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# *MECHANISMS OF IMMUNE EVASION UTILISED BY THE HEPATITIS C VIRUS*

*Danijela Petrovic*

*PhD* *2009*

# *MECHANISMS OF IMMUNE EVASION UTILISED BY THE HEPATITIS C VIRUS*

**A thesis submitted for the degree of Doctor of Philosophy (PhD)**

**by**


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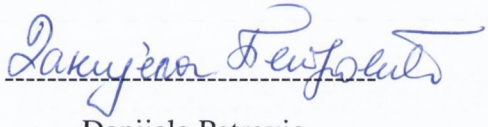

Danijela Petrovic

# *Summary*

Hepatitis C virus (HCV) is a small, enveloped RNA virus which is distributed worldwide and according to the WHO the number of infected individuals is estimated to be approximately 170 million. HCV creates a heavy burden on a public health, since most HCV infections persist, leading in up to 80% of all cases to chronic hepatitis associated with liver fibrosis, cirrhosis and hepatocellular carcinoma. A successful immune response to HCV is characterised by a vigorous, sustained and multi-specific cellular response during the acute phase of infection. However, the persistence of HCV in infected individuals, is due mainly to its ability to evade immune defense, which it does using complex strategies to establish persistence and chronic disease.

The study described in this thesis used a combination of cell and molecular biology techniques to explore and characterise mechanisms of viral evasion in HCV infection. In this study, the effect of HCV Envelope Protein (E2) on T cell IL-2 production was investigated, since IL-2 production is central to the primary responses of the adaptive immune system to pathogens. In a HuT 78 T cell line model and in peripheral blood mononuclear cells, pre-treatment of cells with E2 substantially attenuated IL-2 production in activated cells. Inhibition of IL-2 was at the level of secretion and not transcription as demonstrated by Real Time PCR. Further experiments have demonstrated that the signal delivered via E2-CD81 interaction, targets the enzyme Protein Kinase C-β (PKC-β), previously shown to be essential for IL-2 secretion in HuT 78 cells, to lipid raft microdomains in the cell membrane. This can disrupt the association of PKC-β with the cellular secretory machinery, thereby inhibiting export of IL-2. Furthermore, we also investigated the effect of E2 on the production of other cytokines, implicated in viral infection (IFN-γ, TNF-α, IL-10) or in development of inflammation and liver disease (IL-1α/β, IL-6, TGF-β1). Production of all these cytokines was affected by E2. IFN-γ was inhibited at the level of secretion, TNF-α and IL-10 were inhibited at the level of mRNA, TGF-β1 in HuT 78 cells was also suppressed at mRNA level, while production of IL-1α/β and IL-6 was increased in PBMCs in response to E2. Overall, this part of the study

demonstrated that E2 alters production of these cytokines, creating a cytokine milieu which potentially favours viral persistence and development of chronic disease.

Epitope mutation, as a mechanism of immune evasion by HCV, was also investigated. A detailed model to predict epitopes for HLA Class I and Class II molecules was constructed. The HLA-A3, B7, DRB1 0101 and DRB1 0401 alleles were included in this study, because it had previously been shown that these alleles were significantly associated with viral clearance in an Irish cohort of females infected with HCV 1b from a single source. Viral RNA was then extracted from patients' serum samples, amplified and sequenced. CLC Bio and BioEdit software packages were used to perform analysis of viral sequence data. This involved *in silico* protein translation, alignment with the donor sequence and epitope mapping, where identified putative epitopes were compared to viral sequences obtained from HLA-A3, B7 and DRB1 0401 positive and negative HCV infected individuals. Mutations within the epitopes restricted by protective alleles were identified. In the HLA-A3 restricted epitopes a significant amino acid substitution of lysine (K) to arginine (R) was identified in the KLTPPHSAK epitope. The same K to R substitution was found in the TVYHGAGTK epitope, and this mutation occurred singly or in a combination with a substitution of threonine (T) to alanine (A). In the HLA-B7 restricted epitope LPRLPGVPF, three amino acid substitutions were identified: valine (V) to isoleucine (I) or to leucine (L) and arginine (R) to glutamine (Q). A substitution of V to I was also identified in the HHNMVYATTSRSASQ epitope restricted by HLA-DRB1 0401 allele. All these substitutions were identified in patients infected with HCV, who despite being carriers of protective alleles, developed persistent infection. At the same time these mutations were either present in significantly lower frequency or not present at all in patients who did not express these protective alleles. Therefore, mutations within these HLA restricted epitopes may contribute to viral immune escape and represent a mechanism whereby HCV can establish persistent infection and chronic disease.

The HCV clearly uses a range of strategies to manipulate and evade the human immune response. A complete understanding of the molecular mechanisms underlying these processes, will aid in the design of novel vaccines and therapeutic modalities for this disease.

*To my Mom and Dad,*

*Miladija and Rodoljub Petrovic*

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# *Associated publications*

## Publications

*'The Envelope Protein of the Hepatitis C virus (E2) inhibits IL-2 Secretion from T Lymphocytes'*. Danijela Petrovic, Lucy Golden-Mason, Jennifer Conroy, Eugene Dempsey, Siobhan Mitchell, Yuri Volkov, Cliona O'Farrelly, Dermot Kelleher, Aideen Long. Manuscript submitted.

*'Mechanism of immune evasion utilised by the Hepatitis C virus'*. Danijela Petrovic, Eugene Dempsey, Dermot Kelleher, Aideen Long. Review in preparation.

*'HLA footprinting reveals a novel protective $CD8^+$ T cell response in HCV'* Joint paper Karen Fitzmaurice and Danijela Petrovic, Elizabeth Freitas, Silvana Gaudieri, Eugene Dempsey, Susan McKiernan, Aideen Long, Paul Klenerman, Dermot Kelleher. Manuscript in preparation.

## Oral Presentations

*'Mutations in epitopes restricted by protective HLA-A3 and HLA-DRB1 0401 alleles are associated with the development of persistent hepatitis C infection'*. Danijela Petrovic, Elizabeth Freitas, Silvana Gaudieri, Eugene Dempsey, Karen Fitzmaurice, Susan McKiernan, Paul Klenerman, Aideen Long, Dermot Kelleher. Digestive Disease Week, May 2008, San Diego, CA, USA. Published abstract in Gastroenterology. (International)

*'Mechanisms of Hepatitis C virus immune evasion'*. Danijela Petrovic, IMM Lecture Series, May 2008, IMM, Dublin, Ireland

*'The Envelope Protein of the Hepatitis C virus (E2) inhibits IL-2 Secretion from T Lymphocytes'*. Danijela Petrovic, Lucy Golden-Mason, Jennifer Conroy, Eugene Dempsey, Siobhan Mitchell, Yuri Volkov, Cliona O'Farrelly, Dermot Kelleher, Aideen Long.

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*'The Envelope Protein of the Hepatitis C virus (E2) inhibits IL-2 Secretion from T Lymphocytes'*. Danijela Petrovic, Lucy Golden-Mason, Jennifer Conroy, Eugene Dempsey, Siobhan Mitchell, Yuri Volkov, Cliona O'Farrelly, Dermot Kelleher, Aideen Long. 1st School of Medicine Postgraduate Research Day, September 2007, IMM Dublin, Ireland. (National)

## Poster Presentations

*'Predicted Hepatitis C Virus (HCV) Epitope Mapping for Protective HLA-A3 Alleles'*, Danijela Petrovic, Elizabeth Freitas, Silvana Gaudieri, Eugene Dempsey, Karen Fitzmaurice, Susan McKiernan, Paul Klenerman, Aideen Long, Dermot Kelleher. 45th Annual Meeting, IDSA, October 2007, San Diego, CA. (International)

*'The Envelope Protein of the Hepatitis C virus (E2) inhibits secretion of IL-2 from T lymphocytes'*, Danijela Petrovic, Lucy Golden-Mason, Jennifer Conroy, Eugene Dempsey, Siobhan Mitchell, Yuri Volkov, Cliona O'Farrelly, Dermot Kelleher, Aideen Long. 14th international symposium on Hepatitis C virus and related viruses, September 2007, Glasgow, Scotland, UK. (International)

*'Predicted Hepatitis C Virus (HCV) Epitope Mapping for Protective HLA-A3 Alleles'*, Danijela Petrovic, Elizabeth Freitas, Silvana Gaudieri, Eugene Dempsey, Karen Fitzmaurice, Susan McKiernan, Paul Klenerman, Aideen Long, Dermot Kelleher. Winter Meeting, Irish Society of Gastroenterology, November 2007, Dublin, Ireland. (National)

*'The Envelope Protein of the Hepatitis C virus (E2) inhibits secretion of IL-2 from T lymphocytes'*, Danijela Petrovic, Lucy Golden-Mason, Jennifer Conroy, Eugene Dempsey, Siobhan Mitchell, Yuri Volkov, Cliona O'Farrelly, Dermot Kelleher, Aideen Long. 10th Annual Meeting of the IMM, November 2007, Dublin, Ireland. (National)

*'The Envelope Protein of the Hepatitis C virus (E2) inhibits secretion of IL-2 from HuT 78 T cells'*, Danijela Petrovic, Lucy Golden-Mason, Jennifer Conroy, Eugene Dempsey, Siobhan Mitchell, Yuri Volkov, Cliona O'Farrelly, Dermot Kelleher, Aideen Long. 9th Annual Meeting of the IMM, November 2006, Dublin, Ireland. (National)

# *Abbreviations*

| | |
|---|---|
| A | Alanine |
| ADAR 1 | RNA-specific adenosine deaminase 1 |
| ALD | Alcoholic Liver Disease |
| ALT | Alanine-aminotransferase |
| ANN | Artificial neural networks |
| AP-1 | Activator protein-1 (transcription factor) |
| APC | Allophycocyanin |
| APC | Antigen Presenting cell |
| ATP | Adenosine triphosphate |
| BIMAS | Bioinformatics and Molecular Analysis Section |
| Blimp-1 | B lymphocyte maturation protein-1 |
| BMP-6 | Bone morphogenetic protein-6 |
| BP | Blocking Peptide |
| BSA | Bovine Serum Albumine |
| C | Core Protein of Hepatitis C virus |
| C | Control siRNA |
| C22 | Recombinant Core Protein |
| C33 | Recombinant Protein from NS3 region |
| CDC | Centers for Disease Control |
| CLDN1 | Claudin-1 |
| CLIP protein | Class II-associated Ii (invariante chain) peptide |
| CMV | Cytomegalovirus |
| CTL | Cytotoxic T lymphocyte |
| DAG | Diacylglycerol |
| DC | Dendritic cell |
| DC-SIGN | Dendritic cell specific intracellular adhesion molecule-3-grabbing non-integrin |
| DMSO | Dimethyl Sulfoxide |

| | |
|---|---|
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| dNTP | Deoxynucleotide mix |
| dsRNA | Double-stranded Ribonucleic acid |
| E1 / E2 | Envelope protein 1 / Envelope protein 2 |
| EB/AO | Ethidium Bromide/Acridine Orange |
| EBV | Epstein Barr virus |
| ECM | Extracellular Matrix |
| EDTA | Ethenediamine tetraacetic acid |
| eIF | Eukaryotic Initiation Factor |
| ELISA | Enzyme Linked Immunosorbent assay |
| ELISpot | Enzyme Linked ImmunoSpot assay |
| ER | Endoplasmic reticulum |
| F | Phenylalanine |
| FACS | Fluorescence Activated Cell Sorter |
| FCS | Foetal Calf Serum |
| FITC | Fluorescein Isothiocyanate |
| GAGs | Glycosaminoglycans |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GBV | GB virus (derived from the blood of a surgeon whose initials were G.B.) |
| GFP | Green fluorescent protein |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GA | Golgi apparatus |
| GTP | Guanosine triphosphate |
| HBS | Hepes Based Saline |
| HBV | Hepatitis B virus |
| HCC | Hepatocellular carcinoma |
| HCV | Hepatitis C virus |
| HCVcc | HCV cell culture (system) |

| | |
|---|---|
| HCVpp | HCV pseudopartical (system) |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfoic acid |
| HIV | Human Immunodeficiency Virus |
| HLA | Human Leukocyte Antigen |
| HMM | Hidden Markov model |
| HRP | Horseradish Peroxidase |
| HSC | Hepatic Stellate cells |
| HVR | Hypervariable region |
| I | Isoleucine |
| ICAM-1 | Intracellular adhesion molecule-1 |
| ICOS | Inducible co-stimulator (receptor) |
| IEDB | Immune Epitope Database and Analysis Resources |
| IFN-γ | Interferon-γ |
| IGF-1 | Interferon growth factor-1 |
| Ii | Invariant chain |
| IL | Interleukin |
| IL-2R | Interleukin-2 receptor |
| Io | Ionomycin |
| IP-10 | IFN-inducible protein-10 |
| IRF | Interferon-regulatory factor |
| IRES | Internal Ribosome Entry Site |
| JAK | Janus Kinase |
| JFH-1 | Japanese Fulminant Hepatitis 1 |
| K | Lysine |
| KC | Kupffer cell |
| L | Leucine |
| LCMV | Lymphocytic choriomeningitis virus |
| LDL-R | Low Density Lipoprotein receptor |
| LFA-1 | Lymphocyte function-associated antigen-1 |
| LIKOS | ligand of ICOS receptor |

| | |
|---|---|
| L-SIGN | Liver/lymph node specific intercellular adhesion molecule-3-grabbing integrin |
| M | Methionine |
| MAPK | Mitogen-activated protein Kinase |
| MAPPP | MHC-I Antigenic Peptide Processing Prediction |
| MIP-1α | Macrophage inflammatory protein-1α |
| MCD | Methyl-β-cyclodextrin |
| MCP 1 | Monocyte chemoattractant protein |
| MFB | myofibroblast |
| MFI | Mean Fluorescence Intensity |
| MHC (I and II) | Major Histocompatibility Complex (Class I and Class II) |
| miRNA | micro RNA |
| MMP | Matrix metalloproteinase |
| mRNA | messenger RNA |
| MW | Molecular Weight |
| NANBH | Non-A and non-B hepatitis |
| NE | Non- electroporated cells |
| NF- κB | Nuclear factor κB (transcription factor) |
| NK | Natural Killer cell |
| NKT | Natural Killer T cell |
| NS | Normal serum |
| NS | Non-stimulated cells |
| NS | Non-structural |
| OAS | 2'5'oligoadenylate synthetase |
| ORF | Open Reading Frame |
| PAMPs | Pathogen-associated molecular patterns |
| PaProC | Prediction Algorithm for Proteasomal Cleavages |
| PBC | Primary Biliary Cirrhosis |
| PBMC | Peripheral Blood Mononuclear Cells |
| PBS | Phosphate-buffered Saline |

| | |
|---|---|
| PCR | Polymerase Chain Reaction |
| PD-1 | Programmed death-1 |
| PDGF | Platelet derived growth factor |
| PE | Phycoerythrin |
| peg-IFN | polyethylene glycol-IFN, pegylated IFN |
| PHA | Phytohemagglutinin |
| PI3K | Phosphoinositide 3-kinase |
| PKC | Protein Kinase C |
| PKR | Protein Kinase R |
| PMA | Phorbol 12-myristate 13-acetate |
| PPD | Purified Protein Derivative |
| PRR | Pattern-recognition receptors |
| PSSMs | Position-specific scoring matrices |
| Q | Glutamine |
| R | Arginine |
| RC | Resting cell |
| RdRp | RNA dependant RNA polymerase |
| RISC | RNA-induced silencing complex |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| Rr | Pearson's Correlation Coefficient |
| RT | Reverse Transcription |
| RT-PCR | Real Time-PCR |
| SCR | Scrambled version of BP |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| SFU | Spot forming units |
| siGlo | fluorescent lamin A/C siRNA |
| siRNA | Small Interfering RNA |
| SR-BI | Scavenger receptor B type I |

| | |
|---|---|
| STAT | Signal Transducer and Activator of Transcription |
| SVMs | Support vector machines |
| SVR | Sustained virological response |
| SVRMHC | Support Vector machine Regression MHC |
| T | Threonine |
| TAP | Transporter associated with Antigen Processing |
| TCR | T cell receptor |
| TGF-β1 | Transforming growth factor-beta 1 |
| Th | T helper cell |
| TIMP | Tissue inhibitor of MMP |
| TM | Transmembrane domains |
| TMB | 3,3',5,5'- Tetramethylbenzidine |
| TNF | Tumor necrosis factor |
| Treg | Regulatory T cell |
| TRITC | Tetramethylrhodamine isothiocyanate |
| tRNA | Transport RNA |
| UTR | Untranslated regions |
| V | Valine |
| WHO | World Health Organisation |

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# CHAPTER I

# *GENERAL INTRODUCTION*

# Chapter I

## *1.1 HEPATITIS C VIRUS*

### *1.1.1 Discovery of HCV*

A virus is defined as a subcellular organism which contains proteins and nucleic acid and since it is not metabolically active outside the host cell, it has been described as an obligate parasite. Luis Pasteur was first to introduce term "virus" (Latin word meaning toxin or poison), in the late XIX century, speculating that rabies was caused by a "living thing".

Depending on the nucleic acid within the viral particle, viruses are divided into DNA and RNA viruses, with both groups being involved in etiology of different human diseases. RNA viruses are a highly diverse group causing an array of human diseases such as: viral encephalitis, AIDS, influenza and hepatitis (Talaro, 2006)

Hepatitis has been documented for long time in human history. Since the fifth century B.C. when Hippocrates described jaundice, a common symptom of hepatitis, evidence about hepatitis outbreaks could be traced through the centuries up to modern times. In the early twentieth century, scientists were speculating that hepatitis may be caused by an infectious agent. This was confirmed in the early 1970s when hepatitis A and B viruses were discovered. However, it took more then a decade before the causing agent of non-A and non-B hepatitis (NANBH) was revealed. Namely, in 1987-88 collaboration between researchers from Chiron Corporation and Centers for Disease Control (CDC) made the discovery of Hepatitis C virus. Using reverse transcriptase-polymerase chain reaction (RT-PCR) and molecular cloning they identified a genetic fragment in serum obtained from chimpanzees infected with serum from a patient with post-transfusion NANBH. This fragment did not correspond to chimpanzee, or human DNA, but did correspond to RNA found in NANBH-infected chimpanzee blood and liver, suggesting an RNA virus. In addition, antibodies extracted from NANBH patients were able to recognise part of a protein encoded by this fragment, suggesting that this infectious genetic fragment could induce an immune response (Choo et al,

1989). Hence, for the first time, a virus had been identified before being visualised by electron microscopy or detected by serological techniques; it was described as one of the most significant achievements in virology. Subsequently, full length HCV was cloned (Choo et al, 1991; Kato et al, 1990; Takamizawa et al, 1991), sensitive screening methods for the presence of HCV in blood were developed and, as proved to be very challenging, direct visualisation of HCV particles in serum (Kaito et al, 1994; Prince et al, 1996; Trestard et al, 1998) and liver (De Vos et al, 2002; Falcon et al, 2003; Heller et al, 2005) was achieved.

### *1.1.2 HCV genome organisation*

Hepatitis C virus has been classified as a member of a distinct genus called Hepacivirus in the family Flaviviridae according to the VIII report of the International Committee for Taxonomy of Viruses (Simmonds et al, 2005). It is a small enveloped, icosahedral particle, varying in size between 40 and 70nm in diameter **(Figure 1.1)** (Wakita et al, 2005). It harbors a positive, single stranded RNA genome, which is approximately 9600 nucleotides in length. The genome carries a single long open reading frame (ORF) encoding a polyprotein, of about 3000 amino acid residues, placed between 5' and 3' highly conserved untranslated regions (UTR) (Brazzoli et al, 2007; Wang et al, 1993). These UTRs are essential for viral cycle, with the 5' UTR containing an internal ribosome entry site (IRES) crucial in cap-independent translation of viral RNA (Rijnbrand & Lemon, 2000). The 3' UTR contains a 99-nucleotide element proven to be critical for viral replication (Friebe & Bartenschlager, 2002; Kolykhalov et al, 2000). The polyprotein precursor is proteolytically co and post-translationally cleaved, by host cell and viral proteases, into a set of distinctive products, 4 structural and 6 non-structural proteins **(Figure 1.2)** (Brazzoli et al, 2007; Wieland & Chisari, 2005).

The first cleavage product is the highly basic core protein (C), which exhibits pleiotropic features. It is involved in nucleocapsid formation and it plays a role in the switch between viral polyprotein synthesis and subsequent viral RNA replication (Zhang et al, 2002).

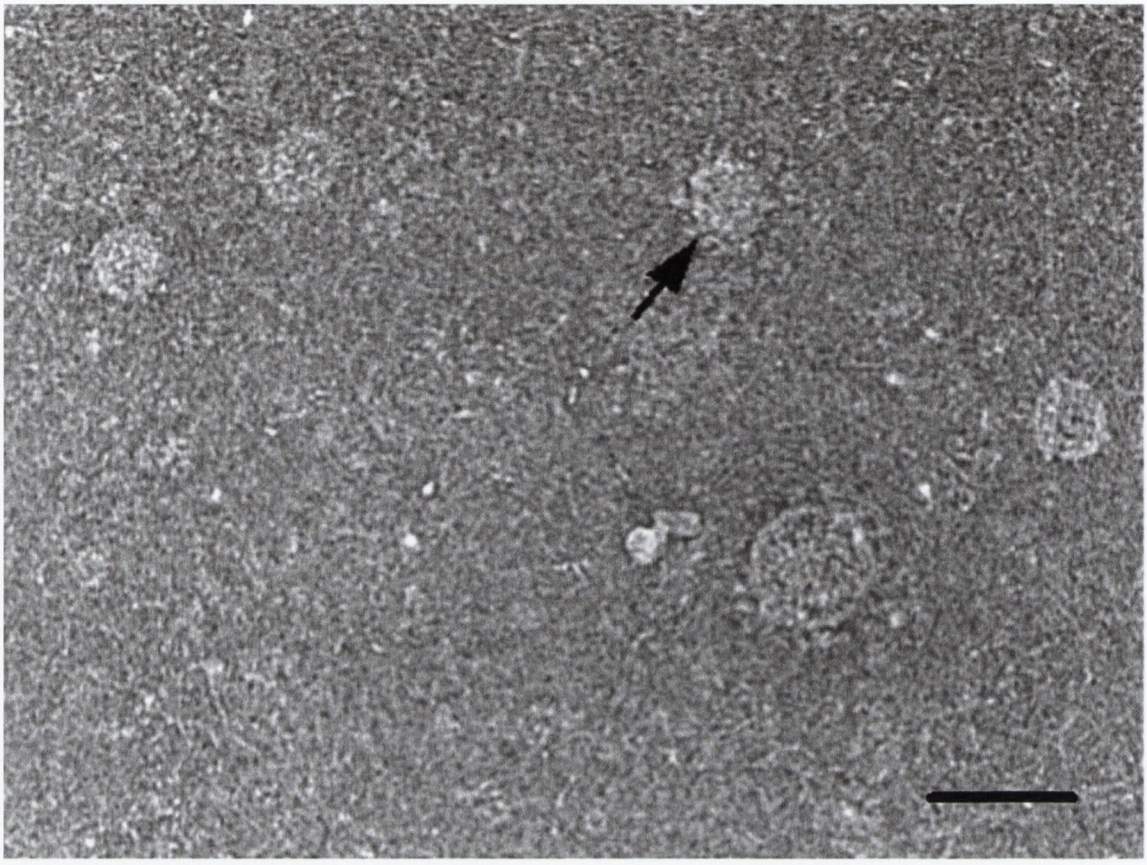


**Figure 1.1 Electron micrograph of hepatitis C particle.** Viral particles were produced in vitro and purified using sucrose gradient centrifugation. They were different in size (approximately 50 and 100nm), with size of 50nm being dominant. Bar is 100nm. Image is taken from Heller et al, 2005.

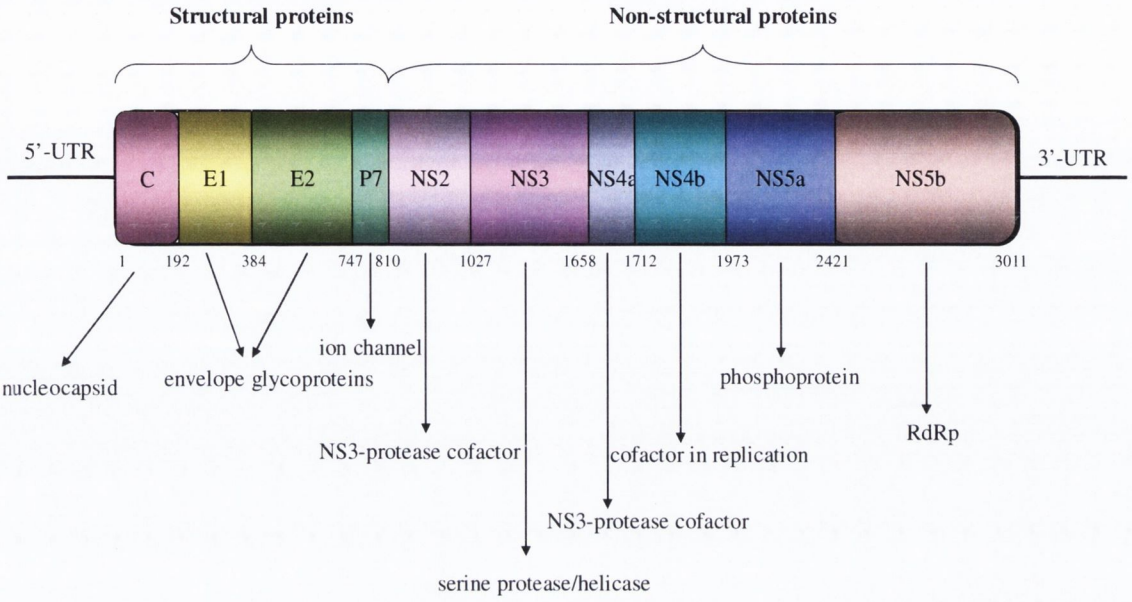



**Figure 1.2 HCV genome organisation.** HCV genome encodes a polyprotein, approximately 3000 amino acids long, that is proteolytically, co- and post-translationally, cleaved into 10 products: 4 structural and 6 non-structural proteins. Core protein (C) forms nucleocapsid, envelope proteins (E1, E2) are involved in formation of viral envelope, while p7 may be a viroporin. Non-structural proteins (NS2-NS5b) are all involved in viral replication. NS3 has protease and helicase activity and for its functioning requires NS2 and NS4a cofactors. NS5b is an RNA dependant RNA polymerase (RdRp) and its cofactors are NS4b and NS5a.

It binds RNA *in vitro* (Kunkel et al, 2001) and attaches on the surface of lipid droplets (Dubuisson et al, 2002), which may affect lipid metabolism and might be associated with development of liver disease, such as steatosis (Asselah et al, 2006; Lerat et al, 2002). Additionally, binding to lipid droplets possibly has a role in viral replication and/or virion development (Moradpour et al, 2007). Moreover, core protein has been shown to transcriptionally regulate several cellular and viral genes and several reports suggest an influence on the transcriptional activity of p53 and the regulation of p53 dependant genes (Herzer et al, 2003; Otsuka et al, 2000).

Envelope proteins (E1 and E2) are key components of the viral envelope. They are highly glycosylated type one transmembrane proteins, with an N-terminal ectodomain and a C-terminal hydrophobic anchor, which form a stable, non-covalent heterodimeric complex (Op De Beeck et al, 2001). A segment long 27 amino acids, positioned at N-terminal side of E2 protein, is termed hypervariable region 1 (HVR1) (Weiner et al, 1991), since it bears numerous mutations and evolves rapidly in infected individuals (Mondelli et al, 2003). However, the basic chemical properties and conformation of this region are highly conserved, with certain amino acid residues located at specific position within the sequence (Muriel Lavie, 2006) . As in other Flaviviridae family members, these proteins are believed to play a key role in viral particle assembly (Penin et al, 2004b). The major envelope glycoprotein E2 is thought to be responsible for initiating virus attachment to the host cell.

The protein p7 is positioned at the junction between the structural and non-structural regions of the viral polypeptide. It is a small, hydrophobic, integral membrane protein, described as viroporin because it has been shown to have a function as an oligomeric ion channel, able to mediate cation flow across the membrane (Griffin et al, 2003; Pavlovic et al, 2003). It possibly has a significant role in maturation and release of viral particles (Moradpour et al, 2007).

Most of the non-structural proteins are required for replication of the viral RNA. Two viral proteases are necessary to regulate complex proteolytic processing of these proteins (Bartenschlager & Lohmann, 2000). NS2 in association with NS3 is the first activated viral protease responsible for the maturation of the residual NS proteins and

involved in autoproteolytic cleavage, separating two components of the protease (Roingeard et al, 2004). In addition, it can be involved in apoptosis inhibition as well as interference with promoters and enhancer elements in cellular gene expression machinery (Franck et al, 2005). When released from NS2, NS3 expresses its multifunctional role. The amino-terminal region of NS3 exhibits serine protease activity allowing it to process the remaining non-structural viral proteins. The carboxy-terminal domain has RNA helicase characteristics and nucleotide triphosphatase activity. It also may have other properties involved in interference with the host cell, for example: inhibition of PKA mediated signal transduction or cell transformation (Bartenschlager & Lohmann, 2000). NS4a is an essential cofactor of the NS3 protease and is required for efficient polyprotein processing. This small protein anchors NS3 to cellular membranes (Wolk et al, 2000). NS4b is a hydrophobic membrane-associated protein that interacts with the viral replicase and colocalises with ER and may interfere with lumen protein interaction with a membrane (Elazar et al, 2004). NS5a is hydrophilic membrane associated protein exhibiting multiple phosphorylation states (Lindenbach & Rice, 2005; Roingeard et al, 2004). The primary function of this protein is unknown, but it appears to be involved in specific protein-protein interactions that are crucial for the construction of a functional HCV replication complex (Penin et al, 2004a) . Moreover, NS5a protein has been shown to be involved in resistance of the infected cell to the antiviral effects of interferon (IFN). NS5b was identified as an RNA dependant RNA polymerase (RdRp), which carries out synthesis of a complementary negative strand of RNA using the genomic RNA as a template, as well as synthesis of a genomic positive RNA molecule utilising this negative strand. Being a crucial component in the process of viral replication, this protein emerged as a major target in antiviral therapies (Moradpour et al, 2007). It is possible that all non-structural proteins, along with p7, 3' UTR and some host cell factors, may be involved in the replication complex (Kolykhalov et al, 2000; Sakai et al, 2003).

### *1.1.3 HCV diversity*

#### 1.1.3.1 HCV classification

HCV has been isolated from all over the world and the main feature of these isolates is genetic heterogeneity. By phylogenetic methods, variants have been classified into 6 genotypes (clades), each containing a different number of more closely related subtypes **(Figure 1.3)**. Genotypes are numbered in order of their identification, while a letter stands for a subtype. Genotypes 7-11 have been also described, but following broad genetic analyses 7, 8, 9 and 11 were classified within genotype 6, while 10 was placed within genotype 3 (International Commettee on Taxonomy on Viruses, ICTVdB index of viruses). At the nucleotide level, genotypes differ by 31-33%, while subtypes differ by 20-25% (Simmonds et al, 2005). Worldwide genotype 1b is the most common; genotype 1a is predominant in Europe and North America; genotype 2 in Mediterranean countries, Japan and the Far East; genotype 3 in South-East Asia; genotype 4 is prevalent in North and Central Africa (Egypt, Congo, Gabon) and Middle East (4a); genotype 5 in South Africa and genotype 6 in South-East Asia (Hong-Kong, Vietnam) and recently Australia **(Figure 1.4)** (Forns & Bukh, 1999; Timm et al, 2007b). In spite of sequence diversity, all these genotypes have a common genome structure containing co-linear genes, similar or identical in size, which encode ten viral proteins.

#### 1.1.3.2 HCV natural history

Most likely, all HCV genotypes and subtypes have the same origin from a common predecessor, with genetic variances occurring over time. Sub-Saharan Africa and South-East Asia are the regions with the largest diversity among HCV variants, thus suggesting that their human population have been exposed to the virus for a longer period of time (Simmonds et al, 2005).

Analysing observed rates of sequence divergence in E1 and NS5b regions of the HCV genome and the geographical distribution of genotypes, a study by Smith at al. estimated

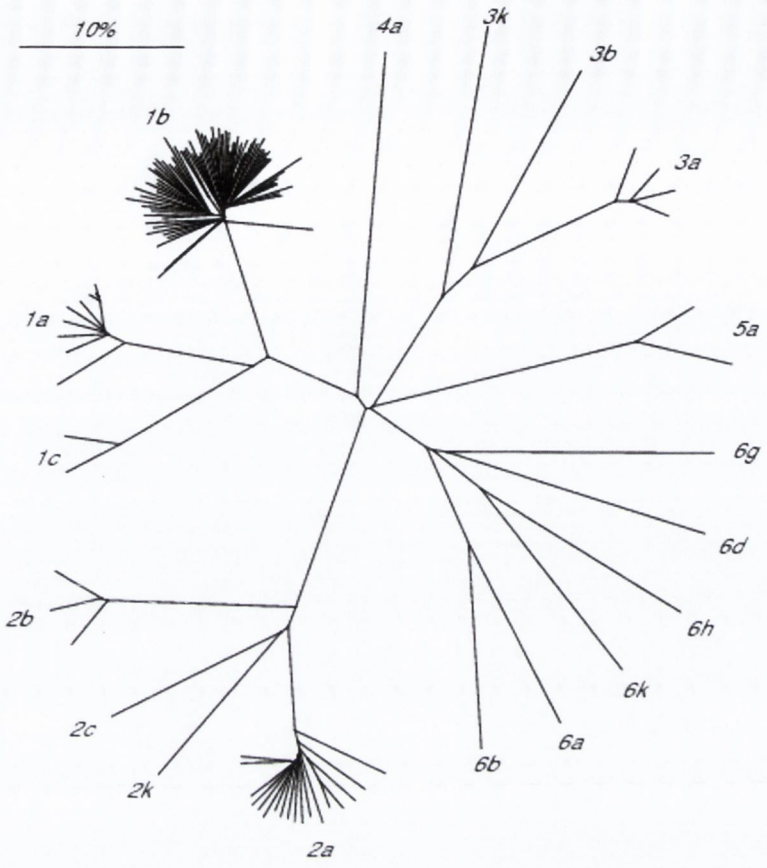



**Figure 1.3 HCV diversity – genotypes and subtypes.** A tree of complete genome sequences was made using the Neighbor-joining method. Genotypes/subtypes represented by less then five sequences are also included in this graph. This image of the genetic tree is taken from HCV sequence database, Los Alamos National Laboratory.

http://hcv.lanl.gov/content/sequence/HCV/variability/HCV_variability.html

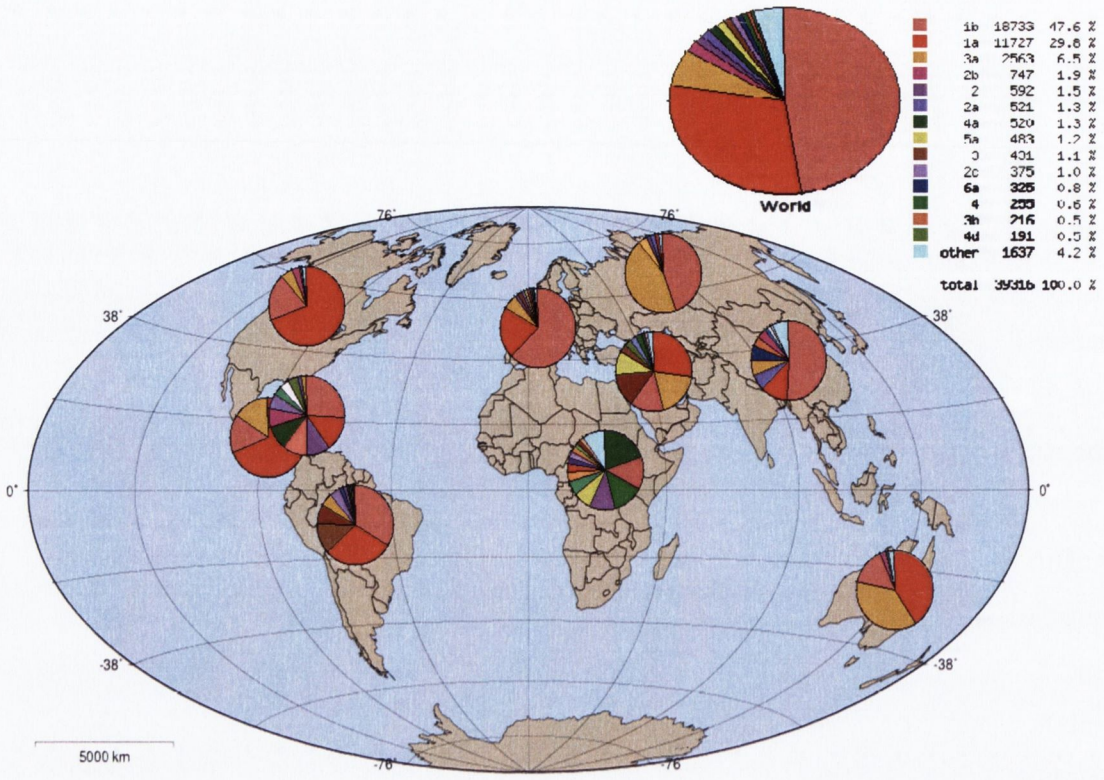



**Figure 1.4 Global distributions of various genotypes of HCV.** Genotype 1b is the most common worldwide. In Europe, Asia (Russia, China, Japan), North and South America and Australia genotypes 1a/b, 2a/b/c and 3a account for more than 90% of HCV infections. Genotype 4 is prevalent in Egypt and Central Africa, while in South Africa genotype 5a accounts for half of infections. Genotype 6 is found in South-East Asia and Australia. Image is taken from HCV sequence database, Los Alamos National Laboratory.

http://hcv.lanl.gov/components/sequence/HCV/new_geography/geography.comp?region=world&form=all&organism=HCV

that HCV genotypes originated at least 500-2000 years ago, while subtypes diverged from each other around 300 years ago (Smith et al, 1997). Another study has suggested that subtypes 1a and 1b were derived approximately 100 years ago, while genotypes 4 and 6 date back hundreds of years (Pybus et al, 2001). In general, the exact time line for the origin of viral genotypes or subtypes is difficult to achieve as all phylogenetic studies have to be based on mathematical models and recent estimations of viral evolutionary rate, since the reconstruction of viral evolution can not rely on material evidence (such as fossils). However, knowledge of viral origin can be valuable, as it could shed light on understanding how it mutates, establishes persistence and causes disease.

Speculation that HCV originated in non-human primates has not been proved, since no primate counterpart or clear viral ancestor has been identified. A potential candidate is GB virus B, which has the closest genetic relationship with HCV (member of genus Hepacivirus) (Beames et al, 2001; Zuckerman, 1995) and which is possibly native to small tamarins monkeys (Lanford et al, 2003; Martin et al, 2003), but this hypothesis has to be further investigated.

### *1.1.4 HCV prevalence and course of infection*

Hepatitis C virus (HCV) is distributed worldwide **(Figure 1.5)** and according to the WHO the number of infected individuals is estimated to be about 170 million, which is 3% of global population, with 3-4 million people newly infected each year (WHO, 2000). At a global level prevalence of HCV varies to a large degree. In general, Northern and Western Europe, North America and Australia have the lowest incident of infection, while the countries with the highest occurrence of HCV are located in Africa and Asia. Germany and Canada have a seroprevalence of less than 1% (Shepard et al, 2005) whilst there is an extremely high number of HCV positive people in the Nile delta of Egypt where figures increase to 60% among individuals over 30 years of age (WHO, 2008).

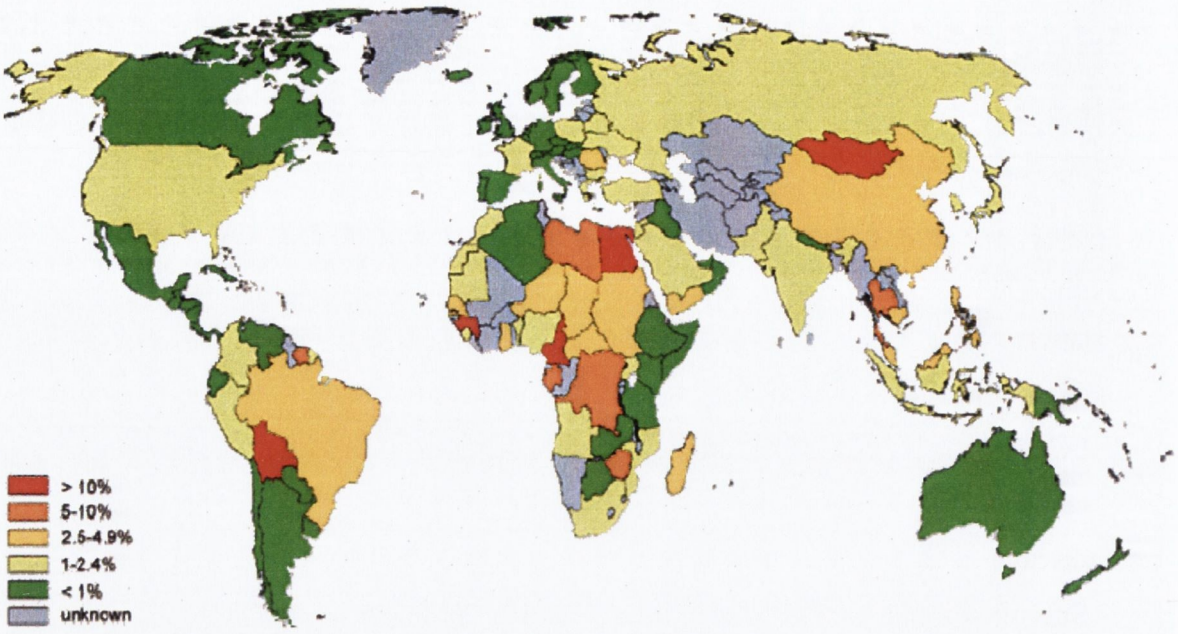



**Figure 1.5 Worldwide prevalence of HCV.** Most countries in Europe, Asia, America and also Australia have HCV prevalence rate lower than 2.5%. Brazil, China, South-East Asia and the region in central Africa have higher prevalence, between 2.5 - 4.9%. In Thailand and parts of Africa prevalence has been shown to range between 5-10%, while it exceeds 10% in some countries such as Bolivia, Egypt, Cameroon, Guinea and Mongolia. The geographic pattern of HCV prevalence is based on WHO Weekly Epidemiological Records 2002; 77: 41-48. www.klinikum.uni-heidelberg.de/HCV.104920.0.html

HCV has been figuratively compared to a "viral time bomb", as most morbidities associated with infection come after some period of time. Since HCV is not directly cytopathic, most HCV infections persist, leading in up to 80% of all cases to chronic hepatitis associated with liver fibrosis, cirrhosis and hepatocellular carcinoma. Circa 20% of infected individuals will develop cirrhosis two decades after initial exposure, while in up to 2.5%, disease will progress to hepatocellular carcinoma (HCC) (Bowen & Walker, 2005) **(Figure 1.6)**. In addition, HCV has also been involved in the pathogenesis of various autoimmune and rheumatic disorders (arthritis, sicca syndrome, lichen planus, mixed cryoglobulinemia, nephropathies, lung fibrosis etc.) as well as development of B-cell proliferative disease such as Non-Hodgkin's lymphoma (Mazzaro et al, 2005; McHutchison, 2004; NIH, 2002) . Chronic infection is declared if HCV RNA in blood is detected for at least 6 months. Chronic hepatitis C is the most common cause for liver transplantation, being responsible for 40-50% of those waiting or having undergone liver transplants (Brown, 2005).

During HCV infection several factors can accelerate the progression of disease. Male gender, obesity, older age at acquisition and particularly co-infections with HIV and/or HBV and alcohol consumption are associated with increased incidence of complications of chronic infection (Shepard et al, 2005). In HIV infected patients, for instance, HCV behaves as an opportunistic infection. It increases liver-related morbidity and mortality among co-infected individuals, despite improvements in highly active anti-retroviral therapy and its beneficial effects on HIV infection (Jaggy et al, 2003; Khalsa et al, 2005; Macias et al, 2002). Similarly, HBV co-infection leads into more severe liver disease, expressing strong correlation with HCC, thus suggesting synergistic effect between these two viruses.

The acute phase of infection is usually asymptomatic with rare cases exhibiting fatigue, malaise and jaundice for a few weeks. Viral RNA can be detected within 1-3 weeks after inoculation, while antibodies to HCV are evident in approximately 90% of infected individuals 3 months later. Elevated levels of alanine-aminotransferase (ALT) inside 4-12 weeks of exposure are indicators of liver cell injury, but subsequently, after several weeks, ALT levels decrease (NIH, 2002).

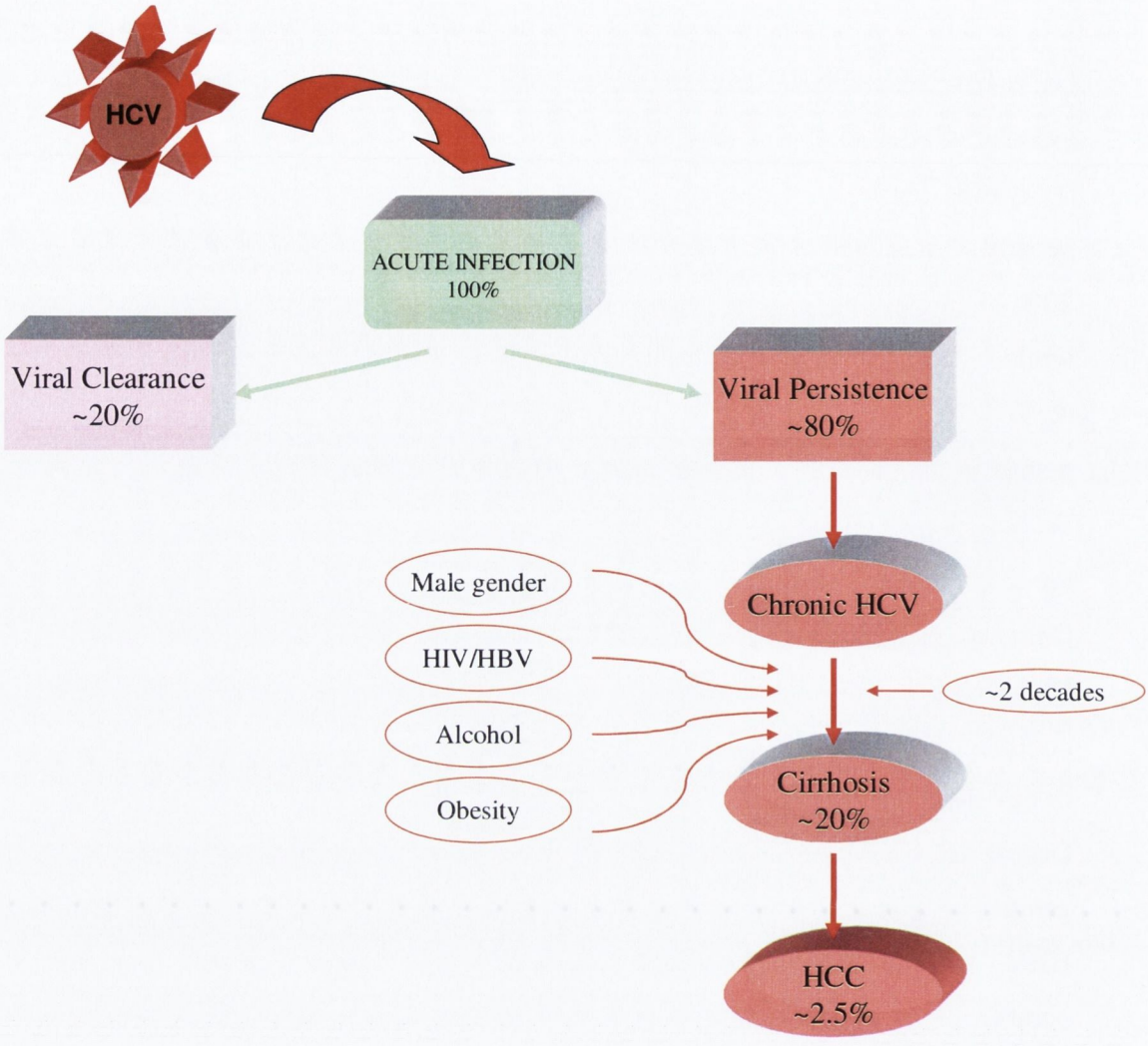



**Figure 1.6 Possible sequelae of HCV infection.** Following acquisition of HCV, approximately 20% of infected individuals manage to resolve the infection spontaneously, while in around 80% virus persists and these individuals go on to develop chronic HCV infection. With time, chronic disease progresses to cirrhosis in about 20% of patients, mainly those who are co-infected with HIV or HBV or with a habit of alcohol consumption (other risk factors are thought to be male gender and obesity). About 2.5% of chronically infected individuals develop malignancy and hepatocellular carcinoma (HCC).

Immune events during this stage of disease are considered to be essential in determining the outcome of infection.

### *1.1.5 Viral life cycle*

Like the other viruses, the main goal of HCV is to replicate itself. Being a hepatotropic virus it primarily replicates in hepatocytes, where after internalisation, all subsequent steps occur in the cell cytoplasm, with synergistic action between viral and host cell proteins. After the discovery of HCV, it was extremely difficult to study viral life cycle since it was not possible to successfully culture HCV *in vitro* or to establish small animal models. About a decade elapsed before some model systems were developed, and nowadays the following are in use (Moradpour et al, 2007):

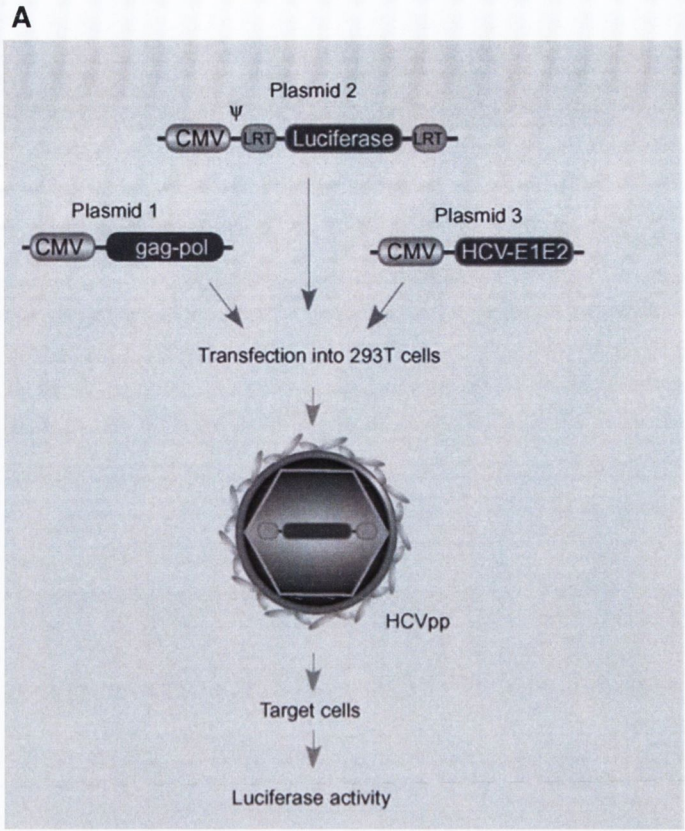
1) *in vivo* models - chimpanzee; transgenic mice; immunodeficient mice and related viruses (for instance GBV-B).

2) *in vitro* models - stable, transfected cell lines; transient cellular expression systems; replicons; related viruses; retroviral pseudoparticles and recombinant infectious HCV.

These systems are used to investigate different aspects of viral life cycle and its interaction with the host. However, "bona fide" progress in determination of viral cell entry and full life cycle was made using the pseudopartical system (HCVpp) and recombinant infectious HCV in tissue culture system (HCVcc).

Bartosch et al. were first to establish a retroviral pseudopartical system. HCVpp were produced by 293T human embryo kidney cells which were transfected with three expression vectors: one encoding E1E2 glycoproteins, one encoding MLV (murine leukemia virus) Gag-Pol core proteins and last one encoding MLV packaging component with reporter GFP gen. Retroviral core proteins easily assemble different viral and cell glycoproteins (Sandrin et al, 2002; Sung & Lai, 2002) and successfully integrate genetic markers into DNA of transfected cells. Thus, generated HCVpp were composed of full length, unmodified E1E2, derived from HCV 1a and 1b, assembled on the retroviral core conjugated with a GFP (green fluorescent protein) marker **(Figure 1.7A)** (Bartosch et al, 2003b). Purified HCV pseudoparticles are capable of infecting hepatic cells (Huh-7 human hepato-carcinoma cells) sufficiently, which certainly has opened new possibilities for investigation of the complex process of the viral entry.

Before the HCVcc system was described, it had been found that full length HCV RNA could replicate in Huh-7 cells only if the adaptive mutations were introduced in those viral genomes. Moreover, structural proteins could be produced, but infectious particles were never released (Blight et al, 2002; Pietschmann et al, 2002). Furthermore, it was reported that full length genomes, lacking culture-adaptive changes, weakly replicated in Huh-7 cells but, at the same time, were able to release core protein and viral RNA in the cell culture medium (Lindenbach & Rice, 2005). This contradiction of adaptive mutations being essential for viral RNA replication, but detrimental for viral production, suddenly was overcome when Kato et al. demonstrated that the JFH-1 (Japanese fulminant hepatitis 1) strain of HCV genotype 2a, could replicate in Huh-7 cells without cell adaptive mutations (Kato et al, 2003). Subsequently, it has been reported that full length RNA from the JFH-1 strain transfected to Huh-7 cells, produced viral particles that were infectious for naïve Huh-7 cells, for the first time demonstrating completion of the viral life cycle (Wakita et al, 2005). Similarly, a chimaeric full length genome, constructed from JFH-1 replicase (5', 3' UTR and NS3-NS5 region) and core-NS2 region derived from J6 (related genotype 2a strain) was shown to replicate and produce infectious virus in Huh-7.5 cells, a subline very tolerant to RNA replication (**Figure 1.7B**) (Lindenbach et al, 2005). This was further supported when it was demonstrated that the full JFH-1 genome produces high levels of viral particles in the same cell subline (Zhong et al, 2005). Viral particles generated in the *in vitro* cell culture system – HCVcc were infectious *in vivo* when chimpanzees were inoculated (Wakita et al, 2005; Lindenbach et al, 2006) as well as immunodeficient mice with partial human livers (Lindenbach et al, 2006).



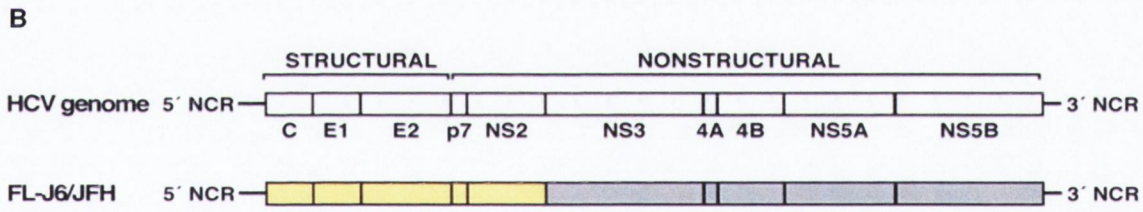




**Figure 1.7 Models to study stages of HCV life cycle. A)** Production of HCV pseudoparticles (HCVpp). Three vectors encoding retroviral core proteins gag and pol, packaging component with reporter gene and encoding E1E2 proteins are transfected in the 293T cells, which then produce HCV pseudoparticles. Figure is taken from Lavie et al. 2006 Hepatitis C Virus genomes and molecular biology. **B)** Construct used to produce infectious HCV in cell culture system (HCVcc) using a chimeric full length genome composed of HCV J6 strain in yellow (C-NS2 region) and HCV JFH strain in grey (5'non-coding region, NS3-3'non-coding region). Figure adopted from Lindenbach et al, 2005.

### 1.1.5.1 Viral entry

The main cell type of the liver, hepatocytes, are the major target of HCV. However, peripheral blood mononuclear cells (PBMCs) also exhibit tropism for the HCV, particularly monocytes, dendritic, T and B cells (Caussin-Schwemling et al, 2001; Goutagny et al, 2003; Rodriguez-Inigo et al, 2000), with even pluripotent progenitors of these white blood cells in a bone marrow being observed to be infected with the virus (Radkowski et al, 2000; Sansonno et al, 1998). It is thought that the degree of HCV infected PBMCs differs among infected individuals and that this may be associated with intravenous drug use (Resti et al, 2002) and HIV co-infection (Laskus et al, 2004).

Initial binding of HCV to the host cell involves a specific interaction between the HCV envelope proteins and receptors required for viral entry. The major envelope glycoprotein, E2, is probably responsible for initiating viral attachment (Rosa et al, 1996) while the role of E1 may be in the membrane fusion, as similarity between hydrophobic domain of E1 with fusion peptides of paramyxovirus and flavivirus has been observed (Flint et al, 1999).

Up to now, a number of molecules have been identified as potential entry sites of HCV including CD81 receptor (Pileri et al, 1998), scavenger receptor B type I (SR-BI) (Scarselli et al, 2002), low-density lipoprotein receptor (LDL-R) (Agnello et al, 1999), and claudin-1 (CLDN1) (Evans et al, 2007). Inhibition of infection of primary human hepatocytes or hepatoma cell lines was achieved by addition of anti-CD81 antibody (Bartosch et al, 2003c; Cormier et al, 2004; McKeating et al, 2004) or after CD81 gene silencing with siRNA (Zhang et al, 2004). Many studies have demonstrated the ability of CD81 to bind E2, but they also revealed that this receptor, although necessary, is not sufficient for viral entry (Bartosch et al, 2003c; Hsu et al, 2003; Zhang et al, 2004). Additionally, internalisation of E2 after binding to CD81 was only partial, as shown by some groups (Germi et al, 2002; Petracca et al, 2000; Roccasecca et al, 2003). This supported a conclusion that CD81 acts as initial attachment molecule as a part of receptor complex responsible for viral entry (Hsu et al, 2003; Pileri et al, 1998).

Many viral particles outside the cells, are found in circulation bound to lipoproteins (Kono et al, 2003; Thomssen et al, 1993) suggesting another, potentially E1E2 independent, mechanism of viral attachment and entry. Complexes of HCV and

lipoproteins have been reported to bind LDL receptor (Agnello et al, 1999; Andre et al, 2002; Germi et al, 2002), but the speculation that it might serve as a collector of HCV particles which facilitates further transfer onto CD81 (Moradpour et al, 2007) has to be investigated further.

Another receptor involved in cholesterol metabolism that binds E2 of HCV is SR-BI (Op De Beeck & Dubuisson, 2003; Scarselli et al, 2002). Like CD81, the interaction between E2 and this high-density lipoprotein receptor was not sufficient to propagate infection in hepatic cells (Hsu et al, 2003). However, cooperation between these two receptors secured viral entry, which could be inhibited with anti-SR-BI antibodies preventing E2 binding (Bartosch et al, 2003). Moreover, along with receptor cooperation, the entry of HCVpp was dependant on cholesterol, which if depleted in the membrane of Huh-7 cells, led to inhibition of HCV infection (Kapadia et al, 2007).

The most recently identified receptor essential for HCV entry is claudin-1 (Evans et al, 2007), a component of tight junctions, which is highly expressed in liver tissue (Furuse et al, 1998). The domain responsible for this process is the first extracellular loop of CLDN1 and antibodies against this part of the molecule inhibit HCV infection. It has also been shown that this receptor functions later in the entry process, after the E2 - CD81 interaction has been established. The hypothesis that CLDN1 may be responsible for lateral migration of the HCV in liver tissue, through cell tight junctions is supported with the findings that coxsackievirus B uses this route to spread to its host cells (Coyne & Bergelson, 2006).

In addition to those mentioned above, there are other molecules described as candidates for HCV entry. It was observed that E2 can bind to DC-SIGN and L-SIGN lectins and it is thought that these molecules act more like facilitators of viral entry to hepatocytes and possibly PBMCs (Hsu et al, 2003) than as an entry sites. Similarly, HVR1 of E2 interacts with cell surface glycosaminoglycans (GAGs), such as heparin or heparin sulphate, but it was shown that this interaction is not sufficient and probably not required for viral entry (Basu et al, 2004).

Despite an array of candidates implicated in the process of viral entry, this topic still needs further investigation and clarification. A possible scenario may be that CD81, SR-BI and CLDN1 are required for viral entry, but it is not completely clear at which

stage of the entry process they may be involved. Other molecules, like lectins, LDL and GAGs may facilitate attachment, uptake and transfer of the HCV particles (Bartosch & Cosset, 2006).

It has been demonstrated that clathrin-mediated endocytosis facilitates HCV entry (Blanchard et al, 2006) and once within endocytic vesicles, HCV requires exposure to low pH (Koutsoudakis et al, 2006; Tscherne et al, 2006) to initiate envelope fusion with endosytic membranes. Subsequently, it may lead to uncoating, which is a process of nucleocapsid degradation and release of viral RNA. However, little is known about this process and it remains to be elucidated. **(Figure 1.8)**

### 1.1.5.2 HCV genome translation and processing of the viral polyprotein

Once released inside the cell cytoplasm, viral RNA serves as a template for production of viral proteins. Translation of the HCV genome is 5' cap-independent, as the viral 5'-UTR does not have a chemical group called the methylated cap which binds eukaryotic initiation factor 4 (eIF4), an essential requirement of cellular mRNAs. Instead, translation of viral RNA depends on IRES, which has a very complex structure of four domains and several loops which facilitate binding of several components necessary for initiation of translation. IRES within the 5'-UTR binds the 40S ribosomal subunit directly and robustly, significantly changing the conformation of the ribosomal subunit (Spahn et al, 2001) and recruiting eIF3 and ternary complex of eIF2-met-tRNA-GTP. This is modified, via 48S intermediate and 60S addition, to active 80S complex (Ji et al, 2004; Otto & Puglisi, 2004). Bypassing eIF4, IRES directly binds ribosome and positions it at the proper initiation site, without ribosomal scanning along the RNA for start AUG codon which is required for most cellular mRNAs. This is an advantage for the virus which increases its protein synthesis during host cell division (Honda et al, 2000; Shimazaki et al, 2002).

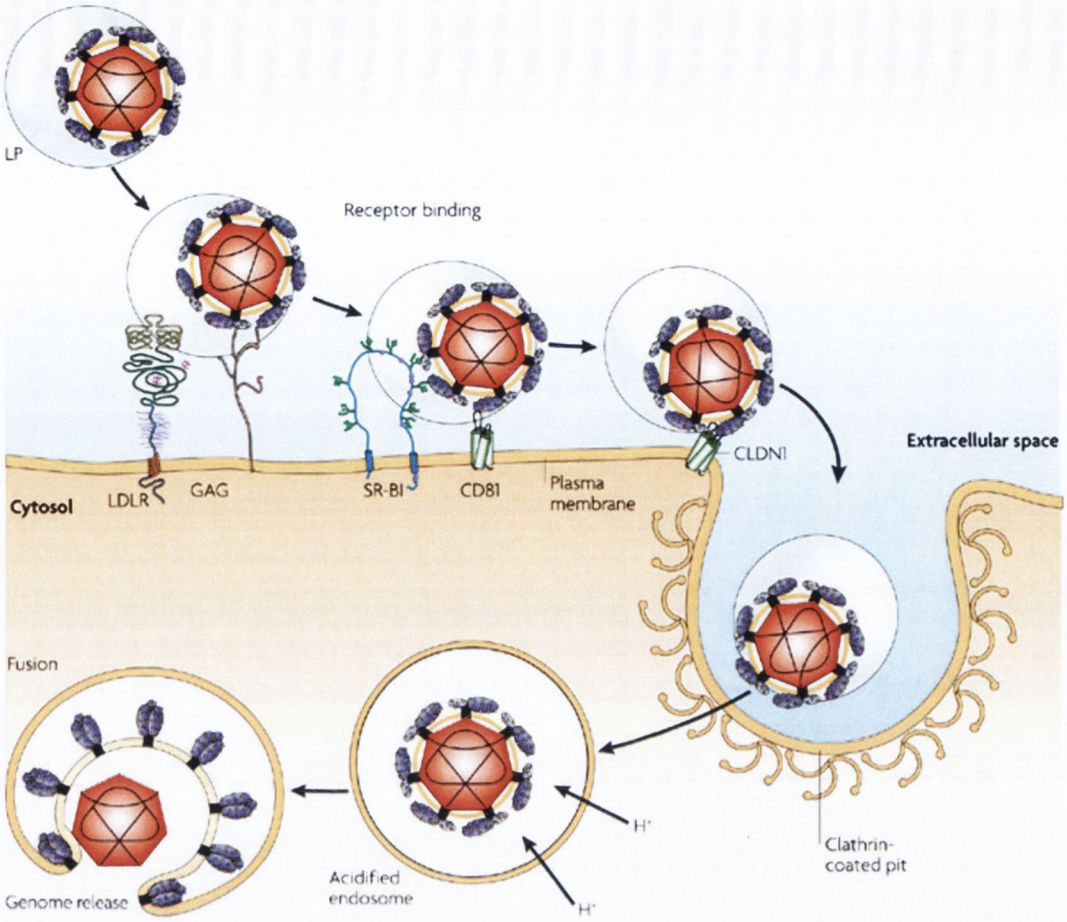



**Figure 1.8 HCV entry.** Viral entry is a multi-level process, which includes cooperative interactions of low density lipoprotein receptor (LDLR), glycosaminoglycans (GAG), scavenger receptor B type I (SR-BI), CD81 receptor and claudin-1 (CLDN1). Internalisation depends on clathrin-mediated endocytosis, while low pH induces fusion of endosome membrane and viral envelope. This is followed by the uncoating process that results in viral RNA release into the cytoplasm. Figure is taken from Moradpour et al, 2007.

Once the process of translation is initiated, the HCV genome produces a single, long polyprotein, that is translocated to the rough ER and is proteolitically cleaved into 10 mature viral proteins. The structural proteins are sliced up by endoplasmatic reticulum (ER) signal peptidase, which processes between C/E1, E1/E2, E2/p7 and p7/NS2. The non-structural region is processed by two viral enzymes, the NS2 autoprotease, which cleaves NS2/3 site and by NS3/4a serine protease which cleaves all remaining sites (Lindenbach and Rice 2005; Moradpour et al, 2007), in the following favoured, but not compulsory order: NS3/4a, NS5a/b, NS4a/b and NS4b/5a (Bartenschlager et al, 1994). **(Figure 1.9)** In addition, HCV 1a also encodes a small protein, up to 160 amino acids in length, called ARFP (alternative reading frame protein) or F (frame shift) which is produced as a result of ribosomal frame shift, which probably occurs near to codon 11 of the core region (Branch et al, 2005).

However, some of the viral proteins have to undergo post-cleavage modifications. E1 and E2 are glycosylated within the lumen of the ER, by addition of oligosaccharide chains on up to 6 sites in E1 and 11 sites in E2. Subsequently, they are coupled into heterodimers (Op De Beeck et al, 2001). These modifications are thought to be critical for viral assembly and infectivity. NS5A is another viral protein that undergoes a chemical modification such as addition of phosphate groups on multiple sites resulting in hyperphosphorylation of the molecule (Reyes, 2002). Since phosphorylation is a conserved characteristic among few viral families, it can indicate the role in HCV life cycle (Reed et al, 1998), where it might be necessary for formation of viral replicon complex or NS5a pathogenesis (Evans et al, 2004; Gao et al, 2004).

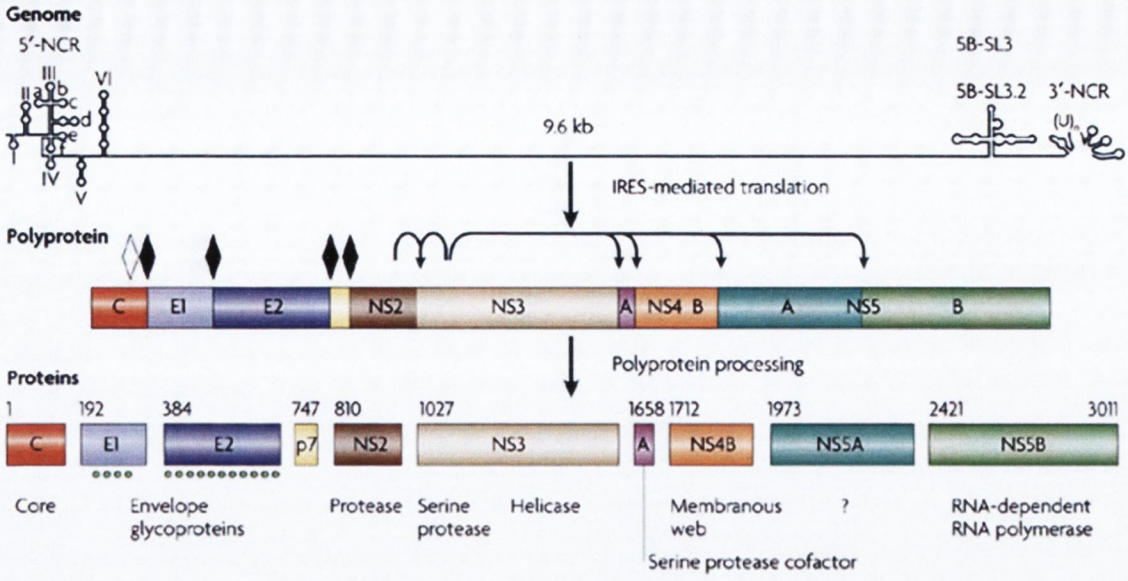



**Figure 1.9 HCV polyprotein processing.** Viral RNA encodes a polyprotein, approximately 3010 amino acids in length, which is proteolitically cleaved into a set of 10 viral proteins. Processing occurs co- and post-translationally and is facilitated by an endoplasmic reticulum signal peptidase (black diamonds), signal peptide peptidase (white diamond) and by viral NS2/3 and NS3/4a proteases (arrows) Figure is adapted from Moradpour et al, 2007.

#### 1.1.5.3 Viral RNA replication

A hallmark of HCV RNA replication is formation of a membrane-associated complex composed of NS proteins, viral RNA, RdRp and altered cytoplasmic membranes, in line with replication described for all positive-strand RNA viruses (Mackenzie 2005; Salonen, Ahola et al. 2005). These membrane alterations are termed "membranous web" and their vesicular ultrastructure was seen in HCV-infected liver in chimpanzees (Egger et al, 2002) and HCV infected Huh-7 cells (Gosert et al, 2003). It has been proposed that NS4b has a crucial role in creation of the web, acting as a scaffold for replicon complex formation (Egger et al, 2002) and that web may be derived from ER membranes (Moradpour et al, 2007). The NS5b RdRp reads the genomic, positive-strand HCV RNA and synthesises the negative-strand of the RNA chain, which being a molecular mirror of the viral RNA subsequently serves as a template to produce multiple nascent, positive-strand genomes.

As many studies have shown, the strand synthesis happens within the replication complex, on the ER membrane (Dimitrova et al, 2003; Pietschmann & Bartenschlager, 2001; Tardif et al, 2002; Waris et al, 2004). Although complementary strands of RNA can bind and form double-stranded RNA (dsRNA), this rarely happens. This is because RdRp can not read dsRNA and it requires positive and negative strands to be separated, which is carried out by the helicase domain of the NS3 proteins (Levin et al, 2004). In addition, the presence of dsRNA is a signal of viral infection for the host cell, which consequently triggers appropriate defence.

It has been noticed that almost the entire 5'-UTR is needed for efficient RNA replication, although a crucial segment for this process is positioned within the first 120 nucleotides. Since this region overlaps with IRES, it is intriguing how this segment can regulate two so different processes. In addition, it was found that a recently discovered conserved structure, 5BSL3.2, within NS5b, interacts and forms long-distance base pairs with 3' 99-nucleotid element that is essential for the replication (Friebe et al, 2005). It is possible that these structures, named cis-acting RNA elements, guide the process of viral replication (Lindenbach & Rice, 2005).

#### 1.1.5.3.1 Generation of quasispecies by error-prone RNA polymerase

Undoubtedly, the key factor during the HCV replication is the enzyme RdRp. This polymerase lacks the proof-reading ability and having no mechanism to correct errors during strand synthesis, it incorporates mutations into newly made RNA (Behrens et al, 1996). Since the estimated production of HCV particles is around $10^{12}$ per day (Neumann et al, 1998), the virus can generate mutations at a very high rate. Several studies, comparing consensus sequences of the isolates obtain from chronically infected individuals at certain time intervals, have revealed that mutational rate might be in the range of 0.816-1.92 x $10^{-3}$ substitutions per genomic site per year (Lu et al, 2001; Rispeter et al, 2000). This could lead to approximately 10 to 20 mutations in HCV RNA genome for each year of infection. Thus, an individual chronically infected with HCV, will harbour a heterogeneous viral population that contains groups of minor genetic variants. These variant sequences, closely related to each other are defined as quasispecies (Gomez et al, 1999). Error-prone RdRp generates viral genome as a swarm of sequences – quasispecies, which may vary from a single mutation to circa 10% within one infected organism (Maggi et al, 1997; Naito et al, 1995).

This dynamic population of quasispecies is possibly responsible for rapid viral evolution. A complex mixture of sequences evolves as a single unit when exposed to the environmental changes, therefore, allowing the virus to amplify the most adapted sequence(s) at any given time, avoiding replicative efforts in unfavourable directions (Cabot et al, 2000).

