Immune Responses against Hepatitis C Virus

by

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Abstract

Hepatitis C virus (HCV) leads to chronic infection in the majority of infected patients presumably due to failure or inefficiency of the immune responses generated. Both antibody and cellular immune responses have been suggested to be important in viral clearance. Non-replicative adenoviral vectors expressing antigens of interest are considered as attractive vaccine vectors for a number of pathogens. In this study, we sought to evaluate cellular and humoral immune responses against HCV NS4 protein using recombinant adenovirus as a vaccine vector expressing NS4 antigen. We have also measured the effect of antigen doses and routes of immunization on the quality and extent of the immune responses, especially their role in viral load reduction, in a recombinant Vaccinia-HCV (Vac-HCV) infection mouse model. Our results show that an optimum dose of adenovirus vector (2×10^7 pfu/mouse) administered intramuscularly (i.m.) induces high T cell proliferation, granzyme B-expressing $CD8^+$ T cells, pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-2 and IL-6, and antibody responses that can significantly reduce the Vac-HCV viral load in the ovaries of female C57BL/6 mice. Our results demonstrate that recombinant adenovirus vector can induce both humoral and cellular protective immunity against HCV-NS4 antigen, and that immunity is intricately controlled by route and dose of immunizing vector.

Adenoviruses (Ad) are commonly used as vectors for gene therapy and/or vaccine delivery. Recombinant Ad vectors are being tested as vaccines for many pathogens. Here, we have made a surprising observation that peptides derived from various hepatitis C virus (HCV) antigens contain extensive regions of homology with multiple adenovirus proteins, and conclusively demonstrate that adenovirus vector can induce robust, heterologous cellular and humoral immune responses against multiple HCV antigens. Intriguingly, the induction of this cross-reactive immunity leads to significant reduction of viral loads in a recombinant vaccinia-HCV virus infected mouse model, supporting their role in antiviral immunity against HCV. Healthy human subjects with Ad-specific pre-existing immunity demonstrated cross-reactive cellular and humoral immune responses against multiple HCV antigens. These findings reveal the potential of a previously uncharacterized property of natural human adenovirus infection to dictate, modulate and/or alter the course of HCV infection upon exposure. This intrinsic property of adenovirus vectors to cross-prime HCV immunity can also be exploited to develop a prophylactic and/or therapeutic vaccine against HCV.

Preface

This thesis is an original work by Shakti Singh. The animal experiments/ procedures (Protocol# AUP212, till Aug 12, 2015) were approved and conducted in accoradance with the University of Alberta Animal Care and Use Committee. The procedures related to human tissue were approved (Protocol# 3983 till Nov 23, 2015) by University of Alberta Human Research Ethics Board.

Recombinant adenoviral vectors expressing Hepatitis C virus antigens were prepared by Dr. Wen Li (University of Alberta), and chimeric vaccinia-HCV viruses were gifted by Dr. Alfred M. Prince (New York University, USA).

Chapter 2 of this thesis has been published as S. Singh, S. Vedi, W. Li, S.K. Samrat, R. Kumar R, and B. Agrawal, "Recombinant adenoviral vector expressing HCV NS4 induce protective immune responses in a mouse model of Vaccinia-HCV virus infection: A dose and route conundrum", *Vaccine*, Vol. 32, issue 23, 2712-2721. I was responsible for the experiment design, execution, data collection and anaylysis, and manuscript writing. S. Vedi and S.K. Samrat assisted with the execution of experiments and data collection. Dr. W. Li prepared recombinant adenovector expressing Hepatitis C virus NS4 antigen. Dr. R. Kumar contributed to concept and manuscript composition. Dr. B. Agrawal was the supervisory and corresponding author and was involved with concept formation, manuscript edits and composition.

Dedicated to my parents

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-Shakti Singh

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List of Abbreviations

AA	: Amino Acid
Ad	: Adenovirus
Ag	: Antigen
ALT	: Alanine Transaminase
ANOVA	: Analysis of Variance
APC	: Allo-phycocyanin
APCs	: Antigen-Presenting Cells
ATCC	: American Type Culture Collection
CCAC	: Canadian Council of Animal Care
CD	: Cluster of Differentiation
cDNA	: Complementary DNA
CFA	: Complete Freund's Adjuvant
CLRs	: C-type Lectin Receptors
CFSE	: 5(6)-Carboxy Fluorescein N-hydroxy Succinimidyl Ester
ConA	: Concanavalin A
CpG	: Cytosine-phosphate-Guanine
CTL	: Cytotoxic T Lymphocyte
CTLA-4	: Cytotoxic T-lymphocyte-Associated Antigen 4
CXCR6	: Chemokine (C-X-C Motif) Receptor 6
GrB	: Granzyme B
DCs	: Dendritic Cells
DAA	: Direct Acting Antiviral
DMSO	: Dimethyl Sulfoxide
DNA	: Deoxyribonucleic Acid
EDTA	: Ethylene Diamine Tetraacetic Acid
ELISA	: Enzyme-linked ImmunoSorbent Assay
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
HCC	: Hepato Cellular Carcinoma
HCV	: Hepatitis C Virus
HDL	: High Density Lipoprotein
HSLAS	: Health Sciences Laboratory Animals Services
HVR	: Hyper Variable Region
ICAM-I	: Intercellular Adhesion Molecule-1
IDU	: Intravenous Drug Use
IFN-γ	: Interferon-gamma
IL	: Interleukin
IM or i.m.	: Intramuscular

IP or i.p. : Intraperitoneal
ISGs : Interferon Stimulatory Genes
JAK : Janus Kinase
kDa : Kilo Dalton
LPS : Lipopolysaccharide
mAb : Monoclonal Antibody
mDCs : Myeloid Dendritic Cells
mg : Milligram
MHC : Major Histocompatibility Complex
min : Minute
ml : Milliliter
NANBH : Non-A Non-B Hepatitis
NK cells : Natural Killer Cells
NF-κB : Nuclear Factor-Kappa B
NS : Non Structural
OD : Optical Density
ORF : Open Reading Frame
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 : Propidium Iodide
Poly I:C : Polyinosinic-polycytidylic Acid
RBCs : Red Blood Cells
PRRs : Pattern Recognition Receptor
RBV : Ribavirin
RIG-1 : Retinoic Acid Inducible Gene-I
RPMI : Roswell Park Memorial Institute
RNA : Ribonucleic Acid
rpm : Revolution Per Minute
STAT : Signal Transducer And Activator Of Transcription
SVR : Sustained Virologic Response
TCR : T Cell Receptor
TGF-β : Transforming Growth Factor-beta
Th : T Helper
TLR : Toll Like Receptor
UTR : Un-translated Region
VLDL : Very Low Density Lipoprotien
µg : Microgram
µl : Microliter

Chapter 1

Introduction to Hepatitis C Virus

1.1 Hepatitis

Hepatitis, inflammation of the liver, can be caused by several agents such as viral infection, alcohol, drugs, toxins, and/or cells of immune system that have gathered to counter an infection. Inflammation is a natural reaction of the body's immune system to injury and is often characterized by swelling and edema due to an infiltration of immune cells. Several hepatitis viruses such as hepatitis A, B, C, D, and E can infect the human liver and cause hepatitis. Hepatitis caused by these hepatic viruses shows similar symptoms but differs in the transmission, duration, and magnitude of the infection. Several non-hepatic viruses such as cytomegalovirus, Epstein-Barr virus, and the virus that causes yellow fever also can cause hepatitis. This chapter describes the current knowledge about hepatitis C virus, its epidemiology, infection, immunity, and other aspects.

1.2 Hepatitis C Virus

Initially, hepatitis A and hepatitis B viruses were thought to be the agents that caused most of the hepatitis cases initiated by blood transfusions. Blood tests were developed for hepatitis B in 1963 and for hepatitis A in 1973, but many of the blood samples taken for post-transfusion illness tested negative for both hepatitis A and B; and this transfusion-associated hepatitis continued to occur. In 1975, the etiological agent for this non-A non-B hepatitis (NANBH) in post-transfusion patients was confirmed as an unknown NANBH infectious agent [1]. In 1989, the genome of this unknown NANBH virus was determined to be a positive sense double stranded RNA and it was partially cloned. This newly identified virus which causes NANBH was named *hepatitis C virus* (HCV) [2]. After identification of this new virus, several anti-HCV antibody and PCR-based detection methods were developed to diagnose cases of NANBH or HCV infection [3]. After

screening of stored blood using these detection methods, it was estimated that approximately 90 to 95% of NANBH cases were actually caused by the hepatitis C virus.

1.3 Transmission and Epidemiology

HCV is mainly transmitted through contaminated blood transfusions, needles (used in occupational health injuries under medical care and in drug injection), syringes, and/ other medical devices. Sexual and perinatal transmission may also occur, although such cases are infrequent. Other less common modes of transmission includes social, cultural, and behavioral practices such as ear and body piercing, circumcision, and tattooing with contaminated tools. HCV transmission associated with blood and blood products was very high before 1991. After 1992, when a number of sensitive screening tests for HCV were developed and instituted for the screening of blood and blood products, the risk of transmission through blood transfusion was quickly reduced (27-80%) and become as low as 1 in 100, 000 per unit of blood transfused in many developed countries [4-6]. Presently, in low prevalence high income regions such as North America, Central and Western Europe, Pacific Asia (Japan and Korea), and Australia, the majority (up to 60-90%) of HCV infections are due to a high number of intravenous drug users (IDU) who share contaminated needles; this number is followed by HCV infections due to un-protected sex and occupational health care workers exposed to blood [7]. HCV transmission through blood transfusions and unsterilized injection is still high in many developing countries that have moderate to high prevalence rate due to lack of proper screening of blood and blood products [4, 7]. Worldwide, approximately two million HCV infections result annually from contaminated therapeutic injections, accounting for up to 40% of all new cases [8]. Transmission through a perinatal route is very low; however, HIV co-infection has been reported to increase the rate of perinatal transmission 4–5 fold [9].

The incidence of HCV in a population can be difficult to determine because acute infections can be mild or asymptomatic. It is estimated that about 2.8% of the world population or 184 million people are chronic carriers of HCV [10, 11]. The majority (80%) of newly HCV infected people enter into a chronic disease state and 20-30% of these further develop liver cirrhosis and hepatocellular carcinoma (HCC) [12] **(Fig 1.1)**. In Canada, about 1.1% of the population (332,000 individuals) are HCV seropositive [13]. The global economic impact of HCV is difficult to estimate. The prevalence of HCV differs not only between countries, but also within geographic regions and by socioeconomic class within a given country [14]. Table 1.1 provides the details of very low (<1.0%), low (1.1–1.5%), moderate (1.5–2.0%), high (2.0–5.0%) and very high (>5.0%) HCV sero-prevalence regions [13].

The genotype distribution in HCV infected individuals also varies by region. Based on genotypic prevalence data available from 98 countries, it has been estimated that genotype (G) 1 or G1 is the most common (46%), followed by G3: 22%, G2: 13%, G4: 13%, G6: 2% and G5: 1% [13]. Subtype 1b accounts for 22% of all infections globally. In North America, Latin America and Europe, G1 is the most common (60–71%), with G1b representing 26%, 39%, and 50% of all infections in these regions respectively. The genotype distribution in Canada is mostly represented by G1a (34.2%), followed by G3: 22.3%, 1b: 20.1%, G2: 15.4% and G4: 2.3% [13]. In the United States, the prevalence of HCV infection is represented by G1a: 46.2%, G1b: 26.3%, G2: 10.7% and G3: 10-12 %. Genotype 1 is also most prevalent in Australasia, Europe, Latin America and North America and ranges from 53–71%. Genotype 4 is the most prevalent in North Africa (71%) and the Middle East. In this region, Egypt alone accounted for 37% of G4 infections; if Egypt is excluded, HCV infections in North Africa comprise 46% genotype 1 and 34% genotype 4. In South Asia and South-East Asia (India, Pakistan and Bangladesh), G3 is the

most prevalent (> 50%), whereas genotype 1b is the most common in Russia, China, Korea, and Japan (>50%) [13].

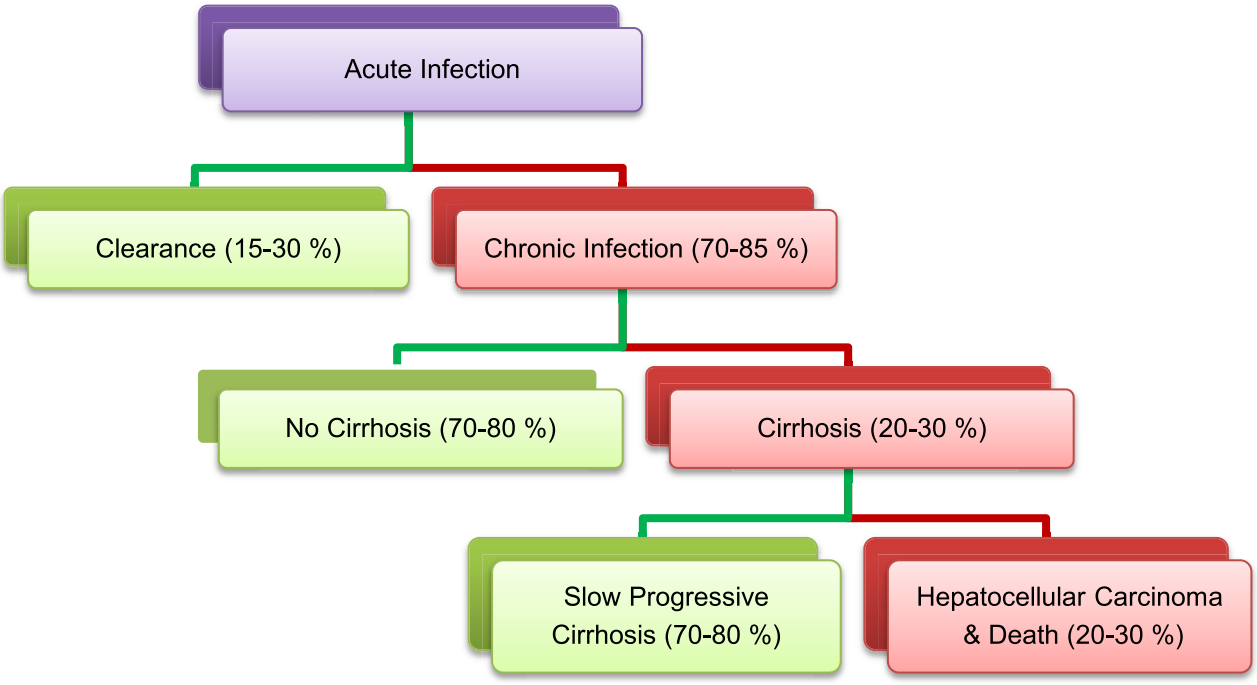

Fig 1.1: Progression of HCV infection. Acute HCV infection may progress to chronic infection in majority of the cases, which may further leads to cirrhosis and hepatocellular carcinoma.

Table 1.1: Global sero-prevalence of HCV [Reference 15]

Incident Rate	% Sero-prevalence	Major Geographical Regions
Very Low	< 1.0	**Asia Pacific**: Korea Republic **Asia, South**: India **Asia, Southeast**: Indonesia, Philippines **Europe, Central**: Czech Republic, Hungary, Poland **Europe, Western**: Austria, Belgium, Cyprus, Germany, Denmark, Finland, France, United Kingdom, Luxemburg, Netherlands, Sweden **Middle East**: Iran, Qatar
Low	1.0- 2.4	**Asia Pacific**: Japan **Asia, East**: China **Asia, South**: Afghanistan, Bangladesh **Asia, Southeast**: Cambodia, Sri Lanka, Myanmar, Malaysia **Australasia**: Australia and New Zealand **Europe, Central**: Bulgaria, Slovakia **Europe, Eastern**: Belarus, Latvia **Europe, Western**: Greece, Switzerland, Spain, Ireland, Israel, Italy, Portugal **America, Latin**: Mexico, Peru, Venezuela, Argentina, Brazil **Middle East**: Algeria, Libya, Morocco, Saudi Arabia, Yemen, Tunisia and Turkey **America, North**: Canada, USA **Africa**: Ethiopia, Madagascar, South Africa, Zimbabwe, Gambia
Moderate	2.5-4.9	**Asia, East**: Taiwan **Asia, Central**: Azerbaijan, Kazakhstan, Kyrgyzstan, Tajikistan **Asia, Southeast**: Thailand **Europe, Central**: Romania **Europe, Eastern**: Russia, Ukraine **Middle-East**: Iraq **Africa**: Congo Republic
High	5.0- 10.0	**Asia, Central**: Georgia, Turkmenistan **Asia, South**: Pakistan **Africa**: Nigeria
Very High	>10.0	**Asia, Central**: Mongolia, Uzbekistan **Africa**: Egypt, Gabon, Cameroon

1.4 Genome and Proteins

HCV is an enveloped virus 50–60 nm in diameter [16]. HCV is thought to adopt a classical icosahedral scaffold in which glycoproteins E1 and E2 are anchored to the host cell-derived double-layer lipid envelope. Within the envelope is the nucleo-capsid which is composed of multiple copies of the core protein, forming an internal icosahedral capsid. The capsid contains genomic positive single-stranded RNA of about 9.6 kb which can be translated into a single polyprotein. The processing of the polyprotein, which occurs co-translationally as well as post-translationally by a cellular and viral protease, makes structural proteins (E1, E2, core) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) [17, 18]. These viral proteins are described in section 1.4.1 and 1.4.2 (**Fig 1.2**).

1.4.1 Structural Proteins

Capsid

The capsid consists of 21–24 KDa protein subunits, which formed from the first 191 amino acids (aa) of the HCV polyprotein. This protein is relatively conserved and contains several antigenic epitopes. It plays a major role in the packaging of viral RNA and also can modulate cellular transduction pathways and also contribute to pathogenicity. In the host cell, HCV core protein is mainly cytoplasmic, located on the endoplasmic reticulum membranes and around lipid vesicles [19-23].

Envelope Glycoproteins (E1 & E2)

The envelope glycoproteins, E1 and E2, are essential components of the HCV virion and necessary for fusion with the host cell membrane and viral entry [24, 25]. E1 and E2 have molecular weights of 33–35 and 70–72 KDa respectively, and assemble as non-covalent heterodimers [26]. E1 and E2 are highly glycosylated, containing up to 5 and 11 glycosylation

sites, respectively. Viral attachment is thought to be initiated via E2 interaction with several components of the receptor complex such as high-density lipoproteins (HDL), scavenger receptor B type I (SR–BI), CD81, occludin, claudin-1 [27-29].

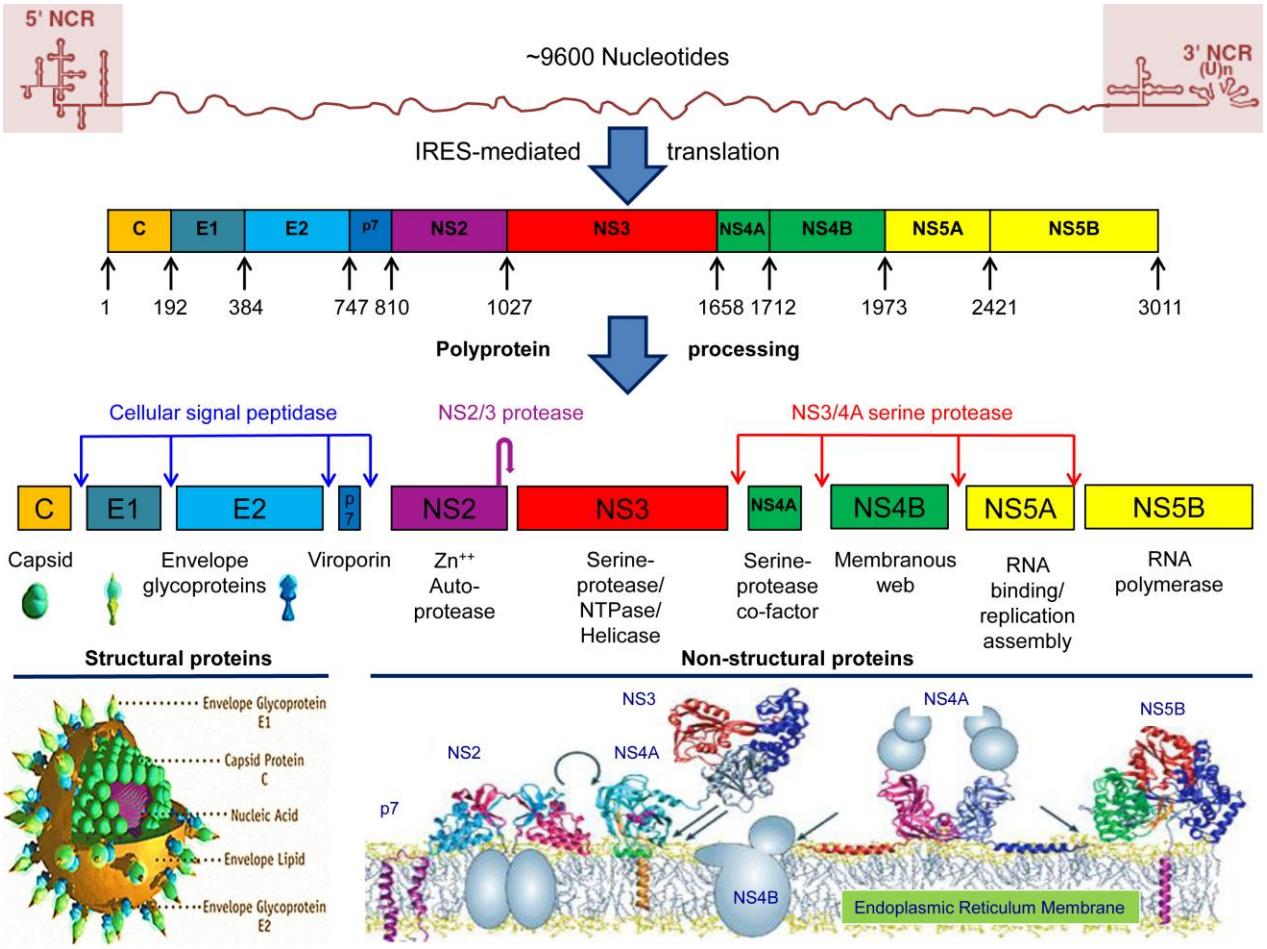

Fig 1.2: Genome and proteins of HCV. HCV RNA genome encodes a single polyprotein of about 3000 amino acid, which is cleaved by host and viral proteases into various structural and non-structural proteins. Cellular signal proteases cleave the polyprotein at Core-E1, E1-E2, E2-P7 and P7-NS2 junctions. This is followed by cleavage at NS2-NS3 junction by NS2-NS3 auto protease. Rest of the polyprotein is cleaved by NS3-4A serine protease. Lower left panel shows the HCV virion structure and right panel shows the arrangement of HCV proteins in endoplasmic reticulum membrane [Lower panels of the figure were modified from references: www.prn.org and 30].

Frame-shift Protein

The F (frame-shift) protein is generated as a result of a –2/+1 ribosomal frame-shift in the N-terminal capsid-encoding region of the HCV poly-protein. The role of F protein in the HCV lifecycle is not defined but it was proposed to be involved in viral persistence [31], and have been found to modulate host immune system [32].

1.4.2 Non-Structural Proteins

NS1 (p7)

NS1 or p7 is a small polypeptide (63 aa) that has been shown to be an integral membrane protein [33]. It belongs to the viroporin family and can act as a calcium ion channel [34]. Mutations or deletions in its cytoplasmic loop suppress the infectivity of HCV cDNA in chimpanzees [35].

NS2

NS2 is a non-glycosylated trans-membrane protein of 21–23 KDa. NS2, together with amino-terminal domain of the NS3 protein, the NS2/NS3 protease, constitutes a zinc-dependent metallo-protease that cleaves the NS2-NS3 junction [17, 18].

NS3

The HCV NS3 protein has serine protease, NTPase, and RNA helicase activities. The serine protease activity remains in N-terminal 1/3 domain (181 aa) and a helicase/NTPase activity in C-terminal 2/3 domain (465 aa). The serine protease domain of NS3 binds to its cofactor NS4A and catalyzes polyprotein cleavage at the NS3–NS4A, NS4A–NS4B, NS4B–NS5A and NS5A–NS5B junctions [36-38]. The 3D structure of the NS3 serine protease domain complexed with NS4A has been determined [39-41], and is one of the most popular targets for antiviral drugs

[42]. The NS3 helicase–NTPase has several functions, including RNA-stimulated NTPase activity, RNA binding, and unwinding of RNA regions of extensive secondary structure by coupling unwinding and NTP hydrolysis [43, 44].

NS4A

The NS4 region of the polyprotein comprises 2 proteins; namely, NS4A and NS4B. The HCV NS4A protein is a 54 amino acid protein, which has a hydrophobic N-terminal region followed by a hydrophilic C-terminal region [45]. The central region (aa 21–34) of NS4A is hydrophobic and required for the activation of NS3 [37, 45, 46] and appears to be an integral part of the protease complex.

NS4B

NS4B is a 27 KDa integral membrane protein containing both hydrophobic trans-membrane and amphipathic helices [47, 48]. It induces the formation of specialized membrane compartment called 'membranous web', which act as RNA replication site [48]. It also binds HCV RNA and help in the formation of replication complex [49, 50].

NS5A

NS5A is 56-58 KDa (447 amino acid) phosphorylated zinc-metallo-protein which does not have any enzymatic property. NS5A exists as multiple phospho-isoforms and is predominantly localized in the cytoplasmic/peri-nuclear compartments of the cell, including the endoplasmic reticulum (ER) and the Golgi apparatus. This pattern of NS5A localization is consistent with the notion that NS5A interacts with multiple host cell and viral proteins. The NS5A plays multiple roles in mediating viral replication, host-cell interactions, and viral pathogenesis [51, 52]. Therefore, this protein is a major target for various direct acting antiviral (DAA) drugs.

NS5B

NS5B is a 68 KDa RNA-dependent RNA polymerase (RdRp). The crystal structure of NS5B revealed that the RdRp has a classical "fingers, palm and thumb" structure formed by its 530 N-terminal aa [53-55] responsible for synthesizing the complementary negative strand of the genome as the replication intermediate and then genomic RNA using the negative strand as the template. Other important features of the HCV NS5B include a β-hairpin loop [56], a GTP-binding site [53, 57] and the ability to homo-oligomerize [58, 59]. A functional interaction between NS5A and NS5B has been reported [60]), and the proposed function of NS5A is to serve as a cofactor for NS5B [61].

1.5 Genetic Variability and Quasispecies

HCV shows a high genetic diversity like other single stranded RNA viruses, which is due to lack of proofreading activity in its RNA-dependent RNA polymerase (RdRp), resulting in the introduction of wrong nucleotides that are not corrected during replication. Hence, any new mutation incorporated during replication, will be inherited in the progeny virus. Because of the high replication rate and a very high frequency of error in replication results in very high mutation rate[62]. Therefore, inside the host, HCV exists as a heterogeneous species called as "quasispecies". The quasispecies nature is distributed throughout the genome except in the 5'UTR, where no variation is observed. Some of the non-structural protein, NS5A in particular, as well as the capsid encoding sequences also show a lower, yet significant, rate of variability. Rest other non-structural proteins are relatively conserved. Some `hot spots' of mutation (1.6%) have been identified in the E2 envelope protein as hypervariable regions: HVR 1 and 2. HVRl is 27 amino acids in length that shows up to 80% diversity in amino acid sequence. However, several positions in HVRl are relatively conserved and have B-cell epitopes, suggesting that a conserved

secondary structure of HVRl is required for the viability of the virus. The complexity of this region does not correlate with the severity of liver disease, HCV genotype, or viral load [18, 63, 64]. HVR2 is only found in the HCV−1b genotype [63] and consists of 7 amino acids, located at positions 91 to 97 of the E2 envelope protein. HVR2 shows up to 100% amino acid sequence diversity and have no B-cell epitopes. The sequence analysis of the HCV genome reveals that the 5' and 3' coding regions are relatively conserved and form secondary structures [65]. The mechanism of frequent genetic mutations in HVRl and HVR2 is not yet fully understood, but it certainly helps HCV in generating variants and escape from immune recognition [66-70].

1.6 Classification and Genotypes

HCV has been classified under *flaviviridae* family as a separate genus *hepaciviruses.* Its genome shows extensive heterogeneity and contains several variable and hypervariable regions in the genome. There are also relatively well-conserved regions, such as capsid, NS3, NS5 coding and 5'-non coding region [71]. On the basis of these conserved regions in genome, HCV can be classified in to seven distinct genotypes (1 to 7) and more than 50 subtypes. It has been showed that anti-capsid or NS4 antibodies can be used to distinguish between many HCV genotypes. However, the serologic differences do not equate much with distinct serotypes based on antibody recognition as seen in most other viruses. It also does not show a regular correlation with specific genotype that can be used as a quicker method to determine genotype using the antibody. Therefore, the most reliable methods used for genotyping of HCV are based on nucleic acid genome rather than antibody based serotyping, and includes: restriction length polymorphism of the 5' NCR, reverse dot blot hybridization analysis of 5'-NCR or LIPA (Line Probe Assay) and nested PCR amplification of the core region using type specific primers. PCR amplification has been found to be the most reliable method of genotyping. Each genotype regularly shows a

difference of more than 20% at the nucleotide level and more than 15% at the amino acid level, although the 5' NCR and capsid protein-encoding region show high homology, i.e., > 90%. However, the clinical and virological data have not shown significant phenotypic differences between various genotypes, but there is a general agreement that the response to interferon therapy depends on the infecting HCV genotype [72].

1.7 Viral Life Cycle

Due to lack of efficient cell culture system, the study of HCV viral life cycle has been delayed and still is not completely understood. The initial step in the viral life cycle involves the attachment and binding of the virus to its cellular receptor(s) on hepatocyte. Several receptors such as CD-81, human scavenger receptors class B type (HSR-B), lectins L–SIGN and DC–SIGN, glycosaminoglycans and low density lipoprotein receptor (LDLR) binds E2 protein and help in attachment and entry of virus in to the host cell [73-78]. Another receptors occludin and claudin-1 which are highly expressed at tight junctions, has also been shown to be involved in the late stage of viral entry [79]. After attachment, the virus enters the host cell by endocytosis and the release of the viral genomic RNA into the cytoplasm is thought to be pH dependent.

Immediately after the release of the genomic RNA into the cytoplasm, translation of the viral proteins starts by binding of 5'–internal ribosome entry site (5'–IRES) to ribosomes. The translation generates the polyprotein that is co– and post-translationally cleaved by cellular and viral proteases into individual structural and non-structural proteins. The capsid protein associates with LDs and recruits non-structural proteins and the replication complex to LDs-ER-associated membranes [80]. The newly synthesized NS5B RdRp along with other viral and host factors form replication complex and makes full-length negative strand RNA intermediate. This negative strand then serves as a template for synthesis of positive strands, used for further translation,

replication and RNA genomes for new viral particles. The NS3/4A helicase/NTPase unwinds the double stranded replication intermediates [44, 81], and NS4B induces the formation of membranous web [48].

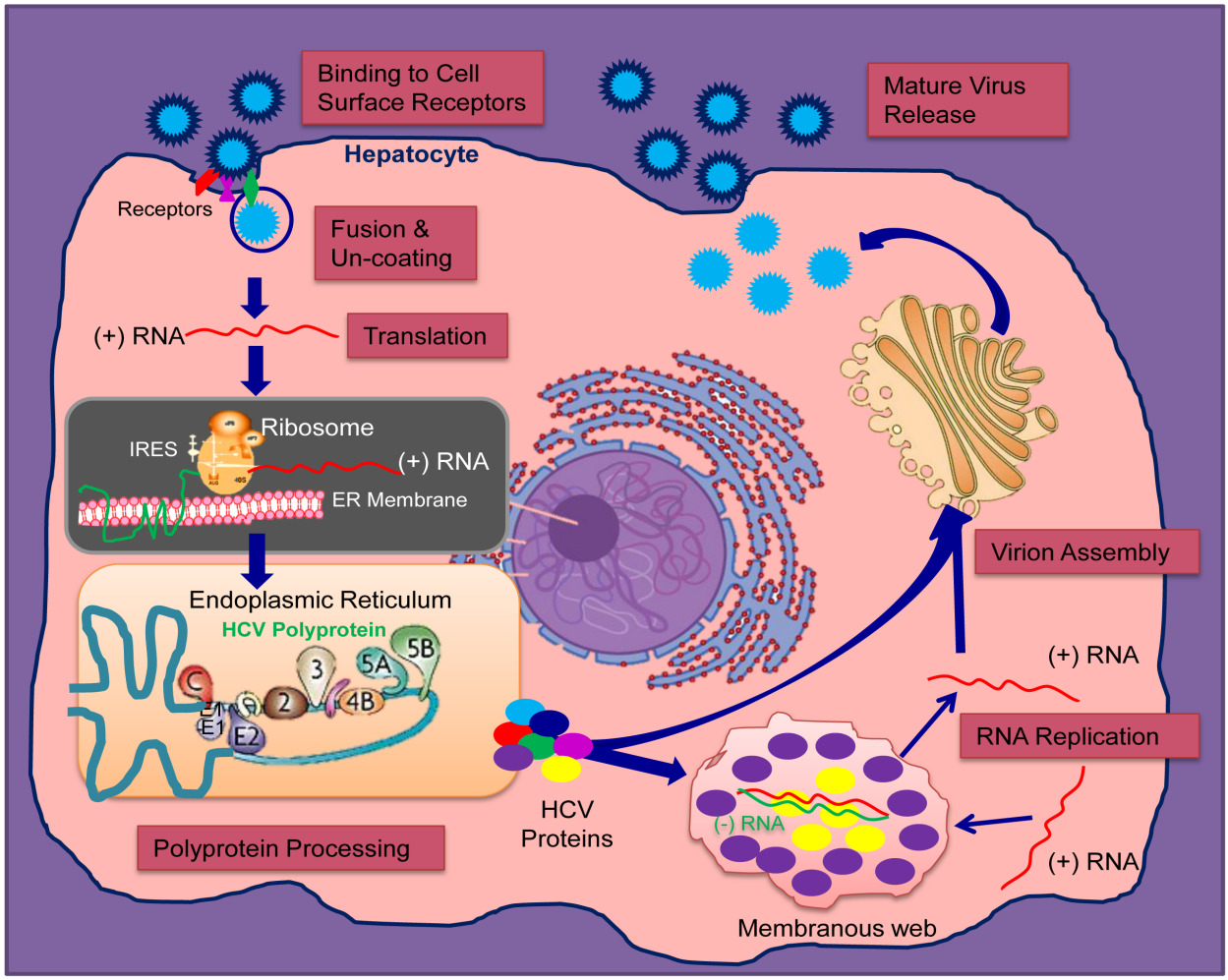

Fig 1.3: Life cycle of HCV. The first step in the HCV life cycle is the attachment of virus to the cell surface. Viral envelope E1 and E2 proteins interact with the cell surface receptors. Envelope fuses with the cell membrane and capsid containing viral genome released inside the cell, where capsid degrades and viral RNA releases in the cytoplasm. Viral RNA translated into polyprotein, which is cleaved into individual viral proteins by cellular and viral proteases [30]. The RNA polymerase (NS5B) synthesizes multiple copies of original ssRNA genome using (-) ssRNA intermediate. The virus assembly takes place in golgi complex from where the virus particles transported through vesicle to the cell surface and bud outside the cell. Viral RNA translation and polyprotein processing takes place in close proximity, which is shown here in separate steps just for the sake of simplicity [This figure was modified from reference 30].

The capsid protein form nucleo-capsid with viral RNA, and further interact with envelope glycoproteins E1 and E2, which is followed by budding of viral particles through ER or other intracellular membranes acquiring envelope membrane. The mature infectious virus particles are released from the host cell through the golgi complex via a secretory pathway [82, 83] **(Fig 1.3)**.

1.8 Progression of HCV Infection

Hepatitis C virus causes acute infections in liver, which can progress to persistent chronic infection.

1.8.1 Acute Infection

Clinical manifestation of acute HCV infection varies widely from mild or asymptomatic infection requiring no treatment to fulminant hepatic failure needing liver transplantation. Only 20% to 30% of patients show jaundice, and 10% to 20% may show non-specific symptoms including anorexia, malaise and abdominal pain [84]. Altogether, the illness generally lasts for 3 to 12 weeks. Serum alanine amino transferase (ALT) is released in bloodstream when liver is diseased or damaged, and can increase up to 10 fold in serum before the clinical symptoms appears. In patients with self-limited hepatitis, serum ALT levels return to normal and HCV RNA becomes undetectable; levels of anti HCV antibodies also decrease progressively and will finally disappear. The concentrations of HCV RNA are low during the acute symptomatic phase.

