisms (SNP) close to the IL28B gene have been studied extensively in acute hepatitis C. Linkage between IL28B and HCV clearance came in picture from studies of large cohorts of patients with chronic HCV infection who were treated with PEG-IFN-α and RBV. These cohorts were investigated in genome-wide association studies (GWAS), which allow an

unbiased sampling of variations in genes across the entire genome (98). Although more than 500,000 SNPs were considered, however, the strongest predictor of sustained virologic response (SVR) was a SNP (rs12979860) located on the long arm of chromosome 19, within the *IFN-λ* gene cluster (99). At this position, people with the CC genotype have SVR rates more than 2-fold higher than those with the minor T allele. However, the role of different SNPs in the IL28B region is currently a matter of debate. Rao *et al.* (100) showed a positive impact of four SNPs -- rs8099917 TT, rs8105790 TT, rs12980275 AA and rs10853728 CC -- on spontaneous clearance of HCV infection. However, further studies are needed to clarify the value of IL28B genotyping in acute hepatitis C and if treatment decisions can be based on distinct SNPs in the IL28B gene (101).

1.6.2 Chronic infection

The existence of HCV RNA in the blood for at least 6 months after the onset of acute infection indicates the possibility of chronic hepatitis C. The rate of the chronic HCV infection depends on many factors, including the patient's age at the time of infection, gender, ethnicity, and the development of jaundice during the acute infection. It has been reported that self-limiting HCV occurs in only 15-25% of patients in whom HCV RNA in the serum becomes undetectable. In these patients, ALT levels return to normal. However, approximately 75-85% of infected patients do not clear the virus by 6 months. These patients develop chronic hepatitis (102).

Chronic HCV infection may also cause extrahepatic manifestations (EHM), which is a leading clinical manifestation of HCV infection and can determine the overall prognosis of the disease. Further, HCV infection is known to be associated with mixed cryoglobulinemia (MC). MC is defined by the presence of cryoglobulins in the blood. Cryoglobulins are immunoglobulins that precipitate at temperatures lower than 37 C. Overall, cryoglobulins can be found in 19–55% of HCV-infected individuals (103, 104). HCV also interferes with the metabolic system. Patients with chronic HCV may develop insulin resistance and type 2 diabetes (105). Insulin resistance in patients with chronic HCV infection may lead to a faster progression of liver fibrosis and a higher prevalence of HCC (106, 107).

1.7 IMMUNE RESPONSE AGAINST HCV

The first defence against any type of infection is the innate immune response. Anatomic barriers including skin and mucous membranes are the first line of the innate immune system which needs to be penetrated to allow an infectious agent to reach to the blood and beyond, to the site of infection. In case of HCV infection, the innate immune response at the liver (site of infection) is represented by Kupffer cells (liver macrophages), natural killer (NK) cells and natural killer T (NKT) cells. The innate immune responses are followed by the activation of adaptive immune responses, which includes humoral and cellular immune response.

1.7.1 Innate immune response

The innate immune system has a number of roles in recognizing and clearing viral infections. The innate immune system is the front line of defence of the human body against HCV immediately after infection. Further, it determines the HCV-specific adaptive immunity that is important for viral clearance. Accumulating evidence suggests that the host has evolved multifaceted innate immune mechanisms to sense HCV infection and elicit defense responses, while HCV has developed elaborate strategies to circumvent host defense mechanisms (108). HCV specific innate immune response is initiated by different pattern recognition receptors, RIG-1, TLR-3, TLR-7 and TLR-8. Viral dsRNAs and 5′-triphosphate-bearing, poly-U rich viral RNAs are recognized by the RIG-I that are constitutively expressed in the cytoplasm and recruits the adapter molecule IFN-β promoter stimulator protein 1 (IPS-1; also called CARD adaptor inducing IFN-β CARDIF], virus-induced signaling adapter [VISA], and mitochondrial antiviral signaling protein [MAVS] leading to activation of IRF3/7 and NF-κB and, ultimately, synthesis of type I and III IFNs and inflammatory cytokines/chemokines (109, 110). TLR3 senses dsRNA in endosomes and trigger a TRIF-dependent pathway activating innate immune responses (111). Viral ssRNAs can also be sensed by TLR-7 or TLR8 in endosomes and activate MyD88-dependent innate immune responses. TLR-7 operates exclusively in pDCs and mainly results in IRF7 activation and subsequent induction of IFN-α and IFN-λs (112, 113), while TLR-8 operates in mDCs and mainly leads to expression of NF-κB-dependent cytokines (111, 114).

Different types of innate immune cells are also involved in generating immune responses against HCV. Because adaptive immunity requires weeks and even months to develop, early host control of the HCV infection relies on the innate immune response that host cells use to combat invading pathogens immediately after infection. Innate immune cells such as dendritic cells (DCs), natural killer (NK) and NKT (115, 116), are major component of the innate immune response against HCV infection. Apart from these cells' induction of IFN-α, IFN-β, IFN-γ, inflammatory cytokines and chemokines from different cells also play important roles in controlling HCV infection.

1.7.1.1 Dendritic cells (DCs)

DCs are professional antigen-presenting cells that sample the environment at sites of pathogen entry and play an essential role in inducing adaptive immune responses. Upon activation by micro-organisms or inflammatory signals, immature DCs migrate to the draining lymph nodes while up-regulating co-stimulatory molecules and developing into mature effector DCs. There, DCs present pathogen-derived peptides in association with major histocompatibility class II (MHC-II) molecules to naive T helper (Th) cells. The Th cells recognize the MHC-II/peptide complex via the T cell receptor (TCR) (117-119). With the appropriate additional interactions mediated by co-stimulatory molecules on the DC, naive Th cells become effector Th cells (120, 121). Strong adhesion between the cells for this crosstalk between DCs and T cells are provided by integrin molecules. The whole complex of molecules interacting between the DC and T

cell is referred to as 'the immunological synapse' (122, 123). Depending on the type of pathogen and the micro-environment of the immature DC, different subsets of effector DC develop, which promote the development of protective effector Th type-1 (Th1) or type-2 (Th2) cells from naive Th cells. In this way, the type of T cell response is adapted to the type of invading pathogen and the infected tissue (124). The distinct subsets of effector DC that bias the polarization of naive Th cells into Th1 cells, Th2 cells or both, are designated DC1, DC2 and DC0, respectively (124, 125).

1.7.1.1.1 The role of DCs in adaptive immunity

DCs identify pathogens through evolutionary-conserved pattern recognition receptors (PRR). PRRs recognize pathogen-derived molecules via pathogen-associated molecular patterns (PAMP). Binding PAMP to PRRs leads to opsonisation, endocytosis, DC activation or apoptosis (126). Toll-like receptors (TLRs) and C-type lectins are two of the major PRR families (127, 128). So far, 11 different TLRs have been identified (110). These bind to a wide variety of microbial compounds, such as RNA or DNA sequences from several pathogens, bacterial cell wall components (LPS and peptidoglycan), bacterial flagella proteins and carbohydrates from the yeast cell wall (128, 129). TLR binding by PAMPs leads to DC activation through NF-κB signalling (128). In contrast to TLRs, binding to C-type lectins can lead the antigen to internalize and be presented (130, 131). C-type lectins include the mannose receptor (MR), BDCA-2, langerin, DEC-205 and DC-specific ICAM-3-Grabbing Nonintegrin (DC-SIGN). DC-SIGN recognizes a variety of micro-organisms, including bacteria

(132), fungi, viruses including HIV-1 and several parasites. Although DC-SIGN binding can lead to internalization and lysosomal degradation, followed by antigen presentation to T cells, most pathogens that bind to DC-SIGN cause long-lasting and chronic infections. This suggests that pathogen-binding to DC-SIGN induces tolerance or immune evasion (133). Besides playing an important role as PRR, C-type lectins have been shown to act as adhesion receptors (134). For instance, DC-SIGN mediates the contact between DC and T cells by binding to ICAM-3, and mediates rolling of DC on the endothelium by interacting with ICAM-2 (134).

1.7.1.1.2 Different DC subsets

DCs make up a heterogeneous group of cells. This group is manifested by differences in location within the body, phenotype and behaviour. Although all currently known DC populations are not well described on the basis of their surface phenotype, in humans, two main DC subsets have been identified: $CD11c^+$ myeloid DC (mDC) and $CD11c^-$ $CD123^+$ plasmacytoid DC (pDC) (135-137). mDCs include Langerhans cells, dermal DC and interstitial DC, and are widely distributed throughout the body. In the blood, at least 4 different types of mDCs can be distinguished on the basis of surface markers. pDCs are primarily located in the blood and in secondary lymphoid organs, but they can be recruited to sites of inflammation (136). Besides the location, the difference between mDCs and pDCs is manifested by TLR expression and cytokine secretion. mDCs express all TLRs except TLR7 and TLR9, which are selectively expressed by pDCs (138,

139). mDCs secrete high levels of IL-12 which help in differentiation of naïve T cells, whereas pDCs are thought to play an important role in the innate immune response to different viruses by producing IFN-α (138, 139).

1.7.1.1.3 DCs in HCV infection

It has been hypothesized that when there is a viral impairment of DCs, HCV-infected individuals are unable to mount an effective T-cell response, leading them to develop a chronic HCV infection (140). Several studies have been reported with contradictory experimental evidence, and there is little overall consensus (141-142). The differing results could be attributed to the selection of patient population, HCV viral load, duration of infection, cell type being studied, *in vitro* expansion, assay being used (143). Further, it is not clear whether DCs become impaired in chronic HCV infection, or if DC impairment is a prelude to inefficient priming and maintenance of HCV-specific T cells, which facilitates the establishment of a chronic carrier state. DCs impairment could also be a consequence of persistent and active HCV infection and associated disease progression (143).

1.7.2 NK cells

NK cells work at the interface of adaptive and innate immune systems. They play an important role in host defence against a number of viruses, including HCV (144, 145). NK cells display both the stimulatory and inhibitory receptors and can distinguish between infected and uninfected cells by recognizing the alterations in MHC class 1 expression induced by viral infection (146). The liver contains more NK cells than any other organ, which itself makes NK cells an important cell

type, which has a role in defence against liver disease such as HCV (147). NK cells are classified on the basis of the level of expression of the CD56 marker. NK $CD56^{dim}$ represents a differentiated subset, which is 90% of the circulating NK cells' pool (148). NK cells residing in the liver produce less IFN-γ and more IL-10 than peripheral blood and splenic NK cells (149). The decreased IFN-γ and the increased IL-10 contribute to the hypo responsive state of the liver microenvironment, making the liver susceptible to viral infection. However, NK cells are capable of secreting IFN-γ to powerfully suppress HCV RNA expression, indicating that NK cells are indeed involved in the host innate immune defense against HCV infection (150). NK cells' function can be modified to improve their antiviral properties by cytokine therapy and by manipulating NK cell receptor expression. The adoptive transfer of TRAIL-expressing NK cells may provide a better treatment option for reducing the recurrence of HCC following a partial hepatectomy and liver transplant (151, 152).

1.7.3 NKT cells

NKT cells, which express both NK cell marker CD56 and T cell marker CD3, also occur in large proportions in the liver. Functional detail for these is not very well known but it is suggested that NKT cells lyse the infected cells by secreting granzyme (proteases), exerting cytotoxic functions and/or producing cytokines such as IFN-γ and IL-4. Functional data suggests that iNKT cells may travel to the liver during HCV infection and acquire a fibrogenic cytokine-producing profile. NKT cells can also secrete IFN-γ to inhibit HCV replication in hepatocytes (153),

and their activity correlates positively with the outcome of acute HCV infection (154) and the efficacy of IFN-α treatment in chronic HCV infection (155). This suggests that NKT cells probably play a role in controlling HCV infection, particularly in the early stages of infection.

1.8 ADAPTIVE IMMUNE RESPONSE

Simultaneous activation both of the humoral and the cellular part of the adaptive immune system appears to be required to the control of HCV infection (115). The result of the complex interactions between the various parts of the adaptive immune system determines the final outcome. Failure of one component may lead to generalised dysfunction. Defective $CD4^+$ T-cells lead to impairment in $CD8^+$ T cell activity and antibody production (156). $CD8^+$ T cell failure leads to high virus replication (157).

1.8.1 Humoral immune response

Chronic infection is characterized by high titers of HCV-specific antibodies. Although studies of the humoral response have shown antibodies for all viral antigens, no antibody conferring immunity to HCV has been detected. A number of investigators have suggested that an early vigorous antibody response to the hyper variable region-I of the E2 glycoprotein is associated with a self-limited course of infection, and HCV persistence follows more complex initial quasispecies distribution (158, 159). However, independent studies in chimpanzees have shown that HVR1 sequence changes are not a pre-requisite to

establish HCV persistence (160). Recently, it has been shown that human monoclonal antibody HCV1 recognizes a highly-conserved linear epitope of the HCV E2 envelope glycoprotein (amino acids 412–423) and neutralizes a broad range of HCV genotypes (161).

1.8.2 Cellular immune response

Clearance of HCV can occur spontaneously during acute HCV infection, and is associated with a vigorous, quick and long-lasting $CD4^+$ and $CD8^+$ cellular immune response against multiple HCV epitopes (116, 162).

1.8.2.1 Role of $CD4^+$ T cells

Approximately 20-30% of HCV-infected people show viral clearance. However, the exact mechanism of viral clearance is not known. $CD4^+$ T cells are critical for adaptive immune responses because they secrete different cytokines which help to augment antibody production by B cells and they also prime the $CD8^+$ T cells that are specific for virus-infected cells (163, 164). Cytokines-like IFN-γ, the most relevant of the Th1 cytokines, promotes neutrophil recruitment and macrophage activation and leads to inflammatory responses, whereas IL-4, IL-10 and other Th2 cytokines limit Th1-mediated inflammatory responses and prevent excessive tissue destruction and restraining inflammation (165). $CD4^+$ T helper (Th) cells are stimulated when they recognize viral peptides in the HLA class II binding groove of professional APCs and provide a helper function for cellular and humoral immune responses. This has been supported by several studies such as those about HLA class II allele DRB1*1101 and DQB1*0301 and DRB1*01,

DRB1*04, and DRB1*15 are associated with virus clearance in diverse populations (166). A strong and sustained response from HCV-specific $CD4^+$ T cells is required for virus control (167-171). In the chimpanzee model of acute infection, high number of $CD4^+$ T cells is associated with a substantial decrease in the viral load (170, 172, 173). Patients with more $CD4^+$ T cells are more likely to clear the virus than those who have less. The loss of early $CD4^+$ T cells causes a relapse of viremia (167, 168, and 174). A recent study demonstrated that broadly directed HCV-specific $CD4^+$ T cell responses are primed irrespective of the outcome of infection. However, these responses rapidly disappear in patients with persistent infection (175). It will be important to identify what causes this rapid collapse of the $CD4^+$ T cell response. Equally important will be defining the functional and transcriptional profiles of these cells before they disappear. Inhibiting or modulating signals from the early $CD4^+$ T cell response could influence other arms of the immune response beyond the time when these cells have already disappeared.

1.8.2.2 Role of $CD8^+$ T cells

MHC class I-restricted peptide-specific cytotoxic $CD8^+$ T cells (CTLs) represent a critical effector limb of the immune system that combats viral infection. $CD8^+$ T cells control viral infection by different mechanisms: by the cytolytic pathway, in which the $CD8^+$ T cell kills the infected cells by secreting perforin and GrB or by expressing cell-bound receptors such as CD-95 and CD-95L; and by the non cytolytic pathway, which is mediated by a secretion of cytokines like IFN-γ and

TNF-α. It is possible that insufficiently activated $CD8^+$ T cells may not be able to clear the viral infection through cytokine-mediated mechanisms, but may still induce immunologically mediated liver injury as a result of cytotoxic effects. It has been shown that the inhibition of HCV replication occurs mainly because of IFN-γ mediated noncytolytic pathways (176). HCV replicon cells are also sensitive to cytolysis by perforin; however, most HCV-infected patients display virus-specific $CD8^+$ T cells with low perforin secretion, indicating that this effector pathway may play a minor role in natural HCV infection (177). The accumulation of virus-specific $CD8^+$ T cells in the liver coincide with elevated levels of liver enzymes and transient control of viral RNA from the plasma (170, 178). There is also direct experimental evidence that $CD8^+$ T cells are the main effector cells against the HCV infection: in an experiment $CD8^+$ T cells were depleted by using an antibody in the chimpanzee which causes a high viral load for a prolonged period of time despite the presence of memory $CD4^+$ T cells. However, after the $CD8^+$ T cells recovered, viral clearance was observed in these animals (179). Further, it has been shown that certain $CD8^+$ T cells alleles such as B27 or B57 are associated with spontaneous resolution of acute HCV infection (166). A different subset of HCV-specific $CD8^+$ T cells have been identified on the basis of the production of IL-17 as well as the high expression of CD161 and the liver homing chemokine receptor CXCR6 (180, 181). This subset has a different epitope repertoire than HCV-specific IFN-γ expressing $CD8^+$ T cells (182). These cells may have a protective effect in the chronic HCV infection,

since they have been found in higher frequencies in patients with mild stages of liver inflammation or fibrosis.

1.8.2.3 Regulatory T cells

Regulatory T cells (Treg) cells are a distinct subset of T cells that play a central role in maintaining the immunological self-tolerance and immune homeostasis. These cells are $CD4^+$ T cells that are characterized based on the presence of forkhead box P3 (FoxP3) transcription factor and the constitutive surface expression pattern of the CD25 that forms part of the high affinity IL-2 receptor (183).

Based on contemporary knowledge, it is generally accepted that Treg cells, IL-10 and TGF-β play a key role in containing HCV-specific immune responses. Treg cells and the cytokines such as IL-10 and TGF-β have been shown to limit clearance of HCV during chronic infections. The mechanism behind limited HCV clearance might be due to the inhibition of the proliferation and effector function of T cells or other immune cells (184). A previous study has shown that in the blood, $CD4^+CD25^+$ Treg cells were able to suppress HCV-specific IFN-γ production of $CD4^+$ and $CD8^+$ T cells and their proliferation capacity in response to HCV (185).

Further studies have shown that Treg cells are present in the liver of chronic HCV-infected patients (186-188) and are virtually absent in the livers of healthy individuals (187). Furthermore, the numbers of intrahepatic Treg cell can be negatively correlated with the development of fibrosis following HCV

infection (187). Besides blocking Treg cells, other studies showed that blocking IL-10 or TGF-β can also augment HCV-specific T cell proliferation and IFN-γ production (184, 189).

The recent data also provides evidence that $CD4^+CD25^+$ Treg cells can be induced in an antigen-specific manner: they can be induced by HCV-derived peptides (190). Interestingly, different HCV proteins/antigens can activate effector T cells or induce Tregs. For example, it has been shown that immunizing mice with the core protein resulted in the induction of Tregs, while immunizing with NS3 protein led to the induction of effector T cells (49). Of note, a recent finding demonstrated that Tregs can be induced by certain viral epitopes, mechanistically linking two different mechanisms of T cell failure, viral escape and enhanced Treg induction (191).

1.9 IMMUNE RESPONSE IN LIVER

A number of studies have attempted to analyze $CD8^+$ and $CD4^+$ T cells responses in the liver of HCV-mono-infected patients. Acute studies revealed that there is a substantial period where the virus is present at high levels, but no tissue damage or inflammatory infiltrate is observed (164). This may be because the virus causes relatively little tissue damage in a short period. It may also explain the lack of a robust link between viral load and disease activity. Another reason for the low amount of tissue damages is the severe delay in mounting the adaptive immune response, despite the presence of a replicating virus, is probably central to HCV's capacity to set up long-term persistence.

Chronic HCV infection studies using major histocompatibility complex class I peptide tetramers, which can directly identify antigen-specific T cells, have also shown relatively enriched populations in liver biopsies compared to blood (192). In some cases, T cell populations may fall below the level of detection in blood but be demonstrated in the liver. However, the majority of the T cell infiltrate in the liver in chronic HCV is apparently not HCV-specific.

The $CD8^+$ T cells in the liver have an unusual cytokine secretion profile, particularly IL-10 secretion (193). Antiviral $CD8^+$ T cells typically secrete IFN-γ, and this has also been shown in T cells derived from the liver. However, a relative increase in IL-10 secretion within liver tissue has been proposed to limit the tissue damage associated with these T cell infiltrates, and an inverse relationship with hepatic inflammation has been shown (194). Relatively little is known about the quality and quantity of virus-specific $CD4^+$ T cells in the liver, although IL-10 secretion in this subset has been reported (195). These data indicate that in chronic HCV infection, antiviral T cells make a home in the infected liver and secrete antiviral cytokines. However, within the hepatic environment, there appears to be a regulatory process occurring, which likely comprises multiple cell subsets. In other words, the T cell infiltrate in the liver probably helps to control virus replication, induction and regulation of hepatic inflammation. Figure 1-5 shows the immune response in the liver.

1.10 ESCAPE FROM IMMUNE RESPONSE

HCV has been shown to be capable of evading the immune response. To

accomplish this, the virus adapts multiple strategies that can help evade the innate as well as the adaptive immune response. Therefore, the HCV can remain in the body of an infected person without being detected. HCV has been shown to evade the innate component of the immune system primarily by interfering with signaling molecules that are required for IFN-γ production and also by modulating the INF-γ signaling pathway (196).

HCV NS3/4A cleaves the adapter molecules TRIF and IPS-1, thereby blocking TLR3 and RIG-I signalling which ultimately inhibit IFNs production (197). HCV core interferes with the JAK/STAT pathway by inducing SOCS1/3 (198) and by inhibiting STAT1 phosphorylation (199). The HCV polyprotein induces protein phosphatase 2A (PP2A), which interferes with STAT1 methylation, thereby increasing the binding of STAT1 to protein inhibitor of activated STAT1 (PIAS) (200). STAT1/PIAS interaction impairs the binding of the ISGF3 complex to the IFN-stimulated response element and blocks the transcription of ISGs. HCV E2 and HCV NS5A inhibit the function of several ISGs (76, 201). Furthermore, the HCV has been shown capable of mounting strategies that can inactivate NK cells and thus prevent adequate production of INF-γ that is vital for the optimal priming and cytolytic function of HCV-specific T cells (197, 202).

The HCV has also been shown to adapt a number of strategies to evade the humoral as well as cellular arms of adaptive immune responses. The HCV virus has a high replication rate and its RNA-dependent RNA polymerase (NS5B) does not have a proof-reading mechanism. As a result of this, multiple quasispecies of

the virus that are slightly different variants have been shown to be present in a single patient, and these variants are capable of escaping recognition by neutralizing antibodies (humoral response) (203). HCV variants are also capable of escaping the virus-specific $CD8^+$ T cell responses and thus can be positively selected in the host (204). Indeed, the HCV can escape from the $CD8^+$ T cells responses based on three different patterns: mutations at the HLA class I binding anchors of $CD8^+$ T cell epitopes can prevent antigen presentation; mutations in the flanking regions of an epitope can disrupt antigen processing and thus result in the lack of antigen presentation (205-207); and mutations within the T cell receptor contact residues in the middle part of epitope can prevent recognition by the $CD8^+$ T cells (208).

In addition to the virus escaping from $CD8^+$ T cells, the dysfunction of HCV-specific T cells is also considered as a hallmark of chronic infection in patients. Specifically, studies have shown that HCV-specific $CD8^+$ T cells have been suboptimal in the context of cytotoxicity, production of antiviral cytokines, and antigen-specific proliferation profile (209, 210). These dysfunctional T cells have been found to have a high expression of the cell-surface-receptor programmed cell death 1 (PD-1) (154, 211-213). Interestingly, the PD-1 expression level was found to correlate with HCV viral loads (211).

1.11 EXPERIMENTAL MODEL SYSTEM

The HCV has been studied using various identified *in vitro* and *in vivo* models. Each model has its own advantages and disadvantages as shown in Table 1 (214).

Further, the development of prophylaxis and novel therapeutics to treat HCV infection has been hampered by the lack of *in vitro* and appropriate *in vivo* model systems to study the antiviral activity of novel compounds, as well as the mechanisms of infection and pathogenesis caused by HCV. However, in the past decade, enormous progress has been made in the establishment of *in vitro* systems and the first chimeric mouse models for HCV infection. These studies may provide essential tools to develop more effective antiviral compounds.

TABLE 1. List of experimental model system

Models	**Advantages**	**Disadvantages**	**Important findings**
Cell culture	Stable HCV-replicating human cell lines HCV-infected human cell lines *In vitro* production of infectious hepatitis C virions (termed HCVcc)	Primarily based in human hepatoma Huh7 cell lines Mainly using genotype 2a JFH1 isolate	IFN-α production by pDCs due to direct contact with HCV-replicating cells HCVcc does not productively replicate in DCs and B cells Non-cytolytic effector functions (via IFN-γ) of HCV-specific $CD8^+$ T cells to inhibit HCV replication Immune escape and viral fitness cost
Chimpanzees	Can be infected by HCV Strain and titer of HCV are defined Serial samples can be obtained before and during the infection	Have a milder hepatitis Have a higher rate of viral clearance Limited by ethical concerns, availability and costs	Induction of hepatic ISG mRNAs in an early phase of HCV infection Association of viral clearance with intrahepatic IFN- γ-producing $CD8^+$ T cells Crucial roles of HCV-specific $CD4^+$ and $CD8^+$

Models	Advantages	Disadvantages	Important findings
	Immune response can be manipulated. Human reagents and tests can be used Close homology between MHC class I HLA and *Pan troglodytes* alleles		T cells for the Viral escape mutations in MHC class I-restricted epitopes HVR1 of E2 as an antibody-neutralized domain
Small animals	Genomic information is available Ease of genetic manipulation Lower cost and ease of maintenance	Not a natural host for HCV infection	Induction of hepatic ISG mRNAs, lipid peroxidation and oxidative stress-inducible genes in the human liver chimeric mice after infection Protection effect of IL-2/OKT3-activated liver allograft-derived lymphocytes (via IFN-γ) Immune-mediated liver injury in the Cre/*lox*P-controlled HCV transgenic mice

1.12 RESOLVING HCV INFECTION

Innate and adaptive immunity are two mechanisms which help in spontaneous clearance of HCV. Rapid induction of nAb early during infection is associated with spontaneous recovery and these antibodies appear to be more cross-neutralizing (215, 216). Induction of neutralizing antibody is less effective

because by the time the virus gets established in the host and leads to development of escape mutants (216). Early strong $CD4^+$ and $CD8^+$ T cell responses are also temporally associated with control of viremia (217).

The emerging consensus is that strong humoral and cellular immune responses accompanied by innate mediators dominate to control infection effectively. From chimpanzee studies it has been shown that with high anti-envelope antibody titers apparently sterilizing immunity was achieved (215, 216, and 218). If the virus is contained in this acute phase, strong memory T cell responses are detectable in the long term, although antibody responses may wane relatively quickly (169).

1.13 CURRENT AVAILABLE TREATMENT

The goal of HCV treatment is to cure the disease by using combination therapy. The standard care and duration of treatment varies from genotype to genotype. Before 2011, the HCV genotype 1 was treated with a combination of pegylated interferon and ribavirin. However, HCV treatment changed dramatically after the approval of two new direct acting antiviral (DAA) drugs – telaprevir and boceprevir – for use in pegylated interferon-based and ribavirin-based triple therapy in mid-2011. This therapy continued for a period of 48 weeks and provided sustained virological response in up to 70 % of patients (219, 220). Genotype 2 and 3 were treated with pegylated interferon and ribavirin for a period of 3 to 12 months depending on the viral load, liver damage, insulin resistance and early response to treatment (221).

HCV genotype 4 is treated for 48 weeks with pegylated interferon and weight-based ribavirin; sustained virological response rates in HCV patients being treated for the first time are as high as 70% (221).

Numerous new drugs targeting various aspects of the HCV life cycle and the host are in development and in clinical trials. Overall, combination therapies will be the rule. New combinations of DAA that have synergistic effects, decrease the risk of resistance, improve antiviral efficacy, are effective in different genotypes and have a favourable safety profile. Interferon-free combination regimens appear to be on the horizon, providing a new option in particular for patients with non-genotype 1 HCV. There will still be treatment failures and resistance issues to overcome, particularly in the treatment-experienced population. However, an HCV prophylactic vaccine is not available. Because of the persistent nature of the virus and an enormous genetic variability, vaccine candidates need to induce both neutralizing antibodies and T cell–mediated responses to achieve broad, long-lasting cross-protection.

1.14 RATIONALE

HCV is a pathogen which causes acute and chronic infection. A minority of infected people (15-30%) are able to clear viral infection naturally, indicating that a strong immune response could be generated against the infection. However, the exact mechanisms of viral clearance as well as the mechanisms leading to viral persistence in the majority of patients are not known.

In order to understand the mechanisms behind the generation of the antiviral cellular immune response against HCV, I investigated the role of HCV-derived F and core proteins in inducing and modulating cellular immune responses in *in vitro* and *in vivo* experiment models. The F-protein has recently been identified and shown to be expressed in HCV-infected patients. F-protein specific antibodies, as well as $CD4^+$ and $CD8^+$ T cells, have been detected in HCV-infected patients (24, 28 and 29). The core protein has been studied extensively; however, its role in immune suppression/modulation is still unclear. With growing support for the investigation of a prophylactic vaccine against HCV based on cellular immune responses, I wanted to examine the role of HCV-derived F and core proteins in modulating DCs, $CD4^+$ and $CD8^+$ T cells. It is evident from the literature that the host immune responses attempt to clear the virus and that the virus and viral proteins modulate the function of these cells to maintain persistence infection in the host. Therefore, a balance between these two opposing forces determines the outcome of initial infection. So, in order to investigate the role of HCV-F, core and NS3 protein, I hypothesize that

1. HCV-derived F and core proteins will modulate the number/functions of DCs *in vitro* and affect the overall cellular immune response.
2. HCV-derived F or core protein will modulate the function of $CD4^+$ and $CD8^+$ T cells *in vivo* and affect their ability to clear infected/target cells.
3. Early immune events will determine the disparate induction of effector T cells HCV-derived core and NS3 proteins.

Each of these hypotheses will be tested through the following three major objectives:

1. Phenotypic and functional characterization of DCs endogenously expressing HCV-derived F or core protein
2. Phenotypic and functional analysis of T cells from mice immunized with HCV-derived F or core protein
3. To investigate early antigen-specific immune response in HCV-derived core or NS-3 immunized mice

1.15 FIGURES

Figure 1-1

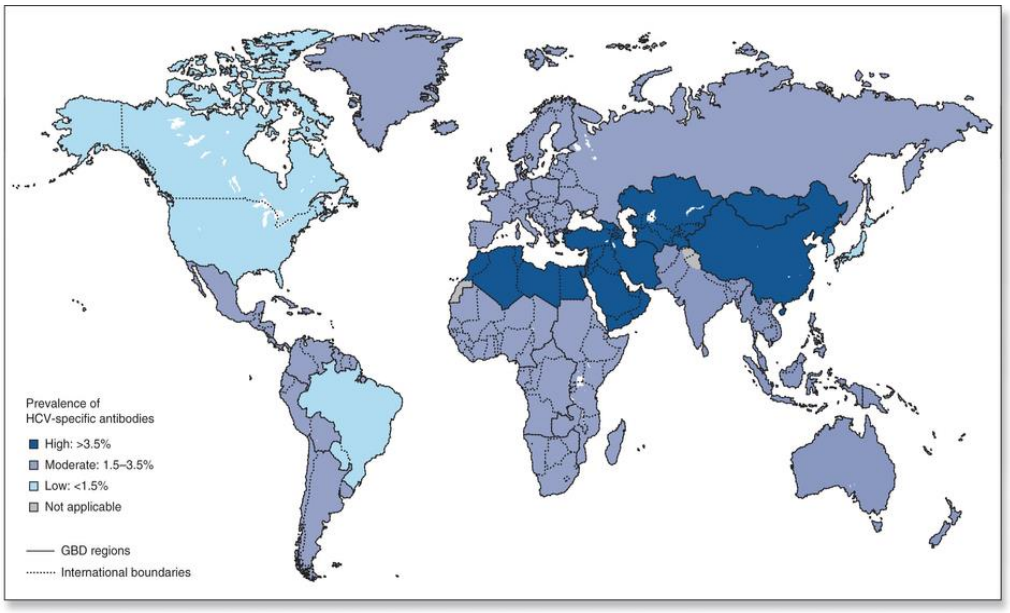

Figure 1-1 Global prevalence of HCV-specific antibodies (222).

Figure 1-2

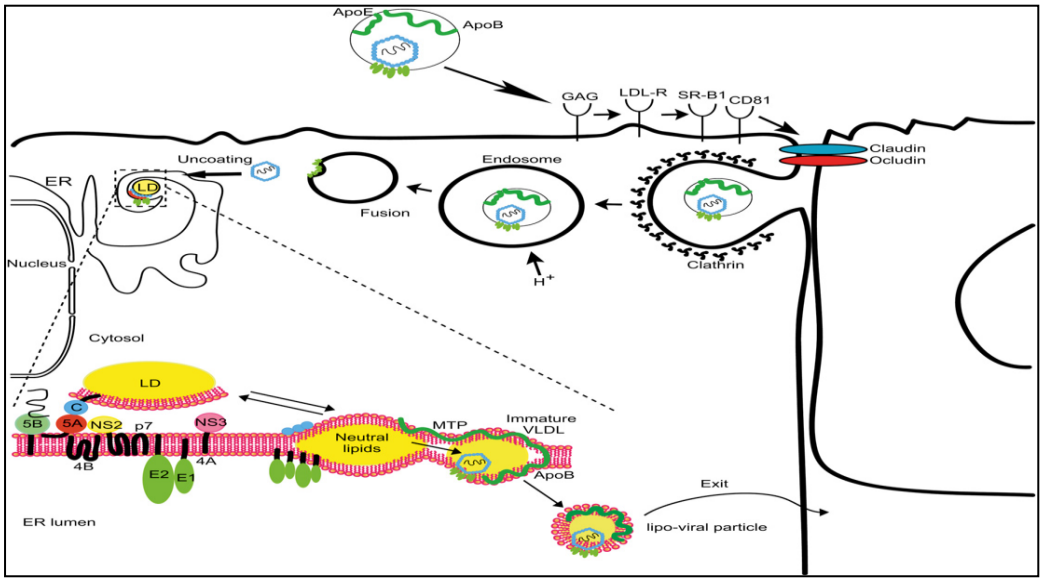

Figure 1-2. The Life Cycle of HCV: Lipo viral particle bind to variety of receptor on the surface including the tetraspanin CD81, the scavenger receptor class B type I. This receptor complex then translocate to tight junction where, co receptor claudin and occludin mediate Catharine dependent endocytosis. Virus particle after clathrin-mediated endocytosis get released from endosome due to pH gradient. HCV translation and replication start in cytosol, where HCV protein translation initiates through an internal ribosomal entry site in the 5' untranslated region (223).

Figure 1-3

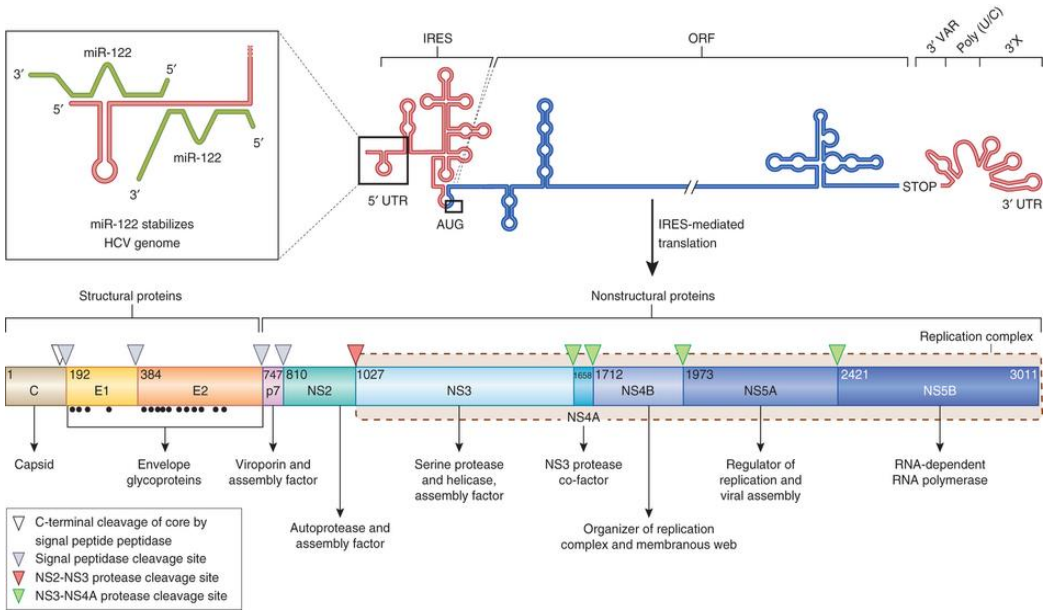

Figure 1-3. HCV proteins: HCV RNA encodes a polyprotein of approximately 3000 amino acids. The polyprotein is cleaved during and after translation by host and viral proteases to yield the ten proteins shown (Core–NS5B). The known functions of each protein are shown adjacent to the colored boxes. ARFP may be produced as a result of a translational frameshift (224).

Figure 1-4

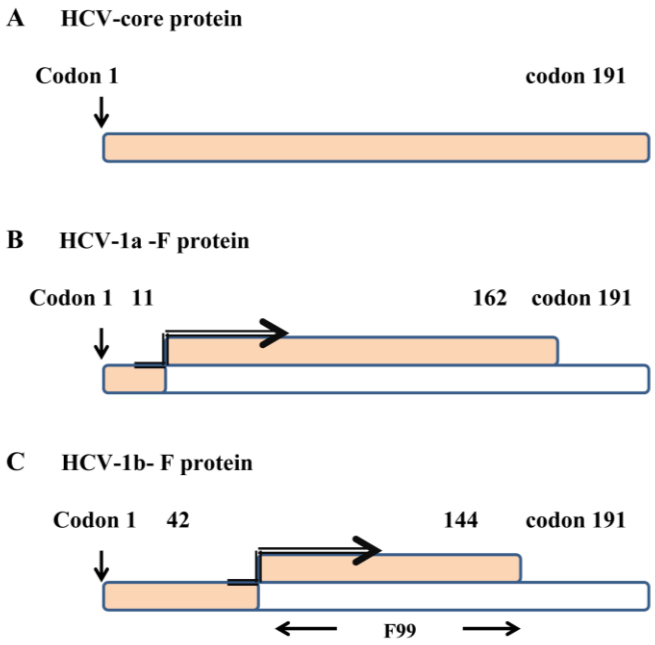

Figure 1-4. F protein encoded from an alternative +1 reading frame overlapping with the core region of HCV genotypes 1a and 1b. The HCV core sequence is illustrated in (A). HCV genotype 1a-derived F protein (B) could be produced as a result of a +1 ribosomal frameshift at or near codon 11, with the protein ending at codon 162. In HCV genotype 1b (C), F protein is located at codon 42. This could be followed by a ribosomal frame shift at the stop codon 144. F99 synthetic peptide (C) is 99 amino acids in length and is located between aa 42 and 141 of the F protein sequence (225).

Figure 1-5

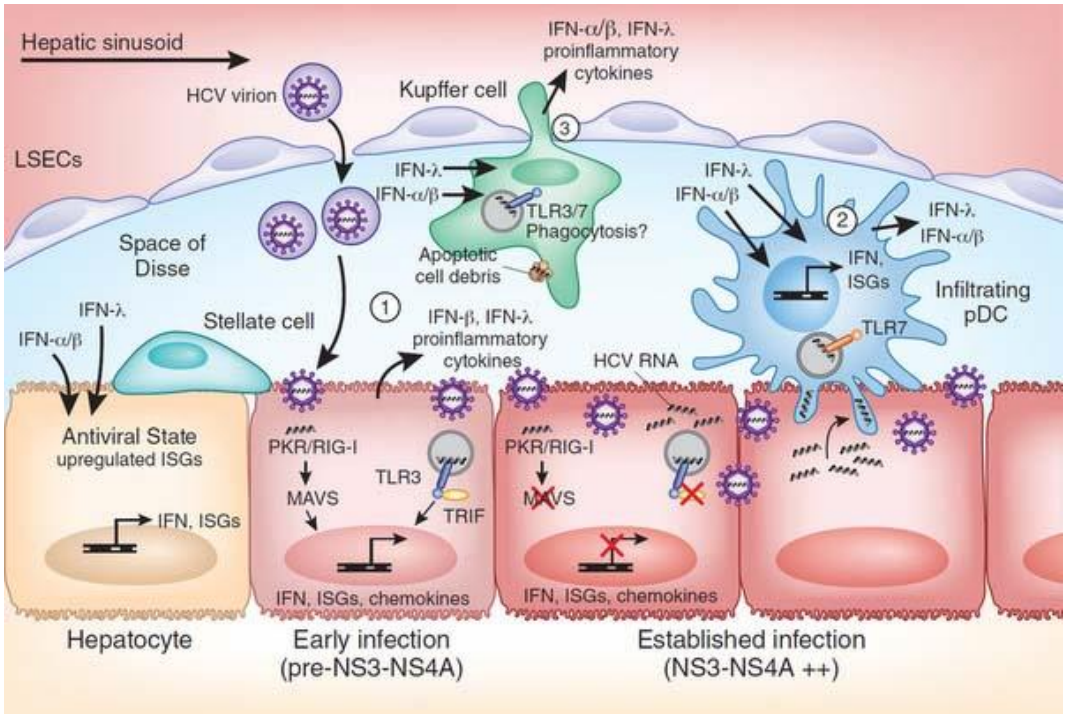

Figure 1-5. IFN induction by HCV in the liver: It shows the different kind of cells that secretes IFN upon stimulation by HCV as well as different mechanism of immune evasion in infected liver (226).

1.16 REFERENCES

1. Tameda, Y. 1996. Hepatitis viruses. *Rinsho byori.* 44: 838–46.

2. Deka, N., M. Sharma, and R. Mukerjee. 1994. Isolation of the novel agent from human stool samples that is associated with sporadic non-A, non-B hepatitis. *J. Virol.* 68: 7810–5.

3. Simons, J., T. Leary, G. Dawson, T. Pilot-Matias, A. Muerhoff, G. Schlauder, S. Desai, and I. Mushahwar. 1995. Isolation of novel virus-like sequences associated with human hepatitis. *Nat.Med.* 1: 564–9.

4. Tabor, E., R. Gerety, J. Drucker, L. Seeff, J. Hoofnagle, D. Jackson, M. April, L. Barker, and G. Pineda-Tamondong. 1978. Transmission of non-A, non-B hepatitis from man to chimpanzee. *Lancet* 1: 463–6

5. Choo, Q., G. Kuo, A. Weiner, L. Overby, D. Bradley, and M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 244: 359–62

6. Kuo, G., Q. Choo, H. Alter, G. Gitnick, A. Redeker, R. Purcell, T. Miyamura, J. Dienstag, M. Alter, and C. Stevens. 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* 244: 362–4.

7. HCV education and support: A Brief History of HCV, 2010

8. Kane, A., J. Lloyd, M. Zaffran, L. Simonsen, and M. Kane. 1998. Transmission of hepatitis B, hepatitis C and human immunodeficiency viruses through unsafe injections in the developing world: model-based regional estimates. *Bulletin of the World Health Organization* 77: 801–7.

9. Tobler, L., and M. Busch. 1997. History of posttransfusion hepatitis. *Clin Chem.* 43: 1487–93.

10. Cook, C., and N Kanaef. 2008. The Global state of Haram of Reduction: Mapping the Response to Drug Related HIV and Hepatisis C Epidemics. London: IHRA.

11. Walsh N. 2009. Scoping Document: A Review of Viral Hepatitis and HIV Co-infection among Injecting Drug Users and Assessment of Priorities for Future Activities. Geneva: WHO

12. Rooney, G., and R. Gilson. 1998. Sexual transmission of hepatitis C virus infection. *Sex Transm Infect*. 74: 399–404

13. Hajarizadeh, B., J. Grebely, and G. Dore. 2013. Epidemiology and natural history of HCV infection. *Nat. Rev. Gastroenterol. Hepatol.* 10: 553-62

14. WHO report 2012

15. Sherman, M., S. Shafran, K. Burak, K. Doucette, W. Wong, N. Girgrah, E. Yoshida, E. Renner, P. Wong, and M. Deschênes. 2007. Management of chronic hepatitis C: consensus guidelines. *Can. J. Gastroenterol.* 21 Suppl C: 25C–34C.

16. Andonov, A., and R. Chaudhary. 1994. Genotyping of Canadian hepatitis C virus isolates by PCR. *J. Clin. Microbiol*. 32: 2031–4.

17. Casey TT, Cousar JB, Collins RD. A simplified plastic embedding and immunohistologic technique for immunophenotypic analysis of human hematopoietic and lymphoid tissues. *Am. J. Pathol*. 1988;131(2):183-9.

18. Moradpour, D., F. Penin, and C. Rice. 2007. Replication of hepatitis C virus. *Nat. Rev. Microbiol.* 5: 453–63.

19. Simmonds, P., J. Bukh, C. Combet, G. Deléage, N. Enomoto, S. Feinstone, P. Halfon, G. Inchauspé, C. Kuiken, G. Maertens, M. Mizokami, D. Murphy, H. Okamoto, J.-M. Pawlotsky, F. Penin, E. Sablon, T. Shin-I, L. Stuyver, H.-J. Thiel, S. Viazov, A. Weiner, and A. Widell. 2005. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 42: 962–73.

20. Kuiken, C., M. Mizokami, G. Deleage, K. Yusim, F. Penin, T. Shin-I, C. Charavay, N. Tao, D. Crisan, D. Grando, A. Dalwani, C. Geourjon, A. Agrawal, and C. Combet. 2006. Hepatitis C databases, principles and utility to researchers. *Hepatology* 43: 1157–65.

21. Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani. 1998. Binding of hepatitis C virus to CD81. *Science* 282: 938–41.

22. Zhang, J., G. Randall, A. Higginbottom, P. Monk, C. Rice, and J. McKeating. 2004. CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. *J. Virol.* 78: 1448–55.

23. Scarselli, E., H. Ansuini, R. Cerino, R. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, and A. Vitelli. 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J.* 21: 5017–25.

24. Evans, M., T. von Hahn, D. Tscherne, A. Syder, M. Panis, B. Wölk, T. Hatziioannou, J. McKeating, P. Bieniasz, and C. Rice. 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* 446: 801–5.

25. Liu, S., W. Yang, L. Shen, J. Turner, C. Coyne, and T. Wang. 2009. Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. *J. Virol.*83: 2011–4.

26. Ploss, A., M. Evans, V. Gaysinskaya, M. Panis, H. You, Y. de Jong, and C. Rice. 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* 457: 882–6.

27. Lozach, P.-Y., H. Lortat-Jacob, A. de Lacroix de Lavalette, I. Staropoli, S. Foung, A. Amara, C. Houles, F. Fieschi, O. Schwartz, J.-L. Virelizier, F. Arenzana-Seisdedos, and R. Altmeyer. 2003. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. *J. Biol. Chem*. 278: 20358–66.

28. Pöhlmann, S., J. Zhang, F. Baribaud, Z. Chen, G. Leslie, G. Lin, A. Granelli-Piperno, R. Doms, C. Rice, and J. McKeating. 2003. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. *J. Viro.* 77: 4070–80.

29. Penin, F., J. Dubuisson, F. Rey, D. Moradpour, and J.-M. Pawlotsky. 2003. Structural biology of hepatitis C virus. *Hepatology* 39: 5–19.

30. Bartenschlager, R., L. Ahlborn-Laake, J. Mous, and H. Jacobsen. 1994. Kinetic and structural analyses of hepatitis C virus polyprotein processing. *J. Virol.* 68: 5045–55.