### 1.1.5.4 Assembly of new viral particles and release from the host cell

Little is known about the late stage of viral life cycle, owing to a lack of appropriate study models. However, this area should now rapidly advance with the models established just 2-3 years ago. Each HCV virion contains positive-single-stranded RNA, capsid shell and the viral envelope, and assembly of these components can be separated into two steps.

It seems that interaction between core protein and 5'-UTR of RNA initiates particle formation, resulting in capsid assembly and RNA enclosure within the capsid shell (Fan et al, 1999; Kunkel & Watowich, 2002; Shimoike et al, 1999). The structure assembled

from capsid shell and viral genomic RNA is known as nucleocapsid. The viral capsid is composed of multiple core protein subunits (at least 420) compiled into an icosahedral shape (Kunkel et al, 2001). Once the nucleocapsid is formed, the second phase of viral assembly may begin, and it results in formation of the viral envelope. However, this process is not yet clear, although retention of E1-E2 heterodimers within the ER prior to viral assembly, indicates that the budding of new HCV virions probably occurs at the ER membrane (Op De Beeck et al, 2001). Moreover, a recent study has shown that core protein may have an important role in directing the budding process, as a single amino acid substitution at the 111 position led to failure of viral particle assembly (Blanchard et al, 2003). However, some viral particles are released without envelope, as they have been found in circulation of chronically infected individuals (Maillard et al, 2001).

The actual release of the viral particles is possibly through the cell secretory pathway, directing the virions from ER through Golgi apparatus towards cell membrane, which has been observed for other members of Flaviviridae family (Serafino et al, 2003). Another route may involve the p7 viral protein, which has been shown to form pores in the cell membrane (Griffin et al, 2003; Pavlovic et al, 2003) in order to increase membrane permeability and facilitate viral release. Similar mechanism has been applied in HIV discharge, where an analogous protein (Vpu) forms an ion channel, resulting in enhancement of viral release (Ma et al, 2002).

## *1.2 IMMUNOBIOLOGY OF HCV*

Acquisition of Hepatitis C virus evokes a very complex immune response in the host, including activation of both innate and adaptive arms of the immune system. As in any other infection, before the initial adaptive response manifests itself, innate immunity is responding at the early stage of infection.

### *1.2.1 Innate immunity*

This part of host defense mechanism is universal, present in plants and animals, with molecular modules having probably evolved before the division into these two kingdoms (Hoffmann et al, 1999).

In humans, when the first line of defense, represented by the physical barrier of skin and mucosa, is compromised, an antigen nonspecific response against the pathogen is delivered by the innate immune system. This system is initiated within a few minutes following the beginning of infection. The innate immune system is composed of many serum proteins (including acute phase proteins and cytokines) and cells, with phagocytes (macrophages, neutrophils, dendritic cells (DC)) and natural killer (NK) cells being essential for functioning of the system (Janeway & Medzhitov, 2002). These cells express pattern-recognition receptors (PRR) responsible for identification of pathogen-associated molecular patterns (PAMPs), which subsequently trigger the cells to perform their effector functions. Since these cells exhibit their functions immediately, without a need to proliferate, the kinetics of the innate immune response is rapid (Medzhitov & Janeway, 2000).

For the innate system it is necessary to develop cooperation among the elements involved, in order to perform highly efficiently. For instance, when a NK cell is triggered, it produces a significant amount of IFN-γ, which then primes macrophages. Activated macrophages in contact with pathogen become hyperactivated and secrete TNF, which binds its receptor displayed on the macrophages, inducing secretion of IL-12. Together TNF and IL-12 further stimulate NK cells to produce more IFN-γ leading to amplification of the initial response. In addition, although NK cells produce IL-2, only after exposure to TNF they express receptors for IL-2, which is an important growth factor that consequently causes proliferation of NK cells (Sompayrac, 2003).

Similarly, interaction between DC and NK cells may be crucial in shaping the innate response. Namely, DCs activate NK cells via production of IL-12 and IL-18 or via expression of NK-activating ligands (Kanto & Hayashi, 2006) **(Figure 1.10)**. In addition, DCs are the most important antigen presenting cells (APC), whose main function is to ingest, process and present antigen to T cells, having a crucial role in networking the innate and adaptive immune responses.

### 1.2.1.1 Type I interferon (IFNs) response and HCV

All nucleated cells in humans are able to produce type I IFNs, which are potent and an early intracellular defense against viruses. Humans with a genetic mutation in both alleles of a protein called signal transducer and activator of transcription-1 (STAT-1) involved in the IFN signaling cascade, developed lethal viral disease at an early age (Dupuis et al, 2003). There are two main members of the Type I IFN family – IFN-α and IFN-β, whose amplification causes cessation or deceleration of viral replication, generating time for host to establish adaptive immune response.

Recently it has been discovered that beside dsRNA, a ssRNA containing 5' triphosphate group induces vigorous activation of IFNs production (Hornung et al, 2006; Pichlmair et al, 2006; Plumet et al, 2007), subsequently leading into activation of more than 300 IFN-stimulated genes involved in antiviral, as well as, antiproliferative and immunomodulatory functions (de Veer et al, 2001; Der et al, 1998). For example, the PAMP-responsive signalling cascade directs nuclear translocation of interferon-regulatory factor (IRF)-3 and nuclear factor κB (NF- κB) to the IFN-β promoter region, leading to transcription and subsequently production of IFN-β by infected cells (Gale & Foy, 2005).

Microarray analyses of liver biopsies, obtained from HCV infected chimpanzees, revealed that at an early stage, HCV induces enhancement in expression of type I IFN genes (Bigger et al, 2001; Su et al, 2002; Thimme et al, 2002). Some studies have shown that IFN-α inhibits HCV subgenomic RNA replication (Frese et al, 2001; Guo et al, 2001), while others have identified IFN-induced proteins with anti-HCV activity, such as protein kinase R (PKR) (Pflugheber et al, 2002),

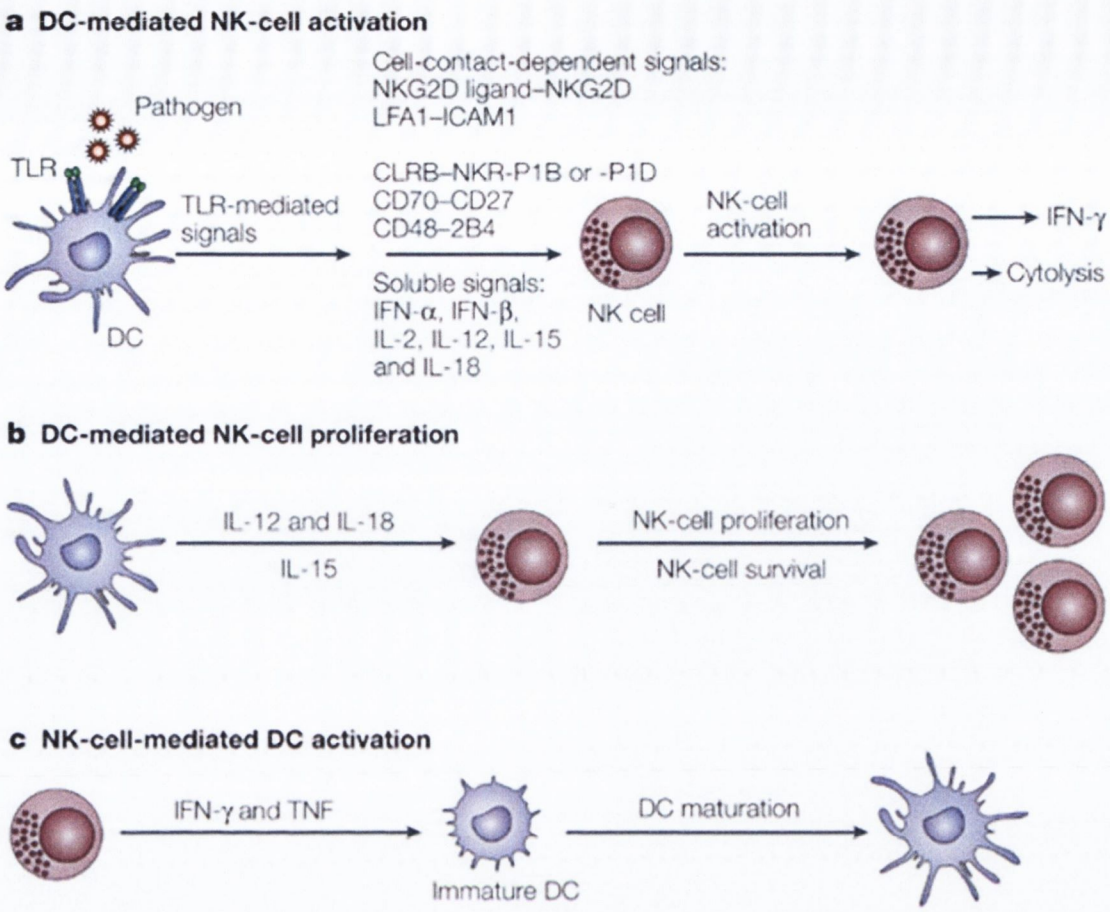



**Figure 1.10 Interactions between dendritic cells (DCs) and natural killer (NK) cells. A)** Activated DCs are potent inducers of NK cells activation. This process is facilitated through different cytokine signals and through a cell-contact dependant mechanism, mediated by adhesion molecules and NK-activating ligands. **B)** Cytokines secreted by DCs are involved in proliferation of NK cells. **C)** Reciprocally, NK cells are implicated in maturation and activation of DCs. Image is adapted from (Degli-Esposti & Smyth, 2005).

RNA-specific adenosine deaminase 1 (ADAR 1) (Taylor et al, 2005), 2'5'oligoadenylate synthetase (OAS)/RNaseL system (Ishii & Koziel, 2008) and p56 (Wang et al, 2003a). PKR has been shown to phosphorylate the α subunit of the eIF2 subsequently leading in the inhibition of the cellular and viral mRNA translation. ADAR 1 catalyses deamination of adenosine to inosine in dsRNA, generating mutations and a less stable form. Endoribonuclease RNaseL, when activated with 2'5'oligoadenilates, degrades viral and cellular RNAs causing viral inhibition. The p56 binds "e" subunit of eIF3, inhibiting ternary complex formation and affecting viral RNA translation (Weber, 2007).

#### 1.2.1.2 NK, NKT cell response in HCV infection

In the first few hours and days during HCV infection, NK and NKT cells are of particular interest, since the human liver contains significantly higher number of these cells than any other organ or peripheral blood (Doherty et al, 1999). NKT cells make up to 30% of all intrahepatic lymphocytes (Guidotti & Chisari, 2001). A study demonstrating that asymptomatic HCV-infected chimpanzees cleared the virus without detectable adaptive immune response, pointed out the important role that NK and NKT cells may have during the acute phase of infection (Thomson et al, 2003). Quickly recruited to the infection site these cells might be involved directly in viral inhibition, through cytolysis of the infected cell, or indirectly, via production of cytokines and chemokines with antiviral activity and through activation of DC and T cells (Ishii & Koziel, 2008). After activation by the type I IFN response, NK cells produce cytokines such as IFN-γ and TNF-α, potent viral inhibitors and chemokines such as MIP-1α and IFN-inducible protein 10 (IP-10), potent NK cell chemoatractants, which cause accumulation of NK cells within the infected liver **(Figure 1.11)**. Recent studies have demonstrated that Huh-7.5 cells co-cultured with NK cells or treated with supernatants obtained from NK cell culture, expressed significantly lower levels of HCV RNA and proteins than untreated cells. This was largely owing to IFN-γ, as this anti-viral activity could be diminished by IFN-γ specific antibody (Li et al, 2004; Wang et al, 2008).
NK cells have an important role in control of the initial phase of viral infection; however, their contribution to arrest of HCV infection has not been fully understood.

#### 1.2.1.3 DC response in HCV infection

DCs have an essential role in connecting innate and adaptive immune responses; hence they are able to uptake and process pathogen and present the derived antigens to the T cells. Based on their precursors, there are two main subsets of DCs: myeloid (mDC) and plasmacytoid (pDC), which produce distinctive cytokines causing difference in CD4+ T cell activation. Triggered mDCs secrete IL-12 and TNF-α, driving CD4+ T cells to a Th1 response, whilst pDCs secrete significant amount of IFN-α causing the expression of Th2 phenotype by CD4+ T cells (Liu, 2001; Liu et al, 2000).

Successful clearance of Hepatitis C virus is associated with maintenance of vigorous T cell responses, with predominantly Th-1 type of immune activity (Kamal et al, 2004; Lechner et al, 2000; Thimme et al, 2001). Taking that T cell activation mainly depends on signals received through antigen presentation (Steinman et al, 2000) and knowing that DCs are possibly the most efficient inducers of immune responses (Szabo & Dolganiuc, 2005), it is likely that DCs may have an important role in determining outcome of HCV infection (**Figure 1.11**).

During HCV infection, immature DCs are activated either through pattern-recognition receptors or via E2 interaction with some of the receptors expressed on the DC (SR-BI, DC-SIGN and CD81) and are subsequently involved in initiation of the immunological cascade and T cell priming. Their importance is confirmed by many studies of chronic HCV infection which have demonstrated that some viral proteins modulated or impaired functions of DCs (Gowans et al, 2004; Pachiadakis et al, 2005) probably facilitating a favourable milieu for viral persistence.

### *1.2.2 Adaptive immunity*

The adaptive immune system is evolutionary younger than innate immunity, developed only in vertebrates and it utilises different principles in immune defense from those implied in innate response (Medzhitov & Janeway, 2000).

Adaptive immunity is alerted while innate immunity is dealing with the pathogen and once the front line of defense fails to eliminate invading organism and when the threshold of antigen is exceeded, adaptive system is fully recruited. The main features of this type of immunity are high antigen specificity and memory of previously

encountered pathogens. During initial infection, the adaptive immune system is slow to start, but rapidly excludes the invader when the organism is rechallenged with the same pathogen. The adaptive immune system is composed of humoral and cellular immunity, which are highly connected and interrelated (Janeway, 2001).

The humoral immune response protects the extracellular space, where antibodies are produced by activated B cells. Antibodies neutralise the pathogen by opsonisation, preparing it for phagocytosis, thus preventing it from cell entry. The naïve B cell is activated when it encounters antigen but, very often, successful triggering requires co-stimulation, which is mostly provided by activated CD4+ T cells when its CD40 ligand (CD40L) molecule associates with CD40 receptor on the surface of B cells.

All antibodies have the same structure derived from paired heavy and light polypeptide chains, which is termed the immunoglobulin (Ig) structure. The antibody molecule contains two heavy chains, linked via disulfide bond, and two light chains, where each of them is linked to heavy chain via disulfide bond. There are two types of light chains: lambda and kappa. Both, heavy and light chains contain variable domain (V), responsible for interaction with the antigen, and constant domain (C). According to their C region, antibodies are classified into five groups: IgA, IgG, IgD, IgE and IgM with IgG representing approximately 75% of all antibodies in the blood (Janeway, 2001; Sompayrac, 2003).

The cellular components of adaptive immunity are mainly CD4+ T cells, otherwise known as helper T cells (Th) and CD8+ T cells, known as cytotoxic T lymphocytes (CTLs).

When naïve Th cells are activated, the major cytokine they produce is IL-2. Following IL-2 induced clonal proliferation, re-stimulation of these cells by APC, leads to secretion of their two subsets of cytokines which determine the fate of the immune response. A Th1 type of response is predominated by the production of IL-2, IFN-γ and TNF. While a Th2 response is characterised by elevated levels of IL-4, IL-5, IL-6, IL-10 and TGF-β (Mizukoshi & Rehermann, 2001). Th1 cells prime macrophages and induce B cell IgG production (IFN-γ), they activate NK cells and previously primed macrophages (TNF) and stimulate proliferation of NK cells, CTLs as well as their own proliferation (IL-2). Th2 cells initiate the humoral immune response and production of

IgM, IgE (IL-4), IgA (IL-5) and inhibits macrophage activation (IL-10). Both types of responses are derived from Th0 cells which have potential to differentiate into Th1 cells when present in an environment rich in IL-12, the cytokine secreted by activated macrophages with engulfed bacterial or viral pathogen. On the other hand, Th0 cells develop into Th2 when exposed to a milieu rich in IL-4, typical for parasitic infection. Interestingly, both Th1 and Th2 cells secrete IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF), which stimulate bone marrow to produce granulocytes, macrophages and DCs, all important in cell-mediated immunity. Of note, there is a negative feedback loop between Th1 and Th2 responses, where IFN-γ inhibits proliferation of Th2 cells, whilst IL-10 prevents proliferation of Th1 cells (Janeway, 2001; Sompayrac, 2003).

The main role of CTL is to induce apoptosis in a target cell in an antigen-specific manner. Upon recognition of antigen, a CTLs release cytolytic granules in a calcium dependant manner. Included in these granules are two cytotoxic proteins: perforin and granzyme. The perforin polymerises to form pores in the cell membrane. Transmembrane pores destroy the integrity of the cell membrane allowing water and ions to pass rapidly into the cells. The granzymes are serine proteases that activate apoptosis once in the cytoplasm of the target cell. These proteases cleave caspase 8 in the cytoplasm, activating downstream enzyme cascades (effector caspases) leading to DNA degradation and programmed cell death (Medema et al, 1997; Sarin et al, 1998). Both enzymes are necessary for efficient cell elimination. However, there is a perforin-independent pathway of cytotoxicity that involves a binding of Fas ligand, expressed on CTLs and even on some Th1 cells, to Fas receptor on infected host cells. This interaction triggers caspases, consequently leading to apoptosis. In addition, activated CTLs also produce certain cytokines, such as IFN-γ that directly inhibits viral replication, activates macrophages and increases the expression of MHC-Class I molecules in target cells, TNF-α/β, which is also involved in macrophage activation and importantly IL-2 that drives clonal cell expansion and differentiation (Janeway, 2001; Sompayrac, 2003).

### 1.2.2.1 Humoral response in HCV infection

In HCV infection, virus-specific antibodies are usually detectable two months after inoculation (Pawlotsky, 1999). Since the most frequently targeted epitopes are situated in HVR1 region of E2, the antibodies probably neutralise homotypic virus, which has been demonstrated in a study on chimpanzees (Farci et al, 1994). However, when the animals were challenged with a different viral genotype, the infection persisted (Gowans et al, 2004). Mutations within the HVR1 region were observed during seroconversion (Farci et al, 2000), and it is thought that they represent viral escape and adaptation to antibody driven immune selection pressure (Bowen & Walker, 2005).

Recent studies using HCVpp system have shown neutralisation of viruses with different genotypes using antibodies isolated from chronically infected individuals. However, minimal levels of these antibodies were detectable in subjects who cleared the virus (Bartosch et al, 2003a; Logvinoff et al, 2004; Meunier et al, 2005), indicating existence of other contributors to the resolution of infection. The role of humoral protection in HCV is still poorly understood and at the same time questioned, as two studies revealed no protection during reinfection of immune chimpanzees or humans (Farci et al, 1992; Lai et al, 1994).

### 1.2.2.2 Cellular response in HCV infection

A large body of evidence suggests that CD4+ and CD8+ T cells have a crucial role in determining the outcome of HCV infection (Diepolder et al, 1995; Lechner et al, 2000; Thimme et al, 2001; Wedemeyer et al, 2002). Numerous studies have shown that vigorous, sustained and multi-epitope specific responses were present in individuals who spontaneously cleared the virus, whilst in chronically infected the T cell responses were late, transitory and focused on a narrow array of epitopes (Lechner et al, 2000; Thimme et al, 2001).

CD4+ and CD8+ T cells have essential, but distinct roles in prevention of chronicity. CD8+ T cells are primary effector cells that mediate immunity, while CD4+ cells have a role in long-term control of the virus and are required for functioning of CD8+ cells **(Figure 1.11)** (Klenerman & Hill, 2005; Shoukry et al, 2003).

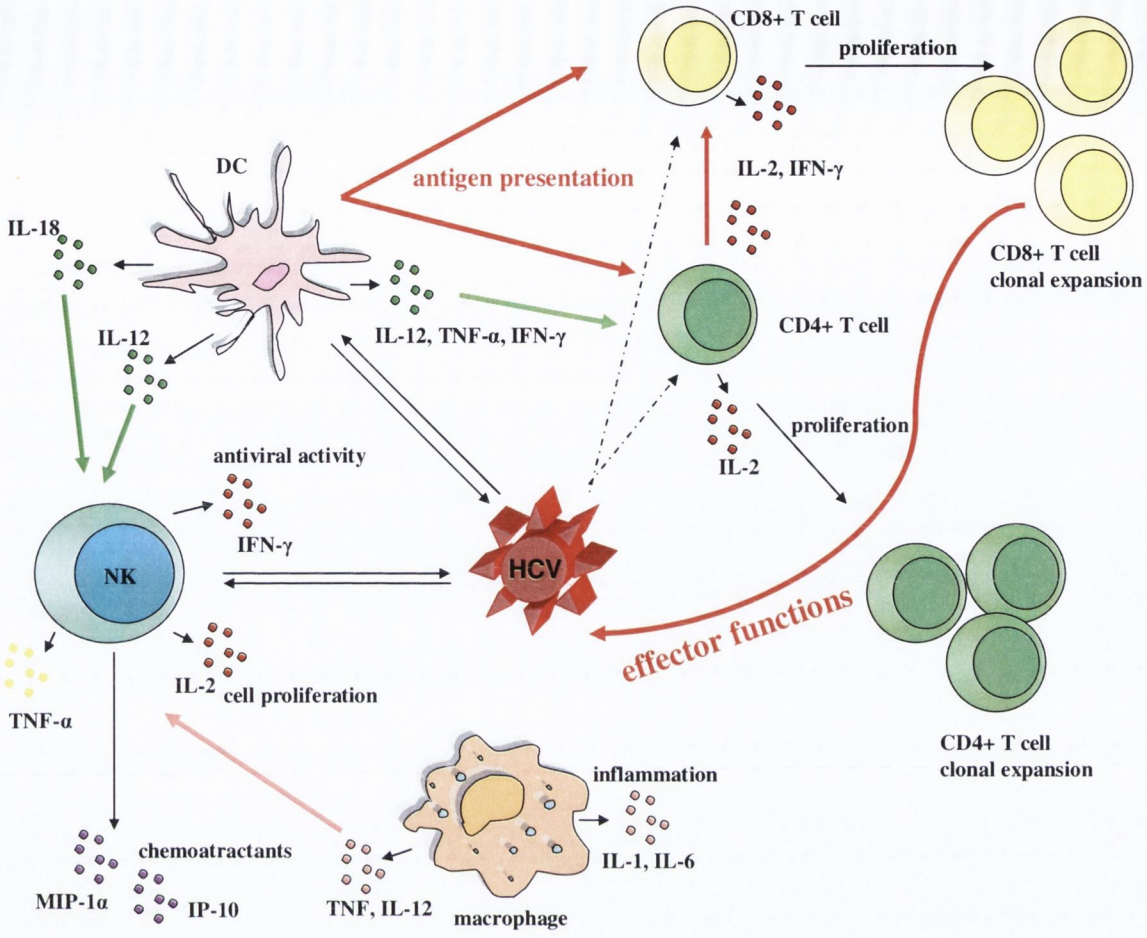



**Figure 1.11 Response of the cells of the innate and adaptive immune system to HCV infection.** The cells of the innate immune system are first to respond to HCV. When they encounter the virus, they become activated and start producing different cytokines, which have direct antiviral activity (IFN-γ, TNF-α), deliver stimulatory signals to other cells of the innate system (IL-12, IL-18), initiate inflammation (IL-1α/β, IL-6) or act as chemoatractants that recruit inflamatory cells to the site of infection (MIP-1α, IP-10). During this cascade of events, the viral antigens are presented to T cells, mainly by DC, which are the professional antigen presenting cells that link the innate and adaptive immune system. When primed, T cells secrete IL-2, which induces their clonal proliferation and development of effector functions. Differentiated effector cells, particularly CD8+ T cells, tackle infection and eliminate the virus.

#### 1.2.2.2.1 CD4+ T cells

CD4+ T cells are believed to be critical in HCV antiviral immunity, providing vital 'help' for both cellular and humoral parts of immune system. These cells deliver co-stimulatory signals to B cells, activate APCs and prime and sustain CD8+ T cell responses.

Animal studies have shown clear evidence of the pivotal role of CD4+ cells in the control of HCV infection. One study has analysed the CD4+ T cells, obtained from liver biopsies of acutely infected chimpanzees. The cells which were able to recognise and proliferate in response to HCV proteins, were not found in animals who failed to clear the virus, but were identified in connection with both transient and permanent viral control (Thimme et al, 2002). This and another similar study, have further demonstrated that HCV specific CD4+ and CD8+ cells were accumulated 8-14 weeks after inoculation, which was synchronised with viral clearance and liver inflammation (Cooper et al, 1999; Thimme et al, 2002).

Grakoui et al. proved the essential role of CD4+ cells in protective immunity, when using antibodies, depleted these cells in recovered chimpanzees before they re-challenged them with the virus. Re-challenged animals were not able to clear the virus and presented persistent low level viraemia, despite expressing a strong virus specific CD8+ response in the liver (Grakoui et al, 2003).

Early studies on patients with symptoms of acute hepatitis C, indicated the important role of HCV-specific CD4+ cells in control of infection (Diepolder et al, 1995; Missale et al, 1996). Furthermore, sequential studies revealed the presence of robust CD4+ T cell responses in self-limited HCV infections (Gerlach et al, 1999; Lechner et al, 2000; Rosen et al, 2002). In some cohorts this immunity was maintained for up to 20 years after exposure to the virus (Takaki et al, 2000).

Data showing that CD4+ cells are essential in modulation of anti-HCV responses were also obtained from studies in settings of allograft transplants for HCV-related end-stage liver disease. Roughly half of the patients receiving the transplant displayed significant CD4+ T cell responses to HCV antigens despite immunosuppression (Rosen et al, 1999; Schirren et al, 2001). Additionally, augmentation in number of CD4+ T cells in

liver recipients was observed with application of antiviral therapy following transplantation. In some patients this response correlated with elimination of the virus (Schirren et al, 2003).

A study on healthcare workers who acquired the virus through needle-stick injuries, also contributed to the dissection of possible CD4+ responses and outcomes of infection. In this study it was observed that some individuals failed to clear the virus and subsequently developed chronic disease, although they exhibited exceptionally vigorous HCV-specific CD4+ response (Thimme et al, 2001). This study suggested three possible paths that can emerged from CD4+ responses (**Figure 1.12**). The first is production of functional and robust CD4+ T cell response, which persists and leads to viral clearance. The second is characterised with the same vigorous, initial response, but owing to yet unelucidated reasons, capitulates to a rebound in viraemia resulting in viral persistence. In the third path no detectable CD4+ responses are present, which certainly leads to HCV chronicity (Shoukry et al, 2004).

A breadth and specificity of CD4+ T cell responses also seem to be critical in determination of infection. Namely, a study by Day et al. demonstrated that an average of 10 epitopes, out of 37 identified, were recognised in individuals with resolved infection, in comparison to no more than one epitope recognised by patients with viral persistence. Circulating virus-specific CD4+ T cells from those who clear the virus, targeted simultaneously a minimum of 4, and up to 14 epitopes located in core, NS3, NS4 and NS5 viral proteins, months and even years, after HCV RNA was no longer measurable in serum (Day et al, 2002). Numerous studies confirmed that the hallmark of self-limited infection was the ability of virus-specific CD4+ T cells to target both structural and non-structural HCV proteins, with the latter being more targeted (Diepolder et al, 1997; Gerlach et al, 1999; Lechner et al, 2000; Rosen et al, 2002; Thimme et al, 2002; Thimme et al, 2001).

A recent study, using a panel of peptides covering the entire viral polyprotein, described the T cell responses in 16 patients with acute infection. Strong, broad and Th1 orientated CD4+ responses were identified in resolvers, unlike weak and narrow responses revealed in those who developed chronic infection (Urbani et al, 2006).

Previously it has been shown that PBMCs obtained from patients who resolved HCV infection expressed a Th1 cytokine profile, whilst those with chronic infection displayed a Th2 profile, suggesting that Th1 rather than Th2 is associated with a successful immune response in the early phase (Tsai et al, 1997b). A Th1 response was dominant when cytokines were measured during the acute phase of infection (Gerlach et al, 1999) and more frequent in resolvers, indicating the correlation between IFN-γ and IL-2 cytokines and favourable outcome of infection (Gramenzi et al, 2005). Studies supporting this evidence have shown that HCV-specific CD4+ cells were impaired in IFN-γ secretion and proliferation (Folgori et al, 2006; Ulsenheimer et al, 2003) or demonstrated loss of IL-2 secretion (Semmo et al, 2005) in chronic infection when compared to cells obtained from subjects who resolved the infection.

#### 1.2.2.2.2 CD8+ T cells

Effector functions of CD8+ T cells incorporate two overlapping mechanisms: cytolytic elimination of infected target cells and non-cytolytic secretion of anti-viral cytokines. Cytotoxicity includes secretion of perforin and granzymes which cause the destruction of target cells, whereas produced cytokines, such as IFN-γ and TNF-α, evoke different pathways to create anti-viral environment within infected organism.

Similar to CD4+ T cells, animal studies have shown that CD8+ T cells migrate to the liver and play an important role in the clinical outcome. Accumulation of virus-specific CD8+ cells coincided with elevated levels of liver enzymes and at least transient clearance of viral RNA from plasma. Chimpanzees that lacked detectable CD8+ responses developed chronic infection (Cooper et al, 1999; Thimme et al, 2002). In a subsequent study, antibody-mediated depletion of CD8+ cells, prior to re-challenge of the animals, demonstrated prolonged viraemia despite presence of memory CD4+ cells. When intrahepatic CD8+ cell were recovered, approximatelly 6 weeks after inoculation, viral replication was terminated (Shoukry et al, 2003).

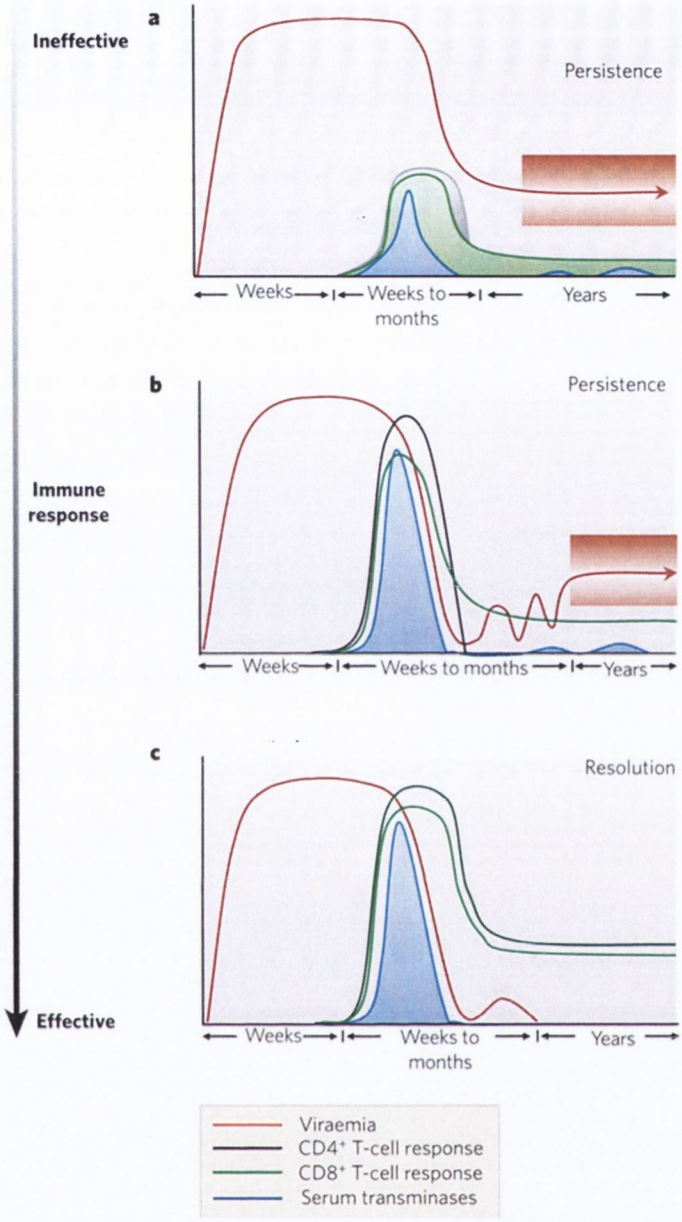



**Figure 1.12 Possible profiles of cellular immune responses to HCV during acute infection. A)** When CD4+ (black line) and CD8+ (green line) T cell responses are weak and insufficient, viraemia (red line) is never controlled. The virus persists and outcome is chronic infection. **B)** When initial CD4+ and CD8+ T cell responses are vigorous and sufficient to control infection, but the CD4+ T cell response becomes reduced, viraemia rebounds and infection persists. **C)** Vigorous and sustained CD4+ and CD8+ T cell responses lead to viral clearance and recovery. Serum transaminase levels (blue line) follow the pattern of cellular responses in all three cases. This image is taken from Bowen & Walker 2005.

In human subjects, accidentally exposed to the virus via needle-stick injury, an appearance of HCV-specific CD8+ population, 7 weeks after the acquisition, correlated with increased levels of transaminases, but not with noteworthy decline of viral load. Of note, at this early stage virus-specific CD8+ cells had no capacity to secrete IFN-γ, but when this was restored, a significant drop in viral titer and resolution of infection was observed (Thimme et al, 2001). Similarly, in a patient with acute infection only around 20% of detected CD8+ T cells secreted IFN-γ upon cognate antigen stimulation, whilst this was enhanced to more than 80% when virus was eradicated, 10 weeks later (Lechner et al, 2000). Moreover, during acute infection the ability of CD8+ cell to exhibit cytotoxicity and proliferative capacity was also impaired, as demonstrated by the same studies. Similar data have been observed in other human and animal studies (Gruener et al, 2001; Shoukry et al, 2003; Urbani et al, 2002) indicating that retention of CD8+ effector features, referred to as 'stunned' phenotype, is a consistent characteristic of acute HCV infection, regardless of the clinical outcome (Shoukry et al, 2004). Several studies have observed that demonstration of effector functions matched with the expression of CD8+ T cell activation markers such as CD38, CD69 and MHC Class II molecules, which decreased once when the infection was either restrained or evolved to persistence (Lechner et al, 2000; Gruener et al, 2001; Thimme et al, 2001).

As with a CD4+ response, the main characteristic of self-limited infection is a broad and multispecific CD8+ T cell response. Targeting of multiple epitopes was demonstrated in the acute phase of resolving infections (Cox et al, 2005; Lechner et al, 2000; Thimme et al, 2001), with CD8+ cells directed against eight to twelve epitopes as revealed by a study from Lechner et al. (Lechner et al, 2000). Equally, a study by Cucchiarini et al. demonstrated higher frequency of CD8+ cells directed against multiple epitopes, in both structural and non-structural viral proteins, in subjects who cleared the virus than in those who developed persistence (Cucchiarini et al, 2000). Similarly, in an animal study it was observed that nine epitopes, spread throughout the whole viral genome, were targeted by intrahepatic CD8+ cells in one chimpanzee (Cooper et al, 1999).

In patients with chronic infections CD8+ responses were weak or targeted a narrow selection of epitopes (Lauer et al, 2004; Lechner et al, 2000). This was also demonstrated in chimpanzee models (Cooper et al, 1999; Thimme et al, 2002). In some persistent infections multispecific responses were also produced as it was observed in several studies in humans (Koziel et al, 1993; Koziel et al, 1995; Koziel et al, 1992; Urbani et al, 2005). In one study, the span of intrahepatic virus-specific CD8+ responses ranged to up to five epitopes in a cohort of chronically infected individuals (Wong et al, 1998) suggesting that viral persistence can occur despite the sporadic multispecificity. Similarly, in an animal study, eight different epitopes were targeted by CD8+ cells in chimpanzees who had been infected for over a decade (Erickson et al, 2001).

Early studies had no evidence that some of the CD8+ T cell epitopes may be more dominant than others. Also, a study analysing the CTL epitope database failed to identified epitopes clustering in any particular region of the HCV genome (Ward et al, 2002). However, a recent study on a well defined cohort infected with HCV, revealed a single epitope restricted by the protective HLA*B27 allele to be associated with viral clearance. Almost three decades after inoculation, this epitope was targeted by CD 8+ T cells in five out of six tested individuals who resolved infection. CTLs obtained from acutely infected HLA*B27 carriers were directed against six epitopes, which also included this epitope, positioned within the NS5b viral protein. Moreover, amino acid substitutions within this epitope led to viral persistence (Neumann-Haefelin et al, 2006) . These findings supported the idea that some epitopes may be immunodominant, preferentially targeted by CTLs in order to clear the infection.

#### 1.2.2.2.3 Memory T cells

Successfully controlled acute infection resulting in viral clearance, establishes robust and enduring memory T cell populations. Maintenance of these cells has a critical role in creation of protective immunity. However, the mechanism that supports the persistence of memory T cell population for many years after exposure to the HCV is not fully understood.

Studies on single source outbreaks have shown that most individuals who resolved the infection had vigorous HCV-specific T cell responses, two or three decades after the inoculation (Takaki et al, 2000; Neumann-Haefelin et al, 2006). A large study on a cohort of intravenous drug users compared the prevalence and persistence of HCV viraemia in individuals who have never been exposed to the virus and subjects who previously resolved the infection. It was observed that those infected for the first time had a 12 times higher probability of developing persistent infection than those who had earlier cleared the virus (Mehta et al, 2002), indicating protective immunity in re-exposed subjects gained through previous infection.

Similar findings were revealed in animal studies, when immune chimpanzees exhibited an increased level of protection from chronic infection upon viral re-challenge (Bassett et al, 2001; Major et al, 2002). Moreover, it was shown that a decrease in duration and peak of viraemia temporally coincided with substantial T cell memory responses with expansion of memory T cells in the blood (Nascimbeni et al, 2003; Shoukry et al, 2003). It was also demonstrated that T cells obtained from liver biopsies of chimpanzees who spontaneously cleared the virus several years ago, were able to recognise HCV antigens, suggesting the establishment of durable intrahepatic memory as well (Shoukry et al, 2003).

During acute HCV infection, naïve antigen specific CD8+ cells become activated, proliferate and acquire effector functions. Most of the effector CD8+ T cells undergo apoptosis, while 5-10% go through differentiation and maturation into a new phenotype known as CD127+CD8+ T cells, which proliferate establishing protective immunity (Urbani et al, 2006). The IL-2 cytokine secreted by the CD4+ T cell subset has a crucial role in expression of CD127 and acquisition of the memory phenotype (Fuller et al, 2005) **(Figure 1.13)**. In addition, intrahepatic effector memory T cells vary from those in the periphery, by expression of the CD69 molecule, thought to be a marker of tissue-resident CD8+ memory cells (Hogan et al, 2001).

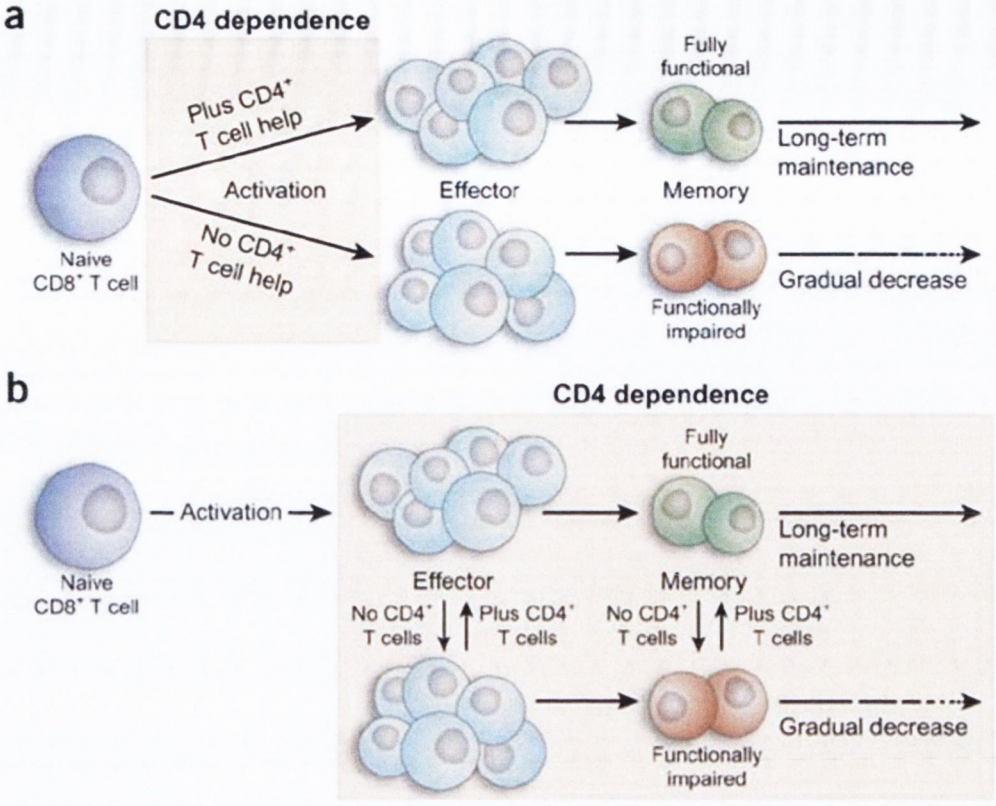



**Figure 1.13 Models of CD4+ T cell-mediated maintenance of CD8+ T cell memory.** **A)** Programming model. CD4+ T cells help is necessary during the CD8+ T cell priming for development of CD8+ T cell effector functions as well as for imprinting the ability to differentiate into potent and useful memory cells. **B)** Environmental model. CD4+ T cell help is required later in CD8+ T cell response. According to this model CD8+ T cells are not irreversibly programmed by CD4+ T cells and preservation of memory function depends on availability of CD4+ cells in the environment. Helped and unhelped CD8+ T cells interconvert through the life of CD8+ T memory cells. Image is taken from (Hamilton et al, 2004).

### 1.2.3 Cytokines and HCV in liver pathogenesis

Hepatitis C virus is involved in a severe liver disease. Cytokines have a role in defining the host response to the HCV and consequently liver pathogenesis. It is though that liver damage is more immune-mediated, caused by infiltrating lymphocytes and cytokines; than by the direct cytopathic effect of the virus (Poccia & Agrati, 2003).
The acute phase of HCV infection does not affect normal liver histology and function (Ramadori & Armbrust, 2001). In the case of failed viral clearance, immune mechanisms are not efficient enough to eliminate the virus, but are sufficient to mediate liver damage during viral persistence. However, this can not exclude the role that the immune system may have in controlling HCV replication during chronic infection, preventing the excessive levels of viral RNA (Spengler & Nattermann, 2007). Data obtained on intrahepatic CD8+ T cells and viral load in chronic infection are conflicting, therefore it is still not clear whether or not they contribute to regulation of viral replication (Bowen & Walker, 2005).

In response to hepatocellular stress, cytokines and chemokines are secreted mainly by Kupffer cells (KCs) (IL-8, IL-1α, IL-1β), but also by sinusoidal endothelial cells (MIP-1), hepatocytes (IL-8), hepatic stellate cells (HSCs) (MCP-1) and lymphocytes (MIP-1), and recruit inflammatory cells to the site of injury. Recruited macrophages, together with KCs and NK cells, secrete proinflamatory cytokines such as IFN-γ and TNF-α. Secreted TNF-α has proved to be a key mediator of inflammation as it induces an increase in proinflamatory cytokines, chemokines and, most importantly, causes changes in expression of cell adhesion molecules on sinusoidal endothelial cells, with augmentation of intracellular adhesion molecule-1 (ICAM-1), leading to employment and sinusoidal transmigration of inflammatory cells (Bissell et al, 1995; Neubauer et al, 1998; Neubauer et al, 2000). Infiltrating cells include T and B cells (Novobrantseva et al, 2005; Salmi et al, 1998), eosinophils, neutrophils, mast cells (Ramadori & Saile, 2004a), macrophages and NK cells (Bioulac-Sage et al, 1996). The cytokine milieu within the liver is crucial to the activation of HSCs (Grappone et al, 1999; Luo et al, 2005), which then secrete bone morphogenetic protein-6 (BMP-6) (Knittel et al, 1997), interferon growth factor-1 (IGF-1) (Scharf et al, 1998), transforming growth factor-beta

1 (TGF-β1) (Kanzler et al, 1999) and platelet derived growth factor (PDGF) (Breitkopf et al, 2005), resulting in an autocrine loop for the activation and differentiation of these cells into myofibroblast-like cells (MFB) (Neuman et al, 2008).

Chronic hepatocellular damage, involving multi-cellular interactions between resident liver and inflammatory cells, leads to development of liver fibrosis and subsequently cirrhosis. Transforming HSCs increase the production of extracellular matrix proteins (ECMs) which is strongly stimulated by TGF-β1 (Kanzler et al, 1999). Moreover, this cytokine inhibits the degradation of the ECMs and promotes the differentiation of HSCs into MFB, resulting in scars formation and loss of tissue function **(Figure 1.14)** (Bataller & Brenner, 2005; Ramadori & Saile, 2004b). When TGF-β1 binds its plasma membrane serine-threonine kinase receptor, the downstream effect is activation of Smad proteins. The Smads are a family of proteins that control gene expression when they translocate to the nucleus. Smad1 and Smad2, which in phosphorylated form assemble heterodimeric complexes with Smad4, translocate to the nucleus, where they activate transcription of collagen types I and II and fibronectin (Cheng et al, 2004). Some viral proteins, such as core and NS3 have been shown to increase the expression of TGF-β1 (Cheng et al, 2004; Shin et al, 2005), stimulating liver pathogenesis.

PDGF is another potent profibrogenic cytokine acting as a strong mitogen for activated HSCs (Bataller & Brenner, 2005; Ramadori & Saile, 2004b). Another cytokine, specific for HCV, (Lau et al, 2005) and considerably overexpressed in chronic infection and cirrhosis, is activin A, strongly induced by IL-1β and TNF-α (Elsammak et al, 2006).

Matrix metalloproteinases (MMPs) are biomarkers also specific for HCV fibrosis. MMP-1, the main collagen type I degrading enzyme, is significantly reduced, whilst the tissue inhibitor of this MMP (TIMP1) is enchansed by TNF-α and IL-1β. In addition, MMP2 and MMP9, involved in ECM modification, are overexpressed in liver pathogenesis (Leroy et al, 2004) and it has been observed that viral core and NS5a proteins induce their production in the liver (Nunez et al, 2004).

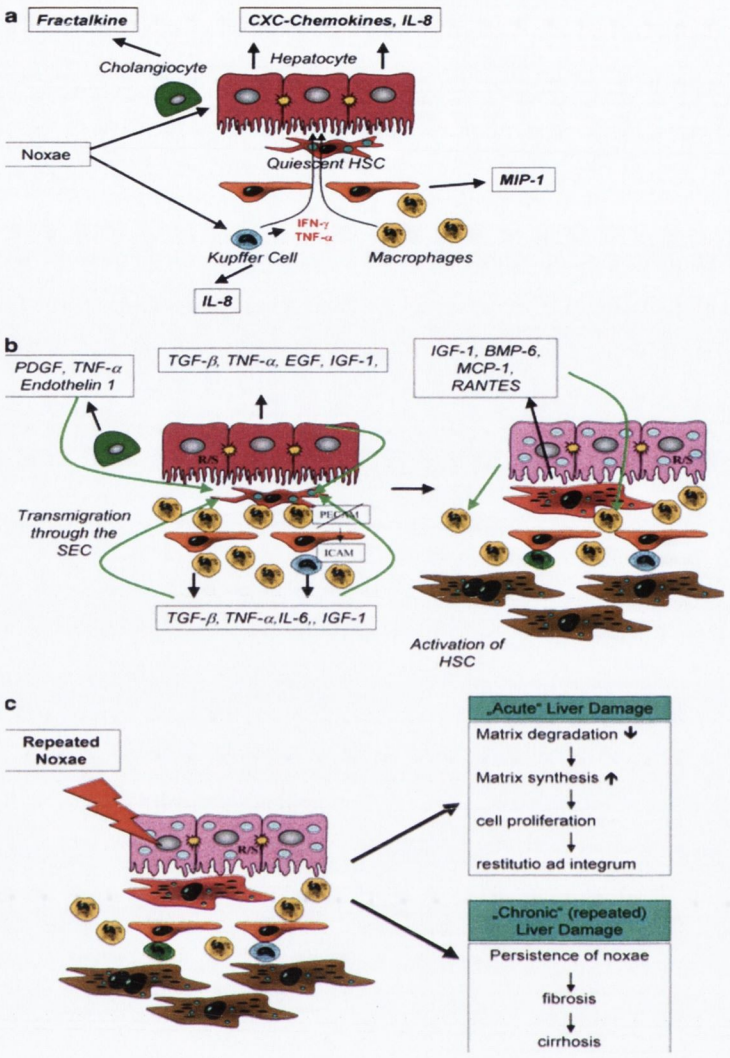



**Figure 1.14 Development of liver injury and fibrogenesis. A)** A hepatocellular stress induces release of cytokines and chemokines by Kupffer cells (KCs) (IL-8), sinusoidal endothelial cells (MIP-1), hepatocytes (IL-8), hepatic stellate cells (HSCs) (MCP-1) and lymphocytes (MIP-1). **B)** This cytokine/chemokine milieu recruits inflammatory cells, mainly macrophages, which then secrete IFN-γ, TNF-α, IL-6 and TGF-β leading to HSC activation. Activated HSCs produce IGF-1, BMP-6, TGF-β1 and PDGF, which further contribute to the differentiation of HSCs into myofibroblast-like cells (MBCs). **C)** TGF-β1 and PDGF stimulate the transformed HSCs to increase secretion of extracellular matrix proteins (ECMs) and to differentiate into MBCs, which results in tissue remodeling, scar formation and fibrosis. This image is taken from Neuman et al, 2008.

Inflammation in the liver is present as long as the virus; and lengthy infection that contributes to prolonged inflammation, results in progression to fibrosis (McCaughan & George, 2004). During this time infiltrated inflammatory cells try to eradicate infected cells via apoptosis, which is followed either by recovery or a new cycle of inflammation and damage, resulting in replacement of the lost tissue through fibrogenesis (Neuman et al, 2008).

Since HCV uses numerous and complex strategies to evade immune defense, the immune system employs different specific and nonspecific mechanisms in order to control the HCV. When all components work appropriately the result is elimination of virus-infected cells and subsequently viral clearance. When the components deal inadequately with the

infection, activation of apoptotic pathways and exclusion of infected cells lead to liver damage, fibrosis and cirrhosis associated with significant morbidity and mortality (Guyot et al, 2006). A balance between viral clearance and chronic inflammation in HCV-mediated immune activation will determine the outcome of infection, possibly in the first few weeks of acquisition (Spengler & Nattermann, 2007).

## *1.3 ANTIGEN PRESENTATION*

### *1.3.1 HLA alleles*

The key players in the field of antigen presentation are molecules of the Major histocompatibility complex (MHC). These molecules have an important role in the immune system and are encoded by a large gene family found in most vertebrates. In humans, these genes are referred to as human leukocyte antigen (HLA) with a coding region located across a 3.6 Mbp on the short arm of chromosome 6 (6p21.31). HLA genes have a crucial role in the determination of immune responses, because these genes encode the products responsible for antigen presentation to T cells. Variant genes occupying the same locus are called alleles, and the combination of different HLA alleles on a single chromosome is termed the HLA haplotype. The more diverse these alleles, the more capable the molecules are of binding and presenting a wide variety of antigens. There are two classes of the genes, with three different genes in each of the classes: HLA Class I with HLA*A, B, C and HLA Class II with HLA*DR, DQ, DP genes and with more then 200 alleles for some of these genes. The products of these alleles can vary from each other by up to 20 amino acids, contributing to a quite significant diversity. Since humans are diploid, we have two copies of each gene, and HLA expression tends to be codominant, which gives us many variables in terms of characterisation of the immune profile (Janeway, 2001; Steinmetz & Hood, 1983).

### *1.3.2 MHC Class I molecules*

MHC Class I molecules are expressed on every nucleated cell in humans, where they display a peptide fragment, derived from an intracellular pathogen (viruses and certain bacteria) or endogenous proteins, to the CD8+ T cells. The MHC Class I molecule is a heterodimer, composed of a 346 amino acid long polypeptide chain, called α or heavy chain and one short, 99 amino acid long chain, called $\beta_2$ microglobulin ($\beta_2$M). A heavy chain, encoded by one of the polymorphic HLA Class I genes, consists 5 domains:

- three extracellular domains α1, α2, α3
- a transmembrane domain
- a cytoplasmic domain

$\beta_2$M, encoded by a monomorphic gene on chromosome 15, is attached to a heavy chain by a number of noncovalent interactions with a function to stabilise the MHC molecule **(Figure 1.15A)** (Janeway, 2001; York & Rock, 1996).

Between the α1 and α2 domains lies a region bounded by a β-pleated sheet (8 antiparallel β- sheets on the bottom) and two α helices on the sides (Bjorkman et al, 1987). This region is called the "groove" and is capable of binding (via noncovalent interactions) a small peptide, 8 – 10 amino acid (aa) residues in length (Rammensee et al, 1995). The side chains of two amino acids insert into the pockets of the MHC molecule which anchors the peptide in the binding cleft. These residues are called anchor residues and their position within the peptide can vary, although the second amino acid at the N-terminus and the carboxy terminal amino acid are the most frequent anchor sites in 9 aa peptides. Different MHC molecules often have diverse anchor residues with the hydrophobic or basic amino acid frequently present at the C-terminus. Substitution of anchor residue can prevent peptides from binding or recognition by T cells, resulting in pathogen persistence **(Figure 1.16)**. This small peptide is "presented" to a CD8+ T cell and defines the antigen, the so-called "epitope", that the T cell specifically recognises. Peptide bound to the groove, becomes an integral part of the MHC molecular structure, which then achieves the most stable state reliably indicating an infection within the cell (Janeway, 2001; Sompayrac, 2003).

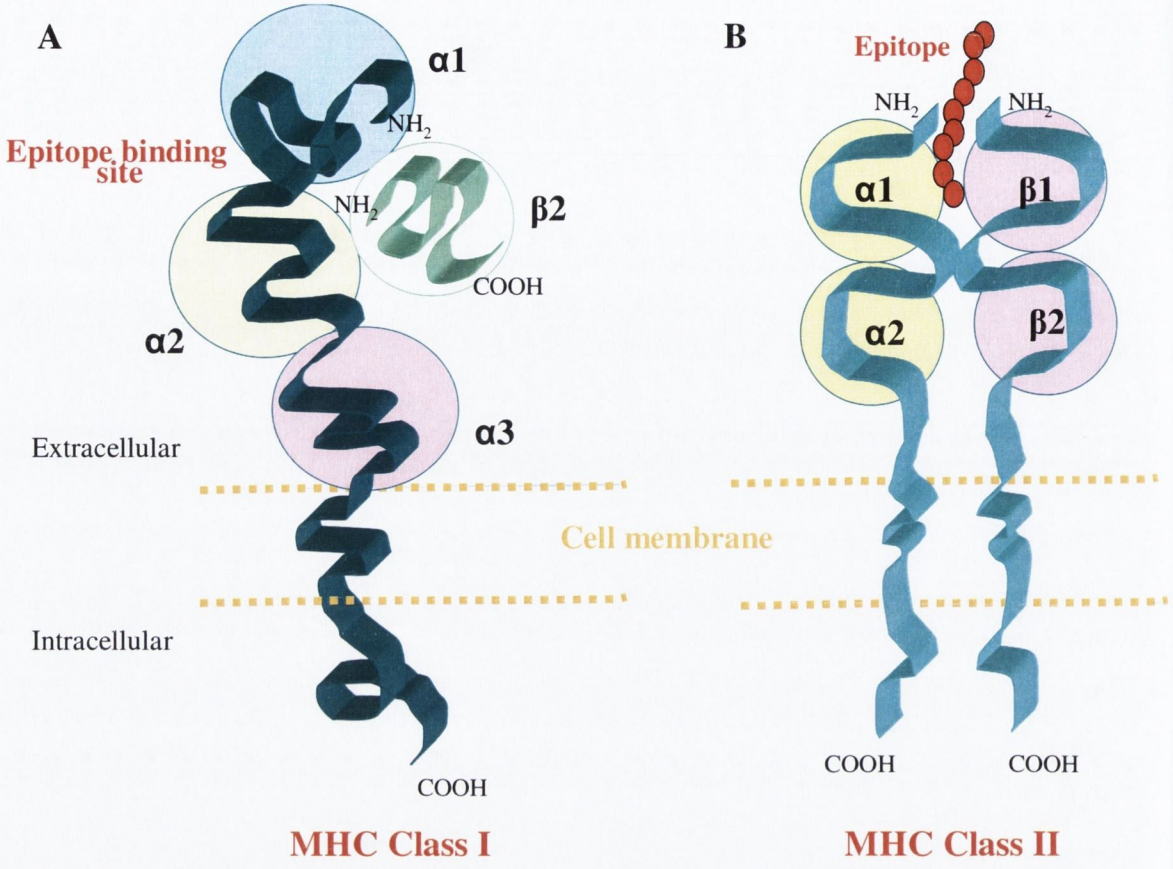



**Figure 1.15 The antigen presenting molecules. A)** MHC Class I molecule is a heterodimer composed of two polypeptide chains: a long, polymorphic heavy chain and a short chain called $\beta_2$ microglobulin. The heavy chain contains intracellular, transmembrane and three extracellular domains (α1, α2 and α3), where α1 and α2 form the binding groove for peptides to be recognised by CD8+ T cells. **B)** MHC Class II molecule is composed of two polymorphic, polypeptide chains, named α and β. Both chains have intracellular and transmembrane domains, as well as, two extracellular domains that form the binding groove for peptides to be presented to CD4+ T cells.

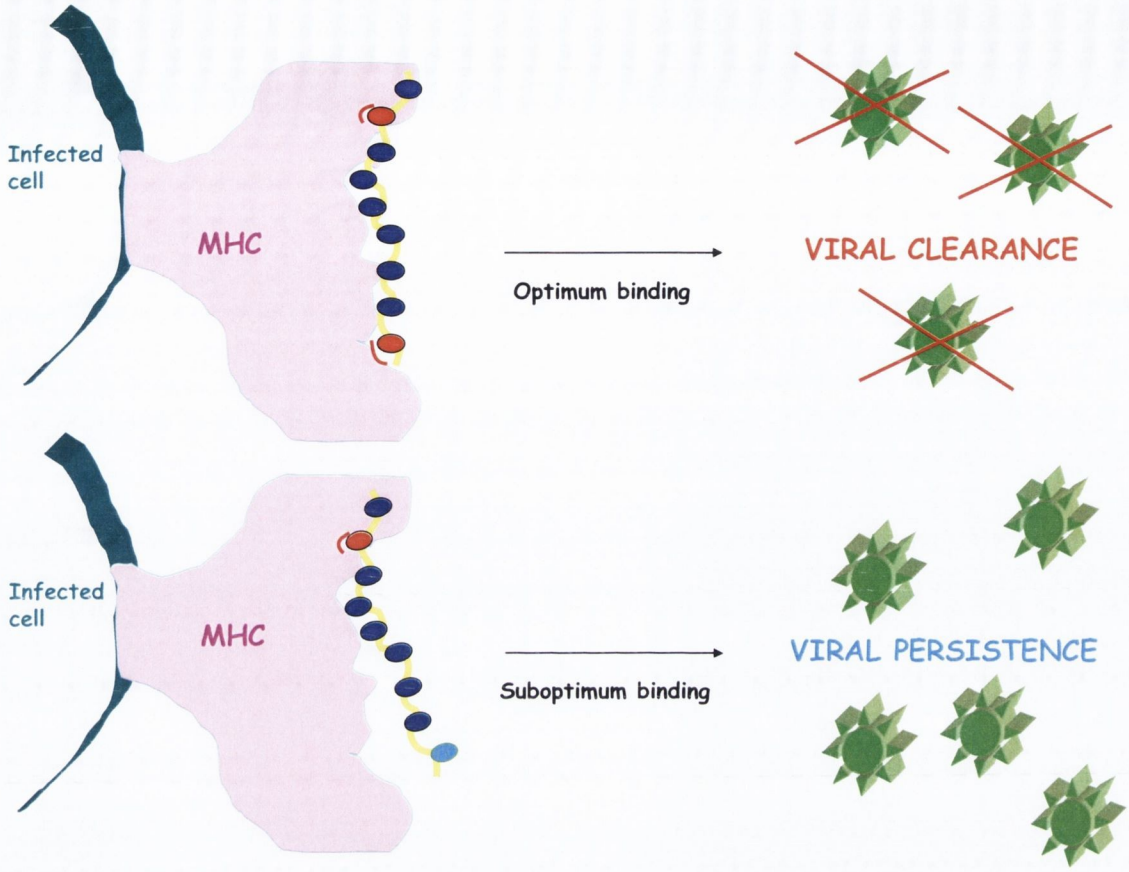



**Figure 1.16 Significance of amino acid substitution(s) within the epitope for MHC-peptide binding.** The residues in the peptides that bind to the MHC molecules are known as anchor residues (in red). Each type of MHC molecules bind specific anchor residues and if they vary, the binding may be disturbed. Substitution of amino acid in the anchor site (in light blue) may result in inadequate binding, which leads to impaired presentation to T cells and inability to generate a T cell response to the virus. If the changed anchor residue binds the MHC groove, recognition by the TCR may fail, which again results in inappropriate immune response and viral persistence.

### *1.3.3 MHC Class II*

MHC Class II molecules are found on specialised antigen-presenting cells such as dendritic cells, macrophages and B cells, which display a peptide from an extracellular pathogen (many bacteria and eukaryotic parasites) or exogenous proteins, to CD4+ T cells. The MHC Class II molecule is composed of a noncovalent complex of one α and one β chain (each about 240 aa, containing subunits 1 and 2) **(Figure 1.15B)** which form a three-dimensional structure similar to Class I. However, the main difference between these two molecules lies in the binding cleft, which is more open in Class II molecules, making them able to bind much longer peptides. The core of the peptide that binds the groove is 9 aa long, with a total length of 13-17 aa in most cases, although the length can vary from probably 12 to up to 30 aa. Defining the anchor residues in these epitopes seems to be more difficult, since Class II binding grooves are more tolerant in their accommodation of diverse amino acid side chains. Hence, some peptides can be bound to more than one MHC, defining them as promiscuous epitopes, which is probably an advantage for a genetically diverse human population (De Groot et al, 2004; Sette & Sidney, 1998).

### *1.3.4 Antigen processing*

The peptides presented by MHC class I are mainly generated in the cytosol by the multicatalytic protease complex called the proteasome. This is a large cylindrical structure composed of four stacked rings, each built from seven subunits, with a cavity, known as the core, which has proteolytically active sites. Intracellular proteins are introduced into the core of the proteasome and are degraded into short peptides, which are subsequently released into the cytosol (Monaco & Nandi, 1995). Expression of proteolytically active subunits within the proteosome is stimulated by interferons, produce in response to viral infection. In intereferon-producing cells there is increased cleavage of the proteins after either hydrofobic or basic residues, resulting in production of peptides with preferred anchor residues for MHC Class I molecules. Following proteasomal processing, peptides are translocated from the cytosol into the ER to assemble with the MHC class I molecule,

whose peptide-binding site is in the lumen of the ER. The peptide translocation is accomplished by the Transporter associated with Antigen Processing (TAP), a heterodimer of TAP1 and TAP2 proteins. Once the peptide is loaded onto the MHC class I molecule, previously assembled within the ER lumen, it leaves the ER to reach the cell surface through the secretory pathway that involves several posttranslational modifications of the MHC molecule (Janeway, 2001; Sompayrac, 2003).

The peptides presented by MHC class II are produced in acidified endocytic vesicles, since extracellular pathogens are engulfed by macrophages or immature DC. The pH in endosomes containing exogenous protein decreases, subsequently activating acid proteases. Cleaved peptides are loaded onto MHC Class II within the vesicles. The pre-synthesised MHC molecule is assembled with a protein called invariant chain (Ii) within the ER, which prevents peptide binding to the Class II molecule. This complex is directed by the Ii to the vesicle with the antigens, where acid proteases cleave Ii to CLIP protein (class II-associated Ii peptide), which is then replaced with a peptide via a reaction catalysed by a class II-like molecule, HLA*DM (Janeway, 2001).

### *1.3.5 T cell receptor (TCR)*

The T cell population expresses around 30,000 T cell receptors (TCR) on their surface. Every T cell has a unique TCR that provides the specificity for an individual T cell to recognize its particular antigen. The vast majority of TCRs are composed of two polypeptide chains termed α (TCRα) and β (TCRβ) chains, linked via disulfide bond within short segments responsible for receptor cell membrane attachment (**Figure 1.17**). Both of the chains have variable (V) and constant (C) domains. The TCR recognises and binds the antigen embedded in the MHC groove. This recognition is "MHC-restricted" because the TCR also interacts with MHC molecule. Also, CD8 molecule on the CTL interacts with both α2 and α3 domain of the MHC Class I molecule, whilst CD4 on the Th cell binds the β2 subunit of Class II in order to strengthen the association between TCR and the antigen-MHC complex (**Figure 1.18**). Both co-receptors enhance the sensitivity of TCR for antigen presented by MHC by approximately 100 fold. The TCR alone, however, can not transduce a signal since the intracellular domains of α and β chains are only a few amino acids in length. To

overcome this, the TCR is linked with a series of accessory chains named the CD3 complex (γ, δ, ε) and with ζ chains, whose cytoplasmatic tails are sufficiently long and contain suitable domains required to signal (**Figure 1.17**). The TCR complex and its co-receptor (CD8 or CD4) are associated with the protein kinases, Fyn and Lck, which upon activation phosphorylate ζ chains, enabling them to bind the cytosolic tyrosine kinase ZAP-70. This kinase phosphorylates different adaptor proteins, which in turn leads to activation of important signalling pathways that result in transcription of genes, involved in differentiation, proliferation and development of effector functions of T cells (Janeway, 2001; Sompayrac, 2003).

Full antigen-specific clonal proliferation of T cells requires an additional, co-stimulatory signal, delivered through the interaction between CD28 receptor on the T cell and B7 molecule on the APC. Furthermore, activated T cells express additional proteins that maintain or modify co-stimulatory signal. For instance, CD40L on T cells binds CD40 receptor on APC, resulting in the enhancement of B7 molecule expression. Once activated, T cells express CTLA-4 receptor that binds B7 with 20 times higher avidity, but this interaction delivers inhibitory signals to the activated T cell. (Induction of a receptor called inducible co-stimulator (ICOS) was also observed, but its association with its ligand LIKOS on the APC has not yet been clarified (Janeway, 2001; Sompayrac, 2003).

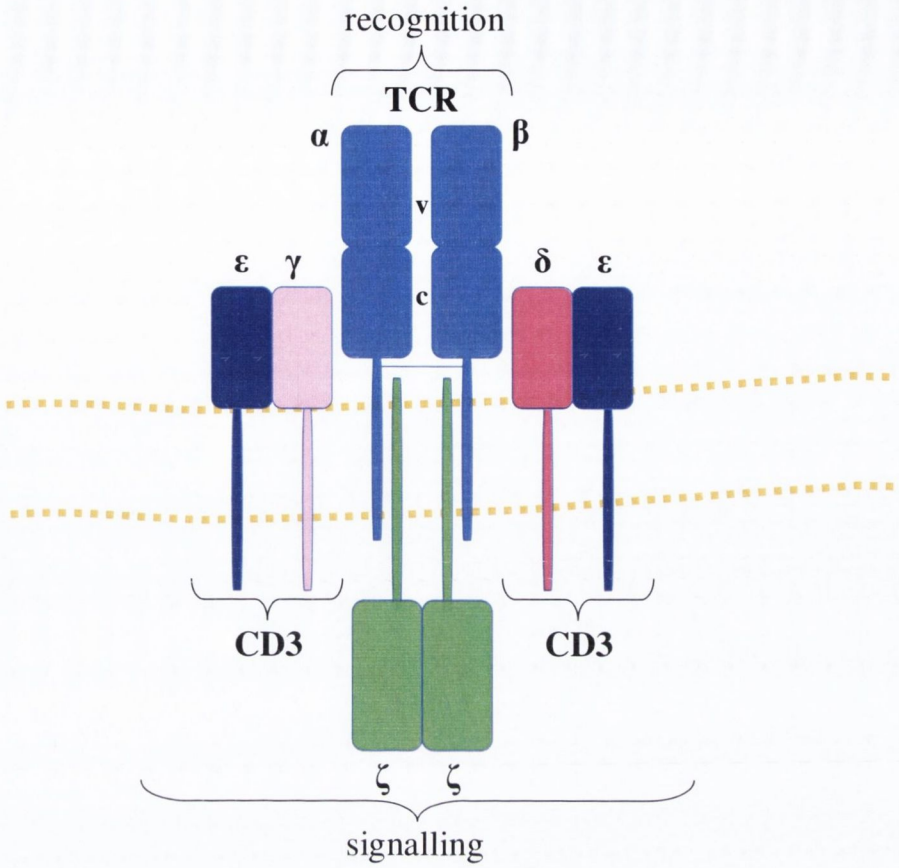



**Figure 1.17 The T cell receptor (TCR) complex.** TCR is found only on the surface of T cells. It is a dimer, composed of α and β polypeptide chains (rarely T cells express TCR that consists γ and δ chains), which both have constant (c) and variable (v) regions. The variable domains of both α and β chains form antigen recognition/binding site. Intracellular domains of TCR α and β chains are only few amino acids long, which is insufficient for signalling. Therefore, to be able to signal the TCR is associated with the CD3 complex (composed of γ, δ and ε chains) and with ζ chains. The cytoplasmatic tails of these molecules are sufficiently long and contain suitable domains required to signal.

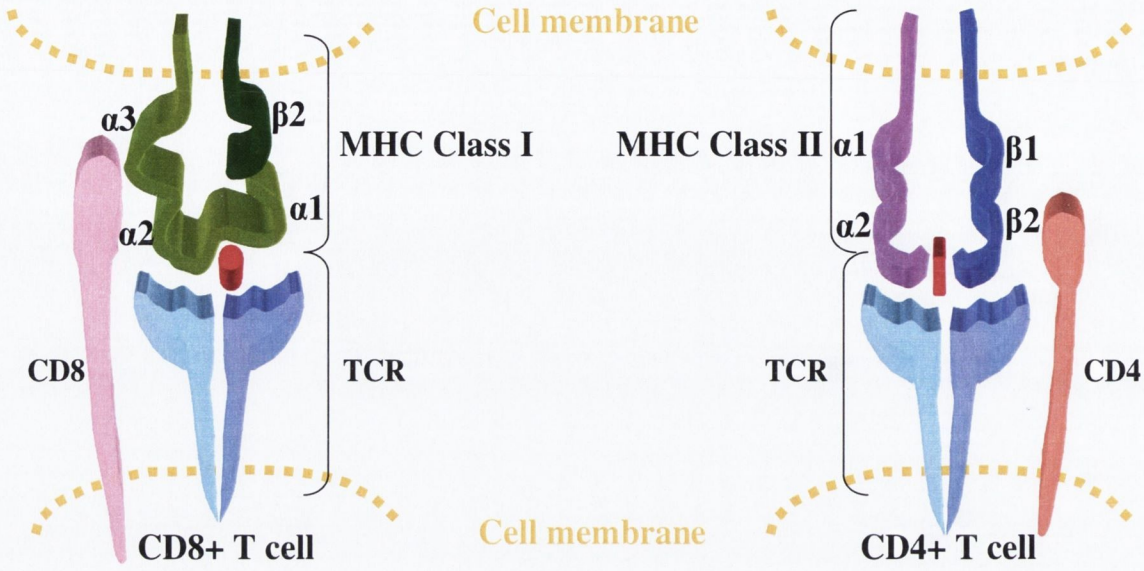



**Figure 1.18 T cell recognition of antigen peptides presented by MHC molecules.** The T cell receptor (TCR) recognises a small peptide that is derived from a pathogen, bound to an MHC molecule and presented on the cell surface. This recognition is MHC restricted. CD8+ T cells express a CD8 molecule, which is a ligand for MHC Class I molecule and interacts with nonpolymorphic regions of α2 and α3 subunits of extracellular domains in the heavy chain of MHC. CD4+ T cells express a CD4 molecule that binds β2 subunit in MHC Class II molecule. Both interactions serve to strengthen and stabilise the interaction between TCR and presented peptide.

## *1.4 HCV IMMUNE ESCAPE*

The Hepatitis C virus is very skillful in subverting the immune system, making itself one of the most successful persistent viruses in humans. HCV applies a complex combination of processes such as interference with signaling pathways, effector cell modulation and generation of genetic variations to evade the host defense at multiple levels. These highly sophisticated strategies have been evolved for both innate and adaptive immune systems (**Figure 1.19**). Besides being able to neutralise host immune response, it resists IFN-based therapy, continuing persistence for the life time of most infected individuals (Gale & Foy, 2005).

### 1.4.1 Subversion of innate immunity

There is large body of evidence suggesting that HCV interferes with type I IFN responses (Thimme et al, 2006). For instance, NS3/4a, a viral serine protease, inhibits activation of viral-induced IRF-3, consequently affecting the transcription of the IFN-β gene (Foy et al, 2003). A recent study has demonstrated that hepatocytes, stained positive for a high abundance of the viral NS3 protein, had IRF-3 excluded from their nuclei (Lau et al, 2008), suggesting impaired type I IFN resposes. HCV core protein blocks activation or translocation of the STAT-1 protein (Bode et al, 2003; Lin et al, 2006), which is required for IFN signalling and also enhances its degradation (Lin et al, 2005; Lin et al, 2006). Moreover, the NS5a viral protein has been shown to annul PKR activation (Gale et al, 1997; Pflugheber et al, 2002) by suppressing its dimer formation (Gale et al, 1998), essential for enzyme activity. The NS5a also stimulates IL-8 production, resulting in partial inhibition of IFN antiviral activity (Polyak et al, 2001) and obstructs the 2'5'OAS/RNaseL pathway (Taguchi et al, 2004), affecting the nucleolytic cleavage of viral RNA.