1.8.2 Chronic Infection

Approximately 80% of newly infected patients progress to develop chronic infection. Such virus infection has potential to cause liver damage and over time, more liver cells are damaged and destroyed, and replaced with scar tissue. This process is called fibrosis. Severe fibrosis can cause the liver to become scarred, and prevents it from working normally. This is called cirrhosis of the liver. In several cases, serious damage to the liver can lead to liver failure

and hepato-cellular carcinoma (HCC). Symptoms are commonly absent, minimal, intermittent or non-specific. Fatigue is commonly reported. Only patients in a more severe or advanced disease stage will suffer from abdominal pain, anorexia, pruritus, weight loss and malaise. Cirrhosis develops in about 20% to 30% of people with chronic infection, and which is followed by HCC in further 20-30% of the patients with cirrhosis. In chronic HCV infections 65-70% of patients show constantly elevated or fluctuating serum ALT level and remaining 30% have normal ALT level despite persistence of viremia [84] **(Figs 1.1 and 1.6)**.

1.9 Immune Responses against HCV

The first defense against any viral infection is the innate immune response. Anatomic barriers, the skin and mucous membranes have to be penetrated to allow the infectious agent to reach the blood flow and subsequently target liver cells. In the liver the innate immune responses are represented by rapid IFN response present in by the infected hepatocytes, natural killer (NK) cells, natural killer T (NKT) cells, and Kupffer cells (liver macrophages). The innate immune responses are followed by the activation of adaptive immune responses.

1.9.1 Innate Immune Responses

Viral infection in target cells initiates a series of intracellular events that establish an antiviral state in infected and neighboring cells [85, 86]. This response depends upon induction of the type I interferons (IFN–α and β), which is triggered by binding of pathogen associated molecular patterns or PAMPs on viral components with the PAMPs receptors expressed on the host cells. HCV RNA and their unique structures such as internal ribosome entry site (IRES), poly-U/UC sequences, 5'–triphosphate of the uncapped RNA act as PAMPs and could be recognized by host intracellular receptors such as TLR–3, protein kinase receptor (PKR) and retinoic acid inducible gene–1 (RIG–I) and melanoma differentiation associated gene 5 (MDA5)

[86-89]. After viral PAMPs' engagement with PKR, RIG–I or TLR–3, the signal is transmitted to downstream signaling molecules [87], which activates various transcription factors such as interferon regulatory factors (IRF-3/7/9) and nuclear factor kappa B (NK–κB), and results in the production and secretion of type I IFNs (IFN–α and IFN–β) and type III IFNs (IL29, IL28A, and IL28B) from the infected cells. Activation of NF-κB also induces the expression of pro-inflammatory cytokines and chemokines that function to amplify the inflammatory response and facilitate leukocyte recruitment. Secreted IFN– α/β binds to IFN receptors in autocrine and/ or paracrine signaling and activates the JAK/STAT pathway which leads to induction of IFN stimulated genes or ISGs. Intracellularly, IFN activates the Mx protein, the 2'5'–oligoadenylate synthase-induced RNAase L that degrades the viral RNAs, and the dsRNA dependent protein kinase (PKR) that inhibits the synthesis of cellular and viral proteins. Type I IFN also stimulates the effector functions of NK cells and $CD8^+$ T cells and triggers the virus specific adaptive response by the up regulation of MHC expression on target cells. Type I IFNs also activates various innate immune cells such as NK cells, NKT cells, Kupffer cells (liver macrophages) and dendritic cells [90], which also can produce type I and III IFNs during HCV infection [91]. Various HCV proteins interfere with the signaling pathways of innate immunity and modulate the subsequent events. The NS3–4A serine protease has been reported to block HCV-induced activation of IRF3 and RIG–I, while core and NS5A inhibit IFN α/β signaling [92-94]. The E2 and NS5A protein of HCV block PKR pathway, which regulates cell proliferation and viability and also has antiviral effect [95-97] **(Fig 1.4)**.

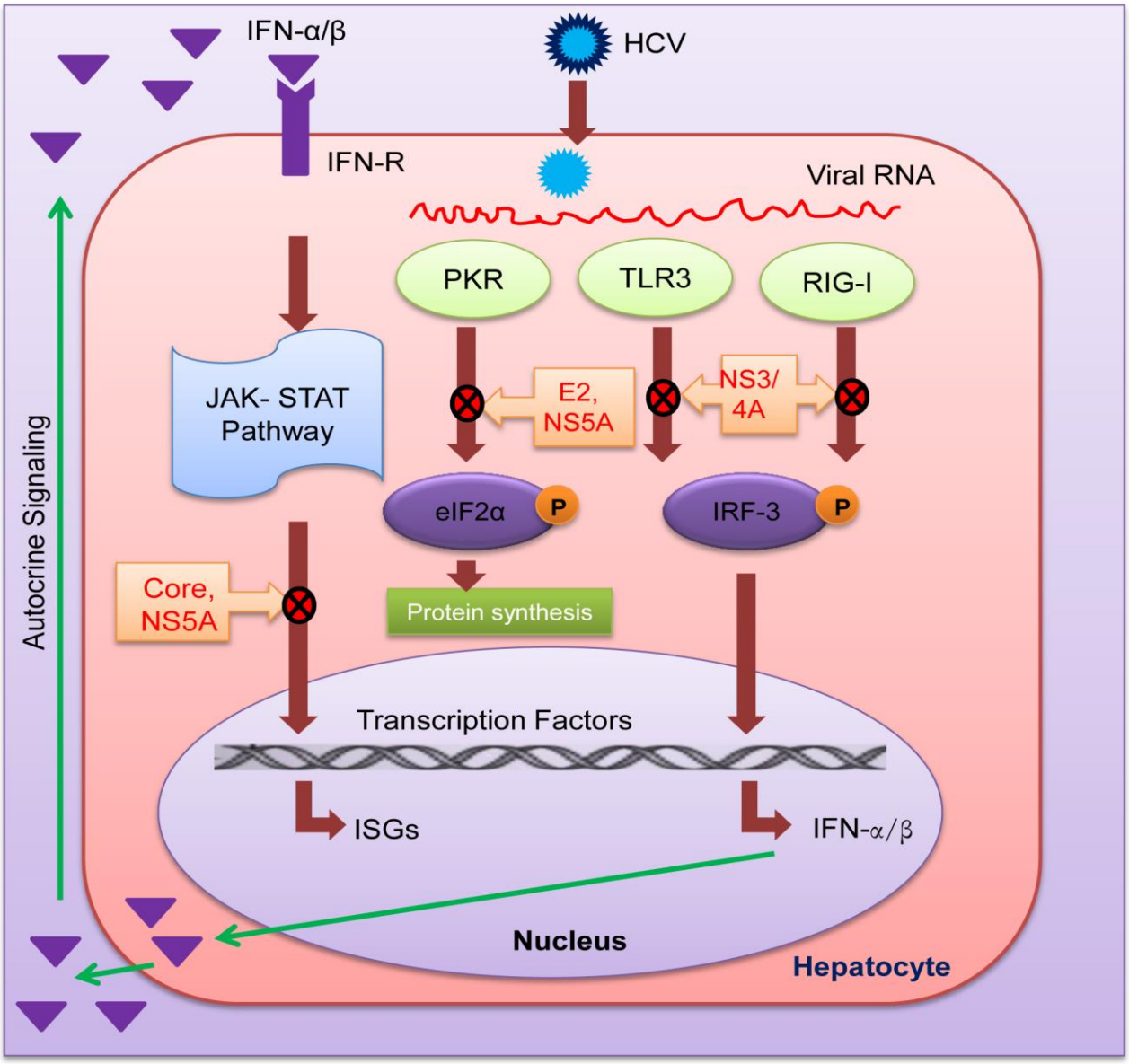

Fig 1.4: Innate immune signaling in HCV infection. Infected cells sense viral RNA through various TLR and produce Type I IFN and pro-inflammatory cytokines, which in turn activate the expression of various ISG in infected and surrounding cells. HCV Core and NS5A inhibit IFN signaling by blocking JAK-STAT pathway. The E2 and NS5A inhibit PKR pathway, which leads to repression of protein synthesis. The NS3/4A inhibits phosphorylation of IRF-3, which leads to blocking of IFN-α and β expression [This figure was modified from reference 98].

Natural Killer (NK) Cells

NK cells recognize and kill virus infected cells. They can be activated by type 1 IFN produced early in infection and by interleukin (IL)−12, produced by the macrophages and dendritic cells. These factors in turn recruit more NK cells and leucocytes in liver. NK cells are significantly higher in number in the liver (30−50% of the lymphocytes) than the peripheral blood or any other organ; therefore these cells also might be of interest in defense against HCV [99]. They produce various cytokines such as GM−CSF, IFN−γ, IL−10, IL−13, TNF−α and TGF−β, which can modulate various DC functions and adaptive immune responses [100]. NK cells themselves are regulated by the expression of various activation and inhibitory receptors.

During acute HCV infection, NK cells have been shown to increase cytotoxic activity, IFN−γ and TNF−α production and up-regulation of activation marker NKG2D. These cytokines (IFN−γ and TNF−α) further activate DCs, and favor Th1 adaptive immune responses [101, 102]. However, during chronic infection NK cells show reduced frequency, cytotoxicity and IFN−γ production; and increased expression of inhibitory receptor NKG2A, cytokines IL−10 and TGF−β [103-110], which results into reduced activation of DCs. Together, these factors favor Th2 biased immune responses. Various HCV proteins interfere with the NK cell functions. HCV E2 protein can directly bind CD81 and inhibit NK cell mediated cytotoxicity [111, 112]. Core protein of HCV can up-regulate TAP1 in p53-dependent pathway, which results in increased MHC class I expression on infected hepatocytes, making them resistant to NK cell killing [113] **(Fig 1.5)**.

Natural Killer T (NKT) Cells

Natural killer T (NKT) cells have both NK and T cell markers and possess both regulatory and effector functions [114]. Upon stimulation they produce immune-stimulatory cytokines such

as IL–4, IFN–γ, TNF–α and may activate both dendritic cells and NK cells. They constitute about 50% of the hepatic lymphocytes in humans [115].

Further, NKT cells are found in decreased frequency in the liver of chronic HCV infected patients [106, 116, 117]. In contrast, sustained response to IFN–α/ribavirin combination therapy results in increased frequency of NKT cells [117]. However, NKT cells also have been correlated with liver damage and fibrosis [118] **(Fig 1.5)**.

Dendritic Cells

Dendritic cells can uptake antigens and present them to T cells after processing. DCs can be activated by antigen uptake or by inflammatory cytokines produced by infected cells, NK and NKT cells. Upon stimulation, they also produce various inflammatory cytokines and IFNs, which recruits other immune cells to the infection sites. Natural killer cells producing TNF- α and IFN-γ help in activation of DCs, which in turn produce IL–12, IL–15, and IL–18 and activate more NK cells. Further, DCs has two subsets: myeloid and plasmacytoid (mDCs and pDCs), which play a crucial role in initial stage of infection and can prime T cells and B cells at the infection site or at distant secondary lymphoid organs. The mDCs secrete IL–10 and IL–12 and express TLR–3 and TLR–8, whereas pDCs are major producers of IFN–α and are specifically equipped to sense viral nucleic acids by TLR–7 and TLR–9 [119].

During chronic HCV infection, mDCs produce low levels of IFN–α, IL–12 and high levels of IL–10, thus creating an immunosuppressive environment which results in impaired T-cell stimulation [120-123]. Further, they also express several inhibitory markers such as PD1–L and induce T_{regs} [124, 125]. Interestingly, pDCs also produce less IFN-α and show reduced frequency in chronic HCV infection. In transgenic mice expressing HCV core and NS3/4A proteins, the

pDCs were found in reduced frequencies and having modulated function [126]. Further, the core and NS3 proteins of HCV have been linked to reduction of IL-29 production by DCs in HCV infected patients [127]. Several *in vitro* studies have been done on behavior of DCs in relation to induction of adaptive immune response when an HCV antigen is endogenously expressed. In one study, DCs were found to induce primary responses against the HCV antigens when these antigens were endogenously expressed [128]; moreover, DCs from HCV naïve individuals endogenously expressing HCV antigens NS4, NS5A/NS5B were able to prime both $CD4^+$ and $CD8^+$ T cells [129]. Expression of Core or NS3 HCV antigen in DCs leads to secretion of pro-inflammatory cytokines [130]. Recently, it has been shown that DCs expressing HCV core and NS3 antigens were able to prime T cells *in vitro,* which becomes effector cells upon activation by TLR ligands and IFN-α and granzyme B [131]. However, HCV genomic RNA has been detected in circulating DCs of HCV patients [132], and was associated with impaired expression of IL–12 and TNF–α [133]. Certainly, DCs are at the forefront in the priming of HCV specific immune responses and play a crucial role in both stimulatory as well as suppressive immune responses and outcome of the infection. During HCV infection, the higher expression of inhibitory markers, suppressive cytokines and poor T cell stimulation capabilities of DCs, collectively may result in exhausted HCV specific T cell responses **(Fig 1.5)**.

Macrophage (Kupffer Cells)

In liver, macrophages are referred to as Kupffer cells, which can phagocytose the pathogen, toxins, and infected dead cells. They act as professional antigen-presenting cells. Kupffer cells express various TLRs, which can sense viral components and secrete various inflammatory cytokines such as TNF–α.

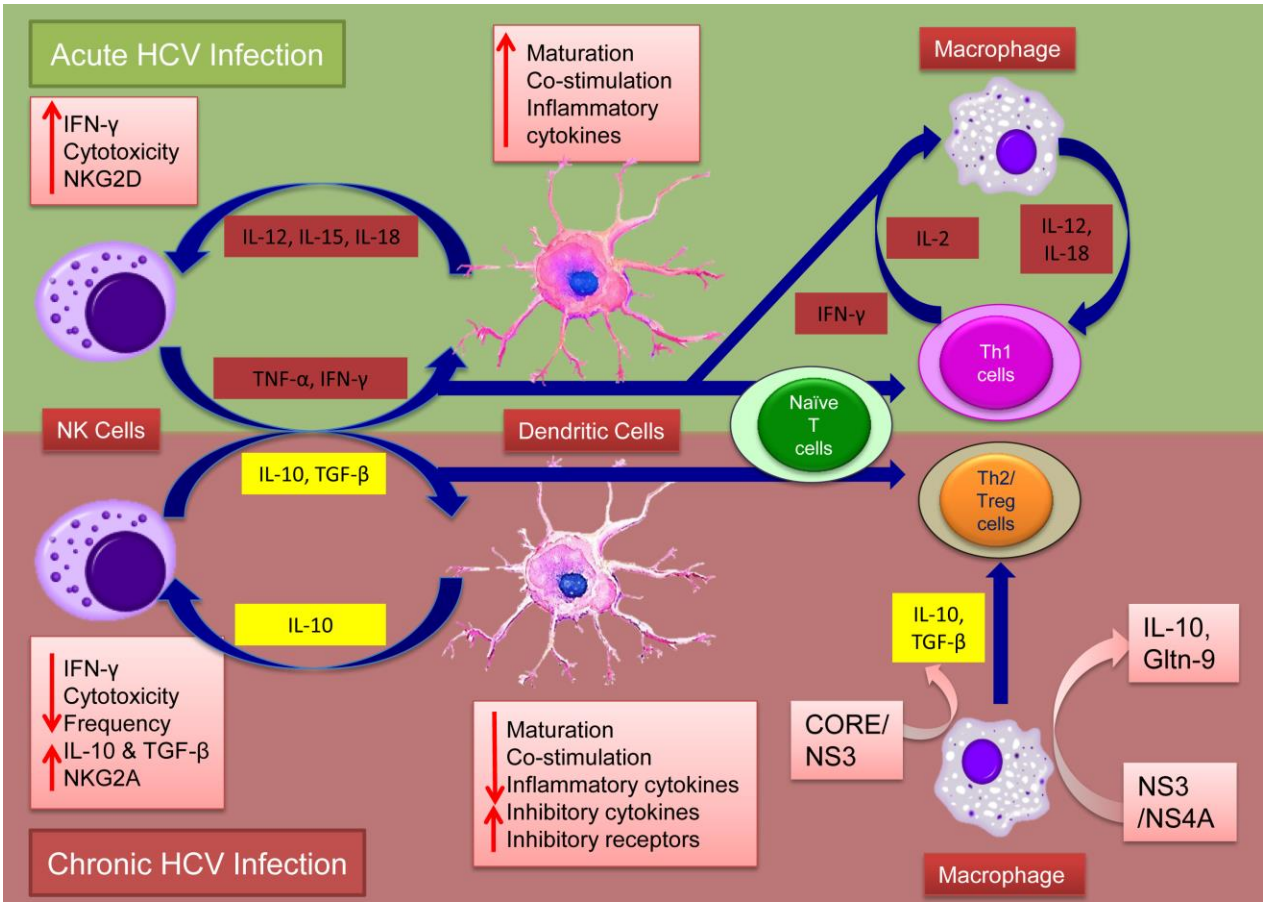

Fig 1.5: Innate immune cells in HCV infection. In acute viral infections, natural killer (NK) cells secrete large amount of IFN-γ and TNF-α, which help in the activation of dendritic cells (DCs). The DCs upon maturation up-regulate various co-stimulatory markers; produce inflammatory cytokines, which activate more NK cells. The TNF-α and IFN-γ produced by NK cells also activate macrophages. Both NK cells and macrophages with such cytokine profiles favors induction of Th1 type immune responses. During chronic HCV infection, these innate immune cells show modulated functions and express inhibitory cytokines and receptors. The DCs show impaired maturation, and become dysfunctional. This kind of innate immune cells producing IL-10 and TGF-β favors Th2 type T cell responses. Various HCV proteins, core, NS3 and NS3-4A induce expression of IL-10 and TGF-β and also expression of various inhibitory receptors on macrophages [This figure was modified from reference 134].

Kupffer cells express a variety of activation markers (CD40, CD80 and MH class-II molecules), inhibitory markers (PD–1 and TIM–3) and inhibitory molecules (galactin-9). Galactin-9 can induce the expansion of T_{regs} and contraction of CD4+ T cells while TIM–3 and PD–1 may impair IL-12 secretion and T cell responses [135, 136]. Transfection of NS3, NS3/4, NS5B or NS5A into mouse macrophages resulted in inhibition of IL–6 induction by various TLR ligands [137]. However, the monocytes and/macrophages from chronic HCV patients secrete pro-inflammatory cytokine upon stimulation with TLR–4 or TLR–2 agonists [138, 139] **(Fig 1.5)**.

1.9.2 Adaptive Immune Response

Activation of innate signaling pathways and cells of innate immune system set the stage for adaptive immune responses. Various antigen-presenting cells such as DCs and macrophages and inflammatory cytokines and chemokines secreted from these innate immune cells recruit and activate both components of adaptive immunity i.e. humoral and cellular.

1.9.2.1 Humoral Immune Response

Humoral immune responses are represented by B cells and antibodies produced by them. Antibodies play a pivotal role in defending the host against pathogens within the extracellular space, with effector mechanisms for viral clearance including neutralization, complement activation, opsonization and antibody-dependent cell-mediated cytotoxicity (ADCC) [140]. Virus neutralization results in the loss of infectivity due to the virus' inability to attach to the cell surface receptors. Further, Fc chain of antibodies bound to virus triggers complement pathway and opsonization, which subsequently taken up by APCs for antigen presentation to B and T cells. Binding of antibodies to the viral antigen expressed on infected cell surface may result in recognition by NK cells and lysis in ADCC pathway.

In the majority of cases, antibodies against HCV are detectable after an average of 23 days (range 7–72), detectable plasma viremia after 39 days (range 23–72) of exposure [141]. These antibodies remain detectable throughout decades of chronic infection. In those who clear the virus, antibody levels gradually diminish in titer. Further, IgG2 responses against Core and NS3 antigens were seen more frequently in those who clear the HCV infection than those who developed chronic infection [142]. This IgG2 predominance has been linked to a Th1 bias in $CD4^+$ T cell responses [143]. Antibody-mediated neutralization of infectivity has been demonstrated *in vitro* for homologous strains in chimpanzees [144]. In some studies, rapid development of high titer neutralization antibodies has been correlated with the viral clearance and those with delayed induction progress to chronic infection [145, 146]. Further, HCV can avoid neutralizing antibodies by several mechanisms such as: by associating with serum lipoproteins (LDL and VLDL) [147], shielding by glycosylation [148, 149], direct cell to cell transfer [150] and escape mutant using HVR1 and 2 in envelope glycoproteins [151, 152].

1.9.2.2 Cellular Immune Responses

Components of cellular immune responses includes $CD4^+$ T helper (Th) cells, $CD8^+$ cytotoxic T cells and regulatory T cells (T_{regs}), which requires antigen epitope presented by MHC class I (to CD8+ T cells) or MHC class II (to CD4+ T cells) molecules on infected host cells or professional antigen-presenting cells (APCs) **(Fig 1.6)**.

$CD4^+$ T Cells

Antigen-presenting cells (APCs) engulf the virus-infected, apoptotic, necrotic cells, cell debris or viral proteins and cleave viral exogenous antigen to 13–18 aa long peptides which can be presented by MHC class II molecule at the cell surface. These MHC class II presented antigens can be identified by $CD4^+$ T cells expressing TCR and the CD4 molecule, which activates the T

helper cells. The $CD4^+$ T cells perform several important functions including direct activation of macrophages, antigen specific B cells and production of cytokines that activate $CD8^+$ T cells [153]. The T helper cells become differentiated into Th1 or Th2 type cells. The Th1 cells produce IL–2, IFN–γ and TNF–α which stimulate activation of $CD8^+$ and NK cells; while Th2 cells produce IL–4, IL–5, IL–6, IL–13 and IL–10 cytokines which stimulate maturation of B cells [154, 155].

Multi-specific and functional $CD4^+$ T cell responses are correlated with HCV clearance while weak and dysfunctional $CD4^+$ T cell responses with the chronic HCV infections [156, 157]. The depletion of $CD4^+$ T cells in chimpanzees that naturally cleared HCV infection have been shown to develop persistent infection upon re-challenge [158]. Further, activated $CD25^+$ HCV-specific $CD4^+$ T cells from patients with chronic HCV have been reported to have impaired capacity to produce IFN–γ and IL–2, and to proliferate *in vitro;* in comparison to cells from subjects who have cleared the infection [159, 160]. The viral clearance is generally associated with Th1 $CD4^+$ T cell responses and viral persistence with Th2 $CD4^+$ T cells response [143]. In the majority of chronic HCV cases, the loss of $CD4^+$ T cell help result in failure of viral clearance by the $CD8^+$ T cells [161, 162]. The cause of the apparent failure of $CD4^+$ T cell responses in patients who develop chronic HCV is not well understood. Current theories include exhaustion or anergy, or possibly both. Exhaustion may result from the high rate of viral production exceeding the capacity of the immune response, resulting in T cells that are over stimulated and therefore cannot be activated further [163]. Anergy, or functional inactivation, may result from high and sustained antigen concentrations that tolerize the immune response [164] **(Fig 1.6)**.

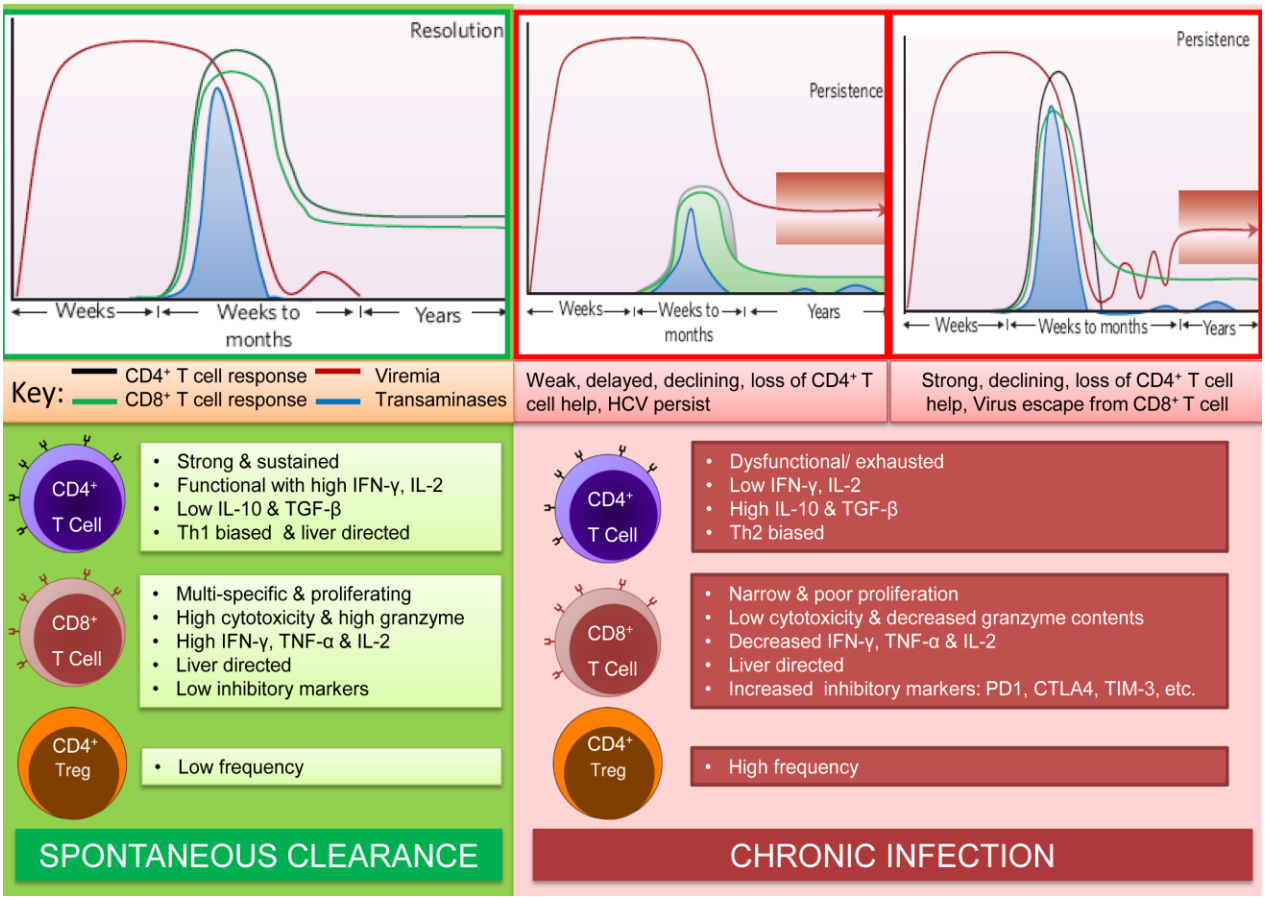

Fig 1.6: T cell immune responses in HCV infection. Both $CD4^+$ and $CD8^+$ T cells responses play a critical role in viral clearance and persistence. This figure displays the T cell immune status in patients who clear the virus and who develops chronic infection. The upper panels of the figure were modified from reference [Upper panels of the figure were modified from reference 165].

$CD8^+$ T cells

A host cell with ongoing infection produces viral proteins that are processed to 8 or 9 aa long epitopes and presented on cell surface by MHC class 1 which can be recognized by $CD8^+$ T cells, expressing the T cell receptor (TCR) and CD8 molecule. This recognition of viral epitopes lead to maturation and activation of effector functions of $CD8^+$ T cells which include lysis of target infected cell (by perforin or granzymes) and secretion of inflammatory cytokines (e.g. IFN–γ and TNF–α). The $CD8^+$ T cells can also be primed by cross-presentation of viral antigens by

DCs. This occurs when DCs acquire exogenous antigens and present them to $CD8^+$ T cells by the endogenous MHC class I pathway.

Similarly as with $CD4^+$ T cells, strong multi-specific $CD8^+$ T cell responses produced early in HCV infection are associated with viral clearance [166-170]. There are controversial reports on the maintenance of HCV-specific $CD8^+$ T cell responses following clearance of HCV infection. In some studies, immune responses have been shown to variably persist for up to several decades after resolution [171, 172], while another study [173] showed that HCV-specific $CD8^+$ T cells persist in chronically infected patients but not in patients with resolved infection, which also suggest that ongoing viremia is important in the maintenance of $CD8^+$ T cell responses. Although, both $CD4^+$ and $CD8^+$ memory T cells are maintained in the absence of HCV antigens in experimental animal models, but this may not occur in the human immune system [174, 175]. Using HLA Class I tetramers, it has been shown that HCV specific $CD8^+$ T cells are detectable at higher frequencies in the liver than in peripheral blood, and in patients with acute resolving infection than in chronic infection [166, 170]. Acutely infected individuals that fail to achieve clearance have similar frequencies [170] but functional assays fail to comparably recognize this population of HCV-specific $CD8^+$ T cells. This strongly suggests a functional impairment of these cells rather than deletion. Impaired production of IFN–γ and TNF–α has been demonstrated from HCV specific $CD8^+$ T cells of patients with acute HCV, who develop chronic infection. This $CD8^+$ T cell dysfunction was HCV specific, as influenza-specific $CD8^+$ T cells from patients with chronic HCV readily secreted IFN–γ. Further, the secretion of IL–10 and TGF–β has been associated with the T cell exhaustion. During chronic infections, $CD8^+$ T cells mainly secret IL–10 and TGF–β [176, 177]. In multiple human and mouse studies, blocking of IL–10/IL–10 receptor and/ TGF–β by antibodies results in the restoration of functional activities of T cells

and subsequent viral clearance [178-181]. Various inhibitory receptors/ ligands such as PD–1/PD–1 ligand, CTLA–4, and TIM–3/galactin–9 are over-expressed by APCs and T cells, and also have been linked to the exhaustion of T cells [182-185] **(Fig 1.6)**.

T_{reg} Cells

Regulatory T cells (T_{reg} Cells) can suppress the induction and proliferation of effector T cells, and help in the maintenance of immune balance. There are mainly two types: natural or induced T_{regs}. Natural T_{regs} are $CD4^+CD25^+$ T cells which develop in the thymus whereas induced T_{regs} develop outside the thymus by acquiring CD25 marker. They become functional under the influence of high levels of IL–10 and TGF–β, and acquire Foxp–3. The T_{regs} functions by secreting immuno-suppressive cytokines such as IL–9, IL–10 and TGF–β; and by directly inhibiting antigen specific effector T cells via apoptosis or co-inhibitory molecules.

Several studies have shown higher frequency and functional activity of HCV-specific T_{reg} in chronic patients with dysfunctional $CD4^+$ T cells compared to individual who cleared the infection [186-189]. The HCV induced secretion of IL-10 and TGF-β, which are characteristic of T_{reg}, has been demonstrated to be prevalent in patients with chronic HCV [159]. Importantly, study of acute HCV infection in chimpanzees demonstrated that the frequency and suppressive activity of $CD4^+CD25^+Foxp3^+$ T cells predicted establishment of chronic infection [190]. Higher frequency of T_{regs} in liver may be due to increased expression of chemokines such as CCL17 and CCL22, which attracts higher number of $CD4^+$ T cells expressing CCR4 receptors. These chemokines could be up-regulated due to NS3/4A [191, 192] **(Fig 1.6)**.

1.9.3 HCV-Specific Heterologous Immunity

The human population is exposed to a plethora of infectious agents and these exposures induce memory T cells and antibodies, which can cross-react with the homologous epitopes

containing antigens of other pathogens, and can modulate immune responses and subsequent outcome of the infection [193]. This type of cross-reactive humoral and cellular immunity is called as heterologous immunity. This occurs due to similarities in amino acid sequences in small stretches of antigen derived peptides and/or promiscuity of T cell receptors (TCR). The ability of a TCR to recognize and bind multiple peptide epitopes which differ in amino acid sequences is called T cell promiscuity [194]. This recognition further depends upon the affinity of MHC-class I or II with the peptide being presented. It has been established that only 2-4 amino acids in the peptide epitopes actively take part in interaction with the TCR, and further strength in peptide-TCR association is provided by co-stimulatory molecules and MHC class I or II molecules [194, 195]. Therefore, it is quite possible that a TCR will still recognize a peptide if its other amino acid(s) is replaced with other similar amino acid(s) [196]. Further, an MHC class I or II molecule can bind to and present a set of epitopes which differ in amino acid composition. Various studies show that $CD4^+$ T cells are more promiscuous than $CD8^+$ T cells in peptide epitope recognition [197]. Studies have shown that if amino acids on a peptide epitope can be substituted by amino acids with similar biochemical property, it will be still recognized by the same T cell clone [196]. Furthermore, some T cell clones could be intrinsically more cross-reactive than the other, which also depends upon the MHC–molecules phenotype it recognizes [198]. Therefore, some individuals may show higher heterologous immunity against certain antigens than others.

Heterologous immunity can modulate the breadth of T cell repertoire and resulting memory T cell pool and immuno-dominance of a specific antigen epitope, which can lead to enhanced or diminished immune response against a pathogen [199-202]. The cross-reactive T cell responses against evolutionary conserved regions among viruses can be seen more frequently, for example among different strains of Influenza viruses or dengue viruses, but are not restricted to

related or evolutionary closed virus groups. Influenza virus has been shown to generate HCV-specific cross-reactive $CD8^+$ T cell responses [200, 203]. Many studies have predicted the change in the immuno-dominant epitopes against a specific virus in individuals which have a pre-existing repertoire of cross-reactive T cells [202].

1.9.4 Summary of HCV-Specific Immune Responses

Both $CD4^+$ and $CD8^+$ T cell responses are crucial for viral clearance as demonstrated by both chimpanzees and human studies. The chimpanzees who successfully resolve primary infection show increased levels of protection from persistent infection upon re-challenge and show decreased viremia and its duration [169]. Depletion of $CD4^+$ and $CD8^+$ T cells before HCV re-challenge in chimpanzees demonstrated that viremia was prolonged, and did not resolve until this subset of cells recovered in the liver, despite the presence of a primed memory $CD4^+$ T cell response [158, 204]. Although caution is needed when extrapolating results from chimpanzee studies to humans given the high rate of natural clearance in these animals [205-208]. The induction of broad sustained and robust $CD4^+$ and $CD8^+$ T cell immune responses in some acutely HCV infected individuals that results in viral clearance is the minority response rather than majority. The reason for this response is unclear. However the development of viral escape mutants resulting from the high replication rate and error prone RNA dependent RNA polymerase is thought to play a major role. Besides, HCV has adopted several ways to down regulate the host immune responses and establish persistence infection. Increasing evidence suggests that several host genetic factors such as certain HLA types and IFN–λ3 (IL–28b) gene phenotypes in human may place some at higher risk and others at low risk of developing a chronic HCV infection [209-211]. Furthermore, pre-existing cross-reactive T cell repertoire against HCV antigens may also be

playing a role in induction of stimulatory or inhibitory immune responses and subsequent viral clearance or persistence.

1.10 Vaccine Development

Despite so many advances in the field of HCV biology, we are still awaiting an effective vaccine against HCV infection. Currently available antiviral drugs have many problems such as side effects, cost and resistance; therefore, HCV vaccine is still a priority. However, vaccine development faced many challenges since the beginning including lack of clear immune-correlate in protection, unusual viral escape capabilities, unavailability of suitable cell culture and infection model. Now much clearer data is available about immune correlates in protection and natural immunity which have helped in the design of an effective vaccine. Broad and sustained virus-specific $CD4^+$ and $CD8^+$ T-cell responses have been correlated with protection against HCV infection [158, 204, 212]. Many studies have now detected neutralizing antibodies against homologous and heterologous strain of HCV [213-216]. Furthermore, rapid appearance of antibodies has been correlated with spontaneous clearance of HCV infection [145, 217, 218]. There are reports where individuals with chronic infection spontaneously cleared the virus upon reversal of poor exhausted immune responses to strong and effective humoral and cellular immune responses [217]. The individuals who spontaneously cleared the HCV infection show reduced levels and duration of viremia with clearance rate of 80% instead of 20% upon re-infection [219]. Together, these reports provide evidence of HCV specific immune memory and possibilities of therapeutic and prophylactic vaccines. There are several prophylactic and therapeutic vaccines, which are currently being tested in human clinical trials and aim to induce broad neutralizing antibodies and/or strong multi-specific and durable T cell immune responses (Summarized in **Table 1.2**).