31. Penin, F., V. Brass, N. Appel, S. Ramboarina, R. Montserret, D. Ficheux, H. Blum, R. Bartenschlager, and D. Moradpour. 2004. Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. *J. Biol. Chem.* 279: 40835–43.

32. Vagu, C., C. Sultana, and S. Ruţă. 2012. Understanding the molecular targets for new therapeutical agents in hepatitis c infection. *Roum. Arch. Microbiol. Immunol.* 72: 5–24.

33. Xu, Z., J. Choi, T. Yen, W. Lu, A. Strohecker, S. Govindarajan, D. Chien, M. Selby, and J. Ou. 2001. Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. *EMBO J.* 20: 3840–8.

34. Walewski, J., T. Keller, D. Stump, and A. Branch. 2001. Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. *RNA* 7: 710–21.

35. Varaklioti, A., N. Vassilaki, U. Georgopoulou, and P. Mavromara. 2002. Alternate translation occurs within the core coding region of the hepatitis C viral genome. *J. Biol. Chem.* 277: 17713–21.

36. Targett-Adams, P., S. Boulant, M. Douglas, and J. McLauchlan. 2010. Lipid metabolism and HCV infection. *Viruses* 2: 1195–217.

37. Tanaka, Y., T. Shimoike, K. Ishii, R. Suzuki, T. Suzuki, H. Ushijima, Y. Matsuura, and T. Miyamura. 2000. Selective binding of hepatitis C virus core protein to synthetic oligonucleotides corresponding to the 5' untranslated region of the viral genome. *Virology* 270: 229–36.

38. Suzuki, T., K. Ishii, H. Aizaki, and T. Wakita. 2007. Hepatitis C viral life cycle. *Adv. Drug Deliv. Rev*. 59: 1200–12

39. Baumert, T., S. Ito, D. Wong, and T. Liang. 1998. Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. *J. Virol.*72: 3827–36.

40. Serafino, A., M. Valli, F. Andreola, A. Crema, G. Ravagnan, L. Bertolini, and G. Carloni. 2003. Suggested role of the Golgi apparatus and endoplasmic reticulum for crucial sites of hepatitis C virus replication in human lymphoblastoid cells infected in vitro. *J. Med. Virol.*70: 31–41.

41. Sato, K., H. Okamoto, S. Aihara, Y. Hoshi, T. Tanaka, and S. Mishiro. 1993. Demonstration of sugar moiety on the surface of hepatitis C virions recovered from the circulation of infected humans. *Virology* 196: 354–7.

42. Santolini, E., G. Migliaccio, and N. La Monica. 1994. Biosynthesis and biochemical properties of the hepatitis C virus core protein. *J. Virol.*68: 3631–41.

43. Suzuki, R., Y. Matsuura, T. Suzuki, A. Ando, J. Chiba, S. Harada, I. Saito, and T. Miyamura. 1994. Nuclear localization of the truncated hepatitis C virus core protein with its hydrophobic C terminus deleted. *J. Gen. Virol*. 76: 53–61.

44. Chang, J., S. Yang, Y. Cho, S. Hwang, Y. Hahn, and Y. Sung. 1998. Hepatitis C virus core from two different genotypes has an oncogenic potential but is not sufficient for transforming primary rat embryo fibroblasts in cooperation with the H-ras oncogene. *J. Virol*.72: 3060–5.

45. Lai, M., and C. Ware. 1999. Hepatitis C virus core protein: possible roles in viral pathogenesis. *Curr. Top. Microbiol. Immunol*. 242: 117–34.

46. Moriya, K., H. Yotsuyanagi, Y. Shintani, H. Fujie, K. Ishibashi, Y. Matsuura, T. Miyamura, and K. Koike. 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J. Gen. Virol*. 78: 1527–31.

47. Moriya, K., H. Fujie, Y. Shintani, H. Yotsuyanagi, T. Tsutsumi, K. Ishibashi, Y. Matsuura, S. Kimura, T. Miyamura, and K. Koike. 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat. Med*. 4: 1065–7.

48. Barba, G., F. Harper, T. Harada, M. Kohara, S. Goulinet, Y. Matsuura, G. Eder, Z. Schaff, M. Chapman, T. Miyamura, and C. Bréchot. 1997. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc. Natl. Acad. Sci*. 94: 1200–5.

49. Krishnadas, D., W. Li, R. Kumar, D. Tyrrell, and B. Agrawal. 2010. HCV-core and NS3 antigens play disparate role in inducing regulatory or effector T cells in vivo: Implications for viral persistence or clearance. *Vaccine* 28: 2104–14.

50. Xu, Z., J. Choi, W. Lu, and J. Ou. 2002. Hepatitis C virus F protein is a short-lived protein associated with the endoplasmic reticulum. *J. Virol.* 77: 1578–83.

51. Dalagiorgou, G., N. Vassilaki, P. Foka, A. Boumlic, A. Kakkanas, E. Kochlios, S. Khalili, E. Aslanoglou, S. Veletza, G. Orfanoudakis, D. Vassilopoulos, S. Hadziyannis, J. Koskinas, and P. Mavromara. 2011. High levels of HCV core+1 antibodies in HCV patients with hepatocellular carcinoma. *J. Gen. Virol.* 92: 1343–51.

52. Bain, C., P. Parroche, J. Lavergne, B. Duverger, C. Vieux, V. Dubois, F. Komurian-Pradel, C. Trépo, L. Gebuhrer, G. Paranhos-Baccala, F. Penin, and G. Inchauspé. 2004. Memory T-cell-mediated immune responses specific to an alternative core protein in hepatitis C virus infection. *J. Virol.* 78: 10460–9.

53. McMullan, L., A. Grakoui, M. Evans, K. Mihalik, M. Puig, A. Branch, S. Feinstone, and C. Rice. 2007. Evidence for a functional RNA element in the hepatitis C virus core gene. *Proc. Natl. Acad. Sci. USA* 104: 2879–84.

54. Deleersnyder, V., A. Pillez, C. Wychowski, K. Blight, J. Xu, Y. Hahn, C. Rice, and J. Dubuisson. 1996. Formation of native hepatitis C virus glycoprotein complexes. *J. Virol.* 71: 697–704.

55. Bartosch, B., J. Dubuisson, and F.-L. Cosset. 2003. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J. Exp. Med*. 197: 633–42.

56. Nielsen, S., M. Bassendine, A. Burt, D. Bevitt, and G. Toms. 2004. Characterization of the genome and structural proteins of hepatitis C virus resolved from infected human liver. *J. Gen. Virol.* 85: 1497–507.

57. Taylor, D. R. 1999. Inhibition of the Interferon- Inducible Protein Kinase PKR by HCV E2 Protein. *Science* 285

58. Carrère-Kremer, S., C. Montpellier-Pala, L. Cocquerel, C. Wychowski, F. Penin, and J. Dubuisson. 2002. Subcellular localization and topology of the p7 polypeptide of hepatitis C virus. *J. Virol.*76: 3720–30.

59. Gonzalez, M., and L. Carrasco. 2003. Viroporins. *FEBS lett*. 552: 28–34.

60. Grakoui, A., D. McCourt, C. Wychowski. S.M. Feinstone and C.M. Rice. 1993. A second hepatitis C virus-encoded proteinase. *Proc Natl. Acad. Sci.* 90:10583-7.

61. Hijikata, M., H. Mizushima, T. Akagi, S. Mori, N. Kakiuchi, N. Kato, T. Tanaka , K. Kimura, and K. Shimotohno. 1993. Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. *J Virol.* 67:4665-75.

62. Tai, C., W. Chi, D. Chen, and L. Hwang. 1996. The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). *J. Virol.* 70:8477-84.

63. Failla, C., L. Tomei, and R. De. 1994. Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. *J. Virol.* 68(6): 3753-60.

64. Kim, J., K. Morgenstern, C. Lin, T. Fox, M. Dwyer, J. Landro, S. Chambers, W. Markland, C. Lepre, E. O'Malley, S. Harbeson, C. Rice, M. Murcko, P. Caron, and J. Thomson. 1996. Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* 87: 343–55.

65. Pawlotsky, J.-M., and R. Gish. 2005. Future therapies for hepatitis C. *Antivir. Ther.* 11: 397–408.

66. Pawlotsky JM, McHutchison JG. Hepatitis C. Development of new drugs and clinical trials: promises and pitfalls. Summary of an AASLD hepatitis single topic conference, Chicago, IL, February 27-March 1, 2003. *Hepatology.* 2004;39(2):554-67.

67. Egger, D., B. Wölk, R. Gosert, L. Bianchi, H. Blum, D. Moradpour, and K. Bienz. 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.*76: 5974–84.

68. Elazar, M., P. Liu, C. Rice, and J. Glenn. 2004. An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. *J. Virol.*78: 11393–400.

69. Gretton, S., A. Taylor, and J. McLauchlan. 2005. Mobility of the hepatitis C virus NS4B protein on the endoplasmic reticulum membrane and membrane-associated foci. *J. Gen. Virol.* 86: 1415–21.

70. Florese, R., M. Nagano-Fujii, Y. Iwanaga, and R. Hidajat…. 2002. Inhibition of protein synthesis by the nonstructural proteins NS4A and NS4B of hepatitis C virus. *Virus Res.* 90(1-2):119-31.

71. Kato, J., N. Kato, H. Yoshida, S. Ono-Nita, Y. Shiratori, and M. Omata. 2002. Hepatitis C virus NS4A and NS4B proteins suppress translation *in vivo*. *J. Med. Virol.* 66: 187–99.

72. Piccininni, S., A. Varaklioti, M. Nardelli, B. Dave, K. Raney, and J. McCarthy. 2002. Modulation of the hepatitis C virus RNA-dependent RNA polymerase activity by the non-structural (NS) 3 helicase and the NS4B membrane protein. *J. Biol. Chem.* 277: 45670–9.

73. Park, J., J. Yang, and M. Min. 2000. Hepatitis C virus nonstructural protein NS4B transforms NIH3T3 cells in cooperation with the Ha-ras oncogene. *Biochem. Biophys. Res. Commun*. 267: 581–7.

74. Kadoya, H., M. Nagano-Fujii, L. Deng, N. Nakazono, and H. Hotta. 2004. Nonstructural proteins 4A and 4B of hepatitis C virus transactivate the interleukin 8 promoter. *Microbiol. Immunol.* 49: 265–73.

75. Koch, J., and R. Bartenschlager. 1999. Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3, NS4A, and NS4B. *J. Virol.* 73: 7138–46.

76. Gale, M., M. Korth, N. Tang, S. Tan, D. Hopkins, T. Dever, S. Polyak, D. Gretch, and M. Katze. 1997. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* 230: 217–27.

77. Blight, K. J. 2000. Efficient Initiation of HCV RNA Replication in Cell Culture. *Science* 290.

78. Lohmann, V., F. Körner, A. Dobierzewska, and R. Bartenschlager. 2001. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J. Virol.*75: 1437–49.

79. Elazar, M., K. Cheong, P. Liu, H. Greenberg, C. Rice, and J. Glenn. 2003. Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication. *J. Virol.* 77: 6055–61.

80. Tellinghuisen, T., J. Marcotrigiano, A. Gorbalenya, and C. Rice. 2004. The NS5A protein of hepatitis C virus is a zinc metalloprotein. *J. Biol. Chem.* 279: 48576–87.

81. Pellerin, M., Y. Lopez-Aguirre, and F. Penin, D. Dhumeaux, and J. Pawlotsk. 2004. Hepatitis C virus quasispecies variability modulates nonstructural protein 5A transcriptional activation, pointing to cellular compartmentalization of virus-host. *J. Virol.* 78:4617-27.

82. Polyak, S., K. Khabar, D. Paschal, H. Ezelle, G. Duverlie, G. Barber, D. Levy, N. Mukaida, and D. Gretch. 2001. Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. *J. Virol.* 75: 6095–106.

83. Tan, S., and M. Katze. 2001. How hepatitis C virus counteracts the interferon response: the jury is still out on NS5A. *Virology* 284: 1–12.

84. Tellinghuisen, T., and C. Rice. 2002. Interaction between hepatitis C virus proteins and host cell factors. *Curr. Opin. Microbiol.* 5: 419–27.

85. Polyak, S. 2003. Hepatitis C virus--cell interactions and their role in pathogenesis. *Clin. Liver Dis.* 7: 67–88.

86. Oh, J., T. Ito, and M. Lai. 1999. A recombinant hepatitis C virus RNA-dependent RNA polymerase capable of copying the full-length viral RNA. *J. Virol.*73: 7694–702.

87. Yamashita, T. 1998. RNA-dependent RNA Polymerase Activity of the Soluble Recombinant Hepatitis C Virus NS5B Protein Truncated at the C-terminal Region. *J. Biol. Chem. 273:15479-86.*

88. Gao, L., H. Aizaki, J.-W. He, and M. Lai. 2004. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. *J. Virol.*78: 3480–8.

89. Schmidt-Mende, J., E. Bieck, T. Hugle, F. Penin, C. Rice, H. Blum, and D. Moradpour. 2001. Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. *J. Biol. Chem.* 276: 44052–63.

90. Watashi, K., N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, and K. Shimotohno. 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol. Cell* 19: 111–22.

91. McCaughan, G., P. McGuinness, G. Bishop, D. Painter, A. Lien, R. Tulloch, B. Wylie, and G. Archer. 1992. Clinical assessment and incidence of hepatitis C RNA in 50 consecutive RIBA-positive volunteer blood donors. *Med. J. Aust.* 157: 231–3.

92. Alter, H., and L. Seeff. 1999. Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. *Semin. Liver Dis.* 20: 17–35.

93. Thimme, R., D. Oldach, K. Chang, C. Steiger, S. Ray, and F. Chisari. 2001. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J. Exp. Med.* 194: 1395–406.

94. Farci, P., H. Alter, D. Wong, R. Miller, J. Shih, B. Jett, and R. Purcell. 1991. A long-term study of hepatitis C virus replication in non-A, non-B hepatitis. *N. Engl. J. Med.* 325: 98–104.

95. Strader, D., T. Wright, D. Thomas, and L. Seeff. 2004. Diagnosis, management, and treatment of hepatitis C.*Hepatology* 39: 1147–71

96. Hoofnagle, J. 2002. Course and outcome of hepatitis C. *Hepatology* 36: S21–9.

97. Farci, P., H. Alter, A. Shimoda, S. Govindarajan, L. Cheung, J. Melpolder, R. Sacher, J. Shih, and R. Purcell. 1996. Hepatitis C virus-associated fulminant hepatic failure. *N. Engl. J. Med.* 335: 631–4.

98. Ge, D., J. Fellay, A. Thompson, J. Simon, K. Shianna, T. Urban, E. Heinzen, P. Qiu, A. Bertelsen, A. Muir, M. Sulkowski, J. McHutchison, and D. Goldstein. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 461: 399–401.

99. Balagopal, A., D. Thomas, and C. Thio. 2010. IL28B and the Control of Hepatitis C Virus Infection. *Gastroenterology.* 139:1865-76

100. Rao, H.-Y., D.-G. Sun, D. Jiang, R.-F. Yang, F. Guo, J.-H. Wang, F. Liu, H.-Y. Zhang, H.-H. Zhang, S.-C. Du, Q. Jin, H. Qin, A.-S.-F. Lok, and L. Wei. 2012. IL28B genetic variants and gender are associated with spontaneous clearance of hepatitis C virus infection. *J. Viral Hepat.* 19: 173–81.

101. Deuffic-Burban, S., H. Castel, J. Wiegand, M. Manns, H. Wedemeyer, P. Mathurin, and Y. Yazdanpanah. 2012. Immediate vs. delayed treatment in patients with acute hepatitis C based on IL28B polymorphism: a model-based analysis. *J. Hepatol.* 57: 260–6.

102. Chen, S., and T. Morgan. 2005. The natural history of hepatitis C virus (HCV) infection. *Int. J. Med. Sci.* 3: 47–52.

103. Sène, D., N. Limal, and P. Cacoub. 2004. Hepatitis C virus-associated extrahepatic manifestations: a review. *Metab. Brain Dis.* 19: 357–81.

104. Pischke, S., M. Cornberg, and M. Manns. 2008. Hepatitis associated cryoglobulinemia. *Der. Internist.* 49: 297–304.

105. White, D., V. Ratziu, and H. El-Serag. 2008. Hepatitis C infection and risk of diabetes: a systematic review and meta-analysis. *J. Hepatol.* 49: 831–44.

106. Veldt, B., W. Chen, E. Heathcote, H. Wedemeyer, J. Reichen, W. Hofmann, R. de Knegt, S. Zeuzem, M. Manns, B. Hansen, S. Schalm, and H. Janssen. 2008. Increased risk of hepatocellular carcinoma among patients with hepatitis C cirrhosis and diabetes mellitus. *Hepatology* 47: 1856–62.

107. Pattullo, V., and J. Heathcote. 2010. Hepatitis C and diabetes: one treatment for two diseases? *Liver Int.* 30: 356–64.

108. Li, K., and S. Lemon. 2013. Innate immune responses in hepatitis C virus infection. *Semin. Immunopathol.* 35: 53–72.

109. Dixit, E., S. Boulant, Y. Zhang, A. Lee, C. Odendall, B. Shum, N. Hacohen, Z. Chen, S. Whelan, M. Fransen, M. Nibert, G. Superti-Furga, and J. Kagan. 2010. Peroxisomes are signaling platforms for antiviral innate immunity. *Cell* 141: 668–81.

110. Seth, R., L. Sun, C.-K. Ea, and Z. Chen. 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 122: 669–82.

111. Kawai, T., and S. Akira. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34: 637–50.

112. Takahashi, K., S. Asabe, S. Wieland, U. Garaigorta, P. Gastaminza, M. Isogawa, and F. Chisari. 2010. Plasmacytoid dendritic cells sense hepatitis C virus-infected cells, produce interferon, and inhibit infection. *Proc. Natl. Acad. Sci.* USA. 107: 7431–6.

113. Zhang, Y.-L., Y.-J. Guo, Bin Li, and S.-H. Sun. 2009. Hepatitis C virus single-stranded RNA induces innate immunity via Toll-like receptor 7. *J. hepatol.* 51: 29–38.

114. Liu, Y.-J. 2004. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. Liu, Y.-J. 2004. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu. Rev. Immunol.* 23: 275–306. 23: 275–306.

115. Dustin, L., and C. Rice. 2006. Flying under the radar: the immunobiology of hepatitis C. *Annu. Rev. Immunol.* 25: 71–99.

116. Rehermann, B. 2009. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. *J. Clin. Invest.* 119: 1745–54.

117. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. Liu, B. Pulendran, and K. Palucka. 1999. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18: 767–811.

118. Banchereau, J., and R. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245–52.

119. Shortman, K., and Y.-J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol.* 2: 151–61.

120. Kapsenberg, M. 2003. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat. Rev. Immunol.* 3: 984–93.

121. Cunningham, A. J., and K. J. Lafferty. 1977. A Simple, Conservative Explanation of the H-2 Restriction of Interactions between Lymphocytes. *Scand. J. Immunol.* 6:1-6.

122. Bromley, S., W. Burack, K. Johnson, K. Somersalo, T. Sims, C. Sumen, M. Davis, A. Shaw, P. Allen, and M. Dustin. 2000. The immunological synapse. *Annu. Rev. Immunol.* 19: 375–96.

123. Dustin, M., and T. Springer. 1989. T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* 341: 619–24.

124. Kaliński, P., C. Hilkens, E. Wierenga, and M. Kapsenberg. 1999. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol. Today* 20: 561–7.

125. Vieira, P., E. de Jong, E. Wierenga, M. Kapsenberg, and P. Kaliński. 2000. Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. *J. Immunol.* 164: 4507–12.

126. Janeway, C. 1988. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor symposia on quantitative biology* 54 Pt 1: 1–13.

127. Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* 2: 675–80.

128. Figdor, C., Y. van Kooyk, and G. Adema. 2002. C-type lectin receptors on dendritic cells and Langerhans cells. *Nat. Rev. Immunol.* 2: 77–84.

129. Boehme, K., and T. Compton. 2004. Innate sensing of viruses by toll-like receptors. *J. Virol.* 78: 7867–73.

130. Engering, A., T. Geijtenbeek, and Y. van Kooyk. 2002. Immune escape through C-type lectins on dendritic cells. *Trends Immunol.* 23: 480–5.

131. Engering, A., M. Cella, D. Fluitsma, E. Hoefsmit, A. Lanzavecchia, and J. Pieters. 1996. Mannose receptor mediated antigen uptake and presentation in human dendritic cells. *Adv. Exp. Med. Biol.* 417: 183–7.

132. Geijtenbeek, T., S. Van Vliet, E. Koppel, M. Sanchez-Hernandez, C. Vandenbroucke-Grauls, B. Appelmelk, and Y. Van Kooyk. 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med*. 197: 7–17.

133. Engering, A., T. Geijtenbeek, S. van Vliet, M. Wijers, E. van Liempt, N. Demaurex, A. Lanzavecchia, J. Fransen, C. Figdor, V. Piguet, and Y. van Kooyk. 2002. The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J. Immunol.* 168: 2118–26.

134. Cambi, A., and C. Figdor. 2003. Dual function of C-type lectin-like receptors in the immune system. *Curr. Opin. Cell Biol*. 15: 539–46.

135. Grabbe, S., E. Kämpgen, and G. Schuler. 2000. Dendritic cells: multi-lineal and multi-functional. *Immuno. Today* 21: 431–3.

136. MacDonald, K., D. Munster, G. Clark, A. Dzionek, J. Schmitz, and D. Hart. 2002. Characterization of human blood dendritic cell subsets. *Blood* 100: 4512–20.

137. Robinson, S., S. Patterson, N. English, D. Davies, S. Knight, and C. Reid. 1999. Human peripheral blood contains two distinct lineages of dendritic cells. *Eur. J. Immunol.* 29: 2769–78.

138. Liu, Y. 2001. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* 106: 259–62.

139. Colonna, M., G. Trinchieri, and Y.-J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat. Immunol.* 5: 1219–26.

140. Liu, B., A. Woltman, H. Janssen, and A. Boonstra. 2009. Modulation of dendritic cell function by persistent viruses. *J. Leukoc. Biol.* 85: 205–14.

141. Bain, C., A. Fatmi, F. Zoulim, J. Zarski, C. Trépo, and G. Inchauspé. 2001. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* 120: 512–24.

142. Pachiadakis, I., G. Pollara, B. Chain, and N. Naoumov. 2005. Is hepatitis C virus infection of dendritic cells a mechanism facilitating viral persistence? *Lancet Infect. Dis.* 5: 296–304.

143. Krishnadas, D., J. Ahn, J. Han, R. Kumar, and B. Agrawal. 2010. Immunomodulation by hepatitis C virus-derived proteins: targeting human dendritic cells by multiple mechanisms. *Int. Immunol.* 22: 491–502.

144. Sullivan, J., K. Byron, F. Brewster, and D. Purtilo. 1980. Deficient natural killer cell activity in x-linked lymphoproliferative syndrome. *Science* 210: 543–5.

145. Harada, S., T. Bechtold, J. Seeley, and D. Purtilo. 1982. Cell-mediated immunity to Epstein-Barr virus (EBV) and natural killer (NK)-cell activity in the X-linked lymphoproliferative syndrome. *Int. J. Cancer* 30: 739–44.

146. Nellore, A., and J. Fishman. 2011. NK cells, innate immunity and hepatitis C infection after liver transplantation. *Clin. Infect. Dis.* 52: 369–77.

147. Corado, J., F. Toro, H. Rivera, N. Bianco, L. Deibis, and J. De Sanctis. 1997. Impairment of natural killer (NK) cytotoxic activity in hepatitis C virus (HCV) infection. *Clin. Exp. Immunol.* 109: 451–7.

148. Cooper, M., T. Fehniger, and M. Caligiuri. 2001. The biology of human natural killer-cell subsets. *Trends Immunol.* 22: 633–40.

149. Lassen, M., J. Lukens, J. Dolina, M. Brown, and Y. Hahn. 2010. Intrahepatic IL-10 maintains NKG2A+Ly49- liver NK cells in a functionally hyporesponsive state. *J. Immunol.*184: 2693–701.

150. Li, Y., T. Zhang, C. Ho, J. Orange, S. Douglas, and W.-Z. Ho. 2004. Natural killer cells inhibit hepatitis C virus expression. *J. Leukoc. Biol.* 76: 1171–9.

151. Ishiyama, K., H. Ohdan, M. Ohira, H. Mitsuta, K. Arihiro, and T. Asahara. 2006. Difference in cytotoxicity against hepatocellular carcinoma between liver and periphery natural killer cells in humans. *Hepatology* 43: 362–72.

152. Ohira, M., H. Ohdan, H. Mitsuta, K. Ishiyama, Y. Tanaka, Y. Igarashi, and T. Asahara. 2006. Adoptive transfer of TRAIL-expressing natural killer cells prevents recurrence of hepatocellular carcinoma after partial hepatectomy. *Transplantation* 82: 1712–9.

153. Ye, L., X. Wang, S. Wang, Y. Wang, L. Song, W. Hou, L. Zhou, H. Li, and W. Ho. 2009. CD56+ T cells inhibit hepatitis C virus replication in human hepatocytes. *Hepatology* 49: 753–62.

154. Golden-Mason, L., N. Castelblanco, C. O'Farrelly, and H. Rosen. 2007. Phenotypic and functional changes of cytotoxic CD56pos natural T cells determine outcome of acute hepatitis C virus infection. *J. Virol.*81: 9292–8.

155. Yamagiwa, S., Y. Matsuda, T. Ichida, Y. Honda, M. Takamura, S. Sugahara, T. Ishikawa, S. Ohkoshi, Y. Sato, and Y. Aoyagi. 2008. Sustained response to interferon-alpha plus ribavirin therapy for chronic hepatitis C is closely associated with increased dynamism of intrahepatic natural killer and natural killer T cells. *Hepatol. Res.* 38: 664–72.

156. Kalams, S., and B. Walker. 1998. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *J. Exp. Med.* 188: 2199–204.

157. Ciurea, A., L. Hunziker, P. Klenerman, H. Hengartner, and R. Zinkernagel. 2001. Impairment of CD4 (+) T cell responses during chronic virus infection prevents neutralizing antibody responses against virus escape mutants. *J. Exp. Med* 193: 297–305.

158. Zibert, A., H. Meisel, W. Kraas, A. Schulz, G. Jung, and M. Roggendorf. 1997. Early antibody response against hypervariable region 1 is associated with acute self-limiting infections of hepatitis C virus. *Hepatology* 25: 1245–9.

159. Farci, P., R. Strazzera, H. Alter, S. Farci, D. Degioannis, A. Coiana, G. Peddis, F. Usai, G. Serra, L. Chessa, G. Diaz, A. Balestrieri, and R. Purcell. 2002. Early changes in hepatitis C viral quasispecies during interferon therapy predict the therapeutic outcome. *Proc. Natl. Acad. Sci.* 99: 3081–6.

160. Bassett, S., D. Thomas, K. Brasky, and R. Lanford. 1999. Viral persistence, antibody to E1 and E2, and hypervariable region 1 sequence stability in hepatitis C virus-inoculated chimpanzees. *J. Virol.* 73: 1118–26.

161. Morin, T., T. Broering, B. Leav, B. Blair, K. Rowley, E. Boucher, Y. Wang, P. Cheslock, M. Knauber, D. Olsen, S. Ludmerer, G. Szabo, R. Finberg, R. Purcell, R. Lanford, D. Ambrosino, D. Molrine, and G. Babcock. 2011. Human monoclonal antibody HCV1 effectively prevents and treats HCV infection in chimpanzees. *PLoS pathogens* 8: e1002895

162. Koziel, M. 2005. Cellular immune responses against hepatitis C virus. *Clin. Infect. Dis.* 41 Suppl 1: S25–31.

163. Shedlock, D., and H. Shen. 2003. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* 300: 337–9.

164. Grakoui, A., N. Shoukry, D. Woollard, J.-H. Han, H. Hanson, J. Ghrayeb, K. Murthy, C. Rice, and C. Walker. 2003. HCV persistence and immune evasion in the absence of memory T cell help. *Science* 302: 659–62.

165. Moser, M., and K. Murphy. 2000. Dendritic cell regulation of TH1-TH2 development. *Nat. Immunol.* 1: 199–205.

166. Schmidt, J., R. Thimme, and C. Neumann-Haefelin. 2011. Host genetics in immune-mediated hepatitis C virus clearance. *Biomark. Med.* 5: 155–69.

167. Missale, G., R. Bertoni, V. Lamonaca, A. Valli, M. Massari, C. Mori, M. Rumi, M. Houghton, F. Fiaccadori, and C. Ferrari. 1996. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. *J. Clin. Invest.* 98: 706–14.

168. Diepolder, H., M. Jung, E. Keller, W. Schraut, J. Gerlach, N. Grüner, R. Zachoval, R. Hoffmann, C. Schirren, S. Scholz, and G. Pape. 1998. A vigorous virus-specific CD4+ T cell response may contribute to the association of HLA-DR13 with viral clearance in hepatitis B. *Clin. Exp. Immunol.* 113: 244–51.

169. Takaki, A., M. Wiese, G. Maertens, E. Depla, U. Seifert, A. Liebetrau, J. Miller, M. Manns, and B. Rehermann. 2000. Cellular immune

responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat. Med.* 6: 578–82.

170. Thimme, R., J. Bukh, H. Spangenberg, S. Wieland, J. Pemberton, C. Steiger, S. Govindarajan, R. Purcell, and F. Chisari. 2002. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc. Natl. Acad. Sci.* 99: 15661–8.

171. Day, C., G. Lauer, G. Robbins, B. McGovern, A. Wurcel, R. Gandhi, R. Chung, and B. Walker. 2002. Broad specificity of virus-specific CD4+ T-helper-cell responses in resolved hepatitis C virus infection. *J. Virol.*76: 12584–95.

172. Shata, M., D. Anthony, N. Carlson, L. Andrus, B. Brotman, N. Tricoche, P. McCormack, and A. Prince. 2002. Characterization of the immune response against hepatitis C infection in recovered, and chronically infected chimpanzees. *J. Viral Hepat.* 9: 400–10.

173. Woollard, D., A. Grakoui, N. Shoukry, K. Murthy, K. Campbell, and C. Walker. 2003. Characterization of HCV-specific Patr class II restricted CD4+ T cell responses in an acutely infected chimpanzee. *Hepatology* 38: 1297–306.

174. Tsai, S., Y. Liaw, M. Chen, C. Huang, and G. Kuo. 1997. Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. *Hepatology* 25: 449–58.

175. Schulze Zur Wiesch, J., D. Ciuffreda, L. Lewis-Ximenez, V. Kasprowicz, B. Nolan, H. Streeck, J. Aneja, L. Reyor, T. Allen, A. Lohse, B. McGovern, R. Chung, W. Kwok, A. Kim, and G. Lauer. 2012. Broadly directed virus-specific CD4+ T cell responses are primed during acute hepatitis C infection, but rapidly disappear from human blood with viral persistence. *J. Exp. Med.* 209: 61–75.

176. Jo, J., U. Aichele, N. Kersting, R. Klein, P. Aichele, E. Bisse, A. Sewell, H. Blum, R. Bartenschlager, V. Lohmann, and R. Thimme. 2009. Analysis of CD8+ T-cell-mediated inhibition of hepatitis C virus replication using a novel immunological model. *Gastroenterology* 136: 1391–401.

177. Jo, J., B. Bengsch, B. Seigel, S. Rau, J. Schmidt, E. Bisse, P. Aichele, U. Aichele, L. Joeckel, C. Royer, K. Sá Ferreira, C. Borner, T. Baumert, H. Blum, V. Lohmann, R. Fischer, and R. Thimme. 2012.

Low perforin expression of early differentiated HCV-specific CD8+ T cells limits their hepatotoxic potential. *J. Hepatol.* 57: 9–16.

178. Cooper, S., A. Erickson, E. Adams, J. Kansopon, A. Weiner, D. Chien, M. Houghton, P. Parham, and C. Walker. 1999. Analysis of a successful immune response against hepatitis C virus. *Immunity* 10: 439–49.

179. Shoukry, N., A. Grakoui, M. Houghton, D. Chien, J. Ghrayeb, K. Reimann, and C. Walker. 2003. Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. *J. Exp. Med.* 197: 1645–55.

180. Northfield, J., V. Kasprowicz, M. Lucas, N. Kersting, B. Bengsch, B. Bengsh, A. Kim, R. Phillips, B. Walker, R. Thimme, G. Lauer, and P. Klenerman. 2008. CD161 expression on hepatitis C virus-specific CD8+ T cells suggests a distinct pathway of T cell differentiation. *Hepatology* 47: 396–406.

181. Billerbeck, E., Y.-H. Kang, L. Walker, H. Lockstone, S. Grafmueller, V. Fleming, J. Flint, C. Willberg, B. Bengsch, B. Seigel, N. Ramamurthy, N. Zitzmann, E. Barnes, J. Thevanayagam, A. Bhagwanani, A. Leslie, Y. Oo, S. Kollnberger, P. Bowness, O. Drognitz, D. Adams, H. Blum, R. Thimme, and P. Klenerman. 2010. Analysis of CD161 expression on human CD8+ T cells defines a distinct functional subset with tissue-homing properties. *Proc. Natl. Acad. Sci.* 107: 3006–11.

182. Grafmueller, S., E. Billerbeck, H. Blum, C. Neumann-Haefelin, and R. Thimme. 2012. Differential antigen specificity of hepatitis C virus-specific interleukin 17- and interferon γ-producing CD8 (+) T cells during chronic infection. *J. Infect. Dis.* 205: 1142–6.

183. Sakaguchi, S., M. Miyara, C. Costantino, and D. Hafler. 2010. FOXP3+ regulatory T cells in the human immune system. *Nat. Rev. Immunol.* 10: 490–500.

184. Cabrera, R., Z. Tu, Y. Xu, R. Firpi, H. Rosen, C. Liu, and D. Nelson. 2004. An immunomodulatory role for CD4 (+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 40: 1062–71.

185. Manigold, T., and V. Racanelli. 2007. T-cell regulation by CD4 regulatory T cells during hepatitis B and C virus infections: facts and controversies. *Lancet Infect. Dis.* 7: 804–13.

186. Ward, S., B. Fox, P. Brown, J. Worthington, S. Fox, R. Chapman, K. Fleming, A. Banham, and P. Klenerman. 2007. Quantification and localisation of FOXP3+ T lymphocytes and relation to hepatic inflammation during chronic HCV infection. *J. Hepatol.* 47: 316–24.

187. Claassen, M. A., R. de Knegt, H. Tilanus, H. Janssen, and A. Boonstra. 2010. Abundant numbers of regulatory T cells localize to the liver of chronic hepatitis C infected patients and limit the extent of fibrosis. *J. Hepatol.* 52: 315–21.

188. Sturm, N., M.-A. Thélu, X. Camous, G. Dimitrov, M. Ramzan, T. Dufeu-Duchesne, P. Bonorino, C. Guillermet, E. Brambilla, P. Arvers, M. Pernollet, V. Leroy, J.-P. Zarski, P. Marche, and E. Jouvin-Marche. 2010. Characterization and role of intra-hepatic regulatory T cells in chronic hepatitis C pathogenesis. *J. Hepatol.* 53: 25–35.

189. Bolacchi, F., A. Sinistro, C. Ciaprini, F. Demin, M. Capozzi, F. Carducci, C. Drapeau, G. Rocchi, and A. Bergamini. 2006. Increased hepatitis C virus (HCV)-specific CD4+CD25+ regulatory T lymphocytes and reduced HCV-specific CD4+ T cell response in HCV-infected patients with normal versus abnormal alanine aminotransferase levels. *Clin. Exp. Immunol.* 144: 188–96.

190. MacDonald, A., M. Duffy, M. Brady, S. McKiernan, W. Hall, J. Hegarty, M. Curry, and K. Mills. 2002. CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons. *J. Infect. Dis.* 185: 720–7.

191. Cusick, M., J. Schiller, J. Gill, and D. Eckels. 2010. Hepatitis C virus induces regulatory T cells by naturally occurring viral variants to suppress T cell responses. *Clin. Dev. Immunol.* 2011: 806061.

192. He, X., B. Rehermann, J. Boisvert, J. Mumm, H. Maecker, M. Roederer, T. Wright, V. Maino, M. Davis, and H. Greenberg. 2000. Direct functional analysis of epitope-specific CD8+ T cells in peripheral blood. *Viral Immunol.* 14: 59–69.

193. Quiroga, J., S. Llorente, I. Castillo, E. Rodríguez-Iñigo, J. López-Alcorocho, M. Pardo, and V. Carreño. 2006. Virus-specific T-cell responses associated with hepatitis C virus (HCV) persistence in the liver after apparent recovery from HCV infection. *J. Med vvirol.* 78: 1190–7.

194. Abel, M., D. Sène, S. Pol, M. Bourlière, T. Poynard, F. Charlotte, P. Cacoub, and S. Caillat-Zucman. 2006. Intrahepatic virus-specific IL-

10-producing CD8 T cells prevent liver damage during chronic hepatitis C virus infection. *Hepatology* 44: 1607–16.

195. Graham, C., M. Curry, Q. He, N. Afdhal, D. Nunes, C. Fleming, R. Horsburgh, D. Craven, K. Sherman, and M. Koziel. 2004. Comparison of HCV-specific intrahepatic CD4+ T cells in HIV/HCV versus HCV. *Hepatology* 40: 125–32.

196. Horner, S., and M. Gale. 2013. Regulation of hepatic innate immunity by hepatitis C virus. *Nat. Med.* 19: 879–88.

197. Xu, X., G. Screaton, and A. McMichael. 2001. Virus infections: escape, resistance, and counterattack. *Immunity* 15: 867–70.

198. Bode, J., S. Ludwig, C. Ehrhardt, U. Albrecht, A. Erhardt, F. Schaper, P. Heinrich, and D. Häussinger. 2003. IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. *FASEB j.* 17: 488–90.

199. Lin, W., S. Kim, E. Yeung, Y. Kamegaya, J. Blackard, K. Kim, M. Holtzman, and R. Chung. 2006. Hepatitis C virus core protein blocks interferon signaling by interaction with the STAT1 SH2 domain. *J. Virol.* 80: 9226–35.

200. Heim, M., D. Moradpour, and H. Blum. 1999. Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. *J. Virol*. 73: 8469-8475.

201. Taylor, D., S. Shi, P. Romano, G. Barber, and M. Lai. 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science* 285: 107–10.

202. Golden-Mason, L., and H. Rosen. 2006. Natural killer cells: primary target for hepatitis C virus immune evasion strategies? *Live Transpl.* 12: 363–72.

203. Neumann-Haefelin, C., and R. Thimme. 2012. Adaptive immune responses in hepatitis C virus infection. *Curr. Top. Microbiol. Immunol.* 369: 243–62.

204. Bowen, D., and C. Walker. 2005. Mutational escape from CD8+ T cell immunity: HCV evolution, from chimpanzees to man. *J. Exp. Med.* 201: 1709–14.

205. Seifert, U., H. Liermann, V. Racanelli, A. Halenius, M. Wiese, H. Wedemeyer, T. Ruppert, K. Rispeter, P. Henklein, A. Sijts, H. Hengel, P.-M. Kloetzel, and B. Rehermann. 2004. Hepatitis C virus mutation affects proteasomal epitope processing. *J. Clin. Invest.* 114: 250–9.

206. Timm, J., G. Lauer, D. Kavanagh, I. Sheridan, A. Kim, M. Lucas, T. Pillay, K. Ouchi, L. Reyor, J. Schulze zur Wiesch, R. Gandhi, R. Chung, N. Bhardwaj, P. Klenerman, B. Walker, and T. Allen. 2004. CD8 epitope escape and reversion in acute HCV infection. *J. Exp. Med.* 200: 1593–604.

207. Kimura, Y., T. Gushima, S. Rawale, P. Kaumaya, and C. Walker. 2005. Escape mutations alter proteasome processing of major histocompatibility complex class I-restricted epitopes in persistent hepatitis C virus infection. *J. Virol.* 79: 4870–6.

208. Söderholm, J., G. Ahlén, A. Kaul, L. Frelin, M. Alheim, C. Barnfield, P. Liljeström, O. Weiland, D. Milich, R. Bartenschlager, and M. Sällberg. 2006. Relation between viral fitness and immune escape within the hepatitis C virus protease. *Gut* 55: 266–74.

209. Wedemeyer, H., X.-S. He, M. Nascimbeni, A. Davis, H. Greenberg, J. Hoofnagle, T. Liang, H. Alter, and B. Rehermann. 2002. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J. Immunol.* 169: 3447–58.

210. Spangenberg, H., S. Viazov, N. Kersting, C. Neumann-Haefelin, D. McKinney, M. Roggendorf, F. von Weizsäcker, H. Blum, and R. Thimme. 2005. Intrahepatic CD8+ T-cell failure during chronic hepatitis C virus infection. *Hepatology* 42: 828–37.

211. Penna, A., M. Pilli, A. Zerbini, A. Orlandini, S. Mezzadri, L. Sacchelli, G. Missale, and C. Ferrari. 2007. Dysfunction and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. *Hepatology* 45: 588–601.

212. Radziewicz, H., C. Ibegbu, M. Fernandez, K. Workowski, K. Obideen, M. Wehbi, H. Hanson, J. Steinberg, D. Masopust, E. Wherry, J. Altman, B. Rouse, G. Freeman, R. Ahmed, and A. Grakoui. 2007. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J. Virol.* 81: 2545–53.

213. Radziewicz, H., C. Ibegbu, H. Hon, M. Osborn, K. Obideen, M. Wehbi, G. Freeman, J. Lennox, K. Workowski, H. Hanson, and A. Grakoui. 2008. Impaired hepatitis C virus (HCV)-specific effector CD8+ T cells undergo massive apoptosis in the peripheral blood during acute HCV infection and in the liver during the chronic phase of infection. 82: 9808–22.

214. Jo, J., V. Lohmann, R. Bartenschlager, and R. Thimme. 2011. Experimental models to study the immunobiology of hepatitis C virus. *J. Gen. Virol.* 92: 477–93.

215. Houghton, Michael. 2007. Prospects for prophylactic and therapeutic vaccines against the hepatitis C viruses. *Immunol. Rev.* 239: 99–108

216. Forns, X., P. Payette, X. Ma, W. Satterfield, G. Eder, I. Mushahwar, S. Govindarajan, H. Davis, S. Emerson, R. Purcell, and J. Bukh. 2000. Vaccination of chimpanzees with plasmid DNA encoding the hepatitis C virus (HCV) envelope E2 protein modified the infection after challenge with homologous monoclonal HCV. *Hepatology* 32: 618–25.

217. Lechner, F., N. Gruener, S. Urbani, J. Uggeri, T. Santantonio, A. Kammer, A. Cerny, R. Phillips, C. Ferrari, G. Pape, and P. Klenerman. 2000. CD8+ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. *Eur. J. Immunol.* 30: 2479–87.

218. Puig, M., M. Major, K. Mihalik, and S. Feinstone. 2004. Immunization of chimpanzees with an envelope protein-based vaccine enhances specific humoral and cellular immune responses that delay hepatitis C virus infection. *Vaccine* 22: 991–1000.

219. Poordad, F., J. McCone, B. Bacon, S. Bruno, M. Manns, M. Sulkowski, I. Jacobson, K. Reddy, Z. Goodman, N. Boparai, M. DiNubile, V. Sniukiene, C. Brass, J. Albrecht, and J.-P. Bronowicki. 2011. Boceprevir for untreated chronic HCV genotype 1 infection. *N. Engl. J. Med.* 364: 1195–206.

220. Jacobson, I., J. McHutchison, G. Dusheiko, A. Di Bisceglie, K. Reddy, N. Bzowej, P. Marcellin, A. Muir, P. Ferenci, R. Flisiak, J. George, M. Rizzetto, D. Shouval, R. Sola, R. Terg, E. Yoshida, N. Adda, L. Bengtsson, A. Sankoh, T. Kieffer, S. George, R. Kauffman, and S. Zeuzem. 2011. Telaprevir for previously untreated chronic hepatitis C virus infection. *N. Engl. J. Med.* 364: 2405–16.

221. Ghany, M., D. Strader, D. Thomas, and L. Seeff. 2009. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 49: 1335–74.

222. Thomas, D. 2013. Global control of hepatitis C: where challenge meets opportunity. *Nat. Med.* 19: 850–8.

223. Joyce, M., and D. Tyrrell. 2010. The cell biology of hepatitis C virus. *Microbes Infect.* 12: 263–71.

224. Scheel, T., and C. Rice. 2013. Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. *Nat. Med.* 19: 837–49.

225. Kong, J., X. Deng, Z. Wang, J. Yang, Y. Zhang, and J. Yu. 2008. Hepatitis C virus F protein: A double-edged sword in the potential contribution of chronic inflammation to carcinogenesis. *Mol. Med. Reports* 2: 461–9.

226. Horner, S., and M. Gale. 2013. Regulation of hepatic innate immunity by hepatitis C virus. *Nat. Med.* 19: 879–88.

CHAPTER-2

Alternate reading frame protein (F protein) of hepatitis C virus: Paradoxical effects of activation and apoptosis on human dendritic cells lead to stimulation of T cells

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2.1 INTRODUCTION

Hepatitis C virus (HCV) was first identified in 1989 as the major causative agent of parenterally transmitted and community-acquired non-A, non-B hepatitis (1). Currently, an estimated 170 million people worldwide are chronically infected with HCV (2). HCV is a major cause of end-stage liver diseases and a high proportion of chronic HCV carriers develop liver cirrhosis and hepatocellular carcinoma (3). Seven major genotypes (genotype 1 to genotype 7) of HCV have been described (based on phylogenetic analyses of the core, E1, and NS5 regions of the HCV genome), with further division of each genotype into several subtypes (1a, 1b, 2c, etc.) (4, 5). HCV contains a single stranded, positive-sense RNA genome 9.6 kb in size. This genome encodes a single open reading frame (ORF) polyprotein. This polyprotein is processed by host and viral proteases into structural (core, E1, and E2) and non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (6). Apart from these ORF proteins, another protein called alternate reading frame protein (F protein) of 162 amino acid in length is translated from within the core encoding region by ribosomal frame shifting (Figure 1-4). During translation, a +1 ribosomal frame shift occurs at codons 9 to 11 to generate F protein with the first 10 amino acids derived from the core (7, 11, and 12). The exact role of F protein in HCV infection is not known but it is suggested that F protein is not required for HCV infection and replication (8). However, its role in virus propagation and development of chronic disease has not been ruled out. Antibodies and cytotoxic T cells specific for the F protein have been detected in HCV infected patients, suggesting its presence during HCV

pathogenesis (9-12).

Dendritic cells (DCs) play a critical role in initiating effective antiviral T-cell responses because DCs are one of the most potent antigen presenting cells *in vivo* (13) and play crucial roles in the enhancement or regulation of cell-mediated immune responses. All DCs express high levels of different Toll-like receptors (TLRs), an important family of pathogen recognition receptors. Ligation of these receptors results in DC maturation and inflammatory cytokine secretion, a key event in the initiation of the innate and adaptive immune response (14). Because DCs strongly express various costimulatory and/or adhesion molecules (13), they can activate naïve T cells in a primary response.