A recent study has demonstrated that NK cells obtained from chronically infected individuals were impaired in their ability to prime DCs, through increased levels of inhibitory receptor CD94/NKG2A, while in healthy controls this was not observed. In addition, NK cells modified DCs to shift the cytokine secretion by CD4+ T cells towards a Th2 response, with production of TGF-β1 and IL-10, central to the establishment of chronicity (Jinushi et al, 2004). Moreover, a study by Meier et al.

described a dramatically decreased number of NK cells and their dysfunction in peripheral blood of chronically infected subjects (Meier et al, 2005), which could be rescued with *in vitro* IL-15 stimulation.

Impairment of DCs by HCV is still controversial, since some studies have shown their dysfunction in HCV infection, while others failed to demonstrate a defect in DCs in settings of chronic disease. It has been reported that mDCs generated from HCV infected patients had impaired capacity to stimulate allogeneic CD4+ T cells (Auffermann-Gretzinger et al, 2001; Bain et al, 2001; Kanto et al, 1999), indicating that inappropriate antigen presentation by DCs might contribute to the incompetent maintenance of sustained virus-specific T cell responses. In contrast, findings by several studies confirmed the presence of functional DCs in persistent HCV infection (Longman et al, 2004; Piccioli et al, 2005), which was also demonstrated in chimpanzees (Larsson et al, 2004). Furthermore, another animal study has confirmed the presence of functionally impaired DCs, but at the same time, they were not a requirement for persistent infection (Rollier et al, 2003). However, the dramatically decreased number, immature phenotype and reduced IFN-α producing capacity of circulating DCs, observed during acute phase of infection (Ulsenheimer et al, 2005), could contribute to the weak and delayed T cell responses, favouring persistence (Bowen & Walker, 2005).

### 1.4.2 Exhaustion of T cells

During many viral persistent infections the effector functions of CD8+ T cells are thought to be greatly impaired. A hierarchical loss of function, starting with defects in IL-2 secretion, proliferation and cytotoxicity, and followed by failure in IFN-γ production was termed cell exhaustion (Wherry et al, 2003). This phenomenon was, for the first time, described in an earlier study that reported deletion of antigen-specific CD8+ T cell populations in an experimental murine model of persistent infection – the LCMV model (lymphocytic choriomeningitis virus) (Moskophidis et al, 1993). Moreover, a study by Zajac et al. in the same model of infection has shown that virus-specific CD8+ cells were present, but they lacked effector functions (Zajac et al, 1998).

More recently several studies of chronic HCV infection have indicated that virus-specific CD8+ cells are possibly functionally impaired (Gruener et al, 2001; Urbani et al, 2002; Wedemeyer et al, 2002) and functional loss of CTLs has been correlated with weak or absent virus-specific CD4+ T cell responses (Khanolkar et al, 2004). In addition, some studies have shown that when viral load was high and CD4+ help absent, virus specific CD8+ cells exhibited complete functional impairment (Fuller & Zajac, 2003; Ou et al, 2001; Wherry et al, 2003). It is thought that CD4+ cells are affected in a similar manner (Brooks et al, 2005; McNeil et al, 2001), demonstrated in a study showing that HCV-specific CD4+ cells were present, but dysfunctional, as they failed to secret IFN-γ and to proliferate (Ulsenheimer et al, 2003).

Notably, the mechanism responsible for HCV-induced T cell exhaustion has not been elucidated. It is believed that numerous immunomodulatory molecules may be involved in this regulation. For instance, the hallmark of chronicity is switching Th responses, with impaired secretion of pro T cell-survival cytokine IL-2 and increased production of anti-inflammatory cytokine IL-10. A recent study demonstrated that IL-2 levels were dramatically increased in individuals with HCV-specific CD8+ cells who successfully resolved the infection compared to those who developed persistence. IL-2 produced by HCV-specific CD4+ cells may have a critical role in priming the CD8+ cells and their proliferation into virus-specific effectors as well as differentiation into fully functional HCV-specific memory cells (Smyk-Pearson et al, 2008). On the other hand, IL-10 has been shown to suppresses Th1 cytokine production and CD4+ and CD8+ T cell proliferation and *in vitro* antibody-mediated inhibition of IL-10 receptor in chronic HCV infection, resulted in an increase in virus-specific CD4+ responses with an antiviral Th1 pattern of secretion (Rigopoulou et al, 2005).

An exhaustion of T cells, common for many chronic viral infections, has been connected with the inhibitory receptor programmed death-1 (PD-1), almost simultaneously, by groups working on the murine LCMV model (Barber et al, 2006) and on HIV infection (Day et al, 2006; Trautmann et al, 2006). These studies revealed that PD-1 was significantly increased on the surface of functionally impaired virus-specific CD8+ T cells and it was positively correlated with viral titer and reduced capacity of cytokine production and T cell proliferation.

Recent evidence has implicated PD-1 in development of persistent HCV infection. Several studies have demonstrated significant upregulation of PD-1 on total and virus-specific CD8+ T cells, in both peripheral blood (Golden-Mason et al, 2007; Penna et al, 2007) and liver (Golden-Mason et al, 2007; Radziewicz et al, 2007), of individuals with chronic HCV infection. Moreover, *in vitro* antibody-mediated inhibition, using antibodies specific to its ligands PD-L1 and PD-L2, led to reconstitution of IL-2 and IFN-γ secretion, and CTL proliferation. This indicates that the PD-1/PD-L1 pathway may be critical in establishment of persistence and a possible novel target for recovery of exhausted CD8+ T cells (Golden-Mason et al, 2007; Penna et al, 2007). A similar study has demonstrated that anti-PD-L1-mediated restoration, with improved expansion and cytokine production of CD8+ cells, was correlated with lower viral load, lower expression of PD-1 and peg-IFN-α treatment (Urbani et al, 2008). However, a more recent study reported that expression levels of the PD-1 molecule on HCV-specific T cells were high early in acute phase and they were sustained on most of the cells in resolved infection period and during the persistence, thus suggesting that this marker alone is independent of infection outcome (Kasprowicz et al, 2008).

A recent microarray study has identified the molecular signature of CD8+ cell exhaustion in the murine LCMV model, comparing dysfunctional with effector or memory virus-specific T cells. Data generated in this study showed overexpression of several inhibitory molecules, including PD-1; key changes in TCR and cytokine signalling pathways; altered expression in genes involved in chemotaxis, adhesion and migration as well as expression of a distinct array of transcription factors (Wherry et al, 2007) indicating the astonishing molecular complexity of the cell exhaustion process.

### 1.4.3 Regulatory T cells

T regulatory (Treg) cells are phenotypically and functionally distinct CD4+ and CD8+ T cell subsets involved in the regulation of the adaptive cellular immune response (Nelson, 2004). They function by maintaining the critical balance between activation and down-regulation of protective immunity (Gremion & Cerny, 2005). These cells suppress activation, proliferation, differentiation and effector functions of immune

cells, thereby controlling immune responses to self-antigens, pathogens and tumours. They can be divided into two major groups: natural Treg cells (nTregs), developed in the thymus and inducible Treg cells (iTregs) which develop in the peripheral circulation (Manigold & Racanelli, 2007). Recent evidence, has implicated regulatory T cells in modulation of immune responses in viral infections, including HCV (Boettler et al, 2005; Manigold et al, 2006)

An early study observed the existence of a HCV-specific CD8+ T cell subset, derived from the liver of a chronically infected individual, which was capable of producing the suppressive cytokine IL-10, indicating a potential inhibition of antiviral T cells (Koziel et al, 1995). These results gained attention when it was recently demonstrated that an intrahepatic CD8+ T cell subset, obtained from chronically infected, inhibited in vitro proliferation of T cells derived from liver, in a HCV-specific and IL-10 dependant manner (Accapezzato et al, 2004). Moreover, a study on HCV liver biopsies revealed that HCV-specific, IL-10-secreting CD8+ T cells were not able to control viral replication, but in contrast attenuated inflammation, hepatocellular necrosis and hepatic fibrosis (Abel et al, 2006).

Besides the intrahepatic CD8+ T regs, it has been observed that HCV infected patients had significantly higher number of CD4+ CD25+ Tregs in peripheral blood, than individuals who cleared the virus. Depletion of these cells enhanced the frequency of HCV-specific CD8+ cells and their IFN-γ production, indicating the possible role of this cell subset in suppression of HCV-specific CD8+ responses (Boettler et al, 2005; Cabrera et al, 2004; Sugimoto et al, 2003). A recent animal study has reported an increased frequency of nTregs, CD4+CD25+FoxP3+ regulatory T cells in chronic infection, however similar levels were present in resolvers. This indicates that Tregs may be involved in regulation of activation and prevention of apoptosis of HCV-specific memory T cells (Manigold et al, 2006). As the authors of the study suggested, it seems that HCV alters this Treg population during the course of infection.

Two mechanisms used by Tregs to mediate their suppressive activity have been described and it is most likely that both of them are involved in this regulation (O'Garra & Vieira, 2004; Shevach, 2002). Several groups have indicated that IL-10 and TGF-β

cytokines may have an important role in Treg suppressive functions (Cabrera et al, 2004; Sugimoto et al, 2003), aligning the predominant cytokines of chronic HCV infection with the characteristic Treg cytokine profile (IL-10, TGF-β). On the other hand, some authors indicate requirement of cell-cell contact for delivery of suppressive function (Boettler et al, 2005; Ng et al, 2001; Rushbrook et al, 2005), but this contact-dependant mechanism is not completely understood.

Interpretation of data obtained on regulatory T cells in HCV infection has proved to be difficult. Some studies have reported that these Tregs could suppress CD8+ T cells specific to other viruses, such as CMV, EBV and influenza (Boettler et al, 2005; Rushbrook et al, 2005), exhibiting a general regulatory ability to inhibit antiviral responses. Moreover, since Tregs have a dual role in HCV infection, limiting collateral tissue damage caused by protective response on one hand, and inhibiting the immune response on the other hand, Tregs would be difficult target to manipulate in therapeutic purposes (Gremion & Cerny, 2005).

### 1.4.4 Escape mutations

HCV, like other RNA viruses, has a low fidelity RNA polymerase that limits the genome size, but at the same time, generates an enormous genomic diversity that can be utilised in the phenomenon of immune evasion. The viral NS5b protein, which is an error-prone RNA-dependent RNA polymerase, lacks proof-reading ability, thereby rapidly creating diverse viral populations. These quasispecies have the potential to contain viral sequence mutation that can escape immune surveillance and contribute to persistence. It is thought that delay of the adaptive immune response facilitates the accumulation of variants, which can be selected before CTLs are expanded and their effector functions acquired (Bowen & Walker, 2005; Rehermann & Nascimbeni, 2005). Despite the multi-specific nature of CTL responses, if they are not supported with CD4+ T cell help, which is common for pre HCV persistence, they might be impaired and insufficient for viral clearance, but adequate to apply immune selective pressure for viral variants (Bowen & Walker, 2005). In addition, escape from CD8+ T cell response also may be supported by TCR diversity (Meyer-Olson et al, 2004). An animal study

demonstrated that the amino acid diversity in a main antigen recognition site within the TCR was reduced in the majority of CD8+ T cells directed against HCV epitopes that mutated and escaped immune recognition when compared with TCRs on CD8+ T cell directed against HCV epitopes with no mutations (Meyer-Olson et al, 2004). Some HCV escape mutants are selected by the humoral response, when antibody selective pressure generates diversity in the HVR1 of E2 that has been associated with development of chronic infection in humans (Farci et al, 2000) and chimpanzees (Shimizu et al, 1994).

The first indication that mutated epitopes did not occur randomly was gained from a study on three chimpanzees persistently infected with HCV virus, who shared HLA Class I haplotypes. Mutations within the epitopes were identified a short time after inoculation, and in some cases were associated with vigorous CD8+ T cell activity. Notably, the rate of nonsynonymus substitutions was statistically increased in MHC-Class I restricted epitopes when compared with nonrestricted epitopes or nearby regions, suggesting that substitutions can occur in multiple HCV epitopes due to selective pressure of CD8+ T cells (Erickson et al, 2001).

The mechanisms that support epitope mutation escape are diverse. Some amino acid substitutions can alter proteasomal cleavage sites, introducing or removing preferential slicing sites, resulting in the loss of the original epitope. Some can impair binding to the MHC molecule, due to amino acid substitutions in the anchor sites, while others modify recognition of variant peptide-MHC complexes (Bowen & Walker, 2005).

Interestingly, further diversity of a mutated epitope is not expected if it no longer binds the MHC molecule, since mutation completely secures evasion of presentation. For epitopes that still possess capacity to bind, it is possible that the mutated epitope preferentially stimulates CD8+ T cells recognising the parental, nonmutated (wild type) epitope, causing the expansion of wild-type specific CD8+ cells, a phenomenon known as the "original antigenic sin". This event diminishes clearance of viral variants, contributing to enhancement of viral escape (Klenerman & Zinkernagel, 1998).

However, another scenario is also possible, where mutated epitopes acting like altered peptide ligands for TCR, deliver an inhibitory signal to CD8+ cells, impairing their activity against wild-type epitopes (Shoukry et al, 2004).

These mutated epitopes can revert after transmission in the absence of a particular restrictive HLA allele in a new host, a phenomenon which was demonstrated in HIV infection. Certain viral variants restricted by protective HLA*B57 allele reverted on transmission to HLA*B57 negative individuals, indicating a "fitness cost" associated with the mutation (Leslie et al, 2004). Studies on HCV-infected chimpanzees (Erickson et al, 2001) and humans (Cox et al, 2005; Ray et al, 2005; Timm et al, 2004) have also demonstrated strong CD8+ T cell-mediated selection pressure for escape mutations and reversion of these polymorphisms in some epitopes.

The protective effect of the allele may be exhibited through induction of significant "fitness cost", i.e. viral variants which are less fit, is beneficial for the host, since it can more sufficiently control viral replication. However, if a "fitness cost" is not involved in sequence substitution, this mutation will stay fixed and could be easily spread through the population, regardless of HLA type, leading to a possible extinction of the wild type epitope (Klenerman & Hill, 2005).

A body of evidence of escape mutations restricted by HLA*Class II molecules in HCV infection is still incomplete as amino acid substitutions are rarely identified (Penna et al, 2002). Some limited data have correlated epitope mutations restricted by the HLA*Class II allele with an altered pattern of cytokine expression (Wang et al, 2003b) and a neutralised and annuled CD4+ T cell activity (Frasca et al, 1999; Wang & Eckels, 1999). However, experimental proof of a statistically significant difference between frequency in polymorphisms within HLA*Class II restricted epitopes and unrestricted epitopes are still to be revealed.

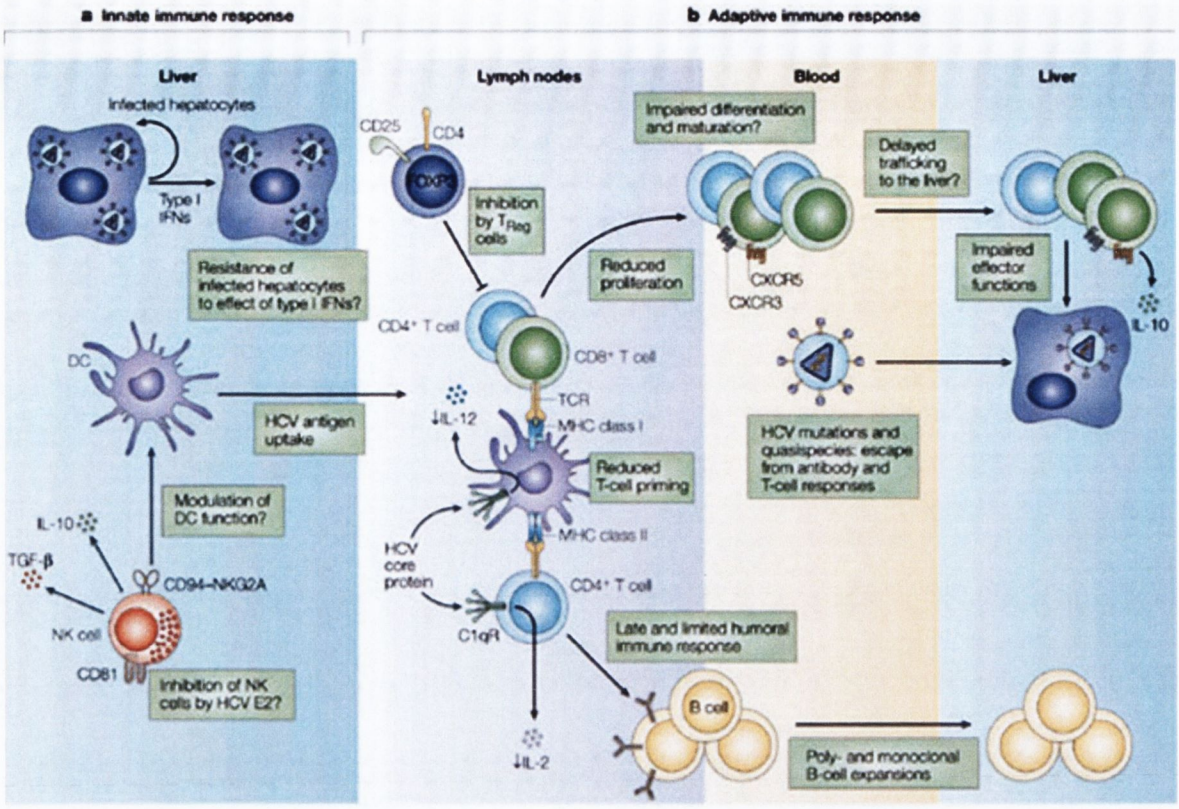



**Figure 1.19 Possible HCV evasion of innate and adaptive immune response. A)** Innate immune response in the liver. *In vitro* evidence shows that NK cells can be suppressed by E2, that E2 can alter their cytokine secretion profile, as well as their ability to activate DCs. Some studies have demonstrated suppression of DC maturation by HCV, which leads to impaired antigen presentation. Type I IFN responses are also affected by viral proteins and, as indicated in a chimpanzee study, HCV is not necessarily sensitive to this type of immune defense. **B)** Adaptive immune response. Viral escape through mutation in antibody and, more importantly, T cell epitopes is a well recognised mechanism of immune evasion. In addition, HCV-specific T cells are impaired in their proliferative capacity and effector functions, which is perhaps caused by exhaustion or reduced T cell priming by APCs. Recently it was reported that Tregs have the ability to inhibit virus-specific T cells, contributing to failure of the cellular immune response. Figure is taken from Rehermann and Nascimbeni 2005

## *1.5 TREATMENT OF HEPATITIS C*

The usual outcome of HCV infection is persistence, which occurs in approximately 80% of infected individuals. This chronicity is incurable in the majority of patients and results in significant liver disease. To date, a vaccine for Hepatitis C has not been discovered and the most widely used therapy is a combination of pegylated IFN-α (peg-IFN) and ribavirin. A cure is associated with sustained virological response (SVR), which is defined as undetectable HCV RNA six months after the cessation of therapy (Bacon & McHutchison, 2007). Initial treatment approach used IFN-α monotherapy, but had limited success with only up to 20% of patients reaching the SVR after a year of treatment. The major improvement was made when a guanosine analogue called ribavirin was introduced in the therapy, which increased SVR to 35-40%. Further improvement came recently when recombinant IFN-α was attached to a large molecule, polyethylene glycol (peg). This modification extended the half-life and improved the pharmacokinetic profile of the molecule; resulting in a better rate of virological response (Feld & Hoofnagle, 2005). Using the combined therapy of peg-IFN and ribavirin, approximately 70-80% of individuals infected with genotype 2 or 3 will achieve SVR. The most prevalent genotypes, 1a and 1b, are the most resistant to this therapy (Zein, 2000) and only 40-50% of infected individuals will achieve SVR (Bacon & McHutchison, 2007). The therapy comprises weekly injection of peg-IFN and an appropriate oral daily dose of ribavarin for 24 weeks, for genotype 2 and 3, and 48 weeks for genotype 1.

Improvements in this therapy are necessary since the treatment is quite invasive, long lasting and with a modest success rate for the most prevalent genotype. Therefore drug development for HCV is evolving. The first drugs to be developed were nucleoside analogues that inhibit NS5b polymerase. The next generation of HCV drugs was NS3-4a serine protease inhibitors. Recently a new approach with immunomodulators, which can enhance innate immunity, has been suggested. Some of these inhibitory or immunomodulatory molecules are in different phases of clinical trials, usually combine with peg-IFN with or without ribavirin. The main target of HCV drug discovery is to increase the success rate of the treatment and to make it more convenient for the patient (Smith, 2006).

In general, the ultimate aim in HCV treatment and prevention is development of a vaccine formulation with immunotherapeutic and prophylactic effectiveness (Houghton & Abrignani, 2005).

## *1.6 THESIS RATIONALE AND OBJECTIVES*

HCV is very successful in evasion of host defence. It has the ability to manipulate different cellular components involved in the immune response in order to escape immune surveillance, as well as to generate genetic mutations which can lead to immune escape.

A recent study demonstrated that patients with chronic HCV infection have preferential loss of IL-2-secreting CD4+ T helper cells (Semmo et al, 2005). The same group also showed that proliferation and IL-2 secretion of CD4+ T cells were impaired in HCV patients when compared to those who spontaneously resolved infection (Semmo et al, 2007). These studies clearly suggest a relationship between a diminished IL-2 response to the virus and development of HCV chronicity. During acute infection, IL-2 is essential for T cell expansion and failure to establish proliferative T cell responses, may contribute to the establishment of persistence. The mechanism of how HCV manipulates IL-2 production is unknown. Other studies have shown that mutations in CD8+ T cell epitopes are associated with viral evasion and that viral escape also plays a central role in establishment of chronic HCV infection (Cox et al, 2005; Neumann-Haefelin et al, 2006; Timm et al, 2004).

Therefore, the major objectives for this thesis were:

- To investigate the effects of the HCV E2 on T cell IL-2 production and to identify possible mechanism(s) that may explain the E2-mediated inhibition of IL-2.
- To investigate production of cytokines involved in immune response to viral infection and cytokines central to the process of inflammation and liver pathogenesis, using a viral component - E2 protein.
- To identify putative epitopes restricted by alleles that are associated with viral clearance in an Irish cohort of females infected with HCV (no information is available on epitopes in the entire HCV 1b genome restricted by HLA*A3, B7, DRB1 0101 and DRB1 0401 alleles and significance of mutations in these epitopes in an Irish cohort in relation to an outcome of infection)

- To evaluate the effect of amino acid substitutions within identified putative epitopes, in relation to immune selective pressure that may lead to viral escape and persistence

# CHAPTER II

# *MATERIALS AND METHODS*

# Chapter II

## *MATERIAL AND METHODS*

### 2.1 REAGENTS

RPMI 1640, foetal calf serum – FCS, HEPES, HBSS, phosphate-buffered saline (PBS), agar (Gibco, Invitrogen Ltd., Paisley, UK); Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway); penicillin, streptomycin, L-glutamine, sodium bicarbonate, glucose and sodium pyruvate, bovine serum albumine (BSA) (Sigma-Aldrich); Phorbol 12-myristate 13-acetate (PMA), phytohemagglutinin (PHA), Ionomycin, dimethyl sulfoxide (DMSO), methyl-β-cyclodextrin (MCD), trypan blue solution, poly-L-lysine hydrobromide, brefeldin A, triton X-100, tween 20, phalloidin-TRITC, paraformaldehyde, sodium chloride (NaCl), TRIS, EDTA, β mercaptoethanol, ethidium bromide, human recombinant IL-2 (Sigma-Aldrich); sodium hydroxide (NaOH) and ethanol (Merck, Darmstadt, Germany); sulphuric acid ($H_2SO_4$) (Riedel-de Haën, Seelze, Germany); hydrochloric acid (HCl) and acetic acid glacial (AnalR, BDH, VWR International); recombinant envelope protein E2, recombinant core protein C22 or protein from NS3 region C33 (recombinant proteins were prepared in Chinese hamster ovary (CHO) cells and were at a purity of > 98%, kind gift of Dr. Sergio Abrignani, Chiron Corporation, Emeryville, CA); Höechst 33342 (Molecular Probes); fluorescent mounting medium (DAKO, Denmark); purified protein derivative (PPD; Statens Serum Institut, Copenhagen, Denmark); Go6976 (Calbiochem, Merck Chemicals Ltd. Nottingham, UK); CSPQYWTGPAC [OH], CPWSAGYTQPC [OH] peptides (Organic Synthesis Core, Institute if Biopharmaceutical Sciences, Royal College of Surgeons in Ireland, Dublin, Ireland); lipid raft Vybrant™ Labeling Kit (Molecular Probes); Human IFN-γ ELISA Ready-SET-Go Kit (eBioscience, San Diego, CA); Human IL-2 DuoSet ELISA development Kit and Human TGF-β1 DuoSet ELISA development Kit (R&D Systems, Inc. Minneapolis, MN); HCV primers (MWG-BIOTECH AG, Germany); TaqMan Master Mix kit and fluorescent labelled TaqMan MGB probes (Applied Biosystems, UK); siRNAs (Dharmacon, Thermo Fisher Scientific, Lafayette, CO); peptides for ELISpot assay (ProImmune Ltd. Oxford, UK); Interferon-γ ELISpot kit

(Mabtech AB, Nacka Strand, Sweden); alkaline phosphatase substrate solution (Protein G - HRP Conjugate Kit, Bio-Rad Laboratories, Inc., Hercules, CA); NucleoSpin RNA II Kit (Macherey-Nagel, Germany); QIAamp UltraSens Virus Kit, RNeasy MinElute Cleanup Kit (Qiagen Ltd., UK); RETROscript kit (Ambion Ltd., UK).

## 2.2 ANTIBODIES

Goat anti-human IL-2 antibody (Sigma Aldrich); purified rat anti-human IL-2 monoclonal antibody, fluorochrome conjugated monoclonal antibodies: PE mouse anti-human CD3, PE mouse anti-human CD19, PE mouse anti-human CD56, APC mouse anti-human CD14, FITC mouse anti-human CD81 (all from BD Pharmingen, San Diego, CA); donkey anti-rat Ig-G antibody Alexa Fluor 488, goat anti-rabbit Ig-G antibody Alexa Fluor 568 (Molecular Probes, Eugene, OR); purified rabbit polyclonal anti-human PKC-β1 antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA); anti-CD3 antibody (produced by hybridoma cell line OKT-3, LGC Promochem, Teddington, UK); anti-CD28 antibody (Ancell Corporation, Bayport, MN); rabbit anti-mouse immunoglobulins (RAM IgG, Dako A/S, Denmark).

## 2.3 CELL CULTURE

The human T cell lymphoma cell line HuT 78 (American Type Culture Collection, Rockville, MD) and freshly isolated human Peripheral Blood Mononuclear Cells (PBMCs) were used in this study.

### 2.3.1 Source of cell line and maintenance of cell cultures

HuT 78 cells are suspension cells derived from peripheral blood of a patient with cutaneous T cell lymphoma (Sezary syndrome) and have the properties of a mature human T cell. HuT 78 were cultured in RPMI 1640 medium containing foetal calf serum - FCS (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), HEPES (10 mM), sodium bicarbonate (1.5 g/l), glucose (4.5 g/l) and sodium pyruvate (1 mM) [Appendix 1]. HuT 78 cells were grown in complete RPMI-1640 medium at 37°C in 5% $CO_2$ in a humidified incubator and were routinely maintained in T-75 $cm^2$ cell culture flasks (Nunclon, Roskilde, Denmark) in the volume of medium

between 15-25 ml. Visual examination was undertaken using phase contrast microscopy and cell density was maintained at 5-8 x $10^5$ cells per ml. HuT-78 cells were passaged three times per week by replacement of medium (1/10 or 1/5 dilution). Supplemented medium was stored at 4°C. FCS was heat inactivated (56°C, 1 hour) to inactivate complement and then aliquoted for storage at -20°C.

### 2.3.2 Isolation of primary blood mononuclear cells (PBMCs)

PBMCs, obtained from healthy volunteers in 10 ml heparinised evacuated tube, were separated on Ficoll-Histopaque density gradient – Lymphoprep, washed with HBSS or RPMI 1640 and used for experiments (Boyum, 1968). Briefly, blood was slowly and carefully layered onto 5 ml of Lymphoprep and centrifuged at 1800 rpm for 30 min at 4°C. Following that, the PBMCs layer, located at the Ficoll-sample interface (**Figure 2.1**), was removed using a sterile pastette, washed with RPMI 1640 and centrifuged at 1500 rpm for 10 min at room temperature. Supernatant was discarded and the same wash step applied twice. After the last wash, PBMCs were resuspended in complete medium for enumeration and either used immediately or cryopreserved. If the PBMCs were obtained from a buffy coat (discarded blood product left over after platelet enrichment, obtained from blood bank), the first step was to dilute the buffy coat with phosphate-buffered saline (PBS) [Appendix 1] at a 1:1 ratio, and then to layer it onto Lymphoprep, followed by the complete procedure as described above.

PBMCs were cultured in RPMI 1640 supplemented with FCS (10%), penicillin (100 U/ml), streptomycin (100µg/ml), L-glutamine (2mM) and HEPES (25mM) [Appendix 1] at 37°and 5% $CO_2$ content in a humidified incubator.

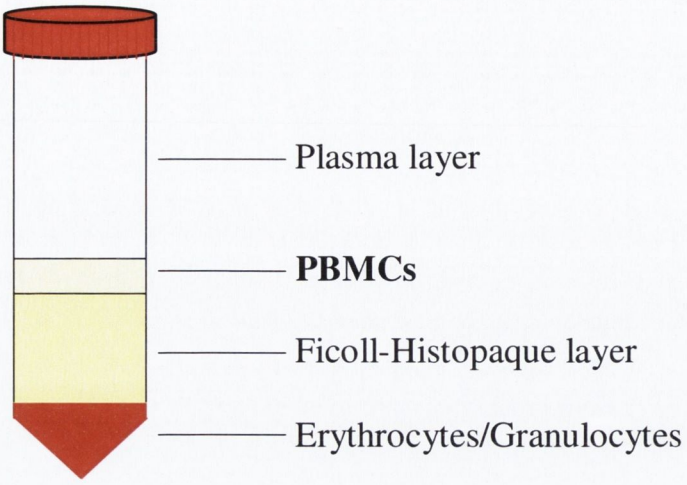



**Fig. 2.1 Different layers of blood separated according to density.** The lymphocytes/monocytes (PBMCs) have lower density then erythrocytes/granulocytes and lower then Lymphoprep (density 1.077g/ml), which allows PBMCs to be retained on the upper gradient surface (Ficoll-sample interface), while precipitating erythrocytes/granulocytes through the gradient to the bottom.

### 2.3.3 Isolation of T cells from PBMCs

Human CD3+ T cell population was separated using Human T Cell Enrichment Column (HTCC Kit, R&D Systems, Inc. Minneapolis, MN) according to the manufacturer's instructions. The PBMC suspension is loaded onto a column and via high affinity negative selection they are purified so that the final eluate contains highly enriched T cell population, with purity ranging between 85% and 95%. Briefly, before loading the cells, the column was placed in a ring stand and prepared by removing the top cap, then the bottom cap and allowing the fluid to drain. The next step was to wash the column with a total of 6 ml of 1x column wash buffer [Appendix 1], again allowing the eluate to drain. The column was then ready to be loaded with the cells. PBMCs, separated on Ficoll-Histopaque density gradient, were resuspended in 1 ml of 1x column wash buffer and applied to the top of the column. The cells suspended within the column were incubated at room temperature for 10 min and afterwards eluted with 4 aliquots (2 ml each) of 1x column wash buffer. Collected T cells were centrifuged at 250 x g for 5 min, supernatant decanted and cells resuspended in the culture medium.

### 2.3.4 Cryopreservation and revival of PBMCs

Freshly made freezing media containing 90% of FCS and 10% of dimethyl sulfoxide (DMSO) [Appendix 1] was shown to be the most effective cryoprotectant. The freezing process had to be gradual to allow the cells to dehydrate, but fast enough to prevent excessive dehydration damage. The freezing media was protected from light (due to DMSO sensitivity) and chilled on ice or placed in refrigerator at 4°C for, approximately, 30 min. Cells were resuspended at a concentration of $5x10^6$ cells/ml in ice cold cryoprotectant. 1 ml of cell suspension was transferred into sterile cryovials (Nunclon, Roskilde, Denmark), which were placed at -80°C (in a styrofoam box or freezing container "Mr. Frosty", Nalgene, Nalge Europe Ltd, UK) for 24 hours. It is critical to obtain a repeatable -1°C/min cooling rate for successful cell cryopreservation and recovery. For long-term storage, the cryovials were transferred in the vapour phase of the liquid nitrogen tank/container (cell storage system at -195.8°C).

The thawing process has to be rapid and carefully carried out. PBMCs were quickly thawed at 37°C in a water bath and washed immediately with culture medium. Briefly, cryovials removed from the liquid nitrogen container were immediately transferred to a 37°C and held in a water bath with an occasional gentle "flick" during thawing. When a small bit of ice remained in the cryovials, they were transferred to the biosafety hood and the content of each cryovials quickly removed into previously prepared sterile centrifuge tubes containing 1 ml of warmed medium. After 1 min, 2 ml, and subsequently after 1 min, 4 ml, of warmed medium were added slowly. Following this, warmed medium was added extremely carefully up to 20 ml and tubes were centrifuge at 1000 rpm for 5 min. Finally, supernatant (with cryoprotectant) was discarded and the cell pellet resuspended in complete medium and transferred to a tissue culture flask. Viability of the cells was checked by a trypan blue dye or ethidium-bromide (as described below).

### 2.3.5 Cell enumeration and viability

To assess cell yield and viability, 10 µl of the cell suspension and 90 µl of 0.4 % trypan blue solution were added into a 0.5 ml microcentrifuge tube, making 1/10 dilution. The content of the tube was mixed carefully and 10 µl was loaded on a Neubauer

haemocytometer (area under the cover slip is filled sufficiently) **(Figure 2.2a)**. Live cells appear white and non-viable cells are blue under a light microscope. Cells were counted in four large squares (1mm x1mm) **(Figure 2.2b)** and calculated using the equation:

$$\text{Cells/ml} = \frac{\text{N° of cells in all 4 square}}{4} \times 10^4 \times D$$

$10^4$ – volume conversion factor to 1 ml
D – specimen dilution factor

Percentage of viable cells was calculated:

$$\% \text{ Viability} = \frac{\text{N° of viable cells}}{\text{Total N° of cells}} \times 100$$

When trypan blue was replaced with Ethidium Bromide/Acridine Orange (0.1%) (EB/AO) [Appendix 1] (Lee et al, 1975) cell viability was determined under ultraviolet (UV) light where live cells appear green and non-viable cells stain red.

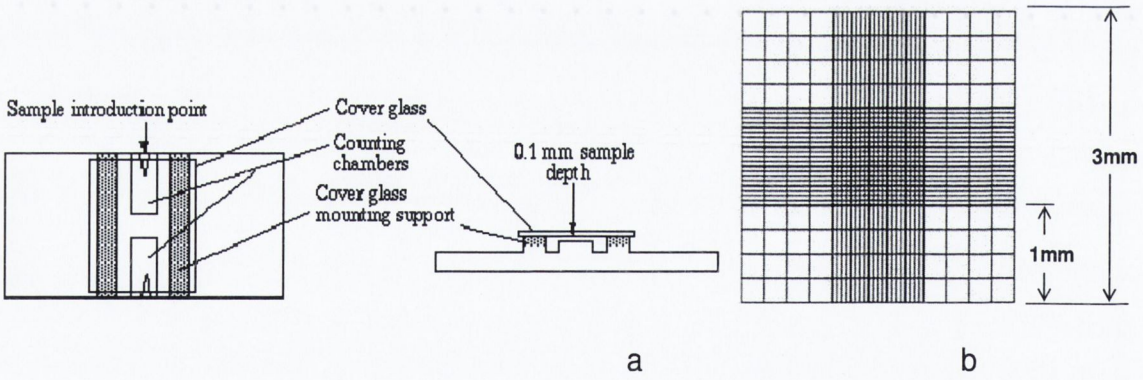



Fig. 2.2 a) Diagram of the hemocytometer and cover glass b) Schematic of a hemocytometer counting grid. Images adapted from Rabinovitch Lab Manual 2005, Department of Pathology, University of Washington, Seattle.

## 2.4 IMMUNOFLUORESCENCE ANALYSIS

### 2.4.1 In situ assay for IL-2 secretion

Immunofluorescence assays were carried out in the LabTek 8 well Permanox chamber slides (Nalge Nunc International, Rochester, NY) coated with poly-L-lysine hydrobromide - high molecular weight (MW>300,000 g/mol) at final concentration of 0.3 mg/ml, for an hour at 37°C. Following that, the wells were washed twice with PBS and coated with 7 μg/ml of anti-interleukin-2 capture antibodies (goat anti-human IL-2 antibody) overnight at 4°C. Unbound antibodies were removed by a double wash with PBS and afterwards, HuT 78 cells at a concentration of $1x10^5$ per ml were added to each well and left at 37°C for 30 min to adhere. Cells were then pretreated with 1 μg/ml of HCV recombinant envelope protein E2 overnight and afterwards stimulated with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) for 5 hours at 37°C. Some cells were treated with 0.2mM methyl-β-cyclodextrin (MCD) for an hour following E2 pretreatment and prior to PMA activation. Other conditions included cells treated just with E2 or PMA or MCD and cells that did not receive any treatment at all. In addition, in some experiments cells were incubated with 20 μg/ml brefeldin A for an hour prior to PMA activation. Subsequently, cells were fixed in 3% paraformaldehyde [Appendix 1] for 30 min at room temperature, gently washed 3 times with PBS, permeabilised or not with 0.5% Triton X-100 detergent and washed. Cells were stained for IL-2 as follows: cells were treated with 7 μg/ml of primary antibody (purified rat anti-human IL-2 monoclonal antibody) for 30 min at 4°C, gently washed twice and next incubated with 20 μg/ml secondary antibody (donkey anti-rat Ig-G antibody Alexa Fluor 488) for 30 min at room temperature, protected from light. The nuclei were stained with Höechst 33342 5 μg/ml for 2 min and, additionally, in some experiments actin filaments were stained with 0.1 mM phalloidin-TRITC for 15 min at room temperature. After the final wash step, stained slides were separated from the upper chamber, dried out completely and mounted with fluorescent mounting medium, which prevents and reduces fading of immunofluorescence.

Confocal microscopy was performed using a 100x oil immersion objective on a Nikon TE2000-U inverted microscope using a Perkin Elmer LSI confocal system, equipped with an Ar/Kr laser (488 nm). Ultraview image acquisition system (Perkin Elmer,

Waltham, MA) and Volocity-2 processing software (Improvision Inc. Waltham, MA) were used for image processing and 3D analyses.

### 2.4.2 Lipid raft assay

For analysis of lipid rafts, HuT 78 cells were added in a concentration of $1x10^5$ per ml, left at 37°C for 30 min to adhere on poly-L-lysine and then either left resting or treated with 1 μg/ml of E2 for 24 hours. Cells were fixed in 1% paraformaldehyde (10 min) and then stained for lipid raft using Vybrant™ Labeling Kit. Firstly, cells are labeled with Alexa Fluor 488 green fluorescence conjugated to cholera toxin subunit B (CT-B) which binds to the ganglioside $G_{M1}$ partitioned in the lipid raft. To achieve this, cells were incubated with CT-B conjugate working solution [Appendix 1] for 10 min at 4°C and afterwards gently washed several times with chilled PBS. Secondly, an antibody recognizing CT-B is then used to cross-link the CT-B labeled lipid rafts into distinct patches on the plasma membrane. Thus, cells were incubated with chilled anti-CT-B antibody working solution [Appendix 1] for 15 min at 4°C, repeating the same wash steps. Following that, cells were labeled with primary antibodies – purified rabbit polyclonal anti-human PKC-β1 antibody (dilution 1:50 for 1 hour at room temperature), washed and then conjugated with goat anti-rabbit Ig-G antibody Alexa Fluor 568, 1: 800 dilution for 30 min at room temperature, protected from light. All the wash steps have to be applied extremely carefully, to prevent cells being washed from the slide.

Stained slides were mounted with fluorescent mounting medium and confocal microscopy was performed using a 63x oil immersion objective on a Zeiss 510 Meta Confocal Laser Scanning Microscope (laser excitation 488 nm and 561 nm).

## 2.5 ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA)

### 2.5.1 Quantification of IFN-γ (IFN-γ ELISA)

Human IFN-γ ELISA Ready-SET-Go Kit was used according to the manufacturer's instructions. Briefly, 96 well Nunc-Immuno Plate MaxiSorp Surface (Nalge Nunc International, Rochester, NY) was coated overnight at 4°C with 100 μl of the capture antibody (dilution 1:250 in 1x coating buffer [Appendix 1], purified anti-human IFN-γ

antibody – clone NIB42). Plates were washed three times with wash buffer (0.05% Tween 20 in PBS) [Appendix 1] and blocked for 1 hour at room temperature with 1x assay diluent (200 µl/well)[Appendix 1]. After repeating the wash step, 100 µl of standards (2-fold serial dilutions in assay diluent of recombinant IFN-γ) or supernatants were added to wells and incubated at room temperature for 2 hours. Wells were washed five times with wash buffer and 100 µl of detection antibody (1:250 in 1x assay diluent, biotin-conjugate anti-human IFN-γ antibody – clone 4S.B3) was added to each well and plate incubated for 1 hour at room temperature. The wash step was repeated (5 times with wash buffer) and 100 µl of avidin-HRP (1:250 in 1x assay diluent, HRP - horseradish-peroxidase) was added to each well and the plate incubated for 30 min at room temperature. Finally, wells were washed seven times and 100 µl of HRP substrate solution (3,3',5,5'- Tetramethylbenzidine – TMB) added per well and incubated for 30 min in the dark. The reaction was stopped by addition of 50 µl 2N $H_2SO_4$ per well [Appendix 1].

Assays were performed in duplicate and absorbance was measured at 450 nm on a Wallac VICTOR plate reader (Perkin Elmer, Boston, MA). The duplicate readings for each standard and sample were averaged and then subtracted for the average of the zero (pg/ml) standard. Cytokine concentrations of the samples were determined from the standard curve (range 0-1000 pg/ml).

### 2.5.2 Quantification of IL-2 (IL-2 ELISA)

An ELISA technique was used to quantify IL-2 secreted from T cells. Human IL-2 DuoSet ELISA development Kit was used according to the manufacturer's instructions. Briefly, 96 well maxisorp plate was coated with 100 µl/well of capture antibody (mouse anti-human IL-2 antibody) at 4 µg/ml in PBS and incubated overnight at room temperature. Plates were washed three times with wash buffer [Appendix 1] and then blocked by adding 300 µl of block buffer (1% bovine serum albumine (BSA) in PBS) [Appendix 1] to each well for a minimum of 1 hour at room temperature. After repeating the wash step, 100 µl of standards (2-fold serial dilutions of recombinant IL-2 in reagent diluent [Appendix 1]) or samples were added to wells and incubated at room temperature for 2 hours. The wash step was repeated and 100 µl of detection antibody

(400 ng/ml in reagent diluent, biotinylated goat anti-human IL-2 antibody) added to each well for 2 hours at room temperature. Following this, wells were again washed three times with wash buffer and 100 µl of streptavidin-HRP (1:200 in reagent diluent) added to each well and plate incubated for 20 min at room temperature in the dark. Wash step was repeated, followed by the addition of 100 µl of HRP substrate solution (TMB) per well. The plates were incubated for 20 min in the dark and the reaction was stopped by adding 50 µl of 2N $H_2SO_4$ per well.

IL-2 concentrations were calculated as described in the IFN-γ ELISA protocol (2.5.1)

### 2.5.3 Quantification of TGF-β1 (TGF-β1 ELISA)

A Human TGF-β1 DuoSet ELISA development Kit was used according to the manufacturer's instructions. Briefly, 96-well maxisorp plate was coated overnight at room temperature with 100 µl/well of the capture antibody (2 µg/ml in PBS, mouse anti-TGF-β1). Wells were washed three times with wash buffer (0.05% Tween 20 in PBS) and blocked for a minimum of 1 hour at room temperature by addition of 300 µl of block buffer (5% Tween 20 in PBS) [Appendix 1] to each well. After repeating the wash step, 100 µl of standards (2-fold serial dilutions of recombinant TGF-β1 in reagent diluent [Appendix 1]) or samples were added to wells and incubated at room temperature for 2 hours. However, before the samples (cell supernatants) were used in the assay they had to go through a procedure outlined below **(Table 2.1)** [Appendix 1]. Namely, this cytokine is secreted in latent complex and has to be functionally released from this complex before it can act as an active molecule.

**Cell Culture Supernatants**

Add 0.1 ml 1N HCl to 0.5 ml sample

Mix well

Incubate 10 min at room temperature

Neutralize by addition of 0.1 ml 1.2N NaOH / 0.5M HEPES

Mix well

Assay

**Table 2.1 TGF-β1 sample activation**

Following 2 hour incubation, plates were washed and 100 µl of detection antibody (300 ng/ml biotinylated chicken anti-human TGF-β1 antibody in reagent diluent,) was added to each well for 2 hours at room temperature. The wash step was repeated and 100 µl of streptavidin-HRP diluted 1:200 in reagent diluent was added per well and incubated for 20 min at room temperature in dark. After repeating the wash step, 100 µl of HRP substrate solution (TMB) was added to each well and incubated for 20 min at room temperature in the dark. The reaction was stopped with 50 µl of 2N $H_2SO_4$.

Assays were performed in duplicates and absorbance was measured immediately at 450 nm on a Wallac VICTOR plate reader. The duplicate readings for each standard and sample were averaged and then subtracted for the average of zero standard. Sample values were multiplied by 1.4, what was the dilution factor in a procedure of sample activation. Cytokine concentration of the samples was determined from the standard curve.

## 2.6 MULTIPLEX CYTOKINE ANALYSIS

HuT 78 cells and PBMCs were stimulated as described in relevant experiments and collected supernatants used to carry out this assay. Multiplex analysis: A Biochip Array Technology system, The Evidence Investigator™ (Randox Laboratories Ltd., Crumlin, UK) **(Figure 2.3a)**, was used to measure multiple cytokines (IFN-γ, TNF-α, IL-1α, IL-1β, IL-4, IL-6, IL-10, MCP1) in cell culture supernatants. The system utilises a sandwich antibody capture immunoassay format on a 1 $cm^2$ solid chip **(Figure 2.3c)**.

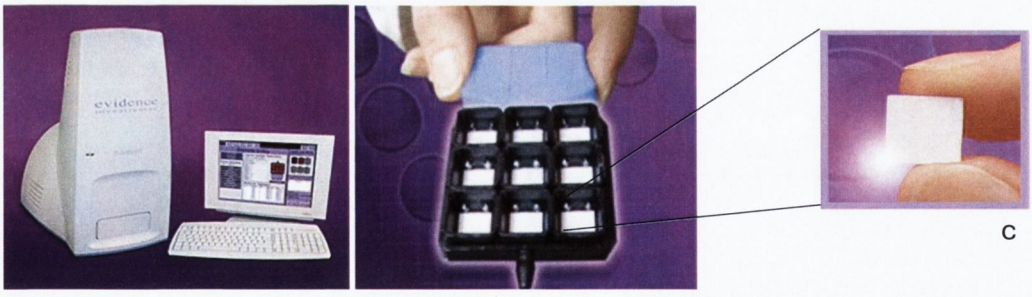



**Fig. 2.3 a) Evidence Investigator system -** instrument with supercooled charge couple device (CCD) camera and unique image processing software. **b) Biochip carrier with nine separate reaction wells c) Biochip that has a surface with recognition sites for multiple parameters.** Images adapted from Evidence Investigator brochure, Version 1, 2007, Randox Laboratories.

There are 9 biochips on a carrier **(Figure 2.3b)** and each biochip contains an array of discrete test regions of immobilised HRP labelled antibodies specific to different cytokines. The light signal, generated from each of the test regions is detected using digital imaging technology and is compared to that from a stored calibration curve. The concentration of each cytokine in the sample is calculated from the calibration curve. Briefly, 200 µl of assay diluent was added to each well and this was followed by addition of 100 µl of calibrator (standards are provided to generate a nine-point calibration curve) or sample per well. The biochips were incubated for 1 hour at 37°C and 370 rpm on the thermoshaker. Following incubation, the wells were washed six times by adding 350 µl of wash buffer [Appendix 1] and after the final wash 300 µl of conjugate solution was added to each well. The biochips were reincubated for 1 hour at 37°C and 370 rpm on the thermoshaker. Afterwards, the wash step was repeated and the wells were left with wash buffer (no longer than 30 min) which was replaced with 250 µl of signal reagent (1:1 luminol and peroxide) in only one carrier at the time, as the incubation in dark takes only 2 min, prior carrier is placed in the instrument for imaging. Capture of images and calculation of cytokines concentrations were defined by the software and processed automatically.

### 2.7 FACS ANALYSIS of CD81 expression

Levels of surface expression of CD81 receptor for different subsets of PBMCs was assessed by flow cytometry. PBMCs subsets were determined using fluorochrome conjugated monoclonal antibodies (mAb) against conventional surface markers: CD3 for T cells (PE mouse anti-human CD3), CD19 for B cells (PE mouse anti-human CD19), CD56 for NK cells (PE mouse anti-human CD56) and CD14 for monocytes (APC mouse anti-human CD14) while CD81 was analysed by staining with FITC labeled mAb (FITC mouse anti-human CD81). Briefly, PBMCs, separated on Ficoll-Histopaque density gradient, were transferred to FACS tubes (BD Falcon Round Bottom Tubes), centrifuged at 1200 rpm for 5 min and resuspended in 500 µl of FACS buffer [Appendix 1]. Cells were centrifuged and resuspended in 100 µl of FACS buffer, the recommended amount of each antibody added, mixed gently and incubate for 20 min on ice in the dark. Following this, 500 µl of FACS buffer was added, cells

centrifuged, again resuspended in 500 µl FACS buffer and centrifuged. The last step was to resuspend cells in 300 µl FACS buffer and then to add 100 µl of 4% paraformaldehyde, mix and store at 4°C protected from light. 30000 events were acquired using a FACSCalibur Flow Cytometer and data was analysed using CELLQuest software (Becton Dickinson).

## 2.8 STUDY POPULATION AND SAMPLES

Subjects for this study were derived from an Irish cohort of females inoculated/treated in 1977 with anti D-immunoglobulin, which was contaminated with HCV, genotype 1b, from a single source. Seventeen years after exposure, when these subjects were identified, 55% of them developed chronic infection and approximately 2% had definite or evidence of probable cirrhosis (McKiernan et al, 2004).

For sequencing purposes we obtained plasma samples from HCV PCR positive ($PCR^+$) individuals, while for ELISpot assays whole blood was collected into heparinised tubes from both non-resolvers (chronically infected individuals, HCV $PCR^+$) as well as resolvers (individuals who cleared the virus, HCV $PCR^-$), all from the cohort. HLA typing was available for all the samples. In the case of CD81 blocking peptide experiments serum was collected from HCV $PCR^+$ patients not necessarily from the cohort.

All samples were obtained from individuals who were/are attending the Hepatology Unit in St. James's Hospital in Dublin. All participants gave written informed consent for this study which has been approved from the Research Ethics Committee at St. James's Hospital.

## 2.9 RNA EXTRACTION AND REVERSE TRANSCRIPTION (RT)

### 2.9.1 HuT 78 RNA

For these experiments, HuT 78 cells were set up in standard culture medium at a concentration of $2x10^6$ cells per ml in 24 well plates (Nunc A/S, Roskilde, Denmark). Cells were pretreated with HCV recombinant envelope protein E2 (1 µg/ml) overnight and afterwards stimulated with 10 ng/ml of PMA for 24 hours. Other conditions included cells treated either with E2 or PMA and cells that did not receive any treatment at all. Total cellular RNA was isolated using a NucleoSpin RNA II Kit

according to the manufacturer's instructions. Briefly, cells were lysed by adding 350 µl of RA1 buffer and 3.5 µl of β-mercaptoethanol to the cell pellet and vortexing vigorously. The lysate was applied on the NucleoSpin Filter unit and centrifuged for 1 min at 11000 x g. Homogenized lysate was mixed with 350 µl of 70% ethanol and loaded on the NucleoSpin RNA II column and centrifuged for 30 s at 8000 x g. Following this, 350 µl of membrane desalting buffer was added onto the column and centrifuged for 1 min at 11000 x g. Afterwards, 95 µl of DNase reaction mixture (10 µl of DNase I and 90 µl of DNase reaction buffer) was applied onto the centre of the column membrane and incubated for 15 min at room temperature. Subsequently, three wash steps were performed: 1$^{st}$ - 200 µl of RA2 buffer was added onto the column and centrifuged for 30 s at 8000 x g; 2$^{nd}$ - 600 µl of RA3 buffer was added and centrifuged for 30 s at 8000 x g; 3$^{rd}$ - 250 µl of RA3 buffer was added and centrifuged for 2 min at 11000 x g. Finally, total RNA was eluted by adding of 60 µl of RNase-free $H_2O$ and centrifuging for 1 min at 11000 x g. Collection tubes were changed after each step. To get a better yield, the RNA eluate was applied onto the column again and centrifuged under the same conditions. The concentration of each sample was obtained from $A_{260}$ measurements on a NanoDrop ND-1000 or NanoDrop ND-8000 spectrophotometer (NanoDrop Technologies, Del). RNA was stored at -80°C to maintain its integrity.

Reverse Transcription (RT) of RNA was performed using equal amounts of RNA (1 µg) from each condition. RT has been done using the RETROscript kit under standard conditions. Briefly, 1 µg of total RNA, 2 µl of Random Decamers and up to 12 µl of Nuclease-free $H_2O$ were mixed in a microcentrifuge tube, spun briefly and heated at 72°C for 3 min. The tube was briefly spun again, placed on ice and mixed gently with RT mix **(Table 2.2).**

| Component | Volume per reaction | Final concentration |
|---|---|---|
| RT Buffer (10x) | 2 µl | 1 x |
| dNTP mix (2.5mM each) | 4 µl | 500 µM |
| RNase Inhibitor (10U/µl) | 0.5 µl | 0.25 U/µl |
| Reverse Transcriptase (100U/µl) | 0.5 µl | 2.5 U/µl |
| $H_2O$ | 1 µl | / |

**Table 2.2 Reverse transcription reaction mix**

Prepared RT reaction (20 µl) was then incubated at 42°C for 1 hour, followed by incubation at 92°C for 10 min. At last, reaction was proceeded to the PCR or stored at -20°C.

### 2.9.2 Isolation of RNA from PBMCs

PBMCs were set up in standard culture medium at a concentration of $4x10^6$ cells per ml in 24 well plates. Cells were pretreated with HCV E2 (1 µg/ml) overnight and then stimulated with anti-CD3/anti-CD28 antibody (as described in the figure legend 3.1). In addition, cells treated either with E2 or anti-CD3/anti-CD28 and cells without treatment were included in these experiments. Total RNA from cells was extracted using a NucleoSpin RNA II Kit according to the manufacturer's instructions and as described above (2.9.1). The concentration of each sample was obtained from $A_{260}$ measurements on NanoDrop ND-8000 spectrophotometer. When RNA yields were not sufficient, samples were concentrated using the RNeasy MinElute Cleanup Kit was used. Briefly, RNA sample volume was adjusted to 100 µl with RNase-free $H_2O$ and mixed thoroughly with 350 µl of RLT buffer. To dilute RNA, 250 µl of 96-100% of ethanol was added, mixed well by pipetting, applied onto RNeasy MinElute spin column and centrifuged for 15 s at 8000 x g. Following this, 500 µl of RPE buffer was added and centrifuged for 15 s at 8000 x g to wash the column. To dry the silica membrane of the column, 500 µl of 80% ethanol was added and centrifuged for 2 min at 8000 x g. The cap of the column was then opened and centrifuged for 5 min at maximum speed. To elute RNA, 14 µl of RNase-free $H_2O$ was added onto the centre of the membrane and centrifuged for 1 min at maximum speed. Collection tubes were changed after each step. Reverse Transcription (RT) of RNA was performed using equal amounts of RNA (0.5 µg) for each condition and using the RETROscript kit under standard conditions (as outlined in 2.9.1).

### 2.9.3 Preparation of Viral RNA

#### 2.9.3.1 Extraction

Clinical plasma samples were collected from the patients belonging to the Irish cohort of females. All samples were tested as HCV PCR positive ($PCR^+$). Viral RNA was

obtained using QIAamp UltraSens Virus Kit according to manufacturer's instructions. Briefly, 1 ml of plasma was mixed with 0.8 ml AC buffer and 5.6 µl carrier RNA by vortexing for 10 s and incubated for 10 min at room temperature. Samples were then centrifuged for 3 min at 1200 x g, supernatant discarded and cell pellet completely resuspended by vortexing in 300 µl of AR buffer (warmed to 60°C) and 20 µl of proteinase K and subsequently incubated for 10 min at 40°C in a mixer incubator at maximum speed (Eppendorf Thermomixer Comfort, Eppendorf UK Limited, Cambridge, UK). Following this, 700 µl of lysate was applied onto the QIAamp spin column and centrifuged for 1 min at 4000 x g. Afterwards, two wash steps were performed: firstly - 500 µl of AW1 buffer was added onto the column and centrifuged for 1 min at 6000 x g; secondly - 500 µl of AW2 buffer was added and centrifuged for 2 min at 20000 x g. The last step was to elute viral RNA by adding 30 µl of AVE buffer and spinning for 1 min at 6000 x g. To ensure maximum recovery of RNA, the last step was repeated and RNA immediately stored at -80°C.

#### 2.9.3.2 RT-PCR

Reverse Transcription (RT) was carried out using RETROscript kit and 10 µl of extracted viral RNA (**Table 2.3**). RT tubes were incubated at 37°C for 90 min in the heating block and following that, the reaction was stopped by increasing the temperature to 95°C for 5 min.

| Component | Amount |
|---|---|
| RT Buffer (10x) | 2 µl |
| dNTP mix (2.5mM each) | 4 µl |
| Random Decamers (50µM) | 2 µl |
| RNase Inhibitor (10U/µl) | 0.5 µl |
| Reverse Transcriptase (100U/µl) | 0.5 µl |
| $H_2O$ | 1 µl |
| Viral RNA | 10 µl |

**Table 2.3 RT reaction components**

RT product (2 µl) was then used in a nested PCR, performed using previously published primers from the 5' non-coding region of the HCV genome (Chan et al, 1992).

PCR mix for the nested PCR was made as shown in **Table 2.4**. The amplified first round PCR product of 306 base pairs and nested PCR product of 251 base pairs were visualized by UV on a 2% agarose gel (2.9.3.3). Primers used for this purpose were:

**S1** 5'-CTGTGAGGAACTACTGTCTT-3' (forward - 1st round PCR)

**S2** 5'-ATACTCGAGGTGCACGGTCTACGAGACCT-3' (reverse – 1st round PCR)

**S3** 5'-TTCACGCAGAAAGCGTCTAG-3' (forward – 2nd round PCR)

**S4** 5'-CACTCTCGAGCACCCTATCAGGCAGT-3' (reverse – 2nd round PCR)

| **First round PCR** | | **Second round PCR** | | **Final** |
|---|---|---|---|---|
| **Component** | **Amount** | **Component** | **Amount** | **concentration** |
| PCR Buffer with $MgCl_2$ (10x) | 5 µl | PCR Buffer with $MgCl_2$ (10x) | 5 µl | 1x |
| dNTPs (10 mM each) | 1 µl | dNTPs (10 mM each) | 1 µl | 200 µM |
| Primer S1 (20 pM) | 0.5 µl | Primer S3 (20 pM) | 1 µl | 0.2 pM/ 0.4 pM |
| Primer S2 (20 pM) | 0.5 µl | Primer S4 (20 pM) | 1 µl | 0.2 pM/ 0.4 pM |
| Red Taq polymerase (1 U/µl) | 1.5 µl | Red Taq polymerase (1 U/µl) | 1 µl | 0.03 U/µl / 0.02 U/µl |
| $H_2O$ | 36.5 µl | $H_2O$ | 39 µl | / |
| cDNA | 5 µl | 1st round PCR product | 2 µl | / |

**Table 2.4 PCR reaction mix for HCV nested PCR**

Both, the first and second round of nested PCR were performed in a 50 µl volume using the same programme: denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and at the end 72°C for 7 min on the PTC-100 Thermal Cycler (MJ Research, Inc. Ramsey, MN).

#### 2.9.3.3 DNA visualisation

The presence of appropriate PCR product was determined by running the samples on a 2% agarose gel containing 0.5 μg/ml ethidium bromide (**Table 2.5**). Samples (10 μl) and 100 bp ladder marker (Invitrogen) (1 μl ladder mixed with 9 μl $H_2O$ and 2 μl 6 x loading dye (Promega, UK)) were loaded on the gel and electrophoresis carried out at 100 volts (V) using 1 x TAE [Appendix 1]. After the dye front had reached the base of the gel, the presence of DNA was visualised using the Kodak Image Station 440CF.

| Components | Amount |
|---|---|
| Agarose | 1 g |
| TAE (1 x) | 50 ml |
| Ethidium bromide (10mg/ml) | 2.5 μl |

**Table 2.5 2% Agarose gel mixture**

### 2.10 REAL TIME-PCR (RT-PCR)

Real Time PCR has been performed to quantify the expression of mRNA for IL-2, IFN-γ, TNF-α, IL-10, TGF-β, IL-6, IL-1α, IL-1β and PKC-β1. The PCR reaction was carried out using the TaqMan Master Mix kit and a mix of primers and fluorescently labelled TaqMan MGB probes for the target gene and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control (1:10). Firstly, 4.5 μl of cDNA (10-100 ng/ml) was added to a 384-well plate (MicroAmp optical 384-well research Plate, Applied Biosystems, Foster City, CA), followed by addition of RT-PCR mix (0.5 μl of primer and 5.5 μl of PCR mix).

This reaction was performed in 10 μl final volume on an ABI Prism 7900HT Sequence Detection System and the programme was as follows: an initial step at 50°C for 3 min, second stage at 95°C for 10 min followed by third stage of 40 cycles of 95°C for 15 sec and 60°C for 1 min. These assays are designed to span exon-exon junctions so as not to detect genomic DNA. Quantitative RT-PCR data was achieved using the comparative $C_T$ method (Livak & Schmittgen, 2001).

## 2.11 SMALL INTERFERING RNA (siRNA)

### 2.11.1 Technology Overview – RNAi and Gene Silencing

RNA interference (RNAi) is evolutionary conserved cellular process observed in most eucaryotic organisms and it probably evolved as a cellular defense mechanism against viruses, transposone expansion and aberrant transcription (Hamilton & Baulcombe, 1999; Sijen et al, 2001; Zamore, 2001). Work by Andrew Fire and Craig Mello has made a crucial discovery in this field and in 2006 was rewarded with a Nobel Prize in Medicine. They reported that injection of double-stranded RNA (dsRNA) into the nematode Chaenorhabditis elegans (C. elegans) induced sequence-specific reduction of mRNA and consequently silencing of the corresponding gene (Fire et al, 1998), referring to the term RNAi. Previous findings in plants demonstrating co-suppression and virus-induced gene silencing (Jorgensen, 1990; Napoli et al, 1990), was retrospectively explained by RNAi. The pathway of RNAi **(Figure 2.4)** is triggered by dsRNA, originated either from replicating viruses or from endogenously expressing, regulatory, non-coding micro RNA (miRNA). dsRNA shares sequence homology with a target mRNA, which might be encoded by, for instance, invading viral genome, transposons or repetitive elements from the host genome. A RNase III-like enzyme, known as DICER, cleaves dsRNA into small interfering RNA(siRNA) duplexes, which are 19-25 base pairs in length with characteristic dinucleotide 3′ overhangs (Ambros et al, 2003; Bernstein et al, 2001; Elbashir et al, 2001a; Elbashir et al, 2001b). The next step involves incorporation of siRNA into a multiprotein structure named RNA-inducing silencing complex (RISC). In an ATP driven reaction, the siRNA strands are separated and the complementary mRNA molecule is recognised by the RISC. Once recognised and bound, mRNA is cleaved and then degraded (Caudy et al, 2002; Hammond et al, 2001; Nykanen et al, 2001).

Since its discovery, the RNAi pathway has been rapidly developed as an innovationary tool for exploration of physiological gene functions. Chemically synthesised siRNAs may be used in vitro experimental conditions to activate the RNAi pathway.

The most common siRNA delivery methods to the mammalian cells are chemical transfection and electroporation. Cationic lipid-based reagents are used for chemical approach, mostly on adherent cell lines, since it eliminates the need for resuspension

and trypsinisation as required for electroporation. The mechanism of this form of transfection is not well understood, but current model suggests that the siRNA:lipid complex enters the cells by endocytosis (Ausubel, 2001). Electroporation, an alternative method that is now widely used, has some advantages over lipid based approach. For instance, gene silencing can be observed within a few hours after the delivery of siRNA, as this method does not depend on cell division (Jarvis R., 2003). Electroporation involves application of short high-voltage pulses, in order to overcome the cell membrane barrier. An electric field pulse induces a transient increase in cell membrane permeability and reversible formation of pores, allowing molecules to pass through the membrane (Gehl, 2003). Electroporation conditions (voltage of the electrical field and duration/frequency of the pulses) have to be optimised for each particular cell type and electroporation buffer (higher ionic content lower resistance for the electrical current), until the fine balance between siRNA uptake and cell viability is achieved.

### 2.11.2 Conduction of siRNA experiments

These experiments were conducted on HuT 78 cells which were transfected with appropriate siRNAs. Briefly, 5 x $10^5$ cells were resuspended in 200 µl of complete medium without antibiotics and mixed gently with 5 µg of either control siRNA (siCONTROL Non-Targeting siRNA Pool) or siRNA for PKC-β1 (siGENOME Smart Pool) in a cuvette (BTX disposable cuvette model 640). Following that, cuvette was placed in the BTX electroporator (ElectroSquarePorator ECM 830, Harvard Apparatus Inc., Holliston, MA ) and cells transfected with the following electroporation settings: *mode* - LV, *voltage* - 300V, *pulse length* - 2 ms, *number of pulses* - 1, *electrode type* - BTX disposable cuvette (4 mm gap), *field strength* - 750 V/cm. Subsequently, cells were transferred to 2 ml of complete warmed medium and harvested at different time points (48, 72, 96 h). During optimisation, in some experiments siGlo (fluorescent form of lamin A/C siRNA) was used to control for transfection efficiency. In addition, the Amaxa Nucleofector transfection system (Amaxa Inc., Gaithersburg, MD) was used in some initial experiments, when cells were resuspended in 100 µl of amaxa solution R, mixed with 1.5 µl of siGlo and then the cuvette placed in the Amaxa apparatus,

programmed on V – 01 (recommended by manufacturer). However, owing to decreased cell viability, only the BTX system was used in further studies. Efficacy of transfection was determined by RT-PCR.

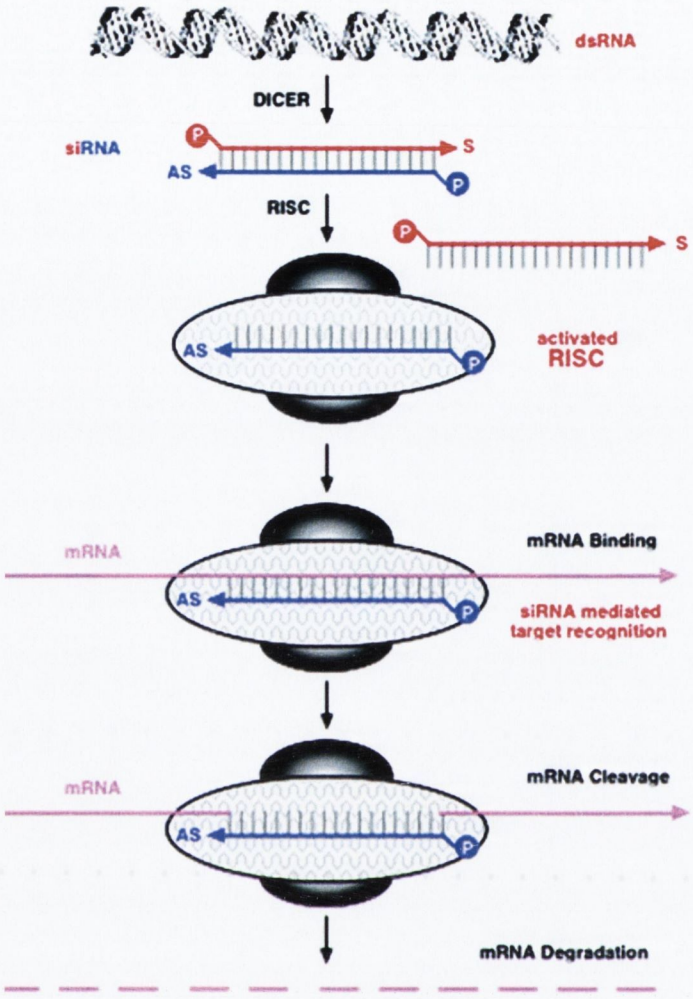



**Figure 2.4 Schematic representation of mechanism of siRNA silencing steps.** siRNA is derived from long double-stranded RNA (dsRNA) through cleavage by DICER. Resulting siRNA molecules can then interact with the RISC complex which, when ATP activated, induces sense (S)/anti-sense (AS) strand separation. It is thought that the AS strand remains in the RISC. Activated RISC complex explores the target mRNA molecules for complementary sequence and when complementary mRNA is bound to AS strand, mRNA degradation ensues resulting in gene silencing. Figure was adopted from (Dykxhoorn et al, 2003).

## 2.12 SEQUENCING OF HEPATITIS C VIRAL RNA

Clinical plasma samples were obtained from the patients belonging to an anti-D cohort and sent to our collaborators in Centre for Clinical Immunology and Biomedical Statistics, Royal Perth Hospital and Murdoch University, Perth, Australia for sequencing. Viral RNA was extracted using the Roche Cobas Amplicor HCV Kit and then used in three one-step reverse transcription-PCR systems (SuperScript III One-Step RT-PCR, Invitrogen) to amplify the viral genome (from core to NS5b region). The first round template was subsequently used in nested second round PCR reactions (Taq DNA polymerase, Roche) containing the combination of HCV genotype 1 generic, specific and adjusted (based on previous sequences from the same cohort) primers, covering the same region. Resultant PCR products were bulk-sequenced using the ABI BigDye Terminator Sequencing Kit (Applied Biosystems). Sequence chromatograms were edited using the program Assign (Conexio Genomix, Australia) (Gaudieri S. et al. 2007. Centre for Clinical Immunology and Biomedical Statistics, Royal Perth Hospital and Murdoch University, Perth, Western Australia).

## 2.13 BIOINFORMATIC ANALYSIS

### 2.13.1 HLA binding epitope prediction

A detailed model was constructed utilising 10 databases, which predict epitopes binding to MHC Class I and Class II molecules. These databases were used to identify amino acid motifs binding to HLA*A3, HLA*B7 (Class I) and HLA*DRB1 0101, HLA*DRB1 0401 (Class II) alleles in the entire viral polypeptide sequence for the HCV-1b genotype. Databases used for epitope predictions were: RankPep, Bimas, Jenner (MHCPred), ProPred 1 (ProPred), MAPPP, Net MHC (Net MHCII), SYFPEITHI, SVRMHC, MHC2Pred and IEDB [Appendix 2]. The HCV polypeptide sequence employed in all prediction databases was from an Irish donor and has been previously published (AF 313916 GenBank/EMBL/DDBJ) [Appendix 3]. Epitopes common to all or most databases were selected and characterised further. NCBI Los Alamos HCV database was utilised as a HCV sequence database (sequence search and background information) and HCV immunology database (CTL epitopes and epitope maps for all HCV-1a proteins).

### 2.13.2 *In silico* protein translation and alignment

HCV sequence results were in cDNA fasta format and these were translated into protein sequences. Obtained sequences were from bulk PCR and sequencing reactions, but mixtures in the sequences, most likely representing quasi-species, could be observed. *In silico* protein translation was carried out using CLC Bio Free Workbench, (Aarhus, Denmark) software. Alignment of the donor sequence with the patient (carriers of protective alleles who develop chronic infection) and control groups (individuals with chronic infection who do not have protective alleles) was performed using BioEdit software package (Ibis Biosciences, Carlsbad, CA). Briefly, selected viral sequences were translated to protein sequences using the choice of six reading frames in CLC Bio programme. Since sequences were not complete, some of them exhibited frame shift and consequently different reading frames, which was overcome by using a choice of multiple translations made at a time. Choosing BioEdit Clustal W multiple alignment algorithms all sequences were aligned with the original donor sequence.

Epitope mapping and identification of amino-acid substitutions was performed using the BioEdit programme. Firstly, each putative epitope was identified in the donor sequence and subsequently in all aligned sequences. The amino acid sequence of the epitope, throughout all sequences, was investigated for potentially significant amino-acid substitutions, all in comparison with the donor sequence (donorwise).