Table 1.2: Prophylactic and therapeutic vaccines in human clinical trials [Reference 220]

Phase I Clinical Trial	Phase II Clinical Trial
Prophylactic vaccines (Clinical trial ID, Sponsor)	
▪ Recombinant glycoprotein E1/E2/ adjuvant MF59 (*NCT00500747, NIAID*) ▪ Ad6NSmut; AdCh3NSmut: target NS3, 4, 5 (*NCT01070407, Okairos*)	▪ Ad3NSmut; MVA-NSmut: target NS3, 4, 5 (*NCT01436357, NIAID*)
Therapeutic vaccines (Clinical trial ID, Sponsor)	
▪ Adch3NSmut /MVA-NSmut and AdCh3Nmut /MVA-NS2,3,5 (*NCT01094873 & NTC01296451, Okairos*) ▪ Virosome formulated CD4/CD8 synthetic peptide and poly-L-arginine (*NCT00445419, Pevion Biotech*) ▪ GI5005 inactivated Saccharomyces cerevisiae encoding NS3-core (*NCT00124215, GlobeImmune*)	▪ TG4040+peg IFN+ Ribavirin MVA encoding NS3, 4, 5b (*NCT01055821, Transgene*) ▪ DNA plasmid expressing HCV NS3/4a (*NCT01335711, Chron Tech Pharma AB*) ▪ IC41 peptide vaccine (core NS3, 4) (*NCT00602784, Intercell AG*) ▪ GI5005+peg IFN+ribavirin (*NCT00606086, GlobeImmune*)

1.10.1 Prophylactic Vaccines

A glycoprotein E1/E2 based vaccine adjuvanted with MF59 has been tested in phase I clinical trial and proven safe as well as demonstrated induction of humoral and $CD4^+$ T cells responses [221]. This vaccine has shown promising results in chimpanzees [220]. It was able to reduce the rate of chronic infection and in some complete protection against homologous viral challenge. Further, it has shown induction of cross-genotype neutralizing activity [216, 222, 223]. Another vaccine approach use a prime

boost strategy using replication-deficient chimpanzee adenoviral vectors and vaccinia Ankara containing HCV NS3, 4 and 5 antigens. This vaccine approach has shown suppression of acute hepatitis upon heterologous challenge with genotype 1a in chimpanzees [224, 225]. The efficacy of this vaccine is being tested in a clinical trial with intravenous drug users in the United States. (**Table 1.2**)

1.10.2 Therapeutic Vaccines

Recombinant HCV core-based vaccine adjuvanted with ISCOMATRIX (IMX, saponin based adjuvant) has shown favorable results in healthy volunteers and modest reduction in viremia in chronic HCV patients during phase I clinical trial [220, 226]. Another approach uses modified vaccinia Ankara vector containing HCV NS3, NS4a, NS5 genes to enhance cellular immune responses against these antigens. In a human clinical trial, this vaccine has shown improvement in T cell responses and reduction in viremia [227]. In a phase II trial, pretreatment of chronic HCV patients with this vaccine followed by INF-α and ribavirin treatment, significantly improved the cure rate [228]. A replication defective chimpanzee adenovirus 3 and modified vaccinia Ankara containing HCV NS3, NS4 and NS5 has been tested in phase I clinical trial (**Table 1.2**). A DNA plasmid based vaccine is also being tested in phase II clinical trial (**Table 1.2**). It contains NS3 and NS4a antigens of HCV. Other therapeutic vaccine approaches which are currently in phase I clinical trials include virosome formulated peptide based vaccines and inactivated bacterial vaccine expressing HCV NS3-core fusion proteins [220] (**Table 1.2**).

1.11 Treatment of HCV Infection

Historically, there was limited success in the treatment of HCV chronic infection due to lack of effective antiviral drugs. Until 2011, pegylated-IFN–α and ribavirin combination therapy was the only treatment option, which provided cure only in 34–54% with genotype 1, 80–82% with genotype 2/3 and 33–50% with genotype 4/5/6 [229]. Overall cure rate with standard IFN

and ribavirin combined therapy was only about 47–54%. In 2011, two directly acting antiviral drugs (DAAs), boceprevir and telaprevir were approved, which target viral NS3 protease. In combination with standard therapy, boceprevir and telaprevir improved the cure rate of genotype 1 up to 75 and 68 % respectively [230-233]. In 2013, two more DAAs were approved which were highly effective even without interferon: Simeprevir (NS3–4A protease inhibitor) and Sofosbuvir (nucleotide analogue, NS5B RNA polymerase inhibitor). Sofosbuvir with interferon and ribavirin for 12 week showed 89% cure rate in chronic HCV patients infected with genotypes 1, 4, 5, or 6 [234, 235]. Simeprevir with interferon and ribavirin, after 12-week of treatment have cured up to 71-80 % of genotype 1a and 82–90% in chronic HCV patients infected with 1b [236, 237]. Apart from these, several other DAAs targeting NS3/4A protease and NS5B RNA dependent-RNA polymerase and NS5A are under clinical trial (**Table 1.3**). Several host factors such as cyclophillin and microRNA–122 which play a critical role in HCV replication, are now being targeted for drug development [172]. Further, as interferon therapy has serious side-effects and show no response in some chronic patients, studies have evaluated the efficacy of these new age DAAs without interferon. Sofosbuvir with ribavirin for 12 weeks has shown cure rates up to 86–97% in genotype 2 chronic patients [235, 238, 239], while in 24 week treatment it shows up to 87–94% cure rate in genotype 3 chronic patients. Further, sofosbuvir and simeprevir +/– RBV for 12 weeks showed >90% cure rate in genotype 1 chronic patients [240]. These new emerging drugs are very promising in terms of cure rate and their effectiveness against broad range of HCV genotypes, and in reducing the time duration of treatment. Various antiviral drugs that are currently under development and licensed, are summarized in **Table 1.3** [230, 233, 235, 236, 238-245].

Table 1.3: Currently approved and future antiviral drugs for HCV chronic infection

Drug regimen	DAA class	Genotype selectivity	Duration in weeks	Cure rate in % (SVR)	Study & References
Interferon/ Ribavirin (RBV) containing					
Boceprevir *	NS3–4A	1	24	69-75	[231]
Telaprevir *	NS3–4A	1	24	68	[232]
Sofosbuvir **	NS5b	1, 4, 5 & 6	12	≥89	NEUTRINO [235]
Simeprevir **	NS3–4A	1a 1b	24 24	71–80 82–90	QUEST 1 [237] QUEST 2 [236]
Faldaprevir	NS3–4A	1a 1b	24 24	64 82	STARTVerso1 [246] STARTVerso2 [246]
Interferon-free					
Sofosbuvir + RBV	NS5b	2 3	12 24	86–97 87–94	FISSION/ FUSION [244] [238] VALENCE [239]
Sofosbuvir & simeprevir +/– RBV	NS5b/ NS3–4A	1	12	≥90	COSMOS [240]
Sofosbuvir & daclatasvir +/– RBV	NS5b/ NS5A	1, 2, 3	12 or 24	89–98	[241]
Asunaprevir & daclatasvir	NS3–4A/ NS5A	1b	24	80.50	[245]
Sofosbuvir & ledipasvir +/– RBV	NS5b/ NS5A	1	12	≥95	LONESTAR [243]
ABT450/ABT267, ABT333 +/– RBV	NS3–4A/NS5A/NS5b	1	8–24	88–96	[242]

*Approved for use by FDA in 2011 (withdrawn from the market due to drug-related side effects),

** Approved for use by FDA in 2014

1.12 Animal models

1.12.1 Chimpanzees

Chimpanzees so far are the best animal model for HCV studies. HCV can infect and replicate in chimpanzees very efficiently, and imitates the infection in humans. They have helped in the studies of HCV transmission, replication and immune responses, antiviral drugs and vaccine development, immensely. However, HCV causes milder liver disease in chimpanzees, which resolve the infection naturally with higher rate, and do not progress to cirrhosis and fibrosis. Further, due to maintenance cost, availability, limited chimpanzee specific reagents, use of chimpanzees in HCV studies is limited. Moreover, many countries have banned the use of chimpanzees in research [247].

1.12.2 Tree Shrew

Tree shrew (*Tupaia* sp.) allows HCV replication, and can be used for the study of HCV replication, pathogenesis and immune responses. However, this animal shows a very low level of viremia and persistent infection could not be established without severe immune-suppression [248]. Moreover, lack of immune-pathological data and research reagents, also restricts the use of tree shrews as model for HCV studies.

1.12.3 Mouse Model

Several strategies have been used to allow HCV infection and replication in mouse. In one, humanized mice were developed by engraftment of hepatocyte progenitors and human $CD34^+$ human hematopoietic stem cells. These mice allowed human hepatocytes and T cells to establish themselves, and they can be infected as well as showed human T cell specific immune responses against HCV antigens. However, these mice did not show viremia in serum and also they lack B-cell immune responses [249].

In another strategy, immuno-deficient (SCID) urokinase plasminogen activator (uPA) transgenic mouse model was developed which show over expression of uPA which causes hepatocyte death with hemorrhagic events due to defects in coagulation system but liver show a continuous regeneration. Such mice have been used to transplant up to 50% of human hepatocytes, followed by infection with HCV with a virus titer up to 10^6 copy/ ml in blood [250, 251]. These mice have been used in direct antiviral drugs' (NS3–4A protease inhibitors, NS5B inhibitors) trials for efficacy, pharmacokinetics and side effects testing and the study of HCV specific innate immune responses [252-254]. However, these mice lack intact immune system and cannot be used for the study of immune responses and vaccine development.

Many transgenic mouse strains have been used to access the effect of individual or co-expression of HCV proteins. The use of transgenic mice can generate important information on HCV proteins and their function. In many of these transgenic mouse models, protein expression levels may be low or even absent, and in some cases may be even higher than in the native HCV infection. Further limitation in the use of transgenic mice includes their immune tolerance to the transgene HCV antigens.

Further, many studies attempt to generate transgenic mice which express HCV entry factors such as human CD81, occludin, SCARB–1, CLDN1. These mice have intact immune system and allow HCV entry and replication. However, due to intact immune system these mice showed only limited viral replication and infection [255, 256].

In several vaccine development and protection studies a vaccinia-HCV chimera has been used to challenge the mice. Vaccinia virus and HCV polyprotein chimera virus causes systemic infection in mouse, specifically in ovaries [257-260]. The mice infected with recombinant replicative vaccinia virus expressing HCV polyprotein have been used as a surrogate of HCV infection.

This is a valuable cost effective model to demonstrate the induction of protective immunity against HCV antigens, as the ability of the induced immunity to kill targets expressing HCV antigens is a critical parameter in antiviral response against HCV.

1.12.4 Rat Model

Rat model is an attractive infection model for the study of several viruses. To develop a rat infection model, fetal rats at 15–17 days of gestation age were injected with Huh7 human hepatoma cells intaperitoneally to induce tissue specific tolerization. Subsequently, Huh7 cells were transplanted successfully in new born rats. These rats were infected with HCV and showed biochemical evidence of hepatic inflammation and human albumin in serum and liver cells. Viral RNA levels peaked at $20x10^3$ copies / ml in serum by week 12 post infection [261, 262]. This model has intact immune system and can be used for antiviral drugs. However, due to low number of transplanted human hepatocytes and relatively low viremia levels, this model still is not as good as chimpanzees.

1.13 Current Study and Hypothesis

Despite the reduction in the number of new infections, HCV still remains a global health threat and an economic burden. There is no HCV vaccine available so far and the available antiviral treatment options are highly costly, , prone to development of resistance and have several side effects. Efforts are being focused on the development of a protective vaccine. Strong humoral and cellular immune responses targeting both structural and non-structural proteins of HCV have been correlated with the protection. Multiple strategies are being employed to improve the quality of HCV vaccine-specific humoral and cellular immunity. One such approach use replication-deficient adenoviral vector which express HCV antigens. However, studies show that pre-existing immunity against these vectors has negative effect on transgene specific immune responses and both route and dose of immunization further impact these immune responses [263,

264]. Further, a number of pathogens have been shown to induce cross-reactive immunity against other un-related pathogens, which may impact the immuno-pathogenesis of the new pathogen.

In order to understand the role of immune responses against a specific HCV non-structural antigen (NS4) in protection, we immunized the mice with Ad vector expressing HCV NS4 and evaluated the induced immune responses and protection from chimeric-HCV challenge in C57BL/6 mice. We also studied the role of different route and dose of immunization in induction of transgene specific immunity. In these experiments, we surprisingly discovered HCV antigen specific immunity in mice immunized with empty Ad vector not expressing HCV antigens. Which lead us to investigate amino acid homology between several Ad proteins and HCV protein derived peptides, and the HCV specific cross-reactive immune responses induced by adenoviral proteins in both mice and humans. A chimeric vaccinia-HCV mouse challenge model has been very helpful in evaluation and characterization of HCV specific immune responses due to its cost effectiveness and ease of study. Our hypotheses in these studies are described below:

I. Ad vector expressing HCV NS4 can induce strong protective immune responses *in vivo*, which can be further enhanced by using optimized dose and route of immunization.
II. Ad vector have some level of amino acid homology with HCV proteins, and therefore induce HCV antigen specific cross-reactive immune responses.
III. HCV naïve human with pre-existing Ad immunity may have HCV cross-reactive antibodies and T cells which will secrete IFN-γ and/or IL-10 upon stimulation with HCV antigens.

These hypotheses will be explored in the following chapters of this thesis.

1.14 References

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Chapter 2

Recombinant Adenoviral Vector Expressing HCV NS4 Induces Protective Immune Responses: A Dose and Route Conundrum

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2.1 Introduction

Hepatitis C virus (HCV) infects human liver and about 150 million people worldwide are infected chronically [1, 2]. The people who clear HCV infection exhibit strong and multi-specific T cell responses, while those who develop persistent infection lack this immunity [3-6]. Exhaustion of the antigen-reactive T cells and modulation of T cells by various HCV proteins have been suggested to play a major role in T cell failure in chronic HCV [7-11]. Further, due to highly evolving antigenic variability in structural proteins, this virus evades recognition by generated antibodies [12]. Currently available treatment includes a combination of ribavirin and direct acting antiviral drugs [13].

From multiple studies, it is clear that a vaccine that would induce broad, robust and effective T cell and antibody responses would be able to protect against HCV infection [14-16]. The vehicle for the delivery of antigens plays a critical role in the induction of strong cellular, as well as humoral responses. Replication-defective recombinant adenoviral vectors have been shown to generate very strong transgene-specific cellular and humoral immune responses [17-21]. However, there have been inconsistencies in results obtained in various studies, where adenoviral vectors have been found to correlate with seemingly contrasting exhausted and chronically over-activated, or sustained and polyfunctional $CD8^+$ T cell responses [22-24].

It is well known that both the quality and quantity of immune responses depend upon antigen doses and routes of immunization [25]. The route of immunization can also influence the trafficking of antigen-specific T cells [26]. Surprisingly, these important aspects of adenoviral vector based immunizations are still not well understood. Intramuscular (i.m.) and intraperitoneal (i.p.) routes are among the most utilized routes of immunization in several mice studies using

recombinant adenoviral vectors [27-34]. These strategies become even more important for pathogens whose clearance depends upon the generation of robust immune responses. Previously, pre-existing immunity against adenoviruses has discouraged the use of adenoviral vectors as a tool for antigen delivery in humans [35]. However, it has been shown that the encounter of the adenoviral vector with pre-existing neutralizing antibodies can be circumvented by choosing the right route of immunization [35, 36].

Various vaccine strategies for HCV have focused on generating strong multi-specific cellular immunity against various structural and nonstructural proteins of HCV because they have been correlated with viral clearance [3, 5, 10, 14-17, 37-42]. Meta-analysis of all chimpanzee studies has suggested that antibodies against structural proteins play a significant role in protection from HCV infection [43]. Therefore, the role of antibody responses cannot be ruled out in protection from HCV infection. In this study, we used replication-defective recombinant adenovirus vectors containing HCV non-structural protein 4 (rAd-NS4) to induce immune responses in C57BL/6 mice. We compared the effects of different doses of adenoviral vector (2×10^5 PFU to 2×10^8 pfu/mouse) delivered i.m. or i.p. on the induction and longevity of both humoral and cellular immune responses, and their subsequent role in reduction of viral load in a Vac-HCV infection model [44]. Our studies demonstrate significant reduction in viral loads that is dependent on both route and dose of rAd-NS4 influencing the induced immune responses.

2.2 Materials and Methods

2.2.1 Recombinant Adenoviral Vector

HCV (genotype 1a) NS4 (amino acids 1658–1972) gene was cloned into replication-defective AdenoVator Vector (QBiogeneInc.) as per the standard method provided in the

QBiogene AdenoVator manual. Recombinant and control adenoviruses were prepared, propagated, purified and stored as reported by our laboratory [45].

2.2.2 Vaccinia Virus

The Western Reserve (WR) strain of VV containing the HCV NS3-NS4-NS5 gene of the HCV BK strain (genotype 1b) (Vac-HCV) was kindly provided by Dr. A. Prince, U.S.A. The virus was grown and assayed in BHK-cells [46].

2.2.3 Mice Immunizations

Six- to seven-week old inbred female C57BL/6 mice were purchased from Charles River laboratories (Sherbrooke, ON, Canada) and housed in the Health Sciences Lab Animal Facility (HSLAS) at the University of Alberta. Groups of six mice were immunized with varying doses of rAd-NS4 (2×10^5 PFU to 2×10^8 pfu/mouse) by either i.m. or i.p. routes on days 0 and 14. Each group was divided in two subgroups (three mice each). One set of mice was euthanized after 8 days of single immunization and the remaining set was euthanized 8 days after second immunizations. The time points of immunizations were chosen from several pilot experiments performed in the lab. Animal immunization experiments were repeated more than three times. All procedures were approved by the University of Alberta Animal Care and Use Committee in accordance with Canadian Council of Animal Care guidelines.

2.2.4 T Cell Proliferation Assay

Antigen-specific T cell proliferation assays were performed using nylon wool purified splenocytes and inguinal lymph node cells as reported previously by us [45]. Briefly, 4×10^5 T cells and equal number of antigen presenting cells (γ-irradiated splenocytes from un-immunized mice) were added in each well in 3–4 replicates, and stimulated with 1 and 5 μg/ml of recall

recombinant HCV NS4 protein antigen (kindly provided by Chiron Corp.). Plates were incubated at 37◦C with 5% CO_2, and on the fourth day, 75 µl culture supernatants were collected from each well and replicates were pooled together and stored at −20◦C until used for cytokine analysis. Thymidine labeled with radioactive tritium (Perkin Elmer, Canada) was added to each well. After overnight incubation, cells were transferred on filters using a harvester (Tomtec Inc. Connecticut, USA) and counted in Microbeta β-scintillation counter (Perkin-Elmer Inc., Massachusetts, USA). Average counts per minute (CPM) of replicates with SD were plotted in bar graphs (representative of three individual experiments).

2.2.5 Cytokine ELISA

Culture supernatants from T cell proliferation assay plates were collected and used to quantify cytokine production using mouse cytokine ELISA kits obtained from eBiosciences (eBiosciences Inc. San Diego, USA) according to the manufacturer's instructions. The plates were read and analyzed in a FluoStar ELISA reader (BMG Labtech GmbH, Ortenberg, Germany). Graphs represent averages of duplicate values.

2.2.6 T Cell Phenotype Analyses By Flow Cytometry

Mouse splenocytes were cultured for 4 days in the presence of 1 µg/ml HCV NS4 antigen. After 4 days of culture, cells were collected, counted and stained for CD3, CD4, CD8, Granzyme B (Bio-legend Inc. San Diego, USA), FoxP3, PD1 and CTLA4 markers with fluorophore-labeled antibodies (eBiosciences Inc. San Diego, USA) using previously reported procedures [47]. Stained cells were run in a FACS Canto and analyzed in FACS Diva software (BD Biosciences, CA, USA).

2.2.7 T Cell Killing Assay

Enriched spleen T cells were cultured in the presence of γ-irradiated splenocytes and 1 μg/ml NS4 protein. After 5 days of culture, the effector cells were collected and washed with RPMI media. EL4 target cells (ATCC TIB 39, VA, USA) were incubated overnight with an NS4 peptide pool (20 amino acid long peptides overlapping in five amino acids at 5 μg/ml of each peptide) obtained from Genscript Inc. After incubation, EL4 targets stained with 3 μM/ml CFSE (CFDA) (Life Technologies Corp., NY, USA) and incubated with effector cells (E:T ratios of 50:1, 25:1 or 12.5:1) for 6 h. Next, 20 μl (20,000 beads) of Count Bright Absolute Counting Beads (Invitrogen, Oregon, USA) were added to each tube. Each sample was run for 10,000 events of count beads in a BD FACS Canto and data were analyzed using FlowJo v.7 software (Tree Star Inc., USA). For the calculation of percentage killing, following formula was used: % Killing = [(Average live cells in PBS control − live cells in immunized group) / Average live cells in PBS control] × 100. The killing assay was done in triplicates and the % killing was calculated for individual replicates and data are shown as avg. ± SD.

2.2.8 Antigen-Specific Antibody ELISA

Blood from mice of the same group was pooled and serum was prepared and stored at −20◦C until use. For the detection of IgG, IgG1, IgG2a and IgM antibodies against NS4 or adenoviral vector, 96-well plates were coated with HCV NS4 protein (1 μg/ml in 1X PBS) or Adeno vector infected HEK-293A cell lysate (100 μl/well), and incubated overnight at 4◦C. Plates were washed and blocked with 1% BSA followed by addition of diluted pooled serum in duplicate. The plates were incubated for 2 h, washed with 1× PBST, and added with secondary antibody (anti-mouse IgG, IgG1, IgG2a or IgM, AP labeled) (Southern Biotech, Alabama, USA) and color was developed with PNPP substrate. Absorbance was read using a FluoStar ELISA

Reader. The background (OD obtained from the wells coated with uninfected HEK-293 A cell lysate) was subtracted from OD values obtained from experimental wells coated with adeno vector infected HEK-293 A cell lysate.

2.2.9 Neutralizing Antibodies against Recombinant Adenovirus Vectors

Ninety-six well plates were seeded with HEK-293A cells at 1×10^4 cells/well and incubated overnight. Serial 10-fold dilutions of Ad-vector (alone) were prepared and incubated with a 1:100 dilution of serum from various groups of immunized mice for 2 h. The hundred µl of neutralized Ad-vector was added in triplicate to the plates. After 10 days, the cell monolayer was fixed by adding 50 µl of 10% formalin to each well and stained with 0.3% crystal violet solution. Reduction in the end-point titer due to neutralization was determined by observing the cytopathic effects of Ad-vector on monolayers (disruption or removal of monolayer). Lower end-point titer of rAd vector indicates more neutralizing antibodies.

2.2.10 Chimeric Vac-HCV Challenge

Vac-HCV (NS3-NS5) chimeric virus was used to challenge the immunized C57BL/6 mice according to procedure reported previously [46, 47]. Briefly, 8 days after immunization, mice were injected with 1×10^5 pfu of chimeric Vac-HCV i.p. Five days after Vac-HCV challenge, mice were euthanized; ovaries were removed and processed individually to quantify the Vac-HCV virus load. Vac-HCV virus samples were titrated by standard plaque assay onTK-1 cell line (ATCC no. CRL 2396).

2.2.11 Statistical Analysis

Data were analyzed by Graphpad Prism software (Graphpad Software Inc., CA, USA). Student's t-test and one-way ANOVA were used to determine the significant difference between

two groups and multiple groups, respectively. The p-value less than 0.05 ($p < 0.05$) was considered to be statistically significant.

2.3 Results

2.3.1 Intramuscular immunization with an optimum dose of rAd-NS4 induces high antigen specific T cell proliferation and cytokine responses

Mice were immunized with four different doses of rAd-NS4 (2×10^8, 2×10^7, 2×10^6 or 2×10^5 pfu/mouse) either i.m. or i.p. at day 0 and 14. Using both i.p. and i.m. routes of immunizations, the two lower doses of rAd-NS4 (2×10^6 and 2×10^5 pfu/mouse) exhibited low NS4-specific responses **(Fig. 2.1)**. We found that the higher doses of immunizations (i.e., 2×10^7 and 2×10^8 pfu/mouse) via the i.m. route induced higher T cell proliferation in comparison to the i.p. route **(Fig. 2.1)**. Further, after both one (**Appendix 1**) and two i.m. immunizations, highest antigen dose (2×10^8 pfu/mouse) induced less T cell proliferation; whereas 2×10^7 pfu/mouse induced significantly higher ($p < 0.05$) T cell proliferation **(Fig. 2.1)**. Therefore, in the next experiments, we compared the effects of only two doses of rAd-NS4 (high 2×10^8 pfu/mouse and low 2×10^7 pfu/mouse) administered through i.m. or i.p. routes. Upon examining the production of various cytokines in culture supernatants, after two immunizations, both the spleen and lymph node T cells produced higher levels of IFN-γ, TNF-α, IL-2 and IL-6 when mice were immunized intramuscularly **(Fig. 2.2).** From splenocytes, the TGF-β levels were higher in high-dose and i.m. route as compared to the i.p. route. In lymph node T cells; TGF-β was higher in the lower dose group given via i.m. route **(Fig. 2.2)**. The TGF-β was detected in antigen un-stimulated groups, but their levels were less than antigen-stimulated groups. These results could be reflective of the

in vivo status of cells independent of *ex vivo* stimulation with antigen. With a lower antigen dose, more IL-10 was detectable in comparison to the higher dose **(Fig. 2.2).**

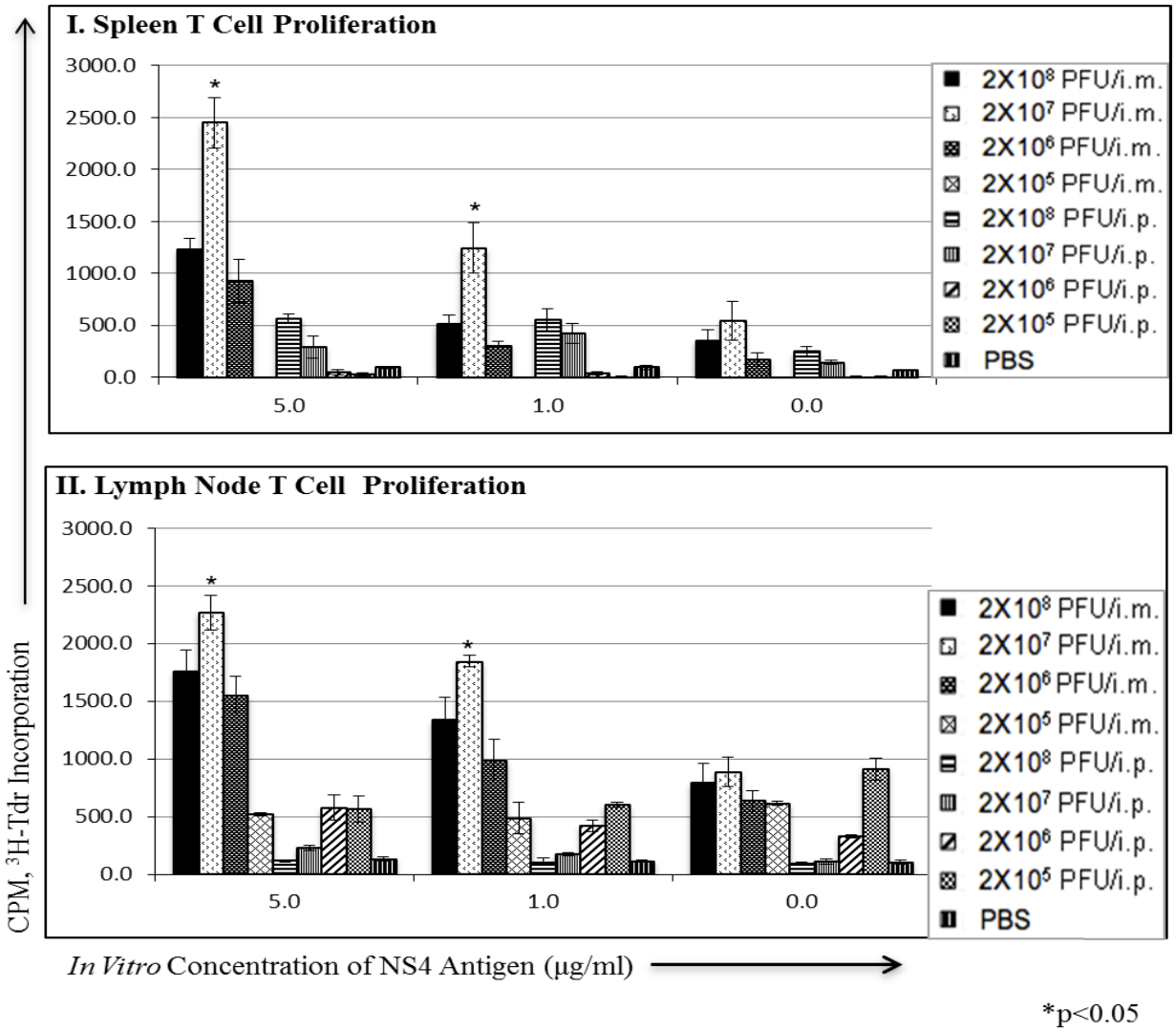


Fig 2.1: T cell proliferation after two immunizations. Antigen-specific T cell proliferation after immunization with various doses (2×10^5 to 2×10^8 pfu / mouse) of recombinant adenovirus vector (rAd) containing HCV-NS4 antigen, by intramuscular (i.m.) and intraperitoneal (i.p.) routes in C57BL/6 mice. After 8 days of two immunizations, mouse splenocytes and lymph nodes were harvested and T cell proliferation was evaluated in each group of immunized mice by a ^{3}H-thymidine-uptake assay. Data with spleen (I) and draining lymph node (II) T cells are shown. Mean ± SD of CPM (counts per minutes) from quadruplicate (I) triplicate (II) wells are shown. The data are representative of 3-5 different repeated experiments.

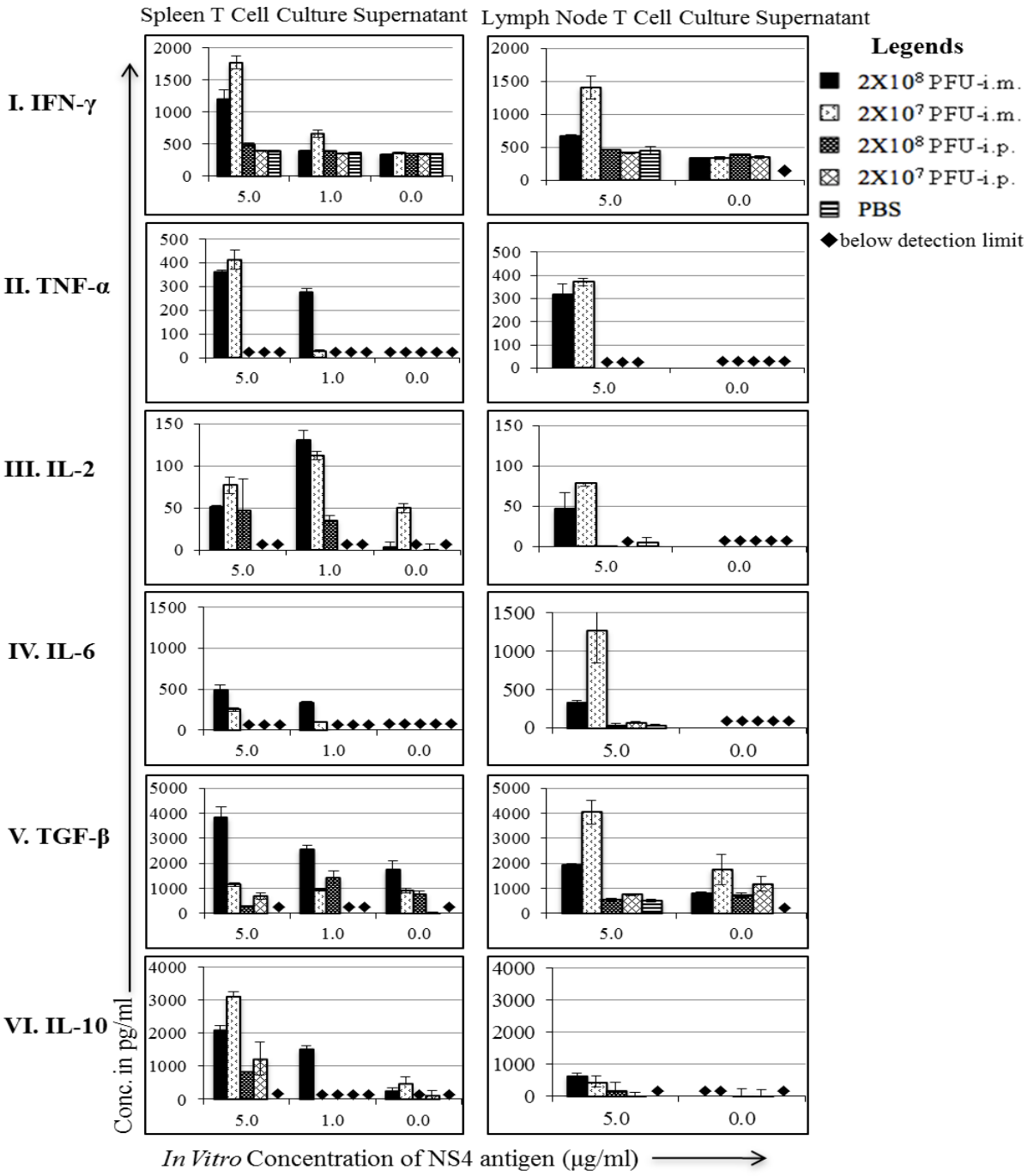


Fig 2.2: Cytokine profile of spleen and lymph node T cells after two immunizations. Spleen and draining lymph nodes were collected 8 days after two immunizations with higher (2×10^8 pfu/mouse) or lower (2×10^7 pfu/mouse) doses of rAd-NS4, followed by culturing the T cells with recombinant NS4 antigens and irradiated syngeneic APCs. Culture supernatants were collected at day 4 and examined for IFN-γ ***(I)*** TNF-α ***(II)***, IL-2 ***(III)***, IL-6 ***(IV)***, TGF-β ***(V)*** and IL-10 ***(VI)***. Mean ± SD of cytokine concentration from triplicate wells / mice group (n = 3) are shown. The data are representative of three different repeated experiments.

2.3.2 Phenotypic characteristics and target killing activity differ in T cells obtained from mice immunized with high vs. low doses ofrAd-NS4 given by intramuscular or intraperitoneal routes

T cells obtained from mice after immunization with low and high doses of rAd-NS4 via i.m. and i.p. routes, were examined for expression of granzyme B, FoxP3, PD-1 and CTLA-4 to determine if effector and /or regulatory T cells are induced, and also if T cells are negatively regulated due to the expression of inhibitory receptors. Low i.m. antigen dose (2×10^7 pfu/mouse) induced the highest levels of Granzyme B expression in CD8+T cells compared to the higher i.m. antigen dose, or both i.p. doses **(Fig. 2.3, panel I)**. Interestingly, the higher i.m. dose generated a higher frequency of CD4+FoxP3+T regulatory cells compared to the lower i.m. dose or to both i.p. doses **(Fig. 2.3, panel II)**. Both i.m. and i.p. routes induced similar patterns of PD1-expressing CD4+T cells; the lower dose of antigen induced less PD1-expressing CD4+T cells compared to the higher dose **(Fig. 2.3, panel III)**. Numbers of CTLA4-expressing CD4+T cells were higher in the mice immunized with the higher i.p. dose when compared to either i.m. dose or the lower i.p. dose **(Fig. 2.3, panel IV)**.

In accordance with Granzyme B expression, T cells induced by i.m. immunization showed higher percent killing of NS4 peptide loaded EL4 target cells in comparison to i.p. immunization **(Fig. 2.4)**.