During HCV infection, the initial interaction between virus or virus-derived proteins and DCs may contribute to either effective cellular immunity resulting in viral clearance or impaired T cell responses leading to viral persistence (15). It has been hypothesized that viral impairment of DCs leads to failure of HCV-infected individuals to mount an effective T-cell response, and thus to develop chronic HCV infection (16). Several studies have been reported with contradictory experimental evidence, and there is little overall consensus (17-30). The differing results could be attributed to the patient population selected, the HCV viral load, the duration of infection, the cell type being studied, the *in vitro* expansion, the assay being used, among other things (15). Further, it is not clear if DCs become impaired in chronic HCV infection, if DC impairment is a prelude to inefficient priming and maintenance of HCV-specific T cells facilitating the establishment of a chronic carrier state, or if DC impairment is a consequence of persistent and active HCV infection and

associated disease progression (15). Therefore, by identifying the mechanisms leading to modulation of DC function and subsequent antigen-specific T cell stimulation against HCV infection; this information will be important in understanding the immunobiology of HCV and in developing therapeutics against HCV. The roles of a number of HCV ORF proteins in modulating human DCs have been extensively studied (15, 31-33). The core antigen of HCV has been found to be associated with a number of immunomodulatory properties (34-37). It has been suggested that most of the core gene products are contaminated with F protein due to the inherent F protein sequence in the HCV core region (38); therefore, the effects ascribed to core proteins can be attributed to F protein or to a combination of F and core proteins. However, it has also been suggested that the production of F protein is negatively regulated by expression of the HCV core protein (38), implying that in an experimental system with abundant expression of core protein, F protein may not be sufficiently expressed. In earlier studies, we investigated the effects of HCV core protein on human dendritic cells, both as exogenously added recombinant protein and as endogenously expressed protein via recombinant adenovirus (15, 36).

In the present study we examined the role of HCV core and F proteins in modulating human DC functions and the resulting antigen specific T cell priming. Recombinant adenoviral vectors containing HCV F or core protein were used to infect DCs obtained from HCV-naive human donors' peripheral blood-derived monocytes. The expression of HCV F and core proteins in DCs led to upregulation of CD-95 and CD-95L, with F protein leading to significantly higher expression of both CD-95 and CD-95L and greater apoptosis of DCs.

Interestingly, the endogenous expression of both core and F proteins also significantly upregulated the expression of CD-40 and TLR-3 on DC surfaces. Our results therefore provided contrasting effects of F and core proteins on DCs. In the presence of HCV-F and core proteins, DCs became activated by expressing CD-40 and TLR-3 while undergoing apoptotic death due to upregulation of CD-95/CD-95L. In the next experiments, we demonstrated that apoptotic DCs were phagocytosed by live DCs; which would result in higher efficiency of DCs to prime and stimulate T cells. This theory was corroborated when these DCs were cocultured with autologous T cells; significant antigen specific priming and peptide-dependent proliferation of $CD4^+$ T cells were observed. Therefore, HCV-derived F and core proteins provide a unique mechanism of DC modulation and apoptosis, ensuing in eventual T cell activation. Our results provide direct experimental evidence to coalesce two apparently contrasting observations in chronic HCV infection: DC modulation and/or apoptosis and induction of antigen specific T cells.

2.2 MATERIALS AND METHODS

2.2.1 Cell line and culture

Monolayers of the 293A cell line (QBiogene Inc., CA, USA), an adenovirus-transformed human embryonic cell line that provides phenotypic complementation of E1 genes, was used for recombinant adenovirus plaque assays, amplification, and virus titration (15, 35). A human monocytic THP-1 cell line was obtained from ATCC (Manassas, VA).

2.2.2 Plasmid construction

The F gene (amino acids 11–164) of the HCV-1 strain (genotype 1a) was PCR amplified from full-length clone of HCV-H77 cDNA using a forward primer 5'-GAA GAT CTA TGC CAA ACG TAA CAC CAA CCG TC-3' and reverse primer 5'- GAA GAT CTC ACG CCG TCT TCC AGA ACC CGG A-3'. The PCR products were cloned into the commercial pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA) and the purified cDNA fragments were cloned into the AdenoVator Transfer vector (pAdenoVator-CMV5-IRES-GFP, QBiogene) generating rAd-F (Appendix Figure-1). The adenovirus construct expressing HCV core protein has been reported earlier (15, 35, and 36).

2.2.3 Recombinant adenovirus vectors

Recombinant adenoviruses were propagated, purified, and stored using the standard method provided in the manual (QBiogene) and as reported by our group (35, 39). The recombinant adenoviral vectors were stored in aliquots at -80°C. Viral particles of Ad5/CMV-LacZ (with no gene insert) obtained from QBiogene were used as a control adenoviral vector (denoted as CV throughout the thesis).

2.2.4 Preparation and infection of human peripheral blood monocyte derived DCs

Peripheral blood samples were obtained from HCV-negative donors (30–60 years of age of both sexes) from phlebotomy clinic after written informed consent. Use of human blood samples and the written consent form were approved by the Health Research Ethics Board at the University of Alberta, Canada. DCs were

generated from human peripheral blood mononuclear cells (PBMCs) as described previously (35, 39). Briefly, PBMCs were isolated from blood by Ficoll-Hypaque (Amersham Biosciences) density gradient centrifugation. The intermediate buffy layer containing PBMCs was collected and 5×10^6 cells/ml were cultured for 2 hours in 6-well plates in RPMI 1640 medium (Invitrogen Life Technologies), supplemented with L-glutamine, 1% human AB serum (Sigma-Aldrich), 1% sodium pyruvate (Invitrogen Life Technologies), and 500 U/ml penicillin/streptomycin (Invitrogen Life Technologies). The nonadherent cells (NACs) which mostly included T cells and B cells were subsequently removed and cryopreserved to use in later assays. Remaining adherent cells were treated with 50 ng ml^{-1} granulocyte macrophage colony-stimulating factor (GM-CSF) and 10 ng ml^{-1} of IL-4 (Peprotech) in RPMI media and cultured for 6 days. On day 6, > 95% of the obtained cells was positive for CD-11c suggesting the differentiation of myeloid DCs (Figure 2-3).

2.2.5 Infection with adenovirus

DCs harvested on day 6 of the culture were infected with replication defective recombinant adenoviruses expressing HCV F or core protein, or CV at a multiplicity of infection (m.o.i.) of 100. Expression of F protein in DCs was confirmed by amplifying F protein mRNA using forward primer 5'-GAA GAT CTA TGC CAA ACG TAA CAC CAA CCG TC-3' and reverse primer 5'- GAA GAT CTC ACG CCG TCT TCC AGA ACC CGG A-3'. HCV core protein expression was confirmed using primers reported earlier (15, 35, and 36).

2.2.6 Indirect immunofluorescence

DCs (3–5 x 10^5 cells) were grown on slides and infected with rAd-F for 48 hours. The slides were washed twice with PBS and fixed in 3.5 % paraformaldehyde solution in PBS for 15 min at room temperature. After washing with PBS, the cells were permeabilized with 0.1 % Triton X-100 in PBS for 10 min. The cells were washed once in PBS and blocked with 2 % BSA (Sigma-Aldrich) in PBS. A 1: 100 dilution of a primary mAb to HCV-derived F protein (Research Diagnostics Inc.) was prepared in PBS containing 1 % BSA and incubated with the fixed cells for 1 hour at room temperature to detect HCV-F protein expression. Following two additional washes with PBS incubation with secondary antibody for 1 hour, cells were washed twice with PBS, counter-stained with DAPI (49, 6-diamidino- 2-phenylindole) (Sigma-Aldrich) and mounted in mounting medium. Stained cells were viewed with appropriate filters under a fluorescence microscope. Alexa 564-conjugated goat anti-mouse IgG diluted 1: 2000 in PBS was used as the secondary antibody (Molecular Probes).

2.2.7 Western blot analysis

Western blot analyses of HCV F and core proteins were performed using reported procedures (36). Briefly, after 48 hours of infection of DCs with rAd-F, rAd-core, and CV, cells were rinsed with phosphate buffered saline (PBS), lysed in 1 × Laemmli buffer [50 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 0.01% bromophenol blue, 100 mM DTT] and boiled for an additional 10 min. Cell lysate from each sample was loaded on a 15% polyacrylamide gel, separated by SDS-

PAGE, and transferred to a nitrocellulose membrane (Bio-Rad) using a Trans-Blot apparatus (Bio-Rad). HCV-F and core proteins were probed with specific mAbs against F protein (gift from Dr James Ou, Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, CA) or core protein (Chemicon Inc.) followed by a horseradish peroxidase-conjugated goat anti-mouse IgG antibody and enhanced chemiluminescence detection reagents (Pierce Biotechnology Inc.), as recommended by the manufacturer, to detect HCV F and core protein.

2.2.8 RNA isolation, cDNA synthesis, and reverse transcription

Total RNA from 2–3 × 10^6 DCs was prepared using an RNA isolation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, followed by cDNA synthesis from 0.5–1 µg of total RNA (36).

2.2.9 Phenotypic analysis of DCs infected with F or core protein containing adenovirus vectors

DCs infected with adenoviral vectors containing F or core protein were harvested after 48 hours and phenotypically analyzed by flow cytometry (Appendix Figure-2). The fluorescently labelled mAbs used include CD-11c (PE-Cy7), DEC-205 (FITC), HLA-DR (FITC), CD-80 (PE-Cy5), CD-86 (PE), CD-40 (PE) and DC-SIGN (APC), CD-95 FITC, CD-95L PE in different combinations (eBioscience, San Diego, CA, USA). Antibodies against various TLRs were obtained from Abcam Inc (Toronto, Canada). For flow cytometry, an intracellular staining protocol provided by the manufacturer was used for TLR-3, and TLR-8, whereas

surface staining was performed for the remaining TLR-1, TLR-2, TLR-4 and TLR-5. The cells were gated on the basis of side and forward scatter and then selected for CD-11c positive cells. More than 95% of the cells were positive for CD-11c confirming the DC phenotype of the preparation (Figure 2-2).

2.2.10 Cell purification

$CD4^+$ and $CD8^+$ T-cells were purified by magnetic cell sorting (EasySep, Human CD4 and CD8 selection kit; StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Briefly, $2–3 \times 10^7$ nonadherent cells were resuspended in 100 µl of PBS containing 2% fetal bovine serum and 1 mM EDTA and incubated with EasySep positive cocktail at 100 µl/ml at room temperature (RT) for 15 minutes. Magnetic nanoparticles were added at 50 µl/ml and the mixture was incubated for 10 min at RT. The volume was brought up to 2.5 ml by adding recommended buffer (PBS) containing 2% fetal bovine serum and 1 mM EDTA. The tube was placed in a magnet for 5 min. After 5 min, the supernatant fraction was poured off. This process was repeated 3 times to get pure $CD4^+$ or $CD8^+$ T cells. The tube was removed from the magnet and the $CD4^+$ or $CD8^+$ T cells that bound to the column were flushed out with 2 ml buffer (described above). Purified populations were found to be at > 97% purity by flow cytometry.

2.2.11 *In vitro* proliferation assay

Proliferative responses of T cells were measured in triplicate cultures in flat-bottom 96-well microtiter plate (Costar). A total of 2×10^5 autologous T cells

were cultured with different concentrations (1×10^3 to 2×10^4) of infected or noninfected DCs in 200 µl of AIM-V medium (Invitrogen Life Technologies) at 37°C for 5 days. Purified $CD4^+$ or $CD8^+$ T cells were used in these assays. The assay included negative (no Ag) and positive (phytohemagglutin, 1µg/ml) controls. The cells were pulsed with 0.5 µCi/well [^{3}H] thymidine (Amersham Biosciences) for 16 h and harvested on nylon fibre filter papers (PerkinElmer). The levels of [^{3}H] thymidine incorporation into the cellular DNA were counted in a liquid scintillation counter (MicroBeta Trilux; PerkinElmer). Tests were run in replicates of three wells (Appendix Figure-3).

To determine the secondary T cell responses against peptides derived from HCV-F or core protein, replica plating assays were performed (35). Initially, 48 wells of 96-well plates were plated with F or core protein containing adenovirus-infected DCs (10^4/well) together with 2×10^5 autologous purified $CD4^+$ T cells in a total 200 µl/well of AIM-V medium for 5 days. On day 5, each well was split into three equal wells on three different 96-well plates. On the first plate, no peptide was added; on the second plate, peptides from F and core proteins were added at 1 µg/ml. On the third plate, peptides from F and core proteins were added at 10 µg/ml. Control Ags, SOD, was added in five to six replicates. Each well was fed with irradiated autologous PBMCs (1×10^5/well) and cultured for another 5 days. At the end of the 5 days, 0.5 µCi/well [^{3}H] thymidine was added, followed by harvesting the cells on day 6 and counting [^{3}H] thymidine levels incorporated in the DNA (Appendix Figure-4).

2.2.12 Detection of apoptosis in DCs induced by CD-95 - CD-95L interactions

Apoptotic cells were detected by their ability to stain with FITC-conjugated annexin V and 7-AAD (eBioscience, San Diego, CA, USA). Briefly, 5×10^5 dendritic cells were infected with 100 m.o.i. of replication deficient recombinant adenovirus containing HCV-F or core protein. Infected cells were cultured for 48 hours. At this time, cells were collected and washed 2 times with PBS and resuspended in 100 µl of 1× Annexin V binding buffer. The cells were then stained with 5 µl of FITC-conjugated Annexin V for 15 min at room temperature in the dark. The cells were washed twice with PBS and diluted with 400 µl of 1× binding buffer. Finally, 5 µl of 7-AAD was added and the cells were analyzed by flow cytometry using a FACSCanto II (BD Biosciences). The stained cells were analyzed within 4 hours of staining. In the blocking experiments, anti-CD-95L antibody (1 µg/ml) was added at the beginning of the culture.

2.2.13 Expression of F or core protein containing adenovector in THP-1 cells

HCV-derived F or core protein containing replication deficient recombinant adenovector was used to infect THP-1 cells for 48 hours and analyzed for CD-95L expression and apoptosis by flow cytometry.

2.2.14 Uptake of apoptotic DCs by live DCs

Live DCs were obtained from a 6-day culture of adherent PBMCs with IL-4 and GM-CSF as described earlier. F and control-adenovector infected DCs were obtained 24 hours after infection with recombinant adenovirus vectors. Infected DCs were stained with CFSE (5-(and 6)-carboxyfluorescein diacetate

succinimidyl ester, Invitrogen, Carlsbad, USA) at 2 μM/ml concentration according to the manufacturer's instructions. For positive control of apoptotic cells, untreated DCs were cultured on a six-well dish and irradiated for 2 min with a UV transilluminator with a peak intensity of 9000 mW/cm^2 at the filter surface and a peak emission of 313 nm (66, Appendix Figure-5). Induction of apoptosis in UV treated DCs was confirmed by staining cells with Annexin V and 7-AAD (eBioscience, San Diego, CA, USA). Live DCs were cocultured at 37°C or 4°C with F protein or control vector induced CFSE labelled DCs at 1:1 ratio. After 2–4 hours, flow cytometry analysis was performed to assess the uptake of CFSE-labelled apoptotic DC ($CFSE^+CD\text{-}11c^-$) by live DCs ($CFSE^-CD\text{-}11c^+$).

2.2.15 Statistical analyses

Statistical analysis was performed by one way ANOVA and t-test using Graphpad Prism (Graphpad Software Inc., La Jolla, CA, USA). '*', '**', and '***' indicate significant differences at $P < 0.05$, $P < 0.0036$, and $P < 0.0001$ respectively.

2.3 RESULTS

2.3.1 Endogenous expression of HCV-derived F or core protein in human PBMC-derived DCs

Human monocyte-derived immature DCs were incubated with recombinant adenovirus vectors containing HCV derived F or core protein or the control (Ad/LacZ) at 100 m.o.i. for 48 h. HCV gene expression in the DCs was determined by mRNA detection (RT-PCR) and western blotting (Figure. 2-2A and 2-2B). The molecular masses of HCV-F and core proteins were 17 and 21

kDa, respectively, corresponding to their putative molecular masses. In two repeated western blot experiments, we observed consistent protein expression at 100 m.o.i. of adenoviral vector. The cells were also stained with anti-F antibody and observed visually under a fluorescence microscope to determine the efficiency of gene expression. As shown in figure 2-1 almost 100% of the cells express HCV-F protein.

2.3.2 Phenotype analysis of DCs expressing HCV-derived F or core protein

We determined the expression of various maturation and costimulation markers such as CD-80, HLA-ABC, HLA-DR, CD-86, Dec-205, DC-SIGN and CD-40 on DCs to investigate whether the expression of HCV-derived F or core antigen led to modulation in the DC phenotype which could affect T-cell stimulation and DC survival. In addition, we analysed the expression of TLR-1, TLR-2, TLR-3, TLR-4, TLR-5, and TLR-8. In the differentiated DC cultures, CD-11c was expressed on > 96% of uninfected DCs indicating the myeloid DC lineage of these cells. Upon infection with control vector, F or core recombinant adenoviral vector, CD-11c was not altered (Figure 2-3). Also, we observed that activation/costimulation markers CD-80, HLA-ABC, HLA-DR, CD-86, Dec-205, and DC-SIGN were unchanged in DCs expressing core or F protein compared to controls (Figure 2-4A and Figure 2-4B). However, we found significant upregulation in CD-40 expression (Figure 2-5A, left panel, and Figure 2-5B). Among TLRs, only TLR-3 was upregulated in DCs infected with HCV-derived F or core protein (Figure 2-5A, right panel, and Figure 2- 5C). The remaining TLRs were unchanged within different experimental groups (Figure 2-6A and Figure 2-6B). During these

experiments, we observed that in F expressing DC cultures, cellular recovery was lower (~ 50%) than in core or control vector groups. However, there was no visual sign of cell death due to toxicity in the cultures. Therefore, we examined the expression of molecules involved in apoptosis, i.e., CD-95 and CD-95L. We found that both CD-95 (Figure 2- 6A, right panel, and Figure 2-7B) and CD-95L (Figure 2-7A, left panel, and Figure 2-7C) expressions were significantly higher in DCs expressing HCV-derived F protein. Expression of HCV-core protein in DCs led to an increase in CD-95 expression, but not to a significant increase in CD-95L expression (Figure 2-7B). The culture incubation time for the phenotype analysis was the same as in the immunofluorescence experiments and western blotting; therefore, at the time of phenotype analysis, the HCV-proteins were being expressed by almost all of the DCs.

2.3.3 Expression of F protein leads to apoptosis in DCs

We wanted to determine whether upregulation in CD-95/CD-95L expression caused apoptosis in monocyte derived dendritic cells expressing F protein. DCs infected with recombinant adenovirus containing HCV-derived F or core protein, or control vector for 48 hours were stained with PE Annexin V and 7-AAD. As shown in Figure 2-8A and 2-8B, apoptosis was significantly higher in DCs infected with HCV-derived F protein expressing adenovirus compared to control vector infected DCs, whereas, apoptosis in core infected DCs were similar to control vector infected DCs. A representative flow cytometry analysis is shown in Figure 2-8A and statistical analysis from three different experiments from three different donors is shown in Figure 2-8B. To confirm that apoptosis is mediated

by CD-95L interaction with CD-95, neutralizing anti-CD-95L antibody was added to the culture at the time of initial infection with adenovirus (Figure 2-9). Addition of neutralizing anti-CD-95L antibody (40) at 1 μg/ml significantly reduced the apoptosis in DCs infected with HCV-derived F protein expressing adenovirus compared to the isotype control antibody-treated group (Figure 2-9).

2.3.4 Expression of F protein in THP-1 cells induces upregulation in CD-95L expression followed by THP-1 cell apoptosis

After observing the effects on CD-95L expression and apoptosis in primary monocyte derived human DCs, we determined if similar effects of HCV derived F protein could be seen in the human monocytic cell line THP-1. The rationale was that in primary monocyte differentiated DC cultures, contaminating cells could provide accessory functions to induce CD-95L and apoptosis, whereas in the THP-1 cell line we would be looking at a direct effect of F protein expression on CD-95L and apoptosis. THP-1 cells were infected with adenovirus vectors containing F or control vector and examined for CD-95L expression after 48 hours by flow cytometry (Figure 2-10). The infection with adenovirus expressing F protein led to an increase in CD-95L expression on the surface of the infected THP-1 cells, whereas the control vector did not influence CD-95L expression relative to untreated THP-1 cells (Figure 2-10, left panel). In accordance with the CD-95L expression, apoptosis, as measured by Annexin V and 7-AAD staining, was enhanced in THP-1 cells infected with adenovirus vector expressing F protein, compared to control vector or untreated cells (Figure 2-10, right panel).

2.3.5 Uptake of apoptotic DCs by live DCs

The induction of apoptosis in DCs by HCV-derived F protein could be both beneficial and detrimental for efficient antigen presentation of HCV antigens. In our experiments (Figure 2-10), however, we observed apoptosis in a limited percentage of DCs or THP-1 cells. We therefore hypothesized that induction of apoptosis in a small number of DCs might better allow their phagocytosis by live DCs, resulting in efficient antigen presentation and T cell stimulation. To assess the phagocytosis of F protein induced apoptotic DCs by viable DCs, F expressing apoptotic DCs were labelled with CFSE and incubated with immature viable DCs not expressing F protein. Eight hours later, flow cytometry analysis was performed to assess the uptake of CFSE-labelled apoptotic DCs by live DCs (PI^- $CD\text{-}11c^+$) (Figure 2-11). The results indicated that in the F protein expressing group with a high percentage of apoptotic DCs, the percentage of viable $CFSE^+CD\text{-}11c^+$ DCs was increased compared to control vector or untreated CFSE labelled DCs (Figure 2-11). The UV treated DC group used as a positive control showed the highest number of $CFSE^+CD\text{-}11c^+$ DCs. To confirm that there were no contaminating $CFSE^+PI^-$ apoptotic DCs, a parallel experiment was performed in which apoptotic DCs were labelled with CFSE, cultured for 2 h, and subsequently stained with propidium iodide (PI); approximately 93% of the DCs were PI^+ (Appendix Figure-6A), indicating that gating for PI^- cells would gate out any $CFSE^+$ apoptotic DCs. Furthermore, to distinguish binding of apoptotic DCs to live DCs from internalization of apoptotic DCs by live DCs, the coculture experiments were carried out at 4°C; under these conditions, phagocytosis was

completely inhibited (Appendix Figure-6B). Collectively, the results indicated that high apoptosis induced in DCs by HCV-F protein led to increased phagocytosis by live DCs.

2.3.6 T cells from HCV-naive donors proliferate upon stimulation with autologous DCs endogenously expressing HCV derived F or core protein

In earlier studies we demonstrated that naive T cells from uninfected donors can be primed *in vitro* against core and NS3 antigens of HCV using autologous DCs expressing these antigens through recombinant adenovirus vectors (35). Here we found that F protein leads to apoptosis in DCs but results in more phagocytosis by live DCs. We wondered if naive T cells from uninfected donors could be stimulated *in vitro* to proliferate against HCV-derived F protein. Core protein was used as a positive control in these experiments. The immature dendritic cells (iDCs) expressing HCV F or core protein were cultured with autologous purified $CD4^+$ or $CD8^+$ T cells for 5 days at DC:T cell ratios of 1:200 to 1:20. Uninfected DCs or control-vector infected DCs were used as negative controls (Figure 2-12). T-cell proliferation was determined as a measure of T-cell stimulation. These experiments were performed with T cells obtained from five individual donors and of these, HCV antigen-dependent T-cell proliferation responses were obtained from four donors. After 5 days of culture *in vitro*, proliferation against HCV-derived F or core protein was significantly higher than uninfected DCs or control vector-infected DCs ($P< 0.05$) at DC:T cell ratios of 1:40 and 1:20 in all four donors). Proliferation against HCV-derived F protein was also significantly higher than proliferation against HCV-derived core protein.

2.3.7 $CD4^+$ T cells primed *in vitro* by autologous DCs expressing HCV F or core protein proliferate in a peptide-specific manner in secondary culture

$CD4^+$ T cells were stimulated with autologous DCs expressing HCV derived F or core containing adenovirus vector, followed by a replica-plating experiment to determine peptide-specific proliferation of $CD4^+$ T cells (35). Peptide-specific proliferation was evident in the purified $CD4^+$ T cells in the secondary cultures with HCV-derived F and core peptides at both 1 µg/ml and 10 µg/ml concentrations (peptides used are shown in Table-1). As controls, we also stimulated naive $CD4^+$ T cells in primary cultures with DCs infected with HCV-derived F or core protein but in secondary culture no peptides were added. $CD4^+$ T cell proliferation from the no peptide group was subtracted in peptide stimulated groups. The experiment was repeated in four different donors (Figure 2-13A and Figure 2-13B). We calculated a cumulative response against F and core derived peptides in all four donors using the following formula: sum of total number of responders for individual peptides/sum of total number of peptides tested from all donors. The results were 39/63 for F peptides and 25/63 for core peptides, suggesting that F protein is either more immunogenic than core protein or it is able to efficiently stimulate T cells.

2.4 DISCUSSION

Hepatitis C is a major blood-borne disease, causing chronic infection in 65–80% of infected patients. The hepatitis C virus (HCV) has been shown to infect several extrahepatic cells including DCs. (41-44). DCs are the major antigen presenting cells; they take up a variety of antigens and process and present them to $CD8^+$ and

$CD4^+$ T cells in the context of MHC molecules. After clonal expansion the activated T cells become effector cells and mount an antigen-specific immune response to clear the infection. It has been proposed that HCV persists in a majority of infected individuals due to lack of efficient adaptive immune responses (45-47). It has been hypothesized that HCV can modulate DC and/or T-cell function, and DC dysfunction is known to be one of the direct mechanisms that enable viral persistence (20). Earlier work by Bain et al. and Kanto et al. (17, 19) provided evidence for DC dysfunction in chronic HCV infection. Several studies show impairment of DC function in HCV-infected individuals (18, 20, 25, and 26); however, other reports suggest that DC functions are not affected (21-23, 27-29). In chronic hepatitis C, factors leading to DC dysfunction are postulated to be first, a direct infection of DCs and second, the presence of HCV proteins that might modulate DC function (37, 48).

In this study we show for the first time that HCV-derived F protein can modulate DC functions and the overall immune response against HCV. F protein is synthesized due to a ribosomal frame shift at codon 11 in the HCV core encoding region. It was suggested that F protein is not required for infection and replication of HCV (8) but its role in viral persistence and development of chronic hepatitis and hepatocellular carcinoma has not been ruled out.

We used recombinant adenovirus vectors containing HCV-derived F or core protein to endogenously express these antigens in DCs. Infection with recombinant adenovirus vectors led to expression of HCV-derived F or core protein, as indicated by immunofluorescence in ~ 100% of the DCs (39). Further,

mRNA detection by RT-PCR (Figure 2-2A) and protein detection by western blotting (Figure 2-2B) confirmed the expression of antigens in DCs. These initial experiments verified our ability to efficiently express HCV-derived F and core proteins in human DCs.

Upon examining the phenotype of DCs expressing HCV-derived F, core protein, or control vector, we did not observe significant differences in the expression of CD-11c, CD-80, CD-86, DEC-205, DC-SIGN, HLA class I, or MHC class II (Figure 2-4A and Figure 2-4B). However, we found significant upregulation in CD-40 (Figure 2-5A, left panel, and Figure 2-5B) in DCs expressing F protein.

Because there was significantly higher expression of CD-40 on F-expressing DCs, we hypothesized that upregulation of CD-40 alone may not be sufficient for efficient stimulation of T cells. Therefore, we decided to look for other markers that might be involved in stimulation of T cells. From previous reports (49, 50) we noted that CD-40 and TLRs together can efficiently stimulate T cells. It has been reported that in situ stimulation of CD-40 and TLR-3 transforms ovarian cancer-infiltrating DCs from immunosuppressive to immunostimulatory cells (49). It has also been shown that combinatorial stimulation of TLRs and TRAF signalling by CD-40 cross–linking generates a 10–20 fold increase in the number of activated $CD8^+$ T cells compared to either agonist alone (50). Synergistic effects of combined CD-40 and TLR-3 agonists produced one of the best T cell responses in healthy mice (50). In murine nonepithelial tumours, CD-40/TLR-7 agonists have been utilized as adjuvants in

vaccination with exogenous tumour antigen, which resulted in stronger and less toxic antitumour memory T-cell responses compared to monotherapy (51). CD-40 signaling synergizes with TLR-2 in the B cell receptor independent activation of resting B cells (52). Therefore, we examined the expression of various TLRs: TLR-1, TLR-2, TLR-3, TLR-4, and TLR-5 on HCV-derived F or core protein expressing DCs. Interestingly, we observed a significant increase in intracellular TLR-3 expression in F protein expressing DCs but not in core expressing DCs compared to control vector expressing DCs or untreated DCs (Figure 2-5A, right panel). The remaining TLRs were unchanged within different experimental groups (Figure 2-6A and 6B).

In contrast to activation molecules such as CD-40 and TLR-3, increased expression of CD-95 and CD-95L can lead to apoptosis in cells (53, 54). It has also been reported that DCs can undergo apoptosis involving several mechanisms, including CD-95/CD-95L upregulation (55, 56). In the case of HCV, it was shown that expression of core protein induced apoptosis in response to anti-CD-95 monoclonal antibody (57) and enhanced the susceptibility of hepatocytes to TNF-α mediated apoptosis (58). Moreover, CHO cells stably expressing HCV core protein were shown to undergo apoptosis in response to serum starvation (59). The apoptotic effect of HCV-NS3 protein on three main subsets of cytotoxic lymphocytes prevalent in liver tissue chronically infected with HCV has been shown (60).

Here we report that the expression of HCV-derived F protein in DCs leads to increased expression of CD-95 and CD-95L (Figure 2-7). HCV-derived core

proteins led to a less substantial increase in CD-95L expression compared to the control vector (Figure 2-7). Further, the endogenous expression of F protein in DCs led to significantly high apoptotic death in DCs, which was detected by staining with PE-Annexin V and 7-AAD (Figure 2-8A and 2-8B). Antibodies against CD-95L are known to block apoptosis mediated via CD-95L, so the DCs were infected with recombinant adenovector expressing F protein and cultured in the presence of anti-CD-95L antibody (Figure 2-9). We observed that the presence of anti-CD-95L blocked the apoptosis induced by HCV-derived F protein in DCs (Figure 2-9).

To confirm our observation in primary DCs we did similar experiments in THP-1 cells, a human monocyte derived cell line. After infecting THP-1 cells with adenovirus vector containing F, we observed upregulation in CD-95L expression (Figure 2-10, left panel) and apoptosis (Figure 2-10, right panel).

In our experiments with primary DCs and the THP-1 cell line, we observed that only a fraction of DCs upregulated CD-95L/95 and underwent apoptosis despite ~ 100% DCs expressing F-protein. The reason for this is not clear but we speculate that DC heterogeneity may be a contributing factor. Although the *in vitro* culture led to differentiation of monocytes into DCs, there are possibly a variety of DCs with individual markers, maturation, and cytokine profiles that could affect their progression to apoptosis under the influence of F protein. At this time it is not clear whether CD-40, TLR-3, CD-95, and CD-95L are upregulated in different DC populations or in the same cells, and the mechanism behind this upregulation is also unclear.

Apoptosis plays a very important role in the generation of strong immune responses (61, 62). Several reports demonstrate apoptosis of DCs in viral and other pathogenic infections (55, 63, and 64). Different groups have suggested different roles for apoptosis; three examples follow. Measles virus induced Fas L mediated apoptosis in DCs helps in release of the measles virus (55). Apoptosis induced in DCs by pathogen *Legionella pneumophila* helps in restricting its intracellular replication (63). Uptake of apoptotic DCs leads to conversion of live DCs into tolerogenic DCs; these tolerogenic DCs are unable to mature, secrete TGF-β1, and induce generation of $Foxp3^+$ Tregs (65). In contrast, it has been shown previously that human DCs can acquire relevant antigens and stimulates MHC class I-restricted cytotoxic T lymphocytes (CTLs) by phagocytosing apoptotic cells (66, 67). Therefore, we examined the internalization of apoptotic DCs induced by F protein by live DCs (Figure 2-11) (65). The results obtained indicated that the number of $CFSE^+CD\text{-}11c^+$ cells is highest in the positive control group where maximum apoptosis was observed (37% in the UV group, Figure 2-11). Similarly, untreated DCs and control vector treated DCs with low numbers of apoptotic DCs, had lower numbers of $CFSE^+CD\text{-}11c^+$ cells (7% and 15%, respectively) compared to F expressing DCs (34% in the F treated group, Figure 2-11). To confirm that there were no contaminating $CFSE^+$ PI^- apoptotic DCs, a parallel experiment was performed in which apoptotic DCs were labelled with CFSE, cultured for 2 h, and subsequently stained with PI; approximately 93% of the DCs were PI^+ (Appendix Figure-6A), indicating that gating for PI^- cells would gate out $CFSE^+$ apoptotic DCs.

So far our results depicted an interesting scenario: endogenous expression of F protein led to substantial upregulation in CD-40/TLR-3 expression, apoptosis in DCs through CD-95/CD-95L expression, and efficient uptake of apoptotic DCs by live DCs. Therefore, we hypothesized that apoptosis induced by F protein in DCs is in fact helping in further activation of DCs and ultimately stimulated antigen specific T cells.

Therefore, we cocultured DCs expressing HCV F or core protein, control vector treated DCs or untreated DCs with autologous $CD4^+$ and $CD8^+$ T cells (Figure 2-12). As an initial measure of T-cell activation, purified $CD4^+$ and $CD8^+$ T cells were used. In 4 out of 5 donors tested; we observed antigen-dependent proliferation in core and F groups, compared with control vector or uninfected DCs. Interestingly, proliferation was significantly higher in the F stimulated group compared to core stimulated group in both $CD4^+$ and $CD8^+$ T cells (Figure 2-12). Also, the stimulation with CV-infected DCs was much lower than in DCs expressing F or core antigen, suggesting that adenoviral antigens may be much less stimulatory than HCV F or core antigen. In an earlier report we demonstrated efficient priming and stimulation of antigen specific T cells *in vitro* using autologous DCs expressing core antigen (35).

In our next experiments we sought to further confirm the antigen specific $CD4^+$ T cell proliferation after initial *in vitro* priming (Figure 2-13A and 2-13B). For these experiments, purified $CD4^+$ T cells were incubated with the DCs expressing HCV F or core antigen in the priming cultures and restimulated with irradiated autologous PBMCs along with synthetic peptides derived (Table-1)

from F or core protein in the secondary cultures. Since it is expected that the frequency of antigen-specific T cells would be very low in HCV-naive individuals, we performed these experiments in replica-plating cultures (35). The proliferative responses of $CD4^+$ T cells in replica-plating experiments provided conclusive evidence of antigen specific T-cell proliferation in the primary *in vitro* cultures and also of *in vitro* priming of T cells against peptides of F or core using autologous DCs expressing these HCV antigens. We calculated a cumulative response for quantification of antigen specific responses as described in the results section and observed that for the four donors tested, much more stimulation of peptide specific T cells was observed with F protein (score = 39) than with core protein (score = 25). It is possible that the higher stimulation of peptide specific T cells is related to the higher immunogenicity of F protein compared to core protein, but the ability of F protein to significantly modulate DCs could be a contributing factor.

Based on our results we propose a model to explain how specific immune response and apoptosis can simultaneously occur after expression of HCV F protein in DCs (Figure 2-14): Endogenous expression of F protein leads to DC activation; activated DCs express CD-40 and TLR-3 and some DCs upregulate CD-95L/95; upregulation of CD-95L/95 leads to DC apoptosis; the apoptotic DCs are phagocytosed by live DCs; the increased DC activity allows better antigen processing and presentation (68).

It is well known that phagocytosis of apoptotic cells by DCs leads to further activation of DCs (66). Coculturing of the activated DCs with autologous

T cells results in efficient priming and stimulation of antigen specific T cells, which can be identified by measuring peptide-induced T cell proliferation. Therefore, HCV derived core and F protein antigens provide a unique mechanism of DC modulation and apoptosis, ensuing in T cell activation. The detailed functional characteristics of stimulated T cells are currently being investigated in our laboratory.

The role of apoptosis in HCV infection is not well defined. The kinetics and the extent of hepatocyte apoptosis as well as the pro- and anti-apoptotic mechanisms involved remain unclear. It remains to be tested whether enhanced apoptosis of hepatocytes in HCV infection is related to viral clearance, and whether it has a therapeutic benefit (69). In addition, apoptosis of DCs *in vivo* in chronic HCV infection has been reported but remains to be clearly established (70). However, these investigations are challenged by the compounding factors and complexity of *in vivo* situations.

Our studies reveal the potential of an HCV antigen (F or ARFP) in DC and T cell activation. A number of studies examining immune responses have used PBMCs and T cells from chronically infected HCV patients, whose function and activation are severely modulated/modified due to the long-term presence of infection. Further, *in vivo*, all antigens of HCV are almost simultaneously present, compounding the overall effects on the immune system. Our experimental system approaches studying HCV through individual antigens and delineates the potential of each in terms of a unique mechanism of immune modulation and stimulation.

Our results provide important steps towards the design of a prophylactic T cell vaccine for HCV.

2.5 FIGURES

Figure 2-1

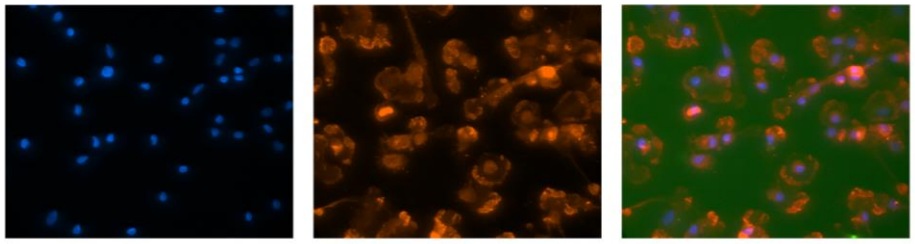


Figure 2-1 Immunofluorescence staining of DCs expressing HCV-F protein. Single staining with DAPI anti-F antibodies and overlap of the two colours are shown. DAPI staining represents the nucleus and anti-F is used to localize HCV-antigen expression.

Figure 2.2

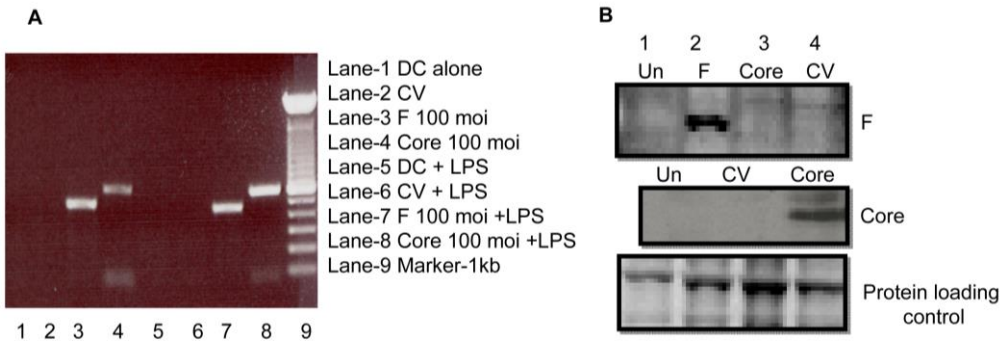


Figure 2-2(A). Expression of HCV-derived F or core mRNA in DCs infected with recombinant adenovirus containing HCV-derived F or core protein. DCs were infected with rAd-F or rAd-core at an m.o.i. of 100. After 48 hours of infection, DCs were harvested, mRNA was extracted, and cDNA was produced as described in Materials and Methods followed by amplification of F or core specific genes. F and core specific genes were expressed in both immature (lanes1–4) and LPS-matured DCs (lanes 5–8). Controls included DCs alone (lanes 1 and 5), and CV treated DCs (lanes 2 and 6) which were amplified with F-specific primers.

Figure 2-2(B). Western blot analysis of the HCV-derived F protein after infection of DCs with a recombinant adenovirus expressing F protein (rAd-F). DCs were infected with rAd-F (lane 2) at m.o.i. 100 or left uninfected (lane 1); a control was infected with rAd (lane 4). 293A cells infected with rAd-core at an m.o.i. of 100 were used as a positive control (lane 3 of top panel and middle panel). Cells were harvested 48 hours after infection. Expression of the F protein (17 kDa) was monitored by using a specific mAb against F, which shows a band

of around 17 kDa from rAd-F infected DCs, corresponding to the putative molecular mass of HCV F. Anti-core antibody shows a band of ~21 KDa from rAd-core infected DCs (middle panel).

Figure 2-3

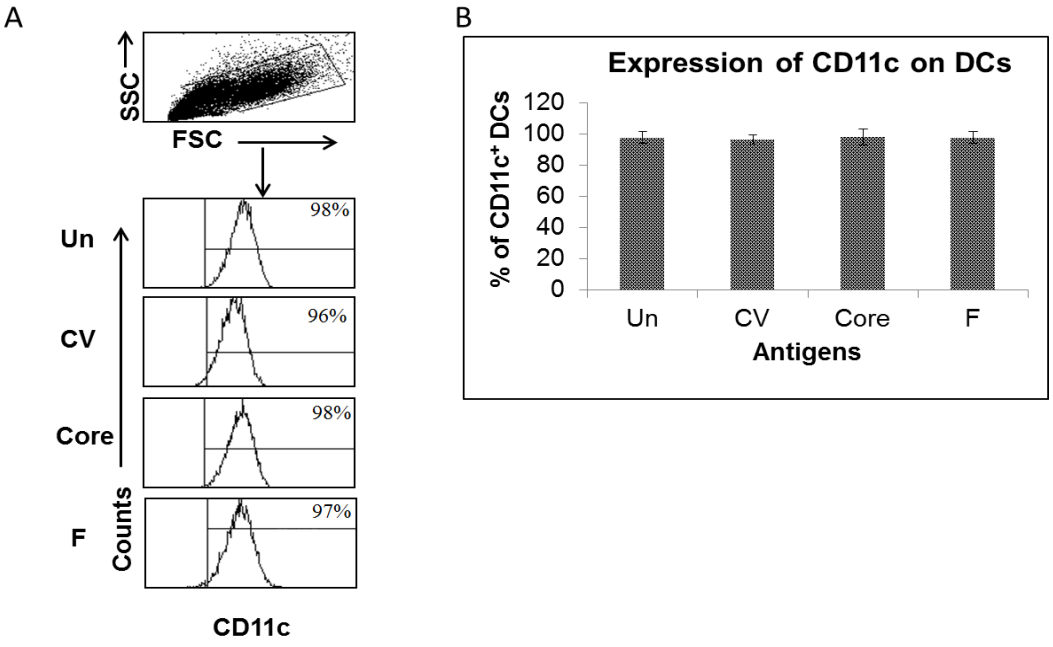


Figure 2-3. Expression of CD-11c on DCs expressing F or core protein. DCs were infected with adenovirus containing HCV-derived F and core protein**.** After 48 hours of infection, DCs were harvested and stained with antibodies against CD-11c. Data shown are representative of 3 repeated experiments from 3 different donors. Bar graph shows the data from 3 different experiments from 3 different donors.

Figure 2-4

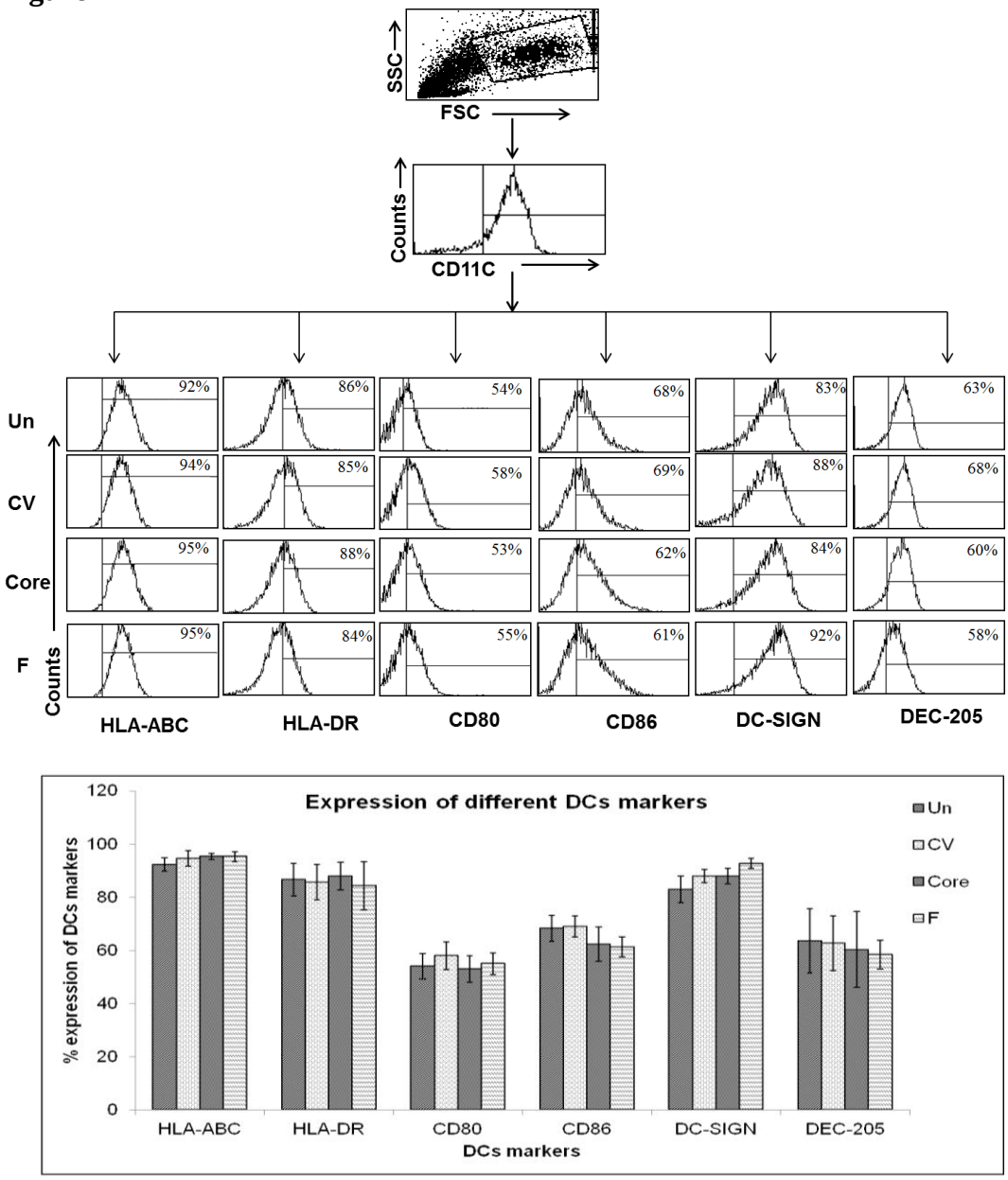


Figure 2-4. Expression of HLA-ABC, HLA-DR, CD80, CD86, DC-SIGN and DEC-205 on DCs expressing F or core protein. After 48 hours of infection, DCs were harvested and stained with antibodies against HLA-ABC, HLA-DR, CD80, CD86, DC-SIGN and DEC-205. Data shown are representative of 3 repeated experiments from 3 different donors. Bar graph shows the data from 3 different experiments from 3 different donors.