## 2.14 QUANTIFICATION OF T CELL RESPONSES USING IFN-γ ELISpot ASSAY

### 2.14.1 HCV peptide preparation

A peptide library, containing panel of short peptides restricted by HLA*A3 allele and control peptides, was purchased from ProImmune Ltd. HLA*A3 allele restricted peptides were derived from HCV donor sequence using bioinformatics, epitope prediction approach (2.13.1) **(Table 5.1)**, while control peptides were derived from commonly encountered viruses, such as Epstein Barr virus (EBV) and cytomegalovirus (CMV) **(Table 2.6)**. Control peptides were previously published: EBV (Benninger-

Doring et al, 1999; Hill et al, 1995) and CMV (Elkington et al, 2003) epitopes and they were suggested by our collaborators, since these viral epitopes restricted by HLA*A3 allele are rarely found in the literature.

| Virus | Viral Protein | Peptide Sequence |
|---|---|---|
| Epstein Barr Virus | BRLF1 | RVRAYTYSK |
| Epstein Barr Virus | EBNA3A | RLRAEAQVK |
| Cytomegalovirus | Pp50 | TVRSHCVSK |

**Table 2.6 Control peptides restricted by HLA*A3 allele**

Peptides were nine amino-acid in length, lyophilised and at a purity of ~86%. The first step in dealing with the peptide set was to dissolve them and obtain a uniform concentration. Therefore, peptides were suspended in 100 µl of DMSO and most of them went into solution. With DMSO toxicity in mind, a maximum of a 50 µl of DMSO was added further to those which did not dissolve. Peptide solutions were further dissolved in RPMI 1640 to a final concentration of 1 mg/ml, aliquoted and stored at –80°C as main stock (as long-term storage). To minimize freeze-thaw cycles, peptides were further diluted ten times (100 µg/ml), stored at 4°C and used in Elispot assays.

### 2.14.2 ELISpot (Enzyme Linked ImmunoSpot) assays

Interferon-γ ELISpot assay was used for quantification of antigen specific T cell responses at a single cell level. Fresh or frozen PBMCs were used in these *ex-vivo* assays. Briefly, MultiScreen-HA 96-well mixed cellulose esters plates (Millipore, Bedford, MA) were coated with 100 µl/well of mouse anti-human IFN-γ antibody, clone 1-D1K, (Mabtech AB, Nacka Strand, Sweden) at 0.5 µg/ml in PBS, and incubated overnight at 4°C. After washing the wells seven times with PBS, plates were blocked for 1-2 hours at 37°C with complete RPMI 1640 medium. Afterwards, PBMCs were seeded in a concentration of $2x10^5$ cells per well and stimulated with HCV peptides at 10 µg/ml. In addition, cells were treated with control peptides (10 µg/ml), PHA (1 and 10 µg/ml) and anti-CD3 (1/1000); all used as positive controls, while wells

with cells in medium alone were used as negative control. All treatments were performed in duplicate. Each plate was then incubated for 18 hours at 37°C and 5% $CO_2$. Following incubation, wells were washed seven times with PBS and 100 µl/well of biotinylated mouse anti-human IFN-γ detection antibody, clone 7-B6-1, (0.5 µg/ml in PBS, Mabtech AB) was added and incubated for 100 min at room temperature. Following this, wells were again washed seven times with PBS and 100 µl of streptavidin-alkaline phosphatase (1:1000 in PBS) added to each well and the plate incubated for 40 min at room temperature in the dark. Repeating the wash step, spots were developed by addition of 100 µl/well alkaline phosphatase substrate solution (Protein G - HRP Conjugate Kit) [Appendix 1] and incubated for 20 min in the dark. Finally, the reaction was stopped by washing the plate with tap water. Plates were allowed to dry thoroughly before they were subjected to analysis. Spots were evaluated with an automated AID Elispot Reader System (AID Diagnostika GmbH, Strassberg, Germany) and IFN-γ producing cells were expressed as spot forming units (SFU) per $2x10^5$ cells. The number of antigen specific IFN-γ secreting cells was calculated by subtracting the SFU average value of negative control wells from the average of experimental wells. If this value was greater then 3 standard deviation above average of negative control wells, it was regarded as significant/positive response.

When cultured ELISpot assays were performed, PBMCs were exposed to the pool of HCV peptides (concentration of each peptide was 10 µg/ml) for three days at 37°C in 5% $CO_2$. Subsequently, medium with peptides was removed and cells resuspended in fresh complete medium and expanded with 20 ng/ml of human, recombinant IL-2. Every three days cells were resuspended in fresh medium with IL-2 until day twelve. After that, cells were rested for a day, washed with medium and used in the ELISpot assay as described above.

## 2.15 DATA ANALYSIS

Statistical analysis was performed using Microsoft Excel and this involved calculation of the summary statistics - mean, standard deviation (SD), standard error of the mean (SEM). Results are expressed as mean ± standard error of the mean (SEM). Student t-

test was used to compare the data and a $p$ value $< 0.05$ was considered statistically significant.

A quantitative assessment of the level of co-localisation between PKC-β and lipid raft was performed using Pearson's Correlation Coefficient. Relevant images were imported to Image Pro® Plus v. 4.5 for co-localisation analysis. Student t-test was used to determine statistically significant differences between Pearson's Correlation Coefficients of compared images.

Differences in sequence variations/amino-acid substitutions within the epitopes between the groups were analysed using Fisher's Exact Test.

# CHAPTER III

# *THE ENVELOPE PROTEIN (E2) OF THE HEPATITIS C VIRUS INHIBITS IL-2 SECRETION FROM T LYMPHOCYTES*

# Chapter III

## *3.1 INTRODUCTION*

Initial binding of HCV to the host cell involves a specific interaction between the HCV envelope protein (E2) and receptor(s) required for viral entry. At the present time, a number of molecules have been identified as potential entry sites of HCV including the CD81 receptor (Pileri et al, 1998), scavenger receptor B type I (SR-BI) (Scarselli et al, 2002), low density lipoprotein receptor (LDL-R) (Agnello et al, 1999) and claudin-1 (CLDN1) (Evans et al, 2007). The interactions are not completely understood and it is likely that some of the receptors cooperate to mediate HCV entry (Evans et al, 2007; Kapadia et al, 2007). One of the best characterised receptors for HCV is the CD81 molecule, a member of the tetraspanin superfamily, a large family of evolutionarily conserved cell-surface proteins (Levy et al, 1998). These molecules facilitate interaction between extracellular and intracellular elements as well as lateral membrane associated molecules and other tetraspanins, in a way which builds a molecular network known as a tetraspanin web (Rubinstein et al, 1996). This web transiently hosts different cell-signalling components, involved in multiple signalling events (Levy & Shoham, 2005). Therefore, tetraspanins are implicated in numerous biological processes, including fertilization of oocytes, metastasis of cancer cells, cell–cell interactions in the central nervous system and in infection and immunity (Boucheix & Rubinstein, 2001; Hemler, 2003). CD81 is a prototypical tetraspanin molecule and has four transmembrane domains, two extracellular loops and a small intracellular domain. The transmembrane domains are responsible for associations with other proteins, which are crucial for the assembly and maintenance of the tetraspanin web. The intracellular domain provides links to cytoskeletal and signalling molecules while extracellular domains promote specific interactions with laterally associated proteins and possibly ligands (Levy & Shoham, 2005). CD81 is expressed on all cells in the immune system. CD81 knockout mice failed to develop Th2 responses (Maecker et al, 1998) and the expression of this molecule on both T and B cells is essential for polarization towards Th2-mediated immunity (Deng et al, 2002). It has been proposed

that CD81 at the cell surface of B cells directly interacts with CD19, which is essential for B-cell activation as a part of CD19–CD21 co-receptor complex (Bradbury et al, 1992; Fearon & Carroll, 2000). Similarly, in T cells, CD81 associates with CD3 and the magnitude of the co-stimulatory response, measured by enhanced cellular proliferation, was similar to CD3-CD28 interaction, as shown in a mouse study (Witherden et al, 2000). It is believed that CD81 associates with TCR complex, since the co-stimulation with HCV E2 failed in cells that lacked downstream signalling molecule Lck. Moreover, it was demonstrated that absence of palmitoylation of CD4 molecules, even in presence of Lck, led to inability of CD4 to interact with CD81 (Levy & Shoham, 2005). Several studies have shown that the state of palmitoylation of certain cysteine residues within intracellular domains, determines the lateral interaction between tetraspanins and their associate proteins (Berditchevski et al, 2002; Kovalenko et al, 2004; Yang et al, 2002). Furthermore, Zhang et al. have reported direct association of CD81 intracellular domains and conventional PKC molecules (Zhang et al, 2001) when PKCs translocate to the plasma membrane.

Interestingly, CD81 is directly involved in human disease, because it has been utilised by two widespread human pathogens: *Plasmodium falciparum* (Silvie et al, 2003) which causes malaria and HCV (Pileri et al, 1998). The larger extracellular loop of CD81 is responsible for HCV attachment, binding E2 with high affinity **(Figure 3.A)** (Petracca et al, 2000). Utilising antibodies that inhibit this interaction, it has been demonstrated that CD81-binding region contains discrete segments of E2 protein, distributed within the same domain for the duration of E2 folding (Callens et al, 2005). In addition, a 27 amino acid long region within HVR1 has been also suggested to have a role in CD81-E2 contact (Penin et al, 2001). Interaction between CD81 and E2 (or an antibody) induced a co-stimulatory signal in CD3-activated T cells (Wack et al, 2001) and in natural killer (NK) cells (Crotta et al, 2002; Tseng & Klimpel, 2002). Furthermore, engagement of CD81 by E2 may have a role in failure of NK cells to clear the virus, as this interaction has been shown to inhibit NK-cell mediated cytotoxicity (Crotta et al, 2002) required for HCV clearance (Khakoo et al, 2004).

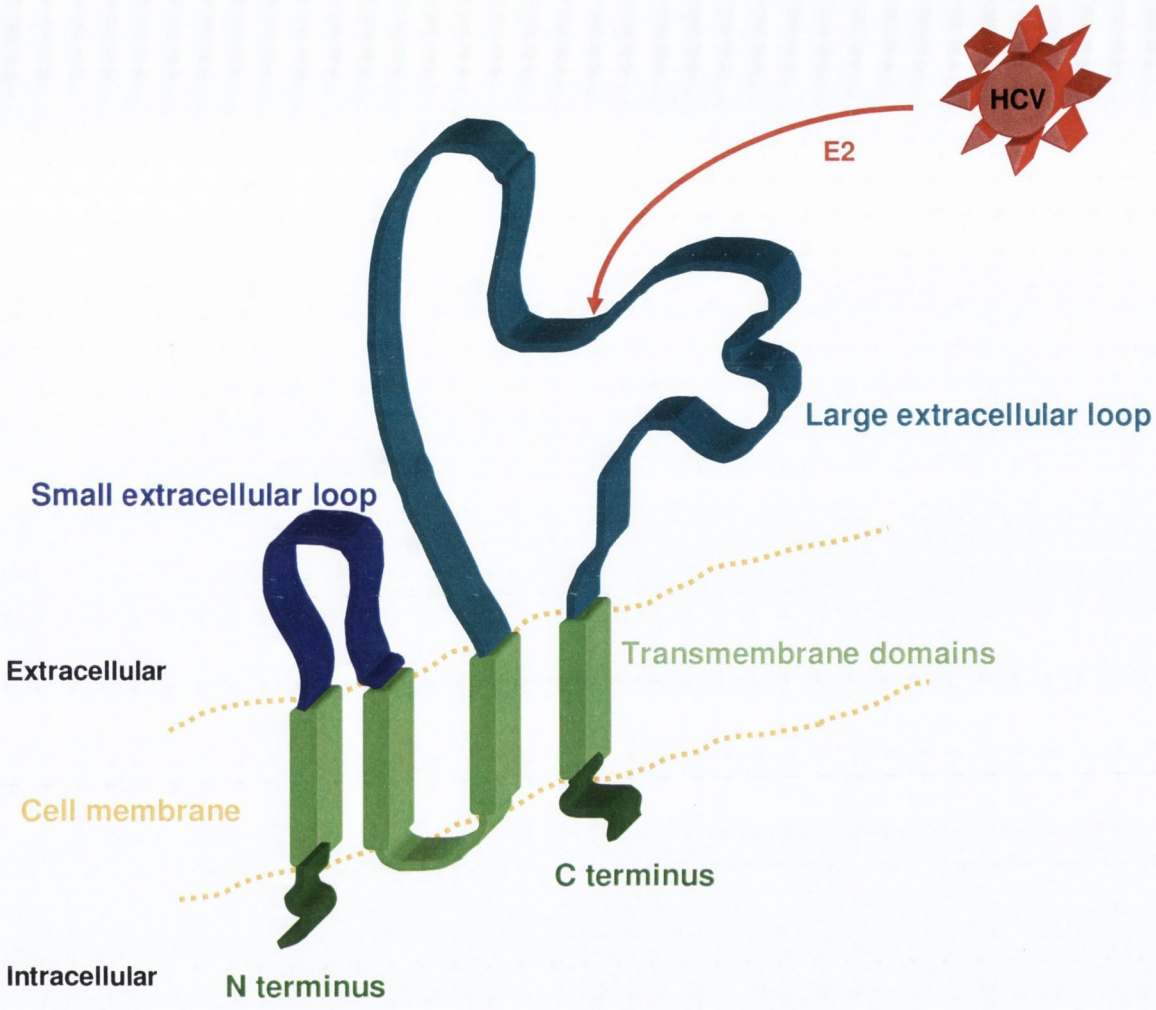



**Figure 3.A Interaction between HCV E2 and CD81.** HCV attaches to the cells via interaction of E2 and the large extracellular loop of the CD81 molecule. CD81 is a typical tetraspanin molecule, with four transmembrane domains (TM), two short cytoplasmic domains and two extracellular loops. TM domains stabilise the CD81 molecule and interact with other tetraspanins and partner molecules. Cytoplasmic domains are involved in its interaction with cytoskeleton elements and signalling molecules, while extracellular domains mediate protein-protein interactions.

In approximately 80% of infected individuals HCV clearance malfunctions, as the virus evades the host immune response through a combination of processes including viral genetic mutation and interference with signalling pathways. Its strategies can undermine both innate and adaptive arms of the immune response (Gale & Foy, 2005). There is evidence that T cell mediated immunity is important for prevention of persistent infection with impaired T cell proliferative responses and effector function associated with chronic infection (Matloubian et al, 1994; Rosenberg et al, 1997; Thimme et al, 2001). It has been previously demonstrated that E2 inhibits T lymphocyte migration, an essential part of the inflammatory process (Volkov et al, 2006). This inhibition of migration could be reversed by treatment of the cells with the lipid raft disrupting agent β-methylcyclodextrin (MCD). Thus, the authors postulated that E2 inhibits this migration by delivering a signal through the CD81 molecule which results in the sequestration of signalling molecules, including the enzyme Protein Kinase C beta (PKC-β) in lipid rafts, preventing its association with the T cell centrosome and microtubule cytoskeleton essential for the mechanism of migration (Volkov et al, 2001).

Protein kinase C is a key enzyme in signal transduction pathways, identified in different mammalian tissues and involved in a variety of cellular responses, such as exocytosis and transformation. The PKC family of protein kinases consists of 10/11 isozymes encoded by 9 genes (Nishizuka, 2003). PKCs are divided into three subfamilies: conventional (or classical), novel and atypical based on their second messenger requirements (Hug & Sarre, 1993; Ohno et al, 1991). Conventional (c)PKCs contain the isoforms α, $\beta_I$, $\beta_{II}$, and γ. These require $Ca^{2+}$, diacylglycerol (DAG), and the presence of phospholipids for activation. Novel (n) PKCs include the δ, ε, η, and θ isoforms, and require DAG, but do not require $Ca^{2+}$ for activation. Thus, conventional and novel PKCs are activated through the same signal transduction pathway (Bell & Burns, 1991; Zidovetzki & Lester, 1992). On the other hand, atypical (a) PKCs (including ζ and ι / λ isoforms) require neither $Ca^{2+}$ nor diacylglycerol for activation. There is a lot of evidence to show that PKCs are involved in many secretory processes; for instance: release of eosinophil cationic proteins from eosinophils in rats (Okumura

et al, 1999), soluble amyloid precursor protein from neurons (Govoni et al, 1996; McLaughlin & Breen, 1999), matrix metalloproteinase-9 from the tertiary granules of neutrophils (Chakrabarti et al, 2006), dense granules from human platelets (Murugappan et al, 2004), insulin from clonal β-cells (Yaney et al, 2002) as well as mucin secretion by bronchial epithelial cells (Abdullah et al, 2003; Park et al, 2007).

IL-2 is cytokine with an important role in maintaining immune homeostasis and tolerance to self (Fontenot et al, 2005) being essential for T cell growth and proliferation. It is produced mainly by activated CD4+ and CD8+ T cells, but activated DCs, NK and NKT cells also produce IL-2. It was among the first cytokines to be discovered in 1975 as a growth-promoting activity for bone marrow-derived T lymphocytes (Morgan et al, 1976). It was also one of the first cytokines characterised at the molecular level with its gene cloned in 1983 (Devos et al, 1983; Taniguchi et al, 1983), and crystal structure solved in 1992 (Bazan, 1992).

The gene for IL-2 is located on the long arm of chromosome 4 and its expression is regulated by several transcription factors. T cell activation through the TCR induces nuclear factor of activated T cells (NFAT) to bind to the promoter region of IL-2 gene and initiates its transcription. However, this signalling does not lead to sufficient IL-2 transcription and additionally requires co-stimulatory signalling. This is provided by CD28-B7 ligation, which further activates AP-1 and NF-κB transcription factor as well as stabilises IL-2 mRNA. Cytokine mRNAs are unstable and short-lived, as this prevents continuous cytokine production. Overall, these transcription factors together with Oct-1, which is constitutively expressed, orchestrate IL-2 gene transcription (Charles A Janeway, 2001; Crabtree & Olson, 2002; Jain et al, 1995; Shapiro et al, 1997). However, IL-2 gene silencing after T cell activation or inhibition of IL-2 transcription is incompletely understood (Malek, 2008). Recently, an auto-regulatory feedback loop of IL-2 has been explained. The antigen activated T cells produce IL-2 and increase expression of high affinity IL-2 receptor (IL-2R) on their surface. Secreted IL-2 binds its receptor, which results in STAT5 activation and induction of B lymphocyte maturation protein-1 (Blimp-1). This Blimp-1 protein is crucial in

downstream repression of the IL-2 gene, but it is not clear whether it directly mediates this inhibition (Gong & Malek, 2007; Villarino et al, 2007).

IL-2 is synthesised as a precursor protein of 153 amino acids and is a processed to a mature, active form by cleavage of a hydrophobic region long 20 amino acids (Eckenberg et al, 2000; Taniguchi et al, 1983). IL-2 is composed of four alpha helices, where the first and the last of the α-helices are connected by long loops **(Figure 3.B)**. Additionally, it has a single disulfide bond between cysteines 58 and 105, which is essential for stability of the molecule and crucial for its biological activity (Gaffen & Liu, 2004). IL-2 was shown to be glycosylated (Robb et al, 1983; Robb et al, 1981), which helps solubility in an aqueous milieu, but the functional significance of this modification is not known (Gaffen & Liu, 2004). The immunological role of IL-2 is highly complex and pleiotropic. It is considered to be the most important factor in T cell expansion, having the central purpose in the regulation of T cell responses (Smith, 1988; Swain, 1991). It also has an effect on other immune cells such as NK cells (Robertson & Ritz, 1990), B cells (Mingari et al, 1984; Nakanishi et al, 1992; Waldmann et al, 1984), monocytes (Espinoza-Delgado et al, 1995), neutrophils (Ferrante, 1992), and glial cells in mice (Eitan et al, 1994; Zhou et al, 2007). Furthermore, IL-2 is essential for downregulation of immune response by eliminating activated T cells, which otherwise can lead into autoimmunity (Kundig et al, 1993; Schorle et al, 1991). In addition, various studies have shown that a key role of IL-2 lies in regulation of development and function of T regulatory cells (Giri et al, 1994; Giri et al, 1995; Khatri et al, 1998; Malek, 2003).

IL-2R is composed of three subunits: IL-2Rα (CD25), IL-2Rβ (CD122) and the common gamma chain - γc (CD132), with all of them being essential for high-affinity IL-2R. The CD122 subunit is expressed on some early lymphoid progenitors, NK, NKT, memory CD8+ T cells, FoxP3+ Tregs and all antigen-activated T cells; and is shared between IL-2R and IL-15R. The subunit γc is expressed on all haematopoietic cells and is a part of IL-4, IL-7, IL-9, IL-15 and IL21 receptors. CD25 subunit is expressed on subsets of immature pre-T and pre-B cells, activated effector cells as well as CD4+FoxP3+ Tregs. However, in *in vivo* conditions only three cell subsets, CD4+ T cells, CD8+ T cells and CD4+FoxP3+ Tregs, co-express all three subunits, which is a

precondition for formation of high-affinity IL-2R and IL-2 signalling (Malek, 2008). IL-2 initially binds CD25, which leads to the association with CD122 and γc resulting in formation of quaternary complex where all subunits interact with IL-2 (Rickert et al, 2005; Stauber et al, 2006). This complex initiates IL-2 signalling cascade via cytoplasmatic tails of CD122 and γc. When in close conntact, Jak-3 via γc and Jak-1 via CD122, phosphorylate crucial tyrosine residues in CD122, which leads to an interaction with the adapter Shc and STAT5 or STAT3 molecules. Shc further activates MAPK and PI3K pathways, essential for cell growth and survival. STATs are further phosphorylated, conformationally changed and translocated into the nucleus, where they regulate expression of genes central to effector T cell functions and cell growth (Gaffen, 2001; Malek, 2008; Nelson & Willerford, 1998). The complex of IL-2 and high-affinity IL-2R has a short life-time and rapidly internalises, a process which is followed by proteasomal degradation (Yu & Malek, 2001; Yu et al, 2000).

Previously, it has been shown that expression of PKC-β is necessary for IL-2 secretion in T cells (Dreikhausen et al, 2003; Long et al, 2001). Furthermore it was demonstrated that PKC-β was specifically involved in secretion (but not transcription) of IL-2 in a HuT 78 T cell line (Long et al, 2001). A characteristic of patients with chronic HCV infection is a failure of IL-2 secretion in the CD4+ T helper cell population (Semmo et al, 2005) and additionally, it has been shown that loss of CD4+ T cell proliferative capacity during acute infection may contribute to the establishment of persistence (Gerlach et al, 1999). These observations lead to a hypothesis that viral interference of IL-2 secretion and attenuation of proper immune response might be through the PKC-β dependent pathway.

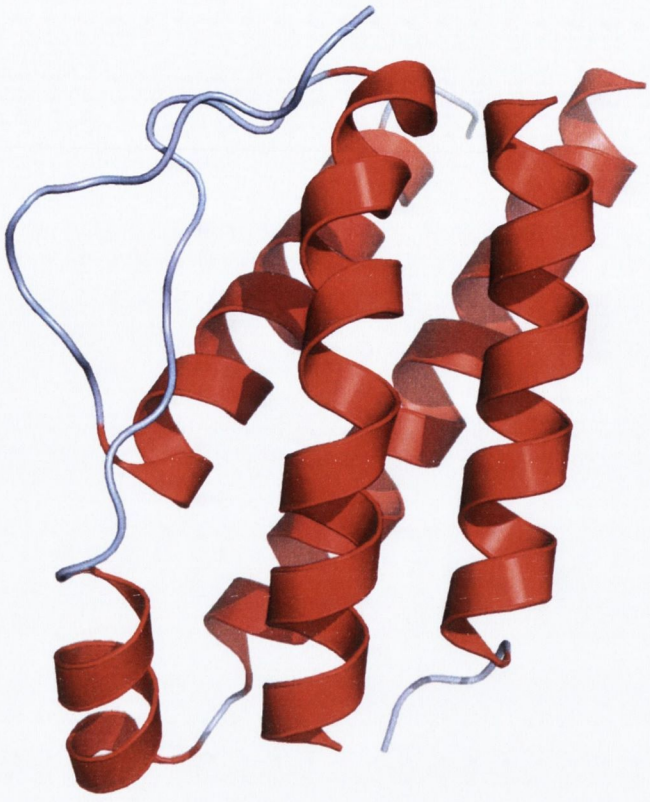


**Figure 3.B Crystal Structure of human IL-2.** This three-dimensional model was published by (Arkin et al, 2003), accessed from the Protein Data Bank (PDB: 1M47) and rendered by Ramin Herati using Pymol.

## *3.2 OBJECTIVES*

IL-2 is an important cytokine required to mount a successful immune response against pathogens, such as viruses. Recently it was demonstrated that patients with chronic HCV infection have preferential loss of IL-2 secreting CD4+ T cells (Semmo et al, 2005) and that proliferation and IL-2 secretion of CD4+ T cells were impaired in these patients when compared to those who spontaneously resolved infection (Semmo et al, 2007). In collaboration with professor Cliona O'Farrelly's group, we have demonstrated that levels of IL-2 in HCV-infected liver is significantly lower than that in livers of patients with other inflammatory diseases such as Primary Biliary Cirrhosis and Alcoholic Liver Disease (unpublished data). The aim of this study is to elucidate how HCV manipulates the ability of lymphocytes to produce this cytokine – a potential mechanism of immune subversion.

Thus the specific aims are:

- To develop *in situ* assay for detection of IL-2 secretion
- To investigate the effects of interactions between specific HCV proteins and lymphocytes on T cell IL-2 secretion (focusing on the Envelope protein 2 (E2))
- To determine a mechanism behind the HCV E2 induced inhibition of IL-2 secretion

## *3.3 RESULTS*

### 3.3.1 HCV PCR$^+$ serum inhibits T cell IL-2 production

It has been observed by our collaborators that levels of IL-2, predicted to reflect the extent of the T cell-mediated immune response, were significantly lower in protein extracts of HCV-infected liver then those detected in the liver of patients with Alcoholic Liver Disease (ALD) and Primary Biliary Cirrhosis (PBC) (Golden-Mason, L. unpublished data). The quantities of IL-2 produced in HCV-infected liver were comparable to those in normal (donor) tissue which was surprising in light of the importance of IL-2 in antigen-specific immune responses as in viral infection.

To further elucidate this finding, we then investigated if the low levels of IL-2, produced by the T cells in the livers of HCV patients, were influenced by the presence of viral particles. Therefore, the ability of lymphocytes to produce IL-2 in response to anti-CD3/anti-CD28 stimulation in the presence of HCV-infectious serum was investigated. PBMCs obtained from healthy donors were exposed to PCR$^+$ or normal serum (NS) for an hour (1 part of serum with 4 parts of serum free medium) and following that serum free medium was removed, cells resuspended in complete medium and stimulated overnight with anti-CD3/anti-CD28 antibody. As shown in **Figure 3.1**, addition of HCV-infectious serum results in significant down-regulation of the production of IL-2 in PBMCs (IL-2 levels in pg/ml: αCD3/αCD28 770.8 ± 5.9 vs αCD3/αCD28/PCR$^+$ 355.6 ± 4.3, p=0.015) (IL-2 levels in pg/ml: resting cells (RC) 0, PCR$^+$ 0, normal serum (NS) 0). This inhibitory effect on IL-2 secretion was not seen for normal serum (αCD3/αCD28/NS 588.5 ± 17.6, p=0.082). These data suggest that exposure to HCV-infected serum prior to stimulation *via* CD3/CD28 results in down-regulation of the ability of the lymphocytes to produce IL-2, a likely *in vivo* scenario. It has been previously demonstrated that the envelope protein of HCV, E2, via its interaction with CD81, inhibits LFA-1-stimulated T lymphocyte polarisation and migration by a mechanism which prevents reorganisation of the microtubule cytoskeleton and centrosome (Volkov et al, 2006). As these cellular components are critical for the secretory process, it was investigated if the presence of E2 in serum was mediating the observed inhibition of IL-2 production. In similar experiments, 5 hours

long pre-incubation of donor cells with a peptide which blocks E2:CD81 interaction (Cao et al, 2003), prior to exposure to $PCR^+$ serum reversed its inhibitory effect on TCR-stimulated IL-2 production (**Figure 3.2 A**; αCD3/αCD28/$PCR^+$ 183.4 ± 1.6 vs BP/$PCR^+$/αCD3/αCD28 358.7 ± 10.4, p=0.0008) (E2-mediated inhibition of IL-2 secretion: p=0.005). This rescue of IL-2 secretion was not observed in the presence of a scrambled control peptide (SCR). Significant inhibition of IL-2 production by $PCR^+$ serum (25.7%, p=0.0001) was partially rescued by blocking peptide to 56.4% of the level of αCD3/αCD28 stimulation across five experiments **(Figure 3.2 B)** (p=0.011).

### 3.3.2 HCV envelope protein E2 inhibits IL-2 production from PBMCs

Knowing that obstruction of E2:CD81 interaction leads to recovery of IL-2 production, we postulated that E2 of HCV might be involved in the process of IL-2 inhibition. We then investigated whether the recombinant E2 could inhibit IL-2 production in PBMCs. Firstly, PBMCs, obtained from healthy donors, were incubated with E2 overnight and following that stimulated with either PMA/Ionomycin (PMA/Io) (10 ng/ml of PMA and 1 μg/ml of Ionomycin) or plate bound anti-CD3/anti-CD28 antibodies. A significant decrease in IL-2 secretion was observed in PBMCs pre-treated with E2 prior to activation (**Figure 3.3**: IL-2 levels in pg/ml: PMA/Io 2297.5 ± 3.7 vs. E2/PMA/Io 115.2 ± 10.9, p=0.002; αCD3/αCD28 1825.3 ± 58.3 vs. E2/αCD3/αCD28 53 ± 1.5, p=0.02; (resting cells (RC) 27.4 ± 1.4)). This data implicates E2 in the inhibition of IL-2 secretion from T lymphocytes.

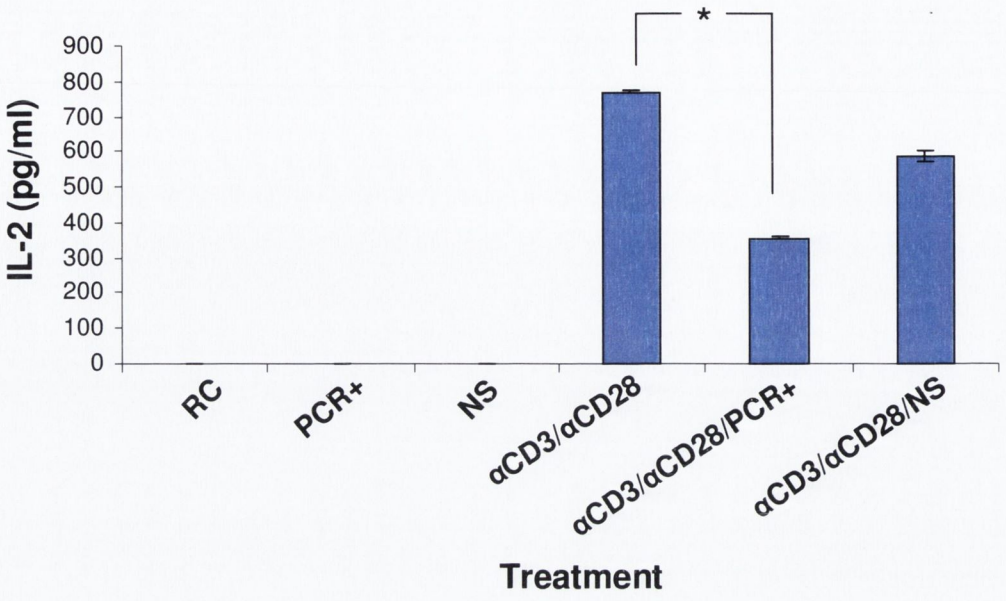



**Figure 3.1 Pre-incubation of PBMCs with HCV-infectious serum down-regulates IL-2 production.** PBMCs were isolated from healthy volunteers and incubated with either normal or HCV $PCR^+$ serum for an hour, prior to αCD3/αCD28 stimulation. The plates were precoated with rabbit anti-mouse immunoglobulins (1/100 dilution) for an hour at 37°C, washed properly in PBS and then coated with a premixed solution of anti-CD3 antibody (1/100 dilution of OKT-3 supernatant in sterile PBS) and anti-CD28 antibody (5 μg/ml) for 2 hours at 37°C. After the wells were washed twice with PBS, the PBMCs were layered in a concentration of $1x10^6$ per ml for 24 hours at 37°C. Following the 24 hour stimulation the supernatants were collected and analysed for IL-2 production. All the experiments were performed in 48 well plates (Nunc A/S, Denmark) in a final volume of 0.5 ml. Data is presented as mean ± SEM of one representative experiment from three independent experiments with similar results. Significant difference is indicated by the asterisk.

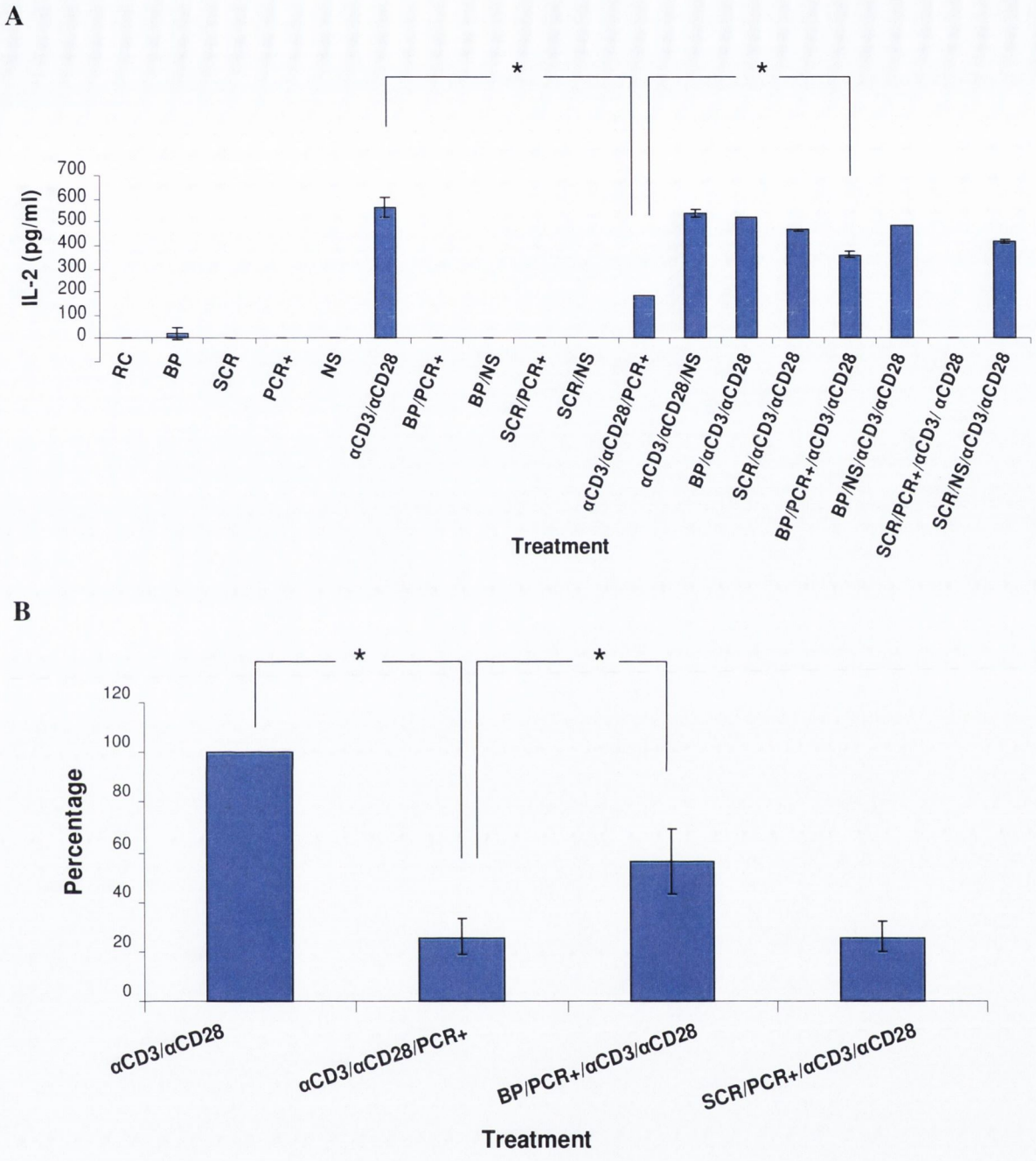



**Figure 3.2 A peptide which blocks CD81-E2 interaction reversed inhibitory effect of HCV-infected serum on TCR-stimulated IL-2 production. A)** PBMCs obtained from healthy donors were pre-treated with a peptide which blocks E2:CD81 interaction (BP) (initially, the BP was added to the cells 5 hours prior any treatment and following that was reapplied reaching the final concentration of 320 μM) prior to incubation with

either normal or HCV $PCR^+$ serum followed by stimulation with αCD3/αCD28 antibodies. A scrambled version (SCR) of the BP was used as a peptide control. Data is presented as mean ± SEM of one representative experiment from five independent experiments with similar results. **B)** This graph indicates the mean percentage recovery in IL-2 secretion in the presence of BP for the five independent experiments. Significant differences are indicated by the asterisks.

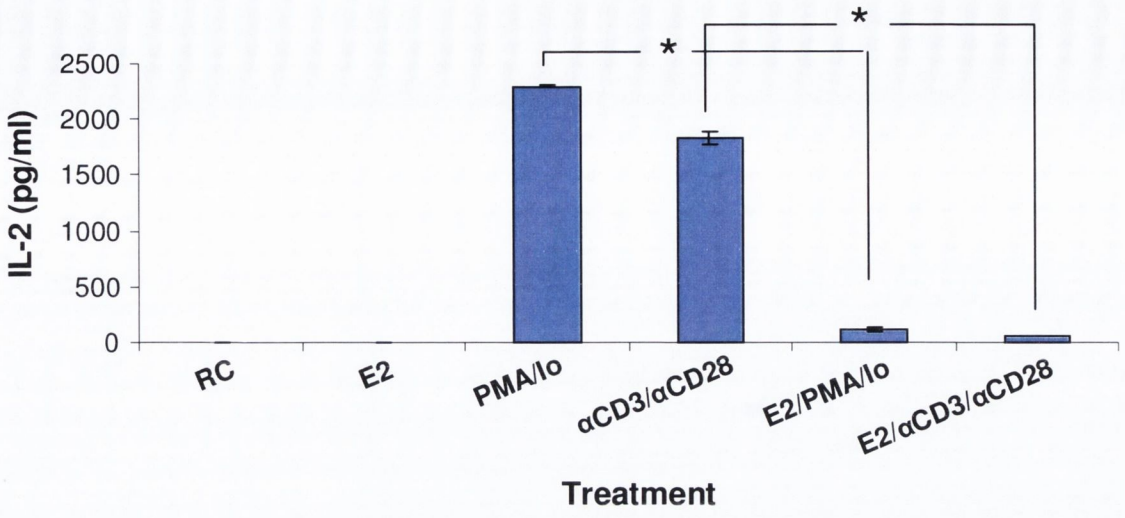



**Figure 3.3 The envelope protein (E2) of the Hepatitis C virus inhibits IL-2 secretion from PBMCs.** PBMCs from the healthy donors were incubated with E2 overnight and after that stimulated. E2-mediated inhibition of IL-2 secretion in PBMCs is significant when stimulated either via PMA and Ionomycin (PMA/Io vs. E2/PMA/Io, p=0.002) or with anti-CD3/anti-CD28 (αCD3/αCD28 vs. E2/αCD3/αCD28, p=0.02). Data is presented as mean ± SEM of one representative experiment from three independent experiments with similar results. Significant differences are indicated by the asterisks. In resting and E2 treated cells, the IL-2 concentration was below the sensitivity of the ELISA assay (<5 pg/ml). The mean percentage inhibition in IL-2 secretion in stimulated PBMCs, pretreated with E2, for three independent experiments is represented in graph 6.1 Appendix 6.

### 3.3.3 *In situ* assay for IL-2 secretion

Based on these findings, we decided to investigate the effect of E2 on IL-2 secretion in a T cell model system - HuT 78 cells. Firstly, we developed and optimized an assay for the analysis of IL-2 secretion. The cells were adhered on the poly-L-lysine coated chamber slides and treated with E2 and PMA, as described earlier (Methods 2.4.1), in the presence of an anti-IL-2 capture antibody. IL-2 (secreted or intracellular) was detected using primary - rat anti-human IL-2 antibody and secondary - donkey anti-rat Ig-G antibody Alexa Fluor 488. Resting cells (RC) and brefeldin A/PMA stimulated cells were used as controls. Brefeldin specifically inhibits translocation of proteins from the endoplasmic reticulum to the Golgi apparatus (GA), through the interaction with Golgi specific proteins that may be involved in maintaining the structural integrity of the organelle. This effect is accompanied by disruption and collapse of the GA which leads to the reduction of the protein secretion. The phorbol ester PMA is a potent stimulator that induces activation in HuT 78 cells and wide variety of responses, including the secretion of IL-2. Cells treated with PMA demonstrated secretion of significant amount of IL-2 **(Figure 3.4)**. In contrast, resting cells exhibited significantly lower amount of secreted IL-2 and that minimal secretion is due to natural ability of resting HuT 78 cells to secrete a small quantity of this cytokine. IL-2 secretion in the cells treated with E2 showed similar pattern to the resting, non-stimulated cells. However, the cells stimulated with E2 overnight prior to PMA activation, demonstrated a dramatically modified phenotype when compared to those treated with PMA alone. Namely, in these cells the IL-2 secretion was significantly attenuated although the cells were treated with PMA **(Figure 3.4)**. Similarly, the IL-2 attenuation was observed in control cells which were treated with brefeldin A for an hour prior to PMA stimulation. HuT 78 cells incubated with brefeldin A secreted significantly lower amount of IL-2 when compared to the PMA stimulated cells and pattern of secretion was similar to that observed in E2 treated cells **(Figure 3.4)**.

To elucidate whether IL-2 was secreted outside the cell and localised on the cell surface and extracellular space, three-dimensional confocal analysis was performed. PMA stimulated HuT 78 cells **(Figure 3.5 A)** were evaluated using 3D confocal imaging and it was shown that IL-2 was localised outside the cell membrane **(Figure**

**3.5 B, C**). When PMA stimulated cells were permeabilised **(Figure 3.5 D)** and analysed using the same method, we observed that fluorescently labeled IL-2 was not exported outside the cell but maintained within the cell body **(Figure 3.5 E, F)**.

Additional immunofluorescence staining for IL-2, actin filaments and nuclei in PMA stimulated HuT 78 cells was performed and images taken at different cell planes. Focussing on the top of the cell, structures located inside the cell lose resolution as they are not at the same level as object that was in focal point **(Figure 3.6 A)**. Therefore, when the focus is on IL-2 secreted outside the membrane, images taken for actin or nuclei are disseminated, whilst keeping the focus on IL-2, showing that these structures are located deeper within the cell body. **Figure 3.6 B** illustrates the same structures but with appropriate sharpness.

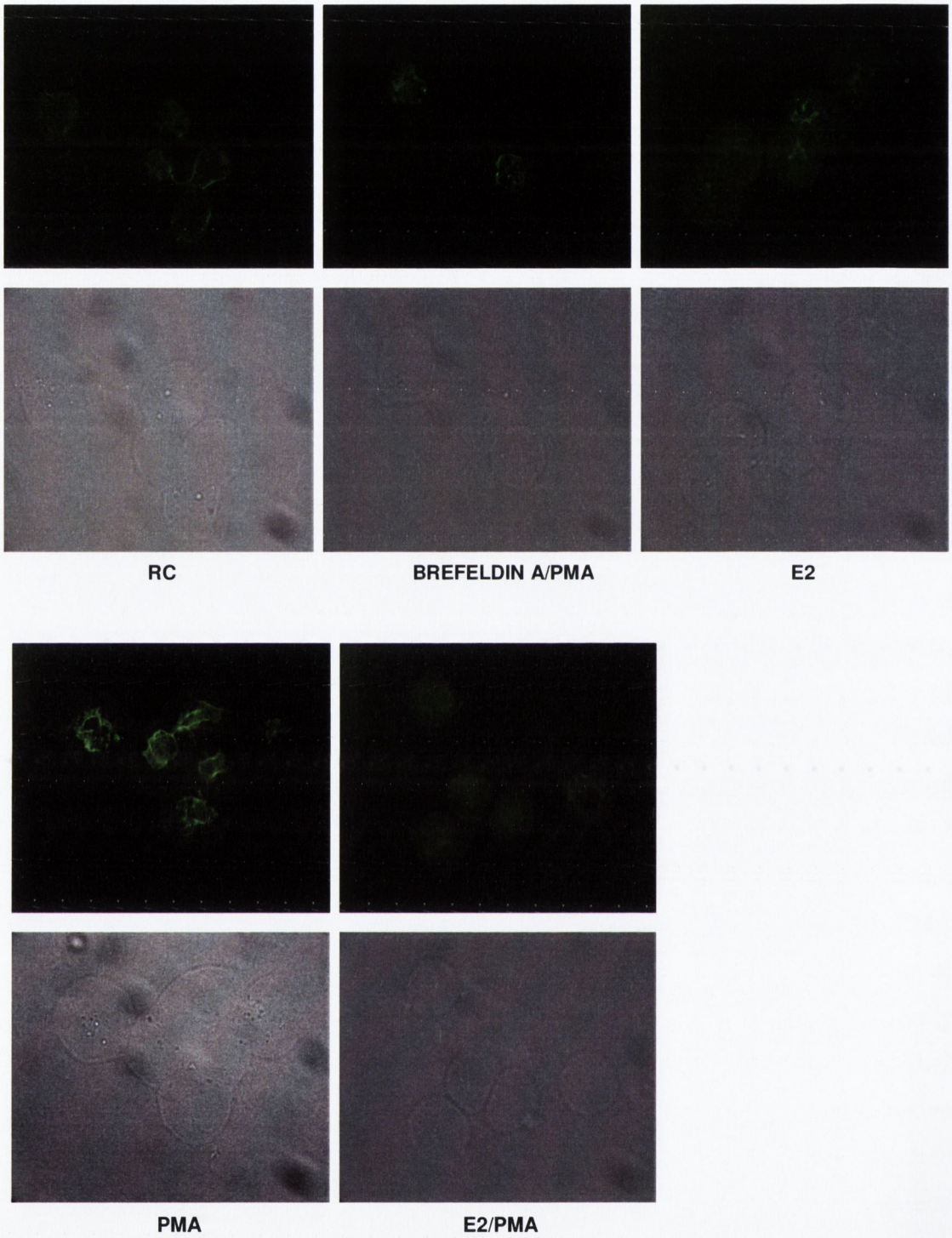



**Figure 3.4 HuT 78 cells stained for IL-2 and corresponding phase image.** HuT 78 cells were pre-treated with E2 overnight or control (brefeldin A 20 μg/ml 1 hour) following PMA (10 ng/ml 5 hours) stimulation. Fluorescently labelled IL-2 in resting, E2, E2/PMA, Brefeldin A/PMA and PMA treated HuT 78 cells and related phase contrast images. This experiment has been conducted three times.

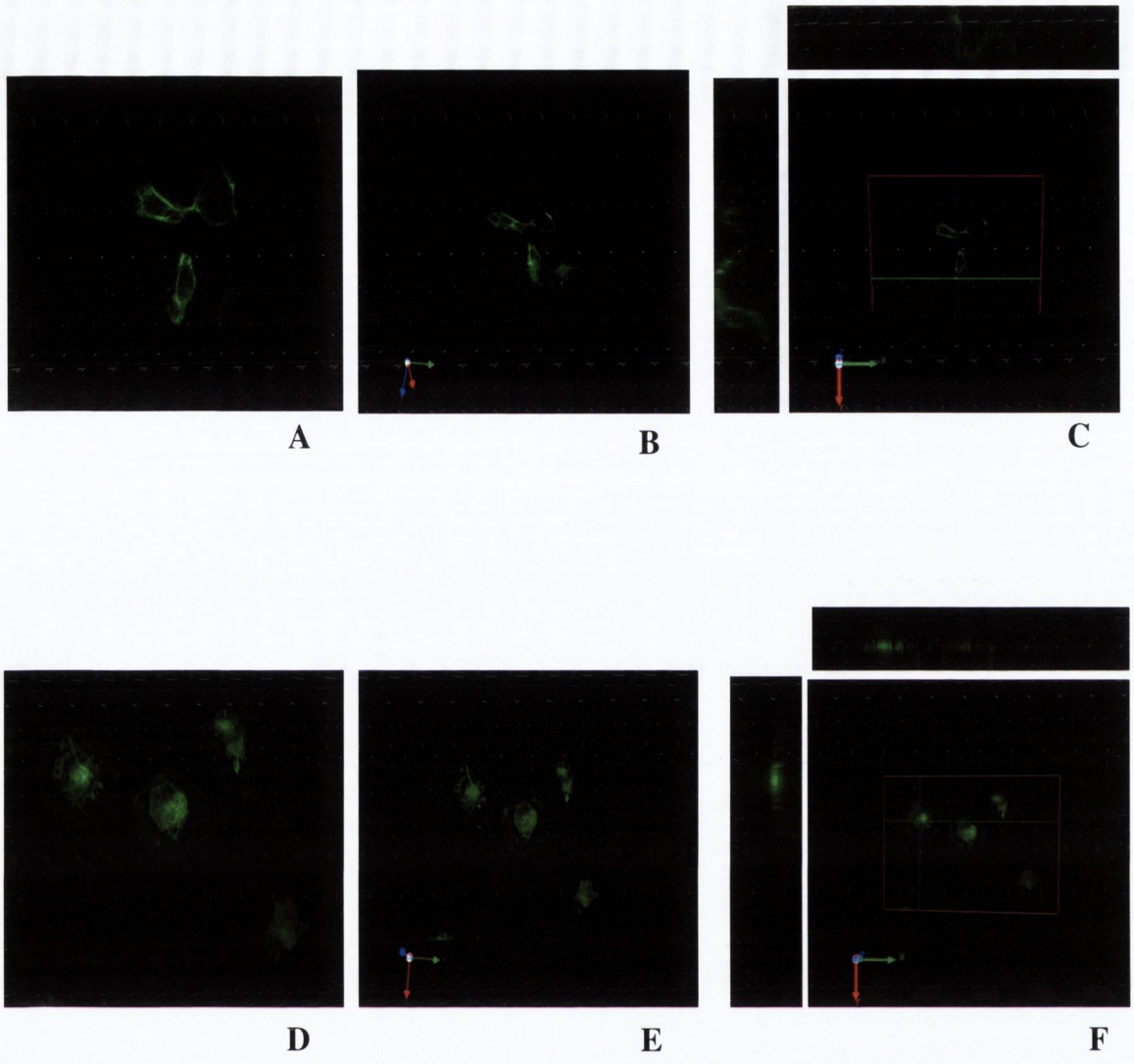
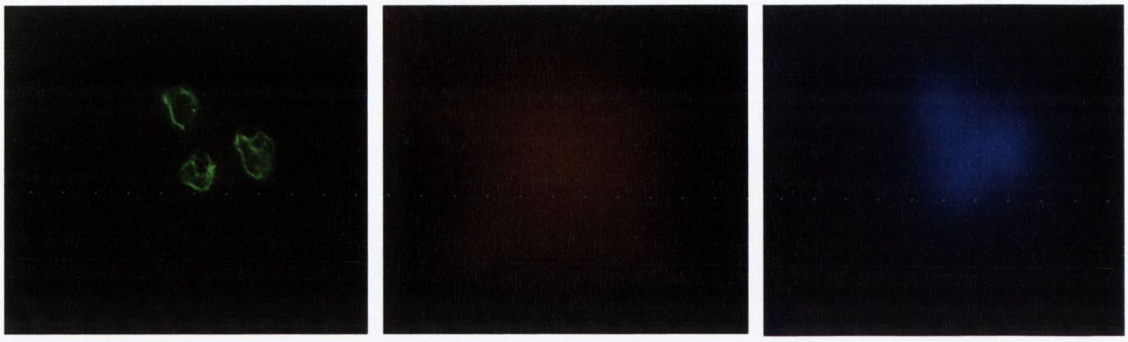



**Figure 3.5 Three-dimensional confocal analysis of IL-2 secretion. A)** PMA stimulated HuT 78 cells stained for IL-2 **B)** 3D projection of the cells indicated by arrows in respective left image **C)** 3D projection of IL-2 distribution in vertical and horizontal plane as indicated by the arrows. Vertical and horizontal dissections proved that IL-2 is secreted outside the cell membrane. **D)** Intracellular staining for IL-2 in permebilised HuT 78 **E)** 3D projection of the cells indicated by arrows in respective left image **F)** 3D projection of IL-2 distribution in vertical and horizontal plane as indicated by the arrows. Confocal analysis was performed twice.

A



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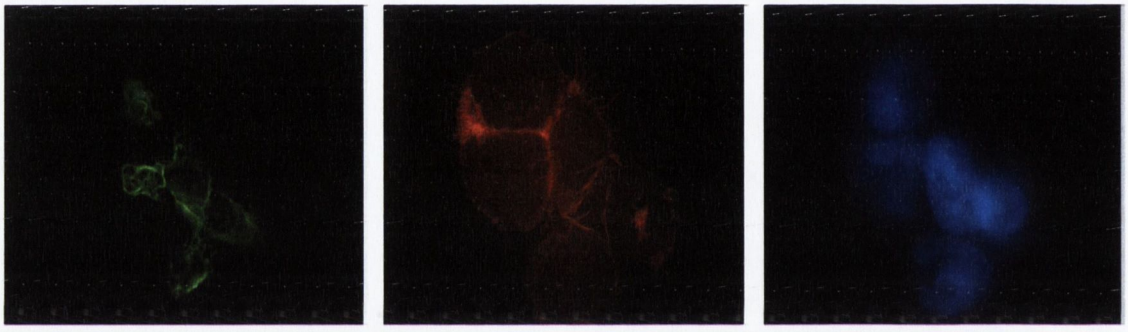


**Figure 3.6 A)** First panel on the left shows fluorescently labeled IL-2 following the PMA stimulation; middle panel illustrates actin filaments stained with phalloidin-TRITC, where images were taken focusing the IL-2 secreted on the top of the membrane, outside the cells (left panel) and similarly last panel where nuclei were stained with Höechst 33342.

**B)** First panel on the left shows IL-2 secreted after PMA stimulation; panel in the middle is image of the same group of cells taken focusing the actin staining where cells boundaries are clearly shown and the last panel displays nuclei of the same group of cells stained with Höechst 33342. This type of actin filaments staining was performed once.

### 3.3.4 HCV E2, but not C22 or C33, inhibits IL-2 secretion

To investigate if E2-mediated inhibition of IL-2 secretion is a unique phenomenon related to E2, the effect of other HCV viral peptides on PMA-stimulated IL-2 secretion was investigated. HuT 78 cells were pre-treated with either viral E2 protein or core protein - C22 or protein derived from the NS3 region - C33 overnight and afterwards stimulated with PMA. Cells pre-treated with E2 demonstrated a significant reduction in IL-2 secretion following PMA stimulation (**Figure 3.7**). In control, PMA-stimulated cells IL-2 levels in pg/ml were 284.9 ± 14.3 while in those pre-treated with E2 followed by PMA, levels of IL-2 were 25.3 ± 6.5, (p=0.0088); (resting cells (RC) 25 ± 3.3). HuT 78 cells pre-incubated with C22 or C33 did not exhibit any inhibition on IL-2 secretion. Since there was no impact on IL-2 secretion by other tested viral proteins it indicated distinctive capability of E2 in this process.

### 3.3.5 Inhibition of E2-CD81 interaction recovers the ability of HuT 78 cells to secret IL-2

The CD81 cell surface molecule, a member of the tetraspanin family, is an important receptor for binding of HCV particles that are infectious in cell culture (Lindenbach et al, 2005) and for cell entry of HCV pseudoparticles (Lavillette et al, 2005). In order to demonstrate the specificity of this interaction in the inhibition of IL-2 secretion, a peptide, previously proven to block E2-CD81 interaction was used (Cao et al, 2003). In these experiments, cells were exposed to blocking peptide (or scrambled control) prior to E2 pre-treatment and subsequently activated with PMA. The specificity of recombinant E2:CD81 interaction mediating this inhibition of IL-2 secretion was confirmed by rescue with the blocking peptide (**Figure 3.8**; IL-2 levels in pg/ml: E2/PMA 60.5 ± 0.4 vs. BP/E2/PMA 866.6 ± 13.5, p=0.01; (PMA 1558.5 ± 22.5; resting cells (RC) 28.8 ± 1.5)). IL-2 secretion was partially restored in HuT 78 cells incubated with the blocking peptide thus providing further evidence that E2-CD81 interaction mediates inhibition of IL-2 secretion.

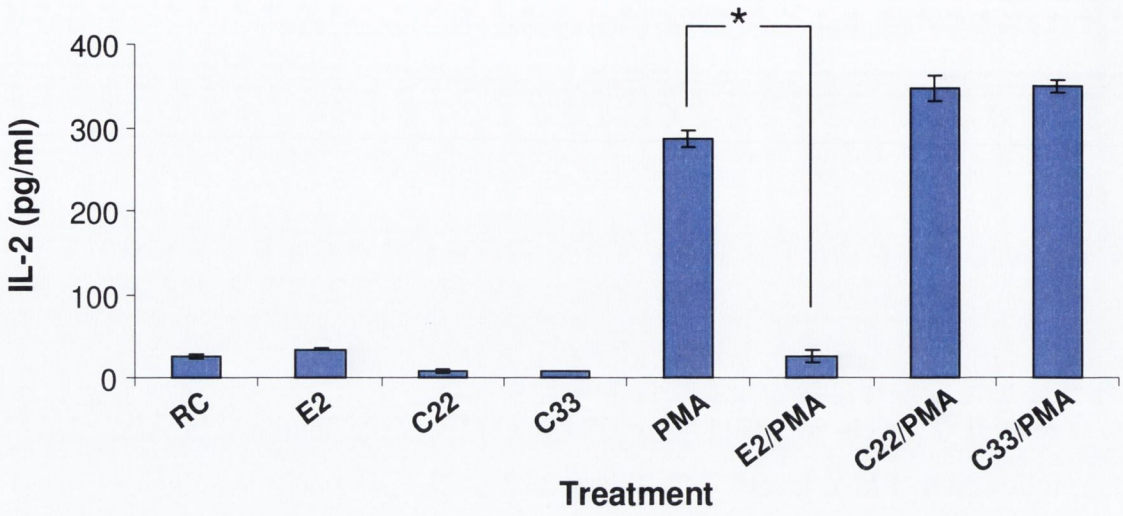



**Figure 3.7 Pre-treatment of HuT78 cells with HCV envelope protein E2 but not core peptide C22 and NS3 peptide C33 inhibited PMA-stimulated Il-2 secretion.** In these experiments HuT 78 cells, with a density of $7x10^5$ per ml, were pretreated with 1 µg/ml of HCV recombinant E2 protein overnight (in control wells E2 was replaced with HCV recombinant core protein C22 or protein from NS3 region C33, both at a final concentration of 1 µg/ml) and afterwards stimulated with 10 ng/ml of PMA for 24 hours at 37°C. The experiments were performed in 48 well plates in a final volume of 0.5 ml. In HuT 78 cells pre-treated with E2 significant reduction in IL-2 secretion is observed (p=0.0088). However, incubation with the other two viral peptides C22 and C33 did not result in any reduction in IL-2 secretion. Data is presented as mean ± SEM of one representative experiment from three independent experiments with similar results. The mean percentage IL-2 secretion in E2 or C22 or C33 pretreated HuT 78 cells stimulated with PMA, for three independent experiments is represented in graph 6.2 Appendix 6.

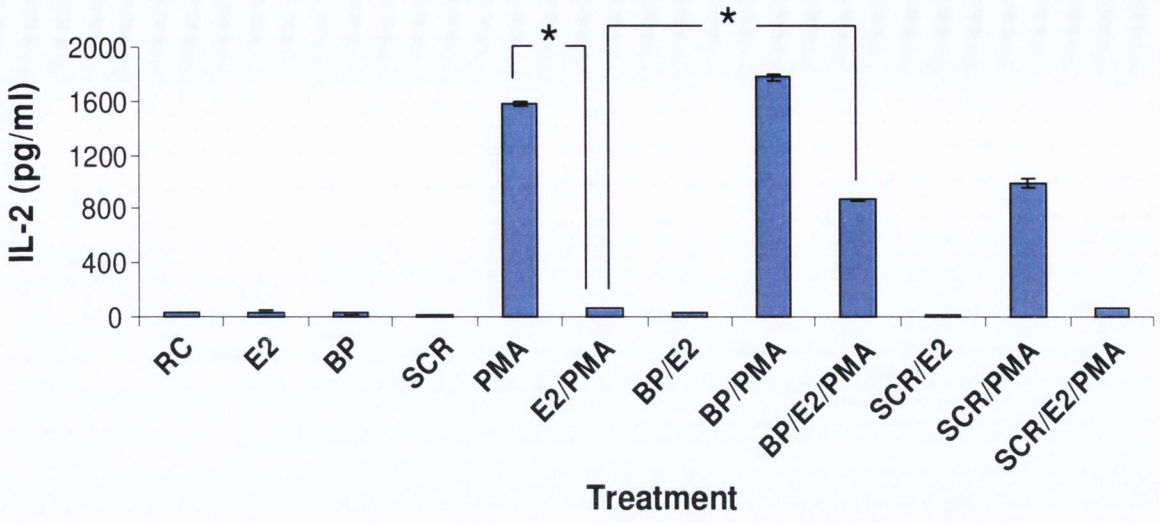



**Figure 3.8 A blocking peptide (BP) which can abolish E2 binding to CD81 can rescue IL-2 secretion.** HuT 78 cells pre-incubated with BP (as described in the figure legend 3.2) and then treated with E2 prior PMA stimulation secreted significantly higher amount of IL-2 in comparison to those pre-treated with E2 but not exposed to BP ($p= 0.01$). Data is presented as mean ± SEM of one representative experiment from three independent experiments with similar results. Significant differences are indicated by the asterisks. As a control a scrambled version of the BP (SCR) was used. The mean percentage recovery in IL-2 secretion in the presence of BP for the three independent experiments is shown in the graph 6.3 Appendix 6.

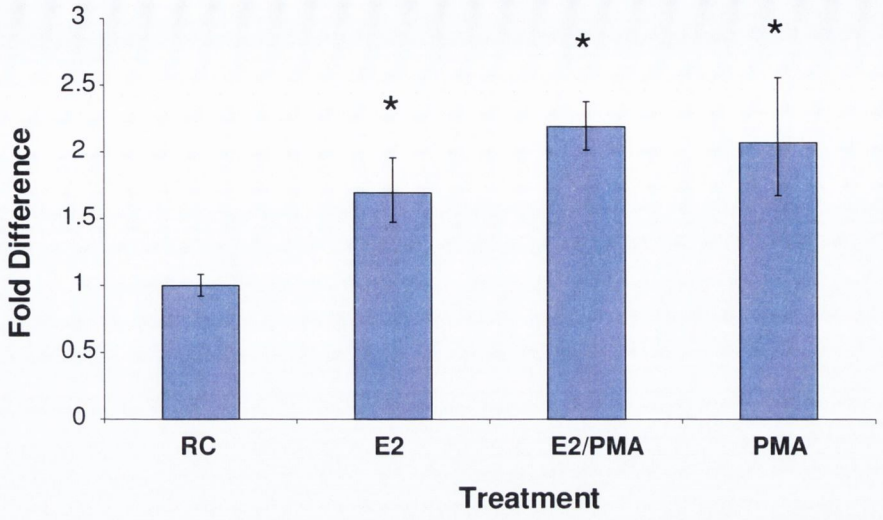
### 3.3.6 HCV envelope protein E2 inhibits IL-2 production at the level of secretion

To further elucidate the mechanism of E2-mediated inhibition of IL-2 production in HuT 78 cells, we then examined whether this process was affected at the level of transcription. For this purpose cells were incubated with E2 overnight and following that stimulated with PMA for 5 hours. This short treatment was sufficient for detection of changes in gene expression. RNA was extracted and reverse transcription performed as described earlier (2.9.1). Quantitative Real Time-PCR for IL-2 mRNA was performed to measure its expression **(Figure 3.9 A)**. In this experiment we demonstrated that E2 did not repress IL-2 gene transcription and IL-2 mRNA levels in PMA stimulated cells increased 2.07 fold over resting cells, ($p=0.01$) while PMA increased IL-2 mRNA 2.19 fold in the presence of E2, ($p=0.0005$) (E2 treated cells increased 1.7 fold over resting cells, $p=0.0087$). The examination of intracellular IL-2 protein expression by immunofluorescence analysis revealed that E2 pre-treated cells stimulated with PMA had significant amounts of IL-2 protein in their cytosol which was not released into the extracellular space/cell surface **(Figure 3.9 B)**. This immunofluorescence assay was developed by incubating the cells in the presence of an anti-IL-2 capture antibody. When cells were pre-treated with E2 prior to 5 hours long PMA stimulation, IL-2 could only be detected in the cytosol of permeabilised cells. Augmentation at the level of mRNA for IL-2 gene after E2/PMA treatment and detection of IL-2 within the cell (but not outside) demonstrated that E2 did not affect the level of transcription of the IL-2 gene as well as level of its translation into a protein.

### 3.3.7 E2 targets PKC-β to lipid rafts

The HuT 78 cell line was utilised further to investigate the mechanism of E2-mediated inhibition of IL-2 secretion. It has been previously demonstrated that the expression of PKC-β is necessary for the export of IL-2 from HuT 78 cells activated with PMA (Long et al, 2001) and in more recent study, it has been postulated that ligation of CD81 with E2 may result in sequestration of PKC-β in lipid raft microdomains (Volkov et al, 2006). Therefore, we proposed that the virus may manipulate this enzyme in its inhibition of IL-2 secretion. To explore this, HuT 78 cells were immunofluorescently stained for lipid

A

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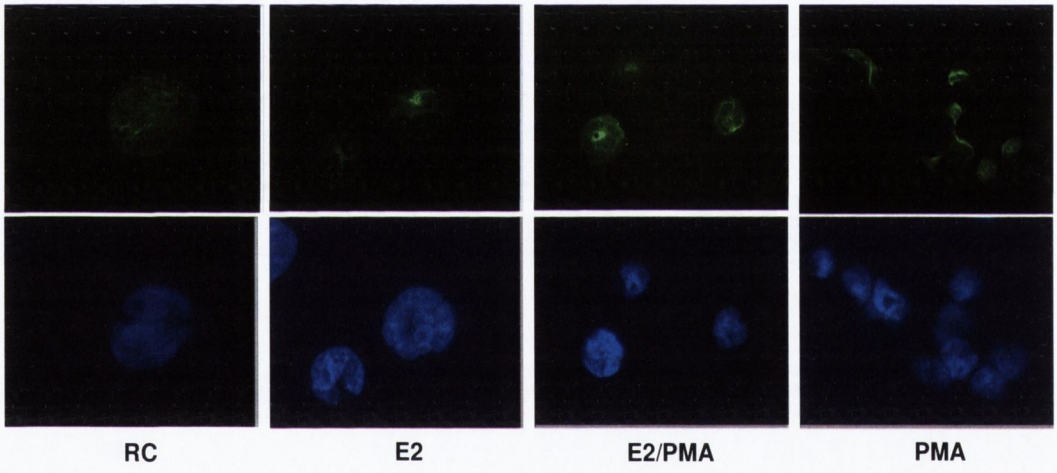


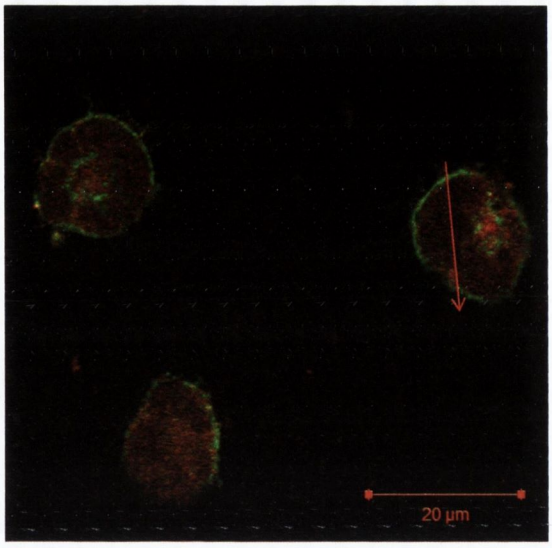

**Figure 3.9 E2 prevents IL-2 production at the level of secretion not transcription.** **A)** Effect of E2 on IL-2 mRNA expression in HuT 78 cells. Quantitative RT-PCR data was achieved using the comparative $C_T$ method. The housekeeping gene GAPDH was used as an endogenous control. Significant differences ($p<0.05$) are indicated by the asterisks. **B)** Immunofluorescence analysis demonstrates that E2 does not affect the production of IL-2 in HuT 78 cells. Cells were stimulated with PMA (10 ng/ml) in the presence or absence of E2 and production of IL-2 detected in permeabilised cells. Upper row shows fluorescently labeled IL-2 while lower images display nuclei of the same group of cells stained with Höechst 33342. These experiments were conducted twice.

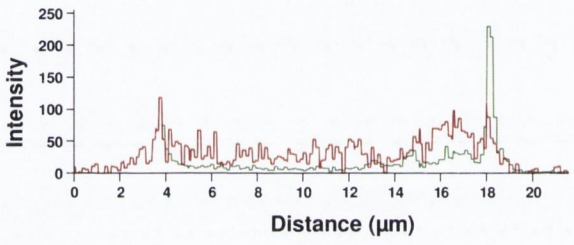
raft (in green) and PKC-β (in red) and confocal microscopy performed. In resting HuT 78 cells, PKC-β displays a cytosolic distribution **(Figure 3.10 A)**; however, when these cells have been incubated with E2, PKC-β is located in the lipid raft compartment where it co-localises with ganglioside GM-1, a well established marker for lipid raft (in yellow) **(Figure 3.10 B)**. Co-localisation, defined as the presence of different types of molecules (fluorochromes) at the same physical position (Smallcombe, 2001), was assessed and quantified using Pearson's Correlation Coefficient (Rr) for resting (Rr = 0.4472 ± 0.05) and E2 treated cells (Rr = 0.6035 ± 0.02) **(Figure 3.10 C)**. Pearson's Correlation Coefficient displays statistical evaluation of the extent of overlap between two molecules of interest, including only the similarity of shapes between images, but not image intensity (Manders et al, 1992). Rr was computed using Image Pro Plus software v. 4.5. Following the analysis, it was clearly shown that degree of PKC-β co-localisation with GM-1 was substantially increased following E2 treatment (p=0.0055).

### 3.3.8 Disruption of lipid rafts recovers IL-2 secretion

Ligation of CD81 with E2 results in translocation of PKC-β into lipid raft microdomains thus preventing its association with the centrosome and microtubule cytoskeleton, which is essential for the process of secretion. Therefore, it was investigated if disruption of lipid rafts could reverse the inhibitory effect of CD81:E2 interaction in IL-2 export. Following the pre-treatment of HuT 78 cells with E2, cells were incubated in the presence of the lipid raft disrupting agent β-methylcyclodextran (MCD) prior to PMA stimulation. Immunofluorescence analysis illustrates that the cells, although exposed to E2, when treated with MCD prior to stimulation, could restore the secretion and IL-2 could be detected on the cell surface **(Figure 3.11)**. Following quantification, the concentration of IL-2 (pg/ml) secreted by HuT 78 cells pre-treated with E2 and followed by PMA stimulation was 60.5 ± 0.4 while in the presence of MCD this was increased to 949.8 ± 32.2, p= 0.0233 (resting cells 28.8 ± 1.5, PMA stimulation 1580.9 ± 22.5) **(Figure 3.12)**. Although E2-mediated inhibition of IL-2 secretion was significant (p=0.0095), treatment with lipid raft disruptor recovered the ability of HuT 78 cells to secrete IL-2 in response to PMA even in the presence of E2.

A

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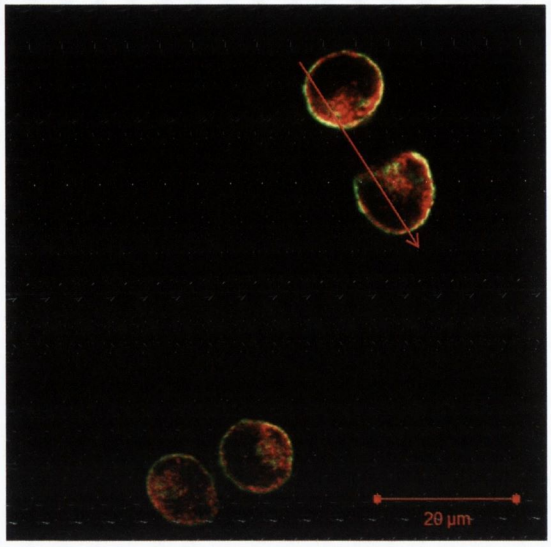
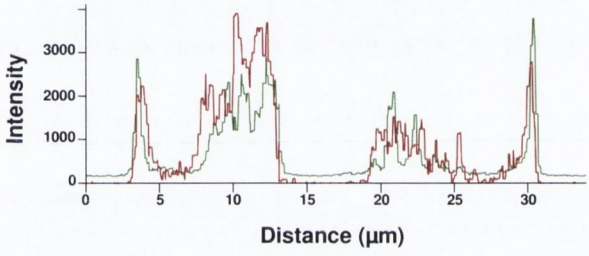



C

| | |
|---|---|
| **Pearson's Correlation Coefficient - Resting Cells** | **Rr = 0.4472** |
| **Pearson's Correlation Coefficient - E2 treated Cells** | **Rr = 0.6035** |
| **T test** | **p = 0.0055** |

**Figure 3.10 E2-CD81 interaction induces the translocation of PKC-β from a cytosolic distribution to associate with lipid raft microdomains.** Subcellular localization of PKC-β (red) and lipid raft/Ganglioside GM-1 (green) in **(A)** Resting cells and **(B)** E2-treated cells. The graphs below each image represent intensity and distribution of green and red fluorescence indicated by the red arrows. Significant difference (p=0.005) in the level of co-localisation was observed when compared Pearson's Correlation Coefficients (means) calculated for both resting and E2 treated cells **(C)**. This experiment has been conducted twice.