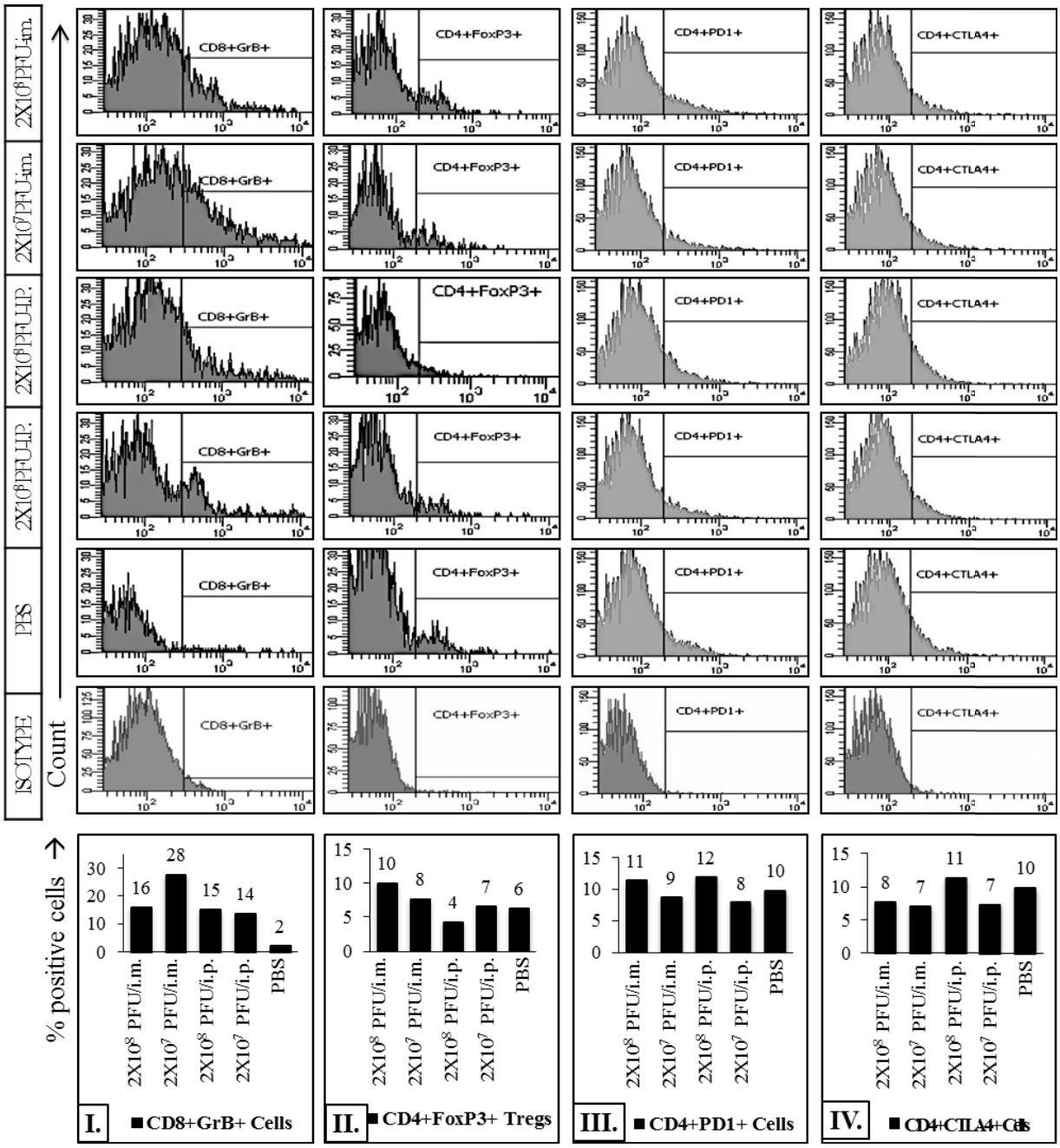


Fig 2.3: T cell phenotyping. Spleens were collected 8 days after two immunization with higher (2×10^8 pfu/mouse) or lower (2×10^7 pfu/mouse) doses of rAd-NS4 and cultured in the presence of 1 µg/ml NS4 antigen for 5 days, followed by staining for various markers such as Granzyme B (GrB), FoxP3, PD1 and CTLA4. ***(I)*** Frequency of Granzyme B-expressing $CD8^+$ T cells ***(II)*** $FoxP3^+$ $CD4^+$ T regulatory (T-regs) cells ***(III)*** $PD1^+CD4^+$ T cells. ***(IV)*** $CTLA4^+CD4^+$ T cells. The data are representative of three different repeated experiments.

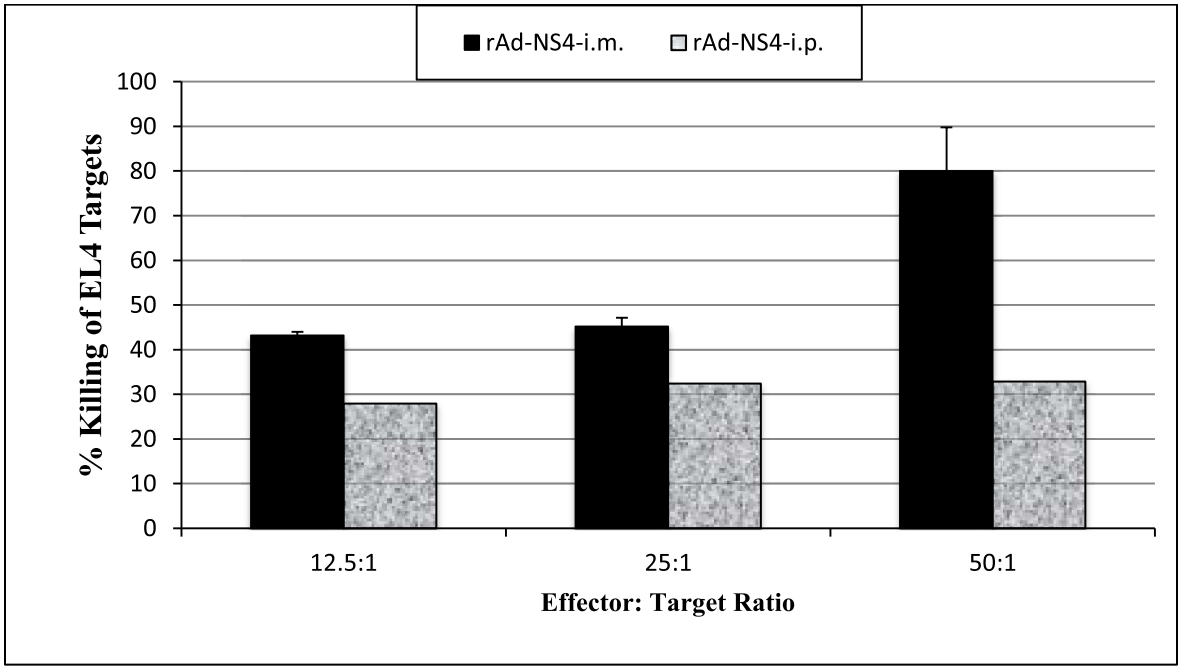


Fig 2.4: Killing activity of effector T cell. Spleen T cells obtained from i.m. and i.p. immunized mice were cultured *in vitro* for 5 days with recombinant NS4 protein and irradiated syngeneic APCs, followed by a CFSE killing assay using EL4 target cells loaded with an NS4 peptide pool. For the calculation of percentage killing, following formula was used: *% Killing = [(Average live cells in PBS control − live cells in immunized group)/Average live cells in PBS control] × 100*. The killing assay was done in triplicates and the % killing was calculated for individual replicates and data are shown as mean ± SD. The data are representative of three different repeated experiments.

2.3.3 Induction of antigen-specific humoral immune responses in mice immunized with higher vs. lower doses of rAd-NS4 given by intramuscular or intraperitoneal routes.

After two immunizations, the high dose of rAd-NS4 by both i.m. and i.p. routes induced significantly higher levels of anti-NS4 IgG antibodies IgG antibodies in comparison to the lower immunizing dose ($p < 0.05$). Low i.m. dose induced slightly higher IgG ($p = 0.1252$) and significantly higher IgG1 ($p = 0.021$) responses than did low i.p. dose **(Fig. 2.5)**. We also determined the induction of adeno vector derived antigens-specific IgG antibodies in each route of immunization. The dose of immunization showed an interesting pattern of anti-adeno vector antibodies. After one immunization, low i.m. dose (2×10^7 pfu/mouse) provided the highest adeno-vector specific IgG responses, whereas after two immunizations, high i.m. dose (2×10^8 pfu/mouse) provided the highest IgG titers ($p < 0.05$, in comparison to all other groups) **(Fig. 2.6)**. IgG1 shows a similar pattern with both one and two immunizations. IgG2a was undetectable after single immunization; however it was detectable after two immunizations, but was not significantly different in various immunization groups ($p > 0.05$). After single immunization, IgM was not significantly different among various groups ($p > 0.05$). However, after two immunizations, adeno-vector specific IgM was comparable in all rAd-NS4 immunized groups but higher in comparison to PBS control ($p < 0.05$). We also analyzed neutralizing antibodies against adeno-vector in mice immunized i.m. or i.p with high and low rAd-NS4, where lower viral titers represent the presence of higher levels of neutralizing antibody titers **(Fig. 2.7)**. Interestingly, sera from mice immunized with rAd-NS4 with both high and low doses and by both routes, demonstrated some neutralization of adenovirus compared to the PBS control group **(Fig. 2.7)**. However, we observed the least neutralization by the sera from the mice

immunized with low i.m. dose and the highest levels of neutralizing antibodies by high i.p. dose ($p < 0.05$).

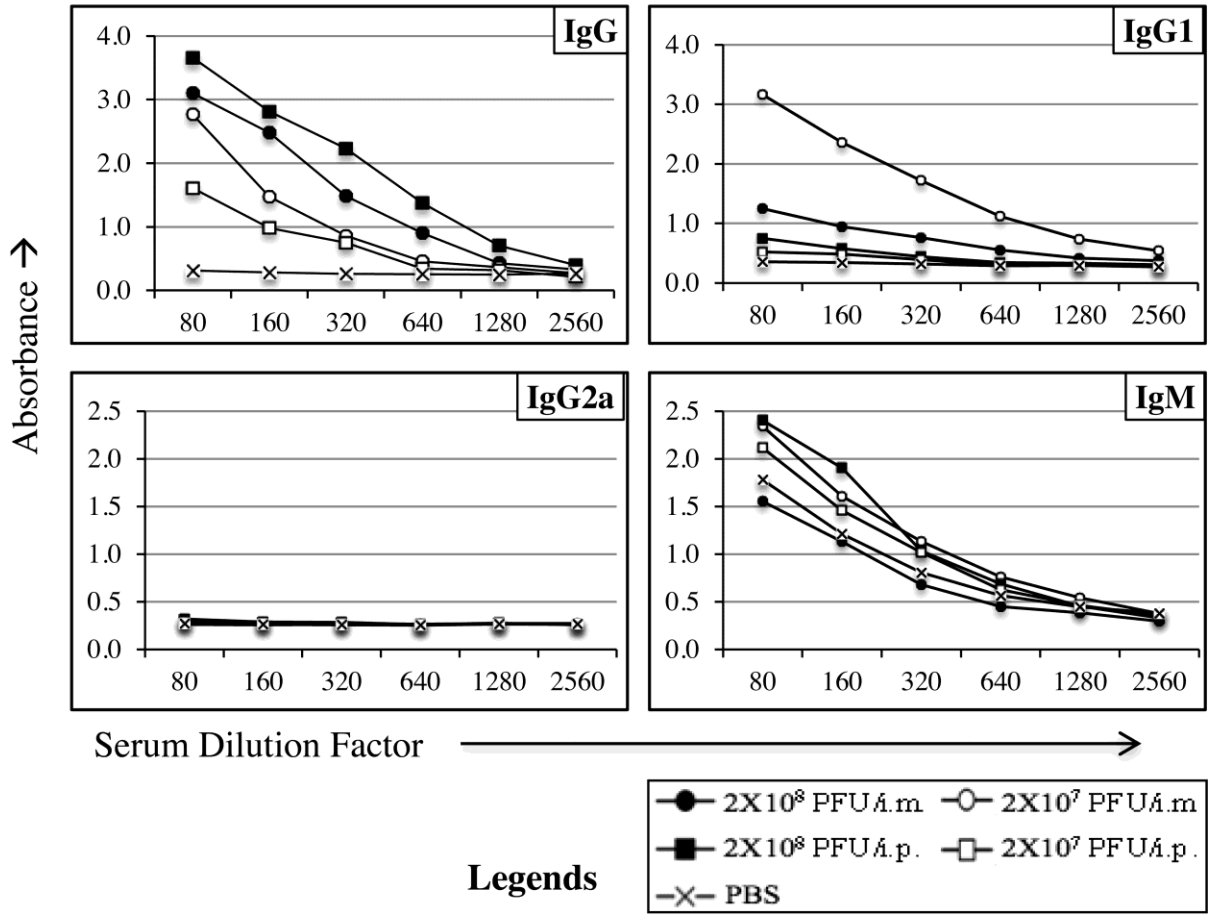


Fig 2.5: Anti-NS4 antibodies. Recombinant NS4 antigen was used as the coating antigen in an ELISA**.** Mean of duplicate OD is plotted against serum dilution factor.

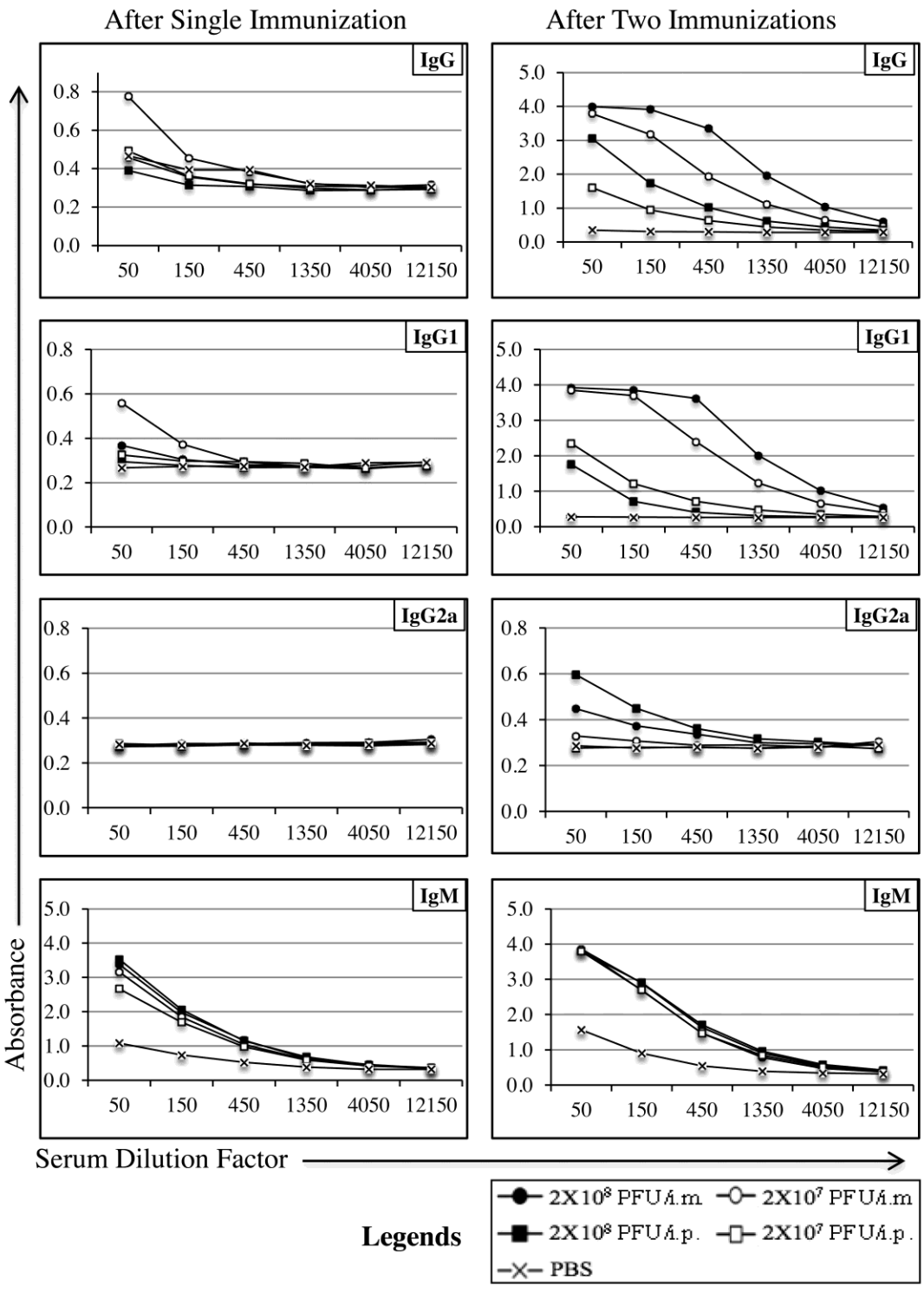


Fig 2.6: Adeno-vector specific antibodies. Lysate from adenovirus-infected HEK-293A cells was used as the coating antigen in an ELISA; Mean of duplicate OD is plotted against serum dilution factor.

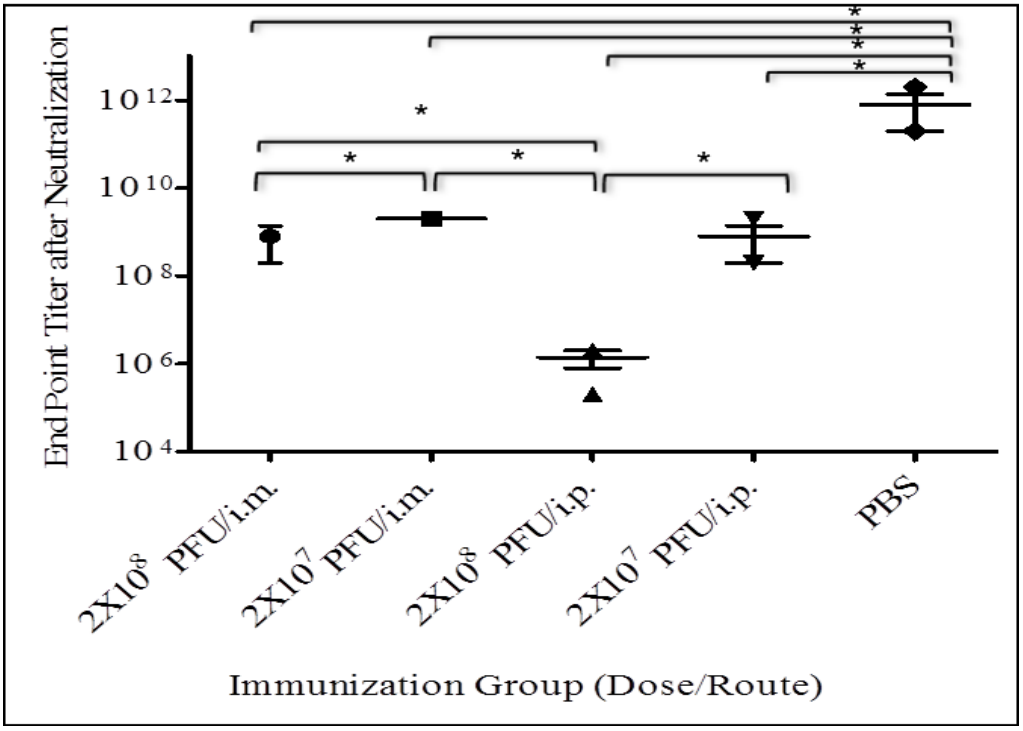
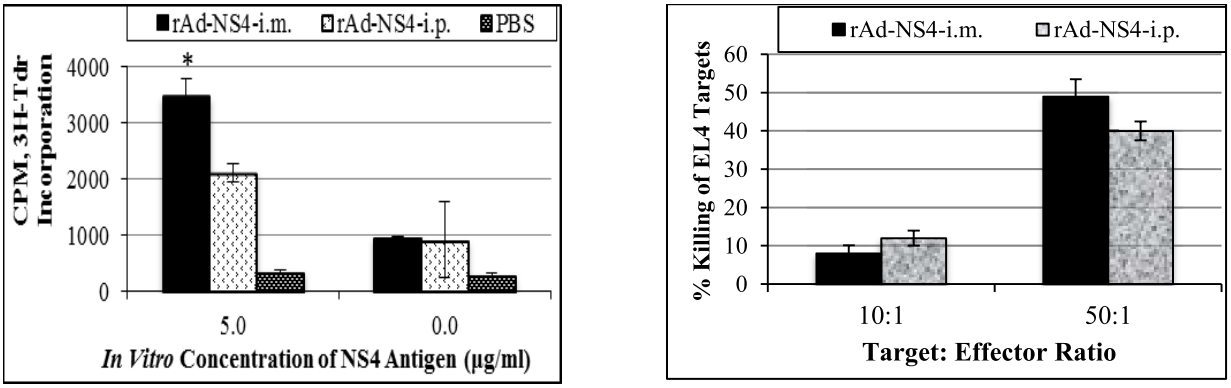


Fig 2.7: Adenovirus vector neutralizing antibodies. Ad virus stock was pre-incubated with sera from various immunized mice, followed by infecting the HEK-293A cells and examining the viral plaques / cytopathic effects for end-point titer determination (lower end-point titer of Ad-vector indicates more neutralizing antibodies). Mean ± SD of viral titers from four replicates is shown (*$p<0.05$). Data are representative of three different repeated experiments.

2.3.4 Intramuscular immunization with rAd-NS4 induces higher long-term cellular and humoral memory responses than intraperitoneal immunization

We examined antigen-specific T cell and antibody responses two months after two i.m. or i.p. immunizations with low doses of rAd-NS4 (2×10^7 pfu/mouse) to determine the longevity of the immune responses induced. The i.m. route of immunization showed higher antigen-specific T cell proliferation in comparison to the i.p. route, however, the target cell killing activity was comparable with T cells obtained from both routes of immunization **(Fig. 2.8 A-B)**. Antigen-specific antibodies were also present two months after immunization. Anti-NS4 IgG and IgG1 were higher in mice immunized i.m., whereas IgM levels were higher in mice immunized i.p. ($p < 0.05$) **(Fig. 2.8 C)**.

(A). T cell proliferative responses **(B). Antigen-specific EL4 target killing**



(C). Anti-HCV NS4 antibodies two months after 2nd immunization

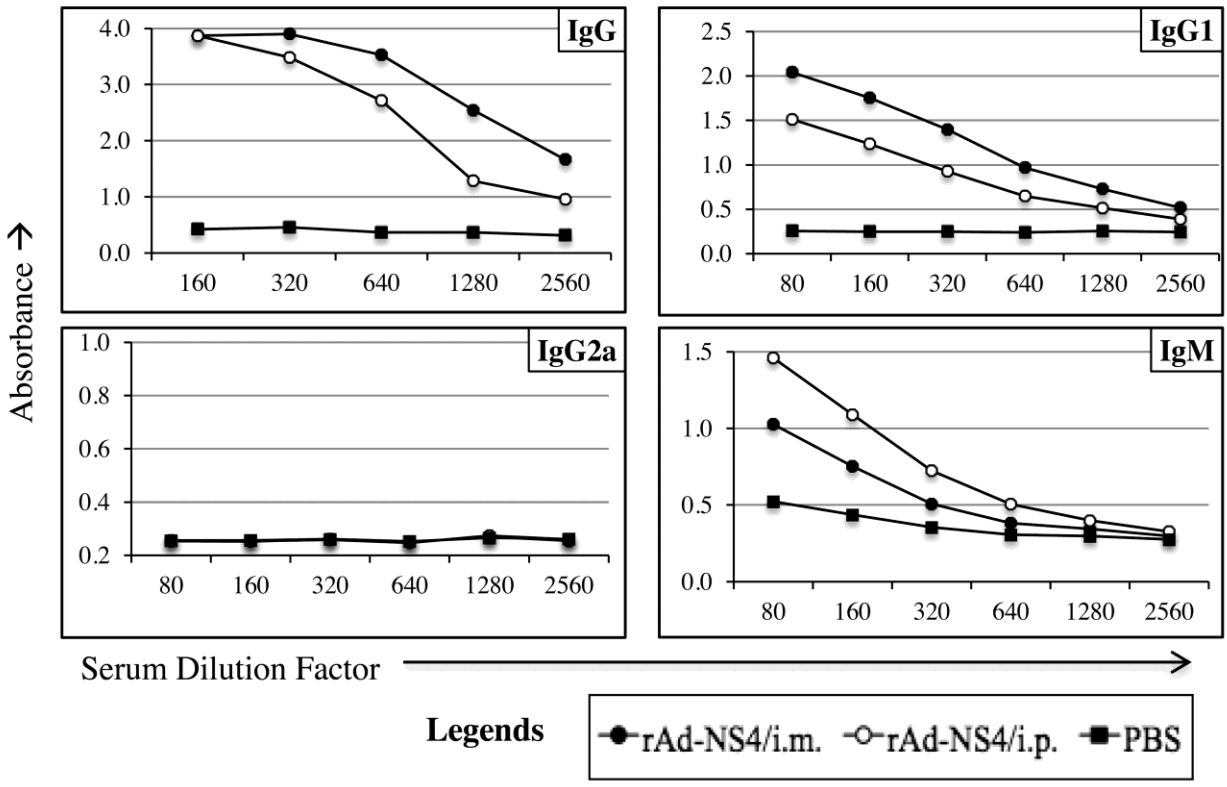


Fig 2.8. Memory responses two months after two immunizations. ***(A). T cell proliferative responses.*** The proliferation assay was done in triplicates and data are shown as mean ± SD (*p<0.05). ***(B). Antigen-specific EL4 target killing.*** The killing assay was done in triplicates and the % killing was calculated for individual replicates, and data are shown as mean ± SD. ***(C). NS4 antigen-specific antibody responses.*** The antibody ELISA was done in duplicates and data are shown as mean ± SD. The data are representative of three different repeated experiments.

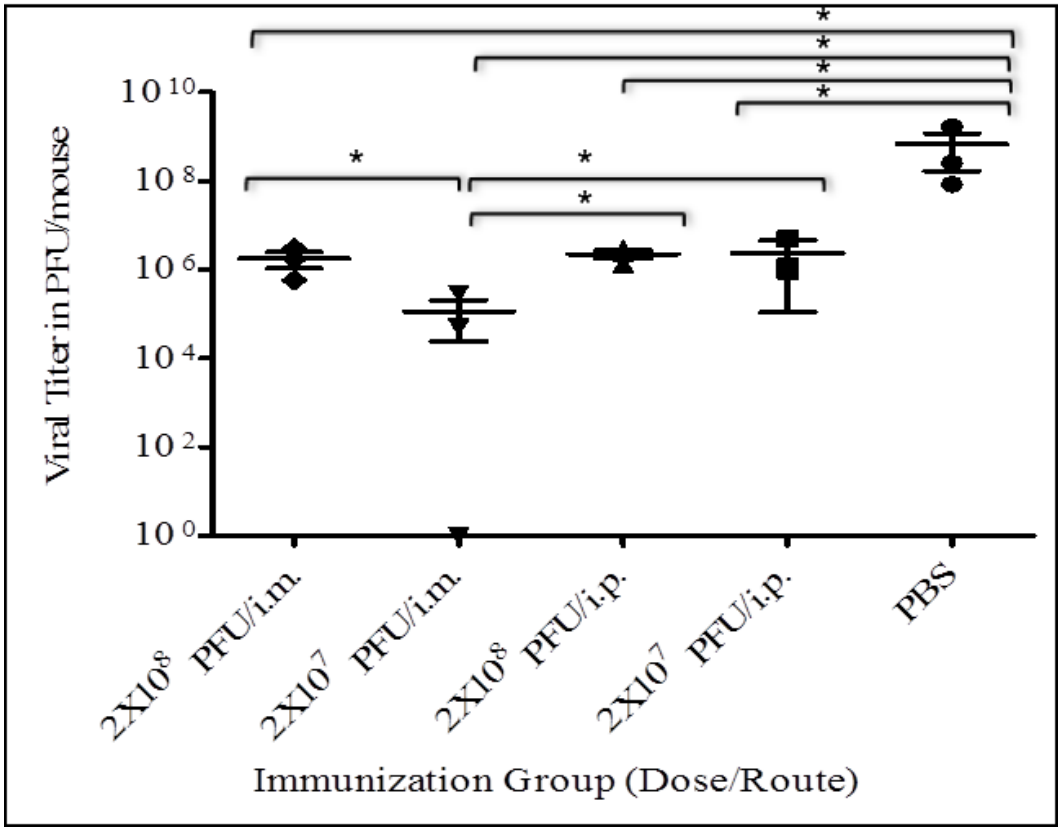


Fig 2.9. Vaccinia-HCV challenge after two immunizations. After two immunizations (Day 0 and 14) with high (2×10^8 pfu/mouse) or low (2×10^7 pfu/mouse) antigen doses via i.m. or i.p. route, mice were challenged with 1×10^5 pfu/mouse i.p. Five days after challenge, mice were euthanized and viral load was determined in the ovaries of individual mouse by plaque assay using TK-1 cell line. Mean ± SD of viral titers from four replicates is shown (*$p<0.05$). The data are representative of three different repeated experiments.

2.3.5 Immunization with low intramuscular dose rAd-NS4 provides the maximum reduction in Vac-HCV viral load

We challenged mice with recombinant Vac-HCV 8 days after first and second immunizations to examine prevention of infection or reduction in viral load after immunization with low and high doses of rAd-NS4, via i.p. and i.m. routes. Mice immunized once only failed to control Vac-HCV load in ovaries compared to controls (**Appendix 2**). However, after two immunizations, all of the groups were able to significantly reduce Vac-HCV load compared to PBS group **(Fig. 2.9)**. Interestingly, maximum reduction in viral load was observed in mice

immunized i.m. with low antigen dose. Moreover, out of three mice, one mouse successfully cleared the virus. Overall, this group was significantly different than groups with immunization by both i.p. doses and high i.m. dose ($p < 0.05$) (Fig. 5). Table 1 summarizes various factors that together impact the reduction in viral load and depicts the overall picture of immune status of mice immunized with higher or lower antigen dose administered by i.m. or i.p. routes.

2.4 Discussion

Adaptive immunity against HCV includes humoral (antibodies), and cellular ($CD4^+$ and $CD8^+$ T cells) responses, and all three components have been shown to be important for viral clearance [3-5, 43]. Therefore, vaccine/immunotherapeutic strategies for HCV should include both types of adaptive immune responses.

Several studies have reported the importance of dose and route for effective immunization by the adeno vector expressing the transgene antigens [25-34]. Both intramuscular and intraperitoneal routes of immunization have been examined using recombinant adenovirus vectors, but not compared directly. In this study, we investigated the induction of humoral as well as cellular responses after immunization with rAd-NS4 by i.m. and i.p. routes and correlated them with reduction of Vac-HCV viral load. The results and various immunization parameters obtained are summarized in Table 1. Our results demonstrated intriguing effects of dose and route of immunizations with replication-defective recombinant adenovirus vector expressing HCV-NS4 antigen. While intramuscular immunization fared better in inducing immune responses and reducing viral load compared to intraperitoneal route, a lower dose of rAd-NS4 vector (2×10^7 pfu/mouse) provided better immunity and protection via i.m. route compared to a higher dose (2×10^8 pfu/mouse) of immunization.

Initially, we optimized the antigen dose by immunizing the mice with 2×10^5 to 2×10^8 pfu/mouse (at one log differences) given by i.m. or i.p. routes **(Fig. 2.1)**. After two immunizations, the lower i.m. dose (2×10^7 pfu/mouse) induced higher proliferation in both spleen and inguinal lymph node T cells in comparison to the higher i.m. dose (2×10^8 pfu/mouse) or both i.p. doses **(Fig. 2.1)**. In our experiments, only inguinal lymph nodes were harvested; the results could have been different if Peyer's patch or mesenteric lymph nodes were harvested for i.p. immunized mice. However, since the systemic spleen T cells also provided very low T cell responses after i.p. immunizations, it is possible that 8 days after immunizations, the local lymph nodes cells may have given a response similar to the response seen in spleen cells. With the i.m. route of immunization, various pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-6, were expressed at higher levels in both the spleen and lymph node T cells **(Fig. 2.2)**. In contrast, IL-10 was produced at comparable levels in both antigen doses via both routes. Higher IL-10 production may be due to an intrinsic property of HCV NS4, as shown in a previous study [48]. Expression of Granzyme B by $CD8^+$ T cells is correlated to effector function [49]. Interestingly, Granzyme B expression was the highest in $CD8^+$ T cells in mice immunized with low i.m. dose **(Fig. 2.3, panel I)**, which again correlated to peptide-loaded target killing activity **(Fig. 2.4)** and antigen-specific proliferation **(Fig. 2.1)**.

Various inhibitory markers such as CTLA-4, PD-1 and $CD4^+FoxP3^+$ T regulatory cells have been found to be up-regulated during persistent infection with HCV [7-11]. In our study, we observed that high antigen dose of immunization via i.m. route led to low T cell response; therefore, we examined the expression of these inhibitory /regulatory markers among various antigen doses and routes of immunization. It was interesting to note that numbers of $CD4^+FoxP3^+$ T regulatory cells were higher in i.m. immunized mice, perhaps representing a

feedback regulatory mechanism **(Fig. 2.3, panel II)**. The expression of negative regulators of T cells, PD-1 and CTLA-4, was higher in the high dose group by both routes and by high i.p dose, respectively. It is possible that due to early timing of examining the inhibitory marker expression, we did not observe a significant difference, and only small differences were observed. In addition, multiple immunizations over a longer time may be necessary to induce stable inhibitory molecule expression on T cells, as is the case in chronic HCV.

High titers of IgG specific to NS4- and adenovirus antigens were obtained after two immunizations with the higher dose of rAd-NS4 by either i.m. or i.p. routes. Interestingly, with lower doses of rAd-NS4, the i.m. route provided significantly higher IgG titers against NS4 and adeno vector derived antigens compared to the i.p. route **(Fig. 2.5 & 2.6)**. These results provided a conundrum as it is generally believed that presence of antibodies against adenoviral vector antigens negatively influences the overall immune responses due to rapid clearance of the vector [35]. We hypothesized that the induction of neutralizing vs. viral protein-specific antibodies may be differentially induced by different routes and doses of rAd-NS4. To test this theory, we also examined neutralizing antibody against adeno vector by using a virus neutralization assay **(Fig. 2.7)**. In this assay, interestingly, the serum from mice immunized with the high i.p. dose of NS4-rAd had the highest virus neutralization ability, in contrast to the antibody responses against NS4 or intracellular adenovirus derived antigens **(Fig. 2.5 & 2.6)**. These data suggest that in the case of intraperitoneal immunization, induction of higher levels of neutralizing antibodies negatively influences the induction of cellular immune responses. However, when the adeno vector is administered in the muscle, leading to rapid intramuscular expression of viral proteins and transgene product, neutralizing antibodies are induced at a lower level and the pre-existing antibodies are not able to access the adenovirus particles immediately. The efficient antigen

expression by the vector is followed by stimulation of T cell responses. Interestingly, after high dose of immunization by both i.m. and i.p. routes, significantly higher levels of IgG were obtained, compared to low dose immunizations, but T cell responses were reduced. Another curious result was the detection of high levels of IgG1 against NS4 in mice immunized with low i.m. dose of rAd-NS4 **(Fig. 2.5)**. IgG2a levels were relatively very low and comparable in all four groups (Fig. 3A). High IgG1 titers are generally considered indicative of a Th2 type immune response [50]. However, in our experiments, high levels of IFN-γ and TNF-α were obtained from splenocyte cultures of these immunized mice, suggesting a Th1 type cellular immunity. It has been reported previously that in chronic HCV patients, IgG1 antibodies are predominant against all of the structural and non-structural proteins [51]. In addition, in chronic HCV patients treated with IFN-γ and ribavirin, HCV-specific IgG1 predominates, irrespective of induction of Th1 cellular responses, suggesting their independence from Th1 and/or Th2 [52]. It has further been suggested that IgG1 production may be IL-2 dependent, and IFN-γ or IL-4 independent [53]. Murine IgG1 has been shown to facilitate phagocytosis of IgG1-bound immune complexes into macrophages and dendritic cells through FcRIII [54], which would allow efficient processing and presentation of antigens to T cells.

We also investigated the longevity of the immune responses in mice after two i.m. or i.p. immunizations with low dose of rAd-NS4 and these showed a pattern of T cell and antibody responses similar to those after 8 days of immunization, with i.m. route showing higher overall immune response compared to i.p. **(Figs. 2.5 to 2.8)**.

Two high or low doses of rAd-NS4 administered through i.m. or i.p. route at two weeks interval significantly reduce Vac-HCV load in challenged mice compared to PBS controls. However, low dose given via i.m. route showed significantly higher reduction in viral loads

compared to both high and low doses via i.p. route and high i.m. dose ($p < 0.05$) **(Fig. 2.9)**. Our results strongly indicated that optimum reduction in the viral load in these mice is associated with strong cellular and humoral immune responses and high levels of pro-inflammatory cytokines induced upon immunization with the low i.m. dose of rAD-NS4 **(Table 2.1)**.

The differences we observed in the induced immune responses by i.m. vs. i.p. routes were significant and have not been reported previously. Upon intramuscular immunization, the transgene is expressed in muscle cells, and start declining after 4 days [21]. There is no clear report on the site of transgene expression upon intraperitoneal immunization with rAd vector. It could be expressed by peritoneal macrophages, followed by processing and presentation of antigens. Alternatively, upon i.p. injection, the adenovirus can gain entry into the blood circulation through mesenteric veins, followed by expression within splenic cells, and /or other distant tissues. However, this may result in a loss of efficiency of antigen expression due to non-replicative nature of the virus vector. It is possible that the efficiency of antigen expression in muscle immediately at the injection site allows for better antigen presentation by APCs and stimulation of T cells.