Figure 2-5

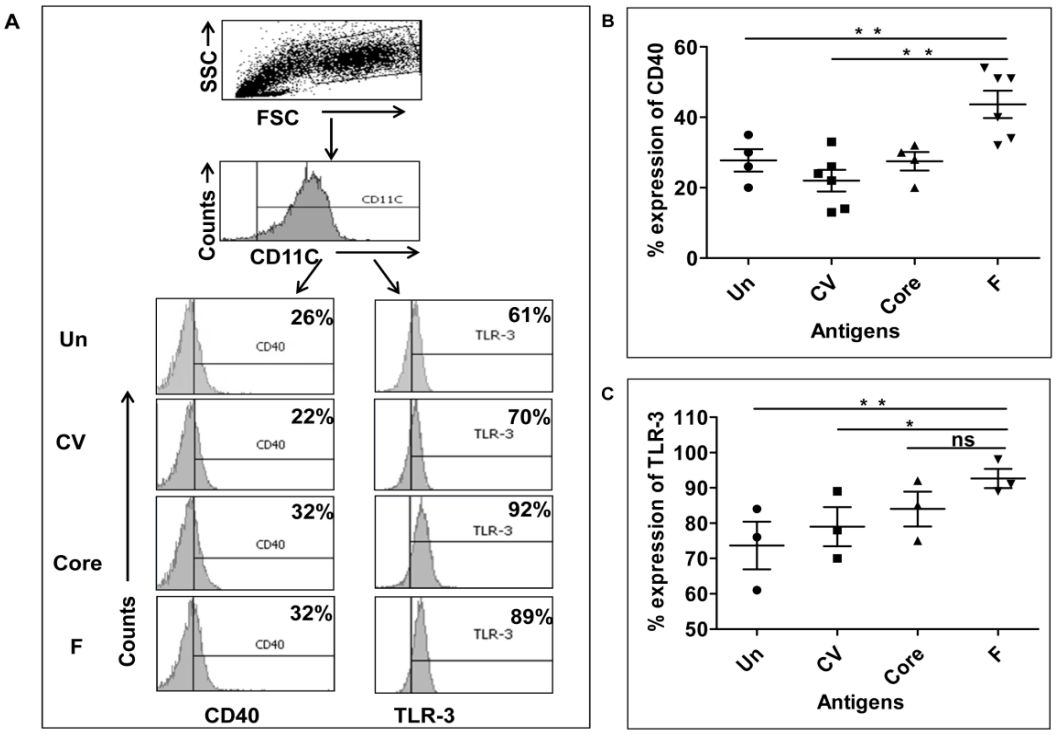
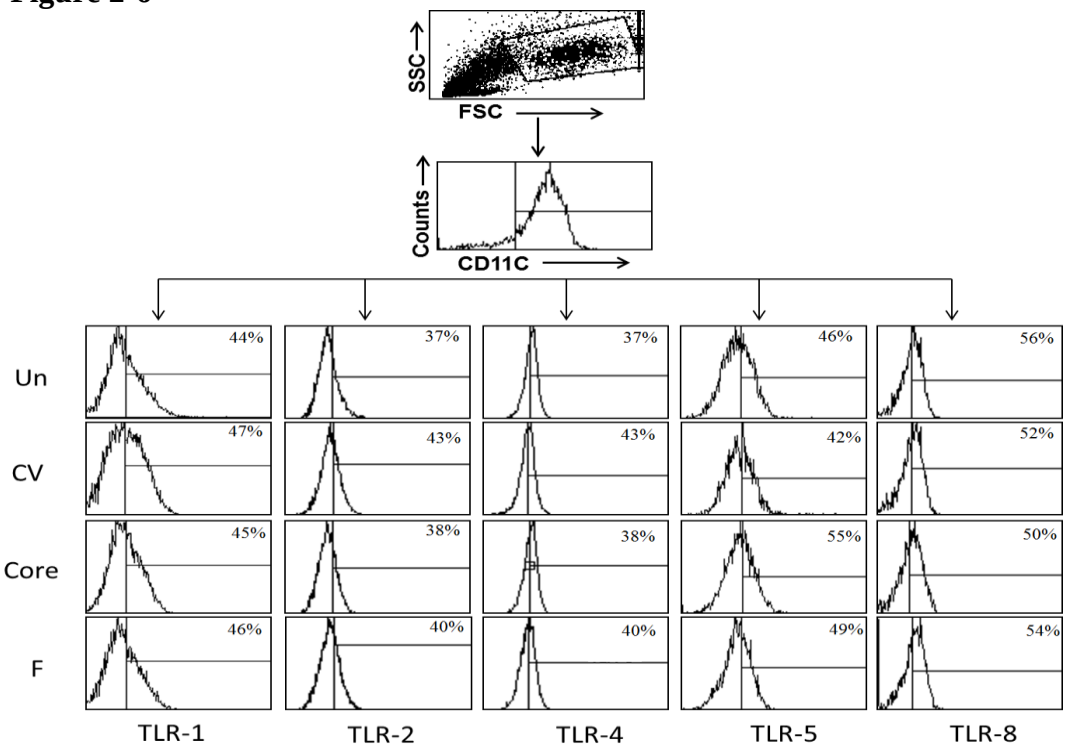


Figure 2-5. Expression of CD-40 and TLR-3 on DCs expressing F or core protein. DCs were infected with adenovirus containing HCV-derived F and core protein**.** After 48 hours of infection, DCs were harvested and stained with antibodies against CD-40 (A, left panel & B) and TLR-3 (2A, right panel & C). Data shown are representative of 4 repeated experiments from 4 different donors. '*' indicates P<0.05 and '**' indicates P<0.0036.

Figure 2-6



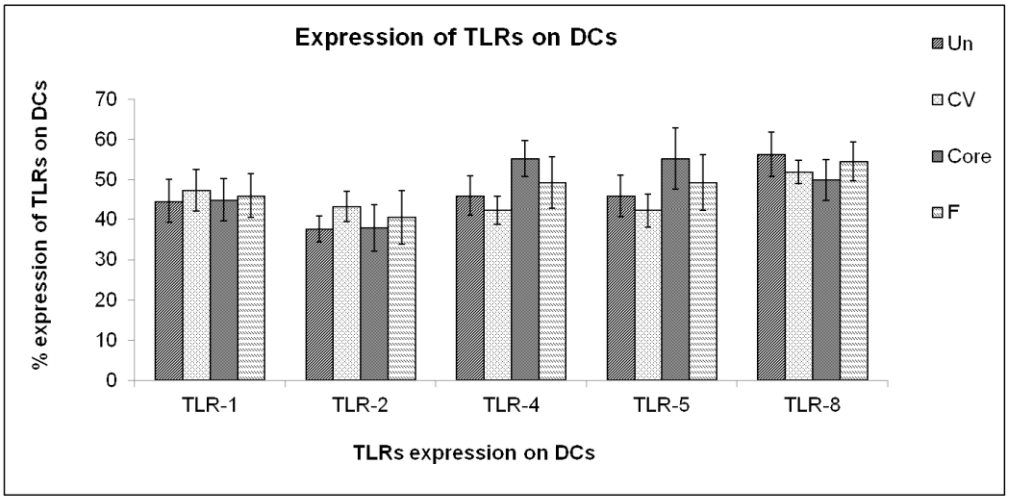


Figure 2-6. Expression of TLR-1, TLR-2, TLR-4, TLR-5 and TLR-8 on DCs expressing F or core protein. After 48 hours of infection, DCs were harvested and stained with antibodies against TLR-1, TLR-2, TLR-4, TLR-5 and TLR-8. Data shown are representative of 3 repeated experiments from 3 different donors. Bar graph shows the data from 3 experiments from 3 different donors.

Figure 2-7

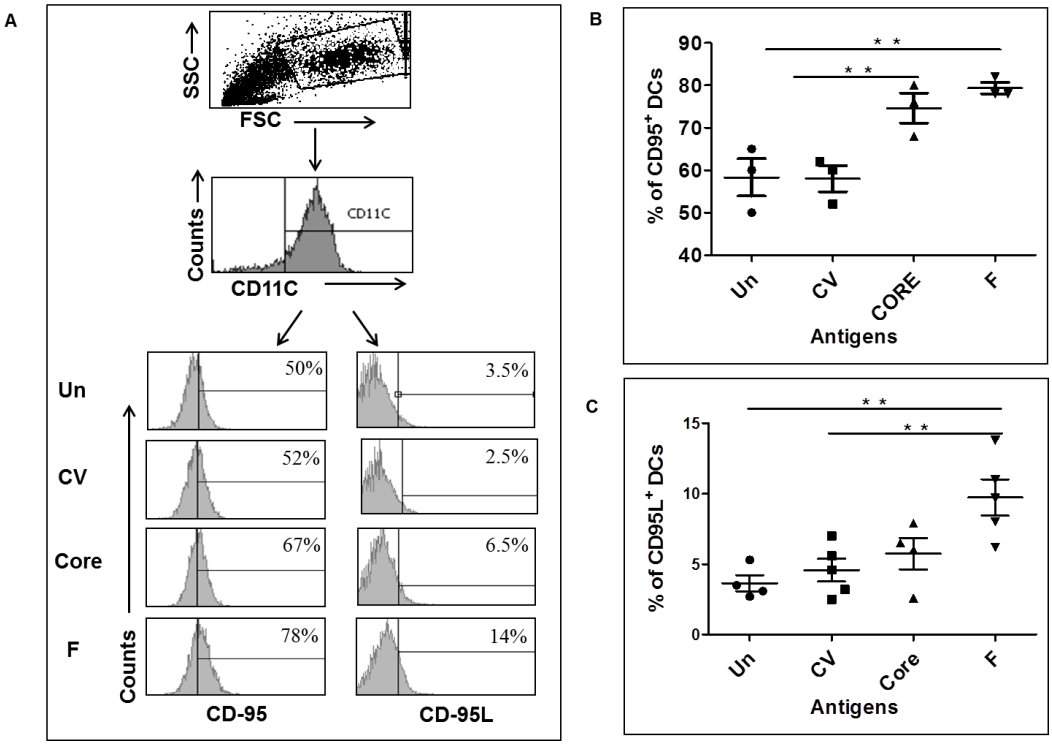


Figure 2-7. Expression of CD-95 and CD-95L on DCs expressing F or core protein. DCs were infected with adenovirus containing HCV-derived F or core protein. After 48 hours of infection, DCs were harvested and stained with antibodies against CD-95 (A, left panel) and CD-95L (A, right panel). Data shown are representative of 5 different experiments done separately in 5 different donors. Cumulative statistical analysis of CD-95 (B) and CD-95L (C) expression is shown from 5 different donors. '**' indicates $P<0.0036$.

Figure 2-8

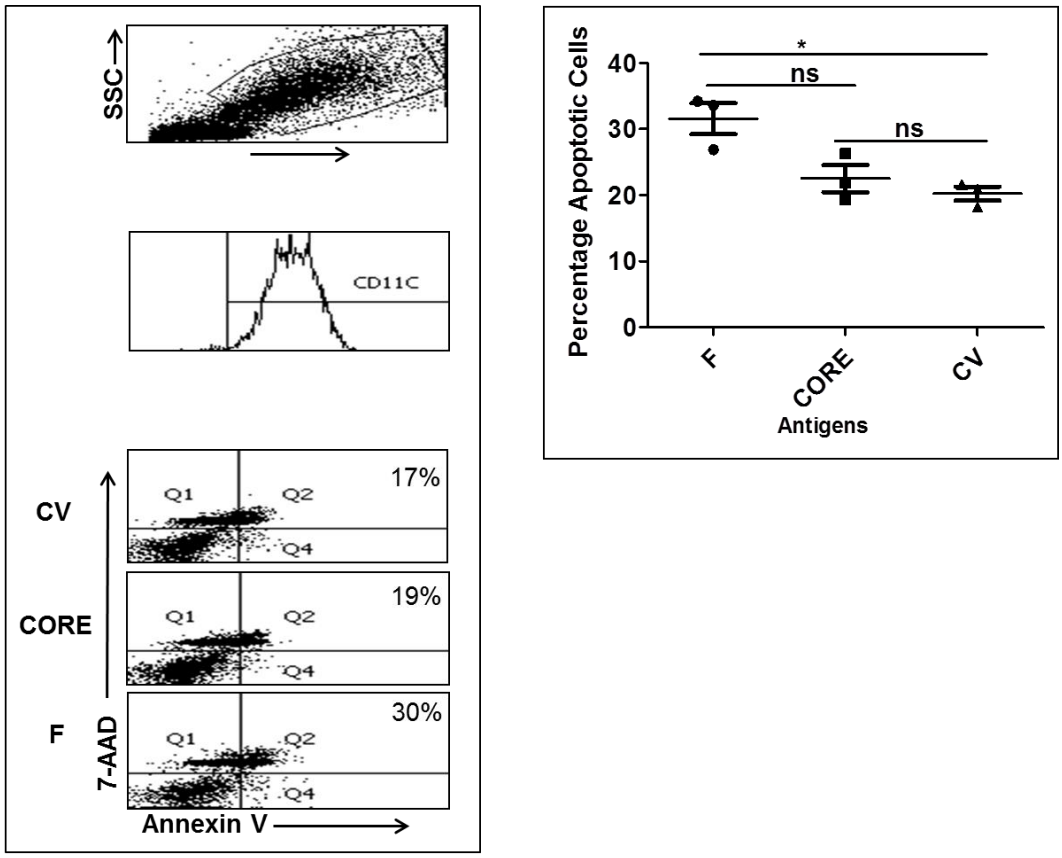
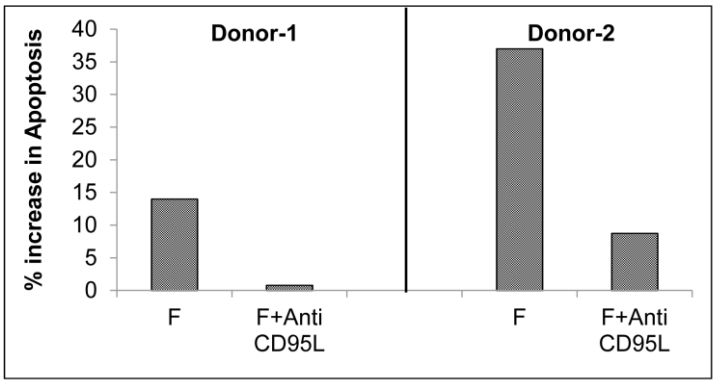


Figure 2-8. Expression of F protein leads to apoptosis in DCs. DCs were infected with rAd-F, rAd-core or control vector at an m.o.i. of 100. After 48 hours of infection, DCs were collected and stained with PE-Annexin V and 7-AAD. Statistical analysis of apoptosis in 3 different donors is shown in Figure B. Data are representative of 3 different experiments from 3 different donors. '*' indicates $P<0.05$.

Figure 2-9



$$\text{\% increase in Apoptosis} = \frac{\text{Apoptosis in F- Apoptosis in CV}}{\text{Apoptosis in CV}} \times 100$$

Figure 2-9. The CD-95/CD-95L apoptotic pathway is involved in rAd-F induced apoptosis in DCs. DCs were infected with rAd-F at an m.o.i. of 100 with and without 1 µg/mL of a neutralizing monoclonal anti-CD-95L antibody. After 48 hours, the cells were stained with PE-Annexin V and 7-AAD and analyzed by flow cytometry. The data are represented as % increase in apoptosis using the formula [100 x (Apoptosis in F- Apoptosis in CV/Apoptosis in CV)]. The experiment was repeated with two different donors.

Figure 2-10

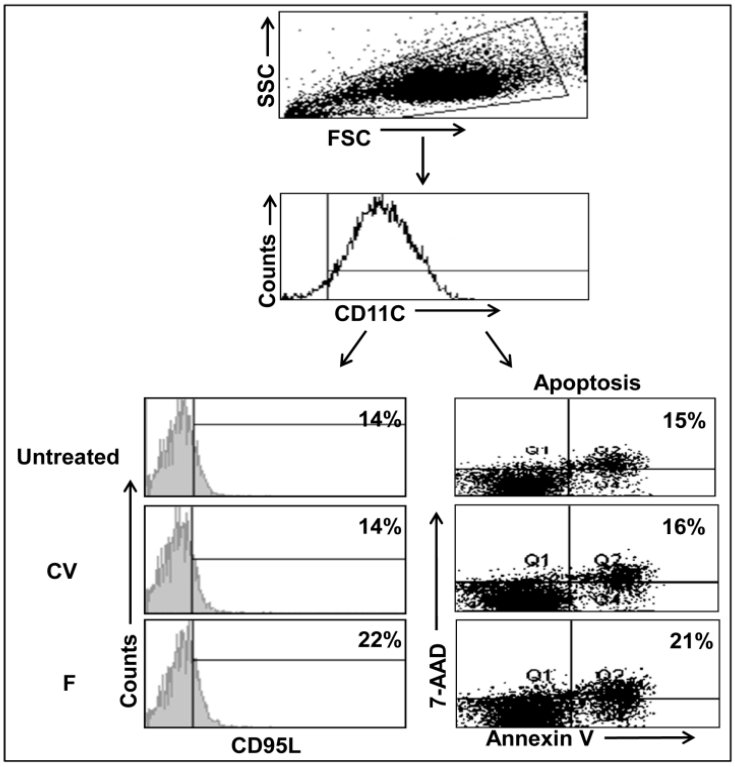


Figure 2-10. rAd-F infection of THP-1 cells induces CD-95L expression and apoptosis. THP-1 cells were infected with rAd-F at an m.o.i. of 100. After 48 hours of infection, the cells were harvested and stained using an anti-human CD-95L PE-conjugated monoclonal antibody and analyzed by flow cytometry (left panel). To measure apoptosis in THP-1 cell, after 48 hours of rAd-F infection, THP-1 cells were harvested, stained with PE-Annexin V and 7-AAD, and examined by flow cytometry (right panel).

Figure 2-11

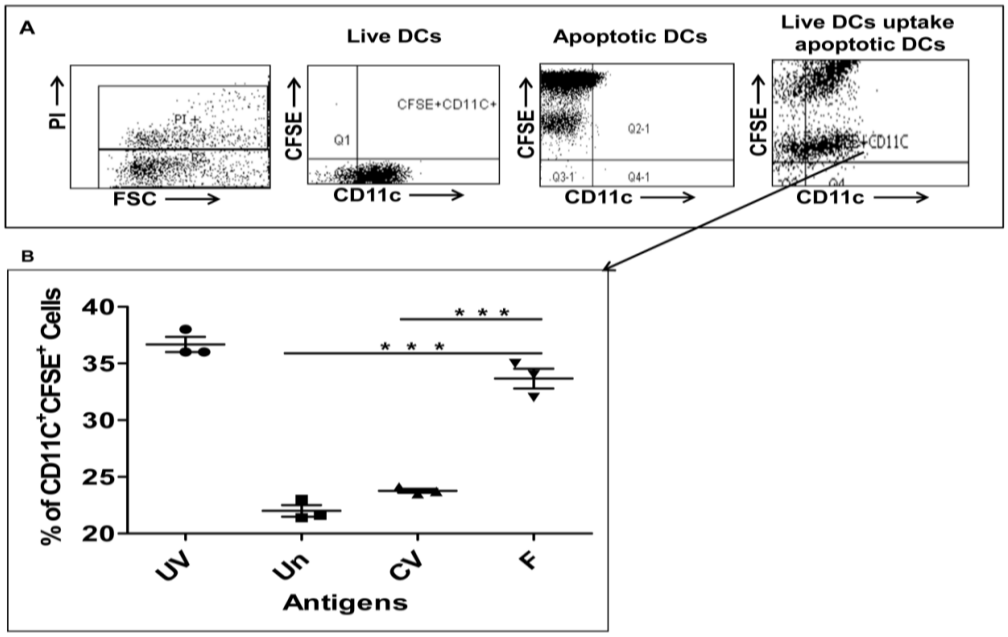


Figure 2-11. F-protein induced apoptotic DCs are efficiently taken-up by live DCs *in vit*ro. CFSE-labelled apoptotic DCs were incubated with viable immature DCs at a ratio of 1:1 for 2 hours. Flow cytometry analyses were conducted to assess uptake of $CFSE^+$ apoptotic DCs by viable $CD\text{-}11c^+$ DCs. Double positive cells indicate the number of viable DCs which have uptaken apoptotic DCs. Figure 7A shows the gating pattern in different groups: viable DCs were gated based on PI exclusion and CD-11c expression (see gate Figure 2-10A) and the proportion of $CFSE^+$ cells was assessed among $CD\text{-}11c^+$ to determine the phagocytosed DCs (Figure 2-10A). $CFSE^+CD\text{-}11c^+$ double positive cells are significantly higher in F group which has more apoptotic DCs induced by rAd-F infection compared to control vector or untreated DCs. UV treated DCs were used as positive control for apoptotic DCs. Data are representative of three different experiments. '***' indicates $P<0.0001$.

Figure 2-12

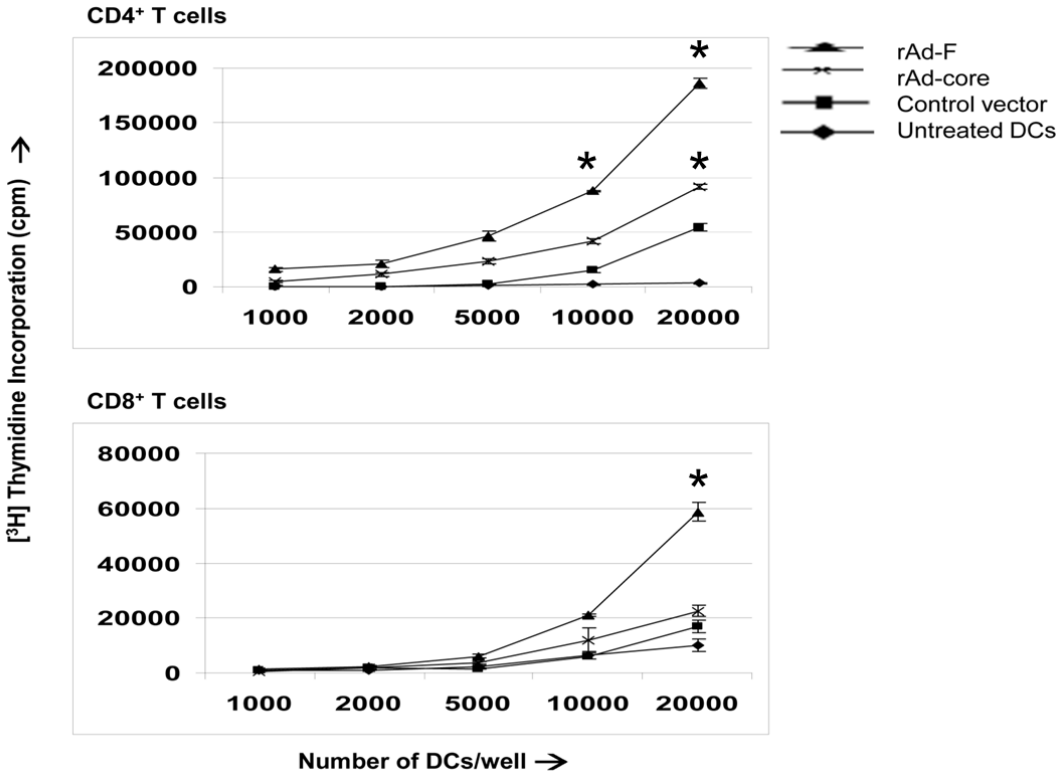


Figure 2-12. Primary proliferative response of naive autologous $CD4^+$ and $CD8^+$ T cells upon stimulation by DCs expressing HCV antigens F and core. Purified $CD4^+$ and $CD8^+$ T cells were stimulated using DCs or expressing HCV antigens at various DC:T cell ratios. The proliferation of T cells was determined by ^{3}H-T incorporation assay. The line on the graphs represent DCs with no antigens (—◆—), DCs expressing control vector (—■—), core (—✳—) and F protein (—▲—). Mean±SE of triplicate wells are shown here. The data shown are representative of 5 repeated experiments with 5 different donors.

Figure 2-13

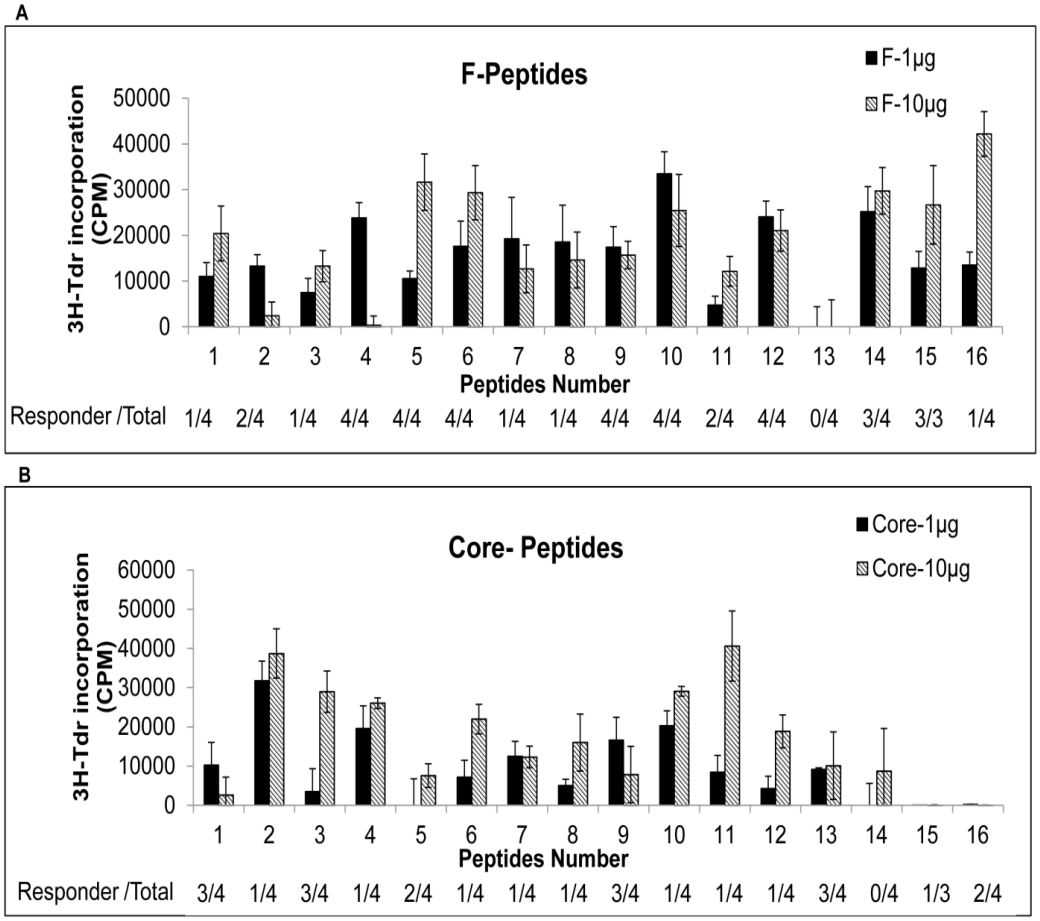


Figure 2-13. *In vitro* priming with DCs expressing F and core leads to peptide dependent proliferation of CD4$^+$ T cells in secondary cultures. The CPMs shown are means±SE of individual CPMs subtracted with no-antigen groups from corresponding wells. The CPMs of the no antigen control plates ranged from 2,000 to 8,000. The data are representative of four repeated experiments from four different donors. Numbers of responders against each peptide are shown at the bottom. A response was considered positive when the CPM in the presence of peptide was at least two-fold as much as that in the absence of peptide.

Figure 2-14

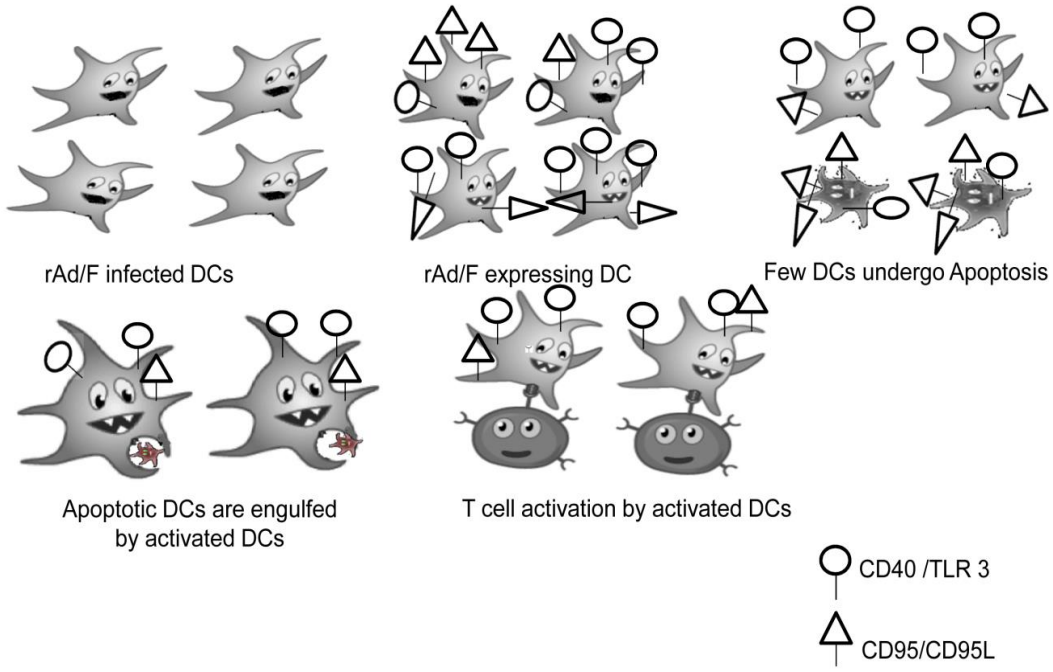


Figure 2-14. A model to explain the paradoxical role of F protein on DCs apoptosis and activation, and T cell stimulation. DCs infected with rAd-F upregulate CD-40, TLR-3, CD-95 and CD-95L. DCs expressing CD-95/95L will undergo apoptosis and DCs expressing CD-40/TLR-3 will be activated. Activated DCs will uptake apoptotic DCs and will further increase the level of their activation which will substantially enhance their ability to stimulate T cells.

TABLE 2

A. Peptides derived from F protein

No.	Position	AA Sequence
1	01-15	PNVTPTVAHRTSSSR
2	11-25	PNVTPTVAHRTSSSR
3	21-35	TSSSRVAVRSLVEFT
4	31-45	LVEFTCCRAGALDWV
5	41-55	ALDWVCARRGRLPSG
6	51-65	RLPSGRNLEVDVSLS
7	61-75	DVSLSPRHVGPRAGP
8	71-85	PRAGPGLSPGTLGPS
9	81-95	TLGPSMAMRVAGGRD
10	91-105	AGGRDGSCLPVALGL
11	101-115	VALGLAGAPQTPGVG
12	111-125	TPGVGRAIWVRSSIP
13	121-135	RSSIPLRAASPTSWG
14	131-145	PTSWGTYRSSAPLLE
15	141-155	APLLEALPGPWRMAS
16	148-162	LPGPWRMASGFWKTA

B. Peptides derived from core protein

No.	Position	AA Sequence
1	C1-15	MSTNPKPQRKTKRNT
2	C13-27	RNTNRRPQDVKFPGG
3	C25-39	PGGGQIVGGVYLLPR
4	C37-51	LPRRGPRLGVRATRK
5	C49-63	TRKTSERSQPRGRRQ
6	C61-75	RRQPIPKARRPEGRT
7	C73-87	GRTWAQPGYPWPLYG
8	C85-99	LYGNEGCGWAGWLLS
9	C97-111	LLSPRGSRPSWGPTD
10	C109-123	PTDPRRRSRNLGKVI
11	C121-135	KVIDTLTCGFADLMG
12	C133-147	LMGYIPLVGAPLGGA
13	C145-159	GGAARALAHGVRVLE
14	C157-171	VLEDGVNYATGNLPG
15	C169-183	LPGCSFSIFLLALLS
16	C177-191	FLLALLSCLTVPASA

2.6 REFERENCES

1. Choo, Q., G. Kuo, A. Weiner, L. Overby, D. Bradley, and M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 244: 359–62.

2. WHO report, 2012

3. Hoofnagle, J. 2002. Course and outcome of hepatitis C. *Hepatology* 36: S21–9.

4. Simmonds, P., J. Bukh, C. Combet, G. Deléage, N. Enomoto, S. Feinstone, P. Halfon, G. Inchauspé, C. Kuiken, G. Maertens, M. Mizokami, D. Murphy, H. Okamoto, J.-M. Pawlotsky, F. Penin, E. Sablon, T. Shin-I, L. Stuyver, H.-J. Thiel, S. Viazov, A. Weiner, and A. Widell. 2005. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 42: 962–73.

5. Simmonds P, Becher P, Collett MS, Gould EA, Heinz FX, Meyers G, Monath T, Pletnev A, Rice CM, Stiasny K, Thiel HJ, Weiner A, Bukh J (2011) Flaviviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds) Virus taxonomy. Elsevier, Oxford, pp 1003–1020

6. Moradpour, D., F. Penin, and C. Rice. 2007. Replication of hepatitis C virus. *Nat. Rev. Microbiol.* 5: 453–463.

7. Xu, Z., J. Choi, T. Yen, W. Lu, A. Strohecker, S. Govindarajan, D. Chien, M. Selby, and J. Ou. 2001. Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. *EMBO J.* 20: 3840–3848.

8. McMullan, L., A. Grakoui, M. Evans, K. Mihalik, M. Puig, A. Branch, S. Feinstone, and C. Rice. 2007. Evidence for a functional RNA element in the hepatitis C virus core gene. *Proc. Natl. Acad. Sci.*104: 2879–2884.

9. Bain, C., P. Parroche, J. Lavergne, B. Duverger, C. Vieux, V. Dubois, F. Komurian-Pradel, C. Trépo, L. Gebuhrer, G. Paranhos-Baccala, F. Penin, and G. Inchauspé. 2004. Memory T-cell-mediated immune responses specific to an alternative core protein in hepatitis C virus infection. *J. Virol.* 78: 10460–10469.

10. Komurian-Pradel, F., A. Rajoharison, J.-L. Berland, V. Khouri, M. Perret, M. Van Roosmalen, S. Pol, F. Negro, and G. Paranhos-Baccalà. 2004. Antigenic relevance of F protein in chronic hepatitis C virus infection. *Hepatology* 40: 900–909.

11. Varaklioti, A., N. Vassilaki, U. Georgopoulou, and P. Mavromara. 2002. Alternate translation occurs within the core coding region of the hepatitis C viral genome. *J. Biol. Chem.* 277: 17713–17721.

12. Walewski, J., T. Keller, D. Stump, and A. Branch. 2001. Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. *RNA* 7: 710–721.

13. Steinman, R. 1990. The dendritic cell system and its role in immunogenicity. *Ann. Rev. Immunol.* 9: 271–296.

14. Creagh, E., and L. O'Neill. 2006) TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends. Immuol.* 27: 352–357.

15. Krishnadas, D., J. Ahn, J. Han, R. Kumar, and B. Agrawal. 2010. Immunomodulation by hepatitis C virus-derived proteins: targeting human dendritic cells by multiple mechanisms. *Int. Immunol.* 22: 491–502.

16. Liu, B., A. Woltman, H. Janssen, and A. Boonstra. 2009. Modulation of dendritic cell function by persistent viruses. *J. Leuk. Biol.* 85: 205–214.

17. Kanto, T., N. Hayashi, T. Takehara, T. Tatsumi, N. Kuzushita, A. Ito, Y. Sasaki, A. Kasahara, and M. Hori. 1999. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J. Immunol.* 162: 5584–5591.

18. Auffermann-Gretzinger, S., E. Keeffe, and S. Levy. 2001. Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. *Blood* 97: 3171–3176.

19. Bain, C., A. Fatmi, F. Zoulim, J. Zarski, C. Trépo, and G. Inchauspé. 2001. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* 120: 512–524.

20. Pachiadakis, I., G. Pollara, B. Chain, and N. Naoumov. 2005. Is hepatitis C virus infection of dendritic cells a mechanism facilitating viral persistence? *Lancet Infect. Dis.* 5: 296–304.

21. Larsson, M., E. Babcock, A. Grakoui, N. Shoukry, G. Lauer, C. Rice, C. Walker, and N. Bhardwaj. 2004. Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. *J. Virol*. 69: 8079–8083.

22. Longman, R., A. Talal, I. Jacobson, M. Albert, and C. Rice. 2004.) Presence of functional dendritic cells in patients chronically infected with hepatitis C virus. *Blood* 103: 1026–1029.

23. Piccioli, D., S. Tavarini, S. Nuti, P. Colombatto, M. Brunetto, F. Bonino, P. Ciccorossi, F. Zorat, G. Pozzato, C. Comar, S. Abrignani, and A. Wack. 2004. Comparable functions of plasmacytoid and monocyte-derived dendritic cells in chronic hepatitis C patients and healthy donors. *J. Hepatol.* 42: 61–67.

24. Yakushijin, T., T. Kanto, M. Inoue, T. Oze, M. Miyazaki, I. Itose, H. Miyatake, M. Sakakibara, N. Kuzushita, N. Hiramatsu, T. Takehara, A. Kasahara, and N. Hayashi. 2006. Reduced expression and functional impairment of Toll-like receptor 2 on dendritic cells in chronic hepatitis C virus infection. *Hepatol. Res.* 34: 156–162.

25. Kanto, T., M. Inoue, H. Miyatake, A. Sato, M. Sakakibara, T. Yakushijin, C. Oki, I. Itose, N. Hiramatsu, T. Takehara, A. Kasahara, and N. Hayashi. 2004. Reduced numbers and impaired ability of myeloid and plasmacytoid dendritic cells to polarize T helper cells in chronic hepatitis C virus infection. *J. Infect. Dis.* 190: 1919–1926.

26. Anthony, D., N. Yonkers, A. Post, R. Asaad, F. Heinzel, M. Lederman, P. Lehmann, and H. Valdez. 2004. Selective impairments in dendritic cell-associated function distinguish hepatitis C virus and HIV infection. *J. Immunol.* 172: 4907–4916.

27. Longman, R., A. Talal, I. Jacobson, C. Rice, and M. Albert. 2005. Normal functional capacity in circulating myeloid and plasmacytoid dendritic cells in patients with chronic hepatitis C. *J. Infect. Dis.* 192: 497–503.

28. Larsson, M., J. Fonteneau, and N. Bhardwaj. 2001. Dendritic cells resurrect antigens from dead cells. *Trends Immunol.* 22: 141–148.

29. Rollier, C., J. Drexhage, B. Verstrepen, E. Verschoor, R. Bontrop, G. Koopman, and J. Heeney. 2003. Chronic hepatitis C virus infection established and maintained in chimpanzees independent of dendritic cell impairment. *Hepatology* 38: 851–858.

30. Lau, A., and A. Thomson. 2003. Dendritic cells and immune regulation in the liver. *Gut* 52: 307–314.

31. Takaki, A., M. Tatsukawa, Y. Iwasaki, K. Koike, Y. Noguchi, H. Shiraha, K. Sakaguchi, E. Nakayama, and K. Yamamoto. 2010.

Hepatitis C virus NS4 protein impairs the Th1 polarization of immature dendritic cells. *J. Viral Hepat.*17: 555–562.

32. Wertheimer, A., S. Polyak, R. Leistikow, and H. Rosen. 2007. Engulfment of apoptotic cells expressing HCV proteins leads to differential chemokine expression and STAT signaling in human dendritic cells. *Hepatology* 45: 1422–1432.

33. Abe, T., Y. Kaname, I. Hamamoto, Y. Tsuda, X. Wen, S. Taguwa, K. Moriishi, O. Takeuchi, T. Kawai, T. Kanto, N. Hayashi, S. Akira, and Y. Matsuura. 2007. Hepatitis C virus nonstructural protein 5A modulates the toll-like receptor-MyD88-dependent signaling pathway in macrophage cell lines. *J. Virol.* 81: 8953–8966.

34. Tu, Z., H. Hamalainen-Laanaya, C. Nishitani, Y. Kuroki, I. Crispe, and M. Orloff. 2012. HCV core and NS3 proteins manipulate human blood-derived dendritic cell development and promote Th 17 differentiation. *Int. Immunol.* 24: 97–106.

35. Li, W., D. Krishnadas, J. Li, D. Tyrrell, and B. Agrawal. 2006. Induction of primary human T cell responses against hepatitis C virus-derived antigens NS3 or core by autologous dendritic cells expressing hepatitis C virus antigens: potential for vaccine and immunotherapy. *J. Immunol.* 176: 6065–6075.

36. Li, W., J. Li, D. Tyrrell, and B. Agrawal. 2005. Expression of hepatitis C virus-derived core or NS3 antigens in human dendritic cells leads to induction of pro-inflammatory cytokines and normal T-cell stimulation capabilities. *J. Gen. Virol.* 87: 61–72.

37. Dolganiuc, A., K. Kodys, A. Kopasz, C. Marshall, T. Do, L. Romics, P. Mandrekar, M. Zapp, and G. Szabo. 2003. Hepatitis C virus core and nonstructural protein 3 proteins induce pro- and anti-inflammatory cytokines and inhibit dendritic cell differentiation. *J. Immunol.* 170: 5615–5624.

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

39. Li, W., D. Krishnadas, R. Kumar, D. Tyrrell, and B. Agrawal. 2007. Priming and stimulation of hepatitis C virus-specific $CD4^+$ and $CD8^+$ T cells against HCV antigens NS4, NS5a or NS5b from HCV-naive individuals: implications for prophylactic vaccine. *Int. Immunol.* 20: 89–104.

40. Iannello, A., O. Debbeche, R. El Arabi, S. Samarani, D. Hamel, F. Rozenberg, N. Heveker, and A. Ahmad. 2011. Herpes simplex virus type 1-induced FasL expression in human monocytic cells and its implications for cell death, viral replication, and immune evasion. *Viral Immunol*. 24: 11–26.

41. Bürgel, B., M. Friesland, A. Koch, M. Manns, H. Wedemeyer, K. Weissenborn, W. Schulz-Schaeffer, T. Pietschmann, E. Steinmann, and S. Ciesek. 2011. Hepatitis C virus enters human peripheral neuroblastoma cells - evidence for extra-hepatic cells sustaining hepatitis C virus penetration. *J. Viral Hepat.*18: 562–570.

42. Fletcher, N., and J. McKeating. 2012. Hepatitis C virus and the brain. . *J. Viral Hepat*. 19: 301–306.

43. Blackard, J., N. Kemmer, and K. Sherman. 2006. Extrahepatic replication of HCV: insights into clinical manifestations and biological consequences. *Hepatology* 44: 15–22.

44. Bain, C., A. Fatmi, F. Zoulim, J. Zarski, C. Trépo, and G. Inchauspé. 2001. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* 120: 512–524.

45. Bowen, D., and C. Walker. 2005. Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* 436: 946–952.

46. Rehermann, B. 2009. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. *J. Clin. Invest.* 119: 1745–1754.

47. Shoukry, N., A. Grakoui, M. Houghton, D. Chien, J. Ghrayeb, K. Reimann, and C. Walker. 2003. Memory $CD8^+$ T cells are required for protection from persistent hepatitis C virus infection. *J. Exp. Med*. 197: 1645–1655.

48. Kanto, T., and N. Hayashi. 2004. Distinct susceptibility of dendritic cell subsets to hepatitis C virus infection: a plausible mechanism of dendritic cell dysfunction. *J Gastroenterol*. 39: 811–812.

49. Scarlett, U., J. Cubillos-Ruiz, Y. Nesbeth, D. Martinez, X. Engle, A. Gewirtz, C. Ahonen, and J. Conejo-Garcia. 2009. In situ stimulation of CD40 and Toll-like receptor 3 transforms ovarian cancer-infiltrating dendritic cells from immunosuppressive to immunostimulatory cells. *Cancer Res.* 69: 7329–7337.

50. Ahonen C, Doxsee C, McGurran S, Riter T, Wade W, et al. (2004) Combined TLR and CD40 triggering induces potent $CD8^+$ T cell expansion with variable dependence on type I IFN. *J. Exp. Med.* 199: 775–784.

51. Ahonen, C., A. Wasiuk, S. Fuse, M. Turk, M. Ernstoff, A. Suriawinata, J. Gorham, R. Kedl, E. Usherwood, and R. Noelle. 2008. Enhanced efficacy and reduced toxicity of multifactorial adjuvants compared with unitary adjuvants as cancer vaccines. *Blood* 111: 3116–3125.

52. Jain, S., S. Chodisetti, and J. Agrewala. 2010. CD40 signaling synergizes with TLR-2 in the BCR independent activation of resting B cells. *PLoS ONE* 6: e20651.

53. Lowin, B., M. Hahne, C. Mattmann, and J. Tschopp. 1994. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* 370: 650–2.

54. Kägi, D., F. Vignaux, B. Ledermann, K. Bürki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 265: 528–30.

55. Servet-Delprat, C., P. Vidalain, O. Azocar, F. Le Deist, A. Fischer, and C. Rabourdin-Combe. 2000. Consequences of Fas-mediated human dendritic cell apoptosis induced by measles virus. *J. Virol.* 74: 4387–4393.

56. Fugier-Vivier, I., C. Servet-Delprat, P. Rivailler, M. Rissoan, Y. Liu, and C. Rabourdin-Combe. 1997. Measles virus suppresses cell-mediated immunity by interfering with the survival and functions of dendritic and T cells. *J. Exp. Med.* 186: 813–823.

57. Ruggieri, A., T. Harada, Y. Matsuura, and T. Miyamura. 1997. Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. *Virology* 229: 68–76.

58. Zhu, N., A. Khoshnan, R. Schneider, M. Matsumoto, G. Dennert, C. Ware, and M. Lai. 1998. Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. *J. Virol.* 72: 3691–3697.

59. Honda, M., S. Kaneko, T. Shimazaki, E. Matsushita, K. Kobayashi, L. Ping, H. Zhang, and S. Lemon. 2000. Hepatitis C virus core protein induces apoptosis and impairs cell-cycle regulation in stably transformed Chinese hamster ovary cells. *Hepatology* 31: 1351–1359.

60. Thorén, F., A. Romero, M. Lindh, C. Dahlgren, and K. Hellstrand. 2004. A hepatitis C virus-encoded, nonstructural protein (NS3) triggers dysfunction and apoptosis in lymphocytes: role of NADPH oxidase-derived oxygen radicals. *J. Leuk. Biol.* 76: 1180–1186.

61. Albert, M., S. Pearce, L. Francisco, B. Sauter, P. Roy, R. Silverstein, and N. Bhardwaj. 1998. Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* 188: 1359–1368.

62. Russo, V., S. Tanzarella, P. Dalerba, D. Rigatti, P. Rovere, A. Villa, C. Bordignon, and C. Traversari. 2000. Dendritic cells acquire the MAGE-3 human tumor antigen from apoptotic cells and induce a class I-restricted T cell response. *Proc. Natl. Aca. Sci.* 97: 2185–2190.

63. Nogueira, C., T. Lindsten, A. Jamieson, C. Case, S. Shin, C. Thompson, and C. Roy. 2009. Rapid pathogen-induced apoptosis: a mechanism used by dendritic cells to limit intracellular replication of Legionella pneumophila. *PLoS Pathog.* 5: e1000478.

64. Nieda, M., A. Kikuchi, A. nicol, Y. Koezuka, Y. Ando, S. Ishihara, N. Lapteva, T. Yabe, K. Tokunaga, K. Tadokoro, and T. Juji. 2001. Dendritic cells rapidly undergo apoptosis *in vitro* following culture with activated $CD4^+$ Valpha24 natural killer T cells expressing CD40L. *Immunology* 102: 137–145.

65. Kushwah, R., J. Wu, J. Oliver, G. Jiang, J. Zhang, K. Siminovitch, and J. Hu. 2010. Uptake of apoptotic DC converts immature DC into tolerogenic DC that induce differentiation of $Foxp3^+$ Treg. *Eur. J. Immunol.* 40: 1022–1035.

66. Albert, M., B. Sauter, and N. Bhardwaj. 1998. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 392: 86–9.

67. Larsson, M., E. Babcock, A. Grakoui, N. Shoukry, G. Lauer, C. Rice, C. Walker, and N. Bhardwaj. 2004. Lack of phenotypic and functional impairment in dendritic cells from chimpanzees chronically infected with hepatitis C virus. *J. Virol.* 78: 6151–6161.

68. Rovere, P., C. Vallinoto, A. Bondanza, M. Crosti, M. Rescigno, P. Ricciardi-Castagnoli, C. Rugarli, and A. Manfredi. 1998. Bystander apoptosis triggers dendritic cell maturation and antigen-presenting function. *J.Immunol.*161: 4467–71.

69. Jahan, S., S. Khaliq, M. Siddiqi, B. Ijaz, W. Ahmad, U. Ashfaq, and S. Hassan. 2010. Anti-apoptotic effect of HCV core gene of genotype 3a in Huh-7 cell line. *Virol. J.* 8: 522.