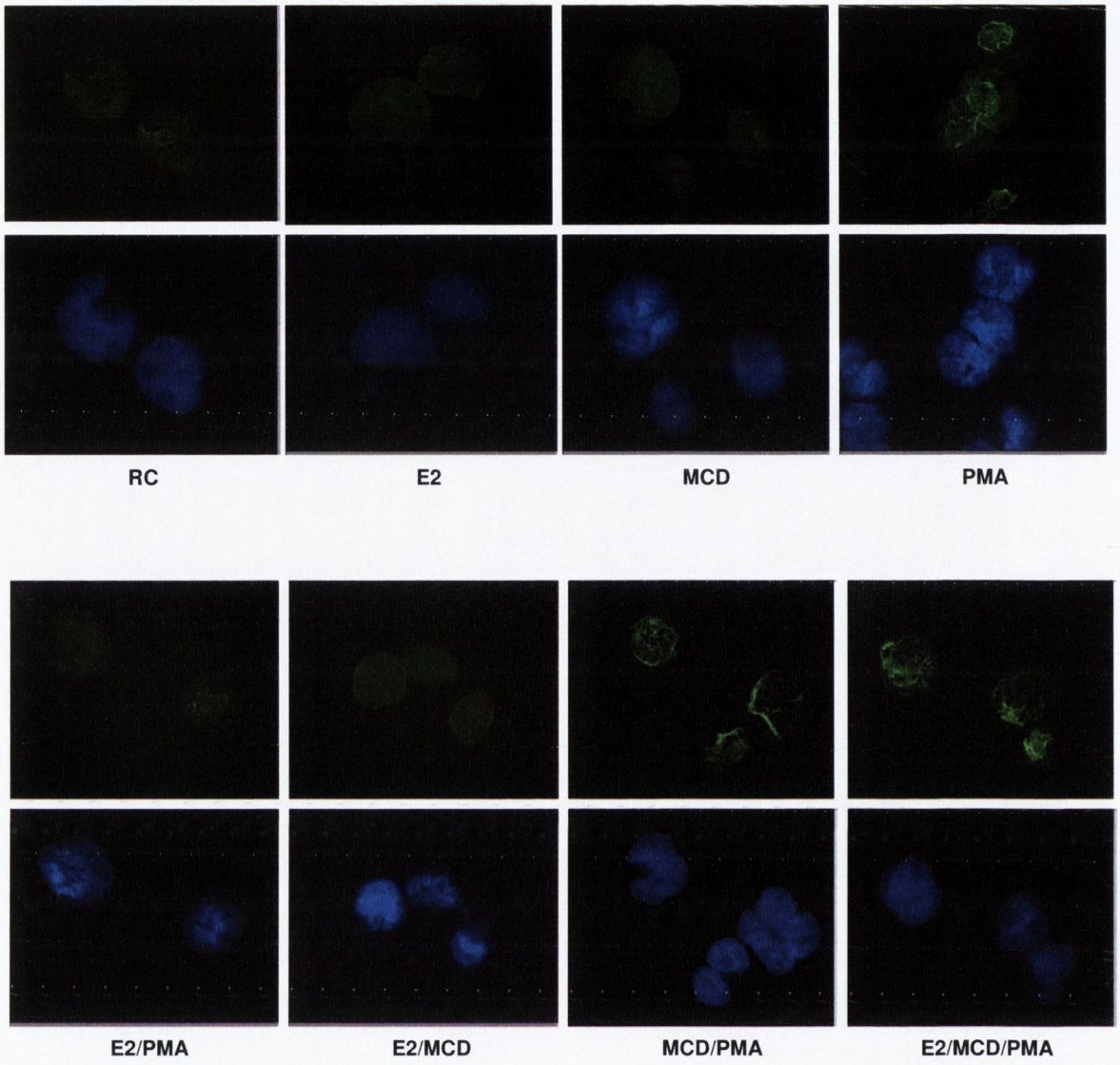



**Figure 3.11 The lipid raft disrupting agent, MCD, recovers the inhibitory effect of E2 on IL-2 secretion.** HuT 78 cells were incubated with E2, MCD and PMA on its own or in different combinations (Methods 2.4.1). PMA stimulated secretion of IL-2 was inhibited once when the cells were previously exposed to E2. However, HuT 78 cells incubated with E2 and subsequently treated with MCD prior to PMA activation, exhibited successful recovery of the IL-2 secretion. Additionally, MCD did not alter the secretory phenotype of the PMA activated cells or E2 treated cells. All conditions are presented with immunofluorescence image of IL-2 and corresponding image of nuclei stained with Höechst 33342. This experiment has been conducted twice.

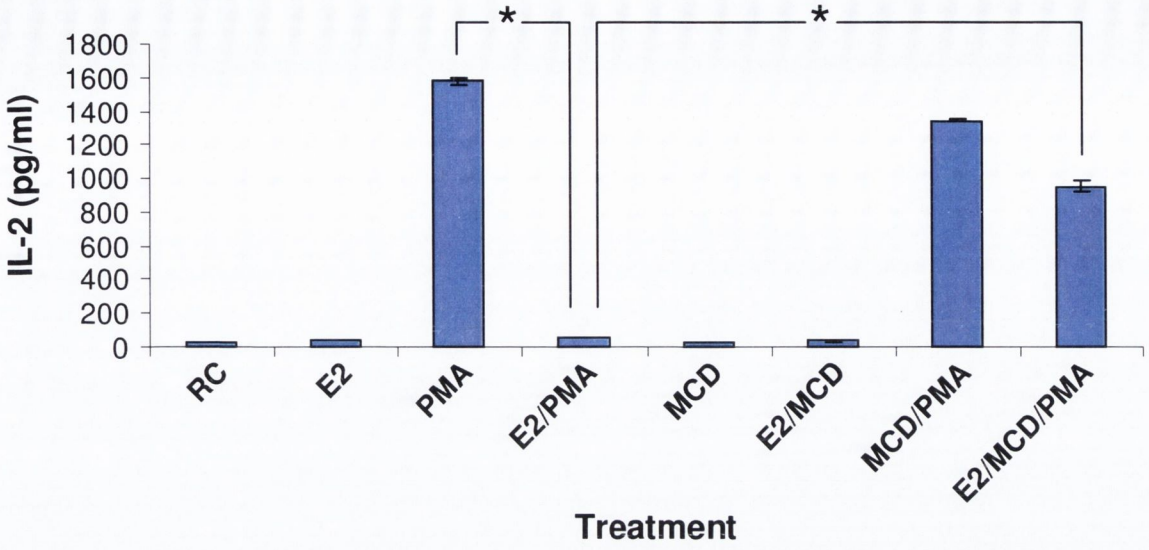



**Figure 3.12 MCD treatment annuls the inhibitory effect of E2 on IL-2 secretion.** Cells were pre-treated with E2 overnight and then incubated +/- MCD (0.2 mM) for an hour prior to PMA stimulation. Those treated with MCD secreted significantly higher levels of IL-2 when compared to those with E2 alone (p=0.02). Data is presented as mean ± SEM of one representative experiment from three independent experiments with similar results. Significant differences are indicated by the asterisks. The mean percentage recovery in IL-2 secretion in the presence of MCD for the three independent experiments is shown in the graph 6.4 Appendix 6.

### 3.3.9 PKC-β as a target molecule in E2 mediated IL-2 inhibition

E2:CD81 interaction directs PKC-β molecule into lipid raft microdomains, thus interrupting its functional role in the process of IL-2 secretion. In order to confirm PKC-β as a major molecule in this inhibition, some other experimental approaches were used.

#### 3.3.9.1 PKC-β siRNA silencing

To further investigate involvement of PKC-β in IL-2 secretion, cells were treated with siRNA targeting PKC-β gene expression. siRNA was delivered into the cells using electroporation as described previously (Methods 2.11). To optimise this procedure siGlo (fluorescent lamin A/C siRNA) was used as a reliable control of siRNA delivery and transfection efficiency. When optimised **(Figure 3.13)** approximately 80 % of HuT 78 cells were successfully transfected, as fluorescence (visualization) correlates well with transfection as well as silencing efficiency.

Before the silencing was assessed by quantitative RT-PCR, the expression of PKC-β in three cell types, while cells were in resting state, was measured **(Figure 3.14)**. PKC-β mRNA level in HuT 78 cells increased 25.18 fold over K4 cells, while in PBMCs increased 876.85 fold. K4 cells are PKC-β-deficient clone derived from a parental HuT 78 cell line (Long et al, 2001). When HuT 78 and PBMCs were compared, the level of IL-2 mRNA in PBMCs was enhanced 34.8 fold over HuT 78 cells.

In order to estimate the success of target gene/protein silencing, quantitative RT-PCR for PKC-β gene expression was performed. A decrease in mRNA levels of the enzyme could be observed at 48 hours after the application of siRNA. **Figure 3.15 A** represents a typical knock-down in PKC-β gene expression with PKC-β mRNA levels of siRNA treated cells being significantly decreased when compared to resting cells (RC, electroporated but not treated with siRNA) ($p=0.0022$) or cells treated with scrambled siRNA control (C) ($p=0.00002$), following the 96 hours long siRNA exposure. At this time point, cells are expected to have reduced levels of the target protein (PKC-β). Viability was approximately 70% for all cells (RC 67.31%, C 68.73%), siRNA treated cells 67.29% in this representative experiment. Subsequently, these cells were stimulated with

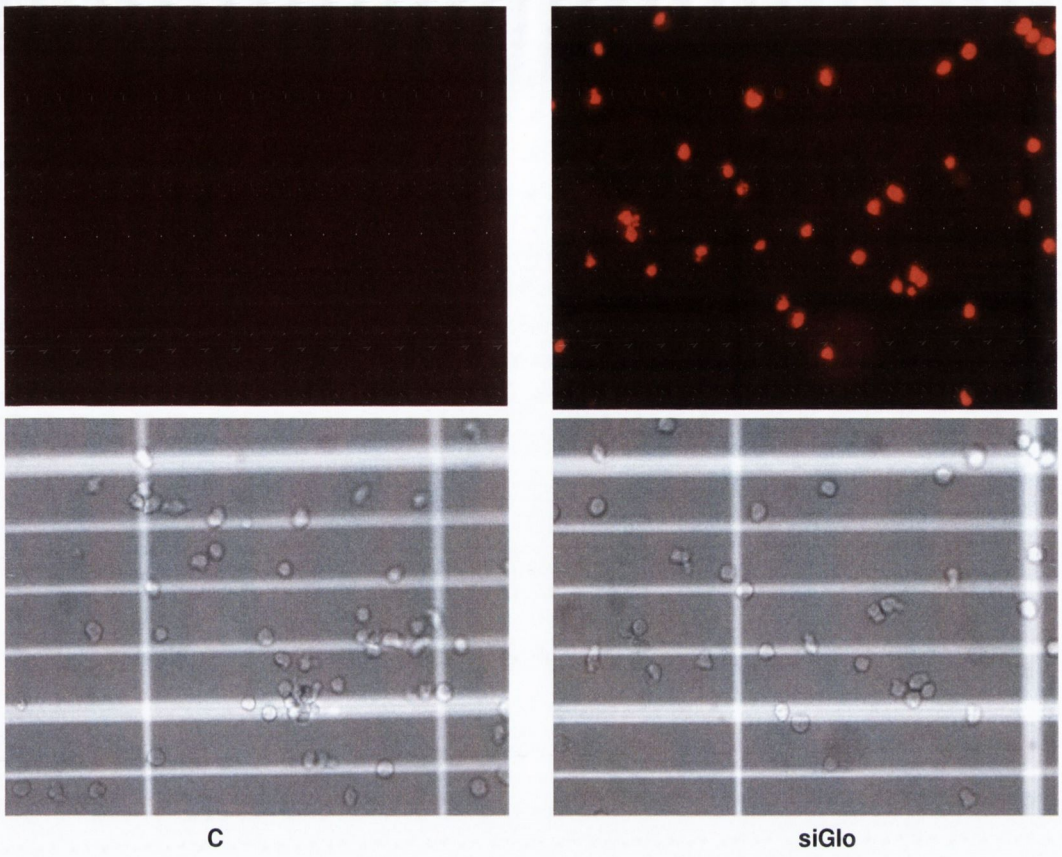



**Figure 3.13 Efficacy of transfection in HuT 78 cells using siGlo and BTX electroporator.** Uptake of siGlo positively correlates with transfection efficiency and target gene silencing. None of the control cells (electroporated cells, no siGlo) exhibit fluorescence, while most of siGlo treated cells were positive (both with respect to phase contrast image of the same microscopic field). Images were taken 24 hours after electroporation.

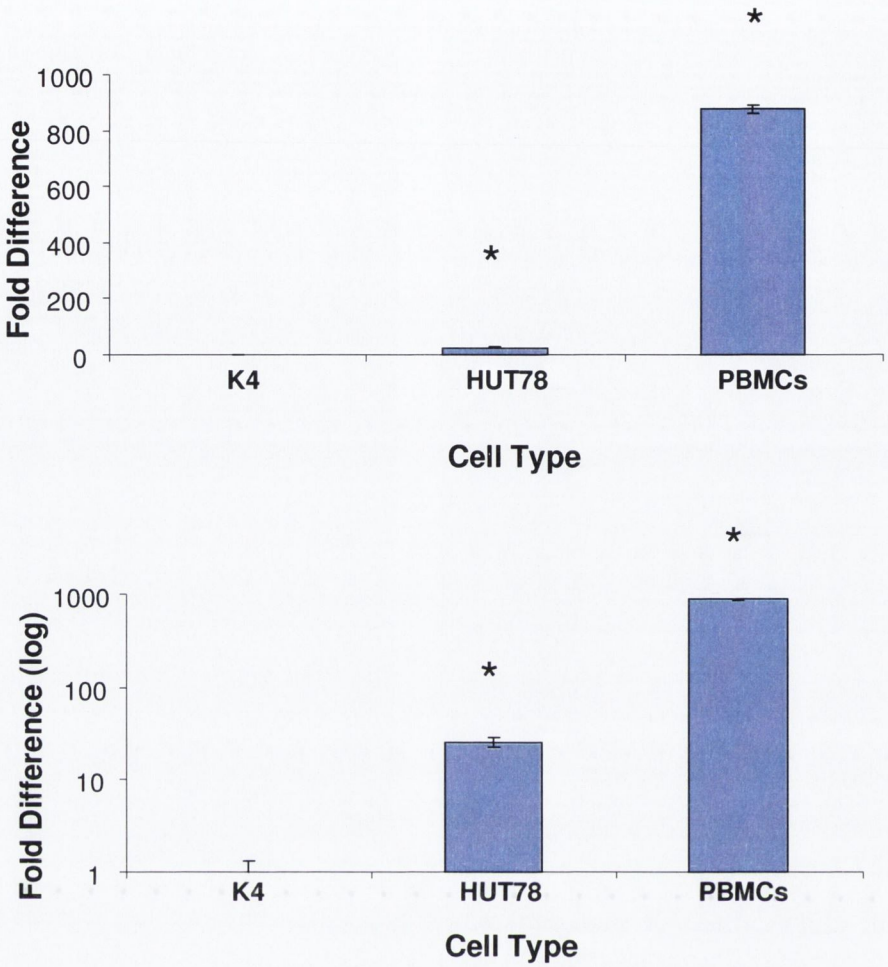



**Figure 3.14 Expression of PKC-β in three cell types.** Both, resting HuT 78 (p=0.0044) and PBMCs (p=0.00007) exhibit significant expression of PKC-β when compared to PKC-β-deficient clone K4. In addition, constitutive expression of the enzyme is considerably higher in PBMCs then in HuT 78 cells (p=0.00005). Fold difference is presented in linear (upper graph) and logarithmic scale (lower graph). This experiment has been done as a test for PKC-β expression and RT-PCR and was conducted once.

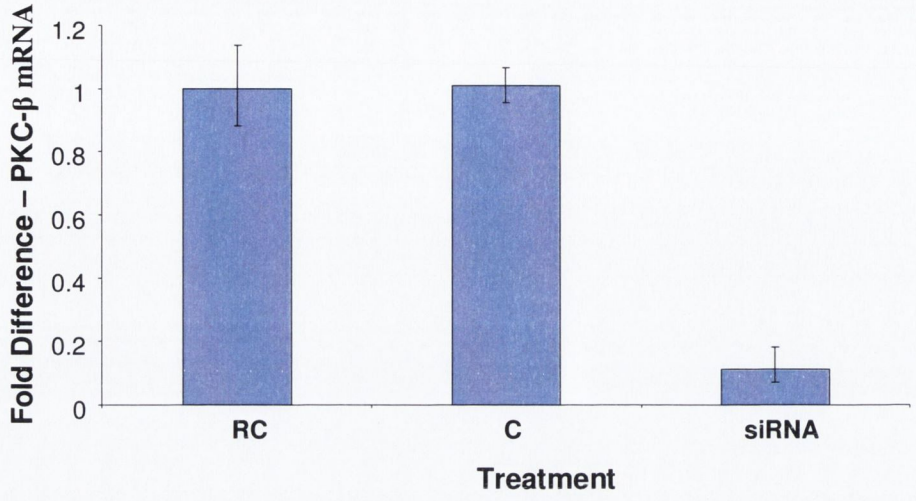
PMA and used for IL-2 secretion assay, which also included non-stimulated cells (NS) and stimulated cells with no electroporation (NE) **(Figure 3.15 B)**. Surprisingly, the expected phenotype of reduction in IL-2 secretion in siRNA treated cells was not observed, although just 10% of PKC-β mRNA was not suppressed (at mRNA level).

### 3.9.2 PKC-β selective inhibition

To try a different approach to further confirm that PKC-β is the critical component required for IL-2 secretion that is sequestered in lipid rafts following E2-CD81 interaction, HuT 78 cells were incubated with a small compound called Go6976. This cell permeable inhibitor selectively inhibits PKC-α/β isoforms, without affecting the activity of other PKC isoenzymes. Cells treated with Go6976, upon lipid raft disruption with MCD, exhibited complete inhibition of IL-2 secretion. In this instance, MCD could no-longer reverse the inhibitory effect of E2 on IL-2 secretion **(Figure 3.16)**. The concentration of IL-2 (pg/ml) secreted by HuT 78 cells which were pre-treated with Go6976 and followed by PMA stimulation was 22.45 ± 1.2 while the cells treated only with PMA secreted 495.55 ± 4.1 pg/ml, p=0.005. In the cells exposed to E2 pre-treatment, followed by MCD incubation and PMA stimulation, IL-2 concentration was 225.86 ± 5.1 while in the presence of Go6976 this was decreased to 24.13 ± 0.4, p=0.01 (resting cells 1.92 ± 0.2, E2/PMA 6.87 ± 0.1 vs PMA, p=0.0036). Hence, inhibition was observed even in the presence of MCD, where PKC-β, disengaged from the lipid raft, was subsequently inhibited by Go6976.

This data indicates that the targeting of PKC-β to lipid rafts microdomains by HCV E2 is a specific mechanism of viral inhibition of secretion of this, and potentially other cytokines.

A

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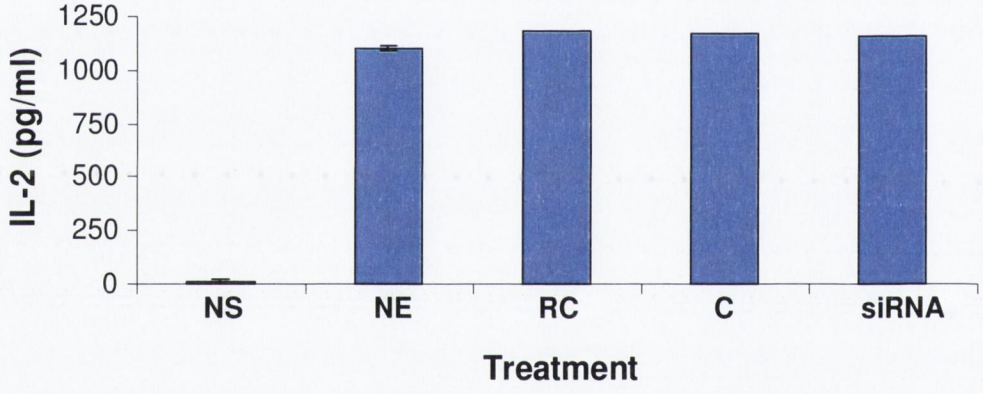



**Figure 3.15 PKC-β silencing and the functional assessment of its phenotype. A)** Reduction of PKC-β mRNA level of 8.9 fold below resting cells (RC) or cells treated with scrambled siRNA control (C), correlating with almost 90% knock-out, was obtained 96 hours after electroporation. **B)** IL-2 secretion was not affected in cells treated with siRNA, although 90% of mRNA for PKC-β was knocked down. Data is presented as mean ± SEM of one representative experiment from three independent experiments with similar results.

NS - non-stimulated cells, NE - PMA stimulated cells with no electroporation, RC - electroporated cells subsequently PMA stimulated, C - cells treated with scrambled siRNA control subsequently PMA stimulated, siRNA - cells treated with PKC-β siRNA subsequently PMA stimulated.

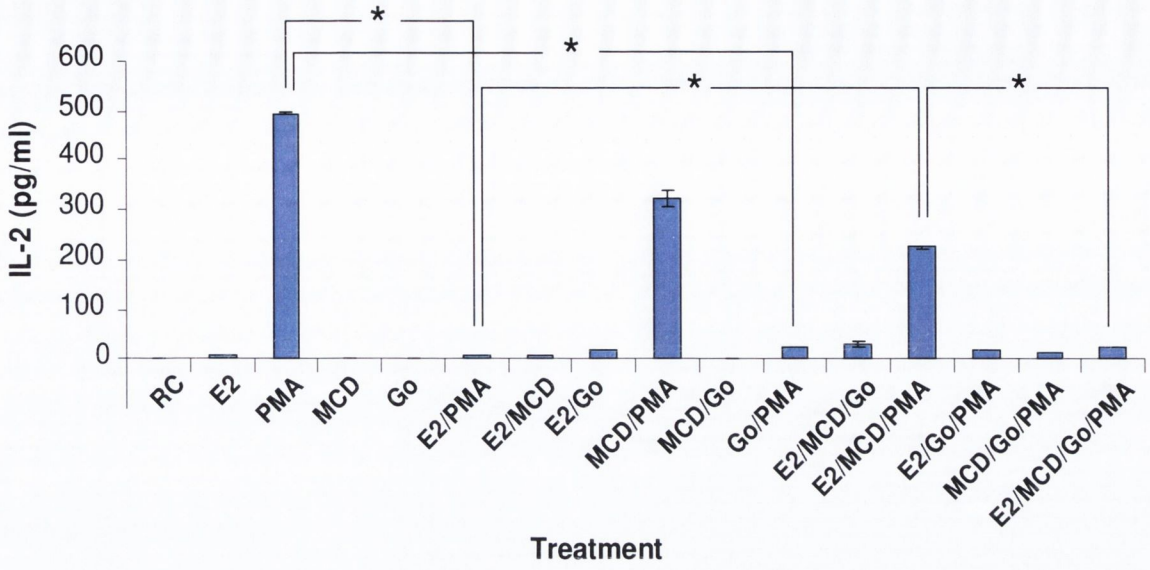



**Figure 3.16 PKC-β selective inhibitor Go6976 prevents the MCD induced recovery of IL-2 secretion.** Selective PKC-α/β inhibitor, Go6976, was added for 30 min at 5 μM final concentration, after E2 and MCD treatments and prior to PMA incubation. The inhibitor Go6976 impedes MCD-induced rescue of IL-2 secretion in cells pre-treated with E2. Data is presented as mean ± SEM of one representative experiment from three independent experiments with similar results. Significant differences are indicated by the asterisks. The mean percentage IL-2 secretion in the presence of Go6976 inhibitor for the three independent experiments is shown in the graph 6.5 Appendix 6.

## *3.4 DISCUSSION*

Effective T cell responses are critical to clear acute viral infection and prevent viral persistence, with robust, early CD4+ T cell responses important in sustaining CD8+ cytotoxic T cell function (Matloubian et al, 1994; Rosenberg et al, 1997; Thimme et al, 2001; Urbani et al, 2006). In a model of chronic viral infection (lymphocytic choriomeningitis virus (LCMV)), Brooks et al. demonstrated that virus-specific CD4+ T cells become functionally inactive during the transition to viral persistence and fail to produce effector cytokines such as IL-2 and TNF-α resulting in an ineffective anti-viral immune response (Brooks et al, 2005). Observations with respect to HCV are consistent with these findings with CD4+ T cell responses important for the outcome of infection. In HCV patients that resolve the infection, a potent CD4+ (Th1-oriented) response precedes the maturation of a protective memory CD8+ T cell response (Urbani et al, 2006). The proliferative capacity of HCV-specific CD4+ T cells is weak or absent in persistent infection. Many theories have been proposed to explain this, including deletion of such cells and induction of anergy. Proliferation of CD8+ effector cells in response to HCV antigen is also weak in patients with chronic infection (Wedemeyer et al, 2002). There are potentially many molecular mechanisms which contribute to this reduced T cell proliferation in patients with chronic disease including the presence of IL-10 and up-regulation of the inhibitory molecule, programmed death-1 (PD-1) (Brooks et al, 2006; Golden-Mason et al, 2007). Additionally, studies in humans and mice have shown loss of proliferative capacity during viral infection to be accompanied by low levels of IL-2 secretion (Fuller et al, 2005; Fuller & Zajac, 2003; Iyasere et al, 2003). Recently, Semmo et al. have shown proliferation of HCV-specific CD4+ T cells from patients with chronic disease to be reduced (on a per cell basis) in parallel to IL-2 secretion (Semmo et al, 2007). These investigators had previously demonstrated loss of IL-2 secreting CD4+ T helper cells in chronic HCV infection (Semmo et al, 2005).

In this study, we have demonstrated that peripheral blood lymphocytes, isolated from healthy donors, pre-incubated in the presence of serum from HCV infected individuals ($PCR^+$), were unable to produce IL-2 following stimulation, while serum isolated from

healthy individuals had no effect. In addition, our collaborators have shown that peripheral blood lymphocytes isolated from HCV patients were capable of secreting IL-2 in response to mitogenic stimulation, demonstrating that failure to secrete IL-2 is not due to genetic pre-disposition, but due to the environment (Golden-Mason, L. unpublished data). These experiments demonstrated that inhibition of IL-2 secretion was mediated by viral particle(s)/factor(s) and that pre-incubation with such factors was necessary for the inhibitory effect to be observed. This inhibitory effect was mediated via E2-CD81 ligation as a peptide previously shown to inhibit this interaction (Cao et al, 2003) and induce an E2-specific immune response could partially reverse this, in both PBMCs and HuT 78 cells. Furthermore, it was demonstrated that the envelope protein of HCV, E2 could inhibit secretion of IL-2 from activated PBMCs. Using other HCV peptides, including core C22 and NS3 region derived C33 peptide, in our HuT 78 cell line model system, the inhibition of IL-2 secretion could not be observed, indicating a specific ability of E2 to cause this phenomenon.

In this study it was validated that E2-CD81 interaction stimulates the translocation of PKC-β from the cytosol to lipid raft subdomains of the cell membrane. These lipid rafts are specific membrane compartments, originally biochemically defined as detergent-resistant membrane fractions composed of cholesterol, glycolipids and protein, which host receptors and signalling molecules involved in different cellular events, such as cell signalling, pathogen invasion or immune responses (Rajendran & Simons, 2005). Translocation of PKC-β and its co-localisation with GM-1 ganglioside within lipid raft, was qualitatively revealed using immunofluorescent microscopy. Quantitative analysis of dual-colour 3D images, using correlation testing, allowed statistical assessment of the degree of overlap between two labeled molecules. The most quantitative estimate is the Pearson's correlation coefficient, (R*r*) (Manders et al, 1992), which depends on the amount of co-localised signals in both channels in a nonlinear manner (Costes et al, 2004) and was significantly higher in E2 treated cells.

PKC-β expression is necessary for export of IL-2 from the HuT 78 T cell line (Long et al, 2001) and its sequestration of PKC-β in lipid rafts prevents its association with the

centrosome and the cellular secretory machinery. In the absence of PKC-β, HuT 78 cells synthesise IL-2 protein but fail to secrete it in response to activation. Another study has shown that introduction of antisense oligonucleotides to PKC-β in Jurkat human leukaemic T cells inhibits the secretion of IL-2 in response to TCR/CD28 co-stimulation (Dreikhausen et al, 2003). More recently, it has been demonstrated that PKC-β is necessary for activation of Cdc42, driving fusion-dependent compartment mixing and exocytosis in a Xenopus model system (Yu & Bement, 2007). Disruption of lipid rafts with MCD reversed the inhibitory effect of E2 on IL-2 secretion again implicating the necessity of PKC-β for this process. Pre-treatment of the cells with the PKC-α/β selective inhibitor Go6976 prevented the MCD-induced rescue of IL-2 secretion. Thus, E2-CD81 stimulated translocation of PKC-β to lipid rafts inhibits the process of IL-2 secretion from T cells. The fact that E2 can inhibit the secretion of another centrosome/synapse-directed cytokine (Interferon-γ) (Huse et al, 2006) *in vitro*, suggests that PKC-β is mechanistically involved in the secretory process. The PKC-β - deficient cell line K-4 also fails to secrete these cytokines in response to PMA stimulation (unpublished data). In targeting PKC-β, HCV is therefore capable of interfering with two microtubule-associated processes – secretion and migration (Volkov et al, 2006), both critical to the T cell mediated immune response.

During this study, when we applied siRNA technology for PKC-β knockdown, the expected phenotype could not be observed. Although the reduction in gene expression was up to 90 %, the HuT 78 cells were able to secrete IL-2 upon PMA stimulation, reaching the level of the untreated control cells. Despite obtaining significant level of gene silencing for PKC-β, we could not achieve knockdown phenotype in our T cell model system. Besides secretion, these cells were also able to migrate, as observed by other colleagues in the group. Previously, it has been demonstrated that PKC-β has an essential role in lymphocyte migration through LFA-1 activation (Volkov et al, 2001). However, HuT 78 cells expressing PKC-β knockdown, still exhibited a migratory phenotype. Reasons for that might lie in a protein turnover, or in ability of enzyme to perform sufficiently even if present in minimal quantity in the cell. Additionally, PKC-β belongs to the superfamily of PKCs, where some other isoforms may have functional

redundancy for complex cellular processes, indicating importance of these enzymes for the particular T cell functions.

In this study we have focused on the modulatory signalling properties of E2 from HCV genotype 1a. As the affinity of E2-CD81 interaction may vary depending on viral genotype (Yamada et al, 2005) this interaction may influence the quantitative and qualitative composition of lipid rafts and also the regulation of activity/down-regulation of signalling molecules such as PKC-β. It is plausible that viral load will also impact on these signalling processes.

The inhibition of IL-2 secretion by $PCR^+$ human serum however, does demonstrate that E2-CD81-mediated attenuation of cytokine secretion is a realistic mechanism of viral immune suppression *in vivo*. Moreover, it has been demonstrated that viruses including the human immunodeficiency virus (HIV) (Djordjevic et al, 2004), herpesvirus saimiri tip (HVS) (Cho et al, 2006), measles virus, (Avota et al, 2004) through the interaction with the lipid rafts target and modulate diverse T cell functions. Depletion of cholesterol enriched plasma membrane microdomains using MCD decreased HCV infection, by causing the mislocalization and depletion of CD81 surface expression (Kapadia et al, 2007). Our study presents a model whereby HCV E2 interaction with CD81 on T lymphocytes can undermine the host immune response and provide a mechanistic explanation for failure of T cells to produce IL-2 during chronic HCV infection. As IL-2 has not proven to be a successful therapy in HCV (Alric et al, 2006; Glesby et al, 2005), targeting the association of PKC-β with lipid rafts may prove to be a more successful and specific strategy.

In conclusion, these data suggest that sequestration of PKC-β to the lipid raft compartment in the T cell membrane, following E2 - CD81 interaction, could be a central mechanism in viral inhibition of IL-2 secretion, contributing to viral evasive strategies. E2-mediated inhibition of IL-2 secretion represents a mechanism whereby HCV can undermine the human immune response to establish persistent infection and chronic disease.

# CHAPTER IV

# *THE ENVELOPE PROTEIN (E2) OF THE HEPATITIS C VIRUS ALTERS THE CYTOKINE PROFILE OF PERIPHERAL BLOOD MONONUCLEAR CELLS*

# Chapter IV

## *4.1 INTRODUCTION*

The host immune response includes the synchronised action of various components of the immune system, with cellular immunity having a central role in mediating viral control and spontaneous resolution of acute HCV infection (Bowen & Walker, 2005). The cellular immune response is mainly characterised by cytotoxicity and secretion of various (antiviral) cytokines, such as IFN-γ and TNF-α (Neumann-Haefelin et al, 2007). Besides having a role in viral clearance, these cytokines are important in intrahepatic inflammation and liver cell injury during chronic HCV infection, largely influencing disease progression (Zeremski et al, 2007).

Hepatocytes express an array of cytokine receptors, making the liver highly predisposed to the effects of these molecules (Ramadori & Armbrust, 2001). Kupffer cells (KCs), macrophages that reside in liver tissue, have been shown to produce pro-inflammatory cytokines such as IL-1(α/β), IL-6 and TNF-α and also growth factors such as TGF-β (Ramadori & Armbrust, 2001). TNF-α and IL-1β are in control of the balance between cell proliferation and apoptosis, but during HCV invasion this balance is disturbed. IL-1β levels are more elevated in liver pathogenesis caused by HCV than by other agents and it gives rise to the inflammatory response (Farinati et al, 2006). IL-6 is present at higher levels in asymptomatic HCV carriers when compared to healthy controls (Zekri et al, 2005). It has been shown that IL-6 and TNF-α are important for normal liver regeneration through the activation of transcription factors NF-κB and STAT3 (Taub, 1996), where TNF-α induces IL-6 and primes hepatocytes (Webber et al, 1998) to respond to growth factors (Fausto et al, 1995; Phaneuf et al, 2000). In addition, cytokines such as IFN-γ, IL-2, IL-4 or IL-10 are secreted from the liver infiltrating T cells or NK cells. In general, Th1 response will be driving inflammation and cell-mediated immunity to direction which controls viral infection (Gramenzi et al, 2005) and accelerates the activation and proliferation of CTLs and NK cells. The acute phase of HCV infection is characterised by an early and vigorous intrahepatic Th1

response. In cases where this response is inefficient and undeveloped it may lead to inflammation and chronic liver immunopathology (Cerny & Chisari, 1999; Missale et al, 1996; Prezzi et al, 2001). A predominant Th2 response, with cytokines such as IL-10 and especially IL-4, has been reported to favour fibrogenesis in liver injury (Gramenzi et al, 2005; Lau et al, 2005). Transplant patients with persistent HCV infection who produced high levels of IFN-γ and low levels of IL-10 exhibited the slowest fibrosis progression (Schirren et al, 2003). In addition, TGF-β1 is considered as one of the most important and potent factors in the process of fibrogenesis (Benlloch et al, 2003; Neuman, 2003). It initiates and stimulates transformation of hepatic stellate cells to myofibroblast (Ramadori & Saile, 2004b) and production of ECM proteins, leading to the formation of scar tissue and loss of liver function (Bataller & Brenner, 2005).

IFN-γ has been classified as a sole member of the type II IFN family. It is a soluble cytokine, in the form of a dimer when biologically active. The dimer is formed by anti-parallel inter-locking of two monomers, each containing a core of six α-helices (Ealick et al, 1991; Thiel et al, 2000). IFN-γ is secreted by Th1 cells, NK cells, CTLs, (Bach et al, 1997; Young, 1996) as well as by B cells (Flaishon et al, 2000), NKT cells (Carnaud et al, 1999) and APCs (Frucht et al, 2001). IFN-γ secreted by NK cells and APCs is involved in an early immune response against infection, while that which is secreted by T cells has a major role in adaptive immunity (Frucht et al, 2001; Sen, 2001). Production of Interferon-γ is stimulated by IL-12 and IL-18 (produced by APCs) whereas, it is inhibited by IL-4, IL-10 and TGF-β (Schroder et al, 2004). IFN-γ plays a central role in coordinating numerous cellular events through transcriptional control over large numbers of genes, increasing immune surveillance and immunoregulation. It has antiviral, antimicrobial, anti-tumour properties, it contributes to up-regulation of antigen processing and presentation and it regulates growth, maturation and differentiation of diverse cell types (Schroder et al, 2004).

The TNF family of proteins contains crucial molecules involved in immunological tolerance, activation, differentiation and apoptosis of immune cells. A member of this family, TNF-α has a highly complex role in T and B cell selection, proliferation and

apoptosis during the immune response in adults as well as neonatal development (Van Parijs & Abbas, 1998). TNF-α receptors (CD 120a/b) are expressed on large variety of cells which contributes to its various downstream effects such as apoptosis, activation, proliferation and survival (MacEwan, 2002; Wajant et al, 2003). In a state of acute or chronic inflammation, TNF-α induces immune cells to produce enhanced levels of other pro-inflammatory cytokines (Mageed & Isenberg, 2002). Having a broad range of, sometimes contradictory effects (Beutler, 1999), there is good indication that disturbance of TNF-α production may have a role in development and pathology of autoimmune diseases (Riminton & Sedgwick, 2000). Deregulation or increased production of TNF-α was reported in a few inflammatory diseases such as rheumatoid arthritis, psoriatic arthritis and Crohn's disease (Kollias, 2005; McDevitt et al, 2002). There are several therapeutic agents currently used in clinics that target and inhibit its activity (Deng et al, 2005; McDevitt et al, 2002).

IL-10 is a homodimer of two interpenetrating polypeptide chains noncovalently bound (Windsor et al, 1993). It exhibits pleiotropic effects and the outcome will depend on dose, location and time of expression (Mocellin et al, 2004). Its prime function is control and termination of inflammatory response, which would assist in minimal tissue damage of the host and elimination of infectious agent (Moore et al, 2001). In general, IL-10 strongly inhibits APCs and as a consequence of this, downregulation of cytokine production in CD4+ T cells and NK cells occurs (de Waal Malefyt et al, 1991; Ding & Shevach, 1992; Fiorentino et al, 1991). In contrast, IL-10 stimulates development of B cells and proliferation, recruitment and cytotoxicity effect of CD8+ cells (Groux et al, 1998; Santin et al, 2000). It is not fully understood how IL-10 expresses immunosuppressive ability on some cell populations and stimulatory effects on others, when all of them utilise similar signalling responses to this cytokine. In addition, engagement of IL-10 in development of T regulatory cells and their ability to produce the same cytokine (Moore et al, 2001) or neutralising the inflammatory effect when secreted by mast cells at the site of allergic reaction (Grimbaldeston et al, 2007), also needs more clarification.

IL-1 family members are IL-1α and IL-1β cytokines synthesised as large precursor peptides, that are subsequently cleaved into their active form of 12-14 β-strands barrel-shaped structure (Dinarello, 1994). They are mainly secreted by macrophages and monocytes, but some other cell types, such as endothelial cells, fibroblasts, microglia cells may produce them (Huising et al, 2004). These two cytokines share a common receptor on target cells and, therefore, share many immunological properties, although Nakae et al. have demonstrated that IL-1β is more potent in activating humoral immune responses (Nakae et al, 2001). IL-1α/β are involved in immunopathology of the CNS such as neurodegenerative disorders (Benveniste, 1998; Patel et al, 2003), induction of fever in rat models (Morgan et al, 2004; Rothwell, 1989) or initiation of hyperexcitability of nerves inducing pain after trauma-activated immune processes (Watkins & Maier, 2002). An important function of these cytokines is to enhance expression of selectins and integrins on endothelial cells facilitating T cell migration to the site of infection (Carlos & Harlan, 1994; Thornhill & Haskard, 1990).

The family members of IL 6-type cytokines trigger target genes involved in differentiation, proliferation, survival and apoptosis (Heinrich et al, 2003). IL-6 molecule has four long α-helical structures similar to some other cytokines (IL-2 for example) (Zilberstein et al, 1986). It exhibits a multifunctional nature and is involved in different aspects of inflammation, immunity and hematopoiesis (Hirano, 1998). IL-6 stimulates B cell differentiation, immunoglobulin production, Th1 type cytokine secretion (Ohtani et al, 2000) and induces development and maturation of myeloid cells, megakaryocytes, osteoclast, neural cells and hepatocytes (Streetz et al, 2000). There are indications that IL-6 has a role in development of autoimmunity (Hirano et al, 1988; Hirano et al, 1987), malignancy (Gado et al, 2000; Seymour et al, 1997; Zhang & Adachi, 1999) and aging (Maggio et al, 2006). Febbraio and Pedersen referred to IL-6 as 'myokine' as they demonstrated elevation of IL-6 levels during physical exercise and muscle contraction (Febbraio & Pedersen, 2005). Secreted mainly by macrophages, in synergistic action with TNF-α and IL-1, it is required for corticosteroid release, fever and hepatic production of mainly protease inhibitors. All these components are features

of the acute phase response, which is probably an attempt to maintain homeostasis (Streetz et al, 2000).

TGF-β1 is one of three isoforms of the TGF-β cytokine that belongs to the TGF-β superfamily of multifunctional growth factors. It is synthesised as a large precursor protein, which is latent and must be proteolytically cleaved into an active form, which is a dimeric polypeptide with many conserved motifs (Herpin et al, 2004; Khalil, 1999). The latent form is located in the extracellular space, where it can be immediately made available as required (Flanders & Burmester, 2003). It regulates cell proliferation and differentiation and therefore has crucial role in embryonic development, angiogenesis and tissue homeostasis in animals (Piek et al, 1999). TGF-β1 is produced by, and has its receptor on endothelial, hematopoietic and connective-tissue cells (Blobe et al, 2000). TGF-β1 knockout mice develop lethal cardiac, pulmonary and gastric inflammation, indicating that TGF-β1 has an important function in inhibiting activation and proliferation of immune cells (Shull et al, 1992). Excessive levels of this protein have been found in serum samples of patients with progressive prostate cancer, colorectal cancer (Ivanovic et al, 1995; Shim et al, 1999) and chronic viral hepatitis (Murawaki et al, 1998) while in urine samples in hepatocellular carcinoma (Tsai et al, 1997a). The essential function of TGF-β1 is induction of extracellular-matrix proteins which leads to wound healing and tissue repair (Singer & Clark, 1999). However, overexpression can cause fibrotic modification directing to the pathogenesis of fibro-proliferative disorders in liver, lungs and kidneys (Blobe et al, 2000; Neuman et al, 2008).

All cytokines analysed in our study exhibit a pleiotropic nature. Their highly complex functions and cell sources are summarised in the Table 4.1.

A recent study has revealed two main pathways of cytokine secretion utilised by CD4+ T cells. Secretion of cytokines such as IL-2, IFN-γ and IL-10 is directed towards the immunological synapse (Huse et al, 2006), which is a formation induced during the process of antigen presentation (Bromley et al, 2001). A contact site of T cell and APC is characterised by clustering of adhesion and signalling molecules (Grakoui et al,

1999; Monks et al, 1998) and the accumulation of several cytokines was observed just beneath the contact site (Kupfer et al, 1991; Kupfer et al, 1994; Reichert et al, 2001). Besides this targeted cytokine secretion at the immunological synapse, Huse et al. have identified that some cytokines, such as TNF-α and IL-4, are secreted in a multidirectional manner. It seems that both of these secretory pathways indicate the appropriate function of released cytokines. Directed secretion at the immunological synapse creates high local concentration of the cytokine, with resultant effects mainly on the cells involved at the immunological synapse, whilst multidirectional release facilitates the dispersion of specific signals targeting a variety of cells (Huse et al, 2006).

Hepatocytes, immune cells within the liver and peripheral blood leukocytes are vulnerable to the virus in the context of their ability to produce cytokines. Local cytokines have an essential role in liver embryogenesis, liver injury or development of liver disease (Ramadori & Armbrust, 2001). In HCV infection these molecules are central to the process of inflammation and to the fate of the immune response. In the first part of this study we have demonstrated that IL-2 is manipulated by HCV E2. Thus, this part of the work will investigate the effect of E2 on production of other cytokines involved in the pathogenesis of HCV.

## *4.2 OBJECTIVES*

- To examine the profile of CD81 expression on PBMC subsets.
- To determine the effect of HCV E2 on secretion of cytokines associated with viral infection and involved in liver pathogenesis, including IFN-γ, TNF-α, IL-10, IL-1α, IL-1β, IL-6 and TGF-β1.
- To determine the effect of HCV E2 on regulation of gene transcription of these cytokines (mRNA level).

| Cytokine | Cell source | Function |
|---|---|---|
| IL-2 | CD4+ T cells, CD8+ T cells | Stimulates growth, proliferation and differentiation of T, B, NK cells and monocytes |
| IFN-γ | CD4+ T cell, CD8+ T cells, NKT cells | Induces the cytokine production of naïve CD4+ T cells towards Th1 type response, stimulates NKT cells and macrophages |
| TNF-α | B cells, T cells, macrophages | Induces proliferation and differentiation of many cell types, essential mediator of inflammation |
| TGF-β | macrophages, epithelial cells | Inhibits Th1 producing CD4+ T cells, stimulates synthesis of extracellular-matrix proteins which leads to wound healing and tissue repair |
| IL-10 | macrophages, CD4+ T cells | Inhibits APCs, suppresses Th1 response and cytokine release from NK cells, stimulates development and proliferation of B cells as well as recruitment and cytotoxic effect of CD8+ T cells |
| IL-4 | CD4+ T cells, mast cells | Induces the cytokine production of naïve CD4+ T cells towards Th2 type response, stimulates B and mast cells |
| IL-1α | macrophages | Stimulates thymocyte proliferation by inducing IL-2 release, B cell maturation and proliferation |
| IL-1β | monocytes/macrophages, DCs | Induces lymphocytes expansion by stimulation of IL-2 secretion, potent inductor of humoral response |
| IL-6 | monocytes/macrophages, T cells, endothelial cells, fibroblasts | Stimulates B cell differentiation and antibody production, induces Th1 response, induces development and maturation of myeloid cells, megakaryocytes, osteoclast, neural cells and hepatocytes |

**Table 4.1 Cytokines and their main cell producers and functions**

## *4.3 RESULTS*

### 4.3.1 CD81 expression on PBMCs subsets

PBMCs contain different cell types and following exposure to HCV E2 their responses may differ. Since CD81 receptor is accountable for E2-host cell interaction, the level of expression of CD81 molecule was assessed in four different cell types in PBMCs, using flow cytometry. PBMCs were obtained from healthy volunteer and double stained for CD81 (FITC) and one of specific cell markers: CD3 for T cells (PE), CD19 for B cells (PE), CD56 for NK cells (PE) and CD14 for monocytes (APC) (**Figure 4.1**). All included subsets highly expressed CD81. The smallest fraction of CD81 positive cells was among CD14 cells (67.9 %), while 99.3 % of CD3 cells expressed CD81 ($p=0.036$), 99.6 % of CD19 positive cells ($p=0.04$) and 99.7 % of CD56 positive cells ($p=0.046$) (**Figure 4.2 A**). When the levels of CD81 were represented as mean fluorescence intensity (MFI) the highest intensity level per cell was observed on CD19+ cells (79.14) followed by CD56+ cells (75.42) and CD14+ cells (68.07). The lowest MFI was noted for CD3+ cells (65.78), indicating the smallest number of CD81 molecules exposed on the cell surface (**Figure 4.2 B**). Furthermore, the proportion of each subset in the PBMCs was determined. The vast majority of cells were CD3+ T cells (48.17 %). The next largest population was made up of CD14+ cells (monocytes) (16.72 %). Substantially smaller proportion of B cells (CD19+ 9.98 %) and NK cells (CD56+ 8.69 %) along with all other cells (16.45 %) built up the rest of PBMCs (**Figure 4.2 C**).

All tested PBMCs subsets expressed CD81 and possibly all could be involved in shaping the cytokine profile after being in contact with HCV E2. Nevertheless, the contribution of each cell population might be related to the proportion of them within total PBMCs.

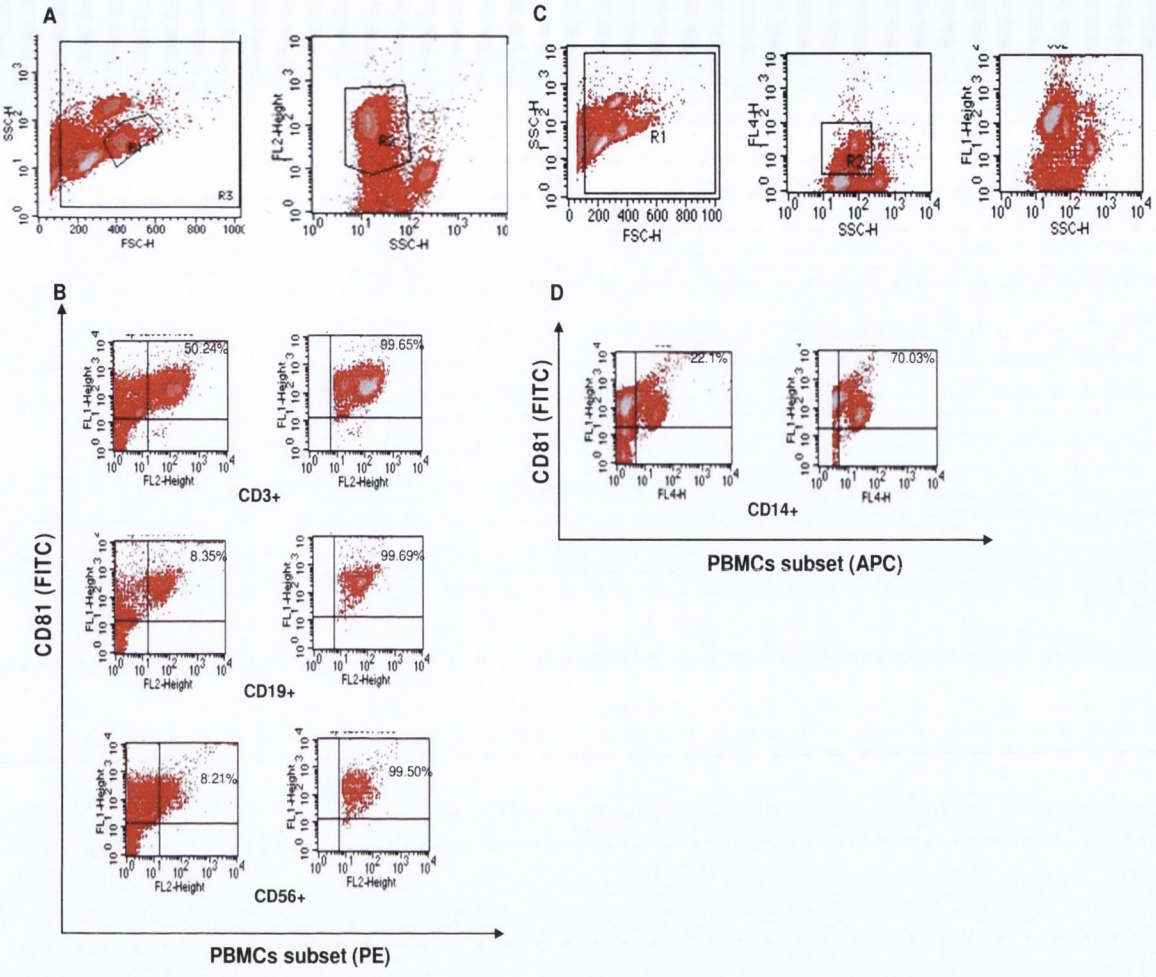



**Figure 4.1 CD81 expressions on PBMCs subsets. A)** On total PBMCs differentiated from cell debris (R3) gated lymphocyte subpopulation (R2). **B)** Panels on the left represent double positive cells (stained for both CD81 (FITC) and either CD3 or CD19 or CD56, specific cell marker (PE)) in total PBMCs population (R3). Panels on the right represent corresponding staining on cells gated on specific PBMCs subset. **C)** On total PBMCs population (R1) gated monocyte subset (R2). **D)** Panel on the left shows CD81 (FITC) and CD14 (APC) positive cells in total PBMCs population (R1), while panel on the right represents double positive cells gated just on monocyte subpopulation (R2).

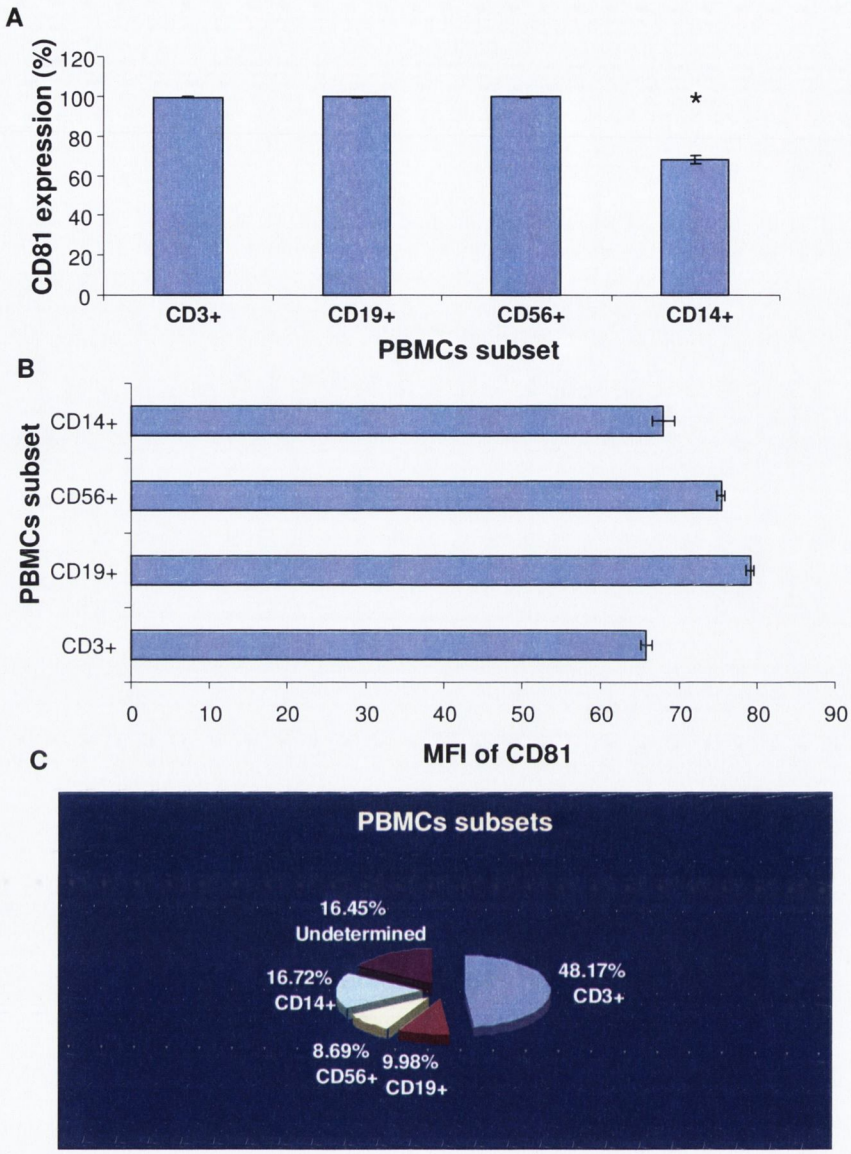



**Figure 4.2 A) Percentage of CD81 expressing PBMCs within each subsets.** Percentage of cells expressing CD81 molecule on their surface is significantly lower in monocytes when compared to the rest of PBMCs populations. **B) Mean fluorescence intensity (MFI) of CD81 specific staining.** MFI shows the amount of CD81 molecules expressed on the surface of different cells population. **C) Fractions of diverse cell types within PBMCs.** The largest subset is made of T cells, followed by monocytes, B and NK cells. Cells that were not determined accounted to 16.5 %. Data is presented as mean ± SEM of results obtained in three independent experiments with similar results.

### 4.3.2 Cytokines inhibited by HCV E2 in HuT 78 cells

To investigate if the E2-mediated inhibition of secretion was unique to IL-2 or if the viral protein was targeting the general secretory machinery, the effect of E2 on secretion of other cytokines was investigated. Supernatants from PMA-activated HuT 78 cells pre-treated with E2 were subjected to multiplex cytokine analysis. These experiments demonstrated that E2 also inhibited secretion of INF-γ, TNF-α and IL-10 from activated HuT 78 cells (**Figure 4.3**) suggesting that E2, via its interaction with CD81 in T cells, is targeting a secretory process. Therefore, incubation with E2 results in significant down-regulation in the secretion of IFN-γ in PMA treated HuT 78 cells (levels in pg/ml were: PMA 2400 ± 0 vs E2/PMA 663.36 ± 53, p=0.019) (resting cells 63.61 ± 0). Similarly, secretion of TNF-α was affected in the same manner (levels in pg/ml were: PMA 1737.2 ± 98.2 vs E2/PMA 133.9 ± 69.5, p=0.004) (resting cells 0) as well as secretion of IL-10 (IL-10 levels in pg/ml: PMA 9000 ± 0 vs E2/PMA 784.3 ± 459.3, p=0.035) (resting cells 1830.6 ± 39.7). Other cytokines measured from the same supernatants were either at very low concentration (IL-4) or undetectable (**Table 4.1**).

### 4.3.3 Cytokines targeted by HCV E2 in PBMCs

After PBMCs, isolated from healthy volunteers, were incubated with E2 overnight and subsequently PMA/Io or anti-CD3/anti-CD28-stimulated, supernatants were collected and analysed for cytokine levels using Evidence Investigator (Methods 2.6). Like Hut 78 cells, pre-treatment with E2 significantly reduced production of IFN-γ, TNF-α and IL-10 in PMA/Io or αCD3/αCD28 stimulated peripheral blood mononuclear cells (**Figure 4.4**). IFN-γ levels in pg/ml were: PMA/Io 3484.95 ± 213.5 vs E2/PMA/Io 0, p=0.038; αCD3/αCD28 295 ± 2.9 vs E2/αCD3/αCD28 0, p=0.006 (resting cells 38.85 ± 1.7); TNF-α levels in pg/ml were: PMA/Io 5149.55 ± 509.1 vs E2/PMA/Io 253.85 ± 12.9, p=0.01; αCD3/αCD28 5418.4 ± 130.1 vs E2/αCD3/αCD28 386.2 ± 47.4, p=0.022 (resting cells 0);

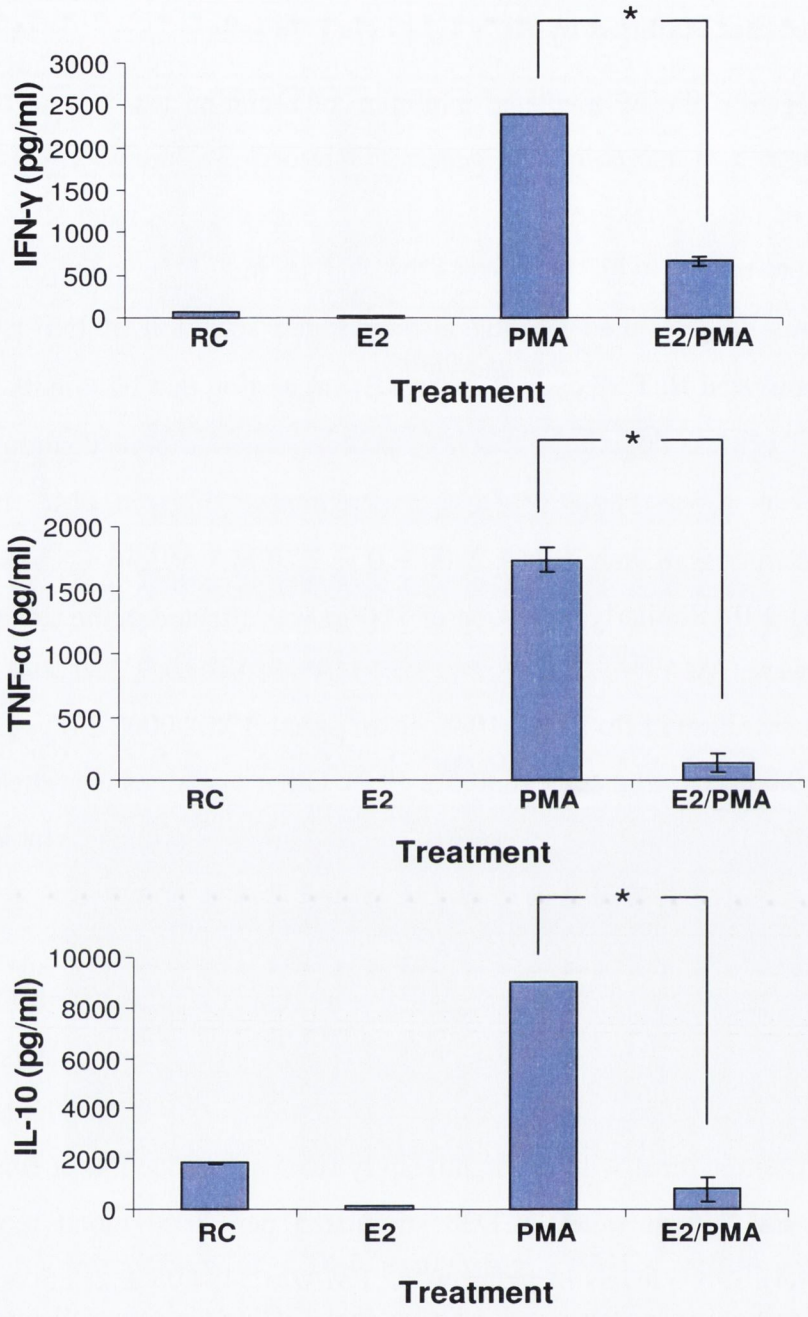



**Figure 4.3 E2 inhibits production of IFN-γ, TNF-α and IL-10 from activated HuT 78 cells.** HuT 78 cells were pretreated with recombinant E2 protein (1 μg/ml) overnight and afterwards stimulated with 10 ng/ml of PMA for 24 hours at 37°C. Cytokines were measured using Evidence Investigator multiplex analysis. Significant decrease in cytokine secretion was observed always after cells were pre-treated with E2. Data is presented as mean ± SEM of one representative experiment from two independent experiments with similar results. Significant differences are indicated by the asterisks.

| | RC | E2 | PMA | E2/PMA |
|---|---|---|---|---|
| **IL-4 (pg/ml)** | 3.19 | 2.95 | 11.33 | 9.38 |
| **IL-6 (pg/ml)** | 0 | 0 | 4.12 | 0 |
| **IL-1α (pg/ml)** | 0 | 0 | 0 | 0 |
| **IL-1β (pg/ml)** | 0 | 0 | 0 | 0 |
| **MCP1(pg/ml)** | 0 | 0 | 0 | 0 |

**Table 4.1 Absent cytokines in HuT 78 cells.** These cytokines could not be calculated by Evidence Investigator due to their absence from the sample or presence at low levels. Data is presented as mean of one representative experiment from two independent experiments with similar results.

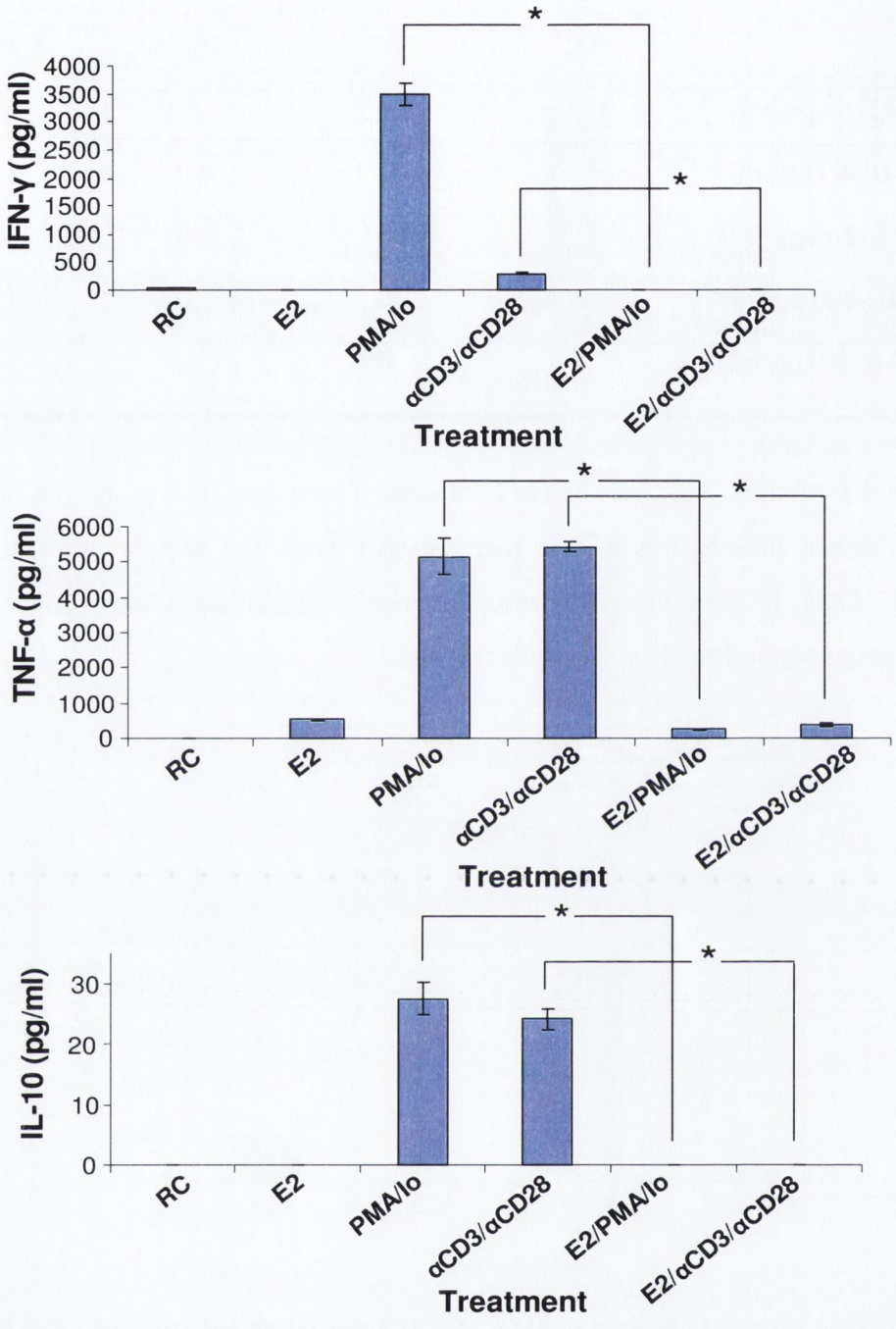
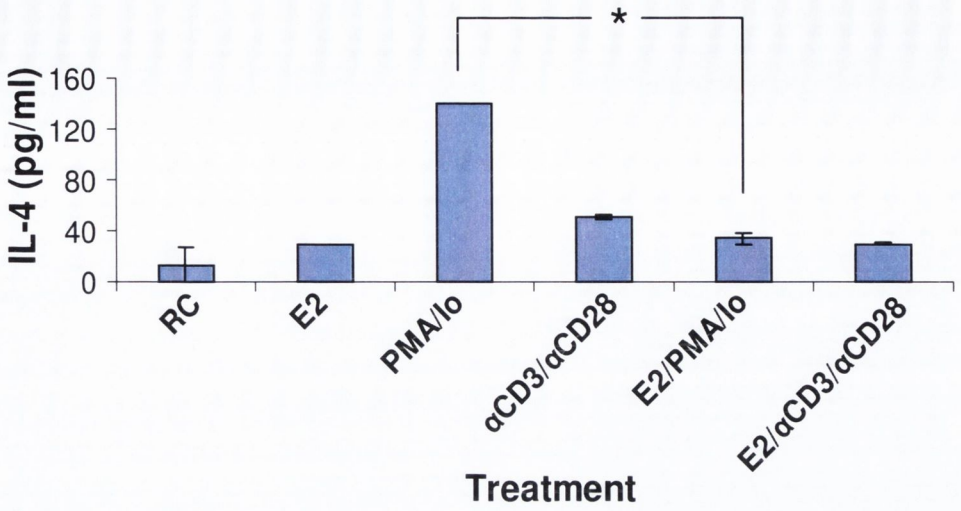



**Figure 4.4 E2 inhibits production of IFN-γ, TNF-α and IL-10 from PBMCs.** PBMCs, obtained from healthy donors, were incubated with E2 (1 μg/ml) overnight and following that stimulated with either PMA/Ionomycin (PMA/Io) (10 ng/ml of PMA and 1 μg/ml of Ionomycin) or plate bound anti-CD3/anti-CD28 antibodies. Cytokines were measured using Evidence Investigator multiplex analysis. A significant decrease in cytokine secretion was consistently observed after cells were pre-treated with E2.

Data is presented as mean ± SEM of one representative experiment from two independent experiments with similar results. Significant differences are indicated by the asterisks.

IL-10 levels in pg/ml were: PMA/Io 27.45 ± 2.6 vs E2/PMA/Io 0, p=0.003; αCD3/αCD28 24.15 ± 1.7 vs E2/αCD3/αCD28 0, p=0.002 (resting cells 0). However, the secretion profile of IL-4 and MCP1 was slightly different (**Figure 4.5**). E2 inhibition of IL-4 secretion in PBMCs was observed only when the cells were stimulated with PMA. PBMCs did not produce IL-4 above basal levels in response to more physiological stimulus anti-CD3/anti-CD28 antibodies. The IL-4 levels of PMA/Io-stimulated cells in pg/ml were 140.3 ± 0 while in those pre-treated with E2 followed by PMA/Io, levels of IL-4 were 33.65 ± 4.2, (p=0.025). When cells were stimulated with αCD3/αCD28, significant reduction of IL-4 secretion could not be observed (p=0.09). A significant decrease in MCP1 secretion was observed in PBMCs pre-treated with E2 prior to activation (MCP1 levels in pg/ml: PMA/Io 4580.95 ± 52.5 vs. E2/PMA/Io 1315.25 ± 0.6, p=0.01; αCD3/αCD28 10000 ± 0 vs. E2/αCD3/αCD28 1317.4 ± 67.4, p=0.005). The fact that MCP1 levels in resting cells (6233.25 ± 132.7) were higher than in PMA/Io stimulated was confusing and requires further clarification. In this set of experiments three cytokines exhibited completely different profile of secretion when compared to the IL-2. Here it was observed that the levels of IL-1α, IL-1β and IL-6 were highly augmented whenever PBMCs were exposed to E2 regardless of stimulation (**Figure 4.6**). Treatment with E2 resulted in elevation of the IL-1α concentration and the levels in pg/ml were: PMA/Io 24.05 ± 4.2 vs. E2/PMA/Io 341.8 ± 2.4, p=0.013; αCD3/αCD28 79.6 ± 4.8 vs. E2/αCD3/αCD28 171.4 ± 10.3, p=0.038 (E2 221.75 ± 0.4; resting cells 0). Levels of IL-1β in pg/ml were: PMA/Io 18.35 ± 1.5 vs. E2/PMA/Io 349.65 ± 3.1, p=0.003; αCD3/αCD28 33.95 ± 1.5 vs. E2/αCD3/αCD28 349.65 ± 3.1, p=0.01 (E2 521.1 ± 24.4; resting cells 0). IL-6 was secreted in the same trend, with the levels in pg/ml as follow: PMA/Io 100.1 ± 5.5 vs. E2/PMA/Io 5318.8 ± 220.9, p=0.026; αCD3/αCD28 31.25 ± 0.7 vs. E2/αCD3/αCD28 4573.15 ± 457.6, p=0.03 (E2 3052 ± 167.1; resting cells 24.05 ± 2.9). E2 stimulation alone was sufficient to induce significant secretion of all three cytokines when compared to both non-stimulated cells (IL-1α p=0.001; IL-1β p=0.029 and IL-6 p=0.034) and stimulated cells. Pre-treatment with E2 prior the stimulation increased the levels of all three cytokines. PMA/Io and αCD3/αCD28 induced IL-6 levels were above the level of E2 pre-treatment, IL-1β induced levels were slightly below the E2 level, while



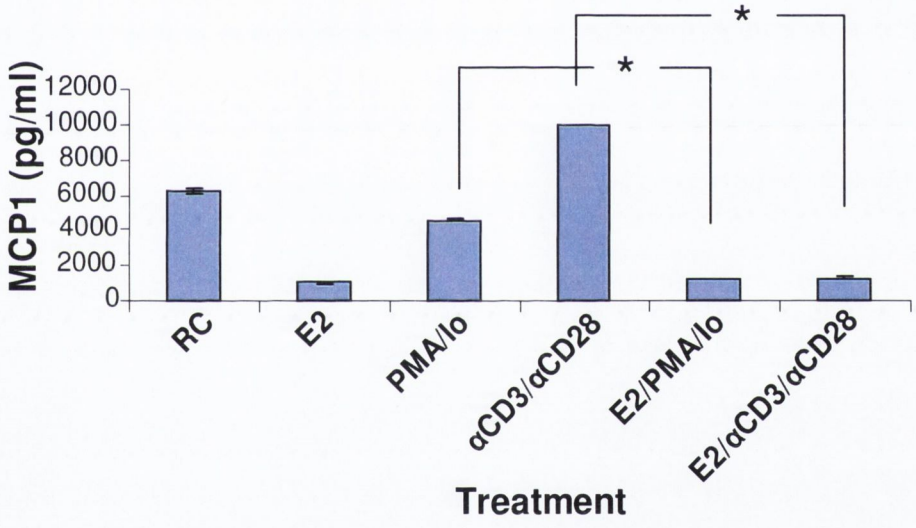




**Figure 4.5 E2 mediated inhibition of IL-4 and MCP1 secretion in PBMCs.** PBMCs, obtained from healthy donors, were incubated with E2 (1 μg/ml) overnight and following that stimulated with either PMA/Ionomycin (PMA/Io) (10 ng/ml of PMA and 1 μg/ml of Ionomycin) or plate bound anti-CD3/anti-CD28 antibodies. Cytokines were measured using the Evidence Investigator. Data is presented as mean ± SEM of one representative experiment from two independent experiments with similar results. Significant differences are indicated by the asterisks. **A)** Decrease in IL-4 secretion was shown when PMA/Io stimulated PBMCs were pre-incubated with E2. **B)** Reduction in the levels of MCP-1 was observed after stimulated cells were pre-treated with E2.