In our study, immunization with rAd-NS4 is shown to induce strong cellular and humoral immune responses, and pro-inflammatory cytokines, which are critically dictated by both route and dose of immunizations. Although, in our studies, pre-immunization with rAd-NS4 did not completely prevent the infection, it was able to significantly reduce viral load. Therefore, rAd-NS4 can be effective as immune-therapeutic vaccine to reduce viremia and viral load.

Table 2.1: Comparison of various immune parameters in mice immunized with rAd-NS4 with different doses and routes of Immunization

Parameters	$2X10^8$ PFU/Mouse i.m.	$2X10^7$ PFU/Mouse i.m.	$2X10^8$ PFU/Mouse i.p.	$2X10^7$ PFU/Mouse i.p.	PBS
T Cell Proliferation	+++	++++ ($p<0.05$)	++	+	+
IFN-γ	+++	++++	++	++	-
TNF-α	+++	++++	-	-	-
IL-2	+++	++++	++	+	-
IL-6	+++	++++	+	+	-
IL-10	+++	++++	+++	++++	-
TGF-β	+++	++	-	-	-
CD8+GrB+ Cells	+++	++++	+++	+++	+
CD4+PD1+ Cells	+++	++	+++	++	++
CD4+CTLA4+ Cells	++	++	+++	++	++
$CD4^+FoxP3^+$ Tregs	+++	++	+	++	++
NS4 specific Antibodies (IgG)	+++	++	++++	+	-
NS4 specific Antibodies (IgG1)	++++	++	-	-	-
NS4 specific Antibodies (IgG2a)	-	-	-	-	-
NS4 specific Antibodies (IgM)	+	+++	+++	+++	-
rAd Vector specific Antibodies (IgG)	++++	+++	++	+	-
rAd Vector specific Antibodies (IgG1)	++++	+++	+	++	-
rAd Vector specific Antibodies (IgG2a)	++	-	+++	-	-
rAd Vector specific Antibodies (IgM)	+++	+++	+++	+++	-
Neutralization of rAd Vector by Serum	++	+ ($p<0.05$)	++++	+++	-
Vac-HCV Load	+++	+ ($p<0.05$)	+++	+++	++++

Key: Symbol + denotes; Strong (++++), medium (+++), poor (++) magnitude of the parameter compared to lowest (+) value among the groups; (-) indicated undetectable or absent.

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Chapter 3

Adenoviral Proteins Induce Heterologous Immune Responses against Hepatitis C Virus Antigens in Mice

A version of this chapter has been used in a patent application *(**Shakti Singh, et al.2015. US Patent**,* Applied*)* and submitted for publication (***Shakti Singh, et al. 2015***).

3.1 Introduction

Chronic infection with hepatitis C virus (HCV) is a serious global health problem. It affects ~170 million people worldwide and can lead to liver cirrhosis, hepatocellular carcinoma and end-stage liver diseases [1-3]. Current treatment of chronic HCV infection is limited to combination drug therapies, which are highly expensive, have serious side effects and have variable success rates in different viral genotypes and patient populations [4]. Development of vaccines to prevent and/or cure HCV infection is of paramount importance.

Natural protection from infection is often used to model strategies to develop new vaccines. The immune mechanisms and correlate of viral clearance and protection from HCV infection have been studied extensively, but they still remain unclear [5-7]. A number of studies have correlated the natural clearance of HCV infection with the appearance of vigorous and multi-specific $CD8^+$ and sustained $CD4^+$ T cell responses [8-10]. In contrast, development of chronic infection is correlated with weak or dysfunctional cellular immune responses [7, 10, 11]. A recent meta-analysis of studies published to date with chimpanzee model concluded that humoral immune responses also play a determining role in protection from chronic HCV infection [12-14]. Further, recent studies support a role of antibodies beyond neutralization of viral particles; extending their function to targeting intracellular pathogens [15]. Therefore, it can be envisioned that a successful prophylactic and/or therapeutic vaccine strategy would encompass the generation of both cellular and humoral responses against multiple antigens of HCV.

Cases of chronic HCV infection have been reported where patients spontaneously clear the infection due to re-activation of functional cellular immune responses [16]. However, the exact

mechanism of this spontaneous reversal of cellular immune responses, from exhausted and/or dysfunctional to functional, remains to be elucidated. In several studies, HCV-specific T cells have been expanded from the peripheral blood T-cell populations in individuals previously not infected with HCV [17, 18]. Among the HCV exposed individuals, 20 to 30% clear the virus after acute infection due to induction of strong multi-specific cellular immune responses [10, 19, 20]. However, it is not known if pre-existing heterologous immunity has any role in the HCV clearance after infection. Influenza A virus infection has been shown to induce narrow heterologous cellular immune response against a single epitope from HCV NS3 antigen [18].

Adenoviruses (Ad) are non-enveloped, icosahedral viruses containing a double-stranded DNA genome of ~26-48 Kbp [21]. They belong to a diverse family (>50 serotypes) of DNA viruses called *adenoviridae*, infect ocular, respiratory or gastro-intestinal epithelium in a diverse range of hosts [21] and induce different levels of immunity [22, 23]. They have gained immense recognition as a vaccine antigen delivery vehicle since their initial use in gene therapy [24], and have also proven to be safe and efficient vaccine vectors for eliciting protective immune responses against the transgene antigen in many animal and human clinical studies [25, 26]. A chimpanzee-adenovirus derived vector expressing HCV antigens NS3-NS5, is currently in clinical trials [27].

We have been studying recombinant adenoviruses expressing various individual HCV antigens for their ability to modulate and induce HCV-specific immunity in an *in vitro* human cell culture system and *in vivo* in mice [28-32]. During our studies, we observed that immunization of mice with control, non-recombinant adenovirus vector (Ad vector) not expressing HCV antigens, also induced HCV antigens-specific T cell responses. To address this

puzzling and unexpected observation, we aligned peptide sequences from different HCV proteins with various adenoviral proteins (Ad proteins) to determine the levels of homology between the proteins of the two viruses. Intriguingly, we discovered varying degrees of homologies (25-53%) between multiple HCV peptide sequences from core, F, NS3, NS4 and NS5 proteins and a number of Ad proteins. In this study, we conclusively demonstrate that immunization of mice with Ad vector not expressing exogenous HCV antigen(s), induces robust cross-reactive humoral and cellular immune responses against multiple HCV antigens. Further, reduction in viral titers in mice infected with vaccinia virus expressing recombinant HCV antigens as a surrogate model corroborated the relevance of cross-reactive immune responses to antiviral immunity. Our study is the first to demonstrate protective heterologous immunity induced by Ad vector against HCV. These studies have significant implications in the design of prophylactic and therapeutic vaccines against HCV. In addition, since recombinant adenovirus vectors are at the forefront as candidate vaccines for several different pathogens including HCV, Ebola virus, Plasmodium, mycobacteria, influenza virus, among others, their widespread use as vaccines could significantly impact the prevalence and natural course of HCV infection.

3.2 Materials and Methods

3.2.1 Adenovirus Vector

Replication-incompetent human adenovirus 5, with no transgene insert, was amplified and titrated in human embryonic cell line 293A (HEK-293A) transformed with adenovirus E1 gene (QBiogene Inc., CA, USA) to provide complementarity for virus production. Recombinant adenoviruses (rAd) expressing HCV antigens core (rAd-core), F (rAd-F), NS3 (rAd-NS3), NS4 (rAd-NS4) or NS5 (rAd-NS5) were prepared and reported earlier by our group [17, 28, 31].

3.2.2 DNA Purification and PCR Amplification

DNA was purified from Ad, rAd-core, rAd-NS3, rAd-NS4, rAd-NS5a, rAd-NS5b vector stocks. Briefly, $1x10^8$ pfu of each vector was taken in individual tubes and DNA was prepared using High-Pure Viral Nucleic Acid Kit^R (Roche Applied Bio). PCR reaction was set up with 10 µl template DNA obtained from the above preparation using 50 µl total reaction volume consisting of 1x PCR buffer, 10 µM dNTP, 25 µM of each primer (forward and reverse, listed in **Table 3.1**) and 1.25 unit of Taq polymerase. PCR tubes containing reaction mixtures were incubated in a thermo-cycler with initial denaturation at 92ºC and 40 amplification cycles (92ºC: 30 Sec, 50-55ºC: 30 Sec, 68ºC: 60 Sec). PCR amplification products were run on 1% agarose gel at 80 volts to resolve amplification products along with 1 Kb sized Quick Load DNA Ladder (NEB Biolab, Germany).

Table 3.1. PCR primers used to detect presence of HCV genes in adenoviral vector stock

Gene	Primer sequence	PCR product size (bp)
Core	Forward: 5′-CGG GAT CCA TGA GCA CGA ATC CTA AAC C-3′ Reverse: 5′-CGG GAT CCT AGG CTG AAG CGG GCA CAG-3′	573
F	Forward: 5′-GAA GAT CTA TGC CAA ACG TAA CAC CAA CCG TC-3′ Reverse: 5′-GAA GAT CTC ACG CCG TCT TCC AGA ACC CGG A-3′	486
NS3	Forward: 5′-CGG GAT CCA TGG CGC CCA TCA CGG CGT AC-3′ Reverse: 5′-CGG GAT CCT ACG TGA CGA CCT CCA GGT C-3′	1893
NS4	Forward: 5′- GAA GAT CTA TGA GCA CCT GGG TGC TCG TT-3′ Reverse: 5′- GAA GAT CTC AGC ATG GAG TGG TAC ACT C-3′	943
NS5a	Forward: 5′- GAA GAT CTA TGT CCG GTT CCT GGC TAA GG-3′ Reverse: 5′- GAA GAT CTC AGC AGC ACA CGA CAT CTT C-3′	1343
NS5b	Forward: 5′- CGG GAT CCA TGT CAA TGT CTT ATT CCT GG-3′ Reverse: 5′- CGG GAT CCT CAT CGG TTG GGG AGG AGG TA-3′	1776

Table 3.2. Description of adenoviral (Ad) proteins, which were compared to determine homologies with HCV proteins' derived peptide epitopes

No	Human Ad5 protein name	Short name	GI number	Accession no.	Trans-cription unit
1	E2A, DNA Binding Protein	DBP	58177697	AAW65516	E2
2	E2B, DNA Polymerase	Pol	58177697	AP_000202	E2
3	E2B, Terminal Protein Precursor	pTP	209846	AAW65504	E2
4	34k, Control Protein	34K	58177724	AAW65531.1	E4
5	ORF1, Control Protein	ORF1	56160565	AP_000232.1	E4
6	ORF2, Control protein	ORF2	56160564	AP_000231.1	E4
7	ORF3, Control protein	ORF3	56160563	AP_000230.1	E4
8	ORF4, Control protein	ORF4	56160562	AP_000229.1	E4
9	ORF6/7, Control Protein	ORF6/7	56160560	AP_000227.1	E4
10	13.6k protein	13.6K	58177696	AAW65503.1	L1
11	52k, Encapsidation Protein	52K	58177700	AAW65507.1	L1
12	pIIIa, Capsid Protein precursor	pIIIa	58177701	AAW65508.1	L1
13	III, Penton Base	Penton	5817770	AAW65509	L2
14	pVII, Core Protein Precursor	pVII	209845	AAA96408.1	L2
15	V, Core Protein	V	209846	AP_000208	L2
16	pX, Core Protein Precursor	pX	58177705	AP_000209	L2
17	pVI, Core Protein Precursor	pVI	209848	AP_000210	L3
18	II, Hexon (Capsid)	Hexon	157879600	1P30_A	L3
19	Protease	Protease	58177708	AP_000212	L3
20	100k, Hexon Assembly Protein	100k	58177710	AAW65517.1	L4
21	22k Protein	22k	58177711	AAW65518.1	L4
22	33k protein	33K	58177712	AAW65519.1	L4
23	pVIII, Capsid Protein precursor	pVIII	454806	AP_000217	L4
24	IV, Fiber (Capsid) Protein	Fiber	209931	AP_000226	L5
25	Encapsidation Protein IVa2	IVa2	56160534	AP_000201.1	IVa2
26	Capsid Protein IX	IX	56160533	AP_000200.1	IX
27	Protein U	U	158536737	ABW72885.1	U

3.2.3 Sequence Alignment

Fifteen to twenty amino acid long sequences from various HCV antigens of genotype 1a (core, F, NS3, NS4 and NS5) were compared with human adenovirus 5 protein sequences (**Table 3.2**) by sequence alignment using ClustalW software (Online version). Sequence homology was documented as pairwise similarity or homology score, and a heat map was prepared by Microsoft Excel to present the distribution of each HCV antigen peptide homology across the different adenovirus proteins. Pairwise scores in the aligned region are the number of identities between the two sequences, divided by the length of the alignment, and calculated as a percentage, so a score of 25 means 25% homology, 30 means 30% homology between aligned sequences and so on. The number of adenovirus proteins showing various levels of homology (25.00 to 30.00, 30.11-35.00, 35.11-40.00, 40.11-45.00, 45.11-50.00 & >50) was calculated from the heat map of scores, and summarized in **Table 3.3** and **Appendix 3-8**.

3.2.4 Mice Immunizations

Six to seven week-old female C57BL/6 mice were purchased from Charles River Laboratory (Charles River, Canada) and immunized twice intramuscularly (at 14-day intervals) using an optimized dose ($2x10^7$ PFU/mouse) of Ad, in presence or absence of poly I:C adjuvant (20 μg/mouse; Sigma-Aldrich, St. Louis, MO, USA). Mice were euthanized 8 days after the second immunization and various tissue samples (e.g. spleens, inguinal lymph nodes, ovaries, serum etc.) were collected.

All animal experiments were approved by the University of Alberta Animal Care and Use Committee in accordance with the Canadian Council of Animal Care guidelines.

3.2.5 T cell Proliferation Assay

Eight days after the second immunization, mice were euthanized, and spleens and/or inguinal lymph nodes were collected. The spleens were pooled from replicates and ground to single cell suspensions and filtered through a Falcon 100 µm nylon cell strainer. The cells were resuspended in 2 ml of media and passed through an equilibrated nylon wool column. The column was washed after 45 min of incubation at 37°C and the flow through contained all the splenic T cells. These T cells were used in the experiments (~90% $CD3^+$ T cells). Lymph nodes were also ground into single cell suspensions and used in the assays. Proliferative responses were measured in triplicate cultures in 96-well flat-bottomed microtiter plates. A total of 4×10^5 T cells from immunized mice and 4×10^5 APCs (spleen cells from unimmunized mice irradiated with 3000 rads) were mixed with different HCV-derived proteins (core = c22-3, NS3 = c33c; NS4 = c100-3; NS5 = NS5 SOD, polyprotein c25 = core+NS3+NS4; polyprotein c200 = NS3+NS4 or control protein = rhSOD, kindly provided by Chiron) or synthetic HCV-derived peptides (**Appendix 3-8**) at different concentrations as described in the figure legends. T cell proliferation was assessed by a radioactive ^{3}H-thymidine incorporation assay. Detailed methodologies for T cell proliferation assays have been reported previously by our group [32].

3.2.6 Cytokine ELISA

Cytokines were assessed in culture supernatants collected from T cell proliferation assays using mouse cytokine ELISA kits supplied by eBiosciences (eBiosciences Inc. San Diego, USA). ELISA was performed according to the manufacturer's instructions. Plates were read and data were analyzed in FluoStar ELISA reader (BMG Labtech GmbH, Ortenberg, Germany). Cytokine concentration per ml of culture supernatant was determined by multiplying the calculated

concentration with the dilution factor. The means of these concentrations (pg/ml) from duplicate wells were plotted in the graphs.

3.2.7 Antibody ELISA

Serum was prepared from the blood of immunized mice (pooled, n = 3 to 5) and stored at -20°C until required. HCV antigen-specific cross-reactive IgG antibodies in Ad vector immunized mice, were measured in 96-well plates coated overnight at 4°C with HCV antigens (core, NS3, NS4 or NS5) at 1 μg/ml in 1x PBS. The next day, after blocking with 1% BSA at room temperature for 1 hour, serial dilutions of serum samples were added to the 96-well plate in 2-3 replicates and incubated again at room temperature for 2 hours. After application of serum, anti-mouse IgG labeled with alkaline phosphatase (AP) (Southern Biotech, Alabama, USA) was added and plates were incubated for 1 hour. Color was developed by adding PNPP substrate (Southern Biotech, Alabama, USA). Plates were washed with 1xPBST (1xPBS with 0.1% Tween-20) after each incubation step. Absorbance was read using FluoStar Optima ELISA Reader (BMG Labtech GmbH, Ortenberg, Germany), and OD values from HCV antigen-coated plates, corrected for background from OD values in SOD-coated plates, were plotted in the graphs.

3.2.8 Flow Cytometry

Mouse splenocytes were cultured with 5 μg/ml of HCV protein antigens or peptides for 5 days in RPMI-1640 media supplemented with 10% fetal bovine serum. On the fifth day, cells were harvested and counted, and $1x10^6$ cells per group were stained for T cell markers. To perform intracellular cytokine staining, splenocytes cultured for 5 days with HCV antigens were treated with ionomycin (1 μg/ml), phorbol 12-myristate 13-acetate or PMA (50 ng/ml) and

brefeldin A (1.5 μg/ml) for 5 hours and subsequently stained for extracellular lineage markers: CD3 (PE Cy7), CD4 [33] and CD8 (APC efluor 780); and intracellular cytokines: IFN-γ (PE) and IL-10 [34]. For T cell proliferation using the CFSE dilution assay, splenocytes were enriched for T cells using nylon wool column and stained with 1 μM CFSE in 1 ml of cell suspension for 7-10 minutes at room temperature. These CFSE stained cells were washed in 1x PBS containing 10 % fetal bovine serum, counted and plated in 24-well plate (2 million / 200 μl volume) with 5 μg/ml of HCV core, NS3, NS4 or NS5 antigen or their representative peptides; and equal number of γ-irradiated syngeneic splenocytes as APCs in a total 500 μl RPMI medium. These cells were cultured for four days and stained for CD3+, CD4+ and CD8+ T cell markers. All stained cells were run in BD FACS Canto, and data were analyzed using FCS Express 4.0 software. Fluorescent-labeled antibodies against various cell markers were purchased from eBiosciences.

3.2.9 T cell Cytotoxicity Assay

Spleen T cells harvested from Ad vector immunized mice were stimulated *in vitro* with the HCV protein antigens core, NS3, NS4, NS5 or polyprotein at 5 μg/ml for 4 days. The target EL4 cells were incubated with corresponding HCV peptides (core peptides #: 2, 14, 17, 25, 27, 28, 32; NS3 peptides #: 8, 10; NS4 peptides #: 3, 4, 8; NS5a peptides #: 1, 2, 16, 20, and NS5b peptides #: 5, 19, 23, 39; or All: a mixture of the above peptides from core, NS3, NS4 and NS5) overnight at 37^{o}C. Next day, peptides loaded EL4 cells were cultured with effectors at 10:1 (effectors: target) ratio for 4-5 hours. CFSE-labeled live targets were quantified by flow cytometry and subtracted from background CFSE-labeled targets to get the numbers of killed targets. Empty (no peptide loaded) EL4 targets were used as a negative control.

3.2.10 Chimeric Vac-HCV Challenge

Eight days after the second immunization with Ad vector, Ad vector + poly I:C or PBS, mice were challenged with $1x10^7$ PFU of Vac-HCV chimeric virus (Vac-C/NS2/NS3 including core, NS2 and NS3 antigens of HCV or Vac-NS3/NS4/NS5 including NS3, NS4 and NS5 antigens of HCV, kindly provided by Dr. Alfred Prince, NY, USA), or wild-type vaccinia (WT-Vac, not containing HCV antigens) virus intraperitoneally. Five days after virus challenge, mice were euthanized and ovaries were removed, homogenized and freeze-thawed three times in 1x PBS. Homogenized samples were stored at -80º C until used for viral titer.

3.2.11 Vac-HCV Titration by Plaque Assay

Serially diluted samples of ovary homogenates were added in duplicate wells in 6-well plates containing 80% confluent monolayers of TK-1 cells (ATCC # CRL 2396) and incubated for 90 minutes. Subsequently, unbound virus was removed and fresh 1x DMEM medium (Gibco by Invitrogen, NY, USA) supplemented with 3% FBS, was added, and plates were incubated for 48 hours. At this time, the medium was removed, and plaques were fixed using 10% formaldehyde (Fisher Scientific, NJ, USA) at room temperature for 30 minutes. Plates were washed with PBS and the monolayers were stained for 30 minutes with 0.5% crystal violet (Sigma-Aldrich Company, MO, USA), followed by further washing. Plaques were counted, averaged and multiplied with the dilution factor to determine the viral load/mouse.

3.2.12 Statistical Analysis

Data were analyzed by Graph-pad Prism software (Graph-pad Software Inc., CA, USA). Two-tailed Student's *t*-test was used to determine the significant difference between two groups, and *P*-values less than 0.05 (<0.05) were considered to be statistically significant.

3.3 Results

3.3.1 DNA isolated from Ad vector does not contain HCV genes

To exclude the possibility of contamination of the non-recombinant Ad vector stock with HCV transgene, we set up PCR with purified Ad DNA using HCV-specific primers **(**listed in **Table 3.1**). None of the primer sets specific for core, F, NS3, NS4, NS5a or NS5b genes of HCV were able to amplify HCV gene products from non-recombinant Ad vector DNA template (**Fig 3.1**). Uninfected HEK cell lysate and recombinant adenoviral (rAd) vectors containing individual HCV genes were used as negative and positive controls, respectively.

3.3.2 Peptide sequences derived from HCV antigens exhibit varying degrees of homology with different adenoviral (Ad) vector proteins

We aligned the amino acid (aa) sequences of peptides (15-20 aa long with 5 aa overlaps) derived from various HCV antigens (core, F, E1, E2, P7, NS2, NS3, NS4a, NS4b, NS5a and NS5b) of genotype 1a with different Ad proteins using the bioinformatics software ClustalW to determine homology scores. Table 3.3 summarizes the range of homologies between HCV-derived peptides and Ad proteins, and the number of HCV peptides and Ad proteins that show these homologies. Intriguingly, almost all of the compared Ad proteins (27, **Table 3.2**) showed homology (scores ranging between 25-40) with several peptides from HCV antigens core, F, NS3, NS4, NS5a and NS5b. The maximum homology scores shown by HCV peptides was core (45-50), F (45-50), NS3 (45-50), NS4 (35-40), NS5a (40-45) and NS5b (40-45) **(Table 3.3, Appendix 3-8)**. HCV antigens E1, E2, P7 and NS2 showed low homology (25-40, except p7) with the lowest number of Ad proteins (**Appendix 9-12**), therefore these were not tested for cross-reactive immune responses induced by Ad vector.

Table 3.3. Summary of HCV peptides showing homology to adenovirus (Ad) proteins

HCV proteins	No. of HCV peptides tested	No. of Ad proteins tested	Number of peptides showing homology (% Homology between HCV peptide and Ad protein sequences)					
			25.00 - 30.00	30.11 - 35.00	35.11 - 40.00	40.11 - 45.00	45.11 - 50.00	>50.00
Core	45	**27**	45	43	27	0	7	1
F	16	**27**	16	15	4	0	3	1
NS3	11	**27**	10	8	8	1	1	0
NS4	20	**27**	15	7	2	0	0	0
NS5a	29	**27**	29	10	7	2	0	0
NS5b	39	**27**	39	7	4	1	0	0

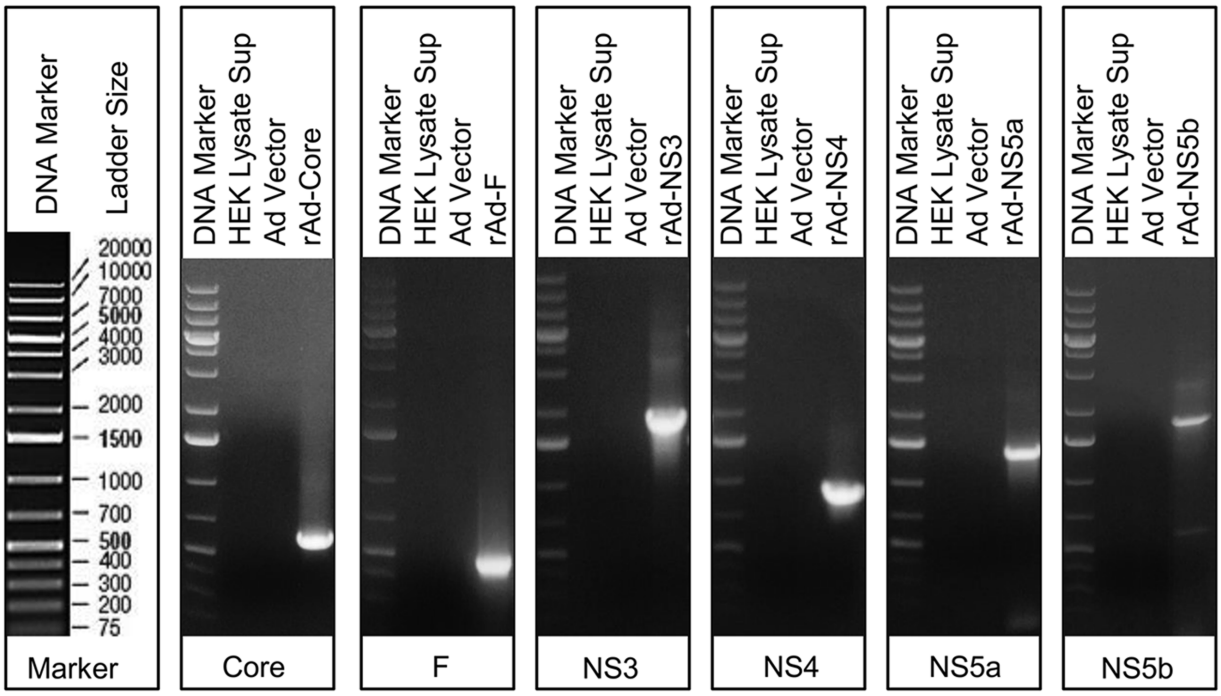

Fig 3.1. HCV genes Core, F, NS3, NS4, NS5a or NS5b are not amplified in Ad vector stock by PCR. First panel shows the DNA ladder, followed by agarose gel electrophoresis of PCR products obtained with HCV Core, F, NS3, NS4, NS5a and NS5b specific primers. HEK lysate supernatant and rAd-HCV vectors were used as negative and positive controls. Data are representative of 2-3 repeated experiments.

3.3.3 Immunization of mice with Ad vector induces cross-reactive cellular and humoral immune responses against multiple HCV antigens

The significant homologies of HCV derived peptides with Ad proteins sequences indicated that adenoviruses might induce cross-reactive immunity against HCV antigens. To test this, female C57bl/6 mice (n = 5/group) were immunized intramuscularly two times (at 14 days interval) with $2x10^7$ PFU/mouse Ad vector, Ad vector + poly I:C or PBS. The proliferation and IFN-γ production from spleen and lymph node T cells were determined in response to HCV core, NS3 and NS5 protein antigens and pools of their selected peptides (**Appendix 3-8**) (core peptides #: 5, 14, 16, 17 & 27; NS3 peptides #: 2, 5, 6, 8 &10; NS5a peptides #: 6, 24; NS5b peptides #: 5, 19, 27) (**Fig 3.2**). Both spleen and inguinal lymph node T cells from Ad vector immunized mice demonstrated high HCV antigens (core, NS3 and NS5)-dependent proliferation and IFN-γ production, which was further increased by co-immunization of Ad vector with poly I:C adjuvant.

Cross-reactive immune responses against core, F, NS3, NS4, NS5a and NS5b derived synthetic peptides in Ad vector immunized mice were evaluated, to characterize and identify the domains of cross-reactivity in various HCV antigens with respect to amino acid sequences. Many of the HCV core, F, NS3, NS5a and NS5b peptides were able to induce T cell proliferation *ex vivo*, which also translated into production of IFN-γ. These peptides had high amino acid sequence homology and multiple high scoring regions with the different Ad proteins. However, some peptides, which showed high homology with respect to high score (>35) and number of homologous regions in Ad proteins, did not show cross-reactive responses in mice immunized with Ad vector (**Fig 3.3** and **Appendix 3-8**).

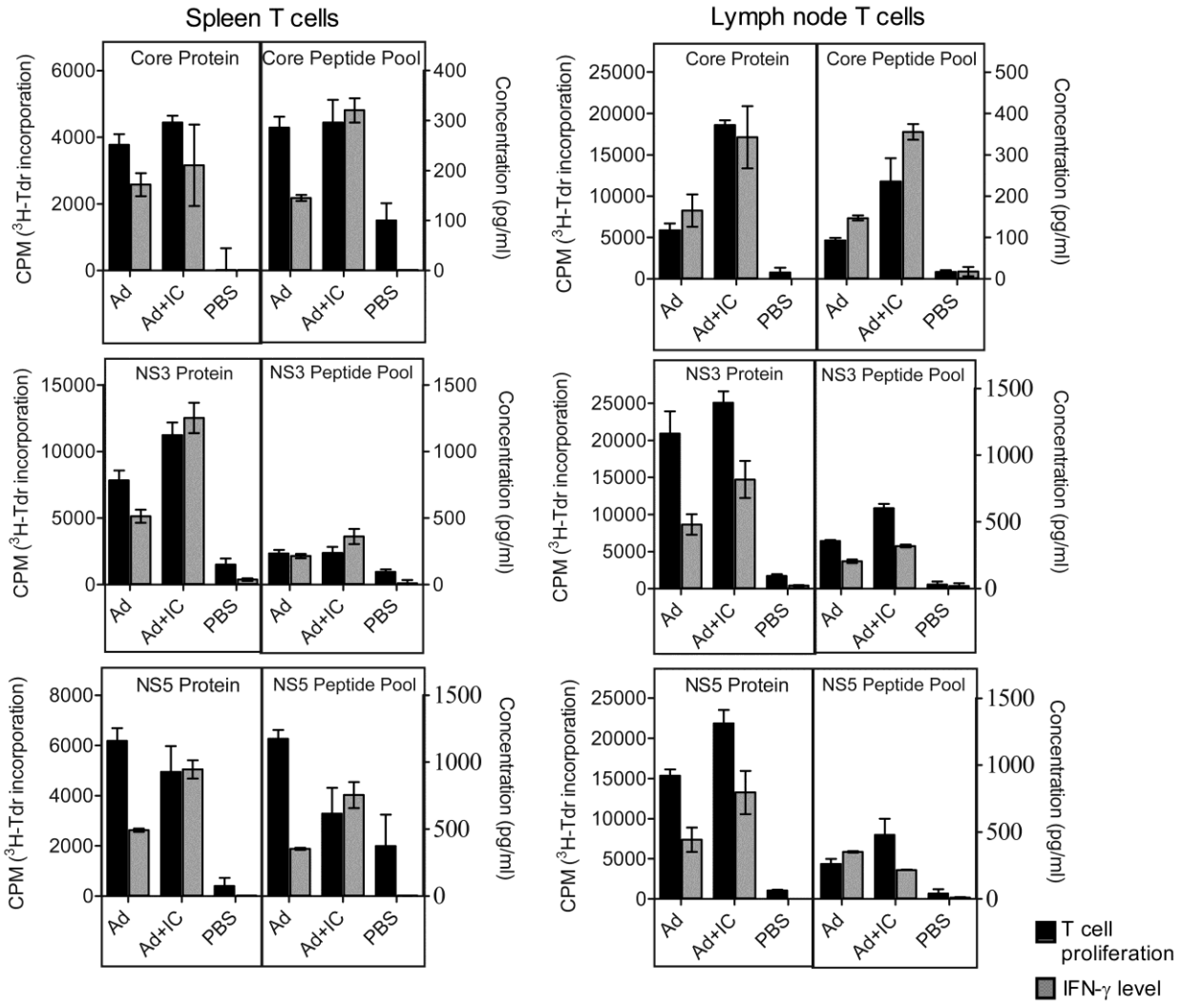

Fig 3.2. Cross-reactive cellular immune responses against HCV antigens in mice immunized with Ad vector (with or without poly I:C adjuvant). Proliferation and IFN-γ production in spleen and lymph node T cells upon *ex vivo* stimulation with HCV antigens, or a pool of 5 peptides showing high homology with Ad proteins (see text for details). Data are presented as mean ± standard deviation of 3-4 replicates, and represent 3-4 independent experiments.

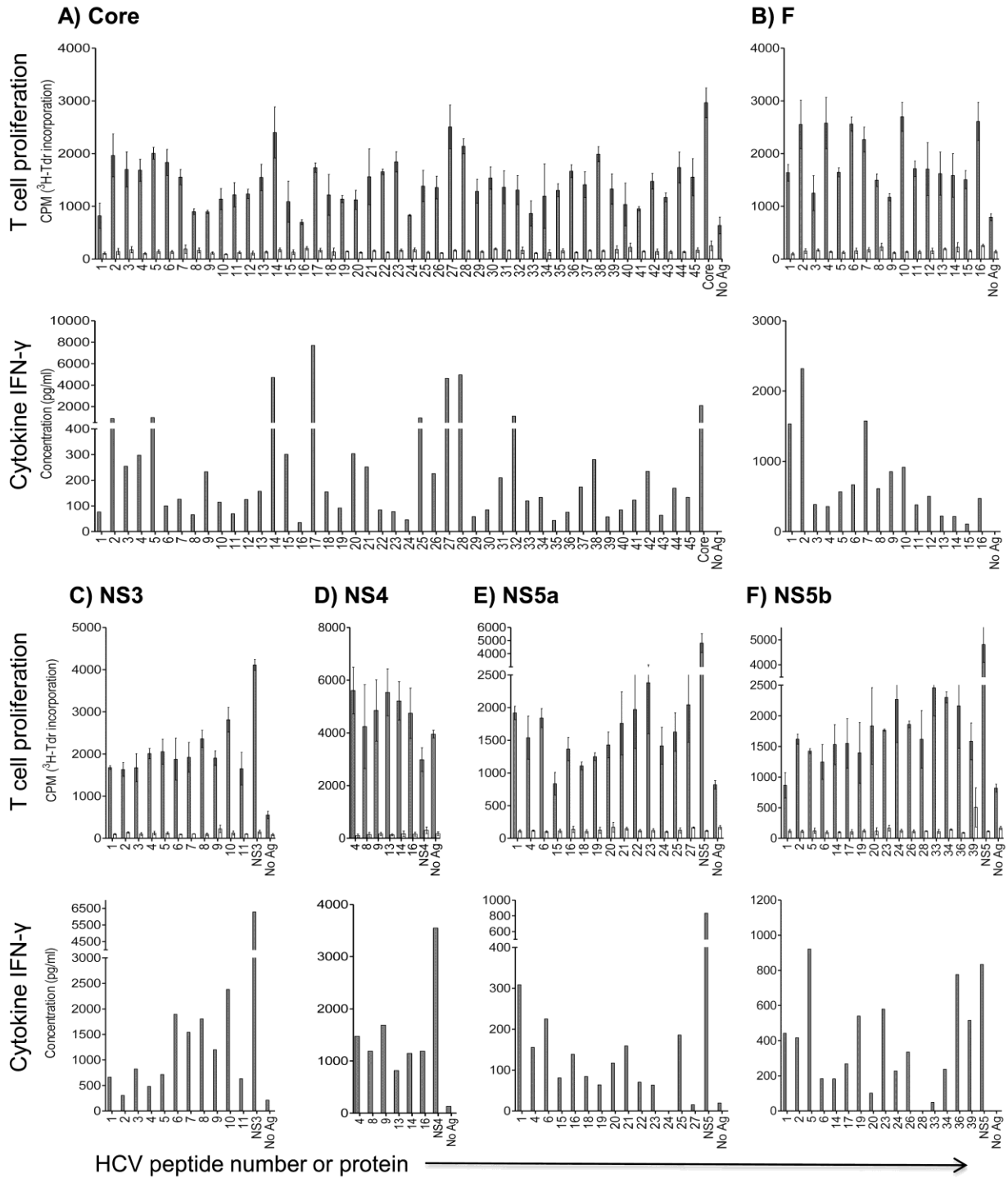

Fig 3.3. Proliferation and IFN-γ production upon stimulation with individual synthetic peptides derived from HCV antigens (Core, NS3, NS4 and NS5) in Ad vector immunized mice. Data are presented as mean + standard deviation of triplicate (counts per minutes, CPM) or duplicate wells (cytokine concentration), and are representative of two independent experiments.

To confirm that the high proliferation responses observed in the ^{3}H-thymidine uptake assay are due to actual proliferation of cross-reactive HCV-specific $CD4^+$ and $CD8^+$ T cells, we performed a CFSE dilution assay along with staining for CD3, CD4 and CD8 markers. Splenocytes obtained from Ad vector immunized mice were stimulated *ex vivo* with HCV protein antigens (core, NS3, NS4 and NS5), or selected representative peptides from these proteins (**Fig 3.4**). Intriguingly, the stimulated splenocytes exhibited remarkable antigen-dependent proliferation of cross-reactive $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells.