70. Dolganiuc, A., and G. Szabo. 2011. Dendritic cells in hepatitis C infection: can they (help) win the battle? *J. Gastroenterol.* 46: 432–47.

CHAPTER-3

Down regulation of granzyme B expression in effector T cells as a new mechanism of immune evasion in hepatitis C virus infection: Potential role of F and core proteins

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3.1 INTRODUCTION

Presently, 170 million people are chronically infected with hepatitis C virus (HCV) (1-5). Chronic HCV infection leads to liver cirrhosis, liver failure, and hepatocellular carcinoma (HCC), and is one of the major causes of liver transplantation in North America (3-5).

HCV is a single-stranded positive sense RNA virus belonging to the *Flaviviridae* family and the *hepacivirus* genus (6). It has seven major genotypes and each genotype has several subtypes (7). Within an individual host, large numbers of quasispecies of HCV can exist. Error prone RNA dependent RNA polymerase causes the emergence of escape mutants resistant to neutralizing antibody and $CD8^+$ cytolytic T lymphocytes (8, 9). Design of a preventive or therapeutic vaccine is a major challenge for HCV due to the high genetic variability of the virus and the existing gaps in our understanding of protective immune responses. However, there is encouraging evidence of protective immunity in HCV pathogenesis: 20–35% of infected people spontaneously clear the virus due to induction of efficient host immune responses, which suggests that the design of a vaccine capable of clearing the virus is possible (10). In chimpanzee studies, it has been shown that both antibody and cellular immune responses are partially effective in controlling HCV infection (11-13). However, chronic/persistent infection in the majority (65–80%) of infected people suggests that this virus has devised multiple strategies to evade the immune system. The factors and mechanisms that allow the virus to circumvent the host's immune

responses and to persist in infected individuals are not clearly understood. Various mechanisms of T cell failure have been suggested, such as impaired primary T cell activation, T cell exhaustion, T cell dysfunction, impaired T cell maturation, suppression of T cell function by viral factors, unresponsiveness due to exposure to high antigen levels, impaired dendritic cell functions, and suppression of T cell function by regulatory T cells (14-21).

The HCV genome encodes a single polyprotein precursor of ~ 3010 aa (1), which, after processing by host and viral proteases, generates 10 different viral proteins: the Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (22). In addition, a protein within the core encoding region—the F protein or alternate reading frame protein (ARFP)—has been reported to be expressed from an alternative +1 reading frame (22-24). The F protein is localized in the endoplasmic reticulum (ER) region, implying that it has a role in viral morphogenesis (25). Previous work suggests that the F protein is expressed in natural HCV infections (26). B and T cell mediated immune responses specific to ARFP have been detected in HCV patients (27). It has also been reported that anti-F antibody is significantly increased in patients with HCC, suggesting a possible role and/or presence of F protein during carcinogenesis (26). The F protein is not essential for viral replication in cell culture or *in vivo* (28), but its role in disease progression and in the development of HCC has not been ruled out.

We have shown that HCV derived antigens NS3 and core play disparate roles in the development of effector T cells and the reduction of viral titers in a

recombinant-HCV-vaccinia infection model (29). In the current study, we examined T cell responses generated in mice against F and core antigens using recombinant adenovirus vectors expressing these proteins. Our results demonstrate that T cells obtained from F and core immunized mice proliferate weakly against the respective antigen derived peptides. Interestingly, high levels of IL-10 and low levels of IFN-γ were produced in peptide-stimulated cultures. We observed that expression of intracellular GrB in $CD4^+$ and $CD8^+$ T cells obtained from F and core immunized mice was much lower than expression in a control vector or in NS3 immunized mice. Diminished expression of GrB by $CD8^+$ T cells from F or core immunized mice correlated with reduced killing of peptide loaded EL-4 target cells. When cultured with exogenous IL-2 *in vitro*, splenocytes from F and core immunized mice increased GrB expression leading to elevated levels of GrB in both $CD4^+$ and $CD8^+$ T cells. *In vitro* treatment of splenocytes with a chemical inhibitor of NF-κB abolished the expression of GrB in T cells in all immunized groups, suggesting that modulation of NF-κB activity might in part be responsible for the lower expression of GrB. In line with this hypothesis, we observed that immunization of mice with a toll-like receptor TLR-3 ligand, poly I:C, along with F or core containing adenovirus, recovered GrB expression levels in T cells. To our knowledge, this is the first study to reveal down regulation of GrB expression as a means of modulating effector T cell responses in a chronic infectious disease; this might represent a new mechanism of immune evasion by the hepatitis C virus.

3.2 MATERIALS AND METHODS

3.2.1 Mice

Six to eight week old male C57bl/6 mice were purchased from Charles River Laboratories Inc. (Canada). All animal experimental protocols were approved by the University of Alberta Animal Care and Use Committee for Health and Sciences, and conducted in accordance with University of Alberta, Edmonton, Canada, guidelines.

Recombinant adenovirus vectors containing NS3, core, and no antigen (control vector) have been described by our group (29, 30). The F protein was PCR amplified from full-length clones of HCV H77 cDNA using forward primer 5'-GCG CGG ATC CATGGG TGC GAG AGC GTC GGT -3' and reverse primer 5'-GCG CGG ATC CTT ATT GTG ACG AGG GGT CGC T-3' and used to prepare recombinant adenovirus vector using methodologies described by our group (30). The adenovirus vectors were propagated and amplified in 293A cells and the presence of the F gene was confirmed by sequencing.

3.2.2 Immunization of mice

Mice were injected intramuscularly in both hind limbs with a total of 2×10^7 replication-deficient recombinant adenovirus particles expressing HCV-F, core, NS3, or control vector in 150 μl volume for immune response studies. For three immunizations, mice were injected on days 0, 10, and 20 (Appendix Figure-7). The NS3 immunization group was used as a positive control in most of the

experiments. For immunization with poly I:C (polyinosinic:polycytidylic acid, Sigma-Aldrich, St. Louis, MO), 2×10^7 replication-deficient recombinant adenovirus particles expressing HCV-F, core, NS3, or control vector and 20 μg of poly I:C were mixed in phosphate buffered saline (PBS) prior to injection (used only for single immunization experiments).

3.2.3 Isolation of splenic T cells

On day 8 after immunization, mice were euthanized to obtain splenocytes. The spleens were pooled from 3 mice from each group and a single cell suspension was prepared by disrupting the spleen between frosted slides and filtering the contents through a Falcon 100 μm nylon cell strainer. After centrifugation, RBCs were lysed and T cells were enriched using nylon wool columns as described previously (29). These enriched T cells (~ 90% $CD3^+$ T cells) were used in various experiments.

3.2.4 T cell proliferation assay

Proliferative responses of splenic T cells were measured in triplicate cultures in 96-well flat-bottomed microtiter plates. A total of 4×10^5 T cells from HCV-F or core immunized mice and 4×10^5 antigen presenting cells (APCs) (spleen cells from naïve unimmunized mice irradiated with 18 Gy) were mixed with 1 μg/ml and 10 μg/ml synthetic peptides derived from HCV-F and core proteins, (Gensrcipt, NJ, USA), and cultured in complete RPMI medium (Hyclone laboratories, South Logan, UT) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) at 37 °C and 5% CO_2 for 4 days. The wells were

pulsed with 0.5 μCi/well [^{3}H]-thymidine (GE Healthcare, Morgan Boulevard, Canada) for 12–18 h and harvested on filter papers (PerkinElmer, Waltham, MA) using a 96-well cell harvestor (Tomtech, Hamden, CT). Measurement of [^{3}H]-thymidine incorporated into the DNA of proliferating cells was performed in a Microbeta Trilux liquid scintillation counter (PerkinElmer, Waltham, MA). Stimulation indices were calculated by dividing the counts per minute (CPM) obtained in presence of peptides with that in the dimethyl sulfoxide (DMSO) control (solvent for dissolving peptides). Proliferation is represented as the mean stimulation index ± SE (standard error) of triplicate cultures from a representative experiment of six repeated experiments.

3.2.5 Cytokine ELISA

Cytokines secreted in the supernatant of proliferating cultures were measured using sandwich ELISA kits as described in the manufacturer's protocol (eBioscience, San Diego, CA) for the presence of IL-10, IFN-γ, TNF-alpha, TGF-β, and IL-12. Dilutions of 1:5 to 1:20 were used for the samples with standards ranging from 15.6 to 2000 pg/ml. ELISA plates were read and the concentrations of cytokines were calculated with an automated ELISA plate reader (Fluostar Optima, BMG Labtech, Allmendgruen, Germany).

3.2.6 Flow cytometry analysis of surface markers and intracellular GrB

A total of 1×10^6 splenocytes from HCV-F, core, NS3, or control vector immunized mice were cultured *in vitro* for 5 days in complete RPMI media. On day 5, cells were collected for intracellular and extracellular staining with

fluorescently labeled mAbs (concentrations according to manufacturer's instructions). The cells were washed with ice-cold FACS-buffer (PBS with 2% fetal bovine serum). Anti-mouse CD3e-PE, CD4-PE-Cy5, CD8aAPC-eflour 780, and CD107b-FITC (eBioscience, San Diego, USA) antibodies were added and cells were incubated at 4°C for 30 min. The cells were washed twice with FACS buffer and fixed in 2% paraformaldehyde in FACS buffer for 10 min. The cells were incubated in cold FACS-buffer + 0.3% saponin (Sigma Aldrich, St. Louis, USA) for 5 min, followed by addition of IL10-FITC, IFN-γ PE (eBioscience, San Diego, CA) or anti-granzyme B antibody labeled with Alexa fluor 647 (Biolegend, San Diego, USA), and further incubated for 30 min at 4 °C. The cells were washed twice with FACS-buffer containing 0.3% saponin and fixed using fixing buffer. The cells were also stained with isotype control antibodies using similar methodologies, read in FACS-Canto II, and analyzed using FACS-DIVA (Becton Dickinson, Mountain View, USA) software. The cells were gated to exclude 98% of isotype-matched control monoclonal antibodies stained cells and analyzed for each marker. To analyze the stained cells, lymphocytes were gated based on side scattered (SSC) and forward scattered (FSC) light, followed by gating for $CD3^+$ T cells. The $CD3^+$ T cells were then gated on either $CD4^+$ or $CD8^+$ T cells, followed by examining GrB expression, which is shown as histograms in most of the figures.

3.2.7 Purification of $CD4^+$ and CD^+ T cells

$CD4^+$ T and $CD8^+$ T cells populations were purified from the 5-day whole splenocyte cultures by magnetic affinity column separation using magnetic beads

(EasySep, Mouse $CD4^+$ and $CD8^+$ T cells selection kit; StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. In brief, ; 2-3 x 10^7 splenocytes were re-suspended in 100 µl of PBS containing (2% Fetal Bovine Serum and 1 mM EDTA) and incubated with EasySep Positive Cocktail at 100 µl/ml at room temperature for 15 minutes. Then added magnetic nanoparticles 50 µl/ml and incubated for 10 min at RT. Further, bring volume up to 2.5 ml by adding recommended buffer (PBS containing 2% Fetal Bovine Serum and 1 mM EDTA). Place the tube in magnet for 5 min. After 5 min, inverted the magnet to pour off the supernatant fraction. Repeat this for 3 times to get gets pure $CD4^+$ and $CD8^+$T cells separately. The cells that bind to the column were flushed out with 2 ml buffer and contained purified $CD4^+$ and $CD8^+$ T. These purified populations were found to be at >97% purity by flow cytometry.

3.2.8 Granzyme (GrB) enzyme assay

GrB was assayed according to methods reported previously (31). In brief, from a 5-day *in vitro* culture of splenocytes obtained from mice immunized with various recombinant adenovirus vectors, a total of 2×10^6 viable splenocytes were resuspended in 50 µl of lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.2, 1% (v/v) Triton X-100) for 10 min on ice. Supernatants were collected following a 10 min microfuge spin (10,000 × *g*). Fifty microliters of each lysate was preincubated with the pancaspase inhibitor Z-VAD-FMK (Enzo life sciences, Farmingdale, NY) for 30 min before addition of the GrB substrate. The paranitroanilide substrate, acetyl-Ile-Glu-Thr-Asp-paranitroanilide (Ac-IETD-pNA; Enzo life

sciences, Farmingdale, NY), was used at 200 μM in reaction buffer containing 50 mM HEPES (pH 7.5), 10% (w/v) sucrose, 0.05% (w/v) CHAPS, and 5 mM DTT. GrB activity was determined by hydrolysis of the substrate at 37 °C in 96-well flat-bottom tissue culture plates (Nalgene Nunc International, Penfield, NY) in a final volume of 100 μl. Released paranitroanilides were measured with a spectrophotometer (Fluostar Optima, BMG Labtech, Allmendgruen, Germany) as absorbance at 405 nm. Enzymatic activity was quantified using a standard curve of recombinant mouse GrB (Enzo life sciences, Farmingdale, NY) activity with dilutions from 2 to 200 U/ml and normalized to total protein content.

3.2.9 CFSE-based EL4 killing assay

The cytolytic activity in 5 day mouse splenocyte cultures was measured against EL4 and peptide-pulsed EL4 target cells at different effector to target ratios in a 4 h CFSE loss assay. EL4 cells were propagated for 2–3 passages and incubated overnight with pools of synthetic peptides (1 μg/ml) derived from F or core protein separately. EL4 cells were harvested and stained with 2 mM of CFSE (5-(and 6)-carboxyfluorescein diacetate succinimidyl ester, Invitrogen, Carlsbad, USA) in PBS for 15 minutes in the dark. CFSE stained target EL4 cells (10,000 cells) were mixed with effector splenocytes in ratios 1:10, 1:30, 1:60, and 1:100. Effector and target cell mixtures were incubated for 4 hours at 37 °C under an atmosphere of 5% CO_2. After 4 hours, 20,000 CountBright beads (Invitrogen, Carlsbad, CA) were added to each tube (Appendix Figure-8). Using FACSDiva to gate the beads, the flow cytometer was run to collect 10,000 beads from each tube

to normalize the amount of cells tested in each tube. The difference in CFSE stained cells between treatment and control tubes indicate the number of cells that were killed. Data are represented as numbers of dead cells in each culture (normalized by a constant number of beads).

3.2.10 Reversal of GrB expression by IL-2 treatment *in vitro*

Splenocytes were cultured with and without murine recombinant IL-2 at 100 pg/ml (Biosource, Grand Island, NY) for 5 days. On day 5, cells were stained with CD3e-FITC, CD4-PE-Cy5, CD-8-APC-eFlour 780, and GrB-Alexa flour 780 (eBioscience, San Diego, CA), followed by flow cytometry analyses of stained cells. For negative control isotype from respective antibody was used.

3.2.11 Suppression of GrB expression by inhibiting NF-κB activity

Splenocytes obtained from mice immunized with various recombinant adenovirus vectors were cultured in the presence or absence of a chemical inhibitor of NF-κB, pyrrolidine dithiocarbamate (PDTC), at 10 μM for five days, followed by staining with fluorescently labeled antibodies and flow cytometry analyses. For negative control isotype from respective antibody was used.

3.2.12 Reversal of GrB expression by polyI:C *in vivo*

Mice were immunized with recombinant adenovirus vectors containing F, core, NS3, or CV (no antigen), along with the TLR-3 agonist poly I:C at 50 μg/ml concentration. Splenocytes obtained from the immunized mice were cultured for five days *in vitro*, and GrB expression was examined in $CD4^+$ and $CD8^+$ T cells.

3.2.13 Statistical Analysis

Statistical analysis was done by one way ANOVA using Graphpad Prism

(Graphpad Software Inc., La Jolla, CA, USA). '*' indicates significant difference at $P<0.05$, and '***' indicates $P<0.0007$.

3.3 RESULTS

3.3.1 *In vivo* priming and stimulation of peptide specific T cell responses against HCV-F and core antigens

To study the generation of immune response against HCV derived F and core antigens, C57Bl/6 mice were immunized with 2×10^7 recombinant adenovirus particles containing HCV-F, core and NS3 proteins or no protein (control vector or CV). Eight days after a single immunization, splenic T cells were isolated and cultured *in vitro* with APCs in the presence of synthetic peptides from respective proteins at 1 and 10 µg/ml concentrations. Synthetic peptides derived from antigens and used in this study are listed in table 1 and table 3. Mice immunized with F or core protein containing adenovirus vectors showed no proliferation or low levels of proliferation against all of the peptides tested. Similar background levels of proliferation were observed in CV immunized mice (Figure 3-1). In contrast, NS3 immunized mice exhibited significantly high proliferation against selected synthetic peptides Figure 3-1, 29). These results suggest that F and core antigens are either poorly immunogenic in mice or that they down regulate immune responses. Proliferative responses against mitogens such as concanavalin

A (con A) were similar in splenocytes obtained from F, core, NS3, or CV immunized mice (Figure 3-1). Interestingly, similar results were obtained after 3 immunizations, where proliferative responses against F and core derived peptides were not substantially increased and were at the same level obtained after a single immunization (Figure 3-2).

We analyzed the production of cytokines IL-10, IFN-γ, TGF-β, IL-12, and TNF-α in the culture supernatants. T cells from mice immunized with F or core, when cultured with respective antigen derived peptides at both 1 and 10 μg/ml concentrations, produced high levels of IL-10 compared to the control group (cytokines were analysed from culture supernatant of T cells cultured with 1 μg/ml of peptides). No IFN-γ or very low levels of IFN-γ were detected in these cultures. The levels of IL-10 produced in cultures from CV immunized mice were below the level of detection or very low against all of the core and F peptides tested (Figure 3-3). Antigen dependent IL-10 expression were also analysed from $CD4^+$ and $CD8^+$ T cells (Figure 3-4). Amounts of IL-12, TGF-β, and TNF-α were below the detection level in all groups against all of the peptides tested.

3.3.2 Phenotypic analysis of T cells from mice immunized with F and core antigens from HCV

In the present study, we examined the expression of several negative regulatory markers (e.g., PD-1 and CTLA-4) on T cells from F and core immunized mice. However, we did not find a significant difference between F, core, CV, and NS3

immunized mice (Figure 3-5). Also, F-antigen immunized mice did not show an upregulation of Tregs (Figure 3-6).

3.3.3 Immunization with F and core antigens of HCV results in reduced GrB expression in $CD4^+$ and $CD8^+$ T cells

To determine the effector function of T cells stimulated after *in vivo* immunization with core or F HCV proteins, we examined the production/expression of GrB within these cells. Whole splenocytes obtained after one or three immunizations were cultured *in vitro* for 5 days in complete media, followed by staining for CD3, CD4, CD8, and intracellular GrB. Percentages of $CD4^+GrB^+$ and $CD8^+GrB^+$T cells were observed to be significantly lower in both F and core immunized mice than in control vector immunized mice (Figure 3-7). In contrast, splenocytes from the positive control—NS3 immunized mice—showed significantly higher expression of GrB in both $CD4^+$ and $CD8^+$ T cells as we reported previously (29). Notably, the results of one and three immunizations were similar in all of the antigens and control vector groups (Figure 3-8). To our knowledge a reduction in GrB expression in T cells with any antigen or pathogen has not been reported, and suggests a new mechanism of T cell modulation by viral (HCV) antigens.

3.3.4 Reduced expression of GrB is not due to degranulation of T cells

Degranulation of GrB containing T cells could reduce intracellular levels of GrB in splenocytes. To determine whether reduced intracellular GrB levels in T cells was due to lower expression of GrB or degranulation, we stained the splenocytes

obtained from HCV-F and core immunized mice with antibodies against CD107b (LAMP-2) after 5 days culture *in vitro*. CD107b is a marker for degranulation of cytotoxic T cells. CD107b expression was similar in $CD4^+$ and $CD8^+$ T cells obtained from F, core, and control vector immunized mice, but were slightly higher in cells obtained from NS3 immunized mice (Figure 3-9). These results confirm that reduction in GrB expression in T cells in F and core immunized mice is due to differential expression of GrB rather than degranulation of GrB containing granules.

3.3.5 Enzyme activity of GrB is reduced in T cells obtained from F and core immunized mice

To confirm that the expression of GrB in T cells corresponds to GrB enzyme activity, we used the synthetic substrate Ac-IEPD-pNA (32). Cell lysates prepared from splenocytes or purified $CD4^+$ and $CD8^+$ T cells obtained from mice immunized with different HCV derived antigens were used in GrB activity assays (Figure 3-10). We observed that splenocytes and $CD4^+$ and $CD8^+$ T cells from F and core immunized mice had slightly reduced GrB activity compared to control vector immunized mice. The splenocytes and T cells obtained from NS3 immunized mice showed higher GrB enzymatic activity compared to F, core, or CV immunized mice, correlating with efficient induction of effector T cells and increased intracellular GrB expression against NS3. These results confirm our observation of differential intracellular GrB expression. The decrease in GrB enzyme activity in T cells obtained from mice immunized with F and core,

although visible, did not appear significantly lower as compared to CV, possibly because of the lower limit of detection by spectrophotometer; background ODs were obtained in these groups.

3.3.6 Reduction in GrB expression in T cells obtained from F and core immunized mice correlates with reduced killing of peptide loaded targets

To determine the ability of T cells with reduced expression of GrB to kill peptide loaded target cells, the reduction in number of dead CFSE labeled target cells was examined. The assay was performed at four different effector to target (E:T) ratios. After 5 days of *in vitro* culture, specific peptide pool loaded and CFSE labeled EL-4 cells were incubated with splenocytes obtained from F, core, CV, or NS3 immunized mice. We observed that, compared to CV immunized mice, splenocytes obtained from F and core immunized mice had significantly reduced ability to kill peptide loaded EL-4 cells at all of the E:T ratios used (data shown with E:T 30:1, Figure 3-11). The positive control—splenocytes from NS3 immunized mice—demonstrated significantly high killing of peptide-loaded targets compared to CV immunized mice (Figure 3-11). These results provide further confirmation that T cells obtained from mice immunized with HCV derived F and core antigens have lower ability to kill target cells than T cells obtained from CV immunized mice.

3.3.7 Addition of exogenous IL-2 partially reverses the decrease in GrB expression in splenocytes obtained from F and core immunized mice

IL-2 is necessary for proliferation, growth, and differentiation of T cells into effector cells. We examined whether treatment with exogenous IL-2 *in vitro* can reverse the reduction in GrB expression in T cells from F and core immunized mice. Splenocytes obtained from F, core, or CV immunized mice were cultured with IL-2 for 5 days and examined for intracellular GrB expression in $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells. In the presence of IL-2, upregulation of GrB expression was significant in $CD4^+$ and $CD8^+$ T cell populations from CV immunized mice (Figure 3-12). T cells obtained from core and F immunized mice also demonstrated higher expression of GrB in the presence of IL-2 compared to cultures with no added IL-2, but GrB expression in the presence of IL-2 did not reach the levels observed in T cells from CV immunized mice (Figure 3-12), suggesting that IL-2 only partially reverses the decrease in GrB expression observed in the T cells of F and core immunized mice.

3.3.8 Modulation of GrB expression is NF-κB mediated

GrB expression is regulated through the NF-κB pathway in activated T cells. IL-2 in human has been shown to activate transcription of GrB through NF-κB activation (33). Also, it has been shown that there is an NF-κB binding site downstream of the GrB gene, which is responsible for GrB gene activation and which is functional *in vitro* and *in vivo* in natural killer (NK) and T cells (33). TLR agonists also activate NF-κB and lead to upregulation of GrB in effector T cells by binding to TLRs expressed on T cells (34). We therefore examined whether T cells isolated from F or core immunized mice and cultured in the

presence or absence of IL-2, poly I:C, and/or PDTC (a known inhibitor of NF-κB) exhibited further reduction in GrB expression (Figure 3-13). In the presence of PDTC, GrB expression was almost completely inhibited in both $CD4^+$ and $CD8^+$ T cells, even under strong NF-κB stimulatory conditions such as the presence of IL-2 and poly I:C (Figure 3-13). These results indirectly suggest that NF-κB modulation may be involved in down regulation of GrB upon immunization with F or core containing recombinant adenovirus vectors. To substantiate this observation, we immunized mice with recombinant adenovirus vectors containing F, core, NS3, or CV (no antigen), along with the TLR-3 agonist poly I:C (Figure 3-10). Splenocytes obtained from the immunized mice were cultured for five days *in vitro*, and GrB expression was examined in $CD4^+$ and $CD8^+$ T cells (Figure 3-14). Intriguingly, the decrease in GrB expression induced by HCV-derived F and or core protein was not only completely reversed in T cells upon co-immunization of mice with F or core and poly I:C, but GrB^+CD4^+ and GrB^+CD8^+ T cell levels were also significantly higher in both F and core immunized mice, compared to CV immunized mice, suggesting the induction of functional effector T cells in response to HCV-F and core antigens. It was interesting to note that the levels of GrB^+CD4^+ and GrB^+CD8^+ T cells in both F and core immunized mice were similar or higher than that of NS3 immunized mice (a positive control) (Figure 3-14). Mice immunized with F and core containing adenovirus vectors along with TLR-3 agonist Poly I:C showed similar level of proliferation (Figure 3-15) as well as IL-10 and IFN-γ expression (Figure 3-16) as compared to mice immunized only with F and core containing adenovirus vectors.

3.4 DISCUSSION

HCV causes acute and chronic infection in humans. HCV is spontaneously cleared in 20–35% of infected patients, but persists in 65–80% of its hosts due to insufficient innate and adaptive immune responses. The mechanisms of immune evasion by HCV have been investigated extensively (21, 35-38) but are not yet clearly understood. In this study we reveal a new mechanism that might contribute to the inefficiency of T cell responses generated in chronic HCV infection—the disarming of effector $CD4^+$ and $CD8^+$ T cells' ability to kill infected target cells. Effector T cells provide antiviral immunity and clearance of viral infection by killing virus-infected target cells. Several viruses and/or viral antigens interfere with the class I antigen processing and presentation machinery, leading to inefficient presentation of viral antigens for recognition by cytotoxic T lymphocytes (CTLs) (39, 40). In an early report, whole irradiated or paraformaldehyde fixed parainfluenza virus type 3 was shown to inhibit GrB mRNA in human PBMCs and NK cells (41). However, protein expression and the functional impact of the decrease in GrB mRNA were not studied.

Expression of co-inhibitory molecules such as PD-1 and CTLA-4 has been shown to be upregulated in chronic HCV infection, suggesting a mechanism of down regulation of T cell responses (29, 42-44). However, these reports have mostly used peripheral blood T cells from chronic HCV infected patients with long-term virus persistence, and may be more representative of T cell exhaustion due to continued antigen stimulation. Our findings of T cell modulation are

distinct and unexpected in that after a single immunization with HCV-F or core antigen, even in the context of recombinant adenoviral vector, substantial reduction in GrB expression was observed after eight days. In contrast, another HCV antigen, NS3, in the context of the same adenovirus vector, induced T cells with greatly upregulated GrB production. The diverse roles of GrB in cellular immunity include apoptosis of intracellular infected cells by caspase dependent and independent mechanisms (45), autoimmune destruction of pancreatic β cells in type 1 diabetes (46), effector T cell mediated clearance of tumors (47)**,** and graft rejection (48). GrB positive $CD4^+$ and $CD8^+$ T cells have also been shown to have a significant role in antiviral immunity (49-52).

Of several HCV strategies to evade the immune system (37), a major mechanism is the modulation of T cell response. A lower expression of GrB upon priming of certain antigen specific T cells could alter the effectiveness of T cell activity in HCV infection. It is possible that intracellular levels of GrB appear to be down regulated because activated effector T cells have undergone degranulation. However, our results with CD107b expression (Figure 3-7) suggested this is not the cause of GrB reduction in F and core immunized mice. Interestingly, splenocytes cultured from NS3 immunized mice showed higher CD107b expression, indicative of their target-cell killing activity. It has been reported that GrB expression can be altered due to different rates of degranulation in activated T cells (55). Cell surface expression of CD107b indicates recent cytotoxic cell degranulation and has been used as a surrogate marker for GrB/perforin mediated killing by effector T cells (56).

Our group have previously reported immune response generated in mice immunized with adenovirus containing HCV core and NS3 proteins. Our group observed that antigen specific splenic T cells from NS3 immunized mice proliferated strongly after secondary stimulation of recombinant NS3 protein or immunodominant peptides derived from NS3 (29). Here, I also demonstrated that immunodominant peptides from core HCV proteins did not show proliferative responses. In the current study, we examined proliferative responses of T cells obtained from F and core immunized mice against a series of overlapping peptides (Figure 3-1). Interestingly, none of the peptides caused significant T cell proliferation above background levels of CV immunized mice. Based on these results, one would assume that F and core antigens are simply not immunogenic in H-2^b background. However, when the supernatants of this peptide stimulated cultures were examined for the presence of cytokines, surprisingly, we found that significantly high levels of IL-10 were produced in response to several of the F and core peptides (Figure 3-3). In further experiment we observed that both $CD4^+$ and $CD8^+$ T cells produce IL-10 in antigen dependent manner (Figure 3-4). However, the stimulation of T cells by specific peptides is the most apparent factor, because there was no IL-10 produced in control (no peptide) group, and there was no or very little IL-10 detected in CV immunized mice (Figure 3-4). Interestingly, in the F and core groups, we did not detect IFN-γ, TNF-α, TGF-β, and IL-12. The cytokine data suggested that F and core proteins are not simply nonimmunogenic, but rather actively modulate T cell responses. We earlier observed induction of a high percentage of Tregs in core-immunized mice (29). In

the present study, we examined the expression of several negative regulatory markers (e.g., PD-1 and CTLA-4) on T cells from F and core immunized mice. However, we did not find a significant difference between F, core, CV, and NS3 immunized mice (Figure 3-5). Also, F-antigen immunized mice did not show an upregulation of Tregs (Figure 3-6), unlike core immunized mice (29).

To determine the physiological significance of modulated GrB expression, we examined GrB enzyme activity in lysates prepared from whole splenocytes or purified $CD4^+$ and $CD8^+$ T cells obtained from F, core, CV, or NS3 immunized mice using a substrate cleavable by active GrB (Figure 3-10). Interestingly, the enzyme activity in NS3 cell lysates was significantly higher than in other lysates, suggesting the functional relevance of high intracellular levels of GrB in NS3 immunized mice. However, the enzyme activity in cell lysates from F and core groups was visibly lower than the CV group and significantly lower than the NS3 group. The enzymatic activity in F and core groups did not seem to be dramatically reduced compared to the CV group as expected from intracellular GrB expression, possibly due to the low sensitivity of the spectrophotometer. Because of the interference of background noise, upregulation of a biological activity was more easily quantified than down regulation. The fact that enzyme activities obtained in F and core lysates were greatly reduced compared to NS3 lysates, however, substantiates our observation and provides a physiological significance to down regulation of intracellular GrB expression. Another parameter of functional significance of GrB expression is the potency of effector T cells in killing HCV infected cells. The $H\text{-}2^b$ restricted peptides are not yet

reported for F and core antigens; therefore, we used EL-4 cells loaded with pools of peptides derived from F and core antigens. Similar to the enzyme activity determination, the killing assay has the limitation of measuring down regulation. We therefore used a CFSE assay to examine the reduction in the number of CFSE loaded cells in the CTL assay, instead of examining the percent of target cells killed (Figure 3-11). We observed that effector T cells generated from F and core immunized mice have lower ability to kill target cells than CV or NS3 immunized mice (Figure 3-11). These results further substantiate the low potency of effector T cells from F and core immunized mice in killing infected cells. In these experiments, target cell killing is not attributed to the presence of cytotoxic cytokines such as IFN-γ because (i) IFN-γ was not detected in F and core immunized mice and (ii) effector cells were washed extensively before they were mixed with target cells in the 4 h killing assay. Increased target cell killing in NS3 immunized mice is further supportive of our observation that $CD107b^+$ T cells were observed in a higher percentage in splenocyte cultures of this group (Figure 3-9).

It has been reported that IL-2 treatment activates and enhances GrB expression in splenocytes (57). We observed that addition of exogenous IL-2 enhanced the expression of GrB in splenocytes obtained from F or core immunized mice in both $CD4^+$ and $CD8^+$ T cells (Figure 3-12). However, the percentage of cells expressing GrB was still lower than the percentage in splenocytes cultured in the presence of IL-2 from CV immunized mice, suggesting that exogenous IL-2 was only partially able to recover the expression

of GrB. GrB expression is also regulated through NF-κB mediated activation (Appendix Figure-9). It has been shown that the GrB gene has a downstream sequence that can bind NF-κB and activate GrB transcription (33). We used the NF-κB inhibitor PDTC to examine if direct inhibition of NF-κB in T cells would lead to inhibition of GrB expression (58). Addition of PDTC to *in vitro* cultures of splenocytes leads to significant or almost complete inhibition of GrB expression in both $CD4^+$ and $CD8^+$ T cells obtained from F, core, CV, and NS3 immunized mice (Figure 3-13). Also, splenocytes cultured with IL-2 and treated with PDTC strongly inhibited GrB expression, suggesting that IL-2 treatment cannot bypass the NF-κB mediated pathway of GrB regulation. These results indirectly support the notion that immunization of mice with HCV-F and core protein could lead to down regulation of NF-κB activity resulting in reduced expression of GrB. It is not clear whether it is the inhibition of NF-κB in T cells or in APCs that leads to modulation of GrB in T cells. Several studies have reported that core and other HCV antigens inhibit NF-κB activation in dendritic cells (DCs) (21, 59, and 60). Also, a defect in NF-κB activation has been demonstrated in DCs obtained from chronically HCV infected patients (61, 62). However, direct inhibition of NF-κB activation in T cells has not been well studied. If NF-κB modulation leads to a reduction in GrB expression in T cells obtained from mice immunized with F and core antigens, the mechanism of this modulation is not clear and is being investigated in our laboratory. To support the plausible mechanism of GrB reduction through modulation of NF-κB, we reasoned that TLR agonists, which are strong activators of NF-κB, might be able to reverse the reduction of GrB

expression if injected together with F and core containing adenovirus vectors. We chose poly I:C, a TLR-3 agonist that represents double stranded RNA (63). We observed that immunization with F or core containing adenovirus vectors admixed with poly I:C dramatically increased GrB expression in both $CD4^+$ and $CD8^+$ T cells (Figure 3-14). In fact, the GrB expression was significantly higher than GrB expression in CV immunized T cells. However there was no change in T cell proliferation (Figure 3-15) and IL10 expression (Figure 3-16). These results suggest that (i) immune modulation of effector T cells caused by F and core proteins can be restored to normal levels with the TLR-3 agonist poly I:C and (ii) a TLR agonist can provide an efficient way to generate effector T cells against HCV antigens F and core. It will be interesting to see if the immune modulatory/suppressive effect of specific viral antigens can be reversed *in vivo*. TLR ligands appear to promote the capacity of DCs to induce T cell responses and are also known to act as adjuvants in the activation of antigen specific T cells (64, 65). Synthetic TLR-3 ligands such as poly I:C has been known to act as potent immune adjuvants by enhancing DC cross-presentation and promoting $CD8^+$ T cell responses (66). However, our study is the first to show that poly I:C can reverse F and core mediated dysfunction in effector T cells, through reversal of the suppression of GrB expression. Whether this reversal is by direct action on T cells or indirectly through DCs or other cells, is not yet clear.

Our work has uncovered a new mechanism of immune modulation by HCV derived F and core proteins, wherein modulation of GrB expression in effector T cells makes them less effective in terms of killing target cells. To our

knowledge**,** the down regulation of GrB by other viral antigens and the implications of this process for antiviral immunity have not been reported. This study opens new avenues in understanding T cell modulation by other pathogens. GrB expression is crucial for effective T cell responses in the clearance of virus-infected cells. Our studies also suggest that adding a TLR-3 agonist as an adjuvant in a vaccine could effectively reverse the T cell function in terms of GrB expression, however, cytokines likes IL-10 and IFN-γ expression was not changed. Our findings are relevant to the design and investigation of prophylactic and/or therapeutic vaccines for chronic HCV infection. Further work is required to understand the mechanisms and signaling pathways involved in the modulation of GrB expression and cytokines expression. It would also be interesting to investigate the reduction of GrB in antigen specific T cell. This could be achieved by tetramer staining for T cells in transgenic mice with HCV-F or core protein specific peptides.

3.5 FIGURES

Figure 3-1

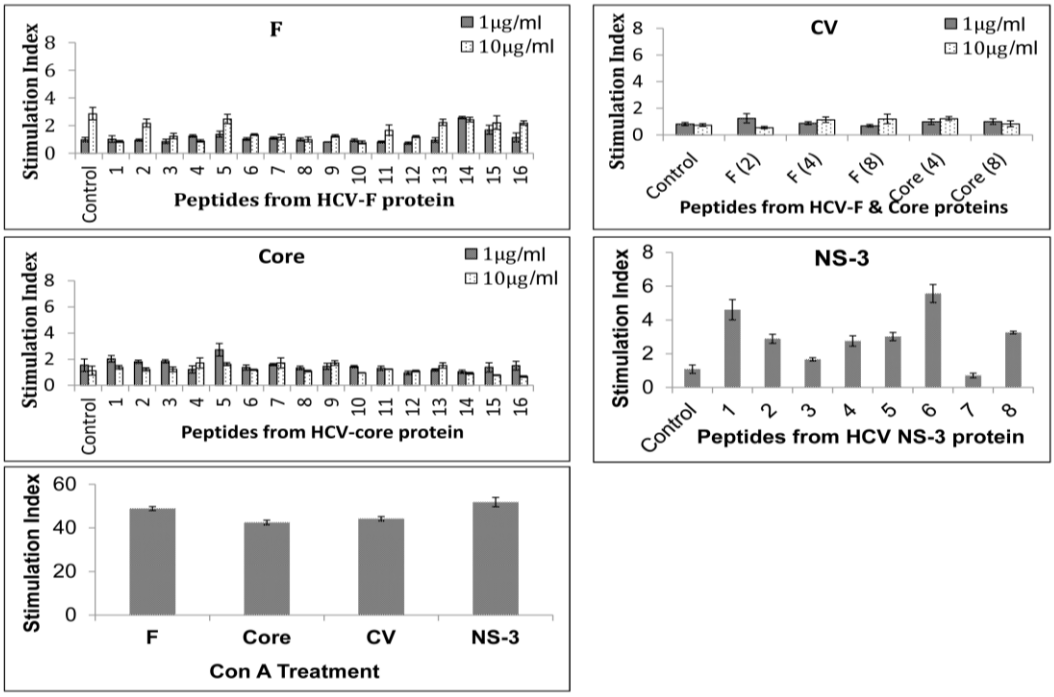


Figure 3-1. Antigen specific proliferation of T cells after single intramuscular immunization. Peptide specific proliferation of T cells after single immunization of mice with 2×10^7 replication deficient adenovirus containing HCV-F, core and control vector (CV). Enriched T cells were cultured *in vitro* with irradiated syngeneic APCs in presence of respective synthetic overlapping peptides at concentration of 1 and 10µg/ml. T cells and APCs in the absence of peptides served as control. Cells cultured in the presence of comparable concentrations of DMSO were used as background to determine the stimulation indices. Stimulation against ConA as well as from NS3 (at concentration 1µg/ml of peptides) group has shown to serve as positive control for proliferation. Data represent average stimulation index±SE from triplicate wells. Results are representative of 4 independent experiments .

Figure 3-2

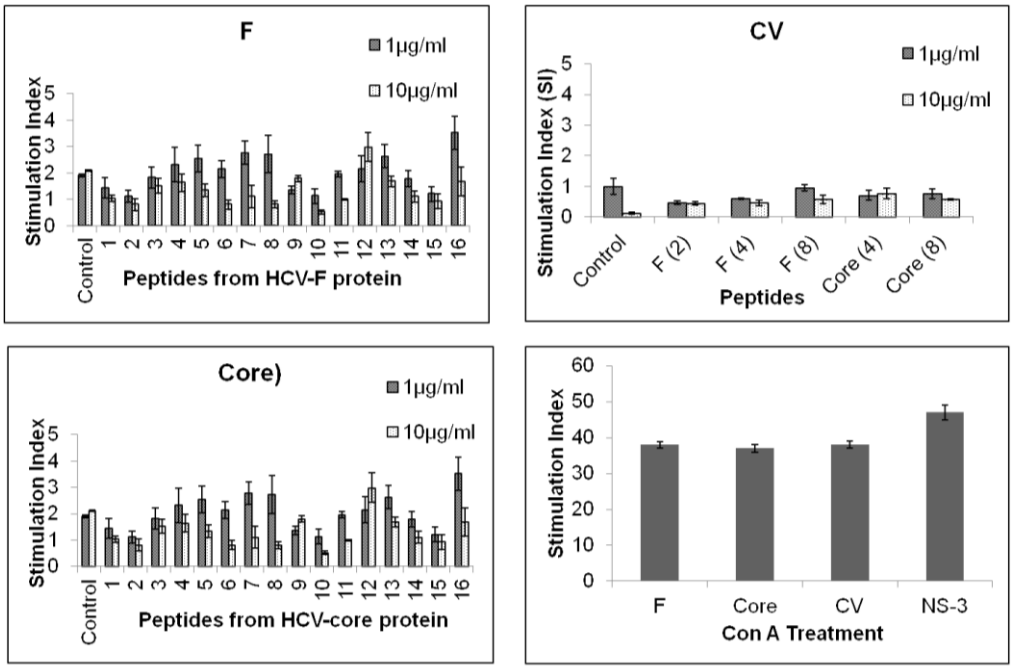


Figure 3-2. Antigen specific proliferation of T cells after third intramuscular immunizations. Peptide specific proliferation of T cells after third immunizations of mice with 2×10^7 replication deficient adenovirus containing HCV-F, core and control vector (CV). Enriched T cells were cultured *in vitro* with irradiated syngeneic APCs in presence of respective synthetic overlapping peptides at concentration of 1µg/ml and 10µg/ml. T cells and APCs in the absence of peptides served as control. Cells cultured in the presence of comparable concentrations of DMSO were used as background to determine the stimulation indices. Stimulation against ConA group has shown to serve as positive control for proliferation. Data represent average stimulation index$\pm$SE from triplicate wells. Results are representative of 3 independent experiments.

Figure 3-3

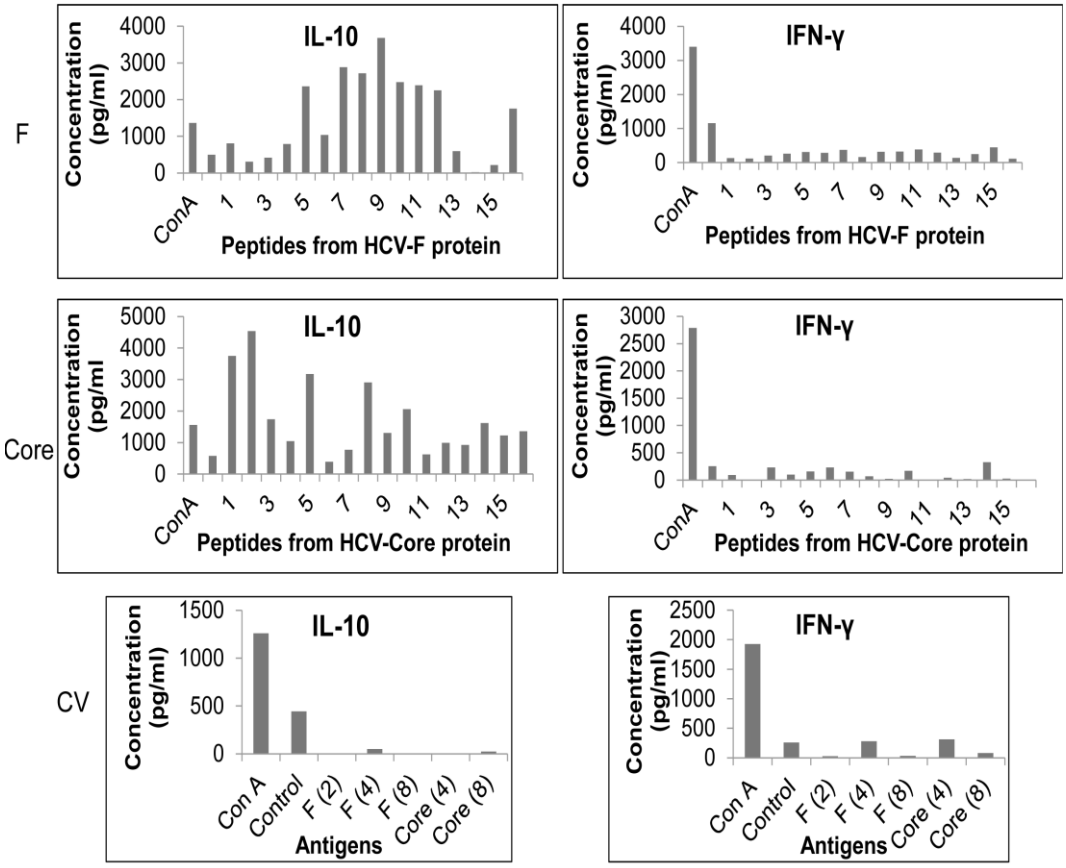


Figure 3-3. T cell cultures stimulated with F and core derived overlapping peptides have high levels of IL-10 and very low or undetectable IFN-gamma. T cells obtained from spleens of immunized mice were cultured with irradiated APCs and 1 and 10 μg/ml of respective peptides. Supernatants from T cell cultures were collected on day 4 and cytokines from 1μg/ml group was analyzed by ELISA. Assays were done in duplicates and average values are shown. Controls represent culture without peptides. Con A represents the positive control for assay. Results are representative of two repeated experiments.

Figure 3-4

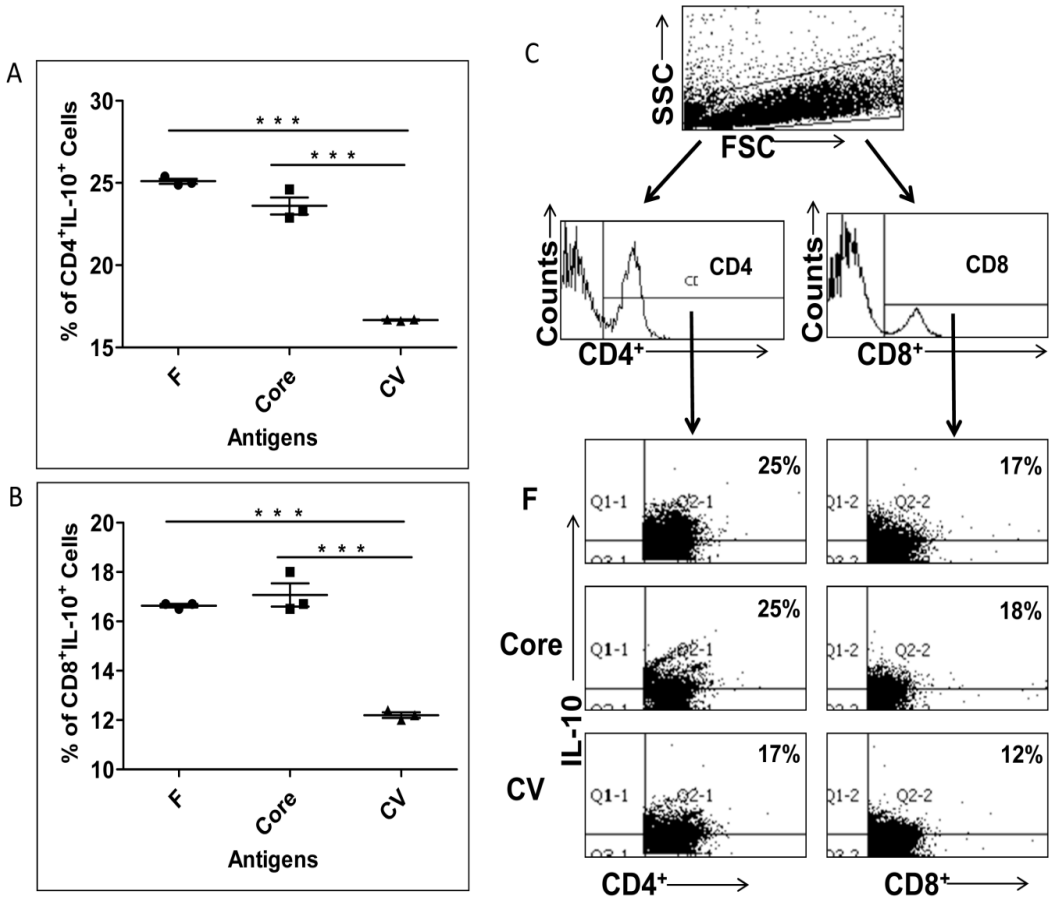


Figure 3-4. Increased expression of IL-10 by T cell from F and core immunized mice. Splenocytes were obtained from immunized mice and cultured for five in days *in vitro*, followed by staining with extracellular CD4, CD8 and intracellular IL-10. T cells were first gated based on $CD4^+$ or $CD8^+$ expression. Intracellular IL-10 expressing cells are shown in $CD4^+$ or $CD8^+$ T cell populations as dot plot. Data shown is from three mice done individually in A and B. Figure 3C shows a representative histogram of $CD4^+IL\text{-}10^+$ T cells (left panel) $CD8^+IL10^+$ T cells (right panel). Data are representative of 2 repeated experiments.