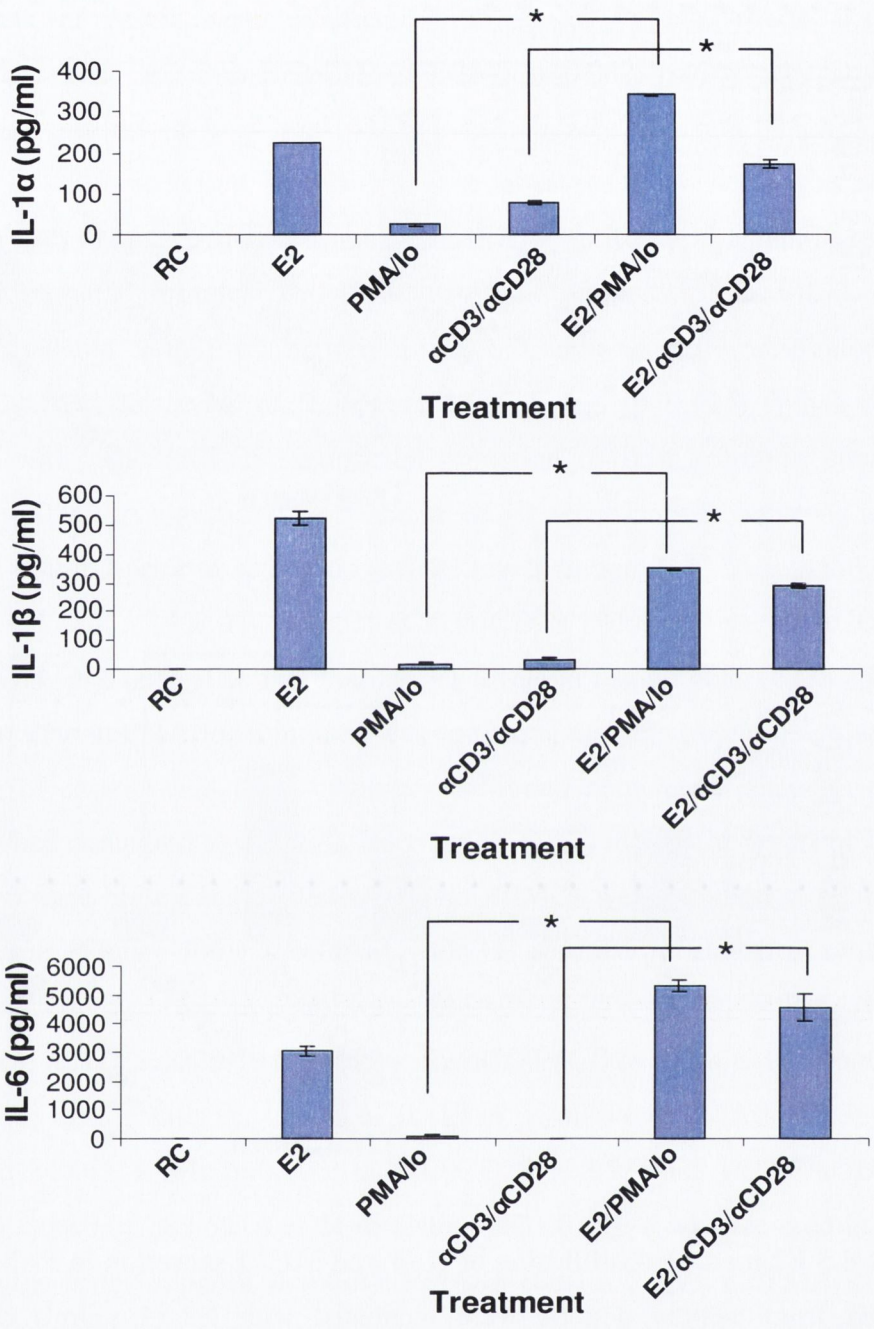



**Figure 4.6 Secretion of proinflamatory cytokines IL-1α, IL-1β and IL-6 by PBMCs in presence of E2.** Cells were activated with PMA and anti-CD3/anti-CD28 following pre-incubation overnight with E2. Cytokines were measured using Evidence Investigator. However, every time when the cells were incubated with E2 a significant augmentation in cytokine secretion was observed. Data is presented as mean ± SEM of one representative experiment from two independent experiments with similar results. Significant differences are indicated by the asterisks.

PMA/Io induced IL-1α level was above and αCD3/αCD28 induced IL-1α level was slightly below the level of E2 pre-treatment.

Like HuT 78 cells, the PBMCs exhibited inhibition of secretion of INF-γ, TNF-α and IL-10 when cells were pre-treated with E2 prior the activation. However, IL-1α, IL-1β and IL-6, which were not produced by HuT 78 cells, were significantly increased every time when PBMCs were exposed to E2.

### 4.3.4 TGF-beta 1 (TGF-β1) production in T cells exposed to HCV E2

TGF-β is an important cytokine, involved in the pathogenesis of liver disease. Therefore, it was investigated whether the recombinant E2 could inhibit TGF-β1 production in HuT 78 cells. Firstly, cells were incubated with E2 overnight and following that stimulated with PMA. A significant decrease in TGF-β1 secretion was observed in HuT 78 cells pre-treated with E2 prior to activation (**Figure 4.7 A** TGF-β1 levels in pg/ml: PMA 529.23 ± 5.1 vs. E2/PMA 0, p=0.005 (E2 158.01 ± 15.1, resting cells 163.15 ± 23.4, medium (M) 0). This data demonstrated implication of E2 in TGF-β1 inhibition. Furthermore, it was examined whether this inhibition can be rescued by MCD treatment and whether PKC-β is involved in this mechanism. HuT 78 cells incubated with the Go6976 inhibitor exhibited partial attenuation of TGF-β1, which suggests some involvement of PKC-β in this secretion. However, MCD treatment could not rescue the E2 mediated inhibition, indicating that PKC-β sequestration in lipid raft is probably not a crucial mechanism required for this process in this model system (**Figure 4.7 B**). The concentration of TGF-β1 (pg/ml) secreted by HuT 78 cells which were pre-treated with Go6976 and followed by PMA stimulation was 529.75 ± 2.5 while the cells treated only with PMA secreted 850.67 ± 4.5 pg/ml, p=0.004. This suggests that PKC-β may contribute to TGF-β1 secretion. In the cells exposed to E2 pre-treatment, followed by MCD incubation and PMA stimulation, TGF-β1 concentration was 258.78 ± 5.4, close to the level of resting cells (resting cells 295.65 ± 15.7, E2/PMA 405.61 ± 0.1 vs PMA, p=0.0003) and reversion of the inhibition could not be observed. This indicates that some other mechanism(s), different to the one implicated in IL-2 secretion, might be involved in this E2 mediated inhibition in HuT 78 cells.

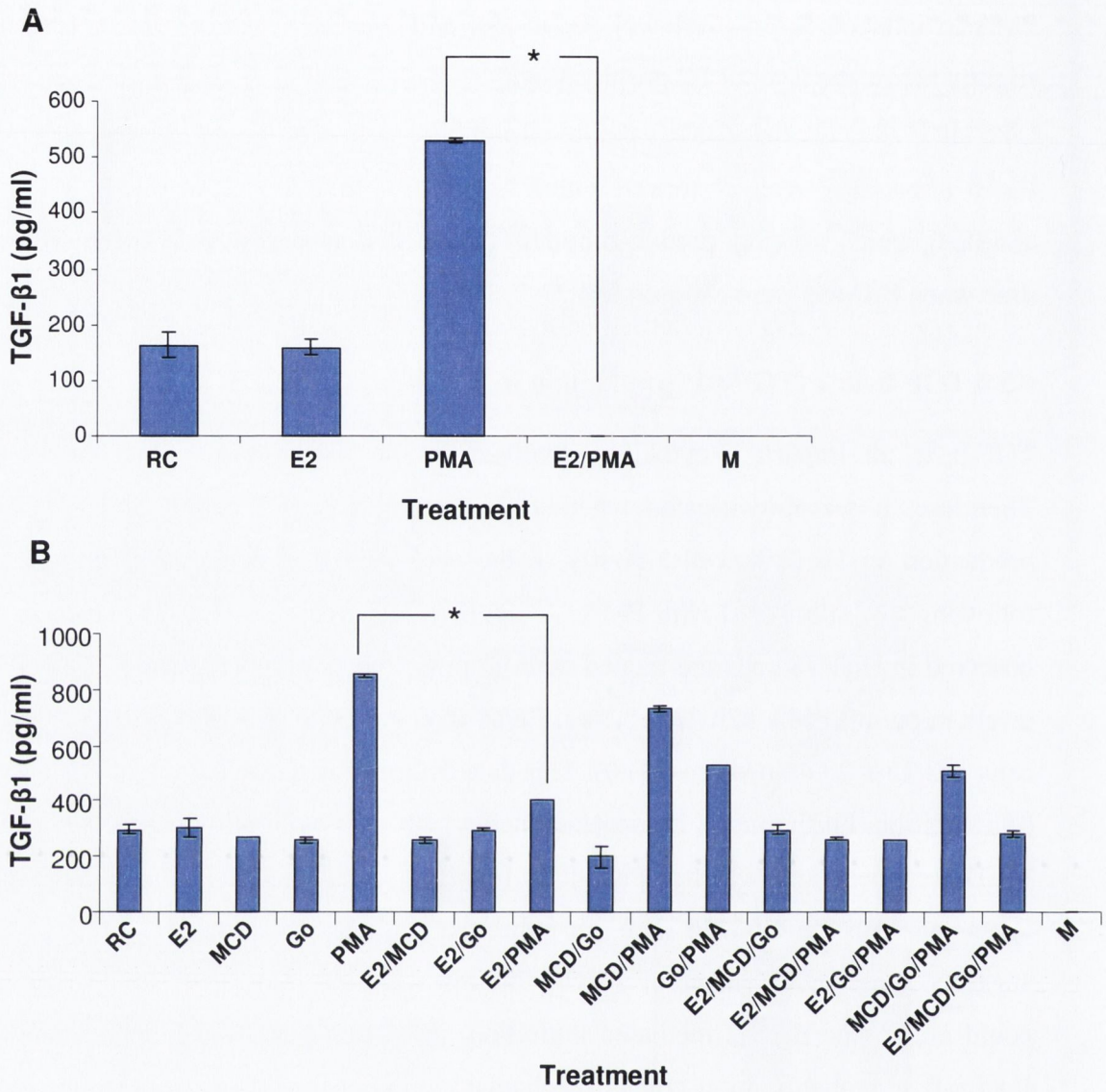



**Figure 4.7 E2 prevents production of TGF-β1 by activated HuT 78 cells. A)** Significant decrease in cytokine secretion was observed when cells were activated with PMA (10 ng/ml, 24 hours) following pre-incubation overnight with E2 (1 μg/ml). **B)** Cells were pre-treated with E2 overnight and then incubated ± MCD (0.2mM) for an hour prior to PMA stimulation and incubated ± PKC-α/β selective inhibitor Go6976 (5 μM 30 min) prior to PMA stimulation and after MCD treatment. When the stimulation was completed, the supernatants were collected and analysed for TGF-β1 production. Cell medium (M) was also assessed for TGF-β1, since medium alone contains certain amount of this cytokine due to presence of FCS. MCD

could not recover TGF-β1 secretion in cells pre-treated with E2. Go6976 just partially inhibited TGF-β1 secretion. Data is presented as mean ± SEM of one representative experiment from three independent experiments with similar results. Significant differences are indicated by the asterisks.

TGF-β1 secretion was also investigated in PBMCs (**Figure 4.8 A**) and interestingly, when these cells were activated with PMA/Io after the pre-treatment with E2, significant inhibition of TGF-β1 secretion could not be observed (levels in pg/ml PMA/Io 1127.09 ± 35 vs E2/PMA/Io 1120.56 ± 77.7). The only reduction was noticed after E2 exposed cells were stimulated with αCD3/αCD28 antibodies (levels in pg/ml αCD3/αCD28 238.93 ± 2.4 vs E2/αCD3/αCD28 3.8 ± 10.9, p=0.027). However, αCD3/αCD28 treatment could produce just low levels of TGF-β1, lower then the basal levels obtained from resting cells (in pg/ml 510.27 ± 27.1).

To assure that these results are not influenced by some other cell population present in PBMCs, the same experiment was conducted on CD3+ T cells purified from PBMCs. T cells exhibited the same profile of TGF-β1 secretion as PBMCs (**Figure 4.8 B**). Levels of TGF-β1 in pg/ml: PMA/Io 797.65 ± 41.7 vs E2/PMA/Io 612.73 ± 24.3; αCD3/αCD28 235.67 ± 3.7 vs E2/αCD3/αCD 0, p=0.029 (resting cells 255.9 ± 9.6). Again αCD3/αCD28 stimulation induced only basal levels in TGF-β1 secretion.

As E2-mediated inhibition of TGF-β1 secretion by HuT 78 cells could not be rescued by MCD treatment, it was then investigated whether the level of transcription was affected. Quantitative RT-PCR was performed to measure TGF-β1 gene expression (**Figure 4.9 A**). E2 did repress TGF-β1 gene transcription and mRNA levels in PMA stimulated cells pre-treated with E2, decreased 3.65 fold of the level of PMA activated cells (p=0.03). However, mRNA levels of TGF-β1 were basal and close to resting cells. When TGF-β1 gene expression was measured in PBMCs, the levels of mRNA were significantly increased over resting cells, regardless on cell treatment. Cells exposed to E2 expressed increase in TGF-β1 mRNA levels of 1.89 fold over resting cells (p=0.03), αCD3/αCD28 stimulated cells enhanced mRNA levels 2.15 fold over resting cells, (p=0.05) while αCD3/αCD28 magnified mRNA 2.75 fold in the presence of E2, (p=0.005).

HuT 78 cells and PBMCs demonstrated noteworthy difference in regulation of gene expression of TGF-β1, which might be explain by pleiotropic functions of this cytokine in different cells, i.e. its role in transformed, carcinogenic cells/cell line and healthy primary cells.

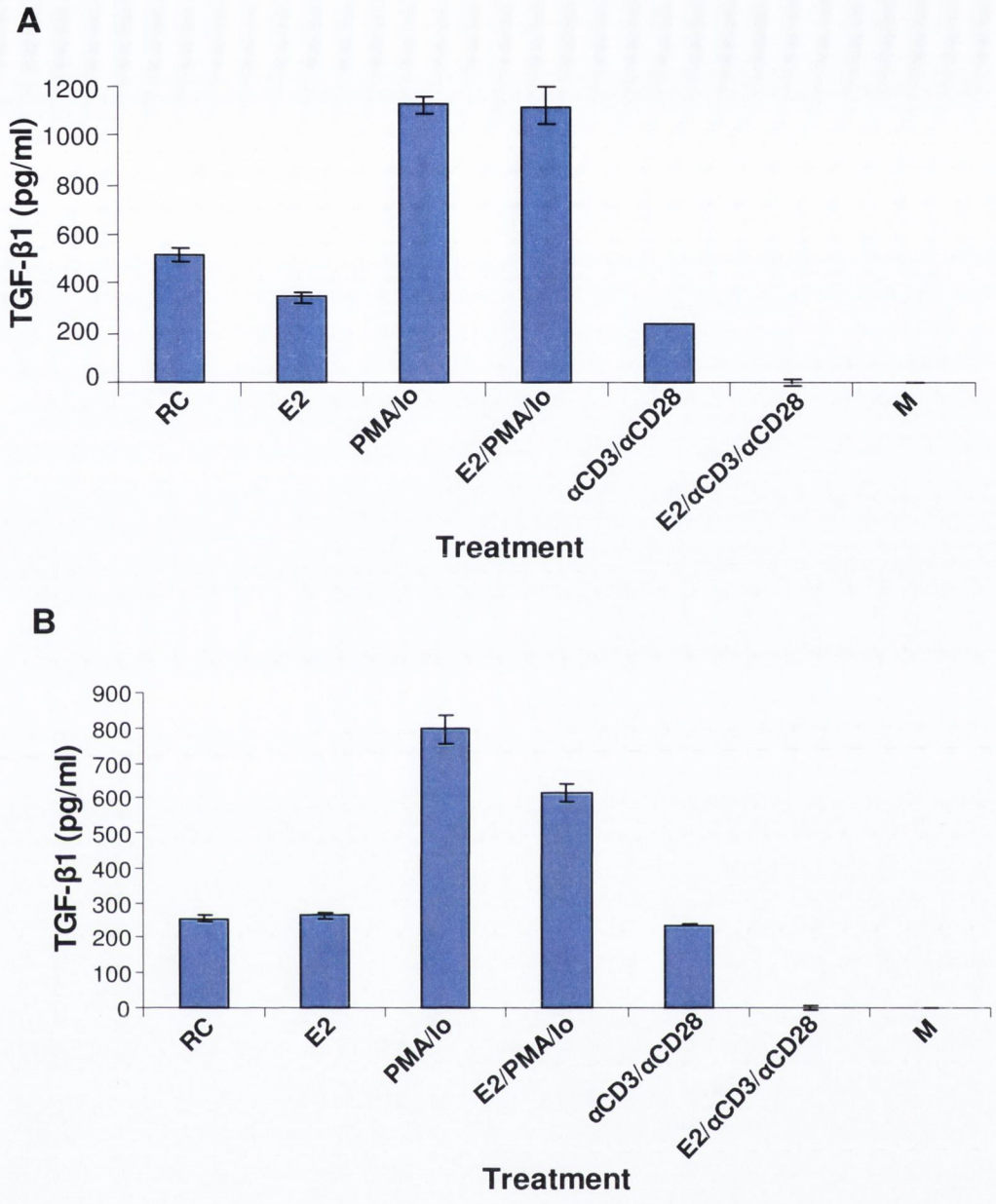



**Figure 4.8 E2 does not prevent secretion of TGF-β1 after PMA/Io stimulation. A)** PBMCs were stimulated with PMA/Io or αCD3/αCD28 following pre-incubation overnight with E2. **B)** CD3+ T cells were stimulated with PMA/Io or αCD3/αCD28 following pre-treatment with E2 overnight. Anti-CD3/anti-CD28 stimulation was at basal levels. Data is presented as mean ± SEM of one representative experiment from two independent experiments with similar results. (M - medium, as medium alone was assessed for the levels of TGF-β1).

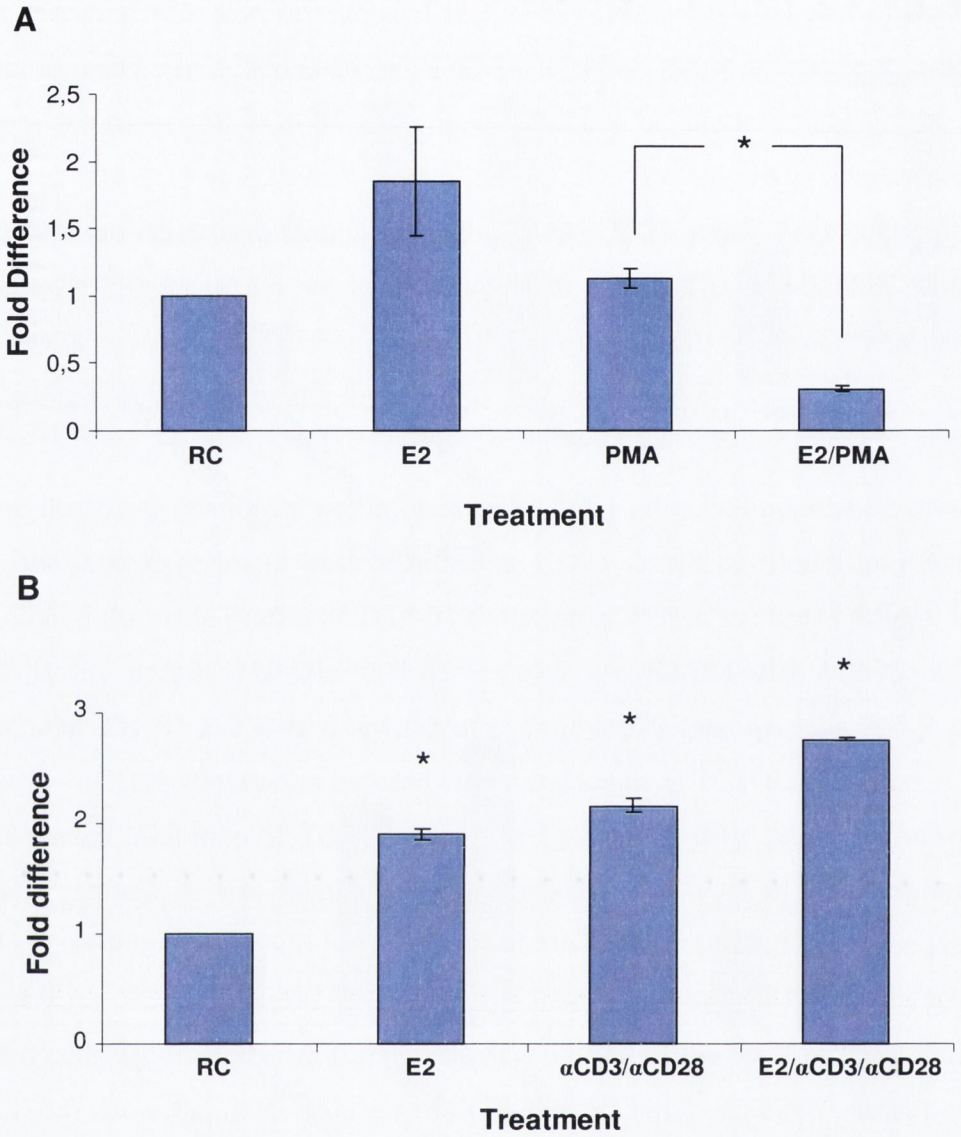



**Figure 4.9 Effect of E2 on TGF-β1 mRNA expression.** mRNA levels were quantified in HuT 78 and PBMCs in response to activation (with or without prior exposure to E2). **A)** HuT 78 cells were activated with PMA following pre-incubation overnight with E2. E2 mediated inhibition of TGF-β1 production is at gene transcription level in HuT78 cells. **B)** PBMCs were pre-incubated overnight with E2 prior activation with anti-CD3/anti-CD28. E2 increases TGF-β1 gene transcription in PBMCs. Quantitative RT-PCR data is presented for one representative experiment from two independent experiments with similar results and was achieved using the comparative $C_T$ method. The housekeeping gene GAPDH was used as an endogenous control. Significant differences ($p<0.05$) are indicated by the asterisks.

### 4.3.5 HCV E2 alters cytokine gene expression in HuT 78

Previous experiments have demonstrated that E2 inhibited secretion of IL-2, IFN-γ, TNF-α and IL-10 from activated HuT 78 cells (sections 3.3.4 and 4.3.2). Furthermore, we investigated the transcription levels of mRNA for the corresponding genes. To this end, cells were exposed to E2 overnight and subsequently activated with PMA for 24 hours. Levels of IL-2 mRNA for all treatments were significantly increased when compared to the resting cells, which is consistent with previous finding, described in section 3.3.6, when cells were stimulated with PMA for 5 hours: E2 pre-treatment alone led to a 140.35 fold increase (p=0.0008), E2/PMA treatment to 103.70 fold increase (p=0.02) and PMA to 125.68 fold enhancement (p=0.02) **(Figure 4.10 A)**. Similarly, mRNA levels for Interferon-γ were considerably elevated over resting cells. For E2 pre-treatment enlargement was 89.97 fold (p=0.0022), for E2/PMA 87.53 (p=0.0028) and for PMA stimulated cells 88.99 fold (0.0023) **(Figure 4.10 B)**.

On the other hand, treatment of HuT 78 cells with E2 prior to activation attenuated stimulated levels of both TNF-α and IL-10 mRNA. E2/PMA treated cells exhibited 6.54 fold decrease in TNF-α mRNA levels when compared to the PMA activated cells (p=0.011) as shown in **Figure 4.11 A**, while the same treatment reduced the IL-10 mRNA level by 87 fold (p=0.02) **(Figure 4.11 B)**.

These data suggest that inhibition of IL-2 and INF-γ is at the level of protein secretion, whereas reduction of TNF-α and IL-10 is due to suppression of mRNA expression.

### 4.3.6 HCV E2 alters cytokine gene expression in PBMCs

As determined previously, pre-treatment with E2 significantly reduced secretion of IL-2, IFN-γ, TNF-α and IL-10 from αCD3/αCD28 stimulated peripheral blood mononuclear cells (section 3.3.2 and 4.3.3). Following E2 pre-treatment IL-2 and IFN-γ mRNA levels in PBMCs were not increased over resting cells, as it was observed in HuT 78. At the mRNA level there was some decrease in both IL-2 (1.72 fold decline, p=0.042) **(Figure 4.12 A)** and IFN-γ (1.8 fold reduction, p=0.048) **(Figure 4.12 B)** in activated PBMCs pre-treated with E2 (this decrease did not reach background levels however). Like in HuT 78 cells,

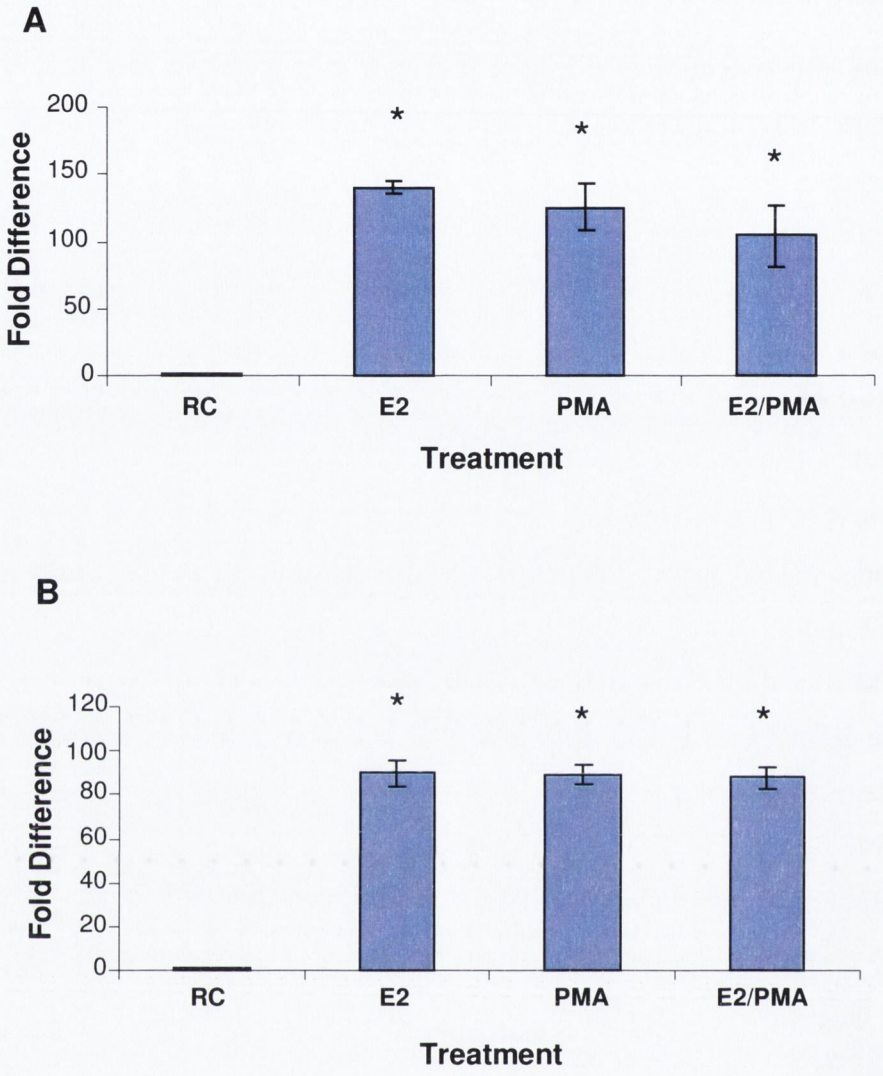



**Figure 4.10 Effects of E2 on IL-2 and IFN-γ mRNA levels in HuT78 cells.** mRNA levels were quantified in HuT 78 in response to activation (with or without prior exposure to E2). HuT 78 cells were activated with PMA (10 ng/ml, 24 hours) following pre-incubation overnight with E2 (1 μg/ml). **A**) Effect of E2 on IL-2 mRNA expression. **B**) Effect of E2 on IFN-γ mRNA expression. Quantitative RT-PCR data is presented for one representative experiment from two independent experiments with similar results and was achieved using the comparative $C_T$ method. The housekeeping gene GAPDH was used as an endogenous control. Significant differences ($p<0.05$) are indicated by the asterisks.

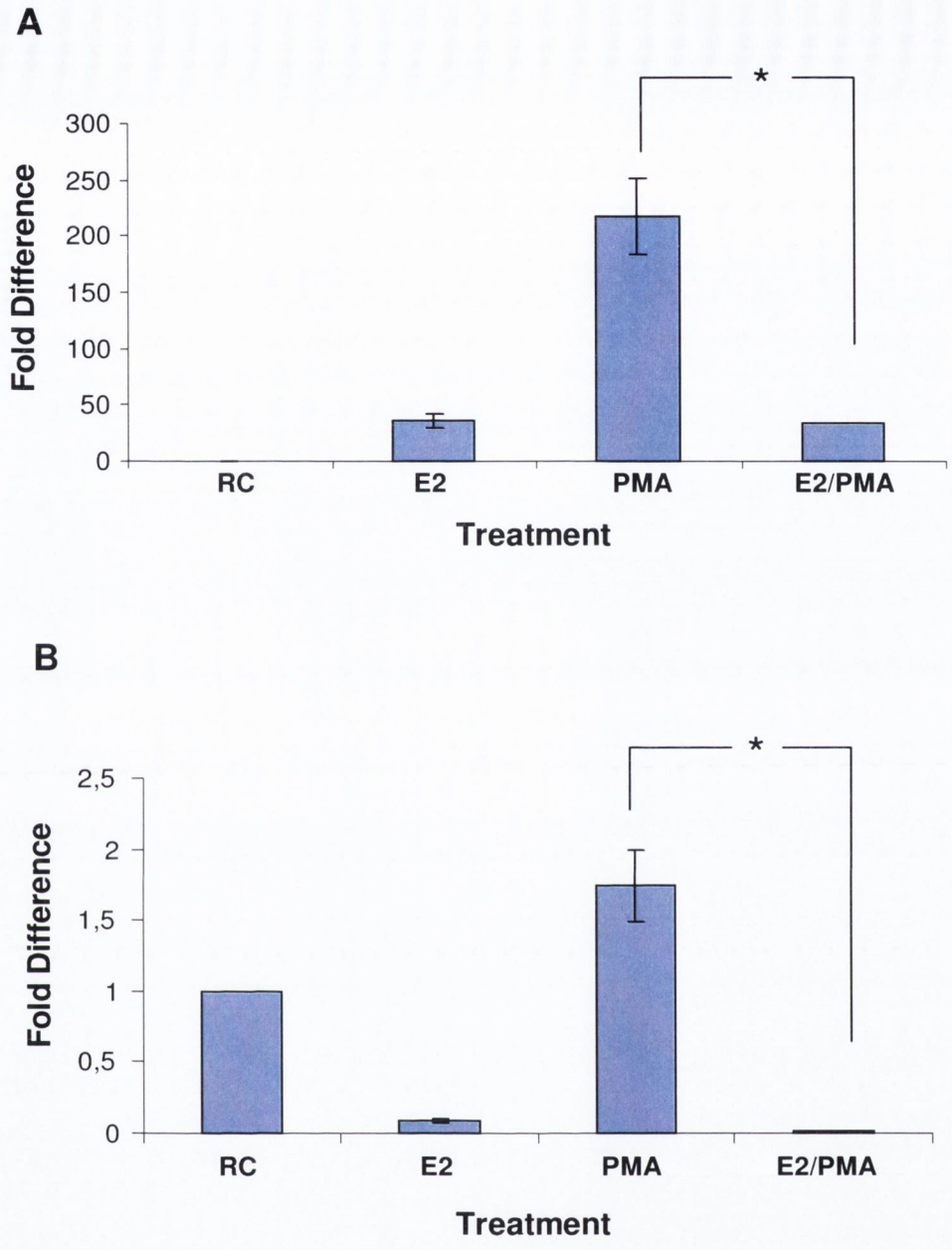



**Figure 4.11 E2 mediated inhibition of TNF-α and IL-10 production is at gene transcription level in HuT78 cells.** HuT 78 cells were activated with PMA following pre-incubation overnight with E2 or were stimulated with E2 or PMA alone. **A)** Effect of E2 on TNF-α mRNA expression. **B)** Effect of E2 on IL-10 mRNA expression. Quantitative RT-PCR data is presented for one representative experiment from two independent experiments with similar results and was achieved using the comparative $C_T$ method. The housekeeping gene GAPDH was used as an endogenous control. Significant differences ($p<0.05$) are indicated by the asterisks.

treatment of PBMCs with E2 prior to activation, diminished stimulated levels of both TNF-α and IL-10 mRNA. E2/αCD3/αCD28 treated cells exhibited 3.98 fold decrease in TNF-α mRNA level when compared to the αCD3/αCD28 activated cells (p=0.038) **(Figure 4.13 A)**, while the same treatment reduced the IL-10 mRNA level by 21.3 fold (p=0.029) **(Figure 4.13 B)**.

At the protein level IL-1α, IL-1β and IL-6 were significantly increased every time when PBMCs were exposed to E2 (section 4.3.3). When gene expression of these cytokines was examined, it was observed that E2 pre-treated cells, subsequently stimulated with αCD3/αCD28 for 24 hours, significantly increased mRNA level when compared to the activated cells not exposed to E2. E2/αCD3/αCD28 treated cells exhibited 2.64 fold rise in IL-1α mRNA level when compared to the αCD3/αCD28 activated cells (p=0.014) as shown in **Figure 4.14 A**, 1.72 fold enlargement in IL-1β mRNA level (p=0.039) **(Figure 4.14 B)** and 84.87 fold enhancement in IL-6 mRNA level (p=0.023) **(Figure 4.14 C)**. Following E2 stimulation alone, mRNA levels increased over resting cells for IL-1α 4.39 fold (p=0.034), for IL-1β 1.61 fold (p=0.06) and IL-6 7.39 fold (0.028).

While E2 mediated inhibition of IL-2 and IFN-γ mainly was at the level of secretion, reduction of TNF-α and IL-10 is due to suppression of gene expression. In contrast, IL-1α, IL-1β and IL-6 were augmented at the gene expression level, and subsequently secretion level in E2 pre-treated activated PBMCs.

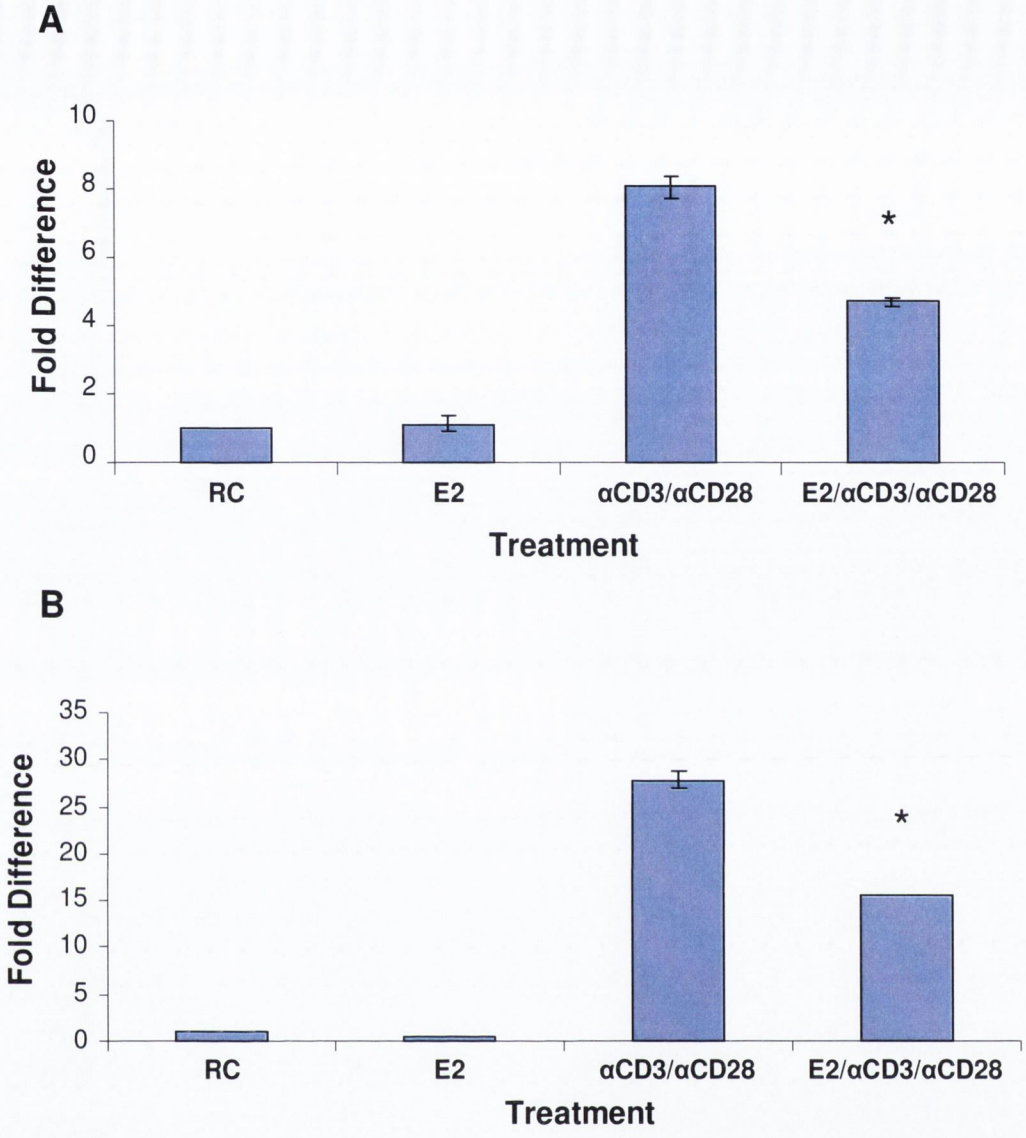



**Figure 4.12 Effect of E2 on IL-2 mRNA and IFN-γ mRNA in PBMCs.** mRNA levels were quantified in PBMCs in response to αCD3/αCD28 activation alone or with prior overnight exposure to E2 and in response to E2 stimulation alone. **A)** IL-2 mRNA expression. **B)** IFN-γ mRNA expression. Quantitative RT-PCR data is presented for one representative experiment from two independent experiments with similar results and was achieved using the comparative $C_T$ method. The housekeeping gene GAPDH was used as an endogenous control. Significant differences ($p<0.05$) are indicated by the asterisks.

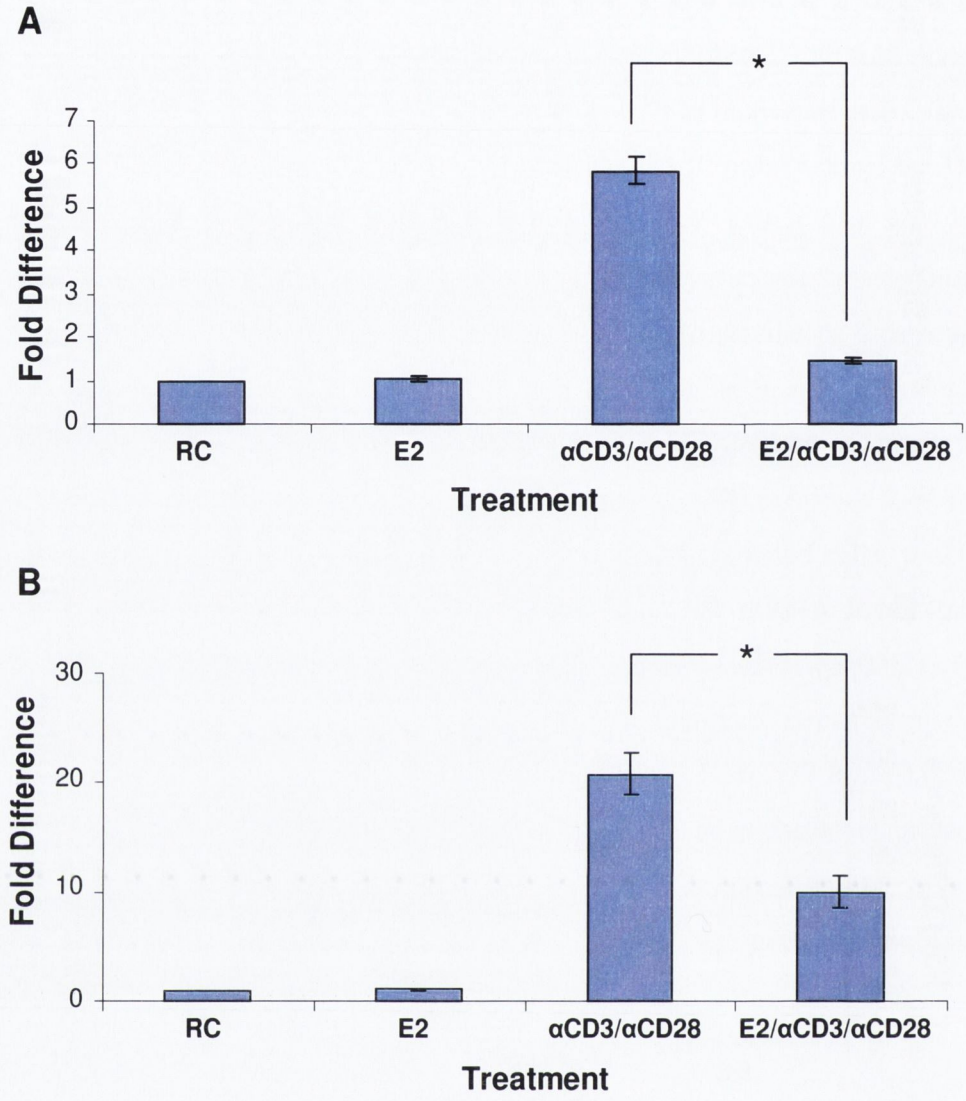



**Figure 4.13 E2 prevents TNF-α and IL-10 cytokine production suppressing gene transcription in PBMCs.** Cells were stimulated with E2 or αCD3/αCD28 alone as well as with αCD3/αCD28 following overnight exposure to E2. **A)** Effect of E2 on TNF-α mRNA expression. **B)** Effect of E2 on IL-10 mRNA expression. Quantitative RT-PCR data is presented for one representative experiment from two independent experiments with similar results and was achieved using the comparative $C_T$ method. The housekeeping gene GAPDH was used as an endogenous control. Significant differences ($p<0.05$) are indicated by the asterisks.

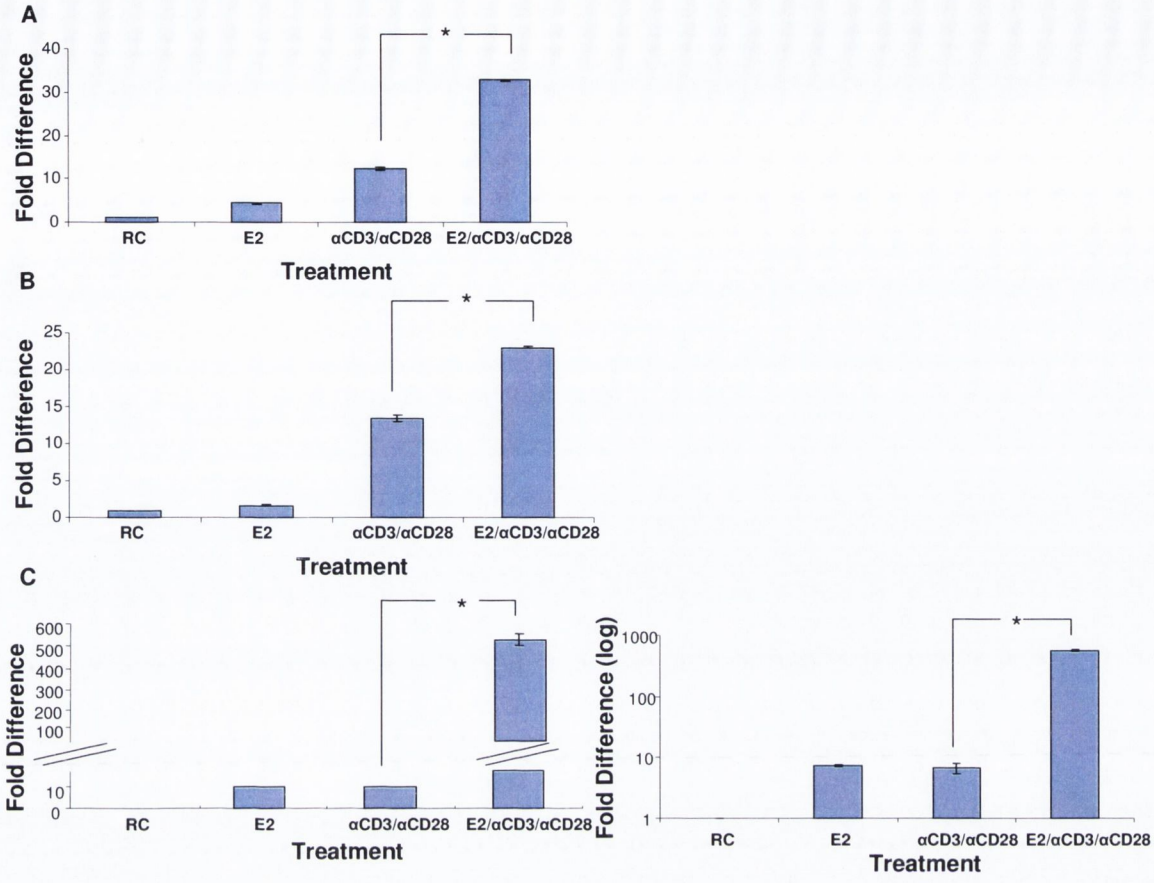



**Figure 4.14 Significant induction of IL-1α, IL-1β and IL-6 cytokines secretion in stimulated PBMCs pre-treated with E2, is due to substantial increase in gene transcription.** mRNA levels were quantified in PBMCs in response to αCD3/αCD28 activation alone or with prior overnight exposure to E2 and in response to E2 stimulation alone. **A)** Effect of E2 on IL-1α mRNA expression. **B)** Effect of E2 on IL-1β mRNA expression. **C)** Effect of E2 on IL-6 mRNA expression, with fold difference presented in linear and logarithmic scale. Quantitative RT-PCR data is presented for one representative experiment from two independent experiments with similar results and was achieved using the comparative $C_T$ method. The housekeeping gene GAPDH was used as an endogenous control. Significant differences ($p<0.05$) are indicated by the asterisks.

## *4.4 DISCUSSION*

Strong and constant CD8 and CD4 T-cell responses are critical in HCV clearance (Lechner et al, 2000; Thimme et al, 2001). If some parts of the complex function of these cells are affected it can lead to an impaired immune response. In the first part of this study it has been demonstrated that E2 of HCV can disturb T cell function causing the inhibition of IL-2 secretion, a possible mechanism of viral persistence. Furthermore, here it has been illustrated that E2 can inhibit the secretion of some other cytokines such as Interferon-γ, TNF-α and IL-10 *in vitro.*

IFN-γ, another centrosome/synapse-directed cytokine (Huse et al, 2006), is affected in the same fashion as IL-2, suggesting that PKC-β is mechanistically involved in both secretory processes. Uninterrupted secretion of IFN-γ is essential for control of infection, as this cytokine produced by T cells infiltrated within the liver, is central to their antiviral activity (Sun et al, 2004). In addition, recovery of CD8+ T cell IFN-γ production is shown to be correlated with viral clearance (Thimme et al, 2001). Likewise for IL-2, gene expression was not affected in HuT 78 cells. However, a slight decrease at IL-2 and Interferon-γ mRNA levels for PBMCs exposed to E2 prior to stimulation is probably due to the mixed population of cells in PBMCs, possibly negligibly contributing to complete inhibition at the protein level, likely to be caused by sequestration of PKC-β into lipid raft. E2 induced weak or absent IFN-γ secretion could subsequently lead to persistent infection, which is consistent with many studies which have shown that antigen specific proliferation and IFN-γ secretion were defective in HCV chronically infected individuals (Neveu et al, 2008; Spangenberg et al, 2005; Ulsenheimer et al, 2003; Wedemeyer et al, 2002). One study has shown that the amount of HCV tetramer-positive CD8 cells which produced IFN-γ ranged from 0.5-17%, while it ranged from 65-98% among CMV-specific CD8 lymphocytes (Penna et al, 2007). Recently, it has been reported that HCV-specific CD4 cells which powerfully proliferated and secreted IFN-γ upon antigenic stimulation, were detected in 3 out of 4 (75%) individuals who had acute HCV infection subsequently resolved,

but not in any of the 10 patients with acute hepatitis with chronic evolution (Kaplan et al, 2007).

The next finding that emerged in this study was that treatment of HuT 78 cells and PBMCs with E2 prior to activation, attenuated both TNF-α and IL-10 at protein level as well as mRNA level. In contrast to IL-2 and IFN-γ, these data suggest that E2-mediated reduction of TNF-α and IL-10 is due to suppression of gene expression. Supporting our findings, Gruner et al have shown that HCV-specific CD8+ T cells expressed reduced levels of TNF-α and IFN-γ when the cells were stimulated with mitogens or HCV peptides, which was not the case with EBV or CMV peptides (Gruener et al, 2001). Similarly, functional defects in HCV-specific CD8+ T cells were associated with decreased production of TNF-α, as well as impaired IFN-γ production and depressed expression of granzyme-A (Penna et al, 2007). In addition, Mendoza et al. observed a reduction in PMA-induced release of TNF-α from PBMCs extracted from patients chronically infected with HCV (Mendoza et al, 1996). However, some studies observed elevated serum levels of TNF-α (Durante-Mangoni et al, 2006) or increased hepatic TNF-α mRNA expression (Gochee et al, 2003) both in chronic HCV infected patients with steatosis. There is evidence which suggests that this TNF-α comes from Kupffer cells and hepatocytes, which were stained strongly-positive for this protein (Neuman et al, 2007), while in our system we were investigating T cells. Even though some studies propose that serum levels of TNF-α are elevated among patients with chronic HCV, they have often provided variable or conflicting results regarding TNF-α production by PBMC in these patients (Larrea et al, 1996; Nelson et al, 1997).

Similarly to TNF-α, IL-10 exhibited the same pattern of expression and E2 caused inhibition at the both protein and gene levels, (although PBMCs produced comparatively low levels of IL-10 protein in this system). IL-10 is an important immunomodulatory cytokine and a recent study in the LCMV murine model of chronic viral infection documented that upregulation of IL-10 led to persistent infection, while neutralising its activity resulted in LCMV clearance (Brooks et al, 2006). Several studies have compared serum levels of IL-10 in HCV-infected and healthy individuals

demonstrating conflicting results. Some have shown levels to be elevated (but at the low level) in chronic HCV infection, while others determined control levels in both groups (Bozkaya et al, 2000; Cacciarelli et al, 1996; Reiser et al, 1997). Nevertheless, none of these studies investigated IL-10 levels during acute phase of infection, which our model system proposed, when it may be crucial in the establishment of chronic infection. It seems clear that IL-10 could suppress CD4+ T cells and their antiviral response promoting increased viremia and development of chronic disease (Kaplan et al, 2008). As demonstrated by several studies, increased levels of intrahepatic IL-10 observed in chronic HCV infection, comes from HCV-specific regulatory T cells, which are distinct subsets of T cells implicated in HCV immune escape (Abel et al, 2006; Accapezzato et al, 2004; Koziel et al, 1995). The role of this cytokine in HCV infection is very complex and has still to be fully elucidated. Results from one study have revealed that PBMCs from uninfected and HIV-monoinfected individuals, after exposure to HCV antigens (core, NS3 and NS4 proteins), exhibited increased IL-10 production, while this IL-10 response was attenuated in PBMCs from individuals with chronic HCV infection (Barrett et al, 2008). This represents possible different roles where in the first instance increased IL-10 response may have a role in establishing chronic HCV infection, whilst attenuating responses in already established chronic infection may favour development of hepatic immunopathology.

In this study it has been documented that the pattern of secretion that was observed for IL-1α, IL-1β and IL-6 was completely different to the one detected for the rest of the analysed cytokines. Namely, at the gene expression level, and subsequently secretion level, these molecules were significantly enhanced every time when PBMCs were exposed to E2. Since E2 pre-treated and/or stimulated HuT 78 T cells did not produce IL-1α, IL-1β and IL-6, we hypothesised that this response may be produced by monocytes. As shown in our experiments, after T cells, monocytes were the second largest subset of PBMCs isolated from healthy volunteer (around 17%), and approximately 67.9% of all monocytes expressed CD81 molecule on the cell membrane, which is in agreement with the literature (Tohami et al, 2004). In addition, monocytes exhibited similar level of expression of CD81 molecules on their cell

surface (MFI 68.07) as T cells (MFI 65.78), showing that they have a potential to be triggered by E2 through CD81, a key mediator of the HCV – host cell interaction. Moreover, it was demonstrated that binding of E2 to some PBMCs subsets, including T cells, was entirely dependent on CD81, while binding to some other subsets, including monocytes, had a CD81-independant component as well, indicating an additional receptor(s) involved in this interaction (Yamada et al, 2005). E2 stimulus alone induces production of proinflamatory cytokines, we believe by monocytes, as previously has been shown that HIV patients have elevated levels of spontaneous production of some monocyte proinflammatory cytokines, such as IL-1β and IL-6 (Baqui et al, 2000; Tilton et al, 2006).

It has been previously reported that HCV-infected patients have elevated levels of IL-6 in serum when compared with healthy controls, as a consequence of virus-induced inflammatory state (Feldmann et al, 2006; Malaguarnera et al, 1997). One study has shown that concentrations of IL-6 in serum was normalised after IFN-α treatment had been conducted and HCV eliminated, supporting the idea that elevated IL-6 serum concentration might come from immune stimulation by HCV (Grungreiff et al, 1999). Previously it has been presented that recombinant HCV core protein triggers IL-6 and IL-8 production in CD14+ cells via stimulation of TLR2 (Feldmann et al, 2006). Similarly, our findings indicated that E2 also can induce production of IL-6 by monocytes.

While IL-6 was consistently found to be increased in liver diseases, such as chronic hepatitis and cirrhosis (Genesca et al, 1999; Kakumu et al, 1991; Lapinski, 2001; Malaguarnera et al, 1997; Rehermann et al, 1992) the literature on IL-1 status in liver disease is conflicting. Some studies have shown that chronically infected HCV patients exhibited lower intrahepatic levels (Dumoulin et al, 1999) or lower levels of spontaneous and stimulated IL-1 expression by the PBMCs (Mendoza et al, 1996; Muller et al, 1993; Ozyilkan et al, 1994) compared to controls. However, some studies suggested that HCV infected individuals had elevated levels of IL-1 when measured in serum (Khoruts et al, 1991; Lapinski, 2001; Muto et al, 1988; Tilg et al, 1993; Tilg et al, 1992) or in liver tissue (Farinati et al, 2006; Kasprzak et al, 2004; Lapinski, 2001).

Since one study has shown that IL-1β can augment IFN-stimulated target gene expression, which plays a role in antiviral activity, it seems that increased levels of IL-1 are desirable for the virus-infected organism (Ichikawa et al, 2002). In addition, another group has suggested that IL-1 has direct antiviral activity demonstrating inhibition of HCV subgenomic RNA replication and viral protein expression by this cytokine (Zhu & Liu, 2003). In our system, which mimics the initial phase of infection, E2 induced significant increase of both isoforms of IL-1 which is in line with a study demonstrating that the numbers of IL-1β positive monocytes increased significantly upon stimulation of isolated monocytes with HCV recombinant core, NS3, NS4, NS5a and NS5b proteins/antigens (Woitas et al, 2002). In general, the precise immunological roles of IL-1 in HCV infection are not defined. It has been proposed that IL-1α and IL-1β are co-stimulatory cytokines for CD4+ T cells which could promote maturation and clonal expansion of B cells (Lama & Planelles, 2007).

The effect of E2 on TGF-β1 production was also investigated in this study, since it is important in development of liver injury/disease and since inflammation associated with chronic HCV infection alters the hepatocytic role of this cytokine from tumor-suppression to fibrogenesis, increasing speed of liver fibrosis and risk of HCC (Gressner et al, 2002; Matsuzaki et al, 2007). It was observed that secretion of this cytokine in HuT 78 cells was completely suppressed after stimulated cells were pre-treated with E2. This could not be rescued through MCD treatment (disruption of lipid rafts), as we showed for IL-2, because the E2-mediated inhibition was at the mRNA level (which was not in the case of IL-2). However, in PMA stimulated PBMCs, TGF-β1 secretion was not suppressed by E2. Anti-CD3/anti-CD28 did not stimulate significant levels of TGF-β1. When the CD3+ T cells were purified from PBMCs, inhibition was observed just when the cells pre-exposed to E2 were stimulated with anti-CD3/anti-CD28 antibody, although stimulation was at the low level. Since the data is unclear, probably due to difficulties in measuring this cytokine at the protein level (TGF-β1 is secreted in inactive form and has to be activated), this should be investigated further. On the other hand, results of mRNA levels in PBMCs undoubtedly showed increased transcription of TGF-β1 gene following incubation with

E2, which is further increased following anti-CD3/anti-CD28 treatment. Similarly, it was described that HCV core upregulates transcription of TGF-β in hepatocytes (Taniguchi et al, 2004) bonding to a promoter region. TGF-β1 levels in liver tissue or hepatic mRNA levels significantly correlated with TGF-β1 serum content, confirming a hepatic source for the elevated serum levels (Kamal et al, 2006; Tsushima et al, 1999). These increased circulating levels have been reported in acute (Flisiak et al, 2005) as well as chronic HCV infection (Ali et al, 2004; Kirmaz et al, 2004; Murawaki et al, 1998).

Different immunological effects that we observed in HuT 78 cells and PBMCs might be due to diverse biological effects depend on the target cells (Barnard et al, 1990). It has been demonstrated that, on one hand, TGF-β can inhibit epithelial, endothelial and haematopoietic cell proliferation and act as a tumor suppressor; while on the other hand, it can promote growth on mesenchymal cells, skin and stromal fibroblasts (Blobe et al, 2000; Roberts & Wakefield, 2003). In addition, TGF-β in normal leukocytes, promotes their differentiation and inhibits their proliferation and activation (Letterio & Roberts, 1998). In transformed cells TGF-β – mediated growth inhibition is lost due to mutation in molecules involved in TGF-β signalling pathways, as shown for 100% of pancreatic cancers and 83% of colon cancers (Grady et al, 1999; Villanueva et al, 1998). As HuT 78 cells are transformed lymphoma T cells and PBMCs are healthy primary cells, it might explain the difference in TGF-β1 expression between these two cell types in this study.

In general, there is controversy with regards to cytokine production and cytokine profiles associated with HCV infection in the literature. This is probably due to multiple factors such as: selection of patients (co-infections, alcohol consumption, co-morbidity), viral characteristics (genotype, viral load), different stage of the disease (which might be associated with varying cytokine types and amounts) and possibly the site of cytokine production. All these factors can create differences and conflicting results, which can be addressed using precisely defined HCV infection settings.

In this study we have shown that E2 from HCV modulates immune cell function leading to effects on cytokine production. The secretion of cytokines such as IL-1α, IL-1β and IL-6 was significantly enhanced after exposure to E2, while secretion of IFN-γ, TNF-α and IL-10 from activated cells was reduced. This inhibition has been shown to be at different levels, affecting secretory machinery (IL-2, IFN-γ) or mRNA levels (TNF-α, IL-10, TGF-β). It is possible that E2 interferes with different pathways in order to disturb the cytokine milieu that is favourable for appropriate immune response and viral clearance.

# CHAPTER V

# *MUTATIONS IN EPITOPES RESTRICTED BY PROTECTIVE HLA*A3, HLA*B7 AND HLA*DRB1 0401 ALLELES ARE ASSOCIATED WITH THE DEVELOPMENT OF PERSISTENT HEPATITIS C INFECTION*

# Chapter V

## *5.1 INTRODUCTION*

Several studies have reported association of HLA alleles with clearance or persistence of HCV infection (Singh et al, 2007). Some of them show that specific HLA Class I alleles are associated with protection against HCV and that protection is linked with stronger class I restricted CD8+ T- cell responses. Thio et al. demonstrated that HLA*A1101, B57, and Cw0102 were associated with viral clearance, whilst HLA*A2301, Cw04 were associated with persistent infection (Thio et al, 2002). Although the population examined in this study was heterogeneous in terms of gender, race and viral genotype, a similar finding which associated the HLA*B57 allele with protection, has been reported in a recent study on a West African cohort, infected mainly with HCV genotype 2 (Chuang et al, 2007). In a Japanese population HLA*B51, B52, B55, B56, B61, B70, Cw1, Cw3 and Cw4 were less frequent in chronically infected individuals (Kondo et al, 2003). A recent study on a Russian cohort demonstrated that HLA*Cw4 was associated with self-resolving HCV infection whereas HLA*B35 correlated with persistence.

In HCV infection, HLA class II alleles have also been associated with resolution of infection. For instance, Thio et al. have reported correlation of HLA*DRB1 0101 and DQB1 0501 alleles with protection against HCV and HLA*DRB1 0301 and DQB1 0201 with viral persistence in American Caucasians (Thio et al, 2001). Additionally, two other studies supported association of HLA*DRB1 0301 and DQB1 0201 alleles with unfavourable outcomes (Alric et al, 2000; Vejbaesya et al, 2000). Alleles HLA*DQB1 0301 and DRB1 1101 have been consistently correlated with spontaneous clearance of HCV infections (Alric et al, 2000; Alric et al, 1997; Harcourt et al, 2001; Harris et al, 2008; Thio et al, 2001; Thursz et al, 1999); and their protective ability was confirmed in meta-analysis of the data published in 11 various studies (Hong et al, 2005). Since most immunodominant epitopes are presented by these alleles (Lamonaca et al, 1999), Hong et al. speculated that presentation of viral epitopes to T helper cells through these alleles might be more effective in leading to viral resolution.

Previous Irish HLA studies and the study described in this chapter are based on a population derived from a specific Irish cohort of females, inoculated with anti D-immunoglobulin in 1977 and 1978, that had been contaminated with HCV from a single source, and was genotype 1b. In early 1994, throughout a "look-back" program, it was discovered that batches of anti-D immunoglobulin used in Ireland during the late 1970s to administer to Rhesus negative women, was contaminated with HCV from a single infected donor. Immediately, a national screening programme was initiated and it has identified that 704 (1.1% of all screened women) had evidence of past or current HCV infection, while more then half (55.4%) of them had positive PCR tests for serum HCV RNA. They were all referred to one of six designated liver centres for clinical assessment and therapy (Barrett et al, 1999; Kenny-Walsh, 1999). After 17 years of exposure 98% had evidence of inflammation, 55% evidence of chronic infection and 51% of fibrosis. Although HCV 1b is known to be more pathogenic than other genotypes (Amoroso et al, 1998; Gordon et al, 1997), the disease progression rate in this cohort was very slow with 2% having probable or definite cirrhosis. It seems that infection at a younger age somehow protects from progression to advanced liver disease (Fanning, 2002; McKiernan et al, 2004).

This unique and homogenous cohort **(Figure 5A)** is an ideal population to study as all individuals were infected with the same viral genotype from a single source, and additionally all were the same gender, with the same ethnic background, had the same route of acquisition and were of a similar, child bearing age. Studies on this cohort have provided valuable data on the contribution of MHC alleles and, since the cohort were exposed three decades ago, could provide important data on the natural history of HCV infection (Barrett et al, 2001).

Study on this cohort done by McKiernan et al. shows that HLA Class I – A3, B27 and Cw01 alleles were significantly associated with viral clearance, being present in 39.5 %, 14 % and 9.3 % of patients clearing the virus as compared to 19.1 %, 2.1 % and 1.4 % with viral persistence. The immune response restricted by these protective alleles is recognized as highly significant in the successful clearance of Hepatitis C virus. Allele HLA*B7 was also more frequent in individuals who resolved the infection (40.7%) than in those who develop chronic disease (25.5%). Alleles HLA*A3 and HLA*B7, both

associated with viral clearance, were part of a haplotype inherited in linkage disequilibrium (McKiernan et al, 2004). In another study it has been observed that HLA*A2, A3 and HLA*B7 restricted HIV epitopes, from conserved viral regions, are predicted to be immunogenic in greater than 85% of individuals infected with HIV. Thus, both HLA*A3 and HLA*B7 (along with A2) alleles are important for epitope-based vaccines designed to induce CTL responses specific for HIV-1 virus (Wilson et al, 2003). Certain HLA Class II alleles were also associated with viral clearance in the same cohort. HLA*DRB1 01 was reported to be associated with self-resolving HCV infection (Fanning et al, 2000). Moreover, 32.3% of those who attained viral clearance were carriers of the HLA*DRB1 0101 allele compared with 8.8% who develop chronic infection (McKiernan et al, 2000). Similarly, HLA*DRB1 0401 allele was present in 29.1% of the population who resolved infection and in 15.6% non-resolvers (McKiernan et al, 2004).

All in all, 75.6% of patients from the Irish cohort who cleared the virus carry either HLA*A3, HLA*DRB1 0101 or HLA*DRB1 0401 allele. This suggests a strong role for host immunogenetic factors in determining outcome in hepatitis C infection.

Since HLA alleles are responsible for antigen presentation to T cells, a perfect match between viral epitope and HLA molecule has to occur in order to initiate a successful immune response. Defining the epitope is one of the crucial steps in understanding the functioning of immune surveillance and vaccine development. Conventional techniques of molecular biology based on ELISpot (Anthony & Lehmann, 2003), flow cytometry (Hoffmeister et al, 2003), binding assays (del Guercio et al, 1995) and HLA*transgenic mice (Sette & Fikes, 2003) are widely used by many groups to study this. In addition, a bio-informatics approach using prediction software has become an important tool in elucidation of epitopes restricted by MHC molecules. Computational methods for prediction include: simple binding motifs, where a "peptide motif" for each MHC allele may be determined from a pool of already sequenced ligands for certain MHC molecules through the interaction of chemically related amino acids in certain positions (Falk et al, 1991; Rammensee, 1995); position-specific scoring matrices (PSSMs), where relative contribution of amino acids at each position in a peptide is used to calculate the prediction (Bhasin & Raghava, 2004a; Bui et al, 2005; Peters & Sette, 2005; Peters et al,

2003); artificial neural networks (ANN) which models amino acid binding motifs that depend on the properties of amino acids in other positions of the peptide (Bhasin & Raghava, 2004a; Bhasin & Raghava, 2004b; Brusic et al, 1998; Milik et al, 1998; Zhao et al, 2003). Similar to ANN, decision trees classifiers (Savoie et al, 1999; Zhao et al, 2003) and hidden Markov model (HMM) (Nielsen et al, 2003; Noguchi et al, 2002); calculate binding affinity of a given amino acid at one position including interactions with amino acids at other positions in the same peptide (Nielsen et al, 2003); and support vector machines (SVMs)(Bhasin & Raghava, 2004a; Bhasin & Raghava, 2004b; Donnes & Elofsson, 2002), a powerful technique that in prediction score includes physicochemical properties (such as α-helix or β-structure preferences, hydrophobicity, pK, 3D form) of each amino acids within the input peptide (Zhao et al, 2003). Prediction algorithms such as ANN, HMM and SVM are also known as "machine learning algorithms" which are able to integrate predetermined qualitative knowledge and to give a quantitative prediction of structure-activity relationships (SAR). Generally, protein prediction can be delivered with accuracy in the range of 70% to 90% (Lara et al, 2008).

There are two approaches in epitope discovery that have been implemented by all these prediction algorithms. The first defines "peptide binding motifs" via positional scanning with random synthetic peptide libraries (binding of synthetic peptides derived from antigens is determined by MHC-peptide binding assays). The other defines "natural ligand motifs" through the search of libraries made of known natural peptide repertoires presented by certain MHC molecules. Thus, natural ligand motifs will reflect, to a certain degree, both antigen processing and binding properties (Schirle et al, 2001).

The first unrestricted web prediction databases available world-wide were BIMAS developed by group at NIH (Parker et al, 1994) and SYFPEITHI developed by a German group (Rammensee et al, 1999). BIMAS outlines potential binders according to the predicted half-time dissociation of MHC-peptide complex based on synthetic peptide libraries. SYFPEITHI ranks predicted epitopes according to the affinity of amino acids in the anchor sites from calculations obtained from a pool of natural ligands (Schirle et al, 2001; Stevanovic, 2005). Besides Bimas and SYFPEITHI, the databases used for epitope predictions in this study were: ProPred 1 (ProPred), Net MHC (Net MHCII), Jenner (MHCPred), MAPPP, RankPep, SVRMHC, MHC2Pred

and IEDB [Appendix 2]. ProPred was initially designed for HLA Class II alleles by Singh and Raghava (Singh & Raghava, 2001) and is based on PSSMs, NetMHC on neural network predictions (Buus et al, 2003); Jenner (MHCPred) is based on machine learning algorithms and quantitative SAR (Guan et al, 2003; Hattotuwagama et al, 2004); MAPPP is based on a score calculated when values for each amino acid within the predicted epitope are multiplied (BIMAS) or added (SYFPEITHI); RankPep uses PSSMs (Reche et al, 2002); IEDB is based on a combination from ANN, stabilised matrix method and relative binding method (Bui et al, 2005; Nielsen et al, 2007; Nielsen et al, 2003; Peters & Sette, 2005) while MHC2Pred and SVRMHC utilise SVM (Lata et al, 2007; Liu et al, 2006; Wan et al, 2006). Overall, what all these databases have in common is that they possess complex algorithms used to screen for probable sequence targets, within the protein of interest, which could bind to the particular MHC molecule. Two other databases - NetChop (Kesmir et al, 2002; Nielsen et al, 2005) and PaProC (Kuttler et al, 2000; Nussbaum et al, 2001) are sources of proteosome cleavage site predictions and they function using ANN and principles used in SYFPEITHI. URL addresses for all databases used in this study are listed in Appendix 2.

This bio-informatics approach is valuable strategy in rapid discovery of epitopes. However, further characterisation of epitopes is necessary in order to reveal essential aspects of viral – host interactions, since generation of viral escape mutation(s) within HLA*restricted epitopes is one of the crucial steps in this interaction. Viral sequence polymorphisms within the epitope region were firstly observed in chimpanzee studies (Erickson et al, 2001; Grakoui et al, 2003; Weiner et al, 1995) and subsequently in humans (Cox et al, 2005; Tester et al, 2005; Timm et al, 2004). These studies have demonstrated that mutations in HLA*Class I restricted epitopes might be responsible for evasion of antiviral CD8+ T cell responses and establishment of viral persistence. In human studies normally it is hard to find direct evidence for viral escape mutations as sequence of infectious inoculum is rarely known. In this study we were able to make ideal comparison between evolving viral sequences derived from Irish cohort and the original donor sequence (AF 313916 GenBank/EMBL/DDBJ) [Appendix 3] identified as a source of the outbreak.

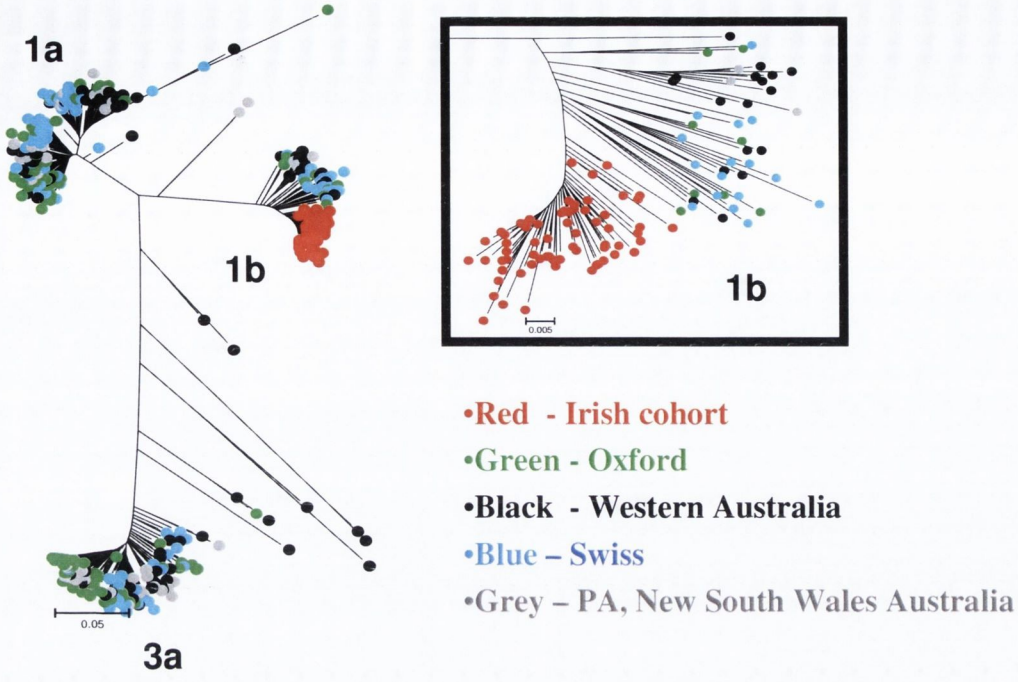



**Figure 5A: Phylogenetic analysis of the HCV NS5b region of viral genomes obtained from different cohorts.** Analysis done on 5 different cohorts reveals heavy clustering of Irish samples (in red) confirming a single source outbreak. Sequences were aligned using ClustalW software. Figure was obtained from collaborator Silvana Gaudieri and incorporated with her permission. (Gaudieri S. et al. 2007. unpublished data; Centre for Clinical Immunology and Biomedical Statistics, Royal Perth Hospital and Murdoch University, Perth, Western Australia)

## *5.2 OBJECTIVES*

The overall aim of this chapter was to identify escape mutations in the Irish cohort which are associated with viral persistence and chronic disease. Previous HLA study has shown that some individuals despite having protective alleles develop chronic HCV infection (McKiernan et al, 2004). We hypothesised that mutation within these HLA restricted epitopes may contribute to viral immune escape and represent a mechanism whereby HCV can establish persistent infection.