To demonstrate that the cytokines produced in culture supernatants are from $CD4^+$ and $CD8^+$ T cells, we performed intracellular cytokine expression analyses of splenocytes obtained from Ad vector immunized mice and stimulated *ex vivo* with HCV core, NS3, NS4 and NS5 protein antigens (**Fig 3.5**) and their selected peptides (**Fig 3.6**). Both $CD4^+$ and $CD8^+$ T cells showed increased expression of IFN-γ and IL-10 simultaneously upon stimulation with HCV proteins when compared to PBS immunized mice (**Fig 3.5**). We noted that HCV core-stimulated $CD4^+$ and $CD8^+$ T cells showed a high frequency of IL-10 expressing cells compared to other HCV antigens. However, IFN-γ producing $CD4^+$ and $CD8^+$ T cells were high against all HCV antigens except HCV NS5 where only IFN-γ producing $CD8^+$ T cells were increased (**Fig 3.5**). Upon stimulation with HCV core, NS3, NS4 and NS5 peptides, cross-reactive $CD4^+$ T cells showed enhanced IFN-γ and IL-10 expression in comparison to the PBS group (**Fig 3.6**). Further, IFN-γ-producing $CD8^+$ T cells were notably high with all the peptides used for *in vitro* stimulation (**Fig 3.6**). However, the frequency of $CD8^+$ T cells, which express IL-10 or both IFN-γ and IL-10, were not increased with any of the selected peptides except HCV core peptide #17, which also showed increased frequency of double positive (both IFN-γ and IL-10) $CD4^+$ T cells (**Fig 3.6**).

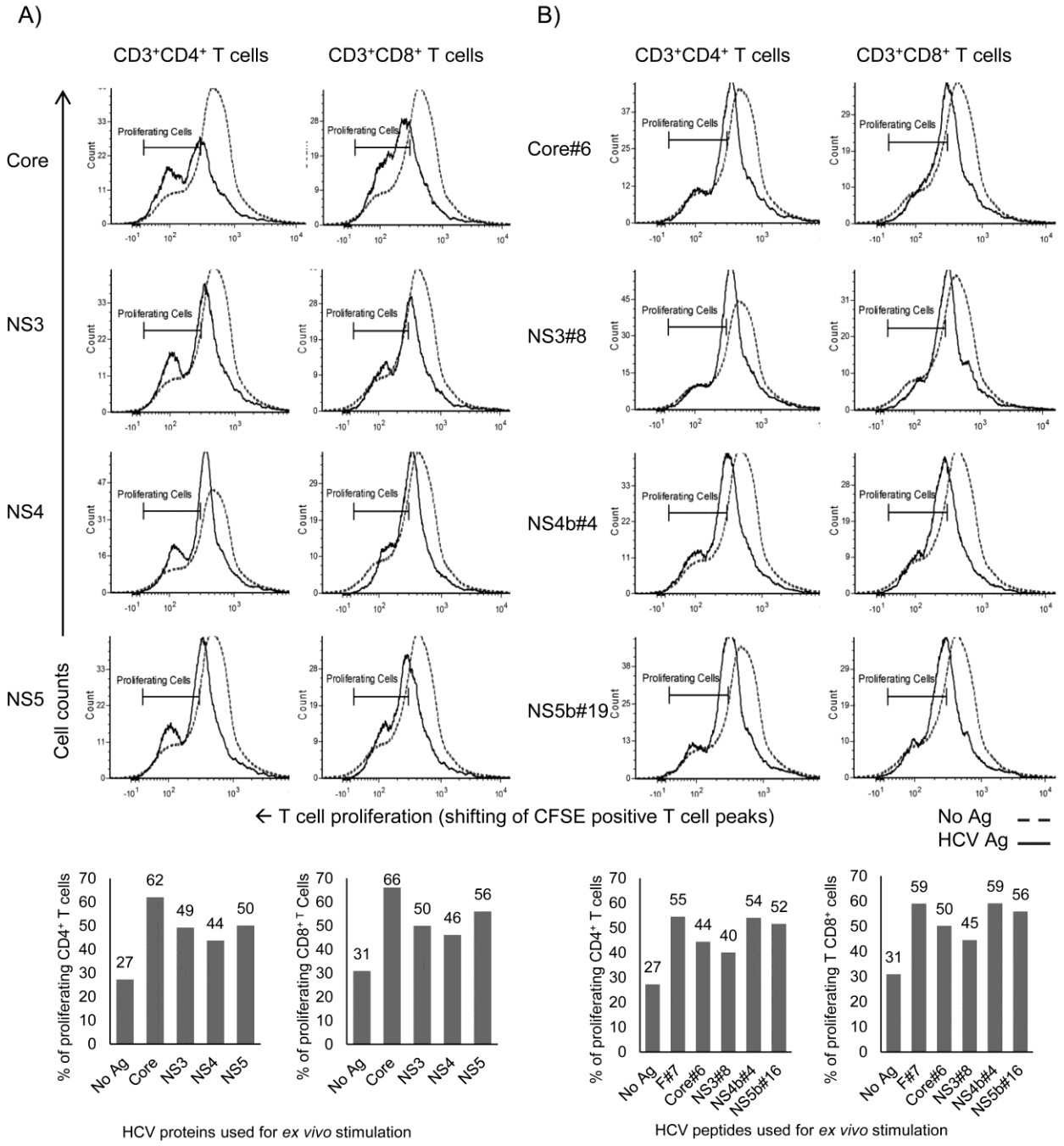

Fig 3.4. Cross-reactive CD4$^+$ and CD8$^+$ T cells obtained from Ad vector immunized mice proliferate in HCV antigens-dependent manner. Splenocytes obtained from Ad vector immunized mice were stimulated *ex vivo* with: **(A)** various recombinant HCV proteins: Core, NS3, NS4 and NS5; or **(B)** their selected respective peptides (at 5 µg/ml each), and analyzed in CFSE-based assay (loss of CFSE due to cell division represented by shift of peak of CFSE$^+$ T cells towards left) by flow cytometry. Data are obtained from a pool (n=5) of spleen cells and are representative of two independent experiments.

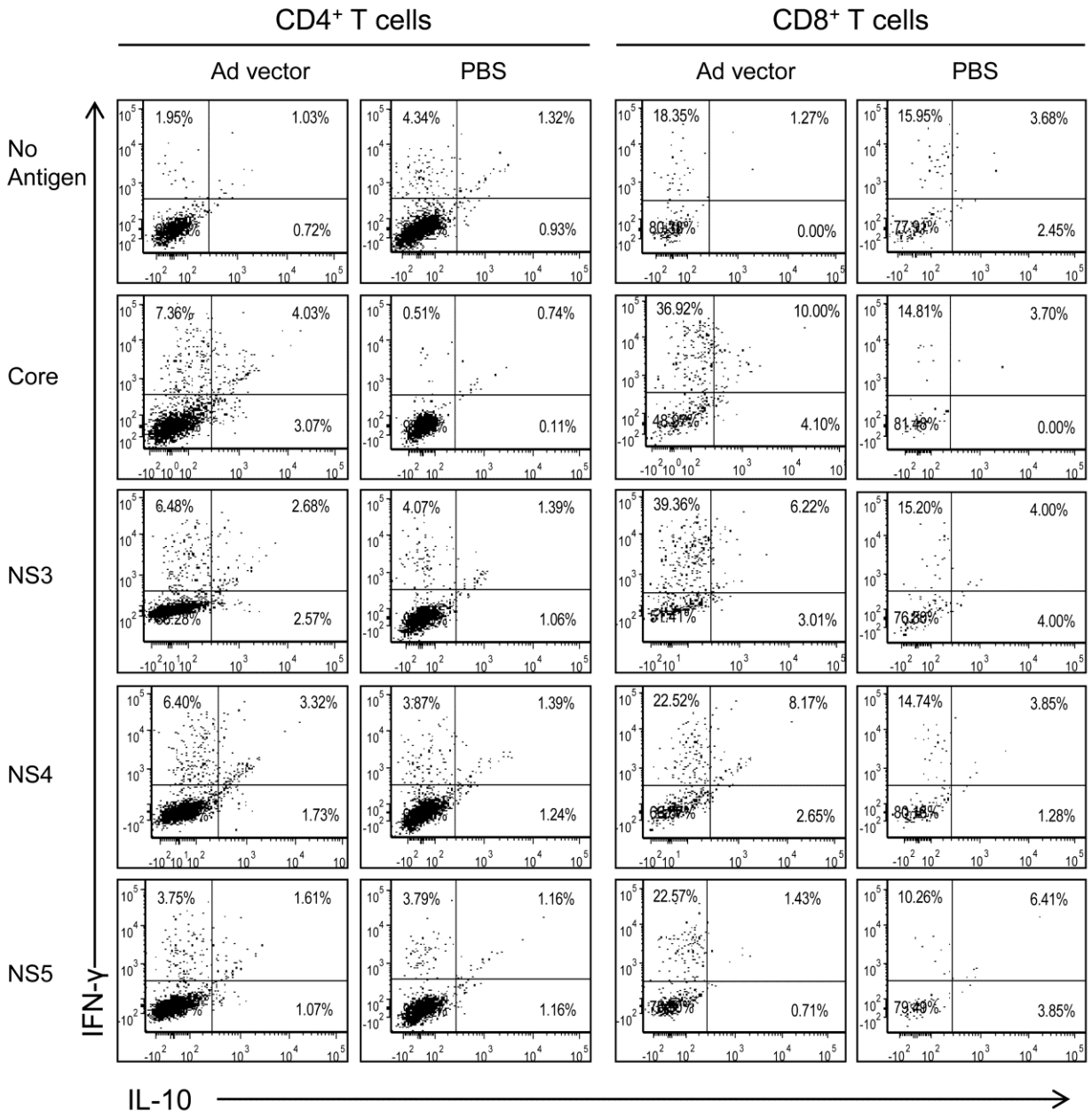

Fig 3.5. Cross-reactive CD4$^+$ and CD8$^+$ T cells obtained from Ad vector immunized mice produce cytokines upon *ex vivo* stimulation with various HCV proteins. Splenocytes obtained from Ad vector immunized mice were cultured with HCV Core, NS3, NS4 or NS5 antigens at 5 μg/ml, and analyzed after 5 days for intracellular IFN-γ and IL-10 expression profile of CD4$^+$ and CD8$^+$ T cells by flow cytometry. Data are obtained from a pool (n=5) of spleen cells and are representative of two independent experiments.

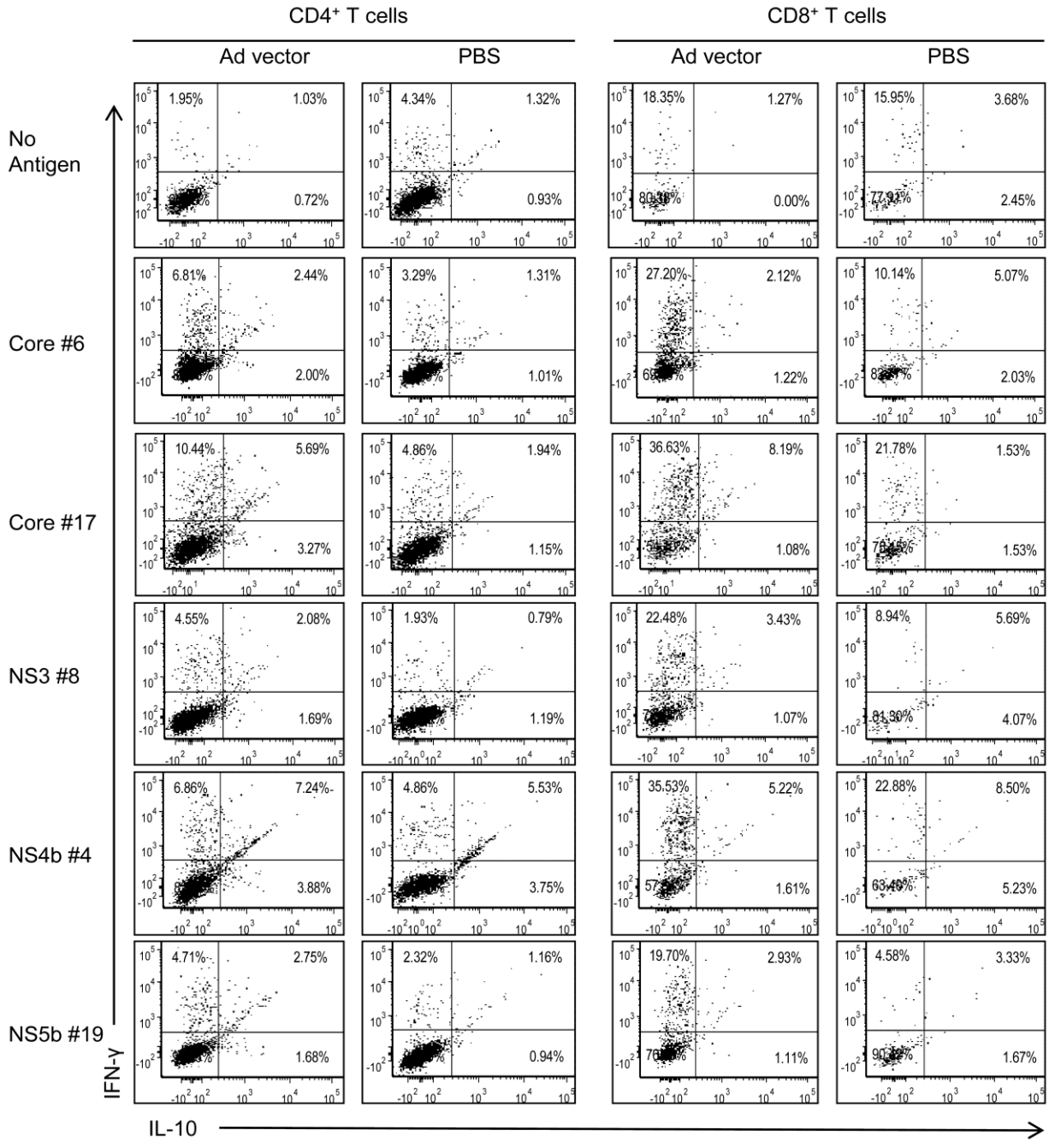

Fig 3.6. Cross-reactive CD4$^+$ and CD8$^+$ T cells obtained from Ad vector immunized mice produce cytokines upon *ex vivo* stimulation with HCV peptides. Splenocytes obtained from Ad vector immunized mice were cultured with representative peptides derived from HCV Core, NS3, NS4 or NS5 at 5 μg/ml each, and analyzed after 5 days for intracellular IFN-γ and IL-10 expression profile of CD4$^+$ and CD8$^+$ T cells by flow cytometry. Data are obtained from a pool (n=5) of spleen cells and are representative of two independent experiments.

We also examined the induction of cross-reactive IgG antibodies against HCV protein antigens (core, NS3, NS4 and NS5) in the serum samples obtained from immunized mice. The Ad vector induces significant amounts of cross-reactive IgG against all of the HCV antigens. Co-administration of TLR agonist poly I:C further enhanced the levels of cross-reactive antibodies. PBS-immunized mice did not show IgG binding to any of the HCV antigens (**Fig 3.7**).

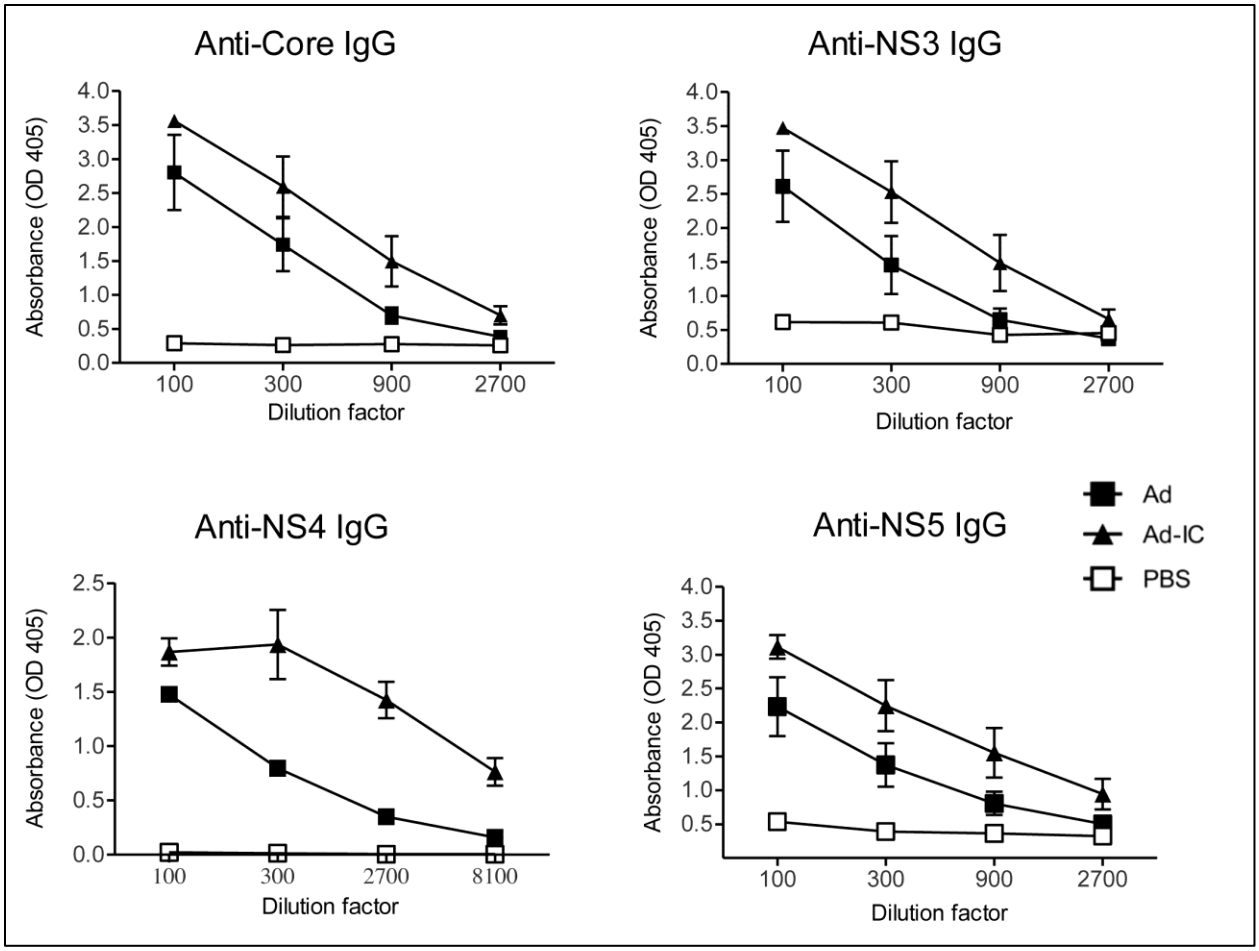

Fig 3.7. Cross-reactive antibody response against HCV core, NS3, NS4 and NS5 antigens in mice immunized with Ad vector (with or without poly I:C adjuvant). Data are presented as mean ± standard deviation of 3-4 replicates, and represent 3- 4 independent experiments.

3.3.4 Cross-reactive effector T cells induced upon immunization with Ad vector exert cytotoxicity to target cells loaded with HCV-derived peptides

The cytotoxic activity of the cross-reactive effector T cells obtained from the spleens of Ad vector immunized mice and stimulated *in vitro* with HCV protein antigens (core, NS3, NS4, NS5 or polyprotein) was examined against EL4 target cells loaded with pools of respective HCV antigen peptides. Different sets of CFSE-stained EL4 targets were prepared by loading them with pools of HCV antigen peptides: core (peptides # 2, 14, 17, 25, 27, 28, 32), NS3 (peptides # 8, 10), NS4 (peptides # 3, 4, 8), NS5 (peptides # NS5a: 1, 2, 16, 20 and NS5b: 5, 19, 23, 39) **(**listed in **Appendix 3-8**), all of the above peptides (ALL) or no peptide loaded (No). These different EL4 targets were cultured with corresponding effector T cells stimulated with HCV protein antigens. The results showed that T cells induced after Ad vector immunization had potent cytotoxic effector T cell function, in an antigen-specific manner (**Fig 3.8**). Interestingly, *ex vivo* stimulation of cross-reactive effectors with core antigen showed the least antigen-specific cytotoxic activity compared to other HCV antigens.

3.3.5 Role of the HCV-specific cross-reactive immune responses induced by Ad vector in reducing viral loads in Vaccinia-HCV challenged mice

To demonstrate the antiviral potential of Ad vector induced cross-reactive immune responses against HCV, a surrogate Vac-HCV infection model [32] was used. Although this is not a direct model of HCV infection, it is a valuable model to assess the ability of the induced immunity to kill host targets expressing HCV antigens. Reduction in Vac-HCV viral titers in this model is a critical parameter for the antiviral response against HCV. After two immunizations with Ad vector with or without poly I:C as adjuvant, the C57BL/6 mice were challenged with wild-type vaccinia (WT-Vac), or Vaccinia-HCV chimeric viruses (Vac-HCV core-NS3 or Vac-HCV NS3-NS4-NS5).

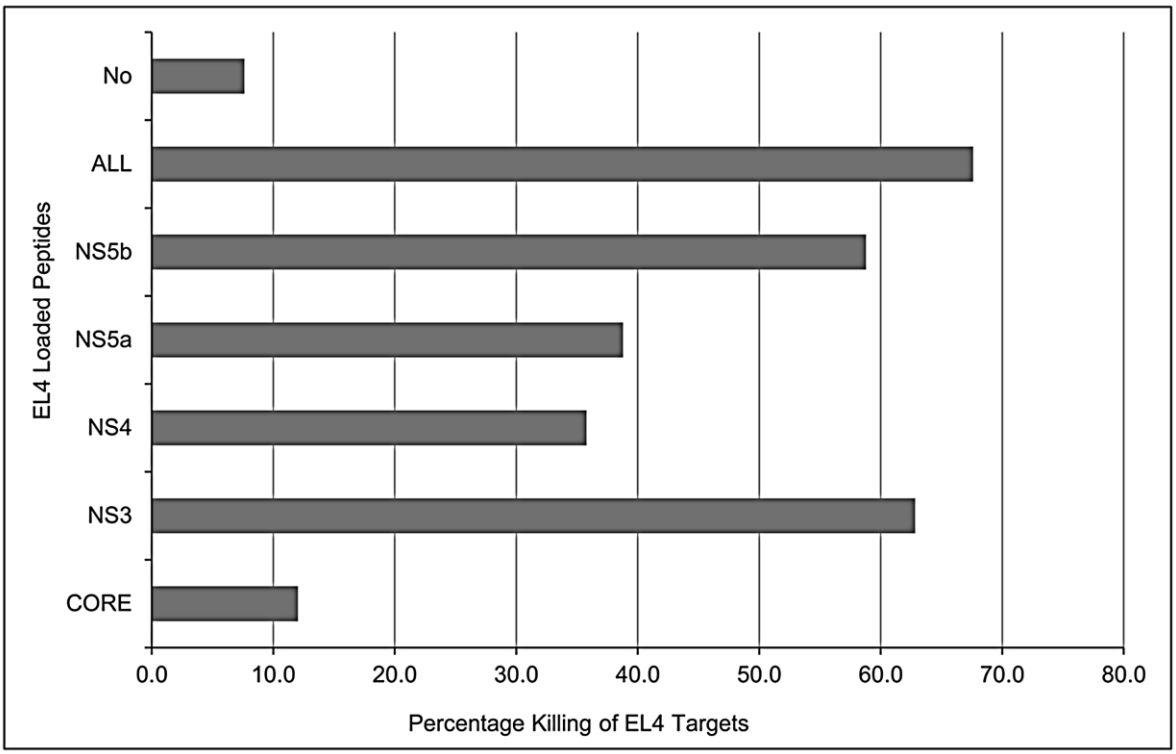

Fig 3.8. Cytotoxic killing of target cells loaded with HCV antigens-derived peptides, by cross-reactive effector T cells obtained from Ad vector immunized mice. Splenocytes harvested from Ad vector immunized mice, were stimulated *in vitro* with the HCV protein antigens Core, NS3, NS4 and NS5 at 5 μg/ml concentration for 4 days. The target EL4 cells were incubated with corresponding HCV peptides each at 1 μg/ml concentration (Core peptides: 2, 14, 17, 25, 27, 28, 32; NS3 peptides: 8, 10; NS4 peptides: 3, 4, 8; NS5a peptides: 1, 2, 16, 20 and NS5b peptides: 5, 19, 23, 39; or All: a mixture of the above peptides from Core, NS3, NS4 and NS5) and peptide-loaded EL4 cells were cultured with effectors at 10:1 (effectors: target) ratio for 4-5 hours. Empty (no peptide loaded) EL4 targets were used as a negative control. CFSE-labeled live targets were quantified by flow cytometry, and % killed targets were calculated using the formula: *% Killing = [(Average live cells in PBS control − live cells in immunized group) /Average live cells in PBS control] × 100)*. Data are representative of three independent experiments.

Five days after challenge, viral loads were determined in ovaries in individual mice (**Fig 3.9**). The mice immunized with Ad vector in the presence or absence of poly I:C and challenged with vaccinia chimeric viruses, had significantly ($p < 0.05$) reduced Vac-HCV (core-NS3 or NS3-NS4-NS5) viral loads in comparison to PBS or HEK cell lysate-immunized mice. In contrast, viral titers in WT-Vaccinia challenged mice were not significantly different in HEK lysate or Ad vector immunized mice ($p = 0.8472$). These results provide strong evidence that the Ad vector induced cross-reactive immunity against specific HCV antigens and not the vaccinia antigens.

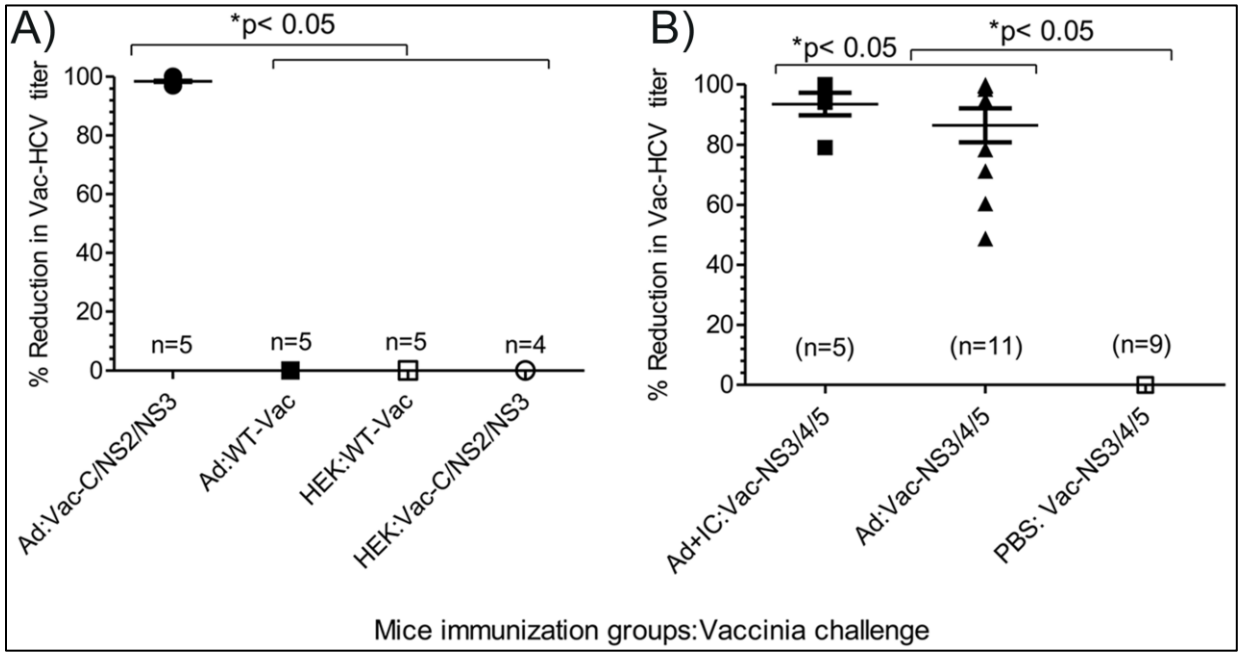

Fig 3.9. Immunization of mice with Ad vector leads to reduced titer of Vaccinia-HCV chimeric virus. Mice immunized twice intramuscularly with Ad vector (with or without poly I:C), PBS, or HEK cell lysate, were challenged 8 days after the second immunization with wild-type Vaccinia (WT-Vac) or chimeric Vaccinia-HCV. Ovaries were harvested 5 days after challenge and viral loads in each mouse were determined by plaque-forming assay using TK-1 cells. **(A)** Challenge with HCV Core-NS2-NS3 (Vac-C/NS2/NS3). **(B)** Challenge with Vaccinia-HCV NS3-NS4-NS5 (Vac-NS3/4/5). The x-axis legends represent various immunization groups (Ad, HEK or PBS) followed by type of vaccinia virus used for challenge (WT or Vaccinia expressing HCV core/NS2/NS3 or NS3/4/5 proteins). Data are presented as mean $\pm$ standard deviation of % reduction in viral titer compared to corresponding unimmunized control group, and statistical comparison was done by two-tailed *t*-test ($p<0.05$ was considered significant).

3.4 Discussion

In the present study, we demonstrate for the first time that Ad vector has the intrinsic ability to induce broad and robust cellular and humoral immune responses against a number of HCV structural and non-structural antigens. The *in silico* sequence alignment studies (**Table 3.3, Appendix 3-8**) exhibited an interesting and unexpected pattern of homologies in the amino acid sequences between HCV and Ad protein sequences. Homology ranged from 25-53% (homologies below 25% are not reported here), with HCV core peptide epitopes showing the greatest amino acid homology to the largest number of Ad proteins. Further, HCV frame-shift protein (F), NS3, NS4, and NS5 also showed significant levels of homology with Ad proteins.

Our results, presented in Figures **3.2 to 3.7**, provide conclusive evidence that Ad vector induced significant levels of cross-reactive immune responses against HCV antigens. These immune responses are mediated by both $CD4^+$ and $CD8^+$ T cells, and cover a broad range of HCV protein epitopes. We also showed that heterologous effector T cells, induced by Ad vector immunization, possess strong cytotoxic effector function (**Fig 3.8**).

Heterologous immunity is the term used when immune responses induced by one virus confer immunity to another unrelated virus and/or its antigens. Indeed, there have been reports showing induction of cross-reactive T cell responses among unrelated viruses [18, 35-37]. Adenovirus and hepatitis C virus are evolutionarily unrelated. They differ in host cell specificity and contain genomes of dsDNA and RNA, respectively. However, the induction of heterologous immunity does not require the entire T cell epitope to be homologous [38]. This immunity can result from similarities in amino acid sequences in small stretches of peptides and/or promiscuity of T cell receptors (TCR) [38-41]. It has been shown that only 2-4 amino acids in the T cell

epitopes actively participate in interaction with the TCR, and stronger associations in peptide-MHC and TCR is provided by co-stimulatory molecules and MHC molecules [41, 42]. Therefore, it is quite possible that a TCR will still recognize a peptide if its non-contact amino acid(s) are replaced with biochemically similar or even different amino acid(s) [38, 41, 42]. Further, MHC class I or II molecules can bind to and present a variety of peptides which differ in amino acid composition [38, 43, 44]. These properties of both MHC molecules and TCR are necessary to process and respond to a vast pool of epitopes originating from a huge number of pathogens and/or antigens [38, 41, 43, 45]. Various studies show that $CD4^+$ T cells are more promiscuous than $CD8^+$ T cells in peptide epitope recognition [42, 44, 46, 47]. Also the MHC class II peptide binding pocket is more flexible than the MHC class I peptide-binding pocket [44, 47]. A higher level of promiscuity occurs at the $CD4^+$ T cell level and flexibility within peptide-MHC II binding allows for greater and less-restrictive $CD4^+$ T cell responses. This property is physiologically relevant as $CD4^+$ T cell help is required to generate both humoral and effector T cell responses [39, 48]. Studies have also shown that if amino acids on a peptide epitope can be substituted by amino acids with similar biochemical property, it will be still recognized by the same T cell clone [44]. Additionally, some T cell clones could be intrinsically more cross-reactive than others. In outbred human population, the repertoire of peptide presentation and T cell recognition becomes even broader due to co-dominant expression of MHC molecules. Therefore, some individuals may show higher heterologous immunity against certain antigens than other individuals [49, 50].

An interesting aspect of Ad vector induced heterologous immunity emerged in responses to core antigen of HCV. We have previously reported that mice immunized with a recombinant Ad vector expressing whole HCV core protein do not generate core-specific T cell responses due to

immunosuppressive effects [29, 30]. In contrast, in the current study we unexpectedly found that an Ad vector without a transgene is able to induce robust core antigen specific humoral and cellular immune responses, due to a high degree of homology between multiple core-derived peptide sequences and various adenovirus proteins. This could be because adenovirus does not encode for a complete sequence of HCV-core but rather exhibits homologies in short peptide sequences, and therefore core-induced immune suppression is not apparent. Interestingly, however, core-stimulated effector T cells showed significantly less killing activity than other effectors (**Fig 3.8).** This might be due to core induced inhibition of granzyme B mediated killing activity of effector T cell upon *ex vivo* stimulation with the core antigen similarly as reported previously [51].

TLR agonists such as poly I:C engage their receptor and trigger innate immune signaling that results in the up-regulation of several co-stimulatory molecules and cytokines by antigen presenting cells and also T cells, enhancing antigen-specific responses. In our studies, co-administration of poly I:C along with Ad vector boosted the intrinsic heterologous immunity of Ad vector against multiple HCV antigens, further endorsing the Ad vector mediated induction of heterologous immunity against HCV (**Fig 3.2, 3.7** and **3.9**).

Humoral immune response is mediated by B cells, which produce antibodies against the pathogen-derived antigens. Antibodies can recognize a linear peptide epitope or a conformational epitope [52, 53]. The cross-reactive antibody response is expected to be contributed by the linear peptide epitopes, which contain identical or biochemically similar amino acids at the Fab' binding site(s) [53]. Therefore, as HCV and Ad vector proteins show a high level of homology at different sites in their proteins, the probability of cross-reactive Ab-binding epitopes is high

and our data suggest that antibodies produced in response to Ad vector immunization also show cross-reactivity with HCV antigens. This was further enhanced upon co-immunization with poly I:C (**Fig 3.7**). These results provide an impetus to modify the diagnosis of HCV infection entirely from serology to PCR based methodologies.

In addition to inducing $CD4^+$ and $CD8^+$ T cell and antibody responses, our data clearly demonstrate the antiviral potential of heterologous immunity induced by Ad antigens. It can significantly reduce the viral load in Vac-HCV challenged mice, but not in wild-type vaccinia challenged mice (**Fig 3.9 A**), and the use of an adjuvant with Ad vector further enhances the cross-reactive antiviral immunity (**Fig 3.9 B**). Whether cellular and/or humoral arms of cross-reactive immunity confer viral reduction in an advanced model of HCV; remains to be determined.

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Chapter 4

Humans with Pre-Existing Adenoviral Immunity Show HCV-Specific Cross-reactive Immunity

A version of this chapter has been submitted for publication (***Shakti Singh, et al. 2015***).

4.1 Introduction

Self-resolving cases of HCV infection are generally associated with the early appearance of broadly neutralizing antibodies and broadly targeting sustained T cell immune responses [1-4]. However, in majority of cases, both humoral and cellular immune responses are weak and dysfunctional; and results in lifelong persistent chronic infections [5, 6]. Many cases of chronic HCV infection have been reported where patients cleared the virus infection due to spontaneous reversal of exhausted and/or dysfunctional T cell immune responses to functional T cell responses [4, 7]. In several studies, HCV-specific T cells have been expanded from the peripheral blood T-cell populations in individuals previously not infected with HCV [8, 9]. Several mechanisms behind the expansion of HCV specific $CD8^+$ T cells from HCV naïve individuals and the reversal of immune responses in cases of chronic HCV infection have been postulated [10, 11]. One possible mechanism could be the presence pre-existing memory B and T cells or generation thereof by natural infection with some other un-related pathogen(s) [12, 13]. Influenza A virus infection has been shown to induce narrow cross-reactive or heterologous $CD8^+$ T cells against NS3 antigen of HCV [9]. We also discovered that adenoviral proteins show extensive amino acid sequence homologies (25-53%) with multiple peptides derived from HCV proteins, and empty Ad vector (with no HCV antigens) can induce robust HCV cross-reactive antibody and T cell immune responses (Chapter 3). In outbred human population, the repertoire of peptide presentation and T cell recognition becomes even broader due to co-dominant expression of MHC molecules. Therefore, some individuals may show higher heterologous immunity against certain antigens than other individuals [14, 15]. This explanation may be further supported by the fact that such HCV-specific T cell responses were eliminated when

memory T cells were depleted from peripheral blood mononuclear cells (PBMCs) of HCV naïve subjects [16].