Figure 3-5

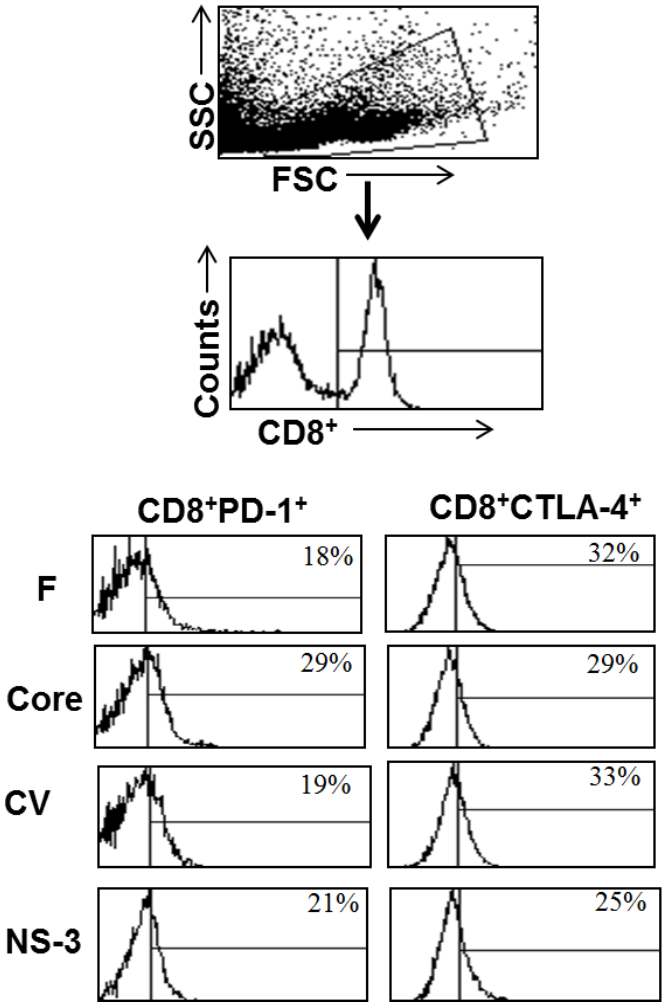


Figure 3-5. Phenotypic analysis of T cell from mice immunized with HCV-derived F, core, NS-3 or control vector for expression of PD-1 and CTLA-4. Splenocytes were obtained from immunized mice and cultured for five in days *in vitro*, followed by staining for CD8, PD-1 and CTLA-4. T cells were first gated based on $CD8^+$ expression. Figure shows a representative histogram of $CD8^+PD\text{-}1^+$ T cells (left panel) $CD8^+CTLA\text{-}4^+$ T cells (right panel). Data are representative of 3 repeated experiments.

Figure 3-6

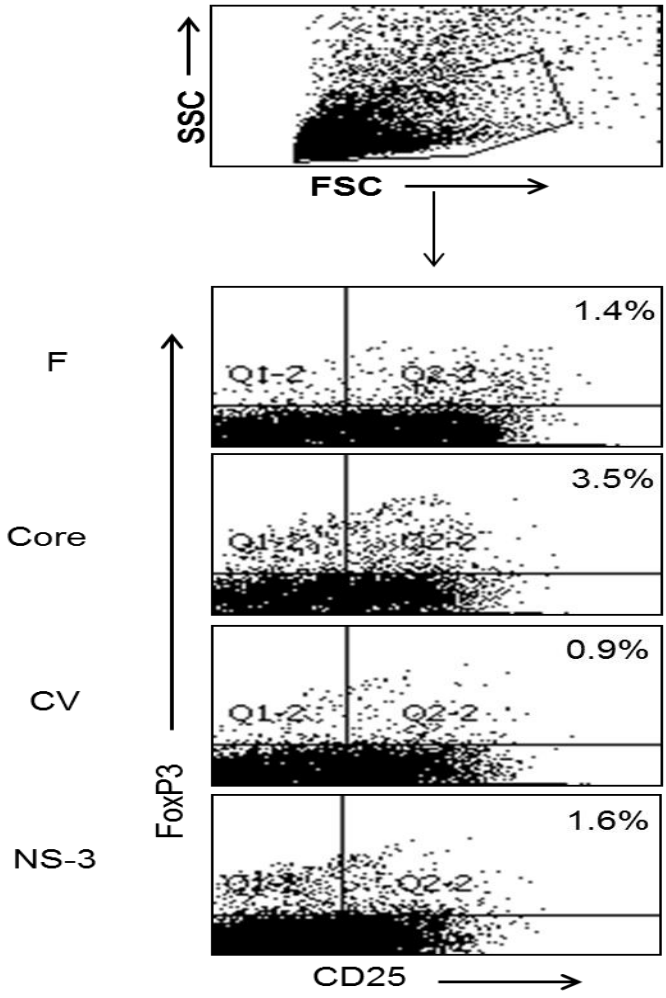


Figure 3-6. Phenotypic analysis of T cell from mice immunized with HCV-derived F, core, NS-3 or control vector for expression of Treg cells. Splenocytes were obtained from immunized mice and cultured for five in days *in vitro*, followed by purification of $CD4^+$ T cells. $CD4^+$ T cells were further stained for FoxP3 and CD-25 markers. Data shown is representative from 3 different experiments.

Figure 3-7

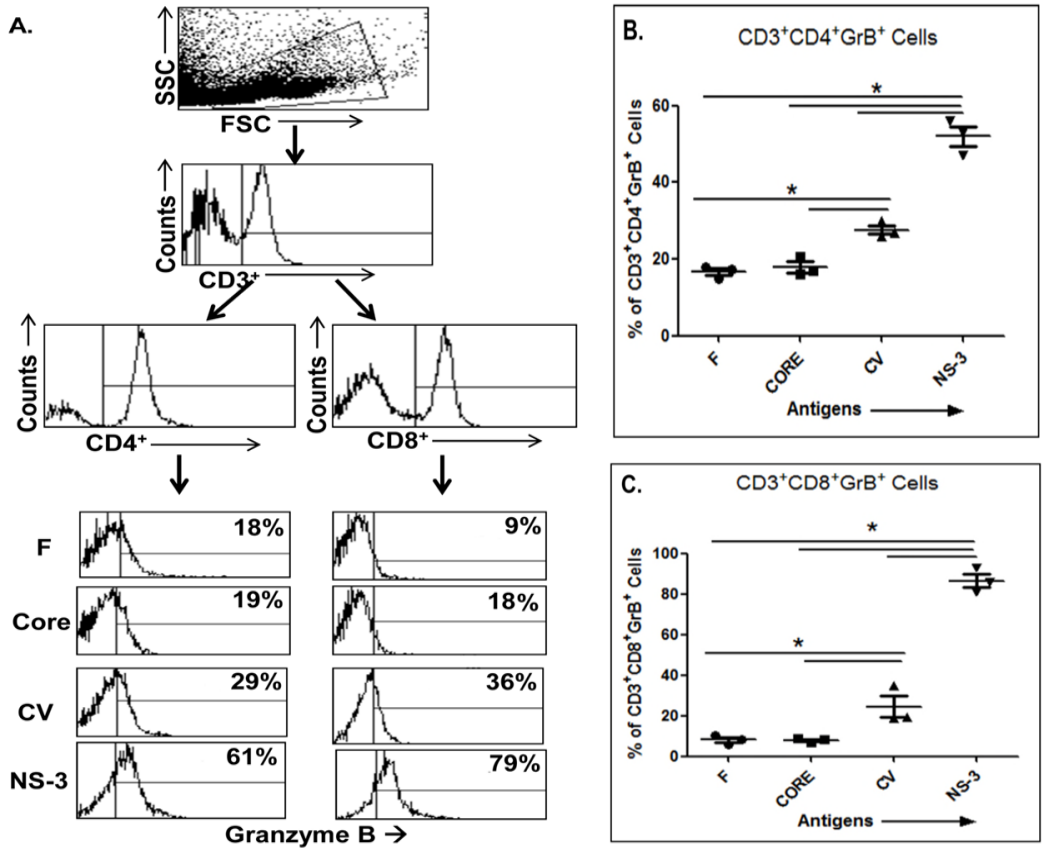


Figure 3-7. Reduced GrB production in T cells after single immunization with F or core containing recombinant adenovirus vectors. Splenocytes were obtained from immunized mice and cultured for five in days *in vitro*, followed by staining with extracellular CD3, CD4, CD8 and intracellular GrB. T cells were first gated based on $CD3^+$ expression and then further gated based on $CD4^+$ or $CD8^+$ expression. Intracellular GrB^+ expressing cells are shown in $CD4^+$ or $CD8^+$ T cell populations as histograms. Data shown is from three mice done individually in A and B. Figure 3C shows a representative histogram of $CD4^+$ GrB^+ T cells (left panel) $CD8^+$ GrB^+ T cells (right panel). Data are representative of 6 repeated experiments.

Figure 3-8

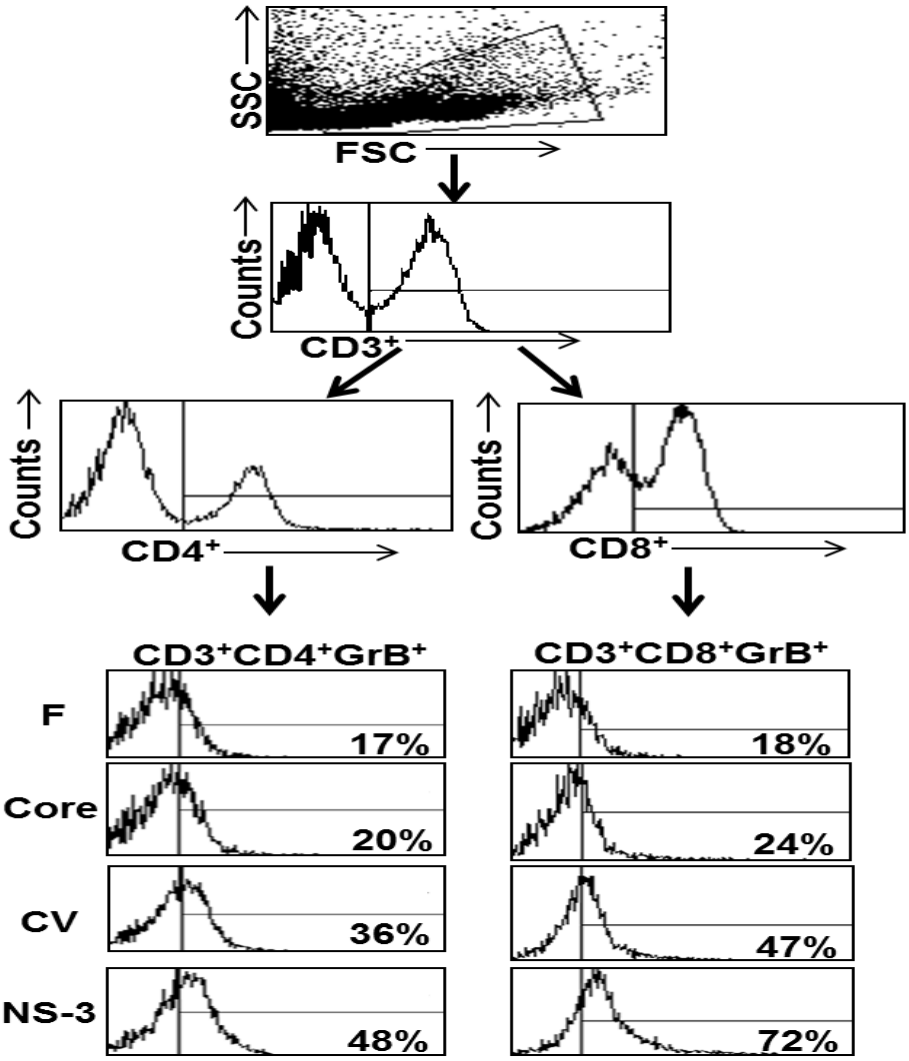


Figure 3-8 Reduced GrB production in T cells after three immunizations with F or core containing recombinant adenovirus vectors. T cells were stained with extracellular CD3, CD4, CD8 and intracellular GrB. T cells were first gated based on $CD3^+$ expression and then further gated based on $CD4^+$ or $CD8^+$ expression. Intracellular GrB^+ expressing cells are shown in $CD4^+$ or $CD8^+$ T cell populations as histograms. Data shown are representative from three different experiments.

Figure 3-9

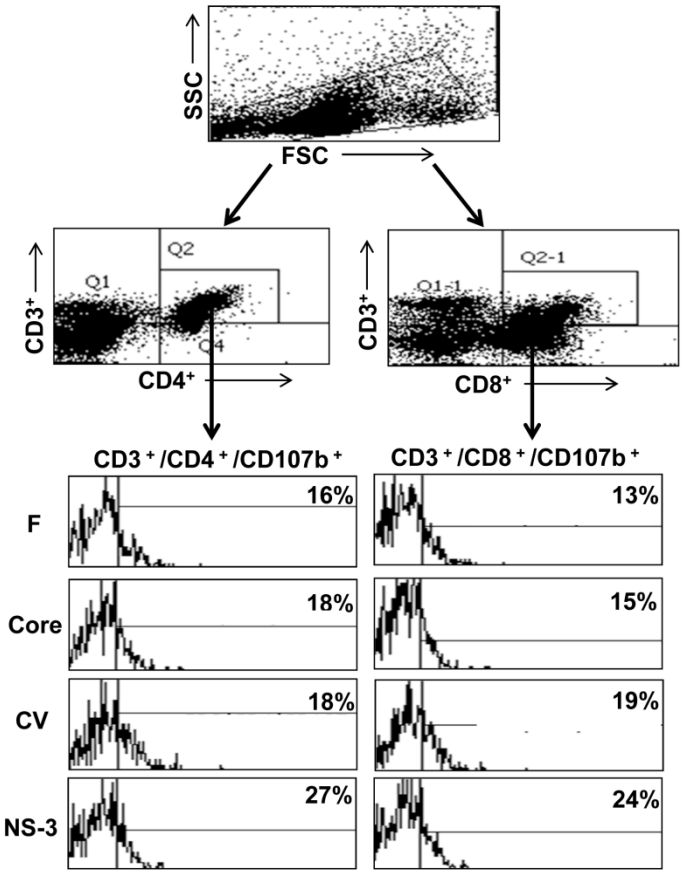


Figure 3-9. Reduction in GrB expression is not due to degranulation. Mice were immunized with recombinant adenovirus vectors ($2x10^7$/mouse) containing F, core, NS3 or no antigen (CV). Spleens were obtained 8 days after immunization and cultured for five days, followed by staining with CD3, CD4, CD8 and CD107b. T cells were gated based on $CD3^+$ and $CD4^+/CD8^+$ expression. $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells were then examined for CD107b expression. Each histogram shows the percentage of $CD3^+CD4^+CD107b^+$ T cells (left panel) and $CD3^+CD8^+CD107b^+$T cells (right panel) in different immunization groups. The data are representative of five independent experiments.

Figure 3-10

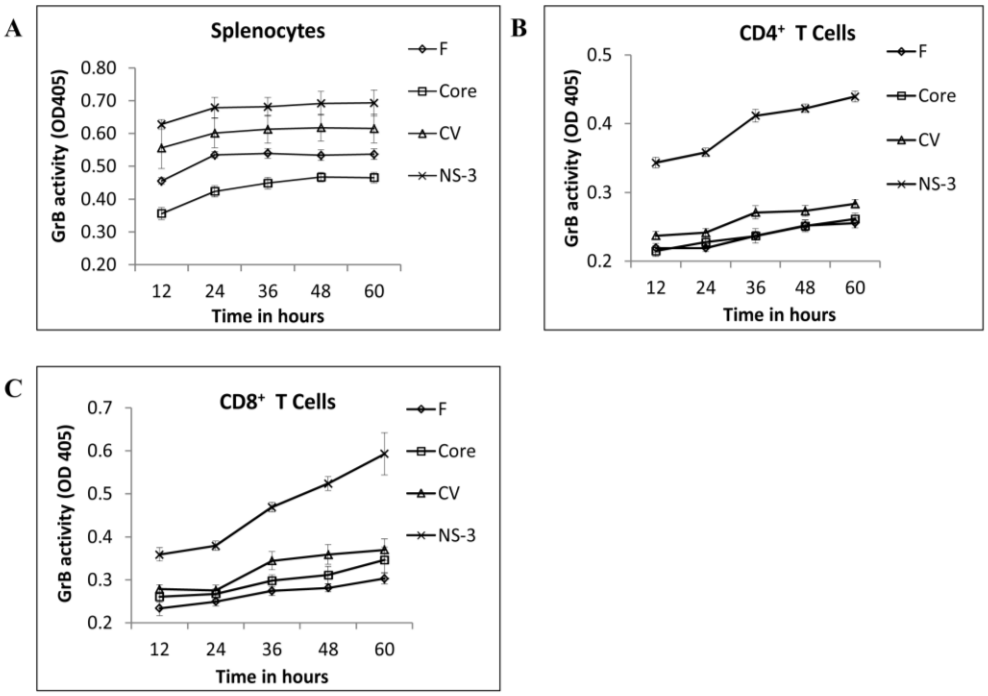


Figure 3-10. T cells from F and core immunized mice have lower enzymatic activity compared to control vector and NS3 immunized mice. Splenocytes obtained from F, core, NS3 and control vector immunized mice were cultured for 5 days *in vitro*. Cell lysates were prepared from 1×10^6 unpurified splenocyte cultures (A), or purified $CD4^+$ (B) and $CD8^+$ (C) T cells, and GrB activity was measured as described in materials and methods using synthetic colorimetric GrB substrate Ac-IEPD-pNA.

Figure 3-11

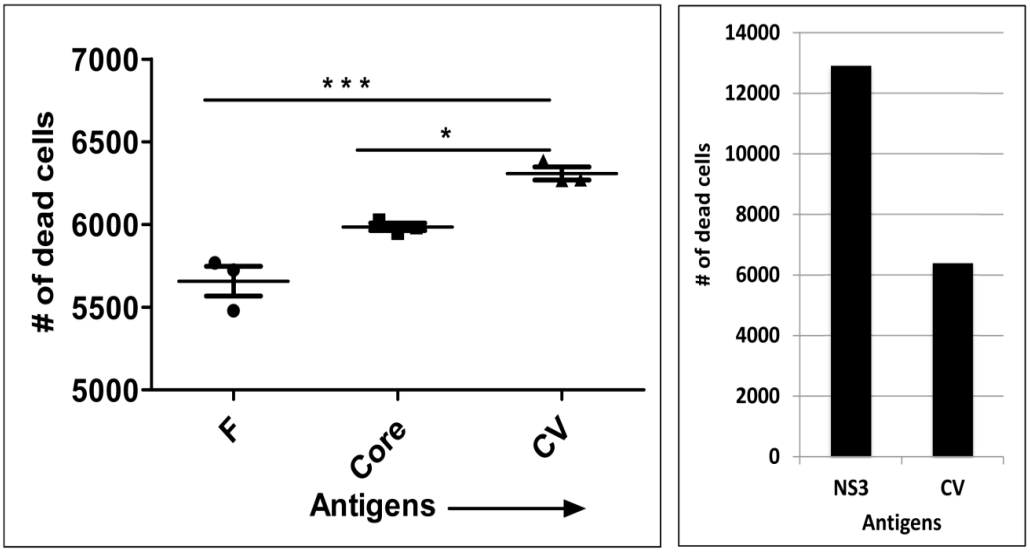


Figure 3-11. Splenocytes obtained from F and core immunized mice have lower ability to kill peptide loaded EL4 target cells. Cytolytic activity of splenocytes was measured against peptide-pulsed EL4 as target cells at 30:1 effectors to target ratio, in a 4-h CFSE loss assay. The data represent mean±SE from triplicate cultures. The experiment is a representative of 3 repeated experiments. '*' indicates $P<0.05$ and '***' indicates $P<0.0007$.

Figure 3-12

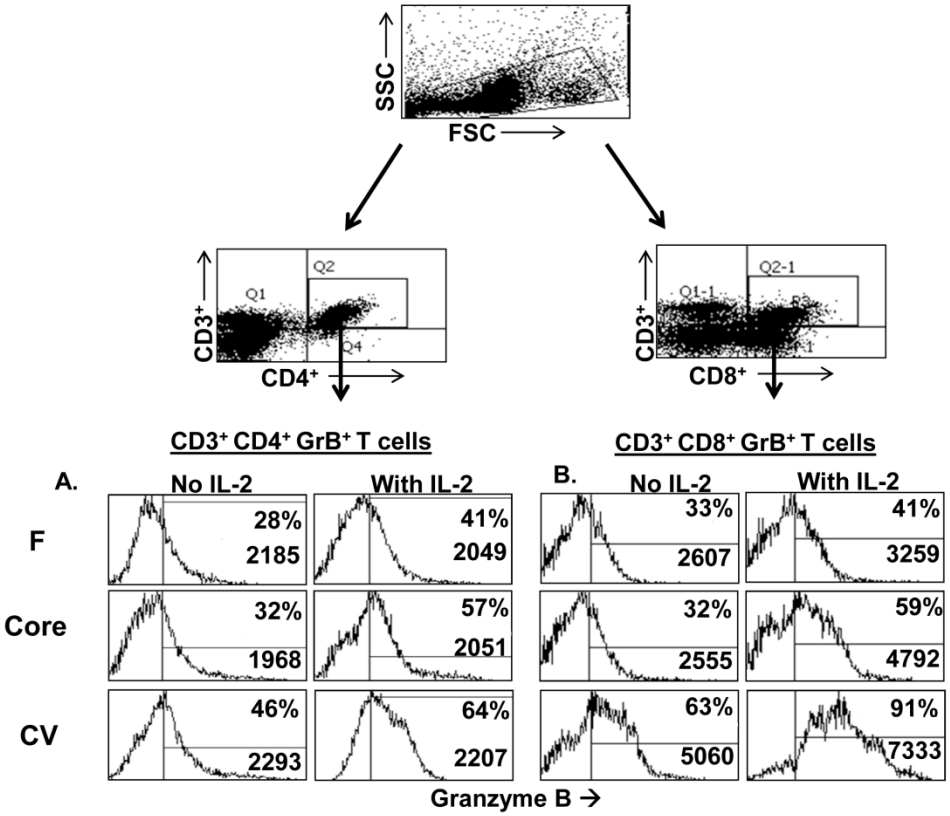


Figure 3-12. Addition of exogenous IL-2 to splenocytes partially reverses the GrB expression. Mice were immunized with replication deficient recombinant adenovirus vectors ($2x10^7$/mouse) containing HCV F, core and no antigen (CV). Splenocytes were cultured in the presence or absence of exogenous recombinant IL-2 for 5 days. Cells were then stained for CD3, CD4, CD8 and intracellular GrB. Each histogram shows the percentage of $CD3^+CD4^+$ GrB^+ T cells (A, left panel without IL-2 treatment, right panel with IL-2 treatment) and $CD3^+CD8^+GrB^+$T cells (B, left panel without IL-2 treatment, right panel with IL-2 treatment).

Figure 3-13

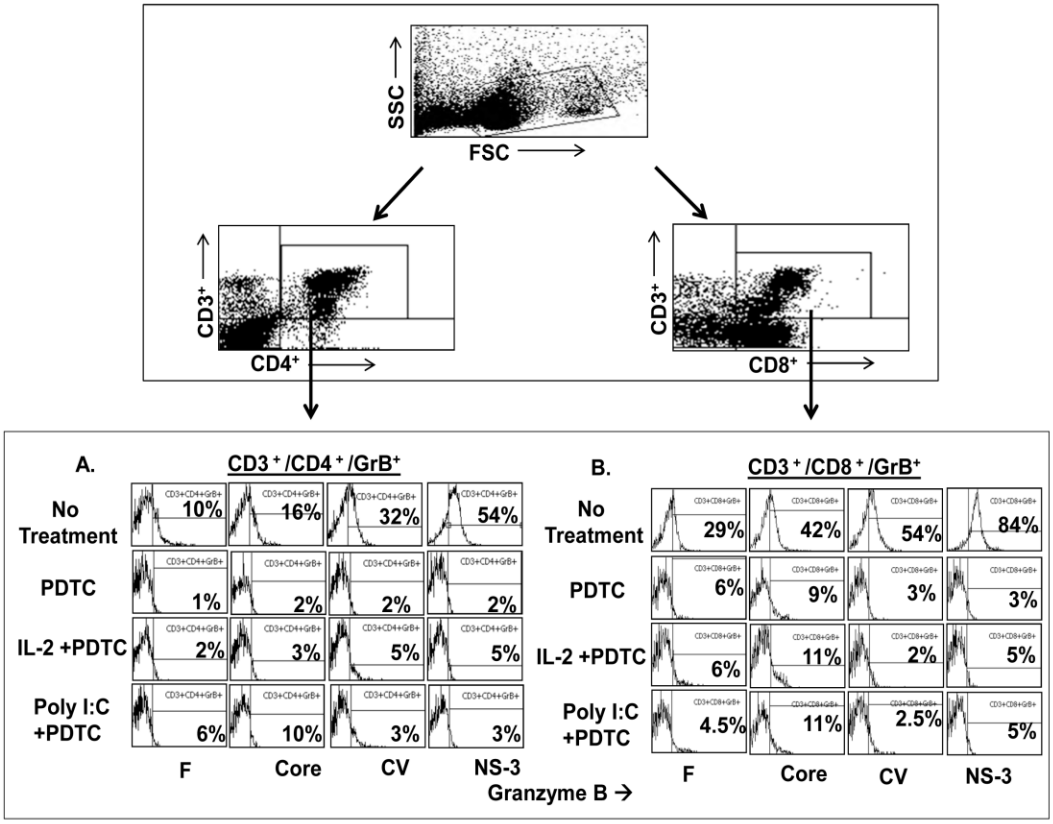


Figure 3-13. Treatment of splenocytes with PDTC in the presence or absence of IL-2 or poly I:C abrogates GrB expression in all immunized groups. Mice were first immunized with recombinant adenovector containing F, core, NS3 and no antigen (CV). Splenic T cells obtained from immunized mice were cultured for five days in the presence of PDTC (a known inhibitor of NF-κB) and presence or absence of IL-2 or polyI:C. Cells were stained and analyzed for intracellular GrB expression on (A) $CD3^+CD4^+$ T cells, and (B) $CD3^+CD8^+$ T cells. These data are representative of three independent experiments.

Figure 3-14

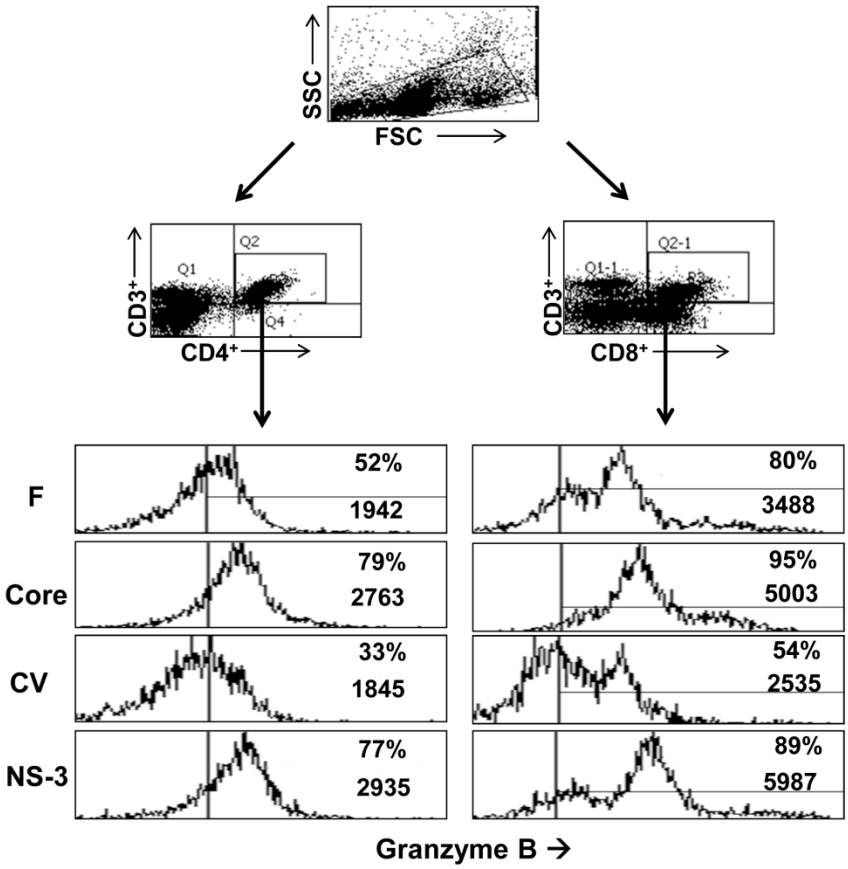


Figure 3-14. Co-immunization of recombinant adenovirus vectors containing F or core antigen with TLR-3 agonist poly I:C reverses the down-regulation of GrB expression: Mice were immunized once with recombinant adenovirus vectors along with poly I:C intramuscularly at 50µg/ml concentration. Eight days after immunization, splenocytes were harvested and cultured for five days, followed by staining with antibodies against CD3, CD4, CD8 and GrB. T cells were gated based on CD3 and then on CD4 or CD8 expression. Each histogram shows the percentage of $CD3^+CD4^+GrB^+$ T cells (left panel) and $CD3^+CD8^+GrB^+$T cells (right panel). The data are representative of three independent experiments.

Figure 3-15

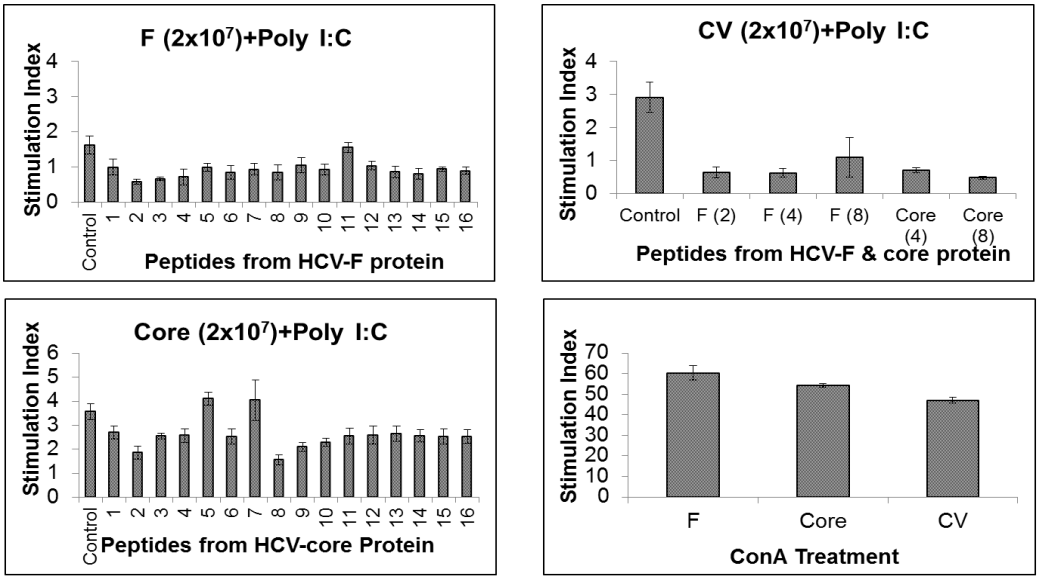


Figure 3-15. Antigen specific proliferation of T cells from mice immunized with recombinant F and core antigen along with Poly I:C after single intramuscular immunization. Peptide specific proliferation of T cells after single immunization of mice with 2×10^7 replication deficient adenovirus containing HCV-F, core and control vector (CV). Enriched T cells were cultured *in vitro* with irradiated syngeneic APCs in presence of respective synthetic overlapping peptides at concentration of 1μg/ml. T cells and APCs in the absence of peptides served as control. Cells cultured in the presence of comparable concentrations of DMSO were used as background to determine the stimulation indices. Stimulation against ConA group has shown to serve as positive control for proliferation. Data represent average stimulation index±SE from triplicate wells. Results are representative of 3 independent experiments.

Figure 3-16

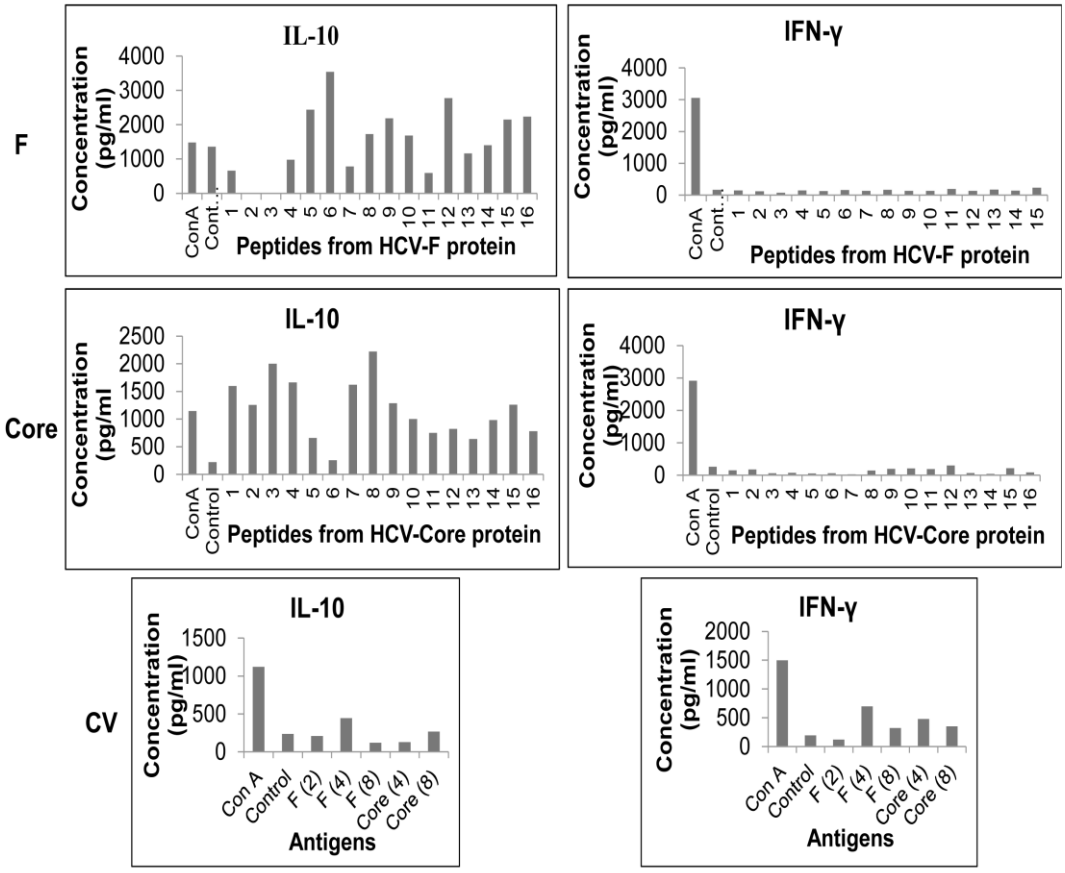


Figure 3-16. T cell from mice immunized with recombinant F and core antigen along with Poly I:C cultured with F and core overlapping peptides have high levels of IL-10 and very low or undetectable IFN-gamma. T cells obtained from spleens of antigen and TLR3 agonist poly I:C immunized mice were cultured with irradiated APCs and 1 μg/ml of respective peptides. Supernatants from T cell cultures were collected on day 4 and cytokines was analyzed by ELISA. Assays were done in duplicates and average values are shown. Controls represent culture without peptides. Con A represents the positive control for assay. Results are representative of two repeated experiments.

3.6 REFERENCES

1. Choo, Q., G. Kuo, A. Weiner, L. Overby, D. Bradley, and M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 244: 359–62.

2. Zaltron, S., A. Spinetti, L. Biasi, C. Baiguera, and F. Castelli. 2011. Chronic HCV infection: epidemiological and clinical relevance. *BMC Infect. Dis.* 12 Suppl 2: S2.

3. Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, and Y. Ohta. 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA* 87: 6547–9.

4. Chiba, T., Y. Matsuzaki, M. Abei, J. Shoda, T. Aikawa, N. Tanaka, and T. Osuga. 1996. Multivariate analysis of risk factors for hepatocellular carcinoma in patients with hepatitis C virus-related liver cirrhosis. *J. Gastroenterol.* 31: 552–8.

5. Mutimer, D., and A. Lok. 2012. Management of HBV- and HCV-induced end stage liver disease. *Gut* 61 Suppl 1: i59–67.

6. Rehermann, B. 2009. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. *J. Clin. Invest.* 119: 1745–54.

7. Gottwein, J., T. Scheel, T. Jensen, J. Lademann, J. Prentoe, M. Knudsen, A. Hoegh, and J. Bukh. 2009. Development and characterization of hepatitis C virus genotype 1-7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. *Hepatology* 49: 364–77.

8. Fafi-Kremer, S., I. Fofana, E. Soulier, P. Carolla, P. Meuleman, G. Leroux-Roels, A. Patel, F.-L. Cosset, P. Pessaux, M. Doffoël, P. Wolf, F. Stoll-Keller, and T. Baumert. 2010. Viral entry and escape from antibody-mediated neutralization influence hepatitis C virus reinfection in liver transplantation. *J. Exp. Med.* 207: 2019–31.

9. Fuller, M., N. Shoukry, T. Gushima, D. Bowen, B. Callendret, K. Campbell, D. Hasselschwert, A. Hughes, and C. Walker. 2010. Selection-driven immune escape is not a significant factor in the failure of CD4 T cell responses in persistent hepatitis C virus infection. *Hepatology* 51: 378–87.

10. Lucas, M., A. Ulsenheimer, K. Pfafferot, M. Heeg, S. Gaudieri, N. Grüner, A. Rauch, J. Gerlach, M.-C. Jung, R. Zachoval, G. Pape, W.

Schraut, T. Santantonio, H. Nitschko, M. Obermeier, R. Phillips, T. Scriba, N. Semmo, C. Day, J. Weber, S. Fidler, R. Thimme, A. Haberstroh, T. Baumert, P. Klenerman, and H. Diepolder. 2006. Tracking virus-specific CD4+ T cells during and after acute hepatitis C virus infection. *PLoS ONE*. 2: e649.

11. Choo, Q., G. Kuo, R. Ralston, A. Weiner, D. Chien, G. Van Nest, J. Han, K. Berger, K. Thudium, and C. Kuo. 1994. Vaccination of chimpanzees against infection by the hepatitis C virus. *Proc. Natl. Acad. Sci. USA* 91: 1294–8.

12. Folgori, A., S. Capone, L. Ruggeri, A. Meola, E. Sporeno, B. Ercole, M. Pezzanera, R. Tafi, M. Arcuri, E. Fattori, A. Lahm, A. Luzzago, A. Vitelli, S. Colloca, R. Cortese, and A. Nicosia. 2006. A T-cell HCV vaccine eliciting effective immunity against heterologous virus challenge in chimpanzees. *Nat. Med.* 12: 190–7.

13. Grakoui, A., N. Shoukry, D. Woollard, J.-H. Han, H. Hanson, J. Ghrayeb, K. Murthy, C. Rice, and C. Walker. 2003. HCV persistence and immune evasion in the absence of memory T cell help. *Science* 302: 659–62.

14. Keck, Z.-Y., A. Saha, J. Xia, Y. Wang, P. Lau, T. Krey, F. Rey, and S. Foung. 2011. Mapping a region of hepatitis C virus E2 that is responsible for escape from neutralizing antibodies and a core CD81-binding region that does not tolerate neutralization escape mutations. *J. Virol.* 85: 10451–63.

15. Gruener, N., F. Lechner, M. Jung, H. Diepolder, T. Gerlach, G. Lauer, B. Walker, J. Sullivan, R. Phillips, G. Pape, and P. Klenerman. 2001. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J. Virol.* 75: 5550–8.

16. Fuller, M., and A. Zajac. 2002. Ablation of CD8 and CD4 T cell responses by high viral loads. *J. Immunol.* 170: 477–86.

17. MacDonald, A., M. Duffy, M. Brady, S. McKiernan, W. Hall, J. Hegarty, M. Curry, and K. Mills. 2002. CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons. *J. Infect. Dis*. 185: 720–7.

18. Cabrera, R., Z. Tu, Y. Xu, R. Firpi, H. Rosen, C. Liu, and D. Nelson. 2004. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 40: 1062–71.

19. Ebinuma, H., N. Nakamoto, Y. Li, D. Price, E. Gostick, B. Levine, J. Tobias, W. Kwok, and K.-M. Chang. 2008. Identification and *in vitro*

expansion of functional antigen-specific CD25+ FoxP3+ regulatory T cells in hepatitis C virus infection. *J. Virol.* 82: 5043–53.

20. Manigold, T., and V. Racanelli. 2007. T-cell regulation by CD4 regulatory T cells during hepatitis B and C virus infections: facts and controversies. *Lancet Infect. Dis.* 7: 804–13.

21. Krishnadas, D., J. Ahn, J. Han, R. Kumar, and B. Agrawal. 2010. Immunomodulation by hepatitis C virus-derived proteins: targeting human dendritic cells by multiple mechanisms. *Int. Immunol.* 22: 491–502.

22. Xu, Z., J. Choi, T. Yen, W. Lu, A. Strohecker, S. Govindarajan, D. Chien, M. Selby, and J. Ou. 2001. Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. *EMBO J.* 20: 3840–8.

23. Walewski, J., T. Keller, D. Stump, and A. Branch. 2001. Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. *RNA* 7: 710–21.

24. Varaklioti, A., N. Vassilaki, U. Georgopoulou, and P. Mavromara. 2002. Alternate translation occurs within the core coding region of the hepatitis C viral genome. *J. Biol. Chem.* 277: 17713–21.

25. Xu, Z., J. Choi, W. Lu, and J. Ou. 2002. Hepatitis C virus f protein is a short-lived protein associated with the endoplasmic reticulum. *J. Virol.* 77: 1578–83.

26. Dalagiorgou, G., N. Vassilaki, P. Foka, A. Boumlic, A. Kakkanas, E. Kochlios, S. Khalili, E. Aslanoglou, S. Veletza, G. Orfanoudakis, D. Vassilopoulos, S. Hadziyannis, J. Koskinas, and P. Mavromara. 2011. High levels of HCV core+1 antibody in HCV patients with hepatocellular carcinoma. *J. Gen. Virol.* 92: 1343–51.

27. Bain, C., P. Parroche, J. Lavergne, B. Duverger, C. Vieux, V. Dubois, F. Komurian-Pradel, C. Trépo, L. Gebuhrer, G. Paranhos-Baccala, F. Penin, and G. Inchauspé. 2004. Memory T-cell-mediated immune responses specific to an alternative core protein in hepatitis C virus infection. *J. Virol.* 78: 10460–9.

28. McMullan, L., A. Grakoui, M. Evans, K. Mihalik, M. Puig, A. Branch, S. Feinstone, and C. Rice. 2007. Evidence for a functional RNA element in the hepatitis C virus core gene. *Proc. Natl. Acad. Sci. USA* 104: 2879–84.

29. Krishnadas, D., W. Li, R. Kumar, D. Tyrrell, and B. Agrawal. 2010. HCV-core and NS3 antigens play disparate role in inducing regulatory

or effector T cells *in vivo*: Implications for viral persistence or clearance. *Vaccine* 28: 2104–14.

30. Li, W., J. Li, D. Tyrrell, and B. Agrawal. 2005. Expression of hepatitis C virus-derived core or NS3 antigens in human dendritic cells leads to induction of pro-inflammatory cytokines and normal T-cell stimulation capabilities. *J. Gen. Virol.* 87: 61–72.

31. Sharma, V., M. Delgado, and D. Ganea. 2005. Granzyme B, a new player in activation-induced cell death, is down-regulated by vasoactive intestinal peptide in Th2 but not Th1 effectors. *J. Immunol.* 176: 97–110.

32. Ewen, C., K. Kane, I. Shostak, P. Griebel, E. Bertram, T. Watts, R. Bleackley, and J. McElhaney. 2003. A novel cytotoxicity assay to evaluate antigen-specific CTL responses using a colorimetric substrate for Granzyme B. *J. Immunol. Methods* 276: 89–101.

33. Huang, C., E. Bi, Y. Hu, W. Deng, Z. Tian, C. Dong, Y. Hu, and B. Sun. 2006. A novel NF-kappaB binding site controls human granzyme B gene transcription. *J. Immunol.* 176: 4173–81.

34. Alexopoulou, L., A. Holt, R. Medzhitov, and R. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413: 732–8.

35. Li, K., E. Foy, J. Ferreon, M. Nakamura, A. Ferreon, M. Ikeda, S. Ray, M. Gale, and S. Lemon. 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc. Natl. Acad. Sci. USA* 102: 2992–7.

36. Gale, M., and E. Foy. 2005. Evasion of intracellular host defense by hepatitis C virus. *Nature* 436: 939–45.

37. Pavio, N., and M. Lai. 2003. The hepatitis C virus persistence: how to evade the immune system? *J. Biosci.* 28: 287–304.

38. Ding, Q., B. Huang, J. Lu, Y.-J. Liu, and J. Zhong. 2012. Hepatitis C virus NS3/4A protease blocks IL-28 production. *Eur. J. Immunol.* 42: 2374–82.

39. Hewitt, E. 2003. The MHC class I antigen presentation pathway: strategies for viral immune evasion. *Immunology* 110: 163–9

40. Hengel, H., and U. Koszinowski. 1997. Interference with antigen processing by viruses. *Curr. Opin. Immunol.* 9: 470–6.

41. Sieg, S., L. Xia, Y. Huang, and D. Kaplan. 1995. Specific inhibition of granzyme B by parainfluenza virus type 3. *J. Virol.* 69: 3538–41.

42. Urbani, S., B. Amadei, D. Tola, M. Massari, S. Schivazappa, G. Missale, and C. Ferrari. 2006. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J. Virol.* 80: 11398–403.

43. Radziewicz, H., C. Ibegbu, M. Fernandez, K. Workowski, K. Obideen, M. Wehbi, H. Hanson, J. Steinberg, D. Masopust, E. Wherry, J. Altman, B. Rouse, G. Freeman, R. Ahmed, and A. Grakoui. 2007. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J. Virol.* 81: 2545–53.