The aim of this study was:

- To utilise a novel bio-informatics approach to identify HCV 1b candidate epitopes restricted by protective alleles HLA*A3, HLA*DRB1 0101, HLA*DRB1 0401 and HLA*B7.
- To generate full length sequence of the viral genome in HCV patients and to investigate if there are mutations within predicted epitopes
- To analyse the association of identified epitope mutations with viral persistence in HCV infected individuals

## *5.3 RESULTS*

### 5.3.1 HLA*A3 Restricted epitopes

We constructed a detailed model utilising 7 databases which predict epitopes binding to MHC molecules. Databases Bimas, Jenner (MHCPred), ProPred 1, NetMHC, RankPep, MAPPP, SYFPEITHI were used to identify amino acid motifs in the entire viral polypeptide sequence which could bind to HLA*A3 molecule. The HCV polypeptide sequence employed in all prediction databases was from an Irish donor and has been previously published (AF 313916 GenBank/EMBL/DDBJ) [Appendix 3]. This donor was infected with HCV genotype 1b and was identified as a source of outbreak. Comprehensive searches yielded epitopes common to all or most of the databases. The most prominent 22 epitopes with a length of nine amino acids (nanomers) and with the highest binding affinity from all databases, were selected and outlined in the **Table 5.1**.

Database Jenner (MHCPred) predicted all 22 epitopes, Bimas and RankPep 19, ProPred 1 18, NetMHC 13 and MAPPP and SYFPEITHI 12 of all common epitopes. The five sequences in red **(Table 5.1)** have been shown previously to have immunogenic features. This was assessed when these epitopes were identified among experimentally proven CTL epitopes within HCV 1a genotype (H77 strain) using Los Alamos HCV Immunology database. In addition, the presence of a proteasome cleavage site at the C terminus was determined either through the utilised databases or, if not available in these databases, using NetChop and PaProC. The vast majority of predicted epitopes were from the non-structural region of the viral sequence with most of them derived from NS5b and NS3 regions.

### 5.3.2 Viral RNA extraction

Having identified candidate epitopes using a novel bio-informatics approach the next step was to compare these predicted epitopes with the viral sequence derived from the donor and patient cohort. In order to sequence the entire viral genome, viral RNA was extracted from chronically infected individuals who carry the HLA*A3 allele. RNA extraction was also performed for the control group, which comprised randomly selected HLA*A3 negative chronically infected patients. RT-PCR was used to confirm

| Rank | Sequence | Start position | Viral protein | No of databases | Cleavage site |
|---|---|---|---|---|---|
| 1 | KLTPPHSAK | 2510 | NS5b | 6 | y |
| 2 | QLFTFSPRR | 290 | E1 | 6 | n |
| 3 | LLTLSPHYK | 829 | NS2 | 6 | n |
| 4 | QLAEQFKQK | 1724 | NS4b | 6 | y |
| 5 | SLTITQLLK | 1951 | NS4b | 6 | y |
| 6 | LLAQEQLEK | 2853 | NS5b | 5 | y |
| 7 | HLIFCHSRK / LIFCHSRKK | 1391 / 1392 | NS3 | 5 | y |
| 8 | GMQLAEQFK | 1722 | NS4b | 5 | y |
| 9 | TVYHGAGTK | 1080 | NS3 | 5 | y |
| 10 | RVFTEAMTR | 2757 | NS5b | 4 | y |
| 11 | RLGVRATRR | 43 | Core | 4 | n |
| 12 | ALVVSQLLR | 332 | E1 | 4 | n |
| 13 | KTSERSQPR | 52 | Core | 4 | y |
| 14 | VVFSDMETK | 975 | NS2 | 3 | n |
| 15 | WMNGTGFTK | 555 | E2 | 3 | y |
| 16 | SVPAEILRR | 2270 | NS5a | 3 | y |
| 17 | AVCTRGVAK | 1183 | NS3 | 3 | y |
| 18 | GLNAVAYYR | 1412 | NS3 | 3 | y |
| 19 | EVFCVQPEK | 2562 | NS5b | 3 | y |
| 20 | SLASSSASQ | 2198 | NS5a | 2 | y |
| 21 | NMVYATTSR | 2455 | NS5b | 2 | y |
| 22 | MVLQAGITR | 901 | NS2 | 2 | y |

**Table 5.1 The candidate epitopes restricted by HLA*A3 allele.** Epitopes are ranked according to the number of databases that have predicted them. Most of the candidate epitopes are derived from NS3 and NS5b region and have proteasome cleavage site. Sequences in red have been previously shown to be immunologically important in HCV 1a genotype.

that the isolation was successful. A nested PCR amplified an abundantly expressed viral target in the 5'non-coding region of 251 base pairs **(Figure 5.1b)** (Methods 2.9.3.2). The first round product of 306 base pairs was run out on the gel to show yield, specificity and negative control (Figure 5.1a). In general, there was no need to perform the second round of nested PCR as the quality of first round PCR products was satisfactory and the presence of RNA in all extracts could be confirmed after the first PCR round **(Figure 5.1c/d/e/f/g)**.

Sequencing was carried out by our collaborators in Centre for Clinical Immunology and Biomedical Statistics, Royal Perth Hospital and Murdoch University, Perth, Australia. Extraction of viral RNA samples was optimised in our facilities, but as RNA is very labile, our colleagues requested plasma samples to be sent instead. Therefore, plasma samples were obtained from our cohort (68 in total) and sent to the collaborators who isolated RNA according to optimised protocol and performed sequencing.

### 5.3.3 Functional analysis of fresh and cryopreserved PBMCs

In the first experiments we carried out some functional validation of PBMCs. To assess the functionality of cryopreserved cells, which were to be used in further experiments, stimulation with phytohemagglutinin (PHA) and purified protein derivative (PPD) was applied in parallel for fresh and frozen cells and IFN-γ levels were determined by ELISA. Cell viability of between 88-94% was achieved following the optimisation of the cryopreservation procedure (as described in 2.3.5). The most consistent results were obtained after 72 hours of stimulation **(Figure 5.2 B)**. IFN-γ secreted from both fresh and frozen PBMCs, were at the similar levels following the PHA stimulation (IFN-γ levels in pg/ml were: fresh cells 1380.04 ± 10.2 vs frozen cells 1336.22 ± 0.4) as well as PPD stimulation (IFN-γ levels in pg/ml were: fresh cells 1365.53 ± 3.6 vs frozen cells 1294.31 ± 0.3). Similar responses between the cell groups were noticed even after 24 hours of treatment with PHA **(Figure 5.2 A)**, (IFN-γ levels in pg/ml were: fresh cells 1202.19 ± 3.3 vs frozen cells 1161.35 ± 0.5). However, although frozen cells strongly responded to PPD stimulation, they could not reach the levels of IFN-γ secreted by fresh cells at 24 hours (IFN-γ levels in pg/ml were: fresh cells 1184.88 ± 0.9 vs frozen cells 429.78 ± 113.9).

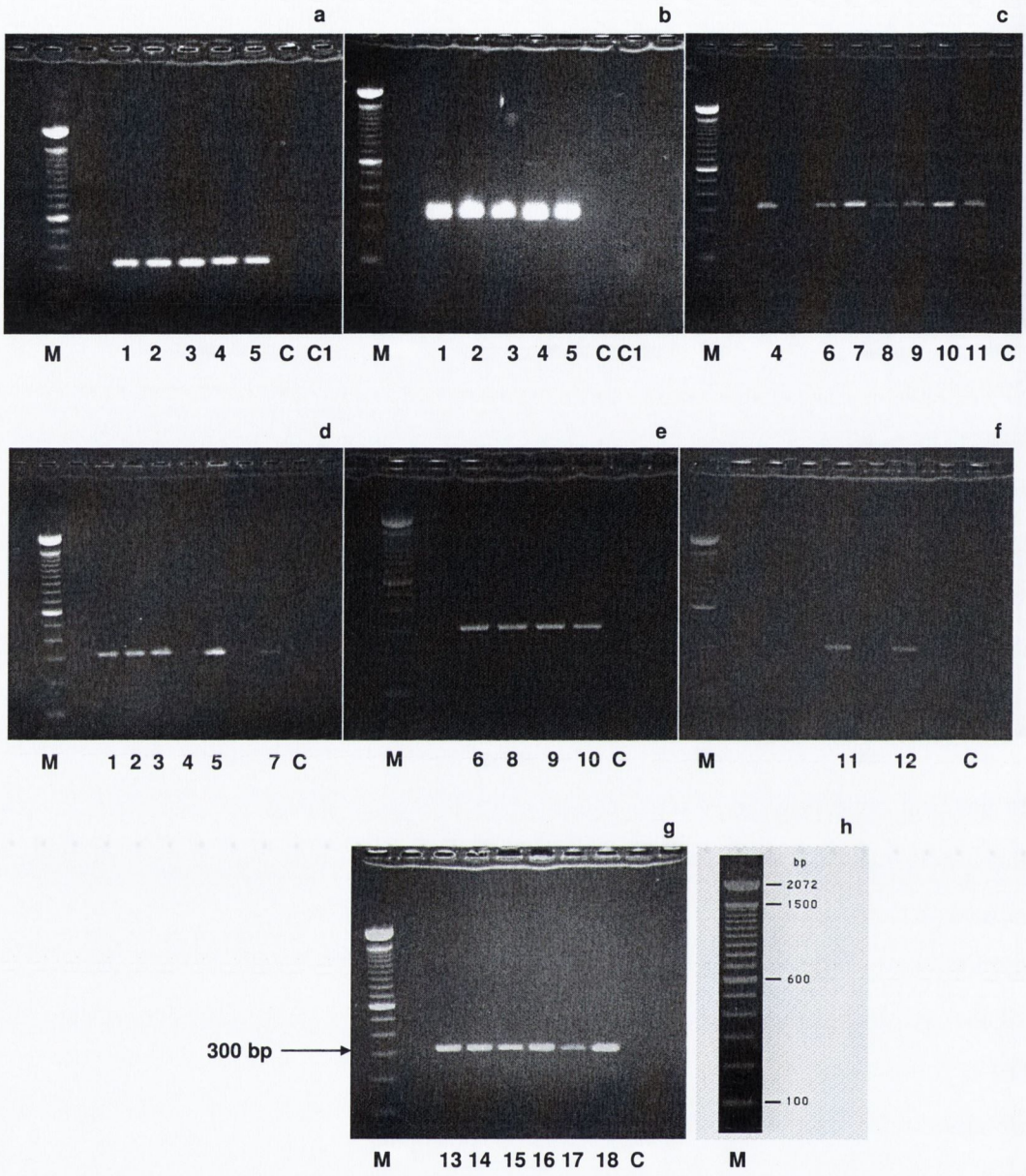



**Figure 5.1 Visualisation of RT-PCR product(s) derived from extracted HCV viral RNA on agarose gel.** Panels **a**, **b** and **c** show amplified part of a 5'non-coding region of viral RNA extracted from control samples (1-11). On **a** and **c** panels samples are run after 1st round of PCR (306 bp), while on **b** panel after nested PCR (251 bp). Panels **d**, **e**, **f** and **g** show amplified part of a 5'non-coding region of viral RNA extracted from HLA*A3 non-resolvers, run on a gel after 1st round of PCR. C-negative control ($H_2O$); C1-negative control (RNA from human cell line); M-molecular weight marker (100 base pairs)(**h**).

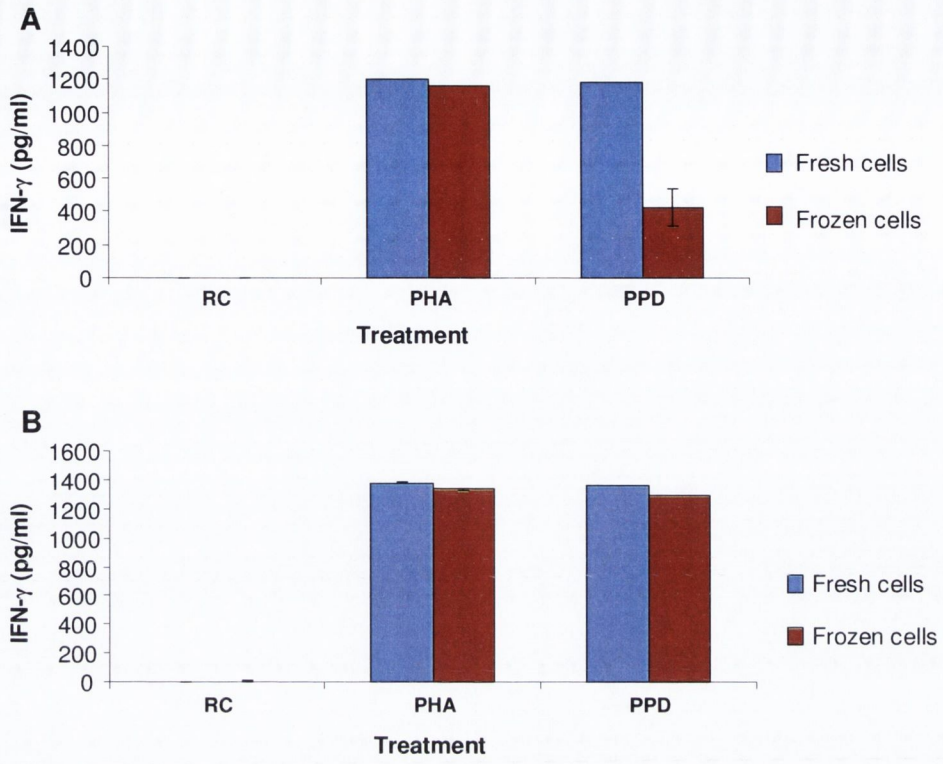



**Figure 5.2 Comparison in the ability of fresh and cryopreserved PBMCs to secrete IFN-γ.** Approximately $5x10^5$ cells per ml were stimulated with PHA at a final concentration of 1, 2, 5 and 10 μg/ml and PPD at a final concentration of 1, 3, 5 and 10 μg/ml in 96 well plates (round bottom, Nunc A/S, Denmark). Prior to stimulation and following the thawing process, frozen cells had a resting period, while they were incubated at 37°C for 24 hours. Following a 24, 48, and 72 hour stimulation period, the supernatants were collected and analysed for IFN-γ production. ELISA technique was used to compare the functional responses of fresh and frozen PBMCs. **A)** Shorter treatment, where cells were stimulated with PHA and PPD for 24 hours, brought some variations between fresh and frozen cells in IFN-γ production, although t-test did not show significance (p=0.096). **B)** The most consistent response was observed after 72 hour long PHA (10μg/ml) and PPD (10μg/ml) stimulation. Data is presented as mean ± SEM of one representative experiment from five independent experiments with similar trend.

Interestingly, while the intensity of PHA responses were very uniform across PBMC samples obtained from 7 blood donors, PPD induced variable responses, possibly depending on the level of previous antigen exposure of each individual.

### 5.3.4 Functional validation of predicted epitopes using ELISpot assay

The preliminary plan in this project was to test the PBMCs (obtained from the patients with protective alleles) in ELISpot assay, using approximately 300 peptides; 20 amino acid in length, overlapping by 10, spanning the entire HCV polyprotein. Following that, peptides giving a positive response in initial screening assay would be truncated and used in further assays to optimise the peptide length, in order to determine viral epitopes. However, instead of performing matrix ELISpot assay and optimising the length of a couple of peptides that induce the best response, it was decided to apply databases predictions based on previously defined binding motifs and set of predefined algorithms. Once the candidate epitopes were obtained they were used further.

Thus, to identify the immunodominant CD8+ T cell epitopes from the panel of HLA*A3 restricted HCV epitopes (Table 5.1), all candidate epitopes were tested using an the IFN-γ ELISpot assay. PBMCs were extracted from six HLA*A3 positive individuals who spontaneously resolved infection and tested using the candidate epitopes predicted by databases. All of tested patients exhibited no detectable CD8+ T cell responses either on the HCV peptides or EBV and CMV control peptides. The PHA and anti-CD3 controls showed that the cells were capable of responding to a non-specific stimulus (**Figure 5.3** and **Figure 5.4**). Stimulation with PHA or anti-CD3 antibody generated a significant number of cells producing IFN-γ (**Figure 5.3a/b**) while stimulation with control EBV, CMV peptides (**Figure 5.3d/e**) and HCV peptides (**Figure 5.3f/g/h**) displayed no measurable T cell specific responses. Furthermore, responses were not observed following bulk stimulation of PBMCs with the same peptides while performing cultured Elispot (Methods 2.14.2). In vitro culture was carried out in order to activate and expand antigen specific populations, but these sets of experiment suffered from problematic background staining and for continuation another approach would have to be adopted.

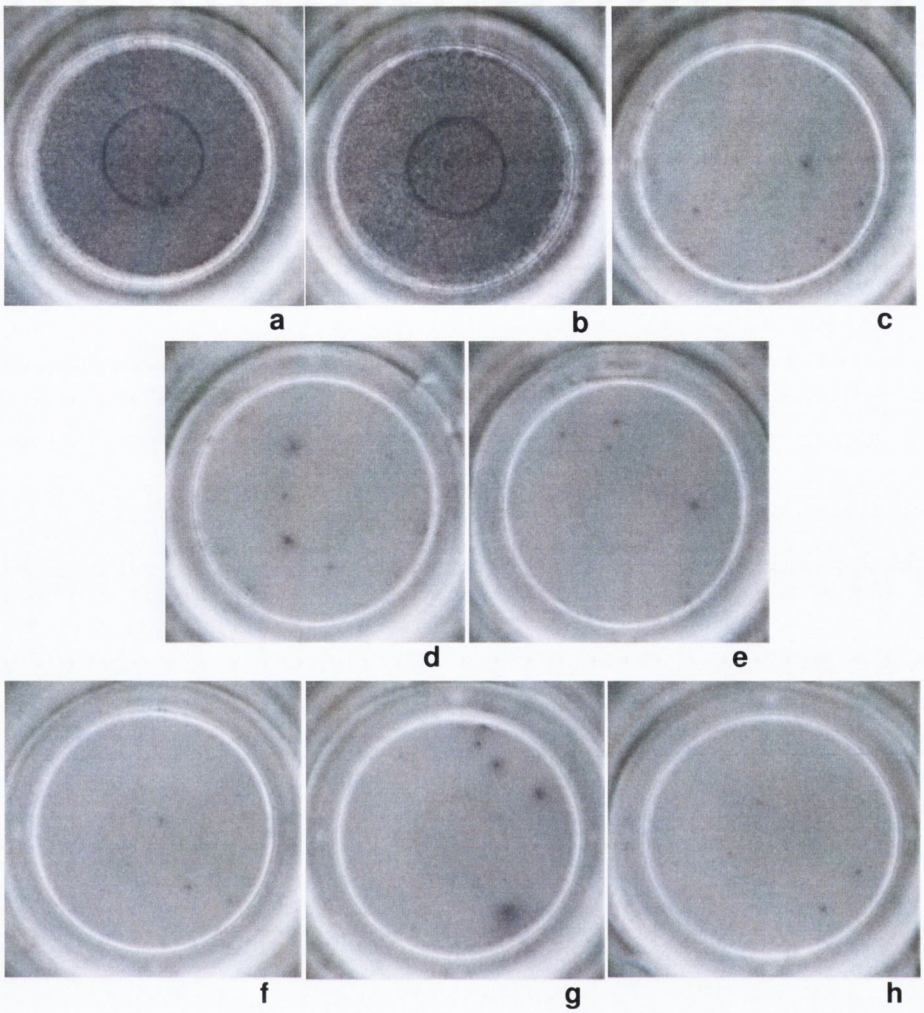



**Figure 5.3 CD8+ T cell-mediated IFN-γ responses in *ex-vivo* ELISpot assay.** The upper three panels show positive and negative controls **a)** IFN-γ responses against PHA, **b)** responses against anti-CD3, **c)** resting cells in RPMI. The two middle panels display wells stimulated with control peptides **d)** EBV (RVRAYTYSK), **e)** CMV (TVRSHCVSK). The lower three panels show IFN-γ responses against HCV peptides **f)** KTSERSQPR, **g)** WMNGTGFTK, **h)** LLAQEQLEK.

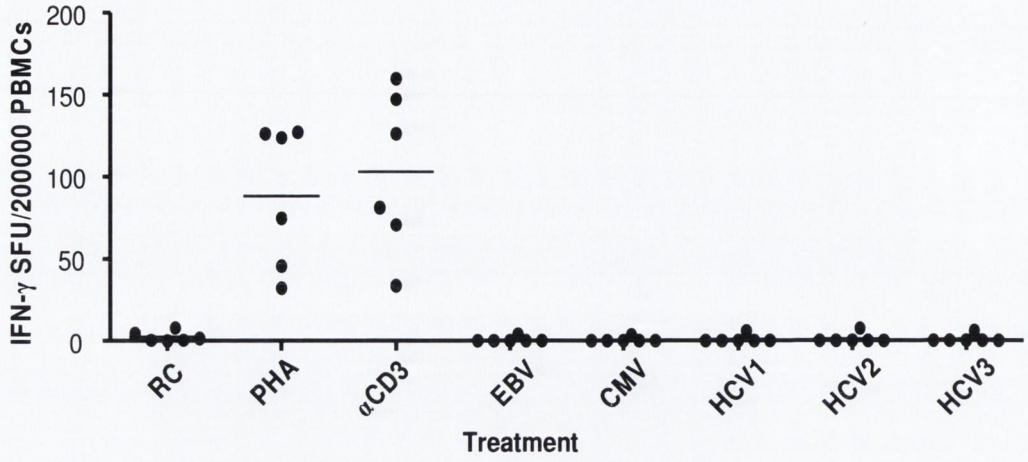



**Figure 5.4 Magnitude of CD8+ T cell responses as determined by IFN-γ ELISpot.** All individuals displayed T cell responses (IFN-γ producing cells) after PHA and anti-CD3 stimulation. However, antigen specific responses stimulated via control peptides (EBV (BRLF1) and CMV (Pp50)) and HCV peptides (HCV1-core region, HCV2-E2 region, HCV3-NS5b region) could not be observed. Responses are presented as mean of duplicate wells after subtraction of a background. Each dot represents one HLA*A3 positive individual who cleared the virus (PCR-). Summation of spot-forming units (SFU) is expressed per $2x10^5$ PBMCs.

### 5.3.5 HLA*A3 restricted epitopes with significant mutations

Since ELISpot assay could not be utilised to identify immunodominant epitopes, we adopted different approach. Plasma samples (68) containing viral RNA were sent to our collaborators for sequencing. 25 samples were 80-100% sequenced, 15 60-80%, 10 samples 40-60% and approximately 33% was sequenced in 12 samples. Sequencing data for the NS5b region was fully available in all samples. *In silico* protein translation was performed using CLC Bio (Free Workbench) software, when viral sequences, in cDNA fasta format, were translated into protein sequences. This programme had an option which allowed the use of multiple reading frames as a starting point; therefore the correct sequence could be deduced from multiple translations made at one time. An appropriate sequence was subsequently aligned with the donor sequence, with the patient group sequences (carriers of protective allele who develop chronic infection) and with control group sequences (individuals with chronic infection who do not have protective allele) using ClustalW multiple sequence alignment in BioEdit software package (Methods 2.13.2).

All candidate epitopes were aligned with sequences obtained from 15 HLA*A3 non-resolvers and 17 non-HLA*A3 non-resolvers used as controls. Amino acid substitutions were observed in three epitopes **(Table 5.2)**. These HLA*driven polymorphisms within epitope region called "footprints" suggest that these regions of viral polypeptide might be important in development of an appropriate immune response. The rest of predicted epitopes were either conserved in both groups or located in the parts of viral genome that has yet not been sequenced or mutated towards consensus HCV 1b viral sequence (AF054247 GenBank/EMBL/DDBJ) [Appendix 4].
It was also noticed that there were six patients out of nine that expressed the protective HLA*A3 allele who did not clear the virus (sequenced for all three epitopes) had mutations in two different epitopes. This may reflect viral adaptation to HLA restricted immune responses in individuals with chronic disease.

| | AF313916 | KLTPPHSAK | TVYHGAGTK | EVFCVQPEK |
|---|---|---|---|---|
| HLA*A3 | P1 | . . . . . R | . . . . . AR | . . . . . |
| | P2 | . . . . . R | XXXXXXXXX | . . . . I . . . . |
| | P3 | M . . . . . | . . . . . AR | . . . . . |
| | P4 | . . . . . | XXXXXXXXX | . . . . . |
| | P5 | M . . . . . R | XXXXXXXXX | . . . . . |
| | P6 | X . . . . . | . . . . . AR | . . . . I . . . . |
| | P7 | M . . . . . | . . . . . AR | . . . . . |
| | P8 | . . . . . R | XXXXXXXXX | . . . . I . . . . |
| | P9 | . . . . . R | . . . . . AR | . . . . . |
| | P10 | M . . . . . R | . . . . . XR | . . . . . |
| | P11 | M . . . . . | . . . . . | . . . . . |
| | P12 | . . . . . R | XXXXXXXXX | . . . . . |
| | P13 | M . . . . . | XX . . . . . R | . . . . . |
| | P14 | R . . . . . | XXXXXXXXX | . . . . . |
| | P15 | R . . . . . R | XXXX . . . . . | . . . . . |
| Non-HLA*A3 | C1 | M . . . . . | . . . . . | . . . . . |
| | C2 | M . . . . . | XX . . . . . | . . . . . |
| | C3 | M . . . . . | XXXXXXXXX | . . . . . |
| | C4 | . . . . . | XX . . . . . A . | . . . . . |
| | C5 | M . . . . . | . A . . . . . | . . . . . |
| | C6 | . . . . . | . . . . . | . . . . . |
| | C7 | M . . . . . | . . . . . | . . . . . |
| | C8 | M . . . . . | . . . . . | . . . . . |
| | C9 | . . . . . R | XXXXXXXXX | . . . . . |
| | C10 | M . . . . . | . . . . . | . . . . . |
| | C11 | M . . . . . | . . . . . | . . . . . . |
| | C12 | . . . . . | . . . . . | . . . . . |
| | C13 | M . . . . . | XXXXXXXXX | . . . . . |
| | C14 | . . . . . | . . . . . | . . . . . |
| | C15 | M . . . . . | XXXX . . . X . | . . . . . |
| | C16 | . . . . . | . . . . . | . . . . . |
| | C17 | . . . . . | . . . . . | . . . . . |

**Table 5.2 Sequence variations corresponding to epitopes derived from donor sequence AF313916 in HLA*A3 positive (P1-P15) and negative patients (C1-C17).** X – sequence not available, dots – consensus with donor sequence, capital letters highlighted – amino acid substitutions. K-lysine, R-arginine, M-methionine, T-threonine, A-alanine, V-valine, I-isoleucine.

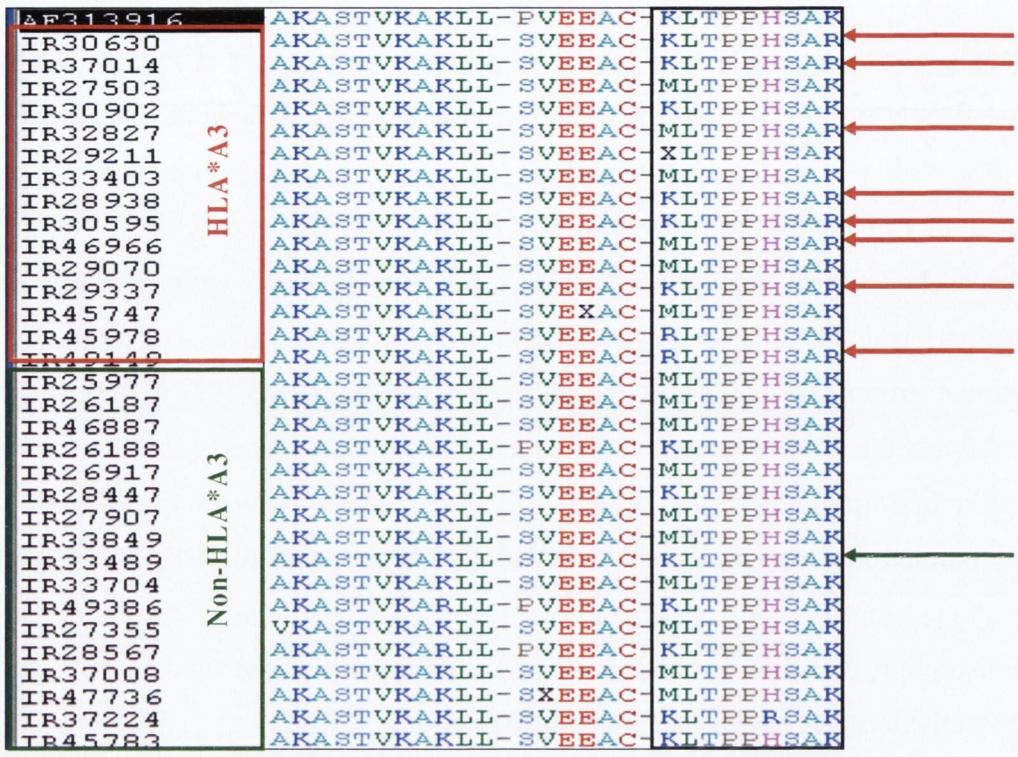
#### 5.3.5.1 KLTPPHSAK epitope

This epitope was predicted to be HLA*A3 restricted by 6 out of 7 tested databases and in all of them was among the epitopes with the highest ranking. It is located in the NS5b region of the viral polypeptide. In the HLA*A3 positive group of non-resolvers, 8 out of 15 patients had a substitution of lysine (K) to arginine (R) (K2518R) situated in the anchor site of the epitope, while in the non-HLA*A3 group just one out of 17 patients had the same mutation **(Figure 5.5 A)**. Thus, 53.33% of individuals in the patient group had a K2518R substitution, whereas only 5.88% in the control group exhibited the same substitution. When calculated using Fisher's exact test a significant difference (p=0.004) was observed between these two groups **(Figure 5.5 B)**. Mutations observed within the KLTPPHSAK epitope in the HLA*A3 patient group may contribute to the viral immune escape and establishment of chronic infection. The substitution of lysine to methionine (M) was equally distributed in both groups and possibly does not have a role in immune evasion.

#### 5.3.5.2 TVYHGAGTK epitope

The TVYHGAGTK epitope was predicted by 5 out of 7 tested databases to be HLA*A3 restricted and in three had the highest ranking. It is situated in the NS3 region of the viral polypeptide. In the TVYHGAGTK epitope, substitution of K with R (K1088R) was present in 7 out of 9 in HLA*A3 patient group while it was not observed among controls. This mutation occurred singly or in a combination with the substitution of threonine (T) to alanine (A) (T1087A). Again, this double mutation was not observed in the control non-HLA*A3 group. Substitution of threonine to alanine was present in 5 HLA*A3 positive patients out of 8 available sequences and in just one of 13 available sequences in the control group **(Figure 5.6 A)**.When these values were compared the K to R substitution, again in the anchor site of epitope, was present in 77.77% of HLA*A3 positive patients and in none of non-HLA*A3 group (p=0.0001). Replacement of T by A was observed in 62.5% of the HLA*A3 patients group and in 7.69% of controls (p=0.014). When these two substitutions were analysed as a double substitution a significant difference between two tested groups was determined with p=0.002 (62.5% with double substitution in HLA*A3 positive patients and none in non-HLA*A3 group of individuals) **(Figure 5.6 B)**.

A




B

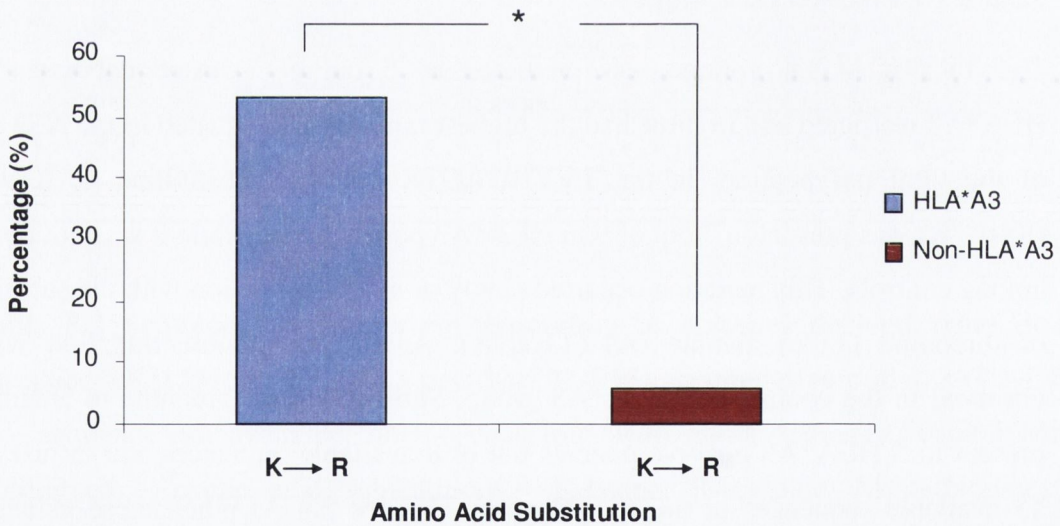



**Figure 5.5 A) Substitutions of lysine (K) to arginine (R) within the KLTPPHSAK epitope in aligned sequences.** In HLA*A3 group of non-resolvers, 8 out of 15 had a K to R substitution, whilst one out of 17 in non-HLA*A3 group exhibited the same substitution (p=0.004). **B) Expression of lysine (K) to arginine (R) substitution within the KLTPPHSAK epitope in patient and control groups.** In the HLA*A3 group of non-resolvers 53.33% had a K to R substitution, while in the non-HLA*A3 group 5.88% expressed the same change (p= 0.004).

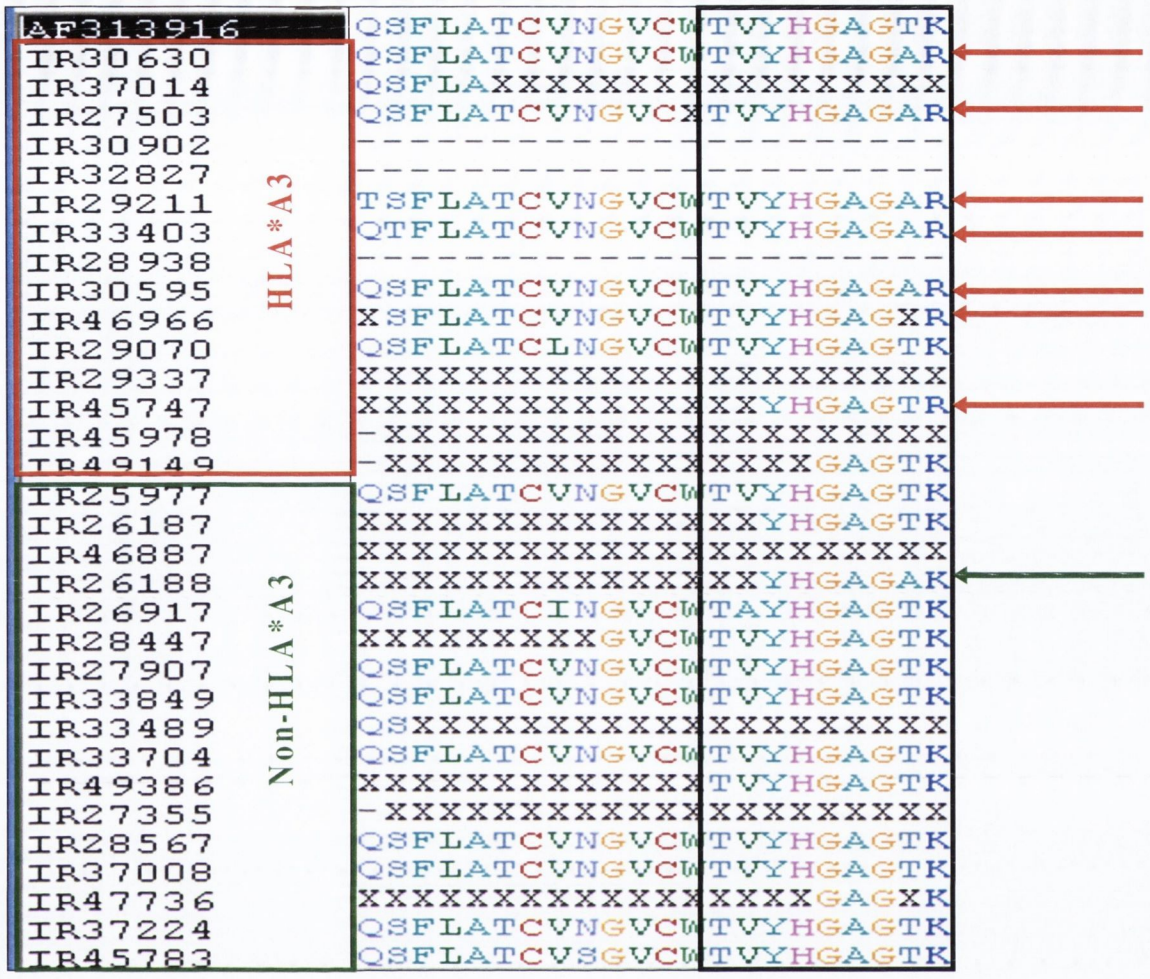



**Figure 5.6 A Substitutions of lysine (K) to arginine (R) and threonine (T) to alanine (A) within the TVYHGAGTK epitope.** Replacement of K with R was present in 7 out of 9 in the HLA*A3 group while was not observed non-HLA*A3 group (P=0.0001). Substitution of T to A was present in 5 individuals in patient group out of 8 available sequences and in just one of 13 available sequences in the control group (p=0.014). In a combination these two substitutions create a double mutation, which was present in 5 HLA*A3 individuals and in none of non-HLA*A3 (p=0.002). Display of this sequences alignment is taken from BioEdit software package (donor sequence is on the top – AF313916; patients samples are in red box; control samples are in green box).

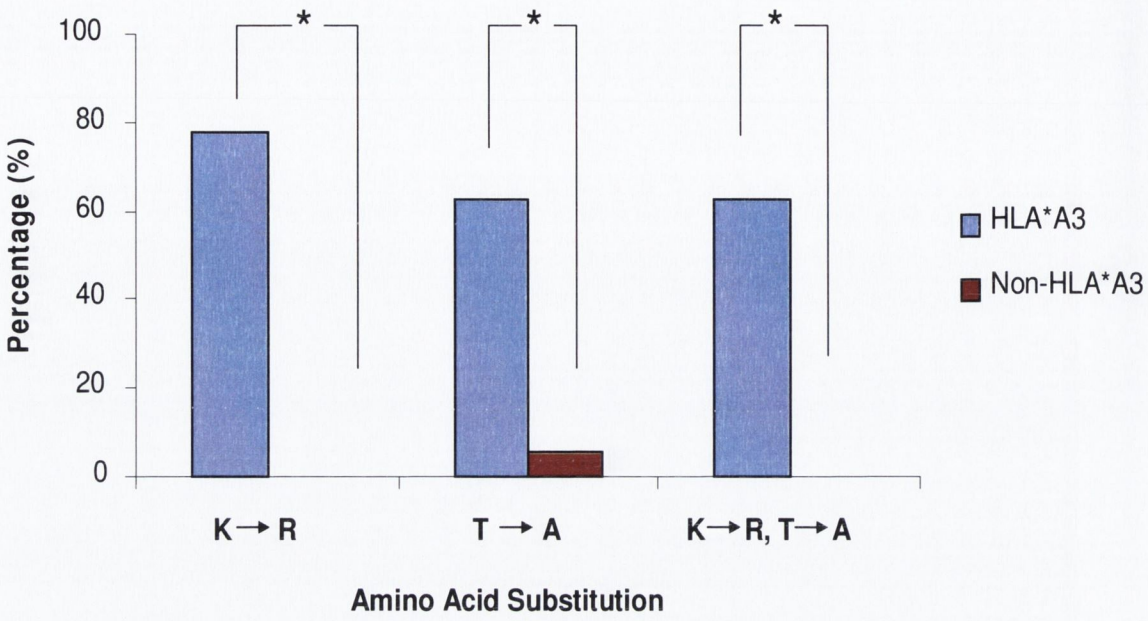



**Figure 5.6 B Polymorphisms observed within the TVYHGAGTK epitope in patient and control group.** 77.77% of patients in the HLA*A3 group had a lysine (K) to arginine (R) substitution, which was not present in the non-HLA*A3 group (p=0.0001). This mutation occurred singly or in combination with substitution of threonine (T) to alanine (A) (62.5%). Again, this double mutation was not observed in the control group (p=0.002). Substitution of T to A was present in 5 HLA*A3 positive patients out of 8 available sequences (62.5%) and in just one of 13 available sequences in the control group (7.69%) (p=0.014)

#### 5.3.5.3 EVFCVQPEK epitope

A valine (V) to isoleucine (I) substitution was observed in the EVFCVQPEK epitope in 3 out of 15 of the HLA*A3 group (20%). None of the non-HLA*A3 individuals have this change **(Figure 5.7 A/B)**. However, when these two groups were compared a significant difference was not observed (p=0.09). To be able to make a final conclusion on this epitope we will need to increase the number in our existing cohort of patients and have them sequenced.

### 5.3.6 HLA*B7

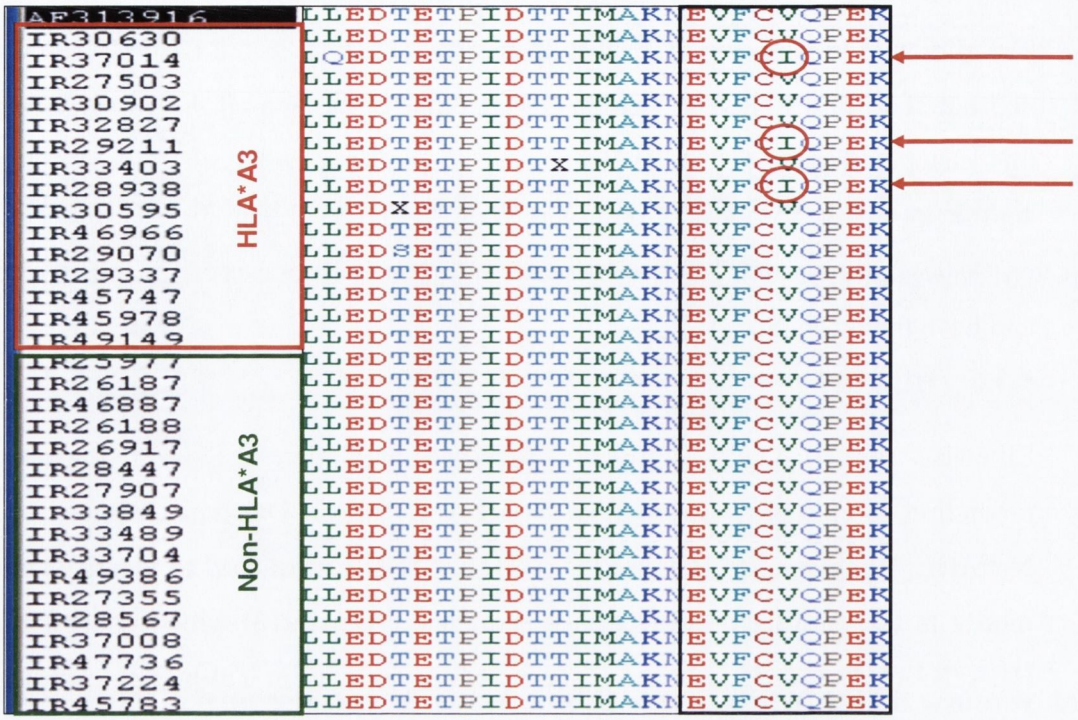
Likewise for HLA*A3 epitopes, 7 epitope prediction databases were used for prediction of epitopes restricted by the HLA*B7 allele. Databases Bimas, ProPred 1, NetMHC, RankPep, MAPPP, SYFPEITHI and IEDB were used to identify amino acid motifs in the entire viral polypeptide sequence (AF313916) which could bind to the HLA*B7 molecule. 25 epitopes (nanomers) are outlined in **Table 5.3** according to the number of databases that have predicted them.

Database SYFPEITHI predicted 24 epitopes, NetMHC and IEDB 22, Bimas and MAPPP 21, RankPep 19 and ProPred 1 12 of all common epitopes. Searching for HLA*B7 restricted CTL epitopes in HCV 1a genotype (H77 strain), using Los Alamos HCV Immunology database, two epitopes were identified to be common for both viral genotypes. Hence, epitopes in red, located in the core region, have been shown previously to have immunogenic features. Additionally, a proteasome cleavage site, if not available in epitope prediction databases, was determined using proteasome cleavage site databases NetChop and PaProC.

Of predicted epitopes, 80% were from the non-structural region of the viral sequence with most of them derived from the NS5b region.

Similar to the HLA*A3 study, i*n silico* protein translation was performed using CLC Bio (Free Workbench) software, when viral RNA(in cDNA fasta format) was translated into a protein sequence, again choosing an option of all six reading frames as a starting point, which results in multiple translations. The resulting appropriate sequence was subsequently aligned with the donor sequence, with the patient group sequences (carriers of protective allele who develop chronic infection) and with control group sequences

A




B

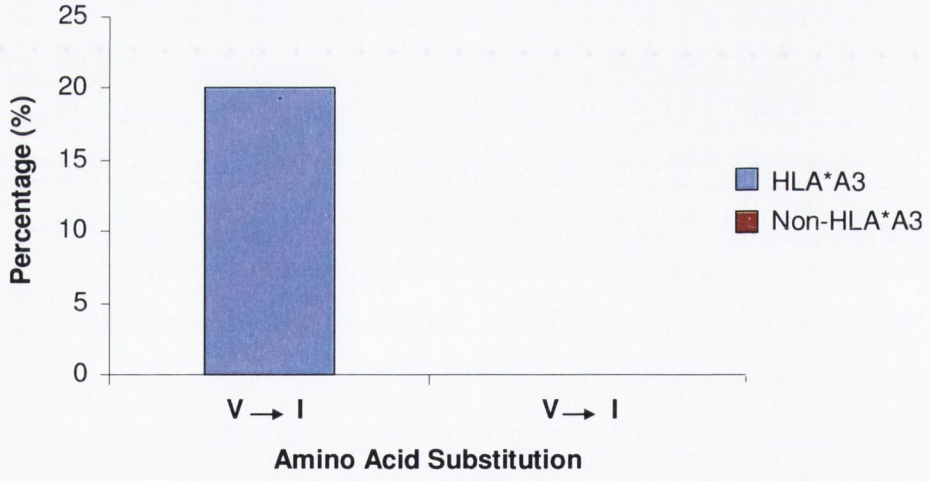



**Figure 5.7 A) Substitution of valine (V) to isoleucine (I) within the EVFCVQPEK epitope in aligned sequences.** Substitution was only observed in patient group (HLA*A3) in 3 sequences out of 15. **B) Expression of valine (V) to isoleucine (I) substitution in the EVFCVQPEK epitope.** In the HLA*A3 group 20% expressed this substitution, whilst none of the non-HLA*A3 controls had this change. However, using Fisher's exact test significant difference between these two groups was not be observed (p=0.09).

| Rank | Peptide Sequence | Start position | Viral protein | No of databses | Cleavage site |
|---|---|---|---|---|---|
| 1 | **RPRWFMLCL** | 2987 | NS5b | 6 | y |
| 2 | **QPRGRRQPI** | 57 | Core | 6 | y |
| 3 | **GPIAYAKPL** | 470 | E2 | 6 | y |
| 4 | **APTLWARMI** | 2835 | NS5b | 6 | y |
| 5 | **TPIPAASQL** | 2956 | NS5b | 6 | y |
| 6 | **IPAASQLDL** | 2959 | NS5b | 6 | y |
| 7 | **GPRLGVRAT** | 40 | Core | 5 | y |
| 8 | **DPRRRSRNL** | 111 | Core | 5 | y |
| 9 | **HPNIEEVAL** | 1359 | NS3 | 5 | n |
| 10 | **TPAETSVRL** | 1531 | NS3 | 5 | y |
| 11 | **SPGQRVEFL** | 2615 | NS5b | 5 | y |
| 12 | **GPWLTPRCM** | 600 | E2 | 5 | y |
| 13 | **TPGERPSGM** | 1503 | NS3 | 4 | n |
| 14 | **SPLTTQHTL** | 1799 | NS4b | 4 | y |
| 15 | **APPSAASAF** | 1821 | NS4b | 4 | y |
| 16 | **LPRLPGVPF** | 2000 | NS5a | 4 | y |
| 17 | **LARGSPPSL** | 2190 | NS5a | 4 | y |
| 18 | **PPRRKRTVV** | 2326 | NS5a | 4 | y |
| 19 | **LPVEEACKL** | 2502 | NS5b | 4 | y |
| 20 | **EARQAIRSL** | 2667 | NS5b | 4 | y |
| 21 | **RARSVRAKL** | 2922 | NS5b | 4 | y |
| 22 | **RARPRWFML** | 2985 | NS5b | 4 | n |
| 23 | **DPPKPEYDL** | 2772 | NS5b | 4 | n |
| 24 | **EPEPDVAVL** | 2164 | NS5a | 4 | y |
| 25 | **SARRGREIL** | 1003 | NS2 | 4 | y |

**Table 5.3 The candidate epitopes restricted by the HLA*B7 allele.** Epitopes are listed according to the number of databases that have predicted them. Most of the candidate epitopes are derived from the NS5b region and have a proteasome cleavage site. Sequences in red have been previously shown to be immunologically important in HCV 1a genotype.

(individuals with chronic infection who do not have the protective allele) using ClustalW multiple sequence alignment in BioEdit software package. All candidate epitopes were aligned with sequences obtained from 18 HLA*B7 non-resolvers and 18 non-HLA*B7 non-resolvers used as controls. Amino acid substitutions were observed in two epitopes **(Table 5.4)**. The rest of the predicted epitopes were either conserved in both groups or displayed substitutions which aligned with consensus viral sequence (AF054247) [Appendix 4] or could not be analysed because of lack of sequencing data for certain parts of viral genome [Appendix 5].

#### 5.3.6.1 LPRLPGVPF epitope

This epitope, located in the NS5a region of the viral polypeptide, was predicted by 4 out of 7 tested databases. In the LPRLPGVPF, epitope substitution of arginine with glutamine (Q) was present in 4 out of 11 available sequences in the HLA*B7 patient group while it was not observed among controls. An additional polymorphism present within this epitope was the substitution of valine to isoleucine or leucine (L) (V2006I/L), occurring on its own (one case) or in a combination with substitution R to Q (two patients). Again, none of these substitutions were observed in the 10 available sequences in the control group **(Figure 5.8 A)**. When tested on its own the R to Q (R2002Q) substitution, present in 36.36% of HLA*B7 positive patients, did not exhibit a significant difference when compared to the non-HLA*B7 group (p=0.09), which is likely to be due to the lack of available sequences in the control group. Interestingly, when all substitutions within an epitope were taken together and analysed a significant difference between two tested groups was determined with p=0.03, where 45.45% among HLA*B7 positive patients had some of the substitutions (or both of them) and 0% in non-HLA*B7 group of individuals. **(Figure 5.8 B)**.

This epitope exhibited a few "footprints" which, when analysed all together, might lead to a conclusion that this part of a viral sequence was under T cell mediated selection pressure and therefore might be an immunodominant epitope.

| | AF313916 | LPRLPGVPF | SARRGREIL |
|---|---|---|---|
| HLA*B7 | P1 | . . . . . L . . | XXXXXXXXX |
| | P2 | . . Q . . . I . . | . . . . . F |
| | P3 | XXXXXXXXX | XXXXXXXXX |
| | P4 | XXXXXXXXX | XXXXXXXXX |
| | P5 | . . . . . | XXXXXXXXX |
| | P6 | XXXXXXXXX | . . . . . |
| | P7 | XXXXXXXXX | . . . . . |
| | P8 | . . Q . . . L . . | . . . . . |
| | P9 | . . . . . | . . . . . F |
| | P10 | . . . . . | . . . . . F |
| | P11 | XXXXXXXXX | . . . . . |
| | P12 | XXXXXXXXX | XXXXXXXXX |
| | P13 | . . . . . | . . . . . F |
| | P14 | . . Q . . . . . | . . . . . F |
| | P15 | XXXXXXXXX | XXXXXXXXX |
| | P16 | . . . . . | . . . . . F |
| | P17 | . . Q . . . . . | . . . . . F |
| | P18 | . . . . . | XXXXXXXXX |
| Non-HLA*B7 | C1 | . . . . . | . . . . . |
| | C2 | . . . . . | . . . . . |
| | C3 | XXXXXXXXX | . . . . . |
| | C4 | . . . . . | . . . . . |
| | C5 | . . . . . | . . . . . |
| | C6 | . . . . . | XXXXXXXXX |
| | C7 | . . . . . | . . . . . F |
| | C8 | . . . . . | . . . . . |
| | C9 | . . . . . | . . . . . |
| | C10 | . . . . . | . . . . . F |
| | C11 | XXXXXXXXX | . . . . . |
| | C12 | . . . . . | . . . . . |
| | C13 | XXXXXXXXX | XXXXXXXXX |
| | C14 | XXXXXXXXX | . . . . . |
| | C15 | XXXXXXXXX | . . . . . |
| | C16 | XXXXXXXXX | XXXXXXXXX |
| | C17 | XXXXXXXXX | . . . . . F |
| | C18 | XXXXXXXXX | . . . . . |

**Table 5.4 Sequence variations corresponding to epitopes derived from donor sequence AF313916 in HLA*B7 positive (P1-P18) and negative (C1-C18) patients.** X – sequence not available, dots – consensus with donor sequence, capital letters highlighted – amino acid substitutions. R-arginine, Q-glutamine, V-valine, I-isoleucine, L-leucine, F-phenylalanine.

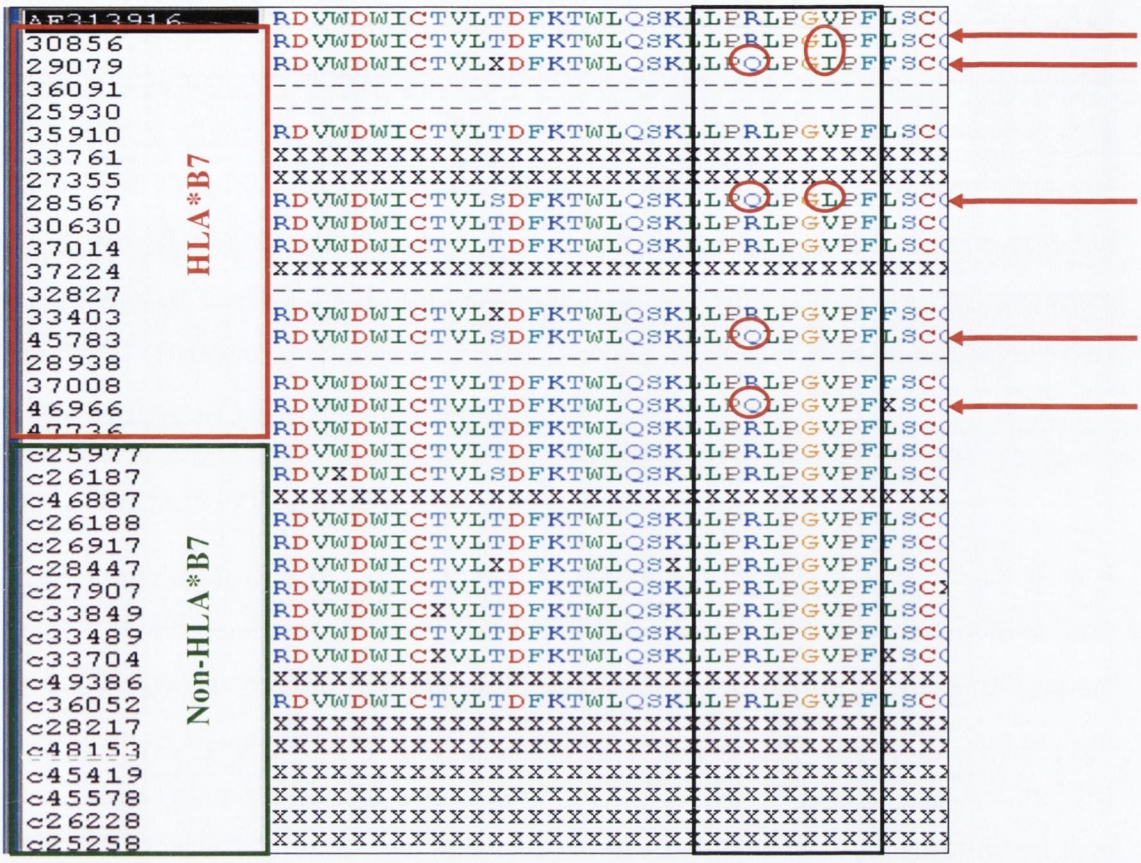



**Figure 5.8 A Substitutions of arginine (R) to glutamine (Q) and valine (V) to leucine (L)/isoleucine (I) within the LPRLPGVPF epitope.** Polymorphisms were observed in 5 sequences out of 11 available in patient group (HLA*B7) and in none out of 10 among controls (non-HLA*B7) (p=0.03). Display of this sequences alignment is taken from BioEdit (donor sequence is on the top – AF313916; patients samples are in red box; control samples are in green box).

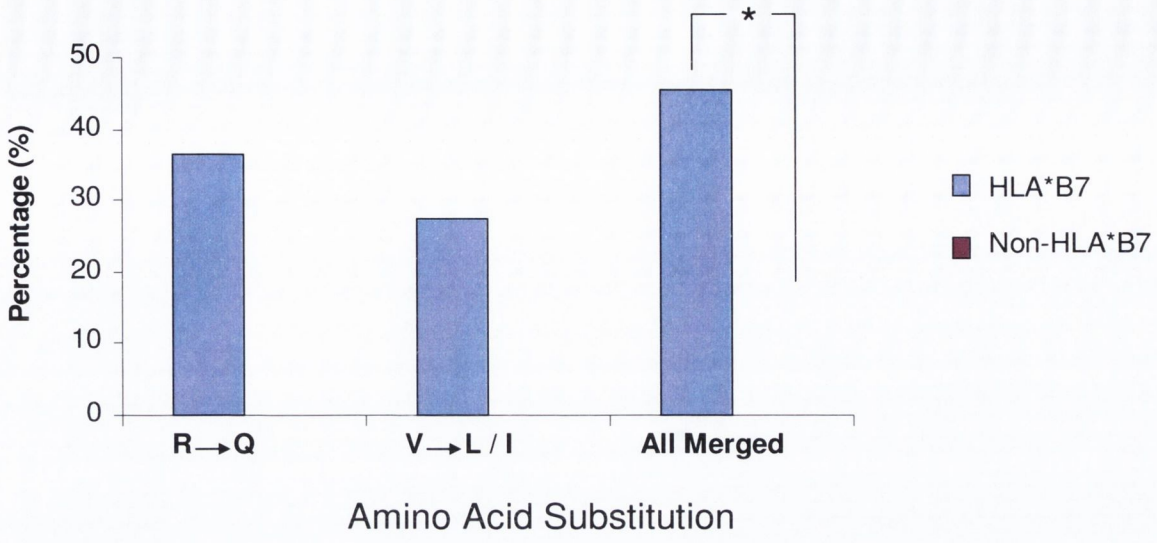



**Figure 5.8 B Expression of different polymorphisms in the LPRLPGVPF epitope in HLA*B7 patient and control non-HLA*B7 groups.** In the patient group (HLA*B7) 36.36% expressed arginine (R) to glutamine (Q) substitution, 27.27% valine (V) to leucine (L)/isoleucine (I) substitution and although none of these were observed in the control group (non-HLA*B7), a significant difference between these two groups could not be observed. However, when all substitutions were analysed jointly 45.45% in HLA*B7 had some of the mutations whereas none of the controls displayed any amino acid changes (p=0.03).

### 5.3.6.2 SARRGREIL epitope

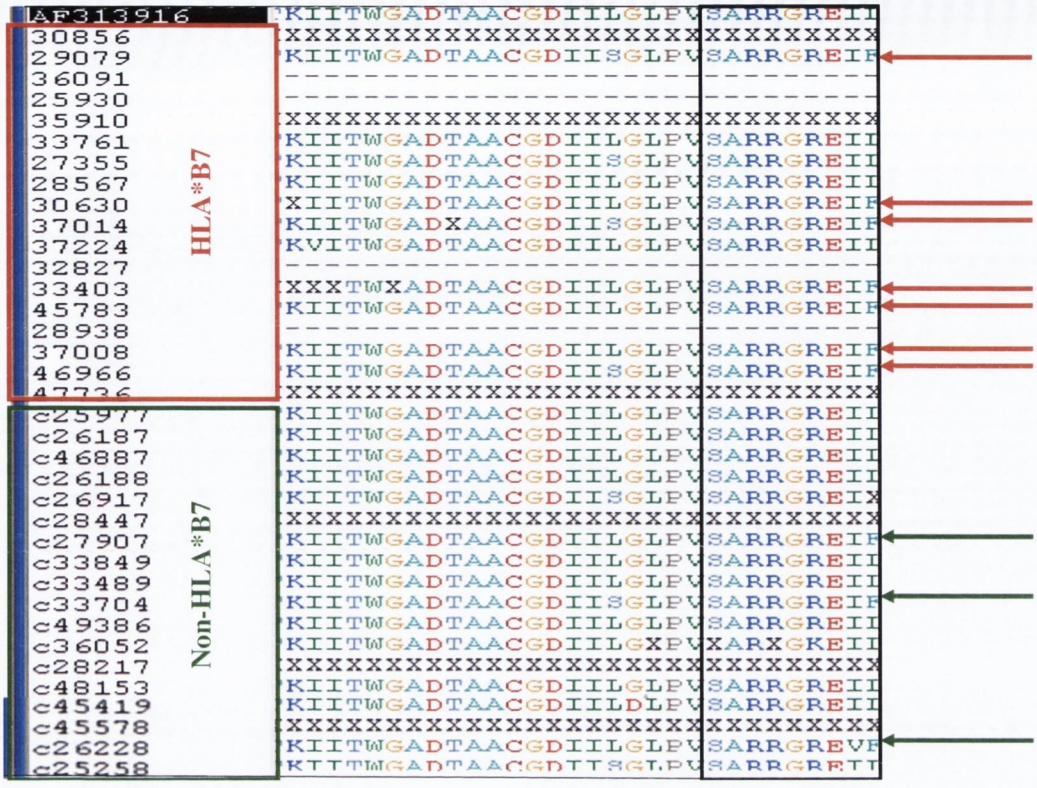
This epitope was predicted by 4 out of 7 tested databases to be HLA*B7 restricted and it is positioned in the NS2 region of the viral polypeptide. In the HLA*B7 group of non-resolvers, 7 out of 11 available sequences among patients had a substitution of leucine to phenylalanine (F) while in the non-HLA*B7 group 3 out of 14 individuals had the same substitution **(Figure 5.9 A)**. In the patient group 63.63% of individuals had an L to F substitution, whereas 21.4% in the control group exhibited the same polymorphism. A marginally significant difference (p= 0.048) was observed between these two groups when calculated using Fisher's exact test **(Figure 5.9 B)**. However, this substitution is consistent with the consensus sequence and, more likely, represents reversion towards the best viral adaptation (Gaudieri et al, 2006).

### 5.3.7 HLA*DRB1 0401

As for previously described epitopes, epitope prediction databases were used to identify amino acid motifs within the entire viral polypeptide sequence (AF313916) which are predicted to bind HLA*DRB1 0401 molecule. 8 databases – Jenner (MHCPred), ProPred, NetMHC II, RankPep, MHC2Pred, SVRMHC, SYFPEITHI and IEDB were used to generate 25 epitopes listed, according to the number of databases that have predicted them, in Table 5.5. These epitopes were predicted either in a form of a nanomer (core of an epitope) or as an extended sequence. Often extended sequences varied in length among different databases (15 - 17 amino acids in length), and such a sequence was marked with an astrisk in the table. Database SYFPEITHI predicted 21 epitopes, Jenner (MHCPred) 19, IEDB and SVRMHC 18, ProPred 17, NetMHC II 12, MHC2Pred 8 and RankPep 7 of all common epitopes. Searching for HLA*DRB1 0401 restricted T helper epitopes using Los Alamos HCV Immunology database, one epitope (in green) was identified to have immunogenic features in the HCV 1a genotype (H77 strain). At the same time this epitope exhibited a promiscuous nature as it could be presented by other alleles: DRB1 1101, DRB1 1201, DRB1 1302 and DRB1 1601.

Again substantial numbers of predicted epitopes were from the non-structural region, mostly NS5b, while all epitopes derived from the structural region were from both envelope protein parts (E1 and E2) of the viral sequence.

A




B

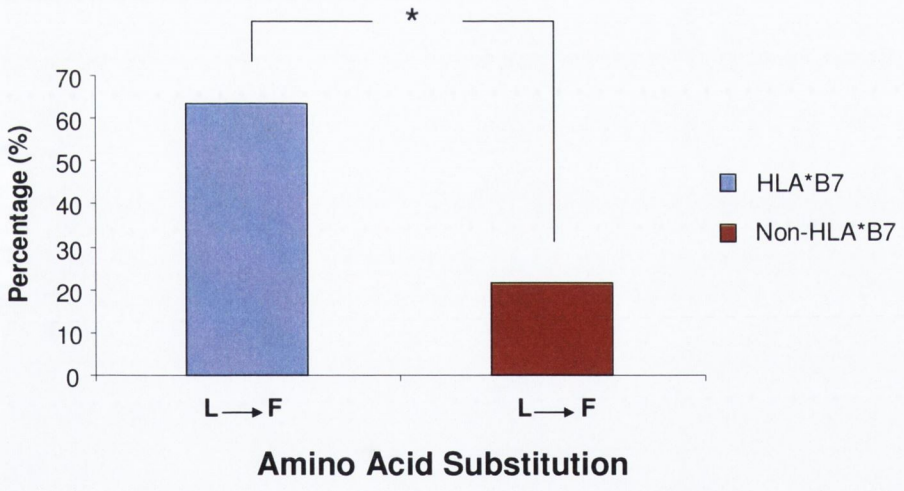



**Figure 5.9 A Substitutions of leucine (L) to phenylalanine (F) within the SARRGREIL epitope in aligned sequences.** Substitution was observed in 7 sequences out of 11 available in patient group (HLA*B7) and in 3 out of 14 among controls (non-HLA*B7) (p=0.048). **B) Expression of leucine (L) to phenylalanine (F) substitution in SARRGREIL epitope.** In the patient group (HLA*B7) 63.63% expressed this substitution, while 21.4% in control group (non-HLA*B7) had this change (p=0.048).

| Rank | Peptide Sequence Core (9) | Start position | Viral protein | Extended Peptide Sequence (15/17) | No of databases |
|---|---|---|---|---|---|
| 1 | **IMYAPTLWA** | 2833 | NS5b | GNIIMYAPTLWARMI | 8 |
| 2 | **MVYATTSRS** | 2456 | NS5b | HHNMVYATTSRSASQRQ * | 7 |
| 3 | **LVLNPSVAA** | 1253 | NS3 | YKVLVLNPSVAATLGFG * | 6 |
| 4 | **YKVLVLNPS** | 1250 | NS3 | YAAQGYKVLVLNPSVAA * | 6 |
| 5 | **SLMAFTASI** | 1790 | NS4b | AIASLMAFTASITSPLT * | 6 |
| 6 | **LLLSTTEWQ** | 666 | E2 | LSPLLLSTTEWQVLP | 6 |
| 7 | **WVALTPTLA** | 240 | E1 | RCWVALTPTLAARNSSI * | 5 |
| 8 | **VAYQATVCA** | 1588 | NS3 | YLVAYQATVCARAQAPP * | 5 |
| 9 | **YGFQYSPGQ** | 2611 | NS5b | YGFQYSPGQRVEFLVNA | 5 |
| 10 | **VTNDCSNAS** | 204 | E1 | VYHVTNDCSNASIVY * | 5 |
| 11 | **FAITKILLA** | 887 | NS2 | AIHPELIFAITKILLAI * | 5 |
| 12 | **FMLCLLLLS** | 2992 | NS5b | PRWFMLCLLLLSVGV | 5 |
| 13 | **LTSMLTDPS** | 2172 | NS5a | DVAVLTSMLTDPSHITA * | 5 |
| 14 | **MMMNWSPTT** | 323 | E1 | AWDMMMNWSPTTALVVS * | 5 |
| 15 | **IQLINTNGS** | 412 | E2 | SQNIQLINTNGSWHI | 4 |
| 16 | **IRVEESIYQ** | 2653 | NS5b | ESDIRVEESIYQCCD | 4 |
| 17 | **VVLTESTVS** | 2334 | NS5a | TVVLTESTVSSALAE * | 4 |
| 18 | **FLSQTKQAG** | 1573 | NS3 | DAHFLSQTKQAGDNF * | 4 |
| 19 | **WHINRTALN** | 421 | E2 | SWHINRTALNCNDSL * | 4 |
| 20 | **FLVNAWKKK** | 2623 | NS5b | RVEFLVNAWKKKKNP * | 4 |
| 21 | **LKEMKAKAS** | 2488 | NS5b | RDVLKEMKAKASTVK * | 3 |
| 22 | **FLADARVCA** | 726 | E2 | LFLFLADARVCACLW | 3 |
| 23 | **YRGLDVSVI** | 1419 | NS3 | VAYYRGLDVSVIPTS | 3 |
| 24 | **WRLLAPITA** | 1024 | NS2 | GQGWRLLAPITAYAQ | 2 |
| 25 | **QLSAPSLKA** | 2206 | NS5a | SASQLSAPSLKATCT | 2 |

**Table 5.5 The candidate epitopes restricted by HLA*DRB1 0401 allele.** Epitopes are outlined according to the number of databases that have predicted them. Most of the candidate epitopes are derived from the NS5b region. The sequence in green has been previously shown to be immunologically important in HCV 1a genotype. Additionally, this epitope is shared with DRB1 1101, DRB1 1201, DRB1 1302, DRB1 1601 alleles. Sequences marked with an asterisk appear in different lengths in different databases.

#### 5.3.7.1 MVYATTSRS epitope

*In silico* protein translation and full alignment were performed as described in the study of HLA Class I alleles using CLC Bio (Free Workbench) and BioEdit software packages (section 5.2.4). All candidate epitopes were aligned with the donor sequence and sequences obtained from 7 HLA*DRB1 0401 non-resolvers and 17 non-HLA*DRB1 0401 non-resolvers used as controls. Epitope HHNMVYATTSRSASQ, positioned in the NS5b region, displayed a "footprint". An amino acid substitution valine to isoleucine was observed in 3 out of 7 in the HLA*DRB1 0401 patient group and in none among 17 controls **(Figure 5.10 A)**. So, in the HLA*DRB1 0401 group, 42.86% expressed V to I substitution while 0% of the non-HLA*DRB1 0401 non-resolvers had this change (p=0.017) **(Figure 5.10 B)**.

The rest of candidate epitopes were either conserved in both groups or could not be analysed due to incomplete sequencing data [Appendix 3.3].

### 5.3.8 HLA*DRB1 0101

In order to identify candidate epitopes restricted by HLA*DRB1 0101 allele, prediction databases were used to recognise amino acid motifs within the entire viral polypeptide sequence (AF313916) which could bind to this molecule. 8 databases – Jenner (MHCPred), ProPred, NetMHC II, RankPep, SVRMHC, MHC2Pred, SYFPEITHI and IEDB were used to generate 25 epitopes outlined in Table 5.6. The order of listed epitopes is according to the number of databases that have predicted them. Similar to HLA DRB1 0401, these epitopes were predicted either in the form of a nanomer (core of an epitope) or as an extended sequence (15 - 17 amino acids), where the variations in length depended on databases. Sequences that vary are marked with astrisk in the table. Database SYFPEITHI predicted 25 epitopes, NetMHC II 24, IEDB 22, Jenner 21, ProPred 2 17, SVRMHC 13, MHC2Pred 9 and RankPep 8 of all common epitopes. A search for immunogenic HLA*DRB1 0101 restricted T helper epitopes within HCV 1a (H77 strain) identified three epitopes present in HCV 1b genotype (in green) and also being common for other alleles: DRB1 0701 (all of them) and DRB1 0102 and DRB1 1301 (epitope15). Epitopes in blue are those overlapping completely (3,6,7 epitopes) or partially (19,20 epitopes) with HLA*

A

B

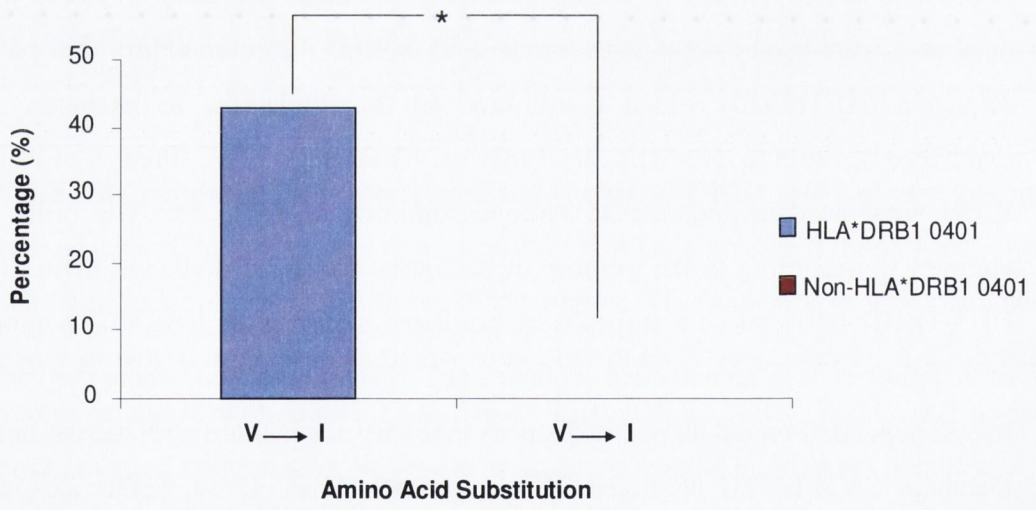



**Figure 5.10 A) Substitutions of valine (V) to isoleucine (I) within the MVYATTSRS epitope in aligned sequences.** Substitution was only observed in the patient group (HLA*DRB1 0401) in 3 sequences out of 7 (p=0.017). **B) Expression of valine (V) to isoleucine (I) substitution in the MVYATTSRS epitope.** In the HLA*DRB1 0401 group 42.86% expressed this substitution. None of the non-HLA*DRB1 0401 individuals had this change (p=0.017).

| Rank | Peptide Sequence Core (9) | Start position | Viral protein | Extended Peptide Sequence (15/17) | No of databases |
|---|---|---|---|---|---|
| 1 | **YVQMALMRL** | 934 | NS2 | GGHYVQMALMRLAAL * | 7 |
| 2 | **LAALAAYCL** | 1668 | NS4a | VGGVLAALAAYCLTT * | 7 |
| 3 | **WVALTPTLA** | 240 | E1 | NSSRCWVALTPTLAARN | 7 |
| 4 | **LVVLNAASV** | 752 | P7 | LENLVVLNAASVAGT * | 6 |
| 5 | **LMRLAALTG** | 939 | NS2 | QMALMRLAALTGTYVYD | 6 |
| 6 | **LVLNPSVAA** | 1253 | NS3 | YKVLVLNPSVAATLG * | 6 |
| 7 | **WRLLAPITA** | 1024 | NS2 | GQGWRLLAPITAYAQ * | 6 |
| 8 | **LKASAACRA** | 2717 | NS5b | LTCYLKASAACRAAK * | 6 |
| 9 | **FIGLALLTL** | 824 | NS2 | GAVFIGLALLTLSPH * | 5 |
| 10 | **LAGLSTLPG** | 1776 | NS4b | SGIQYLAGLSTLPGNPA | 5 |
| 11 | **VNLLPAILS** | 1881 | NS4b | EDLVNLLPAILSPGA * | 5 |
| 12 | **YGIGSAVVS** | 704 | E2 | DVQYLYGIGSAVVSY * | 5 |
| 13 | **VLQAGITRV** | 903 | NS2 | LGPLMVLQAGITRVP * | 5 |
| 14 | **LAILGPLMV** | 894 | NS2 | TKILLAILGPLMVLQ * | 5 |
| 15 | **FNILGGWVA** | 1810 | NS4b | TLLFNILGGWVAAQL * | 5 |
| 16 | **WMMLLIVQA** | 737 | E2 | ACLWMMLLIVQAEAA * | 5 |
| 17 | **FTTLPALST** | 680 | E2 | PCSFTTLPALSTGLI * | 5 |
| 18 | **VRNLSSKAV** | 2528 | NS5b | YGAKDVRNLSSKAVNHI | 4 |
| 19 | **VAVLTSMLT** | 2169 | NS5a | EPDVAVLTSMLTDPS * | 4 |
| 20 | **MNWSPTTAL** | 325 | E1 | AWDMMMNWSPTTALV * | 4 |
| 21 | **FVPVESMET** | 1196 | NS3 | KAVDFVPVESMETTMRS | 4 |
| 22 | **LLALPPRAY** | 801 | P7 | LLLLLLALPPRAYAM * | 4 |
| 23 | **WVLVGGVLA** | 1661 | NS4a | TSTWVLVGGVLAALA * | 4 |
| 24 | **YTWTGALIT** | 2424 | NS5b | SMSYTWTGALITPCSAE | 4 |
| 25 | **AVMGSSYGF** | 2605 | NS5b | AVMGSSYGFQYSPGQRV | 4 |

**Table 5.6 The candidate epitopes restricted by the HLA*DRB1 0101 allele.** Epitopes are listed according to the number of databases that have predicted them. Most of the candidate epitopes are derived from the NS2 and NS5b region. Sequences in green have been previously shown to be immunologically important in HCV 1a genotype and alongside, they are shared with DRB1 0701 (all of them) and DRB1 0102 and DRB1 1301 (FNILGGWVA epitope). Sequences in blue are those shared (completely or partially) with the HLA*DRB1 0401 molecule. Sequences marked with an asterisk appear in various lengths in the different databases.

DRB1 0401 restricted epitopes, which is common for epitopes restricted by HLA Class II alleles. Once more a significant number of predicted epitopes were from non-structural regions, mostly NS2 and NS5b.

*In silico* translation and sequence alignment have been done as described previously. However, analysis could not be performed due to the small number of HLA*DRB1 0101 carriers among available samples. In addition, samples that were available were poorly sequenced so that results on polymorphisms within epitopes could not be obtained.

## *5.4 DISCUSSION*

Escape variations have been documented in various viral infections, such as HIV (Allen et al, 2005; Phillips et al, 1991; Rowland-Jones et al, 1992), SIV (Allen et al, 2000) or influenza (Price et al, 2000). In HCV infection a strong association between escape substitutions and viral persistence was firstly reported in a chimpanzee model (Erickson et al, 2001) with mutations occurring early in infection (acute phase) and remaining stable for years.

The initial focus of this study was to identify immunodominant epitopes using an *ex vivo* ELISpot assay. However, clear CD8+ T cell responses were obtained only after PHA or anti-CD3 stimulation, while control peptides or HCV peptides failed to induce noteworthy IFN-γ secretion from these cells. Control peptides were previously published and they were suggested by our collaborators, since the literature on EBV or CMV epitopes restricted by HLA*A3 allele is rarely found. They demonstrated minimal or no detectable responses. Although the ELISpot technique is very sensitive and can register response at the single cell level, a panel of predicted HCV epitopes also could not evoke CD8+ T cell responses from PBMCs obtained form spontaneous HCV resolvers.

One possible explanation may be that the population of memory CD8+ T cells is probably at such a low level three decades after initial inoculation with the virus, that it can be lost during experimental procedure or can not be detected using this *ex vivo* assay. This can be improved using cultured ELISpot, but owing to time limitations this has not been optimised in this research. Another potential explanation considers the possibility that a small antigen specific population might be sequestrated in the liver, the target organ of HCV and therefore inaccessible in the periphery. Additionally, regulatory T cells may inhibit proliferation and cytokine production of HCV specific T cells which has been suggested by many studies (Boettler et al, 2005; Cabrera et al, 2004; Rushbrook et al, 2005; Sugimoto et al, 2005).

A study by Takaki et al. however, observed *ex vivo* persistent cellular immune response in spontaneous clearers two decades after a single source outbreak of HCV, although they mentioned that the frequency of HCV-peptide-specific T cells among total PBMCs

in blood was very low (0.016%) (Takaki et al, 2000). Other studies applied a cultured assay with peptide specific cell line expansion and with irradiated autologous feeder cells serving as antigen presenting cells (Lancaster et al, 2002; Neumann-Haefelin et al, 2006; Neumann-Haefelin et al, 2008b).

It is difficult to study viral mutations in humans as, very often, the exact viral sequence at the moment of transmission is not available. A study that followed up two patients that were accidentally infected with the same strain of HCV and who shared several HLA alleles, revealed that the one who cleared the infection had multispecific and vigorous T cell responses, whilst the one who developed chronic infection demonstrated mutational escape within a single immunodominant CD8+ epitope (Tester et al, 2005). Another study followed *in vivo* viral evolution of an immunodominant epitope in two acutely infected HLA*B8 positive individuals and compared them with a cohort of HLA*B8 and non-HLA*B8 chronically infected patients. The study identified "footprints" in the analysed sequence caused by HLA*B8-immune selection pressure in both acute and chronic phases of infection (Timm et al, 2004). A recent study by Cox et al. demonstrated a significantly reduced ability of CD8+ cells to recognise mutated epitopes when compared with original sequence, indicating viral escape (Cox et al, 2005). Moreover, the latest study has reported that amino acid substitutions led to a noteworthy decrease or the complete loss of recognition in 17 out of 21 epitopes tested in cellular assays, which was consistent with viral evasion. Furthermore, it was confirmed that most of the mutations, incorporated in variant peptides were not able to induce peptide-specific cell lines, being either poorly immunogenic or not immunogenic at all, and additionally supporting the fact that most of the substitutions are a precondition for viral escape (Neumann-Haefelin et al, 2008b).

In our study population HLA*A3 is the third most common HLA*A allele, while B7 is the most common HLA*B allele (McKiernan et al, 2004). Two epitopes restricted by each of these protective alleles were found to have significant polymorphisms. All epitopes were located in conserved parts of the viral genome **(Figure 5B).** The

TVYHGAGTK epitope has K1088R and T1087A substitutions and bioinformatic analysis shows that the K to R polymorphism is present in the critical anchor site. The epitope is located in the protease domain of the NS3 region and it contains one of two active sites of the enzyme at residue H-1083 as well as one of residues (A-1085) whose side chain atoms make strong hydrophobic bonds with NS4A unit (Kim et al, 1996). Since, this region probably has a limited degree of naturally occurring sequence variations, as an intact protease domain is necessary for NS3 protein to unwind RNA appropriately (Frick et al, 2004), all of the observed polymorphisms might be driven by escape from HLA*A3 restricted immune pressure. The second identified epitope KLTPPHSAK had the same substitution of K to R (K2518R) in the anchor site. This is the most common polymorphism observed in patients with persistent HCV infection (Timm et al, 2004). This epitope, positioned in the NS5b region, was the highest predicted epitope by most of the databases and the mutation K2518R was more frequent in chronically infected HLA*A3 carriers then in non-HLA*A3 individuals ($p=0.004$). This region is also highly conserved with some of mutations affecting enzyme activity and replication capacity (Bressanelli et al, 1999; Lohmann et al, 1997). Therefore, this polymorphism might be the result of HLA*mediated immune pressure and may represent viral escape. In the epitope LPRLPGVPF all substitutions R2002Q, V2006L and V2006I when analysed jointly were associated with HLA*B7 and T cell mediated selective pressure.

These findings are supported by a recent study on the Irish cohort where it was demonstrated that one immunodominant epitope restricted by the protective allele, HLA*B27, exhibited amino acid substitutions in seven out of eight chronically infected HLA*B27 carriers, while mutations were present in two out of nine non-HLA*B27 individuals. These polymorphisms were associated with viral escape as production of IFN-$\gamma$ was significantly diminished when T cells were exposed to these peptide variants (Neumann-Haefelin et al, 2006). Previously it has been shown that each of 22 investigated samples from the same cohort had 41 (median) amino acid substitutions within Core, E1, E2, p7, NS2 and NS3 regions. These polymorphisms if in a known epitope, were as a result of immune-driven selection in individuals carrying a HLA allele associated with this epitope, and the final outcome was viral evasion. However,

polymorphisms detected in those lacking the allele were identical to the consensus sequence (Ray et al, 2005).

Some other studies using a population based approach also acknowledge escape mutations in HLA restricted epitopes and polymorphism towards the consensus sequence when there is no HLA association. A recent study has identified amino acid polymorphisms within published epitopes in the NS3 region, restricted by HLA*Class I alleles, associated with viral escape (Gaudieri et al, 2006). Similarly, another study which assessed a frequently recognised NS3 epitope restricted by HLA*A1, found the same escape substitution spread across two populations infected with different viral genotypes, which subsequently led to impaired MHC-binding (Neumann-Haefelin et al, 2008a). Timm et al. have investigated HLA*driven association with viral persistence and identified mutations within epitopes across the entire viral genome. This highly correlated with viral escape from CD8+ T cell responses also at the population level (Timm et al, 2007a).

In our study it was also observed that some HLA specific epitopes contained polymorphisms which were reverting towards consensus sequence in both tested groups. Such an epitope was HLA*B7 restricted SARRGREIL with L1111F substitution. The polymorphism was consistent with the consensus sequence and dominant in the HLA*positive group. This can be explained with negative selection pressure which, unlike positive selection which drives escape mutation, preserves consensus (wild-type) sequences i.e. operates against mutation. In this context it means that HLA*restricted epitope in HLA*positive individuals would vary considerably less from the consensus sequence than in non-HLA individuals (Moore et al, 2002). According to some other studies, reversion to the consensus sequence may benefit the virus as it was hypothesised that the consensus sequence is the "best adapted" to the T cell responses driven by the most common alleles in the host population, which was the case with HLA*B7 (Gaudieri et al, 2006; Leslie et al, 2005).

In contrast, some mutations may have a high viral fitness cost which, indeed, has been observed in some epitopes with a limited level of sequence variability in the NS3/NS4a region (Soderholm et al, 2006). Therefore, variations within some immunological

epitopes may be restricted due to incompatibility with viral viability. In HIV studies it was shown that polymorphisms resulting in high fitness cost tend to revert to the fitter ancestral (wild-type) sequence, on transmission, if a new host lacks alleles that drive the substitutions (Friedrich et al, 2004; Leslie et al, 2004). A similar observation was made in chronic HCV infection where an escape mutation in HLA*B8 restricted epitope quickly reverted on transmission to non-HLA*B8 subjects (Timm et al, 2004). In addition, a recent study, using a subgenomic replicon, has revealed that some "footprints" within the HLA*B8 associated epitope diminished the viral replication capacity to levels of 60% when compared with prototype sequence, therefore affecting viral fitness (Salloum et al, 2008).

In this study putative epitopes restricted by HLA*DRB1 0101 and DRB1 0401 alleles were also identified using a prediction database approach. It was observed that the core sequences of three epitopes were identical for both alleles, whilst two epitopes shared the sequence with a small frame shift between two of them. These epitopes are described as promiscuous as they can be presented to CD4+ T cells by different HLA Class II molecules. This feature is an advantage for the host as it allows Class II alleles to bind a broad range of peptides, thus facilitating appropriate immune defence.

However, owing to a lack of sequencing data only epitopes restricted by the HLA*DRB1 0401 allele were analysed. Significant single amino acid substitution V2457I was observed within the core (MVYATTSRS) of the HHNMVYATTSRSASQ epitope ($p=0.017$). Although CD4+ T cells are not directly involved in viral elimination, they are essential for the overall immune response. Cytokines released by these cells direct downstream maturation of B and CD8+ T cells contributing to the outcome of the infection. Absence of this cytokine-mediated help was associated with viral sequence variants in certain epitopes as it has been reported by several studies (Eckels et al, 1999; Harcourt et al, 1998; Wang & Eckels, 1999). One of these studies has shown that polymorphisms within an immunodominant epitope from the NS3 region, restricted by HLA*DRB1 15 allele, caused decrease in IL-2 production suggesting that mutations may impair functioning of CD4+ T cells, subsequently leading to viral persistence (Wang & Eckels, 1999). Furthermore, the same group

demonstrated that naturally occurring single amino acid substitutions, within the same epitope, failed to support T cell expansion and were able to shift the antiviral Th1 response to Th2, contributing to viral evasion (Wang et al, 2003b).

Some other studies have suggested that escape mutations associated with HLA Class II are not a common process *in vivo* in HIV (Koeppe et al, 2006) or HCV (Guglietta et al, 2005) and since the body of work on polymorphisms within CD4+ T cell specific epitopes is still limited, their contribution to the viral escape phenomenon will have to be further elucidated.

In this study several amino acid substitutions were identified and replacements were with both similar and different amino acids. Valine was replaced with leucine or isoleucine and from a chemical point of view these amino acids are very similar (**Table 5A**) and often interchangeable. Similarly, substitution of lysine with arginine or arginine with glutamine is considered as a synonymous substitution as these have very similar characteristics (**Table 5A**). However, peptide characteristics do not depend only on physico-chemical properties such as charge or solubility, but also on bond geometry, or shape, volume and side chains of amino acid residues within the peptide. For instance, isoleucine's side chains are quite inactive, not involved in any covalent bonds in enzyme active centres (Baldwin, 2003) and therefore we can expect that the epitope with this residue will exhibit different features to the one with valine or leucine. In a recent study it has been demonstrated that substitution of lysine to arginine in NS3 region led to decreased replication capacity as well as to lost recognition by specific T cells (Salloum et al, 2008) although these amino acids are similar. Two other described substitutions threonine to alanine and leucine to phenylalanine are expected to change the nature of peptides as polar hydrophilic amino acids are replaced with non-polar hydrophobic once (**Table 5A**).

Overall, altered epitopes may affect proteosomal processing (Kimura et al, 2005; Seifert et al, 2004; Timm et al, 2004); binding to HLA molecules (Cox et al, 2005; Erickson et al, 2001; Neumann-Haefelin et al, 2008b) or TCR recognition (Chang et al, 1997; Neumann-Haefelin et al, 2008b). It is clear that interactions between epitopes and MHC molecules or TCR are probably more complicated than can be explained by

change of a few groups. Potentially, all these groups may have a specific role in a functional context.

In summary, utilising a bio-informatics approach candidate epitopes restricted by HLA*A3, B7, DRB1 0101 and DRB1 0401 alleles were identified from the donor sequence. Since all those computational methods have their advantages and disadvantages, 7-8 databases available on the web were used to achieve objective predictions. Data obtained from a range of databases, were combined, integrated and a final collection of candidate epitopes predicted for each allele. Furthermore, polymorphisms within the epitopes restricted by protective alleles were identified. Mutations were identified in patients infected with HCV, who despite being carriers of protective alleles, developed persistent infection. These substitutions in particular regions of the viral genome might be responsible for viral escape and establishment of chronic disease in these individuals.

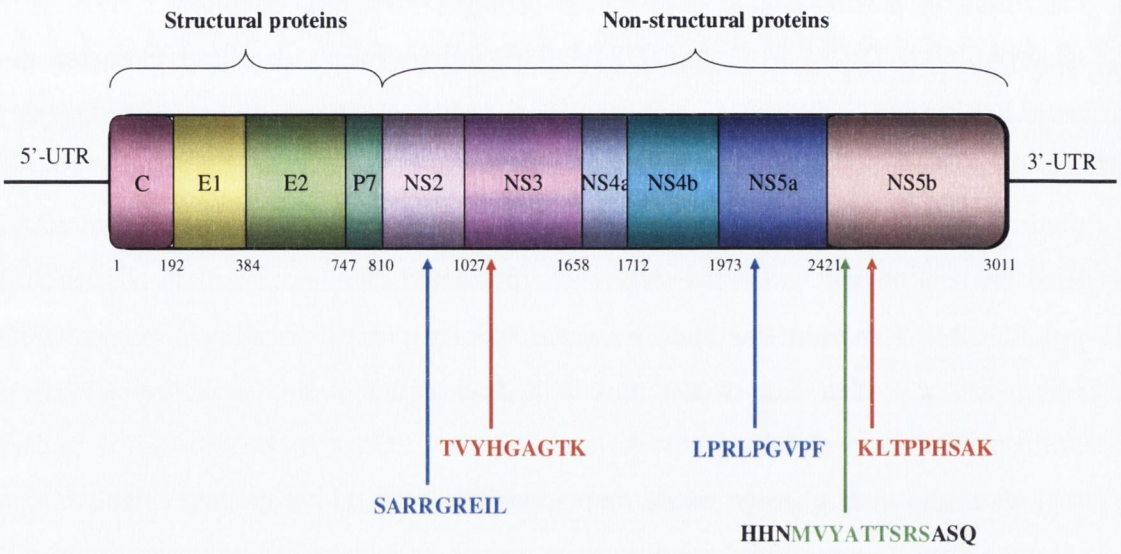



**Figure 5B: HCV genome map with epitopes of interest for this study indicated by arrows.** All identified epitopes are positioned in the conserved part of viral genome, which encodes for non-structural proteins. Epitopes in red are restricted by HLA*A3 allele, in blue by HLA*B7 while epitope in green is specific for HLA*DRB1 0401 allele.

| Amino acid | Letter code | Chemical properties | Physical properties |
|---|---|---|---|
| Alanine | A | aliphatic | nonpolar, hydrophobic |
| Arginine | R | basic | polar, hydrophilic |
| Glutamine | Q | neutral | polar, hydrophilic |
| Isoleucine | I | aliphatic | nonpolar, hydrophobic |
| Leucine | L | aliphatic | nonpolar, hydrophobic |
| Lysine | K | basic | polar, hydrophilic |
| Phenylalanine | F | aromatic | nonpolar, hydrophobic |
| Threonine | T | non-aromatic hydroxyl | polar, hydrophilic |
| Valine | V | aliphatic | nonpolar, hydrophobic |

**Table 5A: Basic physico-chemical properties of amino acids involved in identified substitutions.**

# CHAPTER VI

# *GENERAL DISCUSSION*

# Chapter VI

Hepatitis C virus is a human pathogen infecting approximately 3% of the world's population. During the acute phase most infected individuals are asymptomatic, but only a small proportion eradicate the virus. HCV has been described as "a survival machine and not a killing machine" due to its ability to persists for years, causing, in the vast majority of patients, chronic infection that subsequently leads to serious liver disease (Shoukry et al, 2004). HCV-host interactions have a crucial role in viral survival, persistence, pathogenicity of infection and disease progression. It has been proposed that maintenance of a vigorous, sustained and multi-specific cellular immune response is essential for viral clearance (Lechner et al, 2000; Thimme et al, 2001). However, a large body of evidence suggests that HCV can annul host immune defence, in order to replicate and spread successfully. Applying different strategies, the virus subverts both innate and adaptive arms of the immune system. To escape immune surveillance, HCV alters its epitopes that are recognised by T cells and neutralising antibodies, as well as interferes with cellular components and signalling pathways (Gale & Foy, 2005; Lauer & Walker, 2001). The viral escape mechanisms are so highly developed, that they make HCV one of the most successful persistent viruses in humans (Bowen & Walker, 2005).

In this thesis we explored two different aspects of HCV immune evasion. Firstly, we described a novel pathway that the virus may use to establish persistence. Using a single viral component, envelope protein 2 (E2), we have demonstrated that this structural component altered cytokine production in T cells, which potentially leads to the failure of adaptive immunity. This study identified a novel mechanism how a viral protein, through its interaction with the host cell, manipulates the immune response.
Secondly, in this study it was shown that the generation of escape variants is one of the most potent immune evasion approaches that HCV applies. The work resulted in the identification of novel HCV epitopes restricted by certain HLA alleles, which have been previously shown to be protective in an Irish cohort. Significant single, as well as, multiple mutations were identified within some of these epitopes and they were

associated with chronic infection. This study, therefore, highlights the importance of escape mutations in establishment of viral persistence.

In the first chapter we described the effect of HCV E2 on IL-2 production in T cells. Initially, we observed that pre-treatment of normal PBMCs with serum from HCV $PCR^+$ individuals inhibited their ability to produce IL-2. This IL-2 inhibition was reversed by a peptide blocking the interaction of E2 with CD81. Furthermore, in a HuT 78 T cell line model, pre-treatment of cells with E2 substantially attenuated PMA stimulated IL-2 production. Inhibition of IL-2 was at the level of secretion and not transcription. Subsequent experiments led to identification of a mechanism that facilitates this inhibition. Namely, it was clearly shown that interaction of E2 with the tetraspanin cell surface molecule CD81 results in sequestration of the enzyme PKC-β to the lipid raft compartments of the cell membrane. PKC-β has been previously shown to be essential for IL-2 secretion in HuT 78 cells (Long et al, 2001) and we postulated that its association with lipid raft microdomains can prevent the interaction of PKC-β with the cellular secretory machinery, thereby inhibiting export of IL-2. Our findings were supported by recent study which demonstrated loss of IL-2 secreting CD4+ T helper cells in chronic HCV infection (Semmo et al, 2005). Moreover the same group have shown proliferation of HCV-specific CD4+ T cells from patients with chronic disease to be reduced (on a per cell basis) in parallel to IL-2 secretion, when compared to spontaneously resolved individuals (Semmo et al, 2007).

The immunosuppressive potential of E2 has been described by studies. A recent study has shown that E2 can block host-induced apoptosis of infected cells, through inhibition of cytochrome c release from mitochondria. This facilitates enhancement of viral persistence (Lee et al, 2005). An earlier study has demonstrated that E2 binds interferon inducible PKR and inhibits its kinase activity. When PKR activity is blocked it cannot suppress protein synthesis and viral translation is uninterrupted (Taylor et al, 1999). It has also been reported that recombinant E2 when bound to CD81 on the surface of the NK cells, causes the inhibition of cell proliferation, cytotoxic granule release and cytokine production (Crotta et al, 2002; Tseng & Klimpel, 2002). These studies demonstrated viral evasion strategies that target early antiviral activities of innate immunity.

The E2-mediated inhibition of IL-2 secretion demonstrated in this study, affects the cellular part of the adaptive immune response and possibly occurs early in virus-T cell contact, since the E2 is component that facilitates viral attachment. Alteration of IL-2 secretion may have dramatic consequences on host-virus interaction, since IL-2 is cytokine essential for many aspects of a successful immune response. IL-2 is a well established T cell growth factor. In addition, it triggers T cells to undergo apoptosis when restimulated through the TCR (Lenardo, 1991). IL-2 also stimulates the development of lymphocyte effector functions *in vitro*, promoting differentiation of these cells (Malek et al, 2001). Some murine studies have shown IL-2-independent T cell proliferation and effector cells development resulting in effective primary responses. However, in some model systems these responses were impaired when compared to those in control mice, indicating that IL-2 contributes to development of an appropriate immune response (Malek, 2008). Recent findings suggest a dominant role of IL-2 in formation of memory responses, since long-lasting T cells strictly require the presence of IL-2 (Williams et al, 2006). Similarly, there is data supporting an essential function of IL-2 for the proliferation and homeostasis of regulatory T cells (Malek & Bayer, 2004), which promote self-tolerance by controlling T cell responses in vivo (Nelson, 2004).

Administration of IL-2 has been used therapeutically in a number of conditions including cancer, autoimmune disease and viral infection. Anti-IL-2R immunotherapy has a success in inhibition of unwanted immune responses in autoimmunity and allogeneic transplants (Malek, 2008). Moreover, it has been demonstrated that systemic infusion of IL-2 can improve immune response and boost anti-cancer immunity and increase number of T cells in HIV patients (Dutcher, 2002; Pahwa & Morales, 1998; Yee et al, 2002). This suggests that recovery of IL-2 secretion may be successful therapy in HCV infection. Since injection of IL-2 in chronically infected HCV patients had no success (Alric et al, 2006; Glesby et al, 2005), an approach that targets the liberation of PKC-β from lipid rafts (or prevents association of PKC-β with lipid rafts), may prove to be more successful in restoration of desirable immune response.

Our study indicates that E2 alters IL-2 secretion which may contribute to the impairment of immune defence against HCV infection. To further address the effect of E2 on other cytokines a multiplex cytokine analysis was performed. These experiments demonstrated that E2 also inhibited secretion of IFN-γ, IL-10 and TNF-α from activated HuT 78 cells and PBMCs suggesting that E2, via its interaction with CD81, is targeting a secretory process in T cells. After further analysis it was determined that E2 manipulates these cytokines at different levels. Similarly to IL-2, IFN-γ inhibition was at the level of secretion and not mRNA production. Since both IL-2 and IFN-γ are cytokines shown to be secreted directionally towards the immunological synapse (Huse et al, 2006) and since PKC-β is an essential enzyme in IL-2 export (Long et al, 2001), we postulated that PKC-β may also be involved in IFN-γ secretion. IFN-γ is a cytokine with strong antiviral activity, and several studies have shown that IFN-γ has crucial importance in the control of HCV infection and viral clearance (Neveu et al, 2008; Sun et al, 2004; Thimme et al, 2001). Numerous studies have demonstrated that HCV-specific T cells were impaired in IFN-γ secretion in chronically infected individuals (Folgori et al, 2006; Penna et al, 2007; Ulsenheimer et al, 2003; Wedemeyer et al, 2002). In this study, we proposed that the CD81-E2 - stimulated sequestration in lipid raft is targeting a general (PKC-β - regulated) mechanism, which controls both IL-2 and IFN-γ secretion. Disrupting secretion of these two cytokines through described pathway, represents a novel mechanism whereby HCV can undermine the human immune response to establish persistent infection and chronic disease.

E2-mediated inhibition of TNF-α and IL-10 observed at a protein level, was caused by suppression of mRNA production. TNF-α has a different secretory pathway to that involved in IL-2 and IFN-γ secretion, and is exported in a multidirectional fashion (Huse et al, 2006). Additionally, TNF-α secretion is a complex process and includes proteolytic cleavage of a precursor, which is a membrane-bound protein (pro-TNF-α) (Black et al, 1997; Moss et al, 1997). It suggests that HCV employs different mechanism of TNF-α inhibition targeting at the level of gene expression. Similar to our findings, other groups have demonstrated that HCV-specific CD8+ T cells exhibited decreased production of TNF-α (Gruener et al, 2001; Penna et al, 2007). Besides IFN-γ,

TNF-α is another cytokine with essential antiviral activity, which is secreted early in HCV infection and which orchestrates the process of inflammation within the liver (Neuman et al, 2008). In this study we demonstrated that E2 alters production of this cytokine and indicates that HCV potentially utilises this inhibition of TNF-α as part of its immune evasion strategies. Like TNF-α, IL-10 inhibition was also at the level of gene expression. Supporting our findings, a recent study demonstrated that PBMCs obtained from chronically infected HCV patients, exhibited reduced production of IL-10 when exposed to core, NS3 and NS4 HCV proteins. A study in a murine model of chronic viral infection demonstrated that elevated levels of IL-10 were associated with viral persistence (Brooks et al, 2006). This upregulation of IL-10 was observed in APCs, while in our experiments we focused on T cells, where development of events may be different to that observed in that model system. It is also believed that findings in mouse models may be different in humans, owing to difference in complexity of these two organisms. Also our model mimics an acute infection, where in T cells the initial IL-10 levels are possibly reduced.

Pre-treatment of activated T cells with E2 protein caused suppression at gene expression and/or protein secretion levels for IL-2, IFN-γ, TNF-α and IL-10 cytokines. This study demonstrated that IL-1α, IL-1β and IL-6 were dramatically enhanced whenever PBMCs were exposed to E2. This augmentation in secretion of proinflamatory cytokines in our model is in line with events that occur early in HCV infection and liver inflammation. Namely, resident tissue macrophages, Kupffer cells (KCs), upon activation with viral antigens release IL-1α, IL-1β and IL-6 cytokines, together with TNF-α, which then increase expression of cell adhesion molecules, such as ICAM-1, on sinusoidal endothelial cells. This leads to transmigration and recruitment of inflammatory cells, such as monocytes, NK and T cells, which further increase cytokine production and amplify the inflammatory cascade (Neuman et al, 2008; Ramadori & Armbrust, 2001). We postulate that in our system IL-1α, IL-1β and IL-6 were mainly secreted by monocytes, since these cytokines were not produced by HuT 78 T cell line. Monocytes express similar levels of CD81 molecule on their surface as T cells. This indicates that monocytes have the potential to be activated

through E2-CD81 engagement, which subsequently (possibly utilising different pathways than those employed in T cells) leads to production of these cytokines. However, it is not clear whether elevated levels of IL-1α, IL-1β and IL-6 favour the host defence or viral persistence. It is possible that if the increased levels are sustained, the initial beneficial role for the host switches to the role that supports persistence and liver damage.

During the course of this thesis, we also investigated the effect of E2 on TGF-β1, since it has been proven to be the most potent cytokine that induces fibrogenesis. In HuT 78 cells E2-mediated inhibition of TGF-β1 was observed at both protein and mRNA levels, similar to TNF-α and IL-10. However, E2 pre-treated PBMCs exhibited no suppression at the mRNA level, which is in line with a study that demonstrated elevated levels of intrahepatic mRNA for TGF-β1 following treatment with HCV core protein (Taniguchi et al, 2004). TGF-β1, mainly secreted by activated NK, Kupffer and hepatic stellate cells, triggers synthesis and deposition of ECM proteins in HCV infection. At the beginning of infection this process is balanced with degradation of ECMs (fibrolysis), but as infection continues fibrogenesis overcomes fibrolysis, resulting in excess deposition of ECMs and development of fibrosis (Schuppan et al, 2003). At a protein level neither PBMCs nor purified T cells exhibited E2-mediated inhibition after PMA stimulation. Under more physiological anti-CD3/anti-CD28 stimulation of E2 pre-treated cells some inhibition in TGF-β1 protein secretion was noticed, but significance of that has to be investigate further, since the stimulation was weak and at the levels of control cells. The difference observed between two sets of cells with regard to the effect of E2 on TGF-β1, might be due to mixed population of cells in PBMCs. PMA is a potent stimulator of TGF-β1 production and elevated TGF-β1 levels may be produced by PBMCs subsets, which are not necessarily T cells. Recently it has been demonstrated that monocytes obtained from healthy volunteers, as well as HCV-infected patients, had significantly increased production of TGF-β1 after exposure to NS4 viral protein (Rowan et al, 2008). It must be noted that HuT 78 cells are derived from a lymphoma tumour and may have an altered profile of TGF-β1 secretion when compared to normal T lymphocytes, as TGF-β1 secretion and function

is frequently altered in the cancer setting (Grady et al, 1999; Villanueva et al, 1998), as T cell line.

In summary, we have demonstrated that the envelope protein of HCV, E2, can manipulate cytokine secretion at a number of levels. Cytokines have an essential role in immune response, since they orchestrate different components of innate and adaptive immunity. A HCV viral strategy to manipulate these molecules will lead to the interruption of antiviral defence, which can favour viral survival and persistence.

Another aspect of viral evasion was also analysed in this study. The ability of HCV to generate epitope mutations in order to escape from immune surveillance, was investigated in an Irish cohort of females inoculated with anti-D immunoglobulin contaminated with HCV 1b from a single source. This cohort is ideal to study host – viral interactions, since all individuals were infected with the same viral genotype, they were of the same ethnic background, the same gender and had the same route of infection. In this cohort, HLA*A3, B7, DRB1 0101 and DRB1 0401 alleles were associated with protection from HCV (McKiernan et al, 2004). Utilising a bio-informatics approach this study identified putative epitopes restricted by these protective alleles. These candidate epitopes were derived from the donor sequence and predicted by numerous databases. Data obtained from all these databases were integrated and the most prominent epitopes were listed for each protective allele. Furthermore, these novel epitopes were analysed for amino acid substitutions that are associated with viral persistence. For the HLA*A3 restricted epitopes, significant mutations correlated with chronic disease were observed in two epitopes, KLTPPHSAK (mutation K to R) and TVYHGAGTK (mutations K to R and T to A). In the HLA*B7 restricted epitopes, significant polymorphisms were identified in one epitope LPRLPGVPF (mutations R to Q, V to L and V to I) and in HLA*DRB1 0401 also in one epitope HHNMVYATTSRSASQ (mutation V to I).

In this study, *in vivo* sequence analysis revealed possible mutational escape within these HLA restricted epitopes. Viral evasion through generation of amino acid

substitutions within epitopes is well documented in the literature. The first virus reported to escape the immune surveillance through mutation of epitopes was HIV (Phillips et al, 1991). However, just in recent years this mechanism was recognised as a major influence on clinical outcome and has received a lot of attention. In chronic HCV infection, amino acid substitution that inhibits T cell recognition was initially reported in chimpanzees (Weiner et al, 1995) and subsequently in human studies (Chang et al, 1997; Kaneko et al, 1997; Tsai et al, 1998). Chang et al. have used a panel of HLA*A2 restricted epitopes predicted from a prototypical sequence of HCV-1a genotype and by comparison to viral sequence obtained from chronically infected patients, identified mutations within the epitopes that led to impaired recognition by HLA*A2 restricted CD8+ cells (Chang et al, 1997). These data gained interest when recent studies revealed that mutations within epitopes restricted by HLA Class I alleles were associated with viral persistence. These studies have had access to viral sequences from the source or early phases of infection, and identified escape mutations resulting in development of chronic HCV infection (Tester et al, 2005; Timm et al, 2004). A recent study on the same Irish cohort revealed that a number of mutations within the known epitopes, restricted by HLA Class I alleles, was significantly higher than a number of mutations in other sites. The same group also reported that mutations were more frequent in these epitopes than in the same regions in patients who did not have the particular allele, suggesting immune selection (Ray et al, 2005). Both human and animal studies have suggested that escape substitutions occur early during infection and remain stable for several years of follow up (Chang et al, 1997; Erickson et al, 2001; Weiner et al, 1995)

Potential immunodominant epitopes identified in our study are positioned within parts of the viral genome which are usually conserved in the general population, since they are vital for viral survival. Therefore, the polymorphisms within these epitopes probably will not occur rapidly, allowing the immune system to maintain adequate antiviral CD8+ T cell responses required for viral clearance (Neumann-Haefelin & Thimme, 2007). If within these epitopes, HCV manages to generate mutations that are harmless with regards to its fitness cost, it is more likely that the virus will escape the

host response, establishing persistence and chronic infection. This is probably facilitated by a delay in proliferation of CD8+ T cells and development of their effector functions (Bowen & Walker, 2005). In addition, escape from a CD8+ T cell response also may be supported by lack of adequate CD4+ T cell help. The study of a chimpanzee model demonstrated that generation of escape mutations within epitopes targeted by CD8+ T cells was associated with poor control of viral replication and occurred in the absence of CD4+ T cell help (Grakoui et al, 2003). Since IL-2 is an essential component of CD4+ T cell help, E2-mediated inhibition of IL-2 secretion (which we have demonstrated), may contribute to the generation of viral escape variants. This also may represent a phenomenon of how an initial HCV evasion mechanism (inhibition of IL-2) facilitates the development of subsequent escape strategies.

In this study a bio-informatics approach, which obtains useful information from a substantial amount of data and constructs a good probabilistic model, was utilised in prediction of putative epitopes. *In vivo* sequence analysis was the next stage in identification of immunodominant epitopes and escape mutations. The following step will be to functionally validate these findings in *in vitro* conditions. These strategies are incredibly valuable in rapid discovery of epitopes, as potentional tools for therapeutic and diagnostics purposes. In our study all identified epitopes are derived from conserved parts of the viral genome, which is an advantage for an epitope-based vaccine approach. It is more likely that conserved epitopes will provide broader protection across multiple strains, than those located in the variable regions of the viral genome (Bui et al, 2007). All identified epitopes will further be assessed and validated in the *in vivo* situation, which is a platform for future work including development of vaccines.

## Future work

The work described in this thesis forms a basis for further research which will increase our understanding of how HCV undermines and evades the host immune response.

The observation that E2-CD81 interaction causes sequestration of PKC-β in lipid raft, described in this thesis, could be exploited to determine the intracellular signalling events that lead to this enzyme translocation. Once the molecules involved in this process are identified, it will be possible to better understand this aspect of E2 interference with host cell elements and perhaps to prevent this E2-mediated inhibition of cytokine secretion.

In this study we reported that E2 acts at different levels to alter various cytokines. A range of experiments should be performed to provide further information on E2-mediated modification of signalling pathways, which regulate cytokine gene expression of certain cytokines. The characterisation of these highly complex interactions could in turn provide valuable data on how altered cytokine profiles, caused by E2, contribute to the subversion of immunity or development of chronicity and liver fibrosis.

In this study we focused on the effect of the E2 viral protein obtained from HCV 1a genotype. Genotype variation may affect binding affinity of E2 for CD81 and, subsequently, may exhibit a differential effects on downstream signalling and cytokine production and is worthy of further investigation. As an *in vitro* model system of HCV has only recently been developed, the effect of HCV infection on T cell cytokine production *in vitro* should be investigated. Moreover, little is available in literature on the effects of the entire viral genome on cytokine alteration. Examining the cytokine profiles of T cells exposed to the whole virus, may reveal essential information about this aspect of HCV evasion strategies. The *in vitro* model systems such as HCVpp and HCVcc should be used in these experiments, which may parallel the actual cascade of events in the *in vivo* situations.

In this study we also have identified novel epitopes restricted by protective alleles that may play a role in the early protection facilitated by these alleles. We hypothesised that the mutations observed within these epitopes may be responsible for immune evasion and establishment of viral persistence.

Further experiments will provide data on differences between protective and non-protective responses in tightly defined infection and the nature of protection by Class I alleles associated with successful immune response and viral clearance. The experimental approach should include *in vitro* cellular assays, such as IFN-γ ELISpot and intracellular IFN-γ staining, which will confirm the presence and functionality of CD8+ T cells responses to candidate epitopes in individuals who spontaneously resolved infection. Furthermore, MHC Class I tetramer technology would be a valuable tool in this research. The tetramer complex is composed of a specific HLA molecule and its candidate epitope (in its native form as well as mutated version). This approach will help to determine the avidity of MHC-peptide complex for TCR and to define responses in patients with protective alleles. In addition, tetramers that incorporate mutated versions of epitopes will shed more light on the nature of immune responses in those bearing protective alleles in whom persistence occurred. The hypothesis to be tested here is whether the variation in responses, and subsequently the outcome of infection, are caused by viral sequence variation.

HCV is a bloodborne virus, distributed worldwide, which has serious and sometimes fatal consequences on human health. Morbidity and mortality associated with HCV are the second highest among emerging infections (Strickland T lancet 2008). It is crucial to elucidate different aspects of virus – host interaction, which could lead to more successful treatment and possibly prevention. The current anti HCV treatment has limited success, is long-lasting, toxic and expensive and thus there is an urgent need to improve and develop better immunotherapy for HCV. Therefore, work carried out for this thesis should contribute to a better understanding of the molecular mechanisms underlying viral evasion strategies, which contribute to the failure of the immune response and development of chronic HCV infection. It will also help to better understand the host defence, which may be useful for our understanding of natural and vaccine-induced immunity, leading to the development of more effective therapeutic and prevention strategies.

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# *APPENDICES*

# Appendix 1

## *REAGENTS AND BUFFERS*

### CELL CULTURE

#### Complete Cell Culture Medium - HuT 78

| | |
|---|---|
| RPMI 1640 | 500 ml |
| Heat inactivated Foetal Calf Serum (FCS) | 50 ml |
| L-Glutamine-Penicillin-Streptomycin solution | 5 ml |
| HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (1M) | 5 ml |
| Sodium bicarbonate solution (7.5%) | 10 ml |
| D-(+)-Glucose solution (45%) | 5 ml |
| Sodium pyruvate solution (100mM) | 5 ml |

#### Complete Cell Culture Medium - PBMCs

| | |
|---|---|
| RPMI 1640 supplemented with 25 mM HEPES | 500 ml |
| Heat inactivated FCS | 50 ml |
| L-Glutamine-Penicillin-Streptomycin solution | 5 ml |

#### T cells Enrichment Column Wash buffer 1x

| | |
|---|---|
| 10x column wash buffer | 2 ml |
| Sterile distilled water | 18 ml |

#### PBMCs Cryopreservative Medium

| | |
|---|---|
| FCS | 9 ml |
| Dimethylsulfoxide (DMSO) | 1 ml |

#### Cell viability Ethidium Bromide/Acridine Orange

| | |
|---|---|
| EB (10mg/ml) | 10 µl |
| AO (10mg/ml) | 10 µl |
| PBS | 980 µl |

## IMMUNOFLUORESCENCE ANALYSIS

### Phosphate-Buffered saline PBS 1x

PBS tablets (0.14 M NaCI, 0.01 M $PO_4$ Buffer, 0.003 M KCI) 5 g

Each 5 g tablet should be dissolved in 500 ml distilled water and sterilised by autoclaving.

### Paraformaldehyde – fixation reagent (3% or 1%)

Paraformaldehyde 1.5 g or 0.5 g

Distilled water 50 ml

While heating to dissolve paraformaldehyde, do not exceed 56 °C, otherwise there will be too much autofluorescence.

### Working solution of the fluorescent CT-B conjugate (1µg/ml)-Lipid raft assay

CT-B conjugate stock solution (1mg/ml) 2 µl

Chilled complete medium 2 ml

(CT-B conjugate stock solution was made by adding 100 µl of 1 x PBS to the vial with lyophilised fluorescent CT-B conjugate)
The working solution should be freshly prepared before each use.

### Working solution of the anti-CT-B antibody -Lipid raft assay

Anti-CT-B rabbit serum 10 µl

Chilled complete medium 2 ml

The working solution of the antibody should be freshly prepared before each use.

## ELISA ASSAYS

### Wash Buffer - 0.05% Tween-20 in PBS (pH 7.2-7.4)

1 x PBS 500 ml

Tween-20 250 µl

### 1 x Coating Buffer (IFN-γ Elisa)

10 x Coating Buffer 12 ml

| | |
|---|---|
| Distilled water | 108 ml |

**Block Buffer – 1 x Assay Diluent (IFN-γ Elisa)**

| | |
|---|---|
| 5 x Assay Diluent | 10 ml |
| Distilled water | 40 ml |

**Block Buffer - 1% BSA in PBS (IL-2 Elisa)**

| | |
|---|---|
| Bovine Serum Albumin (BSA) | 0.2 g |
| 1 x PBS | 20 ml |

**Block Buffer - 5% Tween-20 in PBS (TGF-β1 Elisa)**

| | |
|---|---|
| Tween-20 | 2.5 ml |
| 1 x PBS | 50 ml |

**Reagent Diluent – 1% BSA, 0.05% Tween-20 in Tris-buffered Saline (20 mM Tris base, 150 mM NaCl) (IL-2 Elisa)**

| | |
|---|---|
| Tween-20 | 25 µl |
| BSA | 0.5 g |
| Tris base | 0.121 g |
| NaCl | 0.438 g |
| Distilled water | 50ml |

Reagent Diluent should be freshly prepared before each use and adjusted to **pH 7.2-7.4**

**Reagent Diluent (TGF-β1 Elisa)**

| | |
|---|---|
| 0.05% Tween-20 in PBS | 98.6 ml |
| Delipidized Bovine Serum | 1.4 ml |

Reagent Diluent should be freshly prepared before each use and adjusted to **pH 7.2-7.4**

**Stop Solution 2N $H_2SO_4$**

| | |
|---|---|
| $H_2SO_4$ (36.8 N) | 5.43 ml |

| | |
|---|---|
| Distilled water | 94.57 ml |

Always slowly add acid into water.

**TGF-β1 Sample Activation – 1N HCl**

| | |
|---|---|
| HCl (10N) | 10 ml |
| Distilled water | 90 ml |

Always slowly add acid into water.

**TGF-β1 Sample Activation – 1.2N NaOH/0.5M HEPES**

| | |
|---|---|
| NaOH (10N) | 12 ml |
| HEPES | 11.9 g |

Slowly add NaOH to 75 ml of distilled water, mix well and dissolve HEPES and bring final volume to 100 ml.
10N NaOH was made dissolving 40 g NaOH in 100 ml distilled water.

## MULTIPLE CYTOKINES ANALYSIS

**Wash Buffer 1 x**

| | |
|---|---|
| Wash Buffer concentrate (31.25 x) | 32 ml |
| Distilled water | 968 ml |

## FACS ANALYSIS

**FACS Buffer**

| | |
|---|---|
| PBS ($Ca^{2+}$/$Mg^{2+}$ free) | 500 ml |
| Sodium azide (0.02%) | 0.1 g |
| BSA (0.2%) | 1 g |

This buffer has to be filter sterilised.

## PCR - DNA VISUALISATION

**Ladder**

| | |
|---|---|
| Ladder (100 base pairs, 1 μg/ml) | 1 μl |

| | |
|---|---|
| Distilled water | 9 µl |
| Loading Dye (6x) | 2 µl |

**TAE (Tris Acetate EDTA) Buffer 10 x**

| | |
|---|---|
| Tris base | 48.4 g |
| Glacial Acetic Acid | 11.42 ml |
| EDTA (Ethylenediaminetetraacetic acid 0.5M, pH 8.0) | 20 ml |

Bring final volume to 1 l with distilled water.

**TAE Buffer 1 x**

| | |
|---|---|
| TAE Buffer 10 x | 50 ml |
| Distilwater | 450 ml |

**ELISPOT ASSAY**

**Substrate Solution (Protein G - HRP Conjugate)**

| | |
|---|---|
| Buffer (25x) | 400 µl |
| Solution A | 100 µl |
| Solution B | 100 µl |
| Distilled water | 9.4 ml |

# Appendix 2

**Databases used in the study:**

BIMAS - Bioinformatics and Molecular Analysis Section
Computational Bioscience and Engineering Laboratory, Center for Information Technology, National Institute of Health (NIH), Bethesda, MD
http://www-bimas.cit.nih.gov/molbio/hla_bind/

RankPep
Bioinformatics, Cancer Vaccine Center, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA
http://immunax.dfci.harvard.edu/Tools/rankpep.html
http://bio.dfci.harvard.edu/Tools/rankpep.html

SYFPEITHI
Institute for Cell Biology, Department of Immunology, University of Tübingen and Biomedical Informatics Heidelberg, Germany
http://www.syfpeithi.de/home.htm

Jenner (MHCPred)
Bioinformatics Group, The Jenner Institute (The Edward Jenner Institute for Vaccine research), Nuffield Department of Clinical Medicine, Oxford University, Oxford, U.K.
http://www.jenner.ac.uk/MHCpred/

SVRMHC - Support Vector machine Regression MHC
University of Minnesota
http://svrmhc.umn.edu/SVRMHCdb/

MAPPP - MHC-I Antigenic Peptide Processing Prediction
Max Planck Institute for Infection Biology, Berlin, Germany
http://www.mpiib-berlin.mpg.de/MAPPP/

ProPred
Bioinformatics Centre, Institute of Microbial Technology, Chandigarh, India
http://www.imtech.res.in/raghava/propred1/index.html
http://www.imtech.res.in/raghava/propred/

MHC2Pred
Bioinformatics Centre, Institute of Microbial Technology, Chandigarh, India
http://www.imtech.res.in/raghava/mhc2pred/

IEDB - Immune Epitope Database and Analysis Resources
Sponsored by National Institute of Allergy and Infectious Disease, NIH, Department of Health and Human Services

http://tools.immuneepitope.org/analyze/html/mhc_binding.html
NetMHC
Center for Biological Sequence Analysis, Department of Systemic Biology, Technical University of Denmark, Lyngby, Denmark
http://www.cbs.dtu.dk/services/NetMHC/
http://www.cbs.dtu.dk/services/NetMHCII/

NetChop
The Center for Biological Sequence Analysis, Department of Systemic Biology, Technical University of Denmark, Lyngby, Denmark
http://www.cbs.dtu.dk/services/NetChop/

PaProC - Prediction Algorithm for Proteasomal Cleavages
Department of Immunology and Department of Biomathematics, University of Tübingen, Tübingen, Germany
http://www.paproc.de/

Hepatitis C virus Los Alamos Database
Los Alamos National Laboratory, NIH, Department of Health and Human Services
HCV Immunology Database
http://hcv.lanl.gov/content/immuno/immuno-main.html
HCV Sequence Database
http://hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html

# Appendix 3

## AF313916, Irish donor, Full Length Clone

***HEPATITIS C VIRUS POLYPROTEIN*** (3010 amino acid) (9359 base pairs mRNA linear)

MSTNPKPQRKTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATRRKTSERSQPRGRRQPIP
KARRPEGRTWAQPGYPWPLYGNEGMGWAGWLLSPRGSRPSWGPTDPRRRSRNLGKVIDTLTCGFADL
MGYIPLVGAPLGGAARALAHGVRVLEDGVNYATGNLPGCSFSIFLLALLSCLTIPASAIEVRNVSGV
YHVTNDCSNASIVYEAADMIMHTPGCVPCVRENNSSRCWVALTPTLAARNSSIPTTTIRRHVDLLVG
TAAFCSAMYVGDLCGSVFLVSQLFTFSPRRHETVQDCNCSIYPGHVSGHRMAWDMMMNWSPTTALVV
SQLLRIPQAVVDMVAGAHWGVLAGLAYYSMAGNWAKVLIVMLLFAGVDGETYTTGGAQAHTTRGLAT
LFSFGPSQNIQLINTNGSWHINRTALNCNDSLNTGFLAALFYAHRFNASGCPERMASCRPIDKFAQG
WGPIAYAKPLSLDQKPYCWHYAPQPCGIVPASQVCGPVYCFTPSPVVVGTTDRFGVPTYSWGENETD
VLLLNNTRPPRGNWFGCTWMNGTGFTKTCGGPPCDIGGVGNDTLICPTDCFRKHPEATYTKCGSGPW
LTPRCMVDYPYRLWHYPCTVNFTIFKVRMYVGGVEHRLNAACNWTRGERCDLEDRDRSELSPLLLST
TEWQVLPCSFTTLPALSTGLIHLHRNIVDVQYLYGIGSAVVSYAIKWEYVLLLFLFLADARVCACLW
MMLLIVQAEAALENLVVLNAASVAGTHGILSFLVFFCAAWYIKGRLVPGAAYAFYGVWPLLLLLLAL
PPRAYAMDREMAASCGGAVFIGLALLTLSPHYKVFLARLIWWLQYFITRAEAHLQVWIPPLNVRGGR
DAIILLMCAIHPELIFAITKILLAILGPLMVLQAGITRVPYFVRAHGLIRACVLVREVAGGHYVQMA
LMRLAALTGTYVYDHLTPLRDWAHEGLRDLAVAVEPVVFSDMETKIITWGADTAACGDIILGLPVSA
RRGREILLGPADSLEGQGWRLLAPITAYAQQTRGLLGCIVTSLTGRDKNQVEGEVQVVSTATQSFLA
TCVNGVCWTVYHGAGTKTLAGQKGPITQMYTNVDQDLVGWLAPPGARSLTPCTCGSSDLYLVTRHAD
VIPVRRRGDSRGSLLSPRPVSYLKGSSGGPLLCPSGHAVGIFRAAVCTRGVAKAVDFVPVESMETTM
RSPVFTDNSSPPAVPQTFQVAHLHAPTGSGKSTKVPAAYAAQGYKVLVLNPSVAATLGFGAYMSKAH
GVDPNIRTGVRTITTGAPITYSTYGKFLADGGCSGGAYDIIICDECHSTDSTTILGIGTVLDQAETA
GARLVVLATATPPGSVTVPHPNIEEVALSNIGEIPFYGKAIPIETIKGGRHLIFCHSRKKCDELAAK
LSGLGLNAVAYYRGLDVSVIPTSGDVVVVATDALMTGFTGDFDSVIDCNTCVTQTVDFSLDPTFTIE
TTTVPQDAVSRSQRRGRTGRGRTGIYRFVTPGERPSGMFDSSVLCECYDAGCAWYELTPAETSVRLR
AYLNTPGLPVCQDHLEFWEGVFTGLTHIDAHFLSQTKQAGDNFPYLVAYQATVCARAQAPPPSWDQM
WKCLIRLKPTLHGPTPLLYRLGAVQNEVILTHPITKFIMACMSADLEVATSTWVLVGGVLAALAAYC
LTTGSVVIVGRIILSGKPAIVPDREVLYQQFDEMEECATHLPYIEQGMQLAEQFKQKALGLLQTATK
QAEAAAPVVESKWRALESFWAKHMWNFISGIQYLAGLSTLPGNPAIASLMAFTASITSPLTTQHTLL
FNILGGWVAAQLAPPSAASAFVGAGIAGAAVGSIGLGKVLVDILAGYGAGVAGALVAFKVMSGEMPS
TEDLVNLLPAILSPGALVVGVVCAAILRRHVGPGEGAVQWMNRLIAFASRGNHVSPTHYVPESDAAA
RVTQILSSLTITQLLKRLHQWINEDCSTPCSGSWLRDVWDWICTVLTDFKTWLQSKLLPRLPGVPFL
SCQRGYKGVWRGDGIMQTTCPCGAQITGHVKNGSMRIVGPRTCSNTWHGTFPINAYTTGPCTPSPAP
NYSRALWRVTAEEYLEVTQVGDFHYVTGMTTDNVKCPCQVPAPEFFTEVDGVRLHRYAPPCKPLLRE
EVTFQVGLNQYLVGSQLPCEPEPDVAVLTSMLTDPSHITAEAAKRRLARGSPPSLASSSASQLSAPS
LKATCTTRHDSPDADLIEANLLWRQEMGGNITRVESENKVVILDSFEPLRAEEDEREVSVPAEILRR
SRKFPRAMPIWARPDYNPPLVESWKDPDYVPPVVHGCPLPPTKAPPIPPPRRKRTVVLTESTVSSAL
AELATKTFGSSESSAVDSGTATAPPDQPSDDGDAGSDVESYSSMPPLEGEPGDPDLSDGSWSTVSEE
ASEDVVCCSMSYTWTGALITPCSAEESKLPINALSNSLLRHHNMVYATTSRSASQRQKKVTFDRLQV
LDDHYRDVLKEMKAKASTVKAKLLPVEEACKLTPPHSAKSKFGYGAKDVRNLSSKAVNHILSVWKDL
LEDTETPIDTTIMAKNEVFCVQPEKGGRKPARLIVFPDLGVRVCEKMALYDVVSTLPQAVMGSSYGF
QYSPGQRVEFLVNAWKKKKNPMGFAYDTRCFDSTVTESDIRVEESIYQCCDLAPEARQAIRSLTERL
YIGGPLTNSKGQNCGYRRCRASGVLTTSCGNTLTCYLKASAACRAAKLQDCTMLVCGDDLVVICESA
GTXEDAASLRVFTEAMTRYSAPPGDPPKPEYDLELITSCSSNVSVAHDASGKRVYYLTRDPTTPLAR
AAWETARHTPVNSWLGNIIMYAPTLWARMILMTHFFSILLAQEQLEKALDCQIYGACYSIEPLDLPQ
IIQRLHGLSAFSLHSYSPGEINRVAACLRKLGVPPLRVWRHRARSVRAKLLSQGGRAANCGKYLFNW
AVRTKLKLTPIPAASQLDLSSWFVAGYSGGDIYHSLSRARPRWFMLCLLLLSVGVGIYLLPNR

# Appendix 4

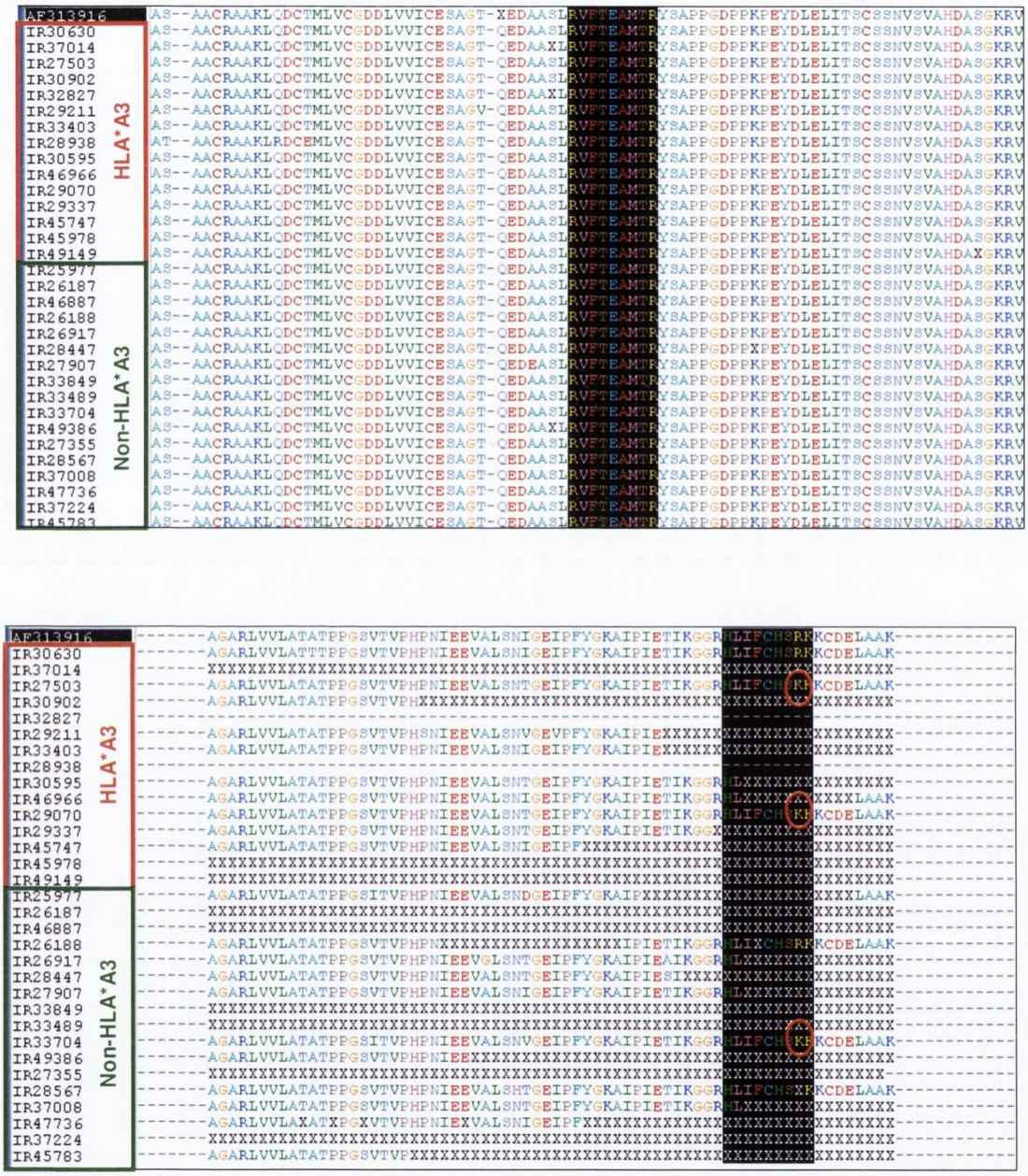
## AF054247, HCV 1b genotype, strain HC-J4, isolate pCV-J4L6S, complete genome

***HEPATITIS C VIRUS POLYPROTEIN*** (3010 amino acid) (9595 base pairs mRNA linear)

MSTNPKPQRKTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATRKASERSQPRGRRQPIPK
ARRPEGRAWAQPGYPWPLYGNEGLGWAGWLLSPRGSRPSWGPTDPRRRSRNLGKVIDTLTCGFADLM
GYIPLVGAPLGGAARALAHGVRVLEDGVNYATGNLPGCSFSIFLLALLSCLTIPASAYEVRNVSGIY
HVTNDCSNSSIVYEAADVIMHTPGCVPCVQEGNSSRCWVALTPTLAARNASVPTTTIRRHVDLLVGT
AAFCSAMYVGDLCGSIFLVSQLFTFSPRRHETVQDCNCSIYPGHVSGHRMAWDMMMNWSPTTALVVS
QLLRIPQAVVDMVAGAHWGVLAGLAYYSMVGNWAKVLIVALLFAGVDGETHTTGRVAGHTTSGFTSL
FSSGASQKIQLVNTNGSWHINRTALNCNDSLQTGFFAALFYAHKFNSSGCPERMASCRPIDWFAQGW
GPITYTKPNSSDQRPYCWHYAPRPCGVVPASQVCGPVYCFTPSPVVVGTTDRSGVPTYSWGENETDV
MLLNNTRPPQGNWFGCTWMNSTGFTKTCGGPPCNIGGVGNRTLICPTDCFRKHPEATYTKCGSGPWL
TPRCLVDYPYRLWHYPCTLNFSIFKVRMYVGGVEHRLNAACNWTRGERCNLEDRDRSELSPLLLSTT
EWQILPCAFTTLPALSTGLIHLHQNIVDVQYLYGVGSAFVSFAIKWEYILLLFLLLADARVCACLWM
MLLIAQAEAALENLVVLNAASVAGAHGILSFLVFFCAAWYIKGRLAPGAAYAFYGVWPLLLLLLALP
PRAYALDREMAASCGGAVLVGLVFLTLSPYYKVFLTRLIWWLQYFITRAEAHMQVWVPPLNVRGGRD
AIILLTCAVHPELIFDITKLLLAILGPLMVLQAGITRVPYFVRAQGLIRACMLVRKVAGGHYVQMVF
MKLGALTGTYVYNHLTPLRDWAHAGLRDLAVAVEPVVFSAMETKVITWGADTAACGDIILGLPVSAR
RGKEIFLGPADSLEGQGWRLLAPITAYSQQTRGVLGCIITSLTGRDKNQVEGEVQVVSTATQSFLAT
CINGVCWTVYHGAGSKTLAGPKGPITQMYTNVDLDLVGWQAPPGARSMTPCSCGSSDLYLVTRHADV
IPVRRRGDSRGSLLSPRPVSYLKGSSGGPLLCPSGHVVGVFRAAVCTRGVAKAVDFIPVESMETTMR
SPVFTDNSTPPAVPQTFQVAHLHAPTGSGKSTKVPAAYAAQGYKVLVLNPSVAATLGFGAYMSKAHG
IDPNIRTGVRTITTGGSITYSTYGKFLADGGCSGGAYDIIICDECHSTDSTTILGIGTVLDQAETAG
ARLVVLATATPPGSVTVPHPNIEEIGLSNNGEIPFYGKAIPIEAIKGGRHLIFCHSKKKCDELAAKL
TGLGLNAVAYYRGLDVSVIPPIGDVVVVATDALMTGFTGDFDSVIDCNTCVTQTVDFSLDPTFTIET
TTVPQDAVSRSQRRGRTGRGRSGIYRFVTPGERPSGMFDSSVLCECYDAGCAWYELTPAETSVRLRA
YLNTPGLPVCQDHLEFWESVFTGLTHIDAHFLSQTKQAGDNFPYLVAYQATVCARAQAPPPSWDQMW
KCLIRLKPTLHGPTPLLYRLGAVQNEVILTHPITKYIMACMSADLEVVTSTWVLVGGVLAALAAYCL
TTGSVVIVGRIILSGKPAVVPDREVLYQEFDEMEECASQLPYIEQGMQLAEQFKQKALGLLQTATKQ
AEAAAPVVESKWRALETFWAKHMWNFISGIQYLAGLSTLPGNPAIASLMAFTASITSPLTTQNTLLF
NILGGWVAAQLAPPSAASAFVGAGIAGAAVGSIGLGKVLVDILAGYGAGVAGALVAFKVMSGEVPST
EDLVNLLPAILSPGALVVGVVCAAILRRHVGPGEGAVQWMNRLIAFASRGNHVSPTHYVPESDAAAR
VTQILSSLTITQLLKRLHQWINEDCSTPCSGSWLRDVWDWICTVLTDFKTWLQSKLLPRLPGVPFLS
CQRGYKGVWRGDGIMQTTCPCGAQIAGHVKNGSMRIVGPRTCSNTWHGTFPINAYTTGPCTPSPAPN
YSRALWRVAAEEYVEVTRVGDFHYVTGMTTDNVKCPCQVPAPEFFTEVDGVRLHRYAPACKPLLRED
VTFQVGLNQYLVGSQLPCEPEPDVTVLTSMLTDPSHITAETAKRRLARGSPPSLASSSASQLSAPSL
KATCTTHHDSPDADLIEANLLWRQEMGGNITRVESENKVVILDSFEPLHAEGDEREISVAAEILRKS
RKFPSALPIWARPDYNPPLLESWKDPDYVPPVVHGCPLPPTKAPPIPPPRRKRTVVLTESNVSSALA
ELATKTFGSSGSSAVDSGTATALPDLASDDGDKGSDVESYSSMPPLEGEPGDPDLSDGSWSTVSEEA
SEDVVCCSMSYTWTGALITPCAAEESKLPINPLSNSLLRHHNMVYATTSRSASLRQKKVTFDRLQVL
DDHYRDVLKEMKAKASTVKAKLLSIEEACKLTPPHSAKSKFGYGAKDVRNLSSRAVNHIRSVWEDLL
EDTETPIDTTIMAKSEVFCVQPEKGGRKPARLIVFPDLGVRVCEKMALYDVVSTLPQAVMGSSYGFQ
YSPKQRVEFLVNTWKSKKCPMGFSYDTRCFDSTVTESDIRVEESIYQCCDLAPEARQAIRSLTERLY
IGGPLTNSKGQNCGYRRCRASGVLTTSCGNTLTCYLKATAACRAAKLQDCTMLVNGDDLVVICESAG
TQEDAAALRAFTEAMTRYSAPPGDPPQPEYDLELITSCSSNVSVAHDASGKRVYYLTRDPTTPLARA
AWETARHTPINSWLGNIIMYAPTLWARMILMTHFFSILLAQEQLEKALDCQIYGACYSIEPLDLPQI
IERLHGLSAFTLHSYSPGEINRVASCLRKLGVPPLRTWRHRARSVRAKLLSQGGRAATCGRYLFNWA
VRTKLKLTPIPAASQLDLSGWFVAGYSGGDIYHSLSRARPRWFPLCLLLLSVGVGIYLLPNR

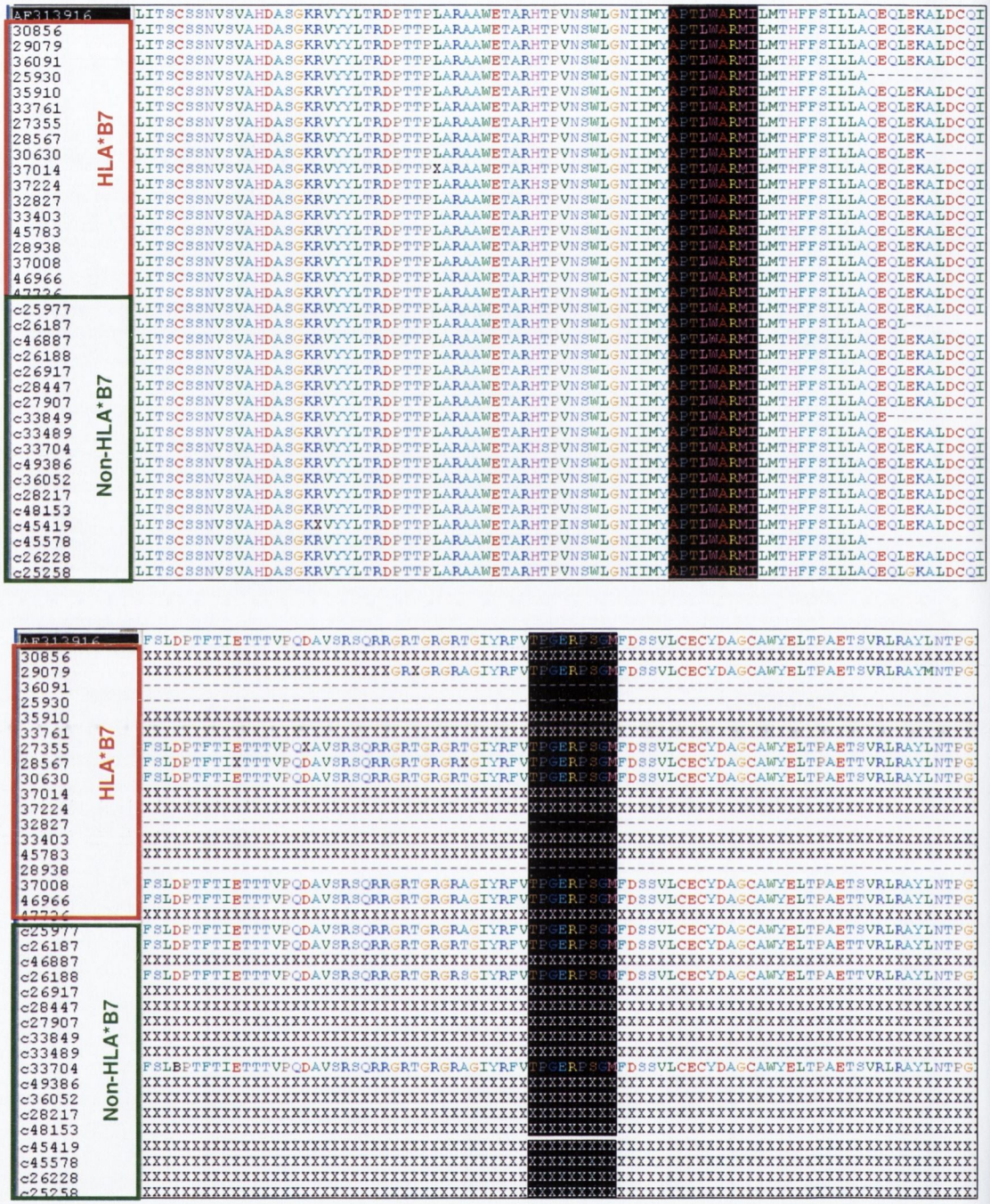
# Appendix 5

## 5.1 HLA*A3 restricted epitopes