Our study further establishes the presence of HCV antigen specific cross-reactive immunity in individuals who never experienced HCV infection, and also corroborates our previous findings in mice (Chapter 3). In this study, we show that normal healthy humans with no known history of HCV infection but seropositive for Ad-specific IgG, demonstrates the presence of cross-reactive antibodies and T cell responses against multiple HCV antigens. The cross-reactive immune responses play dual role at the time of infection. They can influence the dominance of certain T cell repertoire and subsequently affect the breadth of T cell specificities and outcome of the HCV infection either positively or negatively [17].

4.2 Materials and Methods

4.2.1 Human Blood Donors, Plasma and PBMCs

Peripheral blood samples were collected from healthy human donors (aged 23 to 55) of both sexes with no known history of HCV infection. Collected blood was processed immediately for PBMCs isolation using Ficoll gradient separation. The upper plasma layer was frozen in aliquots for IgG ELISA, and the middle PBMCs layer was collected, washed and frozen until use (**Fig 4.1**). All procedures were approved and were in accordance to the University of Alberta Human Research Ethics Board (HREB).

4.2.2 Human IgG Antibody ELISA

Anti-Ad IgG (hexon-specific) antibodies were determined in the plasma of each donor using IBL Adenovirus IgG ELISA Kit (Cat# IB79202, IBL-America Inc. Minneapolis, MN, USA). Plasma samples were assayed at 1:100 dilutions in duplicates according to the manufacturer's

procedure. HCV antigens-specific cross-reactive IgGs were determined by ELISA. Briefly, ELISA plates were coated with recombinant HCV antigens: Core NS3, NS4, NS5 or control protein SOD (superoxide dismutase) at 1 µg/ml overnight at 4°C followed by blocking with 1% BSA (bovine serum albumin) for 1 hour. Plasma samples (1:100 to 1:400) were added in triplicate and incubated for 2 hours at room temperature. Binding of antigen-specific Human IgG was detected by adding anti-human IgG labeled with alkaline phosphatase (AP) (Cat# 2040-04, Southern Biotech, Alabama, USA) for 1 hour, followed by addition of PNPP substrate (Cat#0201-01, Southern Biotech, Alabama, USA) for 30 minutes. Plates were washed with 1x PBST (1x PBS with 0.1% Tween-20) after each incubation step. Absorbance (OD) was measured at 405 nm, and corrected for background by subtracting the OD values obtained in control SOD-coated plates. Absorbance was measured using a FluoStar Optima ELISA Reader (BMG Labtech GmbH, Ortenberg, Germany).

4.2.3 *Ex Vivo* Stimulation of Human PBMCs and Intracellular Cytokine Staining

Frozen PBMCs were thawed and washed with warm PBS twice, counted and cultured in AIM-V serum free medium (Cat# 12055-083, Gibco by Life Technologies, NY, USA) in 96-well plate at $0.5x10^6$ cells/100 µl/well for 2 hours. HCV pooled synthetic peptides (Core peptides # 5, 14, 16, 17 & 27; NS3 peptides # 2, 5, 6, 8 &10; NS4 peptides# 4, 8, 9, 13, 16 or NS5 peptides# NS5a: 6, 24 + NS5b: 5, 19, 27) or individual proteins (Core, NS3, NS4 or NS5) were added to the plates at 5 µg/ml concentration (**Appendix 3-8**). The peptides were selected on the basis of their high sequence homologies with Ad proteins, and detection of cross-reactive responses in mouse experiments. Phyto-hemagglutinin (PHA, 1 µg/ml) and media-treated cells were considered as positive and negative controls, respectively. After three days of incubation, cells were treated with 1.5 µg/ml brefeldin A (Cat. # 00-4506, eBiosciences Inc., San Diego, CA,

USA) for 5 hours, and stained for extracellular lineage markers using fluorochrome-labeled antibodies (purchased from eBiosciences Inc. San Diego, USA): CD3 (eFluor 450, Cat. # 48-0036), CD4 (FITC, Cat.# 11-0049) and CD8a (PE, Cat.# 12-0089); and for intracellular cytokines: IFN-γ (APC, Cat.# 17-7319) and IL-10 (PE-Cyanine 7, Cat.# 25-7108). Stained cells were examined in a BD FACS Canto, and data were analyzed using FACS Diva software. The $CD3^+$ T cell population was gated for $CD4^+$ and $CD8^+$ subsets and evaluated separately for IFN-γ and IL-10 expression. PBMCs stained with isotype antibodies with corresponding flurochromes (Cat. # 48-4714, 11-4714, 12-4714, 17-4714 and 25-4301; purchased from eBiosciences, Inc. San Diego, USA) were used to exclude non-specific binding. Percent increase in cytokine expression was calculated by the formula: [(Antigen treated cells- media control)/ media control] x 100. The percentage of cells positive for IFN-γ, IL-10 and IFN-γ/IL-10 in the media groups ranged from 0.2-10% in different donors.

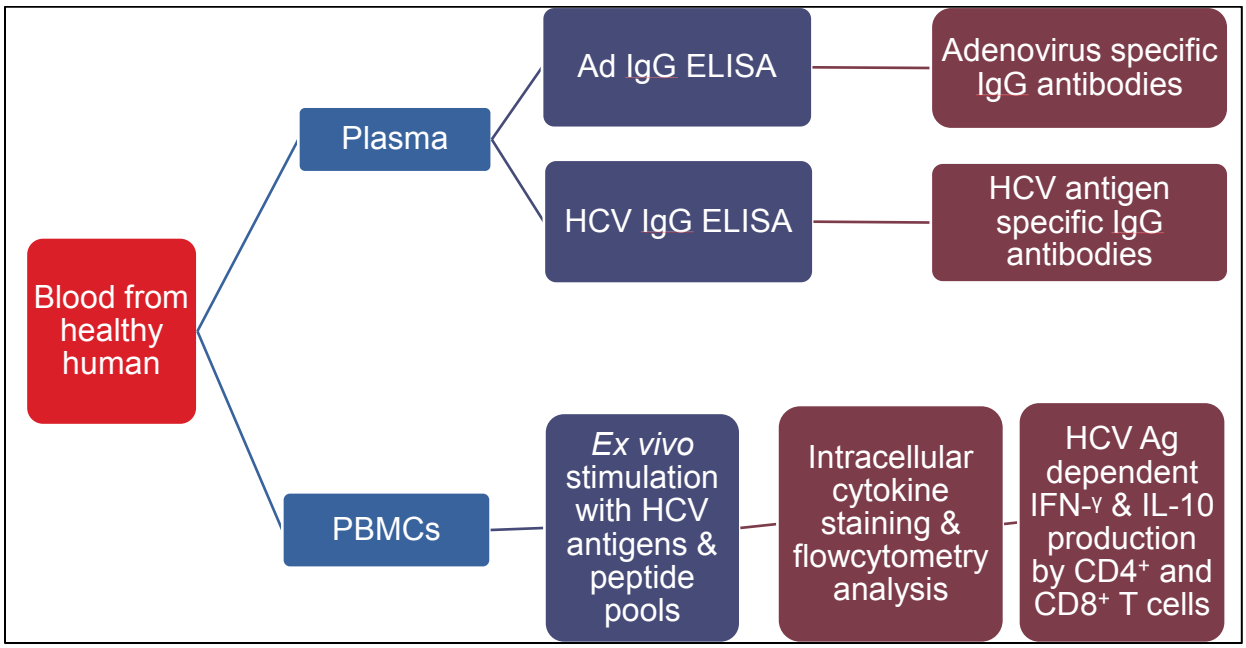

Fig 4.1. Experimental outline of the study of HCV specific heterologous immunity in healthy humans

4.2.4 Statistical Analysis

Correlation coefficients were calculated by Spearman's rank correlation for non-normally distributed data points. Data were analyzed by Graph-pad Prism software (Graph-pad Software Inc., CA, USA). Correlation coefficients, $r \leq 0.35$ were considered to represent weak correlations, 0.36 to 0.67 moderate correlations, and 0.68 to 1.0 strong correlations [18]. *P*-values less than 0.05 (<0.05) were considered to be statistically significant.

4.3 Results

4.3.1 Human healthy donors enrolled in the study were seropositive for Adenovirus

Healthy humans were screened for pre-existing Ad immunity using Ad hexon antigen based IgG ELISA system. Plasma samples collected from the blood of healthy donors were evaluated for Ad specific IgG antibodies, and the mean OD values obtained in Ad IgG ELISA were arranged in their ascending order and plotted in graph. Data show that all of the blood donors were seropositive for Ad virus and had significant levels of Ad specific IgG antibodies (**Fig 4.2**).

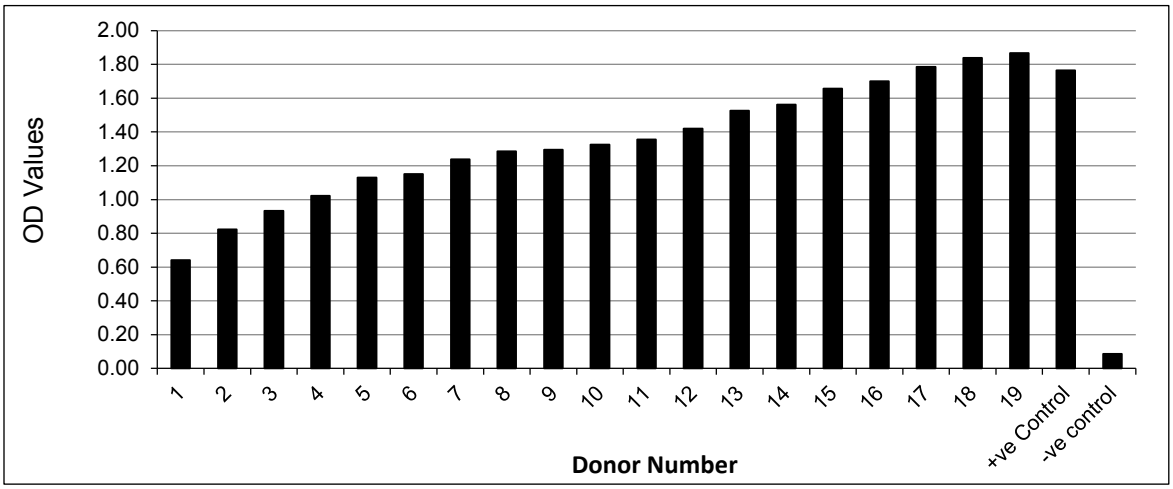

Fig 4.2. Human healthy donors enrolled in the study were seropositive for Adenovirus. Ad - specific IgG antibodies were evaluated by ELISA. Donors are arranged in increasing order of mean OD values. Data are presented as mean ± standard deviation of 2 replicates.

4.3.2 Ad-seropositive human donors have cross-reactive humoral immune responses against HCV antigens which correlate with Ad specific immunity

HCV-antigen specific cross-reactive IgGs were detected and evaluated by ELISA in healthy human blood donors (n=19). The results show that cross-reactive IgG antibodies against HCV antigen core, NS3, NS4 and NS5 were present in majority of HCV-naïve donors at varying levels (**Fig 4.3**). Further, to understand the induction of cross-reactive immunity in HCV-naïve humans, we arranged Ad IgG ODs with matched ODs from corresponding HCV (core, NS3, NS4 or NS5) IgG in ascending order and plotted in scatter graphs. Further, we calculated the correlation coefficients between Ad IgGs and cross-reactive HCV core, NS3, NS4 or NS5 IgGs. There was a significant direct correlation between the presence of Ad-specific IgG with HCV NS3 (Spearman r= 0.500, *p* value = 0.029) and NS4 (Spearman r=0.486, *p* value = 0.035) (**Fig 4.4, II and III**). We also detected cross-reactive IgG against HCV core and NS5 antigens; however the correlations between these antibodies with the Ad hexon-specific IgG (**Fig 4.4, I and IV**) were not statistically significant. A very high percentage of false HCV antibody positive cases have been reported in several studies, where the donors were negative for HCV RNA in their blood and also did not have any known history of HCV infection [19-21]. The presence of anti-HCV antibody without viremia was shown in 72.7% of asymptomatic subjects [22]. Interestingly, Influenza vaccine also has been reported to induce false-positive ELISA for HCV in a number of cases [23]. The induction of HCV antigen specific cross-reactive antibodies upon Ad vector immunization (**Chapter 3**) and detection of HCV specific antibodies correlating with the pre-existing Ad specific immunity in healthy HCV naïve individuals, together can explain the cases of high number of false positive results in HCV antibody based diagnostic assays.

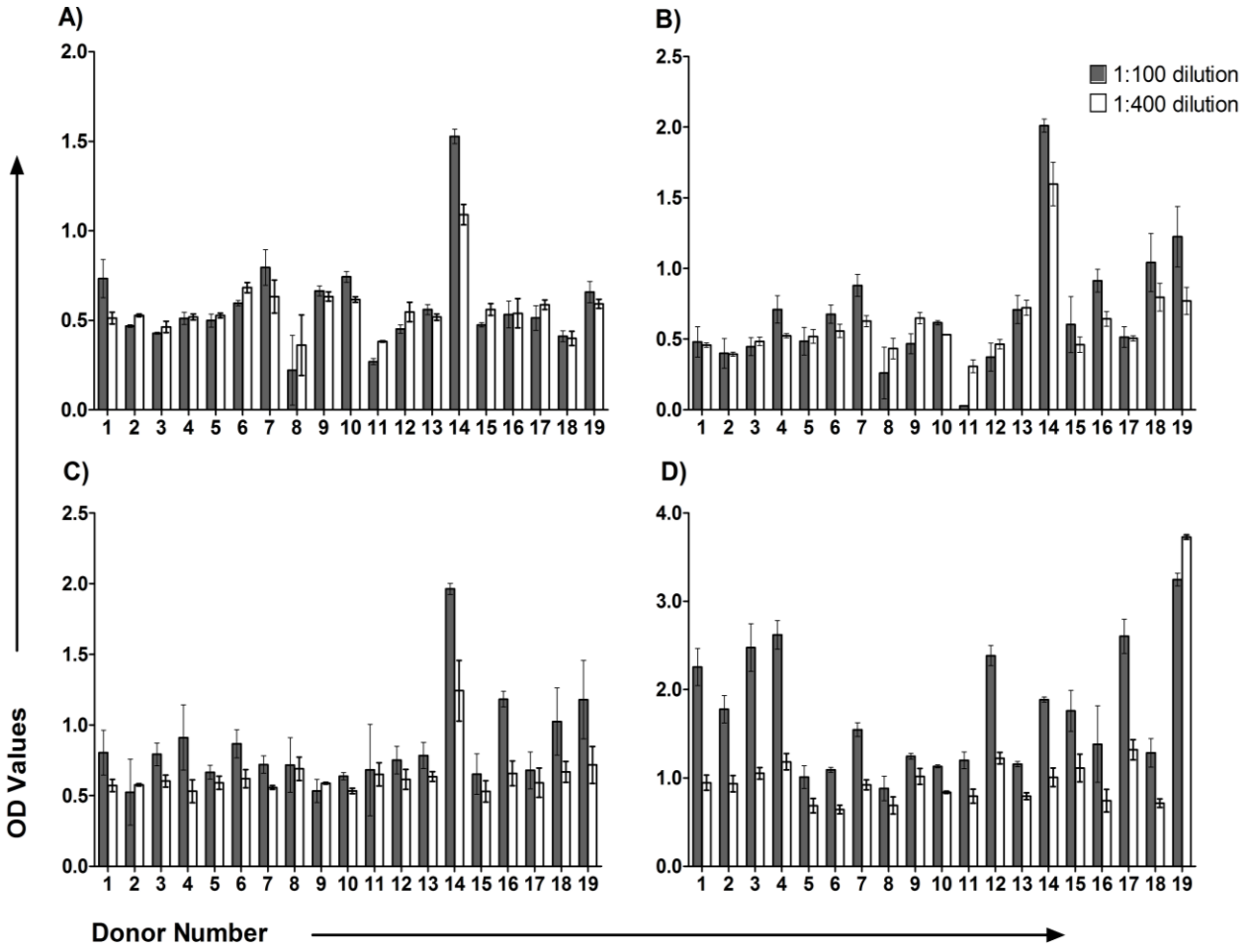

Fig 4.3. Ad-seropositive human donors demonstrate cross-reactive humoral immune responses against HCV antigens. HCV Core (A), NS3 (B), NS4 (C) or NS5 (D) antigen specific IgG antibodies were evaluated in plasma samples prepared from the Ad seropositive healthy blood donors (n=19) by ELISA. Data are plotted as mean ± standard deviation of 3 replicates after SOD antigen OD values subtraction.

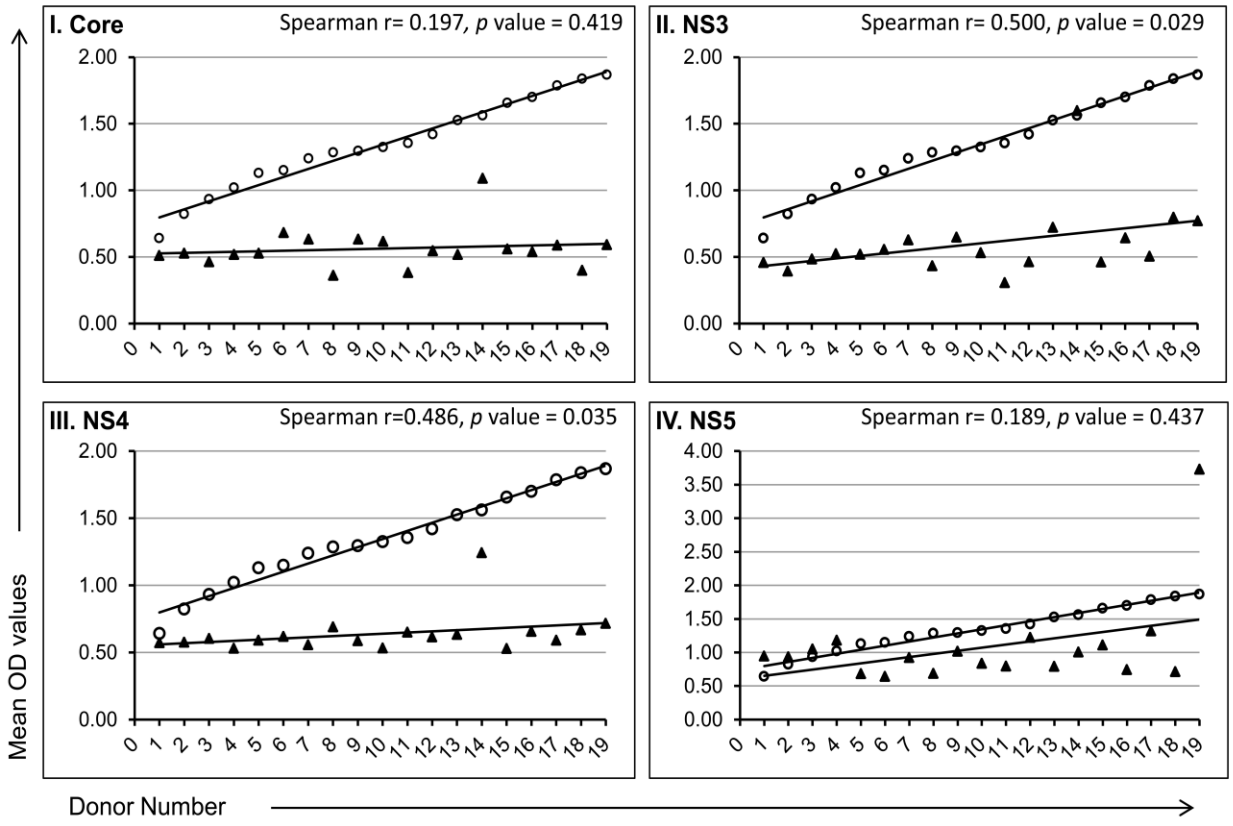

Fig 4.4. Cross-reactive humoral immune responses against HCV antigens correlate with Ad specific immunity in HCV naïve healthy donors. OD values for Ad-specific IgG (open circles) from 19 healthy donors were arranged in ascending order and matched with corresponding OD values for IgG against HCV core, NS3, NS4 or NS5 antigens (solid triangles). The Spearman coefficients for correlation were calculated for the non-normally distributed population.

4.3.3 Humans with pre-existing Ad immunity express IFN-γ and IL-10 in $CD4^+$ and $CD8^+$ T cells in HCV antigen(s) dependent manner

We also determined the intracellular expression of IFN-γ and IL-10 in $CD4^+$ and $CD8^+$ T cell subsets upon *ex vivo* stimulation of PBMCs with HCV peptide pool or protein antigens for 4 days. Interestingly, both $CD4^+$ and $CD8^+$ T cells from these healthy donors (n=17, two donors did not provide enough blood samples to isolate PBMCs) demonstrated induction of HCV antigens-specific IFN-γ and/or IL-10 (**Fig 4.5**). Overall, there was a propensity of IL-10

induction by $CD4^+$ T cells upon stimulation with peptide pools from all four HCV antigens and NS3 and NS4 protein antigens. However, a number of donors showed increased IFN-γ and IFN-γ/IL-10 expression in both $CD4^+$ and $CD8^+$ T cells upon stimulation with HCV antigens (**Fig 4.5**).

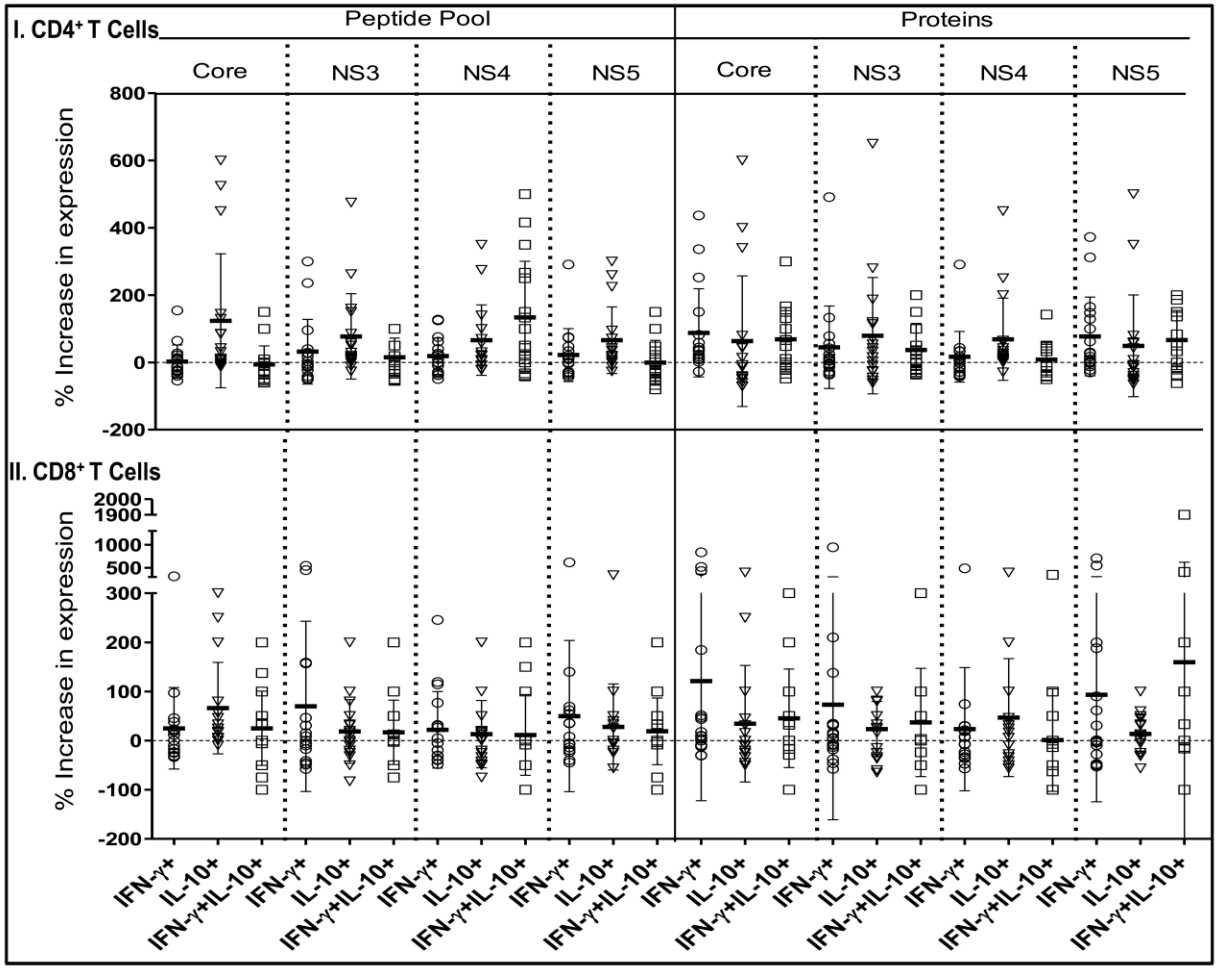

Fig 4.5. Humans with pre-existing Ad immunity express IFN-γ and IL-10 in $CD4^+$ and $CD8^+$ T cells in HCV antigen(s) dependent manner. Expression of IFN-γ and IL-10 in $CD4^+$ and $CD8^+$ T cells upon *ex vivo* stimulation with HCV peptide pools or protein antigens. Data are shown as the percent increase in expression of IFN-γ, IL-10 and IFN-γ/IL-10 in an HCV antigens-dependent manner compared to controls (media). Data for each donor and data points from all donors (n=17, two donors in the cohort did not provide enough blood samples to do cellular assays) are plotted in stacked dot plots as mean ± SD.

4.4 Discussion

Adenoviruses commonly infect humans, and also have been shown to exist persistently as normal gut flora [24]. However, these humans demonstrate high level of immunity against a number of adenoviral proteins in general population. We observed that all of the healthy HCV-naive human donors (n=19) enrolled in this study, were seropositive for anti-Ad IgGs. Further, we sought to investigate HCV-specific cross-reactive immune responses in these human blood donors. We detected varying levels of HCV core, NS3, NS4 and NS5 cross-reactive IgG antibodies (n = 19) in these individuals. Further, we could see moderate but significant direct correlations between Ad specific IgG and HCV antigen specific cross-reactive IgG antibodies, against HCV NS3 and NS4 antigens.

We hypothesized that if cross-reactive $CD4^+$ and $CD8^+$ T cells are present in the PBMCs of these healthy HCV-naïve individuals, they should secrete IFN-γ and/ or IL-10 upon culture with HCV peptide pools or complete protein antigens. Interestingly, in the majority of donors, $CD4^+$ T cells showed higher induction of IL-10 compared to IFN-γ upon stimulation with different HCV antigens, and only a minority of donors (~17 % or 3 out of 17) showed higher levels of IFN-γ expression compared to IL-10 in both $CD4^+$ and $CD8^+$ T cells. These results represent the existence of two discrete courses of natural cross-reactive cellular immune responses against HCV antigens in humans with pre-existing Ad-specific immunity. The reasons for these two courses are not clear and could be dependent upon HLA type, T cell repertoire and/or environmental factors. However, it can be speculated that exposure of such individuals to HCV may also lead to distinct patterns of immune responses culminating in either acute/self-clearing or chronic infections, with a predisposition towards the development of chronic HCV infection in the majority of cases. These results are intriguing and suggest the potential impact of Ad

immunity on the natural course of chronic vs. acute/clearing HCV infection, albeit with a small number of individuals. Prospective immunological studies in a large cohort of individuals will be required to fully predict and correlate Ad immunity to viral clearance vs. persistence upon exposure to HCV.

The heterologous immunity can modulate the breadth of the T cell repertoire and/or the immune-dominance of a specific epitope, which results in increased or decreased immunity to the infecting pathogen [17, 25, 26]. Further, due to high prevalence of adenoviruses, general population may have heterologous immunity to HCV antigens, and that can explain the cases of HCV-specific memory T cells and high prevalence of HCV antibodies in humans with no evidence of HCV infection. We speculate that HCV cross-reactive T cells also play a role in those individuals who fail to induce effective cellular immune responses against HCV infection and develop chronic infections. Recombinant adenovirus vectors are at the forefront as candidate vaccines for several different pathogens including HCV, Ebola virus, Plasmodium, mycobacteria, influenza virus, among others. Rare human and non-human adenoviruses are being developed for Ad vector-based vaccines, to avoid the issues of pre-existing immunity. In fact, these strains of adenoviruses showed a significant level of sero-prevalence in human population [27]. Further, adenovirus-specific T cell immunity against conserved epitopes has been shown across various human serotypes and also across species [27]. Further, this strategy will not completely rule out any pre-existing inter- and intra-species cross-reactive anti-adenoviral immunity. We observed that the homology between HCV epitopes and different adenoviruses (such as chimpanzee Ad) spans across a number of adenoviral proteins (Chapter 3), and thus it can possibly be extended to a number of uncommon human and non-human adenoviruses.

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Chapter 5

General Discussion

In this thesis, I have characterized adaptive HCV-NS4 antigen specific immune responses after immunization with non-replicative recombinant Ad vector expressing HCV NS4 protein. I also examined the effect of route and dose of Ad vector immunization on induction of transgene (HCV NS4) specific immune responses and their subsequent role in viral antigen clearance using a surrogate chimeric vaccinia-HCV (Vac-HCV) mouse infection model. Further, we discovered an unusual and interesting behavior of empty Ad vector, which induced HCV cross-reactive immune responses in mice. Upon further investigation, I found that Ad proteins contain extensive homologies with various HCV protein derived peptide epitopes. These observations led us to investigate the Ad vector induced HCV cross-reactive immune responses in detail in both mice and humans. In mice, we demonstrated that Ad vector alone can induce potent, broad anti-HCV cross-reactive immunity that can significantly reduce viral load upon challenge with infectious Vac-HCV. Further, we also detected HCV cross-reactive antibodies and HCV antigen dependent expression of cytokines (IFN-γ and IL-10) in T cells of a cohort (n=17) of HCV naïve but Ad-immune human individuals. Previous studies also have reported that one pathogen can induce cross-reactive immunity against an unrelated pathogen. This kind of immunity is known as heterologous immunity.

Heterologous immunity is a double-edged sword, which can modulate the breadth of the T cell repertoire, influencing the memory T cell pool and/or the immune-dominance of a specific epitope, leading to enhanced or diminished immune responses against a pathogen. These observations can help in the understanding of immunopathogenesis and disease outcome of HCV infection and also in designing better HCV vaccine.

5.1 Recombinant Ad vector expressing HCV NS4 protein induces protective immunity which critically depends upon dose and route of immunization

Studies have shown that strong multi-specific cellular immune responses against various non-structural proteins of HCV play a critical role in the protection against HCV infection [1-4]. Rapid induction of virus neutralizing antibodies also has been correlated with the resolution of virus infection [5-8]. Moreover, protective and neutralizing potentials of these HCV specific antibodies have been proven in several studies *in vitro* and in chimpanzees [2, 9-12]. Apart from virus neutralization, antibodies also have several effector functions that help in clearing a pathogen from host [13]. Antibodies also have been detected against several nonstructural antigens of HCV antigens [14], which may play an important but yet undetermined role in immune responses against HCV. Therefore, various vaccine development strategies are focused on induction of both humoral and cellular immune responses against multiple (structural and non-structural) proteins of HCV [15, 16].

In this study, we examined the immune responses against HCV NS4 antigen, and their role in the clearance of Vac-HCV infection in C57BL/6 mice. We used human adenovirus 5 derived vector expressing HCV NS4 protein (Ad-NS4) to immunize the mice. Adenoviral vectors can efficiently deliver the vaccine antigens, and also induce robust transgene specific immune responses due to their intrinsic immuno-modulatory properties [17, 18]. In humans, pre-existing immunity against adenoviruses negatively impact the immune responses against transgene antigens delivered by these vectors. Moreover, dose and route of immunization with Ad-vector also influence the quality and quantity of transgene antigen specific immune responses. Several strategies have been explored to overcome these problems. In one such strategy, rare human and non-human adenoviruses have been developed as vectors, which have less pre-existing immunity in human population. However, a recent study have found significant sero-prevalence rate of

these adenoviruses, showing 12% of human subjects seropositive for chimpanzee Ad virus 3, 22% seropositive for rare human Ad virus 6 and 38% seropositive for human Ad virus 5 [19]. Moreover, use of rare adenoviruses as vaccine vectors may not completely rule out any pre-existing inter- and intra-species Ad-specific cross-reactive immunity. In our study, both route and dose of Ad-NS4 immunization showed a significant influence on the induction of HCV NS4 specific protective immunity. We found that an optimum dose of Ad-NS4 administered twice through intramuscular route induces very strong cellular and humoral immune responses which are able to significantly reduce the viral load in Vac-HCV challenged mice. In contrast, high dose intra-peritoneal immunization with Ad-NS4 induced high vector-neutralizing antibodies, which dampened the NS4 antigen specific immune responses and resulted in reduced viral clearance. In conclusion, we demonstrated that Ad-NS4 can induce protective immune responses, which is critically affected by the route and dose of immunization. Inclusion of other HCV antigens in the Ad vector can further broaden these protective immune responses, and can be used as a candidate vaccine against HCV. The effective dose and route of administration of such vaccines should be determined carefully.

5.2 Ad vector derived proteins have extensive amino acid homology with peptide epitopes derived from multiple HCV proteins and induce strong HCV specific heterologous immunity

We all are exposed to vast number of pathogens, and have varying levels of memory B and T cells against them. However, our immune system is equipped with limited antigen epitope recognizing receptors, and cannot have separate B or T cell receptors for each epitope. Therefore, these receptors show some level of flexibility and degeneracy in binding with their antigen epitope. Studies have shown that a single T-cell receptor can recognize quite disparate

peptides [20, 21]. Due to these phenomena, one virus can induce cross-reactive immune responses against other evolutionarily distant virus. The propensity of such immune cross-reactivity among viruses can be further enhanced by the presence of conserved amino acid sequences, which frequently interact with the nucleic acid and various host factors required for establishment of successful infection and replication [22-25]. Several studies have shown induction of cross-reactive immune responses between unrelated viruses for example HCV and influenza virus. [26-31].

In our study, surprisingly we discovered that empty adenoviral vector (with no HCV antigen) can induce broad cross-reactive immune responses against multiple HCV proteins. This finding was further supported by the fact that adenovirus proteins contain extensive regions of homology with multiple peptides derived from various HCV proteins. Further, the mice immunized with empty Ad vector significantly reduced the Vac-HCV load. Therefore, Ad vector may have a potential use as therapeutic/prophylactic vaccine for HCV infection, even without an additional HCV transgene. Further, we observed that the homology between HCV epitopes and different adenoviruses spans across a number of adenoviral proteins which have conserved domains across different adenoviruses, and thus it can possibly be extended to a number of uncommon human and non-human adenovirus derived vectors [19].

5.3 HCV antigen specific cross-reactive immunity in HCV naïve and Ad-immune healthy human blood donors

Adenoviruses usually cause mild asymptomatic infection in upper respiratory tract in humans. Most people experienced adenoviral infection in their life, and have different levels of memory B and T cell immune responses. In our study, we screened 19 healthy human blood donors who never had HCV infection. We could see adenovirus specific antibodies in all 19

donors. Next we sought to investigate whether these Ad immune donors demonstrate the cross-reactive immune responses to various HCV antigens or not. We found that Ad-immune individuals demonstrated the presence of anti-HCV specific humoral (IgG) responses against multiple HCV antigens. Interestingly, anti-NS3 and anti-NS4 IgG showed a moderate correlation with Ad hexon-specific IgG.

Furthermore, all of the Ad-immune individuals demonstrated HCV antigens-dependent induction of cytokines (IL-10, IFN-γ or IL-10/IFN-γ) in $CD4^+$ and $CD8^+$ T cells. However, the majority of cross-reactive T cell stimulation resulted in $CD4^+$ T cells with IL-10 production (n=14/17) and only a minority with IFN-γ production (n=3/17 donors) against multiple HCV antigens. These results support our conjecture observed in mice study (Chapter 3). Further, these cross-reactive immune responses can explain the cases of HCV-specific memory T cells in HCV-naïve individuals, and also explain spontaneous re-activation of HCV-specific functional immune responses and viral clearance. It also suggests that pre-existing Ad immunity cross-reactive to HCV may be playing an important role in the natural course of chronic vs. acute/clearing of HCV infection.