44. Thompson, C., and J. Allison. 1997. The emerging role of CTLA-4 as an immune attenuator. *Immunity* 7: 445–50.

45. Lieberman, J. 2003. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat. Rev. Immunol.* 3: 361–70.

46. Thomas, H., J. Trapani, and T. Kay. 2010. The role of perforin and granzymes in diabetes. *Cell Death Differ.* 17: 577–85.

47. Cao, X., S. Cai, T. Fehniger, J. Song, L. Collins, D. Piwnica-Worms, and T. Ley. 2007. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 27: 635–46.

48. Graubert, T., J. Russell, and T. Ley. 1996. The role of granzyme B in murine models of acute graft-versus-host disease and graft rejection. *Blood* 87: 1232–7.

49. Aubert, R., A. Kamphorst, S. Sarkar, V. Vezys, S.-J. Ha, D. Barber, L. Ye, A. Sharpe, G. Freeman, and R. Ahmed. 2011. Antigen-specific CD4 T-cell help rescues exhausted CD8 T cells during chronic viral infection. *Proc. Natl. Acad. Sci.* 108: 21182–7.

50. Koszinowski, U., M. Reddehase, and S. Jonjic. 1991. The role of CD4 and CD8 T cells in viral infections. *Curr. Opin. Immunol.* 3: 471–5.

51. Aslan, N., C. Yurdaydin, J. Wiegand, T. Greten, A. Ciner, M. Meyer, H. Heiken, B. Kuhlmann, T. Kaiser, H. Bozkaya, H. Tillmann, A. Bozdayi, M. Manns, and H. Wedemeyer. 2006. Cytotoxic CD4 T cells in viral hepatitis. *J. Viral. Hepat.* 13: 505–14.

52. Shoukry, N., A. Grakoui, M. Houghton, D. Chien, J. Ghrayeb, K. Reimann, and C. Walker. 2003. Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. *J. Exp. Med.* 197: 1645–55.

53. Blanco, P., V. Pitard, J.-F. Viallard, J.-L. Taupin, J.-L. Pellegrin and J.-F. Moreau. 2004. Increase in activated CD8+ T lymphocytes expressing perforin and granzyme B correlates with disease activity in patients with systemic lupus erythematosus. *Arthritis and rheumatism* 52: 201–11.

54. Morissette, M., J. Parent, and J. Milot. 2006. Perforin, granzyme B, and FasL expression by peripheral blood T lymphocytes in emphysema. *Respir Res.* 8: 62.

55. Burkett, M., K. Shafer-Weaver, S. Strobl, M. Baseler, and A. Malyguine. 2004. A novel flow cytometric assay for evaluating cell-mediated cytotoxicity. *J. Immunother.* 28: 396–402.

56. Zelinskyy, G., S. Balkow, S. Schimmer, K. Schepers, M. Simon, and U. Dittmer. 2004. Independent roles of perforin, granzymes, and Fas in the control of Friend retrovirus infection. *Virology* 330: 365–74.

57. Janas, M., P. Groves, N. Kienzle, and A. Kelso. 2005. IL-2 regulates perforin and granzyme gene expression in CD8+ T cells independently of its effects on survival and proliferation. *J. Immunol.* 175: 8003–10.

58. Morais, C., H. Healy, D. Johnson, and G. Gobe. 2010. Inhibition of nuclear factor kappa B attenuates tumour progression in an animal model of renal cell carcinoma. *Nephrol. Dial. Transplant.* 25: 1462–74.

59. Joo, M., Y. Hahn, M. Kwon, R. Sadikot, T. Blackwell, and J. Christman. 2005. Hepatitis C virus core protein suppresses NF-kappaB activation and cyclooxygenase-2 expression by direct interaction with IkappaB kinase beta. *J. Virol.* 79: 7648–57.

60. Park, J., W. Kang, S.-W. Ryu, W.-I. Kim, D.-Y. Chang, D. Lee, D. Y. Park, Y.-H. Choi, K. Choi, E.-C. Shin and C. Choi. 2012. Hepatitis C virus infection enhances TNFα-induced cell death via suppression of NF-κB. *Hepatology* 56: 831–40.

61. Zhao, L., J. Shields, and D. Tyrrell. 2010. Functional changes, increased apoptosis, and diminished nuclear factor-kappaB activity of myeloid dendritic cells during chronic hepatitis C infection. *Hum. Immunol.* 71: 751–62.

62. Dental, C., J. Florentin, B. Aouar, F. Gondois-Rey, D. Durantel, T. Baumert, J. Nunes, D. Olive, I. Hirsch, and R. Stranska. 2011. Hepatitis C virus fails to activate NF-κB signaling in plasmacytoid dendritic cells. *J. Virol.* 86: 1090–6.

63. Fortier, M.-E., S. Kent, H. Ashdown, S. Poole, P. Boksa, and G. Luheshi. 2004. The viral mimic, polyinosinic:polycytidylic acid, induces fever in rats via an interleukin-1-dependent mechanism. *Am. J. Physiol. Regul. Integr.Comp. Physiol.* 287: R759–66.

64. Klinman, D. 2004. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat. Rev. Immunol. 4: 249–58.*

65. Ngoi, S., M. Tovey, and A. Vella. 2008. Targeting poly(I:C) to the TLR3-independent pathway boosts effector CD8 T cell differentiation through IFN-alpha/beta. *J. Immunol.* 181: 7670–80.

66. Schulz, O., S. Diebold, M. Chen, T. Näslund, M. Nolte, L. Alexopoulou, Y.-T. Azuma, R. Flavell, P. Liljeström, and C. Reis e Sousa. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 433: 887–92.

CHAPTER-4

Characterization of early immune events influencing the disparate induction of effector T cells by core and NS3 antigens

A modified version of this chapter is in preparation for publication.

4.1 INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic liver disease, cirrhosis, and hepatocellular cancer worldwide (1). Approximately, 20-35 % of the infected people naturally clear the virus, but the remaining majority develop chronic infection. Currently, 150-170 million people worldwide are chronically infected with HCV (2). Development of vaccine against HCV has been slowed due to poor understanding of the type of immunity that naturally controls HCV infection. Since HCV infects only humans and chimpanzees and replicates inefficiently in cell culture, studies aimed at assessing the abilities of individual HCV-encoded proteins to modulate host immune responses during HCV infection have been difficult to perform.

HCV belongs to the *Hepacivirus* genus of the Flaviviridae family. Individual isolates of HCV have considerable sequence variations, and seven major genotypes, each with multiple subtypes are recognized (3, 4). The HCV genome is a single-stranded, positive-sense RNA of approximately 9600 nucleotides, and encodes a polyprotein with a single open reading frame of 3008 to 3033 amino acids. The HCV genome encodes for structural (core, E1 and E2) and non-structural (p7, NS2, NS3, NS4 and NS5) proteins (5). Among these proteins, core and NS3 proteins are highly conserved. Apart from the proteins produced from the open reading frame, an alternate reading protein encoded within the core region is produced due to ribosomal frame shift. In different genotypes, point of ribosomal frame shift is different so length of F protein varies among different HCV genotypes (6-8).

It has been reported that core protein and core protein-derived peptides play a role in inducing T-cell suppression or down regulation (9, 10). Core protein has also been shown to facilitate induction of Fas-mediated apoptosis in JURKAT cells (11). There is significant experimental evidence to suggest that NS3 in contrast to core is an immunodominant protein and that T cells reactive against NS3-derived peptides are very important in clearing the virus (12-14).

In our previous report (14), we examined the T cell responses generated against core and NS3 protein *in vivo* in C57bl/6 mice. Our results suggested that NS3 immunized mice are able to control viral titers in recombinant HCV-vaccinia infected mice in contrast to core immunized mice (14). We reported that core and NS3 have distinct role in terms of T cell activation and viral clearance. *In vivo*, HCV-core leads to the development of Tregs, possibly allowing the viral infection to continue, while HCV–NS3 induces the development of effector T cells, resulting in significant reduction in viral titers. Effector molecules IFN-γ and GrB were significantly down-regulated in core group, compared to control vector or NS3 immunized groups (14). We have further examined the generation of effector T cells against core and F proteins and observed that T cells generated against both core and F proteins are dysfunctional with respect to effector molecules in contrast to T cells generated against NS3 (Chapter 2). This is an intriguing observation but the factors influencing the disparate outcomes in immune response against these two HCV antigens are not clear. It is known that early innate and adaptive immune responses play an important role in generating effective immunity in persistent infection (15). In this report we have analyzed the

very early immune events in the mice immunized with recombinant adenovector containing core and NS3 in a time course manner. Our results demonstrated that despite efficient expression of both antigens at the site of immunization, qualitative differences in T cell and cytokine responses were evident in both groups as early as 12 hours post immunization. T cell proliferation, IL-2, IL-6 and IL-12 production were significantly higher in NS3 immunized mice at 12-48 hours after immunization compared to core immunized mice. All of these cytokines are proinflammatory and known to affect T cell priming, Th1 switch and/or suppressing the Tregs. Therefore, our results suggest that early expression of specific proinflammatory cytokines e.g. IL-2, IL-6 and IL-12 constitutes as important factors to influence the generation T cell effector function.

4.2 MATERIALS AND METHODS

4.2.1 Mice and adenovirus vector

Six to eight week old male C57bl/6 mice were purchased from Charles River Laboratories Inc. (Canada). All animal experimental protocols were approved by the University of Alberta Animal Care and Use Committee for Health and Sciences, and conducted in accordance with Canadian Council of Animal Care (CCAC) guidelines.

Recombinant adenovirus vectors containing NS3, core, and no antigen (control vector) have been previously described by our group (14, 16). The adenovirus vectors were propagated and amplified in 293A cells and the presence of the core and NS3 gene was confirmed by sequencing.

4.2.2 Immunization of mice

Mice were injected intramuscularly in both hind limbs with a total of 2×10^7 replication-deficient recombinant adenovirus particles expressing HCV-core, NS3, or control vector in 150 µl volume. For time point study, mice were euthanized at 12, 24, 48, 72, 96 and 120 hours post immunization.

4.2.3 Immunohistochemistry

After 12, 24 and 48 hours of immunization, mice were euthanized and thigh muscle cells were collected. Ten-micrometer section of quadriceps muscles of hind limbs of immunized mice were stained as described before (17, 18). In brief, slides were washed two times with 0.05% Tween phosphate-buffered saline (PBS) buffer for 2 min, followed by cleaning with Triton x-100 containing PBS. Nonspecific binding of biotinylated secondary antibody, used later in the procedure was blocked by incubation with 5% diluted normal goat serum at room temperature for 30 minutes. Sections were then incubated with anti CD16/32 for one hour followed by two times washing with 0.05% Tween PBS. Slides were incubated with anti-core and anti-NS3 primary antibody in 1:100 dilution for 30 minutes followed by two washes with 0.05% Tween PBS. Endogenous peroxidase activity was depleted by incubating the sections in 3% H_2O_2 with 0.1% sodium azide in 0.05% Tween PBS buffer for 10 min. After two washes in 0.05% Tween-PBS buffer, sections were incubated with 10 pg/ml of biotinylated goat anti-mouse in 1% normal mouse serum (Sigma) for 20 minutes, and washed twice in 0.05% Tween-PBS buffer. The sections were then incubated with DAB for 20

minutes and subsequently washed twice in 0.05% Tween-PBS buffer. Chromogen were added to each section for 5 minutes, and washed twice in 0.05% Tween-PBS buffer. The sections were dried and dehydrated with 95% and 100% ethanol, cleared with xylone and mounted with water-based plastic mount (Polysciences, Inc.).

4.2.4 Isolation of splenic T cells

After 12, 24, 48, 72, 96 and 120 hours of immunization, mice were euthanized to obtain splenocytes. The spleens were pooled from 3 mice from each group and a single cell suspension was prepared by disrupting the spleen between frosted slides and filtering the contents through a falcon 100 μm nylon cell strainer. After centrifugation, RBCs were lysed and T cells were enriched using nylon wool columns as described previously (14). These enriched T cells (~ 90% $CD3^+$ T cells) were used in various experiments.

4.2.5 T cell proliferation assay

Proliferative responses of splenic T cells were measured in triplicate cultures in 96-well flat-bottomed microtiter plates. A total of 4×10^5 T cells from HCV-core, NS3 or control vector immunized mice and 4×10^5 antigen presenting cells (APCs) (spleen cells from naïve unimmunized mice irradiated with 18 Gy) were mixed with 5μg/ml of recombinant core and NS3 protein (kind gift of Chiron/Novartis Inc.) and cultured in complete RPMI medium (Hyclone laboratories, South Logan, UT) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) at 37 °C and 5% CO_2 for 4 days. The wells were pulsed with 0.5 μCi/well [^{3}H]-

thymidine (GE Healthcare, Morgan Boulevard, Canada) for 12–18 h and harvested on filter papers (PerkinElmer, Waltham, MA) using a 96-well cell harvestor (Tomtech, Hamden, CT). Measurement of [^{3}H]-thymidine incorporated into the DNA of proliferating cells was performed in a Microbeta Trilux liquid scintillation counter (PerkinElmer, Waltham, MA). Stimulation indices were calculated by dividing the counts per minute (CPM) obtained in the presence of protein with that in the absence of protein. Proliferation is represented as the mean stimulation index ± SE (standard error) of triplicate cultures from a representative experiment of 3 repeated experiments.

4.2.6 Cytokine ELISA

Cytokines secreted in the supernatant of proliferating cultures were measured using sandwich ELISA kits following the manufacturer's protocol (eBioscience, San Diego, CA) for the presence of IL-2, IL-6, IL-12, IL-10, IFN-γ, TNF-alpha, and TGF-β. Dilutions of 1:5 to 1:50 were used for the samples with standards ranging from 15.6 to 10000 pg/ml. ELISA plates were read and the concentrations of cytokines were calculated with an automated ELISA plate reader (Fluostar Optima, BMG Labtech, Allmendgruen, Germany). Similarly cytokine expression was also measured in supernatant obtained from culturing of splenocytes for 24 or 72 hours from different groups in presence of TLR-3 ligand Poly I:C.

4.2.7 Flow cytometry analysis of surface and intracellular markers

A total of 1×10^6 splenocytes from HCV- core, NS3, or control vector immunized mice were collected for intracellular and extracellular staining with fluorescently

labeled mAbs (concentrations according to manufacturer's instructions). The cells were washed with ice-cold FACS-buffer (PBS with 2% fetal bovine serum). Anti-mouse CD3eFITC, CD25-PE-Cy7, and PD1 PE-Cy5.5 (eBioscience, San Diego, CA) antibodies were added and cells were incubated at 4 ℃ for 30 min. The cells were washed twice with FACS buffer and fixed in 2% paraformaldehyde in FACS buffer for 10 min. The cells were incubated in cold FACS-buffer + 0.3% saponin (Sigma Aldrich, St. Louis, CA) for 5 min, followed by addition of anti-granzyme B (GrB) and anti-FoxP3 antibody labeled with Alexa fluor 647 and PE respectively (Biolegend, San Diego, CA), and further incubated for 30 min at 4 ℃. The cells were washed twice with FACS-buffer containing 0.3% saponin and fixed using fixing buffer. The cells were also stained with isotype control antibodies using similar methodologies, read in FACS-Canto, and analyzed using FACS-DIVA (Becton Dickinson, Mountain View, CA) software. The cells were gated to exclude 98% of isotype-matched control monoclonal antibodies stained cells and analyzed for each marker. To analyze the stained cells, lymphocytes were gated based on side scattered (SSC) and forward scattered (FSC) light, followed by gating for $CD3^+$ T cells. The $CD3^+$ T cells were then gated on different antibody for their expression, which is shown as dot plot/histogram in most of the figures.

4.2.8 Statistical Analysis

Statistical analysis was done by Mann Whitney test using Graphpad Prism (Graphpad Software Inc., La Jolla, CA, USA). '*' indicates significant difference at $P<0.05$.

4.3 RESULTS

4.3.1 Expression of core and NS3 proteins in quadriceps muscles of hind limbs of immunized mice

We reasoned that induction of disparate T cell responses against core and NS3 antigens could be due to differential expression of both antigens at the site of vector immunization. Low, inefficient and/or no expression of antigens *in vivo* could lead to inefficient T cell priming and stimulation. In our *in vitro* expression experiments in human primary DCs using the recombinant adenovirus vectors, both core and NS3 antigens were efficiently expressed to a similar level (16). To examine the *in vivo* expression of both antigens, mice were immunized with replication deficient adenovector containing HCV derived core or NS3 proteins. After 12, 24, and 48 hours of immunization, mice were euthanized and quadriceps muscles of hind limbs was collected for immunohistochemistry. These time points were chosen because we are using replication incompetent recombinant adenovirus vectors. Both at 12 and 24 h post immunization, significant antigen expression was observed in both core and NS3 groups at a qualitatively similar level (Figure 4-1). At 48 hours after immunization, expression of both antigens was reduced, demonstrating the transient nature of antigen expression upon immunization with replication incompetent recombinant adenovirus vectors. As

negative controls we stained both unimmunized and control vector immunized mice with anti-core and anti-NS3 antibody at different time points (Figure 4-1)

4.3.2 Core or NS3 protein specific T cell responses at early time points after immunization

To study the priming of T cell response against HCV derived core and NS3 proteins, the immunized mice were euthanized at early time points after immunization i.e. 12 hours, 24 hours & 48 hours. Spleen T cells were isolated and cultured *in vitro* with syngeneic irradiated APCs in the presence of recombinant core and NS3 protein at 5 µg/ml concentrations for four days followed by ^{3}H-thymidine incorporation assay (Figure 4-2). Mice immunized with core containing adenovirus vectors showed very low proliferation against core protein, whereas mice immunized with NS3 containing adenovector showed high proliferation against NS3 protein at all-time points 12, 24 and 48 hours post immunization (Figure 4-2). Proliferative responses against mitogen such as concanavalin A (con A) was similar in splenocytes obtained from core, NS3, or control vector immunized mice (Figure 4-2). These results suggest that either core is poorly immunogenic in mice or immunization with core is able to down regulate immune responses at early time point similar to what we observed after 8 days of immunization (Vaccine BA). However NS3 protein is highly immunogenic from an early time point after immunization (Figure 4-2).

4.3.3 Cytokine production by splenic T cells stimulated with core and NS3 protein

We analyzed the production of cytokines IL-2, Il-6, IL-4, IL-12, IL-17, IFN-gamma, TGF-beta and IL-10 from supernatants collected from T cell proliferation assays set up at various time points after immunization. We observed that production of IL-2 and IL-12 was significantly higher in NS3 immunized mice stimulated with NS3 protein compared to core immunized mice stimulated with core protein (Figure 4-3). IL-6 production was also high in NS3 immunized mice at early time points of the experiment, which gradually decreases to level similar to core immunized mice. The remaining cytokines were undetectable (IL-17, IL-4 and IL-10) or did not show an indistinguishable pattern (IFN-γ) between core and NS3 groups.

4.3.4 Time course of phenotypic analysis of T cells from mice immunized with recombinant adenovirus vectors containing core or NS3

To examine whether the differences in response against core and NS3 could be attributed to phenotype of T cells and DCs at various time points, splenocytes and draining lymph node cells obtained from core, NS3 or CV immunized mice were analysed for phenotypic changes in T cells and DCs at different time points. In the absence of defined peptide epitopes of NS3 and core antigens to allow tetramer staining, we sought to examine overall gross phenotypes of T cells and DCs by examining CD3, CD25, FoxP3, PD1 GrB, CD11C, CD80, CD86, and PDL1. There was no significant difference in the percentage of $CD3^+$T cells in the lymph nodes or spleen in NS3 immunized mice compared to core. Also, on the $CD3^+$ T cells, the expression of PD-1, CD25 and FoxP3 did not show a difference in

lymph node or spleen of mice immunized with core or NS3 at all of the early time points examined (12-120 hours) (Figure 4-4). We also found that overall GrB expression was higher in NS3 immunized group compared to core immunized group (Figure 4-4). In core immunized mice GrB expression gradually decreased with time but NS3 immunized mice maintained the high expression of GrB. We did not observe any differences in phenotype of DCs from splenocytes or lymph nodes obtained from core or NS3 immunized mice (Figure 4-4).

4.3.5 Cytokine production in supernatants obtained from splenocytes cultured for 24 hours in presence of Poly I:C

We had earlier observed that immunization of mice with rAd-core in the presence of TLR-3 agonist poly I:C reverses the reduction in GrB expression (Chapter 1). In the current study, we noted that splenocytes obtained from core immunized mice have significantly reduced IL-2 and IL-12 production (Figure 4-3). Therefore, we examined if *in vitro* stimulation of splenocytes from core immunized mice with Poly I:C would allow IL-2 and IL-12 production to be recuperated. We observed that both IL-2 and IL-12 production was increased in splenocytes from core immunized group to the level seen in NS3 immunized mice (Figure 4-5). Interestingly, in the poly I:C stimulated splenocytes, IFN-γ production was similar in both core and NS3 immunization groups (Figure 4-5), whereas in the non-polyI:C stimulated cultures, IFN-γ was undetectable in splenocyte cultures from both core and NS3 immunized groups. Cytokines such as IL-4, IL-6, and IL-10 were below detection limit in each of these cultures.

4.4 DISCUSSION

The development of vaccine is ultimately required to protect from chronic HCV infection worldwide. Acute viral clearance in minority of infected patients reinforces the notion that a protective vaccine against HCV is a feasible goal. Further, from chimpanzee studies, it is clear that protective immune response against HCV can reduce the virus load during secondary infection (19). In addition, chimpanzees that clear the virus demonstrate early humoral and cellular immune responses in contrast to those who develop chronic infection (20). From earlier studies, however, it is not clear which antigens need to be targeted in vaccine design, and there are different theories regarding this important question (21). NS3 and core are two such HCV derived antigens which have been implicated in playing contrasting roles in the induction and generation of efficient cellular immunity. While, NS3 has been shown to be an immunodominant protein for the generation of T cells, the role of core has been much debated and thought to be immunosuppressive and/or immunomodulatory (10, 22). Our earlier results with mice immunized with core or NS3 using recombinant adenovirus vectors have implicated that NS3 induces strong T cell responses including effector T cells. However, it was apparent from our studies (14 and Chapter 1) that core protein is not simply non-immunogenic, at least in mice, but rather actively modulates T cell responses. In the core protein immunized mice, high levels of IL-10 in the absence of IFN-γ and significantly reduced levels of GrB producing $CD4^+$ and $CD8^+$ T cells were observed. The final outcome of immune response against a given antigen is dictated by immune events which take place early in

antigen encounter. It has been shown that as early as 6 hours after inoculation of a virus, antigen presentation takes place through DCs to prime naive T cells in the draining lymph nodes (23). In the present study, we sought to examine the early immune events in mice after immunization with recombinant adenovirus vectors containing core or NS3.proteins

First, we reasoned that the disparity of immune responses could be a product of differential antigen expression *in vivo* by replication incompetent recombinant adenovirus vectors. *In vitro,* our studies have shown that both of the recombinant vectors express NS3 or core antigens in primary human dendritic cells to a similar level (16). Now we qualitatively examined the expression of core and NS3 proteins in quadriceps muscles of hind limbs of immunized mice by immunohistochemistry at 12, 24 and 48 hours after immunization (Figure 4-1). These early time points were chosen due to the replication deficient nature of the adenovector used in our studies. We observed the expression of both core and NS3 proteins in mice immunized with core and NS3 containing adenovector to similar levels and with a similar time course suggesting that differences in antigen expression is not the cause of ensuing disparity in induced immune responses (Figure 4-1). Interestingly, at 12 hours post immunization, both of the antigens were expressed to the maximum levels and by 48 hours, the expression was waning to lower levels, but still visible. These experiments allowed us to exclude the non-physiological or technical contribution of the differential immunizations to the difference in outcomes of immune responses generated.

Proliferation of T cells is one of the measures of priming and activation

against a given antigen. We determined the antigen specific proliferation against core and NS3 proteins at 12, 24 and 48 hours post immunization (Figure 4-2). Even at these early time points, there was significant antigen dependent proliferation in NS3 immunized mice whereas virtually no antigen specific proliferation was observed in the core immunized mice. Proliferation against a T cell mitogen ConA was similar in both groups. The relative contribution of $CD4^+$ vs. $CD8^+$ T cells in overall antigen dependent proliferation is not clear at this time. Further, to delineate the immune factors responsible for early response against NS3, we determined the cytokines produced in supernatants collected from T cell cultures. We observed that IL-2, IL-6 and IL-12 were significantly higher by T cells from NS3 immunized mice as compared to core immunized mice at early time points of 12-48 hours post immunization (Figure 4-3). Production of cytokines by DCs and other cells play an important role in generation of effective antigen specific T cell response. IL-12 is a pleiotropic cytokine that is secreted by activated professional APCs (24-26) and plays an important role in T cell activation, skewing towards Th1 response, and protecting T cells from undergoing apoptosis (27-30). Similarly IL-6 also activates T cell proliferation (31) and inhibits proliferation of Tregs (32). It has been shown that early IL-2 production is important for T cell priming and activation, and IL-2 produced by $CD4^+$ T cells are required for stimulation of $CD8^+$ T cells (33). Interestingly, significantly high levels of IL-2 was produced in NS3 immunized mice at 12-48 hours, at which time there was almost no IL-2 produced in core immunized group. However, production of IL-2 in core immunized mice was high at 72-96 h and reached to

the level of NS3 group. Interestingly though, IL-2 production was again undetectable at 120 h in core group, whereas in the NS3 group, IL-2 production was maintained at the high level. Therefore, in the core immunized mice, there is delayed and transient IL-2 production after immunization, in contrast to NS3 immunized mice where continued high levels of IL-2 were detectable.

IL-2 expression not only helps in T cell proliferation but also enhances the expression of GrB. Our results have indicated that GrB expression is higher in T cells from NS3-immunized mice (Figure 4-4, 14) and the addition of IL-2 *in vitro* to splenocytes from core-immunized mice increases GrB expression (Chapter 1). In addition, TLR ligands/agonists have been shown to play an important role in generating effective immune response against apparently less immunogenic antigens (34). Synthetic TLR-3 ligands such as poly I:C has been known to act as potent immune adjuvants by enhancing DC cross-presentation and promoting $CD8^+$ T cell responses (34). We have also observed from our previous studies that immunization with F or core containing adenovirus vectors admixed with TLR-3 agonist poly I:C dramatically increased GrB expression in both $CD4^+$ and $CD8^+$ T cells (Figure 2-11 Chapter 2). Therefore, we examined if exogenous addition of polyI:C to splenocyte cultures from core immunized mice would lead to enhanced ability to produce cytokines such as IL-2, IL-12 and IFN-γ. The data obtained suggested that indeed *in vitro* stimulation of splenocytes with poly I:C can enhance the expression of cytokines like IL-2, IL-12 and IFN-γ, which would play a crucial role in generating early immune response (Figure 4-5).

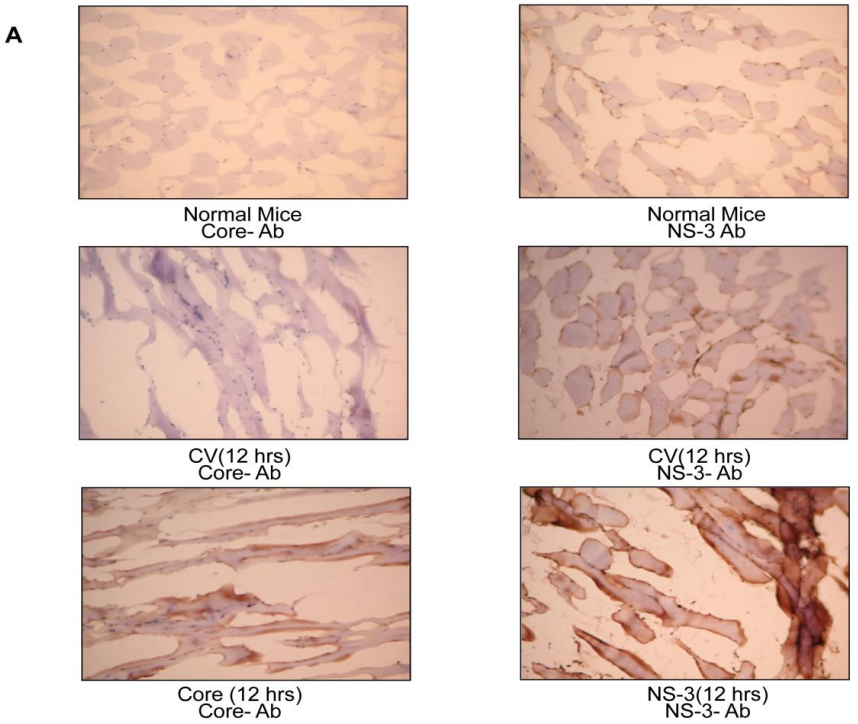
In our studies, we examined a number of T cell and dendritic cell markers

e.g., CD3, CD11c, FoxP3, CD25, PD-1, PD-1L, GrB, CD80 and CD86 in a time course study to examine if the differences obtained in the immune responses could be attributed to a significant modulation of T cell and dendritic cells compartments of spleens and draining lymph nodes (Figure 4-4). In these experiments examining gross phenotypes, except in GrB expression, we did not observe any significant difference in core vs. NS3 immunized groups. In fact, it is known that in chronic HCV patients, there is no gross immune defect or deficiency, and only antigen specific immune compartments are modulated. Our studies would also support this contention. Due to lack of knowledge in specific epitopes of core and NS3 restricted to H-2^b background of mice, we were not able to perform more detailed analysis of peptide epitope specific T cell compartment and specific antigen loaded DCs in mice. However, these studies need to be conducted in future to delineate the exact mechanism and early immune events leading to modulation of T cells following immunization with core.protein

In conclusion, our data suggest that early immune events dictate the induction and generation of effective T cell responses. The two antigens of HCV studied in this report, core and NS3, upon immunization have been shown to lead to two distinct outcomes in terms of generation of effector T cells. Now, our studies have demonstrated that early events in antigen encounter imprint the subsequent immunity and the final outcome; two distinct patterns of early events in immunity can be demonstrated for the antigens core and NS3. However, the final outcome can still be modulated by targeting certain innate immune pathways, such as TLRs mediated signalling.

4.5 FIGURES

Figure 4-1



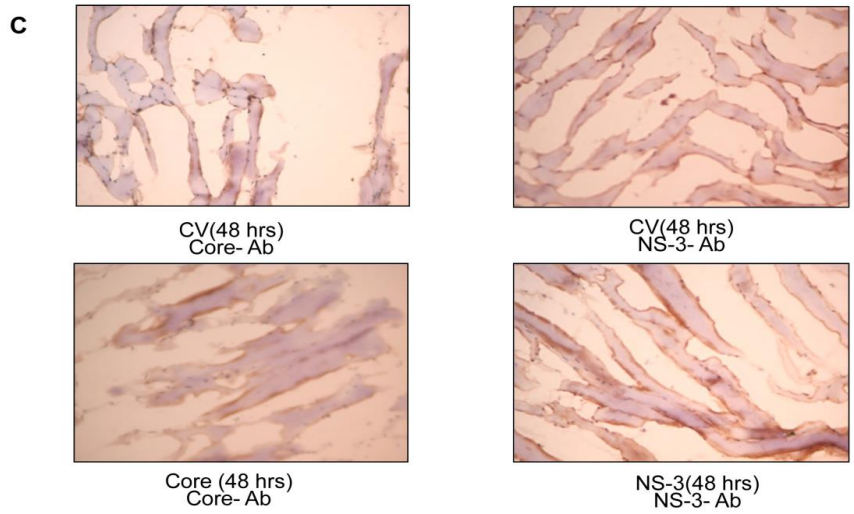


Figure 4-1 A, B and C. Detection of core and NS3 expression in mouse quadriceps muscles of hind limbs by immunohistochemistry: Mice were immunized with 2×10^7 replication deficient adenovirus containing HCV-core, NS3 and control vector (CV) intramuscularly. After 12, 24 and 48 hours of immunization, thigh muscle were isolated and cut into thin slices as described in materials and method. Immunohistochemistry was done to confirm expression of core and NS3 protein. Thigh muscle sections from PBS immunized mice after 12 hours was stained with anti-core antibody (A, left upper pane) and anti-NS3 antibody (A, right upper panel). Thigh muscle section from mice immunized with CV, core or NS3 containing adenovirus were stained with anti-core and anti-NS3 antibody after 12 hours (A), 24 hours (B) and 48 hours (C). At each time point CV immunized mice were stained with anti-core or anti-NS3 antibody as negative controls.

Figure 4-2

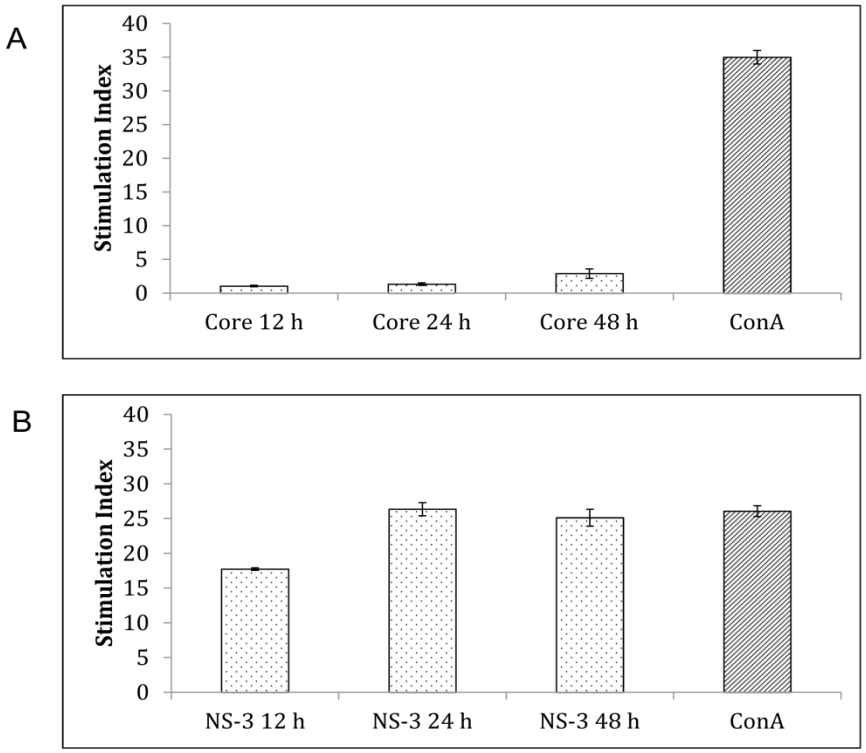


Figure 4-2. Antigen specific proliferation of T cells after 12, 24 and 48 hours of immunization. Mice were immunized with 2×10^7 replication deficient adenovirus containing HCV-core, NS3 and control vector (CV) intramuscularly, and spleens were harvested 12, 24 and 48 hours after immunization. Enriched T cells were cultured *in vitro* with irradiated syngeneic APCs and the relevant recombinant protein antigens NS3 or core at 5μg/ml (Figure A, B). CPMs (200-300) from T cells and APCs in the absence of antigen were used as background to determine the stimulation indices. Stimulation against ConA was used to serve as positive control for proliferation (CPM 15,000-20,000). Data represent average stimulation index±SE from triplicate wells. Results are representative of 3 independent experiments.

Figure 4-3

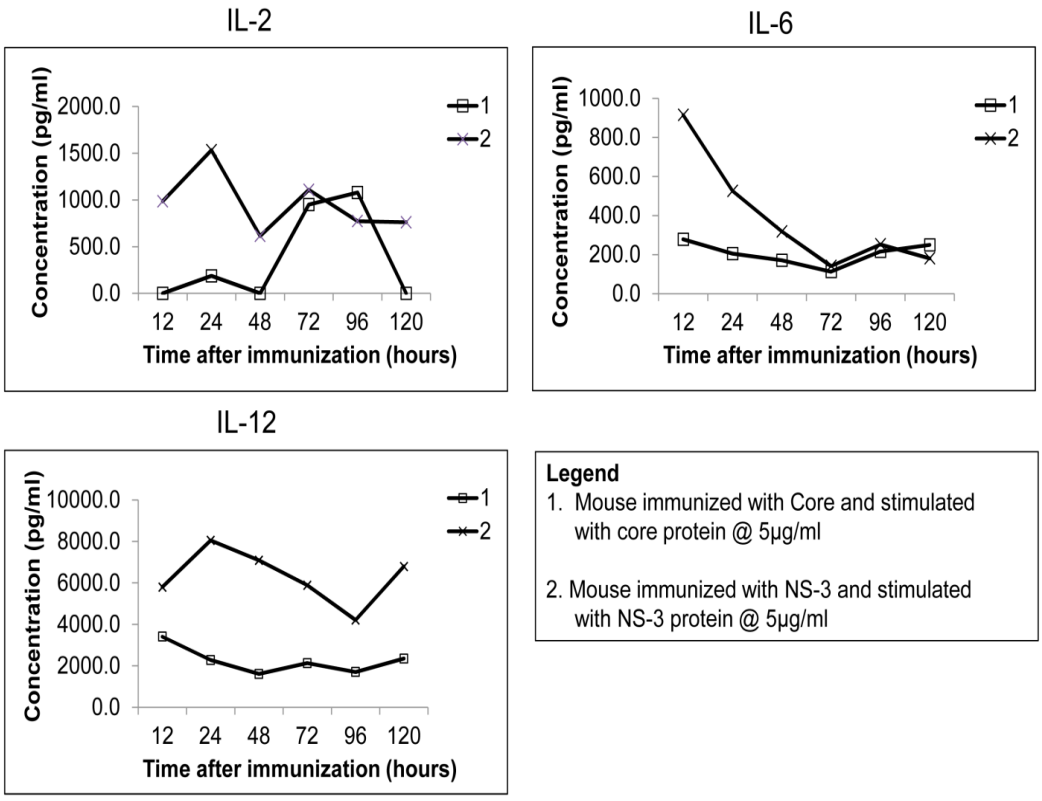


Figure 4-3. T cell cultures obtained from NS3 immunized mice and stimulated with recombinant NS3 protein produce high levels of IL-2, IL-6 and IL-12 in contrast to core. T cells obtained from spleens of immunized mice were cultured with irradiated APCs and 5µg/ml of recombinant protein as shown in figure legend. Supernatants from T cell cultures were collected on day 4 and cytokines were analyzed by ELISA. Assays were done in duplicates and average values are shown. Results are representative of two repeated experiments.

Figure 4-4a

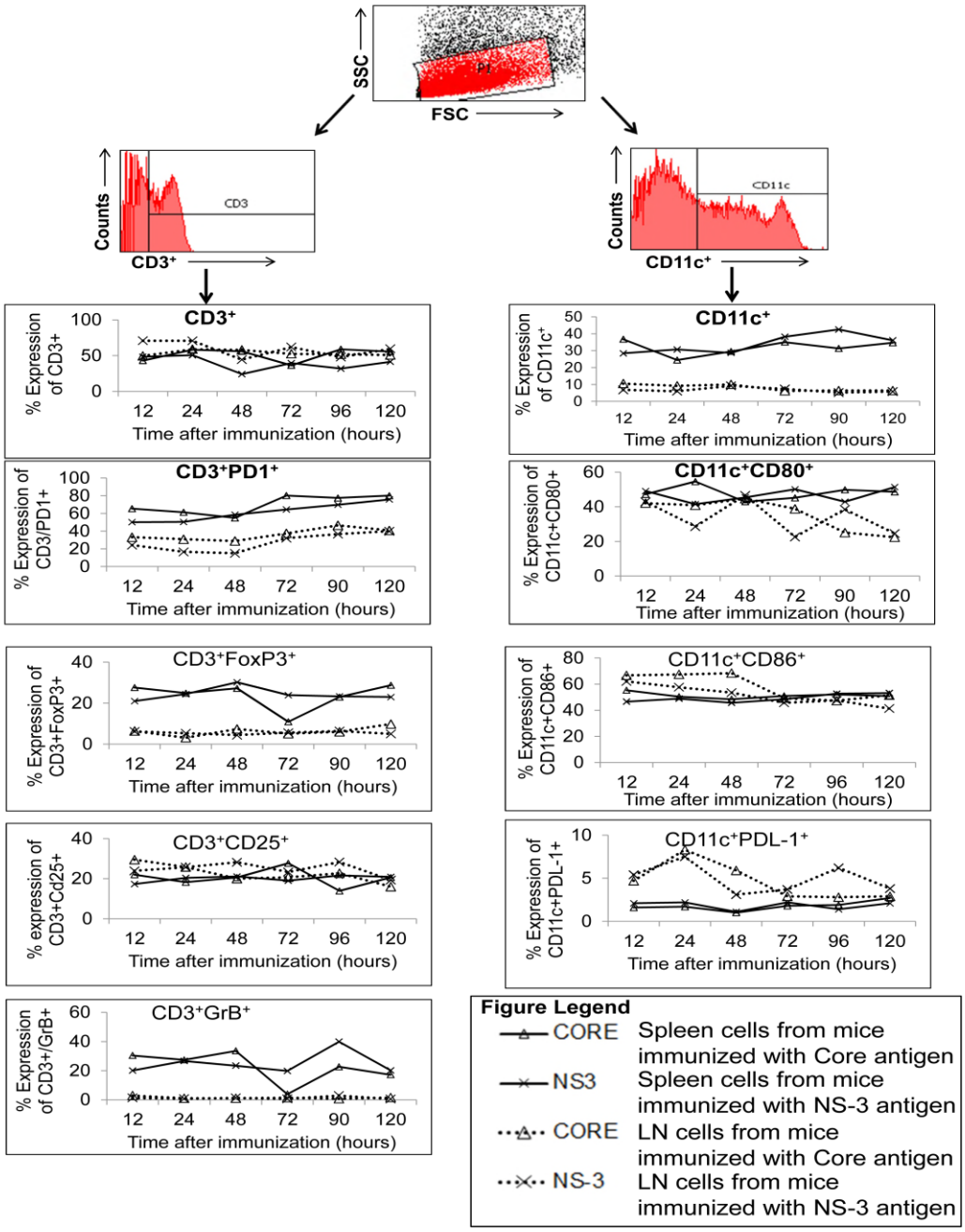


Figure 4a. Time course analysis of phenotypes of splenocytes from mice immunized with 2×10^7 replication deficient adenovirus containing HCV-core or NS3. Splenocytes were obtained from immunized mice at 12-120 hours after immunization and stained for T cell markers CD3, CD25, PD1, FoxP3 and GrB,

and DC markers CD11c, CD80, CD86 and PDL1. T cells were first gated based on $CD3^+$ expression and then analyzed for other markers. DCs were gated based on CD11c expression and then analyzed for CD80, CD86 or PDL1 expression. Data are representative of 3 repeated experiments.

Figure 4-5

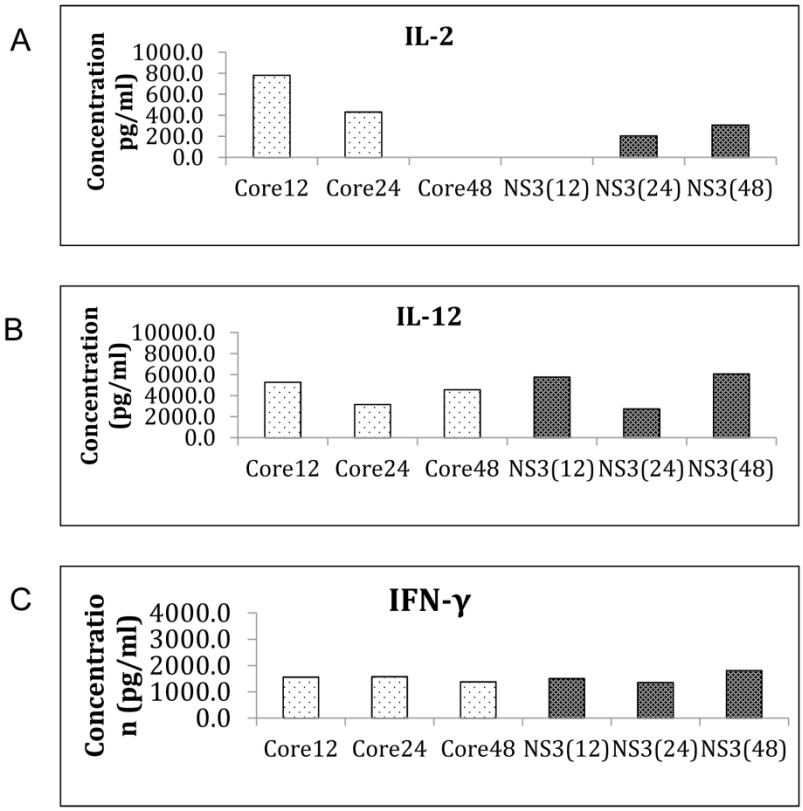


Figure 4-5. ***In vitro*** **stimulation of spleen cells with polyI:C reverses the downregulation of IL-2 and IL-12 in core immunized mice:** Mice were immunized with recombinant adenovirus vectors containing core or NS3 and spleens were collected 12, 24 or 48 hours after immunization. Splenocytes were stimulated with poly I:C and supernatants were collected after 24 hours incubation for cytokine analysis by ELISA. Assays were done in duplicates and average values (within 10% of each other) are shown. Controls represent supernatant from CV immunized mice. Results are representative of two repeated experiments.

4.6 REFERENCES

1. Alter, M. 1997. Epidemiology of hepatitis C. *Hepatology* 26: 62S–65S.

2. WHO report July, 2012.

3. Simmonds P, Bukh J, Combet C, Deléage G, Enomoto N, et al. (2005) Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 42: 962–973.

4. Simmonds P, Becher P, Collett MS, Gould EA, Heinz FX, Meyers G, Monath T, Pletnev A, Rice CM, Stiasny K, Thiel HJ, Weiner A, Bukh J (2011) Flaviviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds) Virus taxonomy. Elsevier, Oxford, pp 1003–1020

5. Dustin, L., and C. Rice. 2006. Flying under the radar: the immunobiology of hepatitis C. *Ann. Rev. Immunol.* 25: 71–99.

6. Xu, Z., J. Choi, T. Yen, W. Lu, A. Strohecker, S. Govindarajan, D. Chien, M. Selby, and J. Ou. 2001. Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. *EMBO J.* 20: 3840–8.

7. Walewski, J., T. Keller, D. Stump, and A. Branch. 2001. Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. *RNA* 7: 710–21.

8. Varaklioti, A., N. Vassilaki, U. Georgopoulou, and P. Mavromara. 2002. Alternate translation occurs within the core coding region of the hepatitis C viral genome. *J. Biol. Chem.* 277: 17713–21.

9. Langhans, B., M. Lechmann, H. Ihlenfeldt, G. Jung, G. Giers, S. Schweitzer, M. El Bakri, T. Sauerbruch, and U. Spengler. 2000. A hepatitis C virus (HCV) core protein derived peptide inhibits HCV specific lymphocyte proliferation. *Eur. J. Med. Res.* 5: 115–20.

10. Large, M., D. Kittlesen and Y. Hahn. 1999. Suppression of host immune response by the core protein of hepatitis C virus: possible implications for hepatitis C virus persistence. *J. Immunol.* 162: 931–8.

11. Hahn, C., Y. Cho, B. Kang, I. Lester, and Y. Hahn. 2000. The HCV core protein acts as a positive regulator of fas-mediated apoptosis in a human lymphoblastoid T cell line. *Virology* 276: 127–37.

12. Diepolder, H., R. Zachoval, R. Hoffmann, E. Wierenga, T. Santantonio, M. Jung, D. Eichenlaub, and G. Pape. 1995. Possible mechanism

involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet* 346: 1006–7.

13. Diepolder, H., J. Gerlach, R. Zachoval, R. Hoffmann, M. Jung, E. Wierenga, S. Scholz, T. Santantonio, M. Houghton, S. Southwood, A. Sette, and G. Pape. 1997. Immunodominant CD4+ T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection. *J. Virol.* 71: 6011–9.

14. Krishnadas, D., W. Li, R. Kumar, D. Tyrrell, and B. Agrawal. 2010. HCV-core and NS3 antigens play disparate role in inducing regulatory or effector T cells *in vivo*: Implications for viral persistence or clearance. *Vaccine* 28: 2104–14.

15. Houghton, Michael. 2007. Prospects for prophylactic and therapeutic vaccines against the hepatitis C viruses. *Immunol. Rev.* 239: 99–108

16. Li, W., J. Li, D. Tyrrell, and B. Agrawal. 2005. Expression of hepatitis C virus-derived core or NS3 antigens in human dendritic cells leads to induction of pro-inflammatory cytokines and normal T-cell stimulation capabilities. *J. Gen. Virol.* 87: 61–72.

17. Casey, T., S. Olson, J. Cousar, and R. Collins. 1990. Plastic section immunohistochemistry in the diagnosis of hematopoietic and lymphoid neoplasms. *Clin. Lab. Med.* 10: 199–213

18. Casey, T., J. Cousar, and R. Collins. 1988. A simplified plastic embedding and immunohistologic technique for immunophenotypic analysis of human hematopoietic and lymphoid tissues. *Am. J. Pathol.* 131: 183–9.

19. Feinstone, S., D. Hu, and M. Major. 2012. Prospects for prophylactic and therapeutic vaccines against hepatitis C virus. *Clin. Infect. Dis.* 55 Suppl 1: S25–32

20. Shata, M., D. Anthony, N. Carlson, L. Andrus, B. Brotman, N. Tricoche, P. McCormack, and A. Prince. 2002. Characterization of the immune response against hepatitis C infection in recovered, and chronically infected chimpanzees. *J. Viral Hepat.* 9: 400–10.

21. Sautto, G., A. W. Tarr, N. Mancini, and M. Clementi. 2013. Structural and Antigenic Definition of Hepatitis C Virus E2 Glycoprotein Epitopes Targeted by Monoclonal Antibodies. *Clin. Dev. Immunol.* 2013.

22. Zabaleta, A., D. Llopiz, L. Arribillaga, L. Silva, J. Riezu-Boj, J. Lasarte, F. Borrás-Cuesta, J. Prieto, and P. Sarobe. 2007. Vaccination against hepatitis C virus with dendritic cells transduced with an adenovirus encoding NS3 protein. *Mol. Ther.* 16: 210–7.

23. Norbury, C., D. Malide, J. Gibbs, J. Bennink, and J. Yewdell. 2002. Visualizing priming of virus-specific CD8+ T cells by infected dendritic cells *in vivo*. *Nat. Immunol.* 3: 265–71.

24. Wolf, S., P. Temple, M. Kobayashi, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, and R. Hewick. 1991. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J. Immunol.*146: 3074–81.

25. Kobayashi, M., L. Fitz, M. Ryan, R. Hewick, S. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* 170: 827–45.

26. Gately, M., L. Renzetti, J. Magram, A. Stern, L. Adorini, U. Gubler, and D. Presky. 1997. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Ann. Rev. Immunol.*16: 495–521.

27. Estaquier, J., T. Idziorek, W. Zou, D. Emilie, C. Farber, J. Bourez, and J. Ameisen. 1995. T helper type 1/T helper type 2 cytokines and T cell death: preventive effect of interleukin 12 on activation-induced and CD95 (FAS/APO-1)-mediated apoptosis of CD4+ T cells from human immunodeficiency virus-infected persons. *J. Exp. Med.* 182: 1759–67.

28. Radrizzani, M., P. Accornero, A. Amidei, A. Aiello, D. Delia, R. Kurrle, and M. Colombo. 1995. IL-12 inhibits apoptosis induced in a human Th1 clone by gp120/CD4 cross-linking and CD3/TCR activation or by IL-2 deprivation. *Cell. Immunol.* 161: 14–21.

29. Marth, T., M. Zeitz, B. Ludviksson, W. Strober, and B. Kelsall. 1999. Extinction of IL-12 signaling promotes Fas-mediated apoptosis of antigen-specific T cells. *J. Immunol.*162: 7233–40.

30. Palmer, E., L. Farrokh-Siar, J. Maguire van Seventer, and G. van Seventer. 2001. IL-12 decreases activation-induced cell death in human naive Th cells costimulated by intercellular adhesion molecule-1. I. IL-

12 alters caspase processing and inhibits enzyme function. *J. Immunol.*167: 749–58.

31. Lin, G., J. Wang, X. Lao, J. Wang, L. Li, S. Li, J. Zhang, Y. Dong, A. Chang, Q. Li, and S. Li. 2012. Interleukin-6 inhibits regulatory T cells and improves the proliferation and cytotoxic activity of cytokine-induced killer cells. *J. Immunother.* 35: 337–43.

32. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 299: 1033–6.

33. Lai, Y.-P., C.-C. Lin, W.-J. Liao, C.-Y. Tang, and S.-C. Chen. 2008. CD4+ T cell-derived IL-2 signals during early priming advances primary CD8+ T cell responses. *PloS ONE* 4: e7766.

34. Schulz, O., S. Diebold, M. Chen, T. Näslund, M. Nolte, L. Alexopoulou, Y.-T. Azuma, R. Flavell, P. Liljeström, and C. Reis e Sousa. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 433: 887–92.

CHAPTER-5

GENERAL DISCUSSION

In my thesis I have focused on various mechanisms of immune modulation by HCV-derived F and core proteins. I have examined the modulation of human DCs expressing the F or core protein and their effect on DC survival and T cell activation in cell culture. Further, I have investigated the immune modulation of $CD4^+$ and $CD8^+$ T cells *in vivo* in mice by the F and core proteins. I have also investigated the early immune events post-immunization with the core and NS3 proteins in order to determine the factors responsible for generating an effective immune response by the NS3 protein. Several interesting observations were made, providing insight into the role of the F and core proteins in HCV infection. In the following sections I have provided an overall discussion of the results obtained during my research and their implications in our understanding of HCV persistence and clearance.

5.1 Modulation of DCs by HCV-derived F and core proteins

The HCV is one of the major blood-borne diseases causing chronic infection in 65-80% of infected patients. However, approximately 20-35% of infected patients clear the virus, although the exact mechanisms of viral clearance are not yet known. The HCV primarily infects the liver cells; however, it can infect several extra hepatic cells including DCs (1-4). DCs are the major antigen-presenting cells; they take up a variety of antigens, and process and present them to $CD8^+$ and $CD4^+$ T cells in the context of MHC molecules. The activated T cells, after clonal expansion, become effector cells and mount an antigen-specific immune response to clear the infection. It has been hypothesized that the HCV can

modulate DC and/or T-cell function, and that DC dysfunction is known to be one of the direct mechanisms enabling viral persistence (5). My work is the first to show that HCV-derived F protein can modulate DC functions and overall immune response. DCs infected with recombinant adenovirus vectors containing the F or core protein exhibit up-regulation of CD-40 and TLR-3 on the surface. As was previously reported, CD-40 and TLRs together can efficiently stimulate T cells (6, 7); I observed a higher proliferation of T cells primed with F protein expressing DCs. In contrast to activation molecules such as CD-40 and TLR-3, increased expression of CD-95 and CD-95L can lead to apoptosis in cells (8, 9). I have observed that the expression of HCV- derived F protein in DCs leads to increased expression of CD-95 and CD-95L which ultimately causes apoptosis in DCs. Apoptosis is not always harmful but can also play a very important role in the generation of strong immune responses (10, 11). Apoptosis in DCs through CD-95/CD-95L expression resulted in an efficient uptake of apoptotic DCs by live DCs. Further, I analyzed antigen-dependent proliferation in core and F groups and observed that proliferation was significantly higher in the F stimulated group compared to the core group in both $CD4^+$ and $CD8^+$ T cells in both primary and secondary cultures. Based on the results obtained I proposed a model to explain how a specific immune response and apoptosis can simultaneously occur after expressing HCV-derived F protein in DCs. On one hand, DCs become activated by expressing CD-40 and TLR-3, and on the other hand some of the DCs undergo apoptotic death due to up-regulation of CD-95/CD-95L. Further, the apoptotic DCs are phagocytosed by live DCs, which could allow better antigen processing

and presentation by DCs. Co-culturing these DCs with autologous T cells results in efficient priming and stimulation of antigen-specific T cells, which could be identified by measuring peptide-specific proliferation. Therefore, HCV- derived F protein provides a unique mechanism of DC modulation and apoptosis, but lead to eventual T cell activation.

5.2 T cell modulation by HCV-derived F and core proteins

Presently, 170 million people are chronically infected with the HCV (12). Chronic HCV infection leads to liver cirrhosis, liver failure, and hepatocellular carcinoma (HCC), and is one of the major causes of liver transplants in North America (13-15).

The factors and mechanisms that allow the virus to circumvent the host's immune responses and to persist in infected individuals are not clearly understood. Various mechanisms of T cell failure have been suggested, such as impaired primary T cell activation, T cell exhaustion, T cell dysfunction, impaired T cell maturation, suppression of T cell function by viral factors, unresponsiveness due to exposure to high antigen levels, impaired DC functions, and suppression of T cell function by regulatory T cells (16-23).

In the current study, I examined T cell responses generated in mice against the F and core antigens upon immunization with recombinant adenovirus vectors expressing these proteins. My results demonstrate that T cells obtained from the F- or core-immunized mice proliferate weakly against the respective antigen-derived peptides. Interestingly, high levels of IL-10 and low levels of IFN-γ were

produced in peptide-stimulated cultures. I observed that expression of intracellular GrB in $CD4^+$ and $CD8^+$ T cells obtained from the F and core immunized mice was much lower than expression in a control vector or in NS3 immunized mice. Diminished expression of GrB by $CD8^+$ T cells from the F- or core- immunized mice correlated with reduced killing of peptide-loaded EL-4 target cells. When cultured with exogenous IL-2 *in vitro*, splenocytes from the F and core immunized mice show elevated levels of GrB in both $CD4^+$ and $CD8^+$ T cells. *In vitro* treatment of splenocytes with a chemical inhibitor of NF-κB abolished the expression of GrB in T cells in all immunized groups, suggesting that modulation of NF-κB activity might in part be responsible for the lower expression of GrB. In line with this hypothesis, I observed that immunizing mice with a TLR-3 ligand, poly I:C, along with the F or core containing adenovirus, recovered GrB expression levels in T cells. To our knowledge, this is the first study to reveal down-regulation of GrB expression as a means of modulating effector T cell responses in a chronic infectious disease; this might represent a new mechanism of immune evasion by the hepatitis C virus. Our work has uncovered a new mechanism of immune modulation by HCV- derived F and core proteins, wherein modulation of GrB expression in effector T cells makes them less potent in terms of killing target cells.

5.3 Early immune events influencing the disparate induction of effector T cells by core and NS3 antigens

From our previous studies it became evident (23 and Chapter 3) that the core is not simply non-immunogenic, at least in mice, but rather actively modulates T

cell responses (chapter 3). The core and NS3 have distinct roles in terms of T cell activation and viral clearance. In the core-immunized mice, high levels of IL-10 in the absence of IFN-γ and significantly reduced levels of GrB producing $CD4^+$ and $CD8^+$ T cells were observed. *In vivo*, HCV-derived core leads to the development of Tregs, possibly allowing the viral infection to continue, while HCV-derived NS3 induces the development of effector T cells, resulting in significant reduction in viral titers. This is an intriguing observation but the factors influencing the disparate outcomes in immune response against these two HCV antigens are not clear. It is known that early innate and cellular immune responses play an important role in generating effective immunity in persistent infection. In this section we have analyzed the very early immune events in the mice immunized with recombinant adenovector containing core and NS3 in a time-course manner. My results demonstrate that despite efficient expression of both antigens at the site of immunization, qualitative differences in T cell and cytokine responses were evident in both groups as early as 12 hours post immunization. T cell proliferation and IL-2, IL-6 and IL-12 production were significantly higher in the NS3-immunized mice at 12-48 hours after immunization compared to core-immunized mice. All of these cytokines are proinflammatory and known to affect T cell priming, Th1 switch and/or suppressing the Tregs. Therefore, my results suggest that early expression of specific proinflammatory cytokines -- e.g., IL-2, IL-6 and IL-12-- constitutes an important factor influencing the generation of the T cell effector function. The two HCV antigens studied in this report, core and NS3, upon immunization have

been shown to lead to two distinct outcomes in terms of generating effector T cells. Now, my studies have demonstrated that early events in antigen encounter imprint the subsequent immunity and the final outcome: two distinct patterns of early events in immunity can be demonstrated for the antigens core and NS3.

5.4 Future Directions

Several studies have been reported with contradictory experimental evidence, and there is little overall consensus about modulating DC function in HCV infection (4-5, 24-26). It is not clear whether DCs become impaired in chronic HCV infection, whether DC impairment is a prelude to inefficient priming and maintenance of HCV-specific T cells facilitating the establishment of a chronic carrier state, or whether it is a possible consequence of persistent and active HCV infection and associated disease progression (17). Work in this thesis suggests that a small percentage of DCs expressing HCV-derived F protein undergo apoptosis which leads to further activation of DCs and T cells. Further work needs to be done to examine why only 10-15% of DCs undergo apoptosis even though ~99% of DCs expressed the HCV-derived F protein *in vitro*. It is possible that only certain sub-types of DCs are undergoing apoptotic death after expression of HCV-derived F protein. This could be achieved by expressing HCV-derived F protein in purified subpopulations of DCs, or examining in depth the functional and phenotypic characteristic of the F- expressing DCs undergoing apoptosis. It would be interesting to investigate how F protein might get taken up by DCs in HCV-infected patient. It has been reported that HCV infection draws the DCs, and other immune cells, into the liver (27, 28); however, role of DCs is not very

well defined in HCV infection. There might be a possibility that DCs uptake F protein *in vivo* and undergo apoptotic death by expressing several apoptotic marker including CD-95 and CD95L as shown in chapter-2. These apoptotic DCs will further get phagocytosed by live DCs which ultimately help in proliferation of antigen specific T cells. It would be interesting to investigate the frequency of HCV-F protein specific T cells in HCV infected patients. Antibody response against HCV-F protein has also been reported.in HCV infected patients (29, 30). Further work is required to know how exactly F protein would be helpful in designing vaccine against HCV

HCV is spontaneously cleared in approximately 20–35% of infected patients, but persists in 65–80% of its victims due to insufficient innate and adaptive immune responses. The mechanisms of immune evasion by HCV have been investigated extensively (23, 31-33) but are not yet completely understood. Of several strategies to evade the immune system by HCV (33), a major mechanism is the modulation of T cell responses. GrB expression is crucial for effective T cell responses in clearing virus-infected cells. Our work has uncovered a new mechanism of immune modulation by HCV-derived and core proteins, wherein modulation of GrB expression in effector T cells makes them dysfunctional, which is reflected by their reduced ability to kill target cells. Future work needs to be done to determine whether interaction between T cells and the F protein is direct or mediated though other immune cells. My studies also suggest that adding a TLR-3 agonist as an adjuvant in a vaccine could effectively reverse the T cell dysfunction induced by the F and core proteins and enhance the T cell's

effector function. Future studies should look at whether this reversal is by direct action on T cells or indirectly through DCs or other cells.

My studies on individual antigens of HCV have delineated interesting immune modulatory mechanisms induced by different antigens. However, in an individual with HCV infection, all of the antigens are simultaneously expressed. Therefore, in the next study, it would be very interesting to look at the effect of some of the disparate antigens together, such as core and NS3, F and NS3, F and core, or F, core and NS3, using both *in vitro* and *in vivo* experiments as described in Chapters 2 and 3.

Progress in the development of an HCV vaccine has been slow due to poor understanding of the type of immunity that naturally controls HCV infection. Chimpanzees that clear the virus demonstrate early humoral and cellular immune responses in contrast to those who develop the chronic infection (34). Our earlier results with mice immunized with the core or NS3 using recombinant adenovirus vectors have implied that NS3 induces a strong T cell response including effector T cells. In this study I showed that early events in antigen encounter imprint the subsequent immunity and the final outcome; two distinct patterns of early events in immunity can be demonstrated for the antigens core and NS3. Future work is required to determine the cell type secreting cytokines like IL-2, IL-6 and IL-12, which dictate the immune response at a later stage of infection. It will be important to investigate whether the early immune events could be altered such that effective cellular immune responses are generated to both core and NS3

antigens of HCV. This can be done by tinkering with innate immunity through TLRs agonists.

5.5 REFERENCES

1. Bürgel, B., M. Friesland, A. Koch, M. Manns, H. Wedemeyer, K. Weissenborn, W. Schulz-Schaeffer, T. Pietschmann, E. Steinmann, and S. Ciesek. 2011. Hepatitis C virus enters human peripheral neuroblastoma cells - evidence for extra-hepatic cells sustaining hepatitis C virus penetration. *J. Viral Hepat.*18: 562–570.

2. Fletcher, N., and J. McKeating. 2012. Hepatitis C virus and the brain. *J. Viral* Hepat. 19: 301–306.

3. Blackard, J., N. Kemmer, and K. Sherman. 2006. Extrahepatic replication of HCV: insights into clinical manifestations and biological consequences. *Hepatology* 44: 15–22.

4. Bain, C., A. Fatmi, F. Zoulim, J. Zarski, C. Trépo, and G. Inchauspé. 2001. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* 120: 512–524.

5. Pachiadakis, I., G. Pollara, B. Chain, and N. Naoumov. 2005. Is hepatitis C virus infection of dendritic cells a mechanism facilitating viral persistence? *Lancet Infect. Dis*. 5: 296–304.

6. Scarlett, U., J. Cubillos-Ruiz, Y. Nesbeth, D. Martinez, X. Engle, A. Gewirtz, C. Ahonen, and J. Conejo-Garcia. 2009. In situ stimulation of CD40 and Toll-like receptor 3 transforms ovarian cancer-infiltrating dendritic cells from immunosuppressive to immunostimulatory cells. *Cancer Res.* 69: 7329–7337.

7. Ahonen C, Doxsee C, McGurran S, Riter T, Wade W, et al. (2004) Combined TLR and CD40 triggering induces potent $CD8^+$ T cell expansion with variable dependence on type I IFN. *J. Exp. Med.* 199: 775–784.

8. Lowin, B., M. Hahne, C. Mattmann, and J. Tschopp. 1994. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* 370: 650–2.

9. Kägi, D., F. Vignaux, B. Ledermann, K. Bürki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 265: 528–30.

10. Thorén, F., A. Romero, M. Lindh, C. Dahlgren, and K. Hellstrand. 2004. A hepatitis C virus-encoded, nonstructural protein (NS3) triggers dysfunction and apoptosis in lymphocytes: role of NADPH oxidase-derived oxygen radicals. *J. Leuk. Biol.* 76: 1180–1186.

11. Albert, M., B. Sauter, and N. Bhardwaj. 1998. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 392: 86–9.

12. WHO report 2012

13. Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, and Y. Ohta. 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA* 87: 6547–9.

14. Chiba, T., Y. Matsuzaki, M. Abei, J. Shoda, T. Aikawa, N. Tanaka, and T. Osuga. 1996. Multivariate analysis of risk factors for hepatocellular carcinoma in patients with hepatitis C virus-related liver cirrhosis. *J. Gastroenterol.* 31: 552–8.

15. Mutimer, D., and A. Lok. 2012. Management of HBV- and HCV-induced end stage liver disease. *Gut* 61 Suppl 1: i59–67.

16. Keck, Z.-Y., A. Saha, J. Xia, Y. Wang, P. Lau, T. Krey, F. Rey, and S. Foung. 2011. Mapping a region of hepatitis C virus E2 that is responsible for escape from neutralizing antibodies and a core CD81-binding region that does not tolerate neutralization escape mutations. *J. Virol.* 85: 10451–63.

17. Gruener, N., F. Lechner, M. Jung, H. Diepolder, T. Gerlach, G. Lauer, B. Walker, J. Sullivan, R. Phillips, G. Pape, and P. Klenerman. 2001. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J. Virol.* 75: 5550–8.

18. Fuller, M., N. Shoukry, T. Gushima, D. Bowen, B. Callendret, K. Campbell, D. Hasselschwert, A. Hughes, and C. Walker. 2010. Selection-driven immune escape is not a significant factor in the failure of CD4 T cell responses in persistent hepatitis C virus infection. *Hepatology* 51: 378–87.

19. MacDonald, A., M. Duffy, M. Brady, S. McKiernan, W. Hall, J. Hegarty, M. Curry, and K. Mills. 2002. CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons. *J. Infect. Dis.* 185: 720–7.

20. Cabrera, R., Z. Tu, Y. Xu, R. Firpi, H. Rosen, C. Liu, and D. Nelson. 2004. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 40: 1062–71.

21. Ebinuma, H., N. Nakamoto, Y. Li, D. Price, E. Gostick, B. Levine, J. Tobias, W. Kwok, and K.-M. Chang. 2008. Identification and *in vitro* expansion of functional antigen-specific CD25+ FoxP3+ regulatory T cells in hepatitis C virus infection. *J. Virol.* 82: 5043–53.

22. Manigold, T., and V. Racanelli. 2007. T-cell regulation by CD4 regulatory T cells during hepatitis B and C virus infections: facts and controversies. *Lancet Infect. Dis.* 7: 804–13.

23. Krishnadas, D., J. Ahn, J. Han, R. Kumar, and B. Agrawal. 2010. Immunomodulation by hepatitis C virus-derived proteins: targeting human dendritic cells by multiple mechanisms. *Int. Immunol.* 22: 491–502.

24. Kanto, T., N. Hayashi, T. Takehara, T. Tatsumi, N. Kuzushita, A. Ito, Y. Sasaki, A. Kasahara, and M. Hori. 1999. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J. Immunol.* 162: 5584–5591.

25. Auffermann-Gretzinger, S., E. Keeffe, and S. Levy. 2001. Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. *Blood* 97: 3171–3176.

26. Lanford, R., D. Chavez, F. Chisari, and C. Sureau. 1995. Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. *J. Virol.* 69: 8079–83.

27. Diana, J., T. Griseri, S. Lagaye, L. Beaudoin, E. Autrusseau, A.-S. Gautron, C. Tomkiewicz, A. Herbelin, R. Barouki, M. von Herrath, M. Dalod, and A. Lehuen. 2009. NKT cell-plasmacytoid dendritic cell cooperation via OX40 controls viral infection in a tissue-specific manner. *Immunity* 30: 289–99.

28. Nattermann, J., H. Zimmermann, A. Iwan, M. von Lilienfeld-Toal, L. Leifeld, H. Nischalke, B. Langhans, T. Sauerbruch, and U. Spengler. 2006. Hepatitis C virus E2 and CD81 interaction may be associated with altered trafficking of dendritic cells in chronic hepatitis C. *Hepatology* 44: 945–54.

29. Gao, D.-Y., X.-X. Zhang, G. Hou, G.-D. Jin, Q. Deng, X.-F. Kong, D.-H. Zhang, Y. Ling, D.-M. Yu, Q.-M. Gong, Q. Zhan, B.-L. Yao, and Z.-M. Lu. 2008. Assessment of specific antibodies to F protein in serum samples from Chinese hepatitis C patients treated with interferon plus ribavarin. *J. Clin. Microbiol.* 46: 3746–51.

30. Komurian-Pradel, F., A. Rajoharison, J.-L. Berland, V. Khouri, M. Perret, M. Van Roosmalen, S. Pol, F. Negro, and G. Paranhos-Baccalà. 2004. Antigenic relevance of F protein in chronic hepatitis C virus infection. *Hepatology* 40: 900–9.

31. Li, K., E. Foy, J. Ferreon, M. Nakamura, A. Ferreon, M. Ikeda, S. Ray, M. Gale, and S. Lemon. 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc. Natl. Acad. Sci. USA* 102: 2992–7.

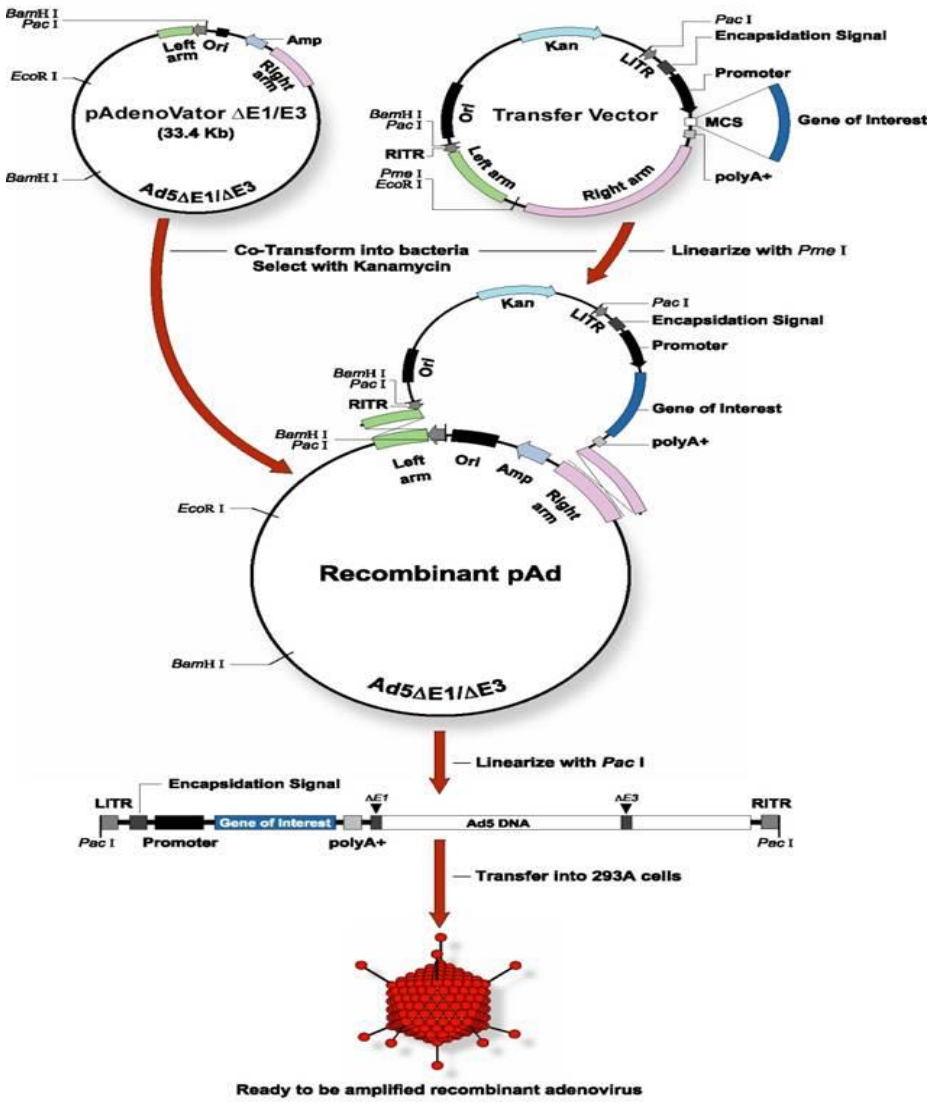
32. Gale, M., and E. Foy. 2005. Evasion of intracellular host defense by hepatitis C virus. *Nature* 436: 939–45.

33. Pavio, N., and M. Lai. 2003. The hepatitis C virus persistence: how to evade the immune system? *J. Biosci.* 28: 287–304.

34. Shata, M., D. Anthony, N. Carlson, L. Andrus, B. Brotman, N. Tricoche, P. McCormack, and A. Prince. 2002. Characterization of the immune response against hepatitis C infection in recovered, and chronically infected chimpanzees. *J. Viral Hepat.* 9: 400–10.

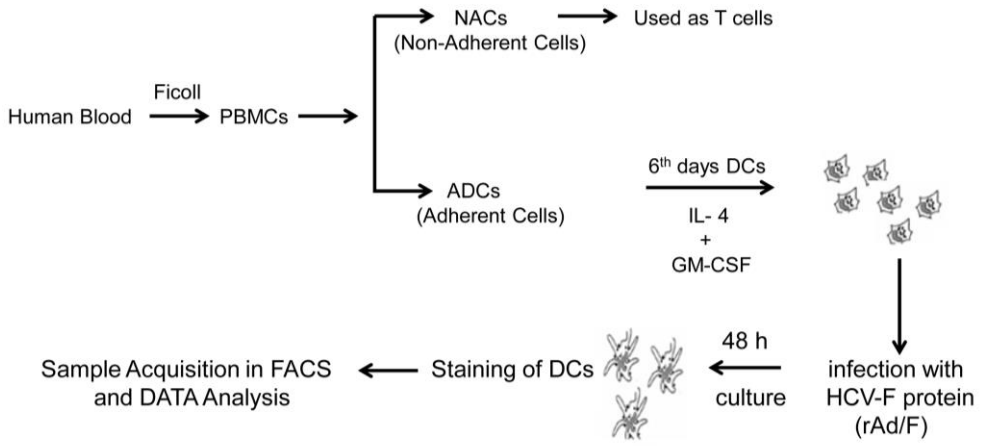
APPENDICES

Appendix Figure-1



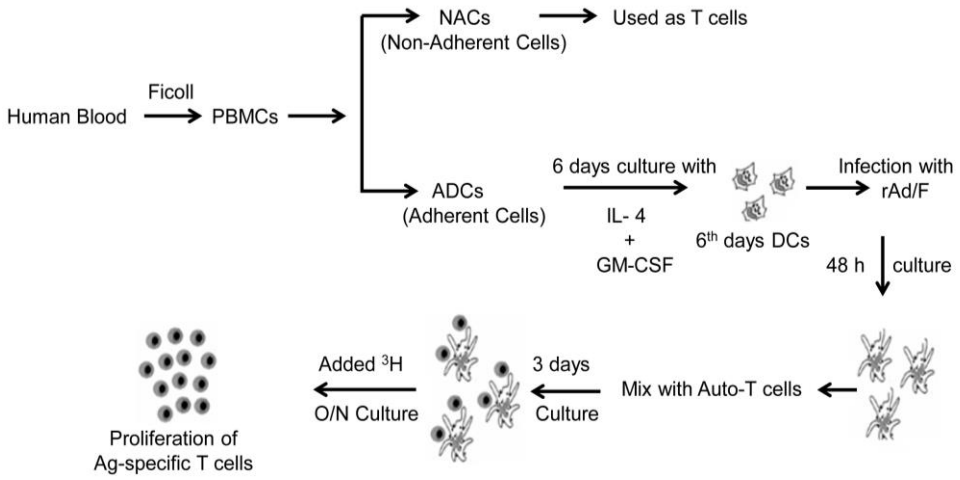
Appendix Figure-1. Cloning of HCV-derived F protein in adenovector virus. First F protein was cloned in transfer vector. Then transfer vector were linearized with PmeI and cotransformed with pAdeno Vator ΔE1/E3 DNA into BJ 5183-competent cells. The Positive recombinants were selected for transformation and propagation in DH5α cells. Further positive clone were transfer in HEK-293A cell line for recombinant adenovirus vector production.

Appendix Figure-2



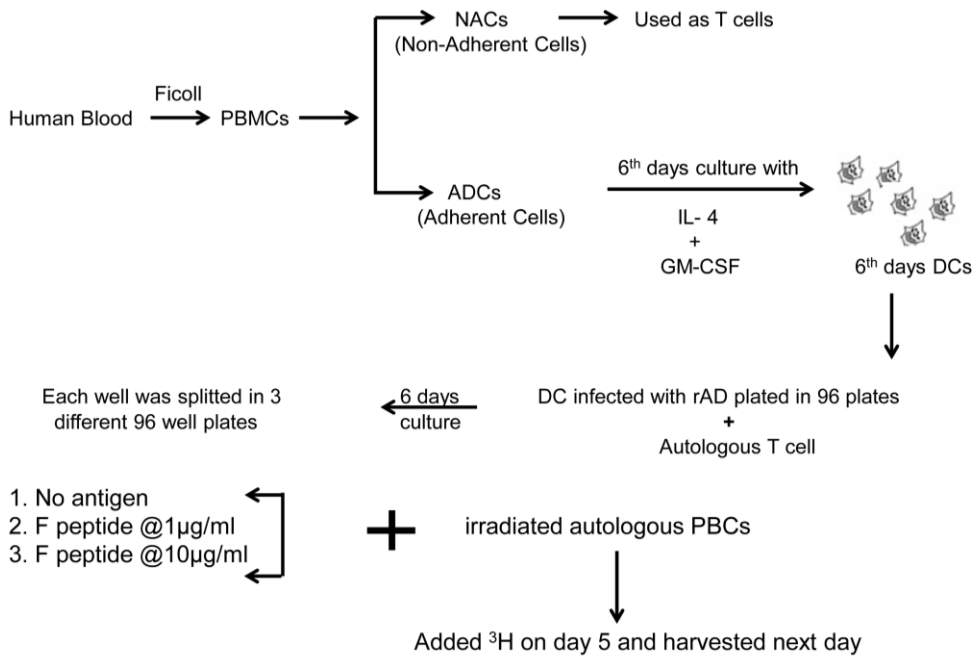
Appendix Figure-2. Experimental plan for analysis of DCs phenotype. PBMCs were isolated from blood by Ficoll-Hypaque density gradient centrifugation. The intermediate buffy layer containing PBMCs was collected and 5×10^6 cells/ml were cultured for 2 hours in 6-well plates in RPMI 1640 medium, supplemented with L-glutamine, 1% human AB serum, 1% sodium pyruvate, and 500 U/ml penicillin/streptomycin. The nonadherent cells (NACs) which mostly included T cells and B cells were subsequently removed and cryopreserved to use in later assays. Remaining adherent cells were treated with 50 ng ml^{-1} granulocyte macrophage colony-stimulating factor (GM-CSF) and 10 ng ml^{-1} of IL-4 (Peprotech) in RPMI media and cultured for 6 days. DCs infected with adenoviral vectors containing F or core protein were harvested after 48 h and used to assess the phenotype by flow cytometry.

Appendix Figure-3



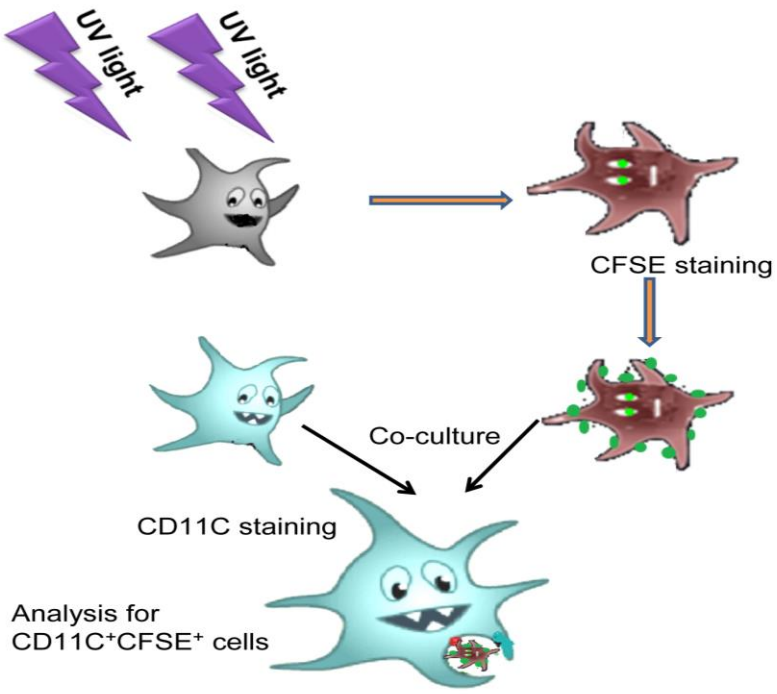
Appendix Figure-3. DCs infected with adenoviral vectors containing F or core protein were harvested after 48 h and cultured with 2×10^5 autologous T cells in different concentrations (1×10^3 to 2×10^4) at 37°C for 5 days. Purified $CD4^+$ or $CD8^+$ T cells were used in these assays. The cells were pulsed with 0.5 µCi/well [^{3}H] thymidine for 16 h and harvested on nylon fibre filter papers (PerkinElmer). The levels of [^{3}H] thymidine incorporation into the cellular DNA were counted in a liquid scintillation counter (MicroBeta Trilux; PerkinElmer). Tests were run in replicates of three to five wells.

Appendix Figure-4



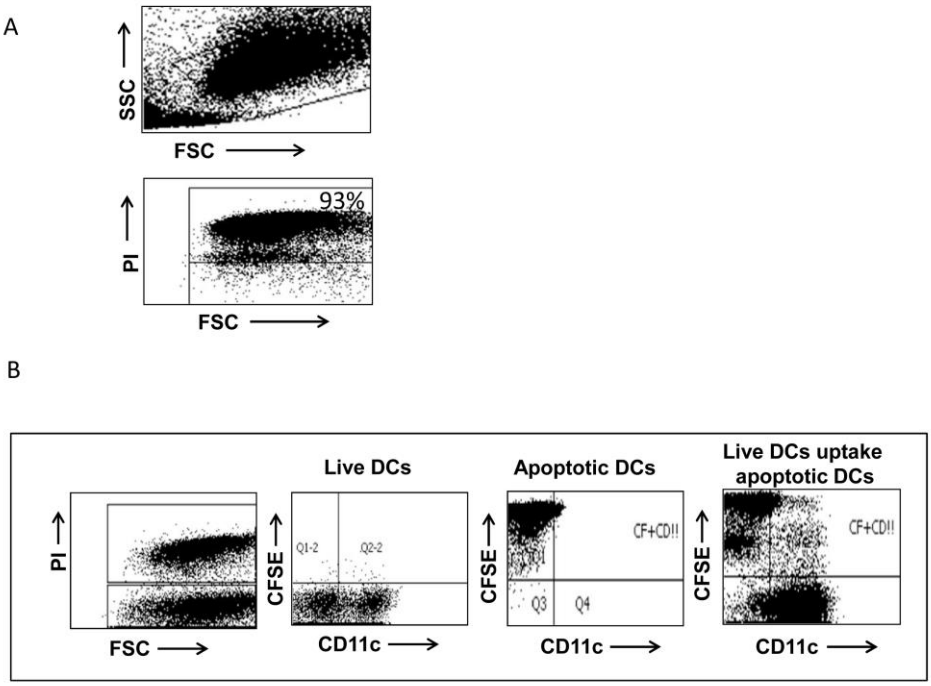
Appendix Figure-4. Experimental plan for secondary T cell proliferation assay. Initially, 48 wells of 96-well plates were plated with F or core protein containing adenovirus-infected DCs (10^4/well) together with 2×10^5 autologous purified $CD4^+$ T cells in a total 200 µl/well of AIM-V medium for 5 days. On day 5, each well was split into three equal wells on three different 96-well plates. On the first plate, no Ag (antigen) was added; on the second plate, the peptides from F and core proteins were added at 1 µg/ml. On the third plate, peptides from F and core proteins were added at 10 µg/ml. Control Ags, SOD, was added in five to six replicates. Each well was fed with irradiated autologous PBMCs (1×10^5/well) and cultured for another 5 days. At the end of the 5 days, 0.5 µCi/well [^{3}H] thymidine was added, followed by harvesting the cells on day 6 and counting [^{3}H] thymidine levels incorporated in the DNA.

Appendix Figure-5



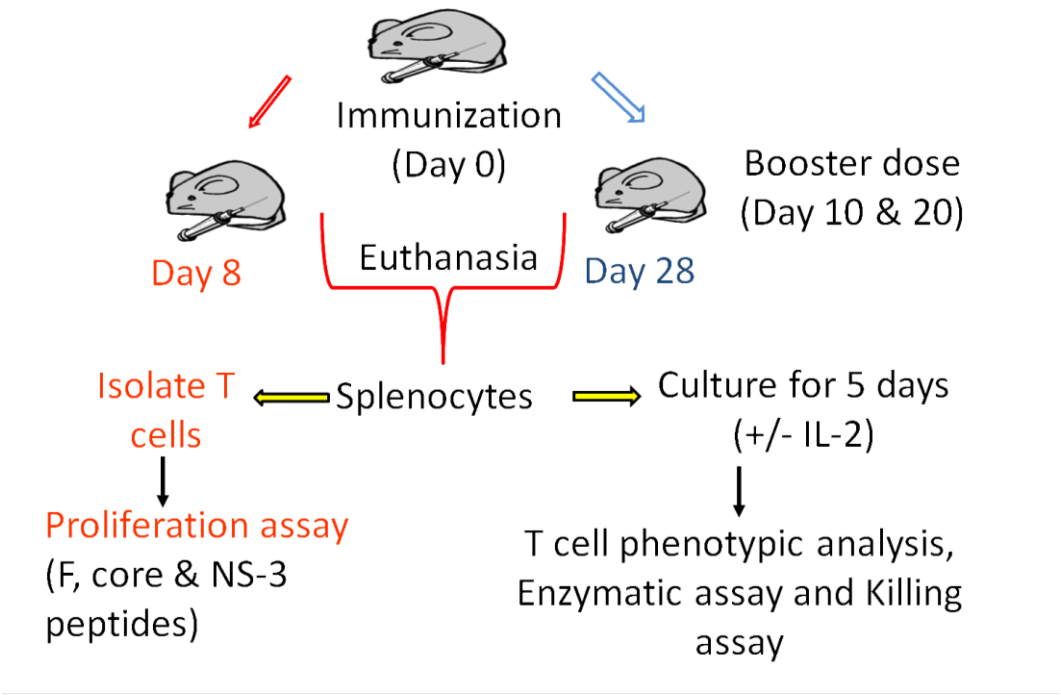
Appendix Figure-5. Phagocytosis of apoptotic DCs by live DCs. For positive control DCs were uv-irradiated for 2 min to induce apoptosis and these apoptotic DCs were labeled with CFSE and incubated with immature CD11c stained viable DCs. Further, FACS analysis was done after 2 hours to assess uptake of CFSE-labelled apoptotic DC ($CFSE^{+}CD\text{-}11c^{-}$) by live DCs ($CFSE^{-}CD\text{-}11c^{+}$).

Appendix Figure-6



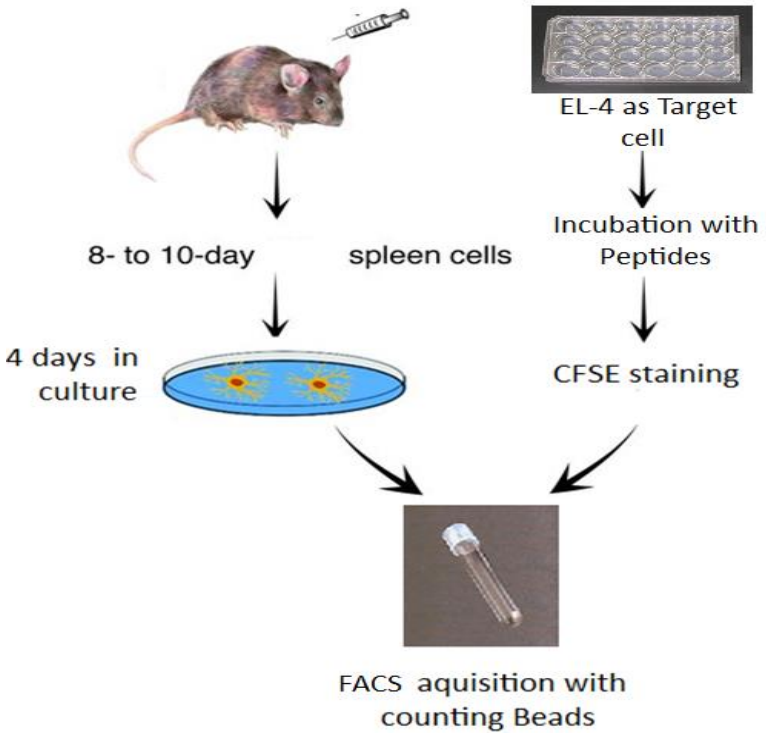
Appendix Figure-6. Uptake of apoptotic DCs by live DCs. For positive control of apoptotic cells, untreated DCs were cultured on a six-well dish and irradiated for 2 min with a UV transilluminator. Induction of apoptosis in UV treated DCs was confirmed by staining cells with Annexin V and 7-AAD (Figure 7A). DCs were cocultured at 37°C or 4°C with F protein or control vector induced CFSE labelled DCs at 1:1 ratio. After 2–4 hours, flow cytometry analysis was performed to assess the uptake of CFSE-labelled apoptotic DC ($CFSE^{+}CD\text{-}11c^{-}$) by live DCs ($CFSE^{-}CD\text{-}11c^{+}$) (Figure 7B).

Appendix Figure-7



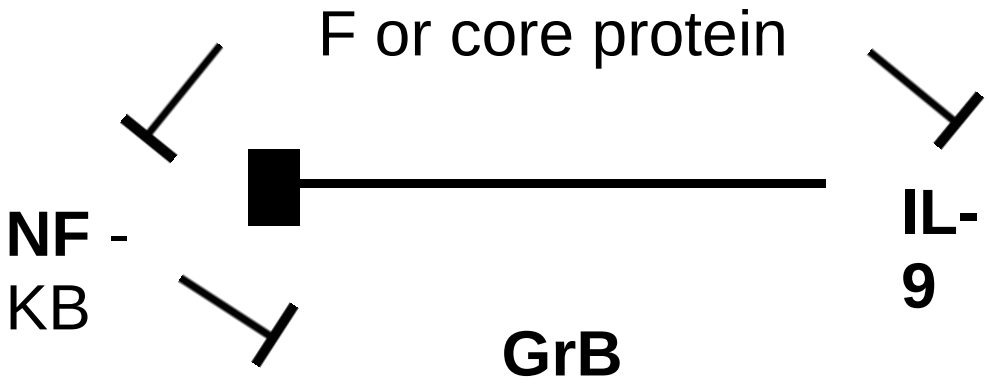
Appendix Figure-7. Experimental plan for immunization of mice and splenocyte culture. Mice were injected intramuscularly in both hind limbs with a total of 2×10^7 replication-deficient recombinant adenovirus particles expressing HCV-F, core, NS3, or control vector in 150 µl volume for immune response studies. On day 8 after first immunization or day 28th after 3rd immunization, mice were euthanized to obtain splenocytes. The spleens were pooled from 3 mice from each group and a single cell suspension was prepared. Whole splenocytes were cultured with and without IL-2 for phenotypic analysis and T cells were enriched using nylon wool column for T cell proliferation assay.

Appendix Figure-8



Appendix Figure-9. Experimental plan for killing assay. EL4 cells were propagated for 2–3 passages and incubated overnight with pools of synthetic peptides (1 μg/ml) derived from F or core protein separately. EL4 cells were harvested and stained with 2 mM of CFSE in PBS for 15 minutes in the dark. CFSE stained target EL4 cells (10,000 cells) were mixed with effector splenocytes in ratios 1:10, 1:30, 1:60, and 1:100. Effector and target cell mixtures were incubated for 4 hours at 37 °C under an atmosphere of 5% CO_2. After 4 hours, 20,000 CountBright beads (Invitrogen, Carlsbad, CA) were added to each tube (Appendix Figure-8). Using FACSDiva to gate the beads, the flow cytometer was run to collect 10,000 beads from each tube to normalize the amount of cells tested in each tube. The difference in CFSE stained cells between treatment and control tubes indicate the number of cells that were killed.

Appendix Figure-9



Appendix Figure-9. **Flow chart for NFκB regulation**. NFκB regulates the expression of GrB by T cells. We hypothesized that F or core protein modulates the activation of NFκB which ultimately reduce GrB expression.

TABLE 3

Peptides derived from NS-3 protein

No.	Position	AA Sequence
1	1067-1081	QTFLATCINGVCWTV
2	1127-1142	SSDLYLVTRHADVIP
3	1187-1201	RGVAKAVDFIPVENL
4	1372-1386	EIPFYGKAIPLEVIK
5	1411-1425	GINAVAYYRGLDVS
6	1467-1482	RRGRTGRGKPGIYRF
7	1607-1622	MWKCLIRLKPTLHGP
8	1621-1635	PTPLLYRLGAVQNEV