5.4 Conclusions

Adenoviral vectors no doubt have many advantages as vaccine antigen delivery vehicle due to their intrinsic adjuvant properties and efficient antigen delivery. Our studies have focused on understanding of the role of adaptive immune responses against non-structural protein (NS4) of HCV in viral clearance. We have conclusively shown that Ad vector expressing HCV antigen can induce protective humoral and cellular immune responses. Further, the problem of pre-existing Ad vector-specific immunity can be avoided and transgene specific immune responses can be further optimized by utilizing suitable dose and route of immunization. Further, we

discovered a significant homology in amino acid sequence between Ad proteins and multiple HCV antigen epitopes, which also reflected in terms of HCV cross-reactive antibody and T cell immune responses in mice immunized with Ad vector. Furthermore, Ad-immune healthy HCV naïve individuals also showed significant level of HCV cross-reactive immune responses. Ad vector (expressing vaccine antigens) based vaccines are being tested for several pathogens including HCV. Our studies further suggest that widespread use of these Ad vector based vaccines could significantly impact the prevalence and course of HCV infection.

5.5 Future directions

We have shown that Ad vector containing the single non-structural antigen (NS4) of HCV can induce strong protective immunity in mice and optimizing the vector dose and route of administration can further enhance this immunity. We speculate that, inclusion of other non-structural and structural proteins will further broaden and enhance the HCV specific immune responses. Such Ad vector based strategies need to be explored in future studies. In our study, we used vaccinia-HCV mice challenge model and showed reduction in viral load in their ovaries. The protective potential of the immune responses induced by Ad-vector containing HCV antigens should be further tested in more relevant humanized animal models that can be infected with HCV.

Our studies demonstrated that Ad vector alone can induce protective cross-reactive immune responses against a number of HCV antigens. We further showed that HCV naïve people with pre-existing Ad immunity also showed cross-reactive antibodies and antigen dependent IFN-γ and IL-10 secretion from T cells. Further, anti-Ad hexon IgG was moderately correlated with the HCV NS3 and NS4 specific IgG in a cohort of 19 blood donors. However, such correlation study requires extensively large cohorts of HCV naïve and infected individuals

and should be investigated in another prospective study. Our studies have significant implications in understanding HCV immunity and have enormous inference in the use of recombinant adenovirus vaccines. We believe that most individuals have some levels of pre-existing Ad specific immunity. It will be interesting to see whether boosting this immunity with Ad vector alone could also boost the cross-reactive immune responses, and whether these responses will help in protection against HCV infection. Furthermore, it needs to be established, how pre-existing immunity to adenoviruses, and prevalent use of Ad vectors as vaccines for other diseases, shape the course of natural infection with HCV in the human population.

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Chapter 4: Humans with Pre-Existing Adenoviral Immunity Show HCV-Specific Cross-reactive Immunity

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Chapter 5: General Discussion

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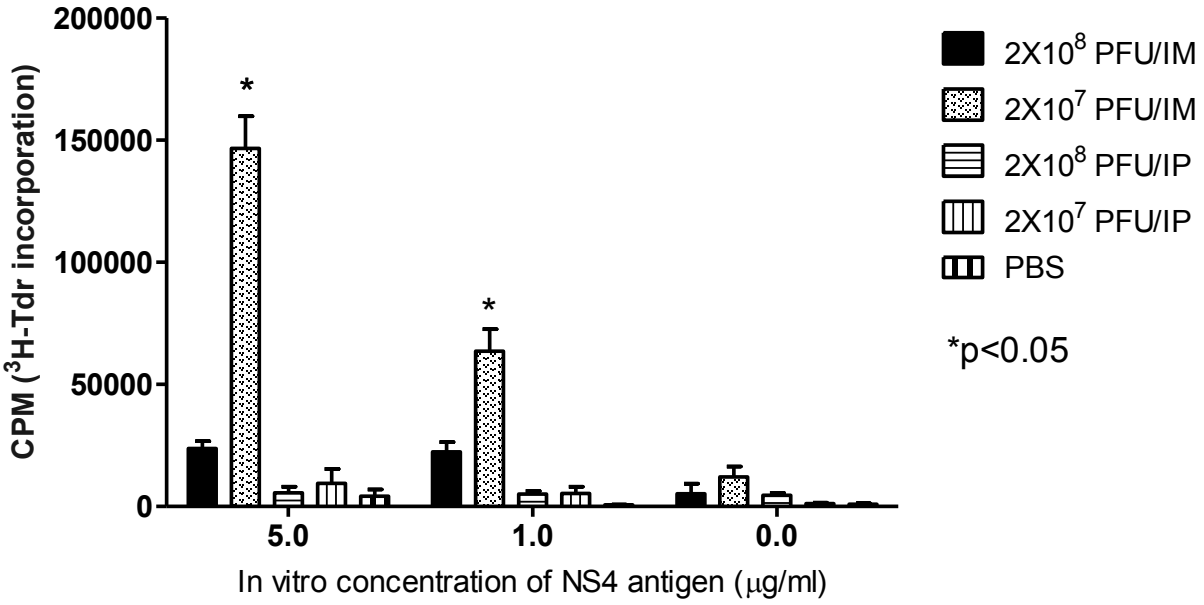
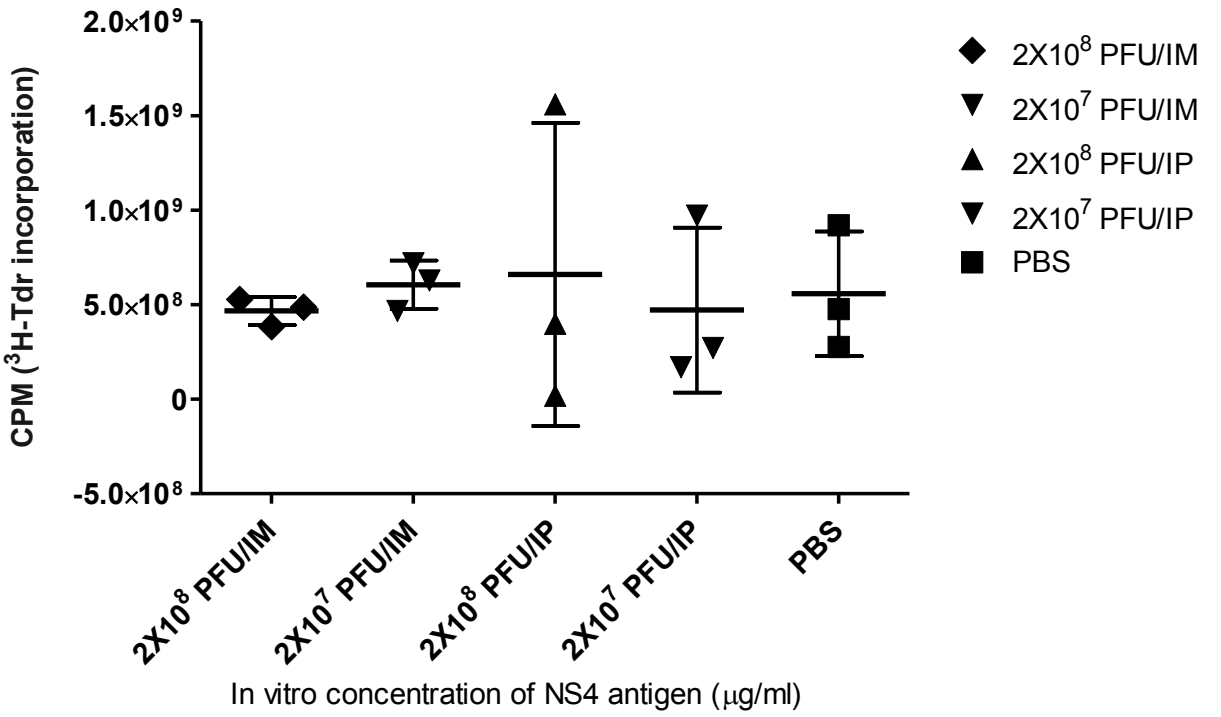
Appendices

Appendix 1:



T cell proliferation after single immunization. Antigen-specific T cell proliferation after immunization with high (2×10^8 pfu / mouse) and low (2×10^8 pfu / mouse) dose of recombinant adenovirus vector (rAd) containing HCV-NS4 antigen, by intramuscular (i.m.) and intraperitoneal (i.p.) routes in C57BL/6 mice. After 8 days of immunization, mouse splenocytes were harvested and T cell proliferation was evaluated in each group of immunized mice by a ^{3}H-thymidine-uptake assay. Mean ± SD of CPM (counts per minutes) from quadruplicate wells are shown.

Appendix 2:



Vaccinia-HCV challenge after single immunization. After 8 days of immunization with high (2×10^8 pfu/mouse) or low (2×10^7 pfu/mouse) antigen doses via i.m. or i.p. route, mice were challenged with 1×10^5 pfu/mouse i.p. Five days after challenge, mice were euthanized and viral load was determined in the ovaries of individual mouse by plaque assay using TK-1 cell line. Mean ± SD of viral titers from each mice group (n=3) are shown. The mean viral titter in immunized mice groups were not significantly different ($p>0.05$) from unimmunized group.

Appendix 3: List of HCV Core derived peptides and number of Ad proteins showing homology

HCV-1a Core peptides (15 amino acid each)				Number of Ad proteins showing homology (range of score in %)					
No.	Name	Location on polyprotein	Amino acid sequence	25.00 - 30.00	30.11 - 35.00	35.11 - 40.00	40.11 - 45.00	45.11 - 50.00	>50.00
1	Core-1	1-15	MSTNPKPQRKTKRNT	5	2	0	0	0	0
2	Core-2	5-19	PKPQRKTKRNTNRRP	10	2	1	0	0	0
3	Core-3	9-23	RKTKRNTNRRPQDVK	8	3	0	0	0	0
4	Core-4	13-27	RNTNRRPQDVKFPGG	13	1	1	0	0	0
5	Core-5	17-31	RRPQDVKFPGGGQIV	10	2	0	0	0	0
6	Core-6	21-35	DVKFPGGGQIVGGVY	5	3	0	0	0	1
7	Core-7	25-39	PGGGQIVGGVYLLPR	10	3	0	0	0	0
8	Core-8	29-43	QIVGGVYLLPRRGPR	13	6	3	0	0	0
9	Core-9	33-47	GVYLLPRRGPRLGVR	10	7	1	0	2	0
10	Core-10	37-51	LPRRGPRLGVRATRK	8	9	0	0	2	0
11	Core-11	41-55	GPRLGVRATRKTSER	12	3	2	0	0	0
12	Core-12	45-59	GVRATRKTSERSQPR	10	5	1	0	0	0
13	Core-13	49-63	TRKTSERSQPRGRRQ	8	6	1	0	0	0
14	Core-14	53-67	SERSQPRGRRQPIPK	7	6	1	0	0	0
15	Core-15	57-71	QPRGRRQPIPKARRP	7	7	2	0	0	0
16	Core-16	61-75	RRQPIPKARRPEGRT	8	10	0	0	0	0
17	Core-17	65-79	IPKARRPEGRTWAQP	6	1	1	0	0	0
18	Core-18	69-83	RRPEGRTWAQPGYPW	6	2	1	0	0	0
19	Core-19	73-87	GRTWAQPGYPWPLYG	3	3	0	0	0	0
20	Core-20	77-91	AQPGYPWPLYGNEGC	4	1	1	0	0	0
21	Core-21	81-95	YPWPLYGNEGCGWAG	6	3	1	0	0	0
22	Core-22	85-99	LYGNEGCGWAGWLLS	5	4	0	0	0	0
23	Core-23	89-103	EGCGWAGWLLSPRGS	5	3	1	0	0	0
24	Core-24	93-107	WAGWLLSPRGSRPSW	5	2	1	0	0	0
25	Core-25	97-111	LLSPRGSRPSWGPTD	9	3	1	0	0	0
26	Core-26	101-115	RGSRPSWGPTDPRRR	11	2	1	0	0	0
27	Core-27	105-119	PSWGPTDPRRRSRNL	8	7	2	0	0	0
28	Core-28	109-123	PTDPRRRSRNLGKVI	12	5	1	0	0	0
29	Core-29	113-127	RRRSRNLGKVIDTLT	12	3	0	0	1	0
30	Core-30	117-131	RNLGKVIDTLTCGFA	3	2	0	0	0	0
31	Core-31	121-135	KVIDTLTCGFADLMG	3	2	0	0	0	0
32	Core-32	125-139	TLTCGFADLMGYIPL	4	1	0	0	0	0
33	Core-33	129-143	GFADLMGYIPLVGAP	4	4	0	0	1	0
34	Core-34	133-147	LMGYIPLVGAPLGGA	4	3	0	0	1	0
35	Core-35	137-151	IPLVGAPLGGAARAL	9	4	3	0	0	0
36	Core-36	141-155	GAPLGGAARALAHGV	7	6	4	0	1	0
37	Core-37	145-159	GGAARALAHGVRVLE	11	8	2	0	0	0
38	Core-38	149-163	RALAHGVRVLEDGVN	10	3	0	0	0	0
39	Core-39	153-167	HGVRVLEDGVNYATG	8	2	0	0	0	0
40	Core-40	157-171	VLEDGVNYATGNLPG	9	1	0	0	0	0
41	Core-41	161-175	GVNYATGNLPGCSFS	4	2	1	0	0	0
42	Core-42	165-179	ATGNLPGCSFSIFLL	5	1	1	0	0	0
43	Core-43	169-183	LPGCSFSIFLLALLS	3	0	1	0	1	0
44	Core-44	173-187	SFSIFLLALLSCLTV	4	1	1	0	0	0
45	Core-45	177-191	FLLALLSCLTVPASA	7	0	2	0	0	0
Cummulative number of Ad proteins showing homology with each HCV Core peptide				331	154	39	0	9	1
Total number of Core peptides showing homology				45	43	27	0	7	1

Appendix 4: List of HCV F derived peptides and number of Ad proteins showing homology

HCV-1a F peptides (15 amino acid each)				Number of Ad proteins showing homology (range of score in %)					
No.	Name	Location on polyprotein	Amino acid sequence	25.00 - 30.00	30.11 - 35.00	35.11 - 40.00	40.11 - 45.00	45.11 - 50.00	>50.00
1	F-1	1-15	MSTNPKPQRKPNVTP	2	8	1	0	0	0
2	F-2	11-25	PNVTPTVAHRTSSSR	11	2	0	0	0	0
3	F-3	21-35	TSSSRVAVRSLVEFT	7	1	0	0	0	0
4	F-4	31-45	LVEFTCCRAGALDWV	5	1	0	0	0	0
5	F-5	41-55	ALDWVCARRGRLPSG	6	3	0	0	0	0
6	F-6	51-65	RLPSGRNLEVDVSLS	7	3	0	0	0	0
7	F-7	61-75	DVSLSPRHVGPRAGP	9	3	1	0	0	0
8	F-8	71-85	PRAGPGLSPGTLGPS	9	6	1	0	0	0
9	F-9	81-95	TLGPSMAMRVAGGRD	5	4	0	0	1	0
10	F-10	91-105	AGGRDGSCLPVALGL	10	3	0	0	1	0
11	F-11	101-115	VALGLAGAPQTPGVG	6	6	1	0	0	0
12	F-12	111-125	TPGVGRAIWVRSSIP	7	2	0	0	0	0
13	F-13	121-135	RSSIPLRAASPTSWG	8	3	0	0	0	0
14	F-14	131-145	PTSWGTYRSSAPLLE	4	2	0	0	0	0
15	F-15	141-155	APLLEALPGPWRMAS	7	5	0	0	1	0
16	F-16	148-162	LPGPWRMASGFWKTA	4	0	0	0	0	1
Cummulative number of Ad proteins showing homology with each HCV F peptide				107	52	4	0	3	1
Total number of F peptides showing homology				16	15	4	0	3	1

Appendix 5: List of HCV NS3 derived peptides and number of Ad proteins showing homology

HCV-1a NS3 peptides (15 amino acid each)				Number of Ad proteins showing homology (range of score in %)					
No.	Name	Location on polyprotein	Amino acid sequence	25.00 - 30.00	30.11 - 35.00	35.11 - 40.00	40.11 - 45.00	45.11 - 50.00	>50.00
1	NS3-1	1367-1381	LSTTGEIPFYGKAIP	7	1	2	0	0	0
2	NS3-2	1411-1425	GINAVAYYRGLDVS	5	0	1	3	0	0
3	NS3-3	1415-1429	VAYYRGLDVSVIPTS	6	4	1	0	1	0
4	NS3-4	1372-1386	EIPFYGKAIPLEVIK	5	2	0	0	0	0
5	NS3-5	1450-1464	SVIDCNTCVTQTVDF	3	0	1	0	0	0
6	NS3-6	1127-1142	SSDLYLVTRHADVIP	5	1	1	0	0	0
7	NS3-7	1621-1635	PTPLLYRLGAVQNEV	6	3	1	0	0	0
8	NS3-8	1467-1482	RRGRTGRGKPGIYRF	10	3	2	0	0	0
9	NS3-9	1187-1201	RGVAKAVDFIPVENL	4	1	1	0	0	0
10	NS3-10	1607-1622	MWKCLIRLKPTLHGP	3	2	0	0	0	0
11	NS3-11	1067-1081	QTFLATCINGVCWTV	0	0	0	0	0	0
Cummulative number of Ad proteins showing homology with each HCV NS3 peptide				54	17	10	3	1	0
Total number of NS3 peptides showing homology				10	8	8	1	1	0

Appendix 6: List of HCV NS4 derived peptides and number of Ad proteins showing homology

HCV-1a NS4 peptides (20 amino acid each)				Number of Ad proteins showing homology (range of score in %)					
No.	Name	Location on polyprotein	Amino acid sequence	25.00 - 30.00	30.11 - 35.00	35.11 - 40.00	40.11 - 45.00	45.11 - 50.00	>50.00
1	NS4a-1	1658-1677	STWVLVGGVLAALAAYCLST	7	0	0	0	0	0
2	NS4a-2	1673-1692	YCLSTGCVVIVGRIVLSGKP	0	1	0	0	0	0
3	NS4a-3	1688-1707	LSGKPAIIPDREVLYQEFDE	3	1	0	0	0	0
4	NS4a-4	1703-1721	QEFDEMEECSQHLPYIEQG	0	1	0	0	0	0
1	NS4b-1	1712-1731	SQHLPYIEQGMMLAEQFKQK	3	0	0	0	0	0
2	NS4b-2	1727-1746	QFKQKALGLLQTASRHAEVI	2	0	0	0	0	0
3	NS4b-3	1742-1761	HAEVITPAVQTNWQKLEVFW	2	0	0	0	0	0
4	NS4b-4	1757-1776	LEVFWAKHMWNFISGIQYLA	2	2	0	0	0	0
5	NS4b-5	1772-1791	IQYLAGLSTLPGNPAIASLM	6	0	0	0	0	0
6	NS4b-6	1787-1806	IASLMAFTAAVTSPLTTGQT	6	0	0	0	0	0
7	NS4b-7	1802-1821	TTGQTLLFNILGGWVAAQLA	5	0	0	0	0	0
8	NS4b-8	1817-1836	AAQLAAPGAATAFVGAGLAG	5	0	1	0	0	0
9	NS4b-9	1832-1851	AGLAGAAIGSVGLGKVLVDI	10	1	0	0	0	0
10	NS4b-10	1847-1866	VLVDILAGYGAGVAGALVAF	3	0	0	0	0	0
11	NS4b-11	1862-1881	ALVAFKIMSGEVPSTEDLVN	0	1	0	0	0	0
12	NS4b-12	1877-1896	EDLVNLLPAILSPGALVVGV	6	0	0	0	0	0
13	NS4b-13	1892-1911	LVVGVVCAAILRRHVGPGEG	0	0	1	0	0	0
14	NS4b-14	1907-1926	GPGEGAVQWMNRLIAFASRG	5	2	0	0	0	0
15	NS4b-15	1922-1941	FASRGNHVSPTHYVPESDAA	0	0	0	0	0	0
16	NS4b-16	1937-1956	ESDAAARVTAILSSLTVTQL	10	0	0	0	0	0
Cummulative number of Ad proteins showing homology with each HCV NS4 peptide				75	9	2	0	0	0
Total number of NS4 peptides showing homology				15	7	2	0	0	0

Appendix 7: List of HCV NS5A derived peptides and number of Ad proteins showing homology

HCV-1a NS5a peptides (20 amino acid each)				Number of Ad proteins showing homology (range of score in %)					
No.	Name	Location on polyprotein	Amino acid sequence	25.00 - 30.00	30.11 - 35.00	35.11 - 40.00	40.11 - 45.00	45.11 - 50.00	>50.00
1	NS5a-1	1972-1991	CSGSWLRDIWDWICEVLSDF	3	0	1	0	0	0
2	NS5a-2	1987-2006	VLSDFKTWLKAKLMPQLPGI	3	0	0	0	0	0
3	NS5a-3	2002-2021	QLPGIPFVSCQRGYRGVWRG	5	2	1	0	0	0
4	NS5a-4	2017-2036	GVWRGDGIMHTRCHCGAEIT	3	1	2	0	0	0
5	NS5a-5	2032-2051	GAEITGHVKNGTMRIVGPRT	5	0	0	0	0	0
6	NS5a-6	2047-2066	VGPRTCRNMWSGTFPINAYT	3	0	0	0	0	0
7	NS5a-7	2062-2081	INAYTTGPCTPLPAPNYKFA	5	0	0	0	0	0
8	NS5a-8	2077-2096	NYKFALWRVSAEEYVEIRRV	3	0	0	0	0	0
9	NS5a-9	2092-2111	EIRRVGDFHYVSGMTTDNLK	3	0	0	0	0	0
10	NS5a-10	2107-2126	TDNLKCPCQIPSPEFFTELD	1	0	0	0	0	0
11	NS5a-11	2122-2141	FTELDGVRLHRFAPPCKPLL	4	0	0	0	0	0
12	NS5a-12	2137-2156	CKPLLREEVSFRVGLHEYPV	6	0	0	0	0	0
13	NS5a-13	2152-2171	HEYPVGSQLPCEPEPDVAVL	5	0	0	0	0	0
14	NS5a-14	2167-2186	DVAVLTSMLTDPSHITAEAA	5	1	0	0	0	0
15	NS5a-15	2182-2201	TAEAAGRRLARGSPPSMASS	15	0	0	0	0	0
16	NS5a-16	2197-2216	SMASS SASQLSAPSLKATCT	4	0	0	1	0	0
17	NS5a-17	2212-2231	KATCTANHDSPDAELIEANL	7	0	0	0	0	0
18	NS5a-18	2227-2246	IEANLLWRQEMGGNITRVES	4	0	0	0	0	0
19	NS5a-19	2242-2261	TRVESENKVVILDSFDPLVA	4	1	0	0	0	0
20	NS5a-20	2257-2276	DPLVAEEDEREVSVPAEILR	4	1	1	0	0	0
21	NS5a-21	2272-2291	AEILRKSRRFARALPVWARP	8	1	1	0	0	0
22	NS5a-22	2287-2306	VWARPDYNPPLVETWKKPDY	4	0	0	0	0	0
23	NS5a-23	2302-2321	KKPDYEPPVVHGCPLPPPRS	9	1	2	0	0	0
24	NS5a-24	2317-2336	PPPRSPPVPPPRKKRTVVLT	14	4	0	1	0	0
25	NS5a-25	2332-2351	TVVLTESTLSTALAELATKS	8	0	0	0	0	0
26	NS5a-26	2347-2366	LATKSFGSSSTSGITGDNTT	6	0	0	0	0	0
27	NS5a-27	2362-2381	GDNTTTSSEPAPSGCPPDSD	7	2	0	0	0	0
28	NS5a-28	2377-2396	PPDSDVESYSSMPPLEGEPG	9	1	1	0	0	0
29	NS5a-29	2392-2420	EGEPGDPDLSDGSWSTVSSG ADTEDVVCC	1	0	0	0	0	0
Cummulative number of Ad proteins showing homology with each HCV NS5a peptide				158	15	9	2	0	0
Total number of NS5a peptides showing homology				29	10	7	2	0	0

Appendix 8: List of HCV NS5B derived peptides and number of Ad proteins showing homology

HCV-1a NS5b peptides (20 amino acid each)				Number of Ad proteins showing homology (range of score in %)					
No.	Name	Location on polyprotein	Amino acid sequence	25.00 - 30.00	30.11 - 35.00	35.11 - 40.00	40.11 - 45.00	45.11 - 50.00	>50.00
1	NS5b-1	2421-2440	SMSYSWTGALVTPCAAEEQK	2	0	0	0	0	0
2	NS5b-2	2436-2455	AEEQKLPINALSNSLLRHHN	5	0	0	0	0	0
3	NS5b-3	2451-2470	LRHHNLVYSTTSRSACQRQK	3	0	0	0	0	0
4	NS5b-4	2466-2485	CQRQKKVTFDRLQVLDSHYQ	2	0	0	0	0	0
5	NS5b-5	2481-2500	DSHYQDVLKEVKAAASKVKA	4	1	0	0	0	0
6	NS5b-6	2496-2515	SKVKA NLLSVEEACSLTPPH	6	0	0	0	0	0
7	NS5b-7	2511-2530	LTPPHSAKSKFGYGAKDVRC	1	0	0	0	0	0
8	NS5b-8	2526-2545	KDVRCHARKAVAHINSVWKD	3	0	0	0	0	0
9	NS5b-9	2541-2560	SVWKDLLEDSVTPIDTTIMA	2	0	0	0	0	0
10	NS5b-10	2556-2575	TTIMAKNEVFCVQPEKGGRK	7	0	0	0	0	0
11	NS5b-11	2571-2590	KGGRKPARLIVFPDLGVRVC	4	0	0	0	0	0
12	NS5b-12	2586-2605	GVRVCEKMALYDVVSKLPLA	3	0	0	0	0	0
13	NS5b-13	2601-2620	KLPLAVMGSSYGFQYSPGQR	9	0	0	0	0	0
14	NS5b-14	2616-2635	SPGQRVEFLVQAWKSKKTPM	3	0	0	0	0	0
15	NS5b-15	2631-2650	KKTPMGFSYDTRCFDSTVTE	3	0	0	0	0	0
16	NS5b-16	2646-2665	STVTESDIRTEEAIYQCCDL	2	0	0	0	0	0
17	NS5b-17	2661-2680	QCCDLDPQARVAIKSLTERL	6	1	0	0	0	0
18	NS5b-18	2676-2695	LTERLYVGGPLTNSRGENCG	10	0	0	0	0	0
19	NS5b-19	2691-2710	GENCGYRRCRASGVLTTSCG	7	0	1	0	0	0
20	NS5b-20	2706-2725	TTSCGNTLTCYIKARAACRA	3	0	0	0	0	0
21	NS5b-21	2721-2740	AACRAAGLQDCTMLVCGDDL	3	0	1	0	0	0
22	NS5b-22	2736-2755	CGDDLVVICESAGVQEDAAN	3	0	0	0	0	0
23	NS5b-23	2751-2770	EDAANLRAFTEAMTRYSAPP	6	1	0	0	0	0
24	NS5b-24	2766-2785	YSAPPGDPPQPEYDLELITS	9	0	0	0	0	0
25	NS5b-25	2781-2800	ELITSCSSNVSVAHDGAGKR	4	0	0	0	0	0
26	NS5b-26	2796-2815	GAGKRVYYLTRDPTTPLARA	8	0	0	0	0	0
27	NS5b-27	2811-2830	PLARAAWETARHTPVNSWLG	3	0	0	0	0	0
28	NS5b-28	2826-2845	NSWLGNIIMFAPTLWARMIL	2	0	0	0	0	0
29	NS5b-29	2841-2860	ARMILMTHFFSVLIARDQLE	1	0	0	0	0	0
30	NS5b-30	2856-2875	RDQLEQALNCEIYGACYSIE	1	1	0	0	0	0
31	NS5b-31	2871-2890	CYSIEPLDLPPIIQRLHGLS	4	0	0	1	0	0
32	NS5b-32	2886-2905	LHGLSAFSLHSYSPGEINRV	6	0	0	0	0	0
33	NS5b-33	2901-2920	EINRVAACLRKLGVPPLRAW	7	1	1	0	0	0
34	NS5b-34	2916-2935	PLRAWRHRARSVRARLLSRG	10	0	0	0	0	0
35	NS5b-35	2931-2950	LLSRGGRAAICGKYLFNWAV	2	0	0	0	0	0
36	NS5b-36	2946-2965	FNWAVRTKLKLTPITAAGRL	3	0	0	0	0	0
37	NS5b-37	2961-2980	AAGRLDLSGWFTAGYSGGDI	7	1	0	0	0	0
38	NS5b-38	2976-2995	SGGDIYHSVSHARPRWFWFC	5	0	0	0	0	0
39	NS5b-39	2991-3015	WFWFCLLLLAAGVGIYLLPN	5	1	1	0	0	0
Cummulative number of Ad proteins showing homology with each HCV NS5b peptide				174	7	4	1	0	0
Total number of NS5b peptides showing homology				39	7	4	1	0	0

Appendix 9: List of HCV E1 derived peptides and number of Ad proteins showing homology

HCV-1a E1 peptides (20 amino acid each)				Number of Ad proteins showing homology (range of score in %)				
No.	**Name**	**Location on polyprotein**	**Amino acid sequence**	**25.00 - 30.00**	**30.11 - 35.00**	**35.11 - 40.00**	**40.11 - 45.00**	**>50.00**
1	E1-1	192-211	YQVRNSSGLYHVTNDCPNSS	7	0	0	0	0
2	E1-2	207-226	CPNSSIVYEAADAILHTPGC	6	1	0	0	0
3	E1-3	222-241	HTPGCVPCVREGNASRCWVA	5	0	0	0	0
4	E1-4	237-256	RCWVAVTPTVATRDGKLPTT	6	1	0	0	0
5	E1-5	252-271	KLPTTQLRRHIDLLVGSATL	2	0	0	0	0
6	E1-6	267-286	GSATLCSALYVGDLCGSVFL	3	2	0	0	0
7	E1-7	282-301	GSVFLVGQLFTFSPRRHWTT	6	0	0	0	0
8	E1-8	297-316	RHWTTQDCNCSIYPGHITGH	7	0	0	0	0
9	E1-9	312-331	HITGHRMAWDMMMNWSPTAA	1	0	0	0	0
10	E1-10	327-346	SPTAALVVAQLLRIPQAIMD	6	1	0	0	0
11	E1-11	342-361	QAIMDMIAGAHWGVLAGIAY	1	0	0	0	0
12	E1-12	357-383	AGIAYFSMVGNWAKVLVVLLLFAGVD	0	0	0	0	0
Cummulative number of Ad proteins showing homology with each HCV E1 peptide				**50**	**5**	**0**	**0**	**0**
Total number of E1 peptides showing homology				**11**	**4**	**0**	**0**	**0**

Appendix 10: List of HCV E2 derived peptides and number of Ad proteins showing homology

HCV-1a E2 peptides (20 amino acid each)				Number of Ad proteins showing homology (range of score in %)				
No.	Name	Location on polyprotein	Amino acid sequence	25.00 - 30.00	30.11 - 35.00	35.11 - 40.00	40.11 - 45.00	>50.00
1	E2-1	384-403	ETHVTGGSAGRTTAGLVGLL	6	1	0	0	0
2	E2-2	399-418	LVGLLTPGAKQNIQLINTNG	6	0	0	0	0
3	E2-3	414-433	INTNGSWHINSTALNCNESL	1	0	0	0	0
4	E2-4	429-448	CNESLNTGWLAGLFYQHKFN	3	0	0	0	0
5	E2-5	444-463	QHKFNSSGCPERLASCRRLT	4	1	0	0	0
6	E2-6	459-478	CRRLTDFAQGWGPISYANGS	3	0	0	0	0
7	E2-7	474-493	YANGSGLDERPYCWHYPPRP	4	3	0	0	0
8	E2-8	489-508	YPPRPCGIVPAKSVCGPVYC	7	2	0	0	0
9	E2-9	504-523	GPVYCFTPSPVVVGTTDRSG	8	0	1	0	0
10	E2-10	519-538	TDRSGAPTYSWGANDTDVFV	2	0	0	0	0
11	E2-11	534-553	TDVFVLNNTRPPLGNWFGCT	7	0	0	0	0
12	E2-12	549-568	WFGCTWMNSTGFTKVCGAPP	3	0	0	0	0
13	E2-13	564-583	CGAPPCVIGGVGNNTLLCPT	9	0	0	0	0
14	E2-14	579-598	LLCPTDCFRKHPEATYSRCG	3	0	0	0	0
15	E2-15	594-513	YSRCGSGPWITPRCMVDYPY	3	0	0	0	0
16	E2-16	609-628	VDYPYRLWHYPCTINYTIFK	5	0	0	0	0
17	E2-17	624-643	YTIFKVRMYVGGVEHRLEAA	1	1	0	0	0
18	E2-18	639-658	RLEAACNWTRGERCDLEDRD	8	0	0	0	0
19	E2-19	654-673	LEDRDRSELSPLLLSTTQWQ	7	1	0	0	0
20	E2-20	669-688	TTQWQVLPCSFTTLPALSTG	4	0	1	0	0
21	E2-21	684-703	ALSTGLIHLHQNIVDVQYLY	5	1	0	0	0
22	E2-22	699-718	VQYLYGVGSSIASWAIKWEY	3	0	0	0	0
23	E2-23	714-733	IKWEYVVLLFLLLADARVCS	4	0	0	0	0
24	E2-24	729-746	ARVCSCLWMMLLISQAEA	1	0	0	0	0
Cummulative number of Ad proteins showing homology with each HCV E2 peptide				**107**	**10**	**2**	**0**	**0**
Total number of E2 peptides showing homology				**24**	**7**	**2**	**0**	**0**

Appendix 11: List of HCV p7 derived peptides and number of Ad proteins showing homology

HCV-1a p7 peptides (20 amino acid each)				Number of Ad proteins showing homology (range of score in %)				
No.	Name	Location on polyprotein	Amino acid sequence	25.00 - 30.00	30.11 - 35.00	35.11 - 40.00	40.11 - 45.00	>50.00
1	P7-1	747-766	ALENLVILNAASLAGTHGLV	6	1	0	0	0
2	P7-2	762-781	THGLVSFLVFFCFAWYLKGR	1	1	0	0	0
3	P7-3	777-796	YLKGRWVPGAVYAFYGMWPL	2	0	0	0	0
4	P7-4	792-809	GMWPLLLLLLALPQRAYA	3	1	0	1	0
Cummulative number of Ad proteins showing homology with each HCV p7 peptide				**12**	**3**	**0**	**1**	**0**
Total number of p7 peptides showing homology				**4**	**3**	**0**	**1**	**0**

Appendix 12: List of HCV NS2 derived peptides and number of Ad proteins showing homology

HCV-1a NS2 peptides (20 amino acid each)				Number of Ad proteins showing homology (range of score in %)				
No.	**Name**	**Location on polyprotein**	**Amino acid sequence**	**25.00 - 30.00**	**30.11 - 35.00**	**35.11 - 40.00**	**40.11 - 45.00**	**>50.00**
1	NS2-1	810-829	LDTEVAASCGGVVLVGLMAL	8	0	0	0	0
2	NS2-2	825-844	GLMALTLSPYYKRYISWCMW	2	0	0	0	0
3	NS2-3	840-859	SWCMWWLQYFLTRVEAQLHV	2	0	0	0	0
4	NS2-4	855-874	AQLHVWVPPLNVRGGRDAVI	4	1	0	0	0
5	NS2-5	870-889	RDAVILLMCVVHPTLVFDIT	3	0	0	0	0
6	NS2-6	885-904	VFDITKLLLAIFGPLWILQA	2	0	0	0	0
7	NS2-7	900-919	WILQASLLKVPYFVRVQGLL	4	0	0	0	0
8	NS2-8	915-934	VQGLLRICALARKIAGGHYV	4	0	0	0	0
9	NS2-9	930-949	GGHYVQMAIIKLGALTGTYV	5	0	0	0	0
10	NS2-10	945-964	TGTYVYNHLTPLRDWAHNGL	6	1	0	0	0
11	NS2-11	960-979	AHNGLRDLAVAVEPVVFSRM	4	1	0	0	0
12	NS2-12	975-994	VFSRMETKLITWGADTAACG	2	0	0	0	0
13	NS2-13	990-1009	TAACGDIINGLPVSARRGQE	4	1	0	0	0
14	NS2-14	1005-1026	RRGQEILLGPADGMVSKGWRLL	1	0	0	0	0
Cummulative number of Ad proteins showing homology with each HCV NS2 peptide				**51**	**4**	**0**	**0**	**0**
Total number of NS2 peptides showing homology				**14**	**4**	**0**	**0**	**0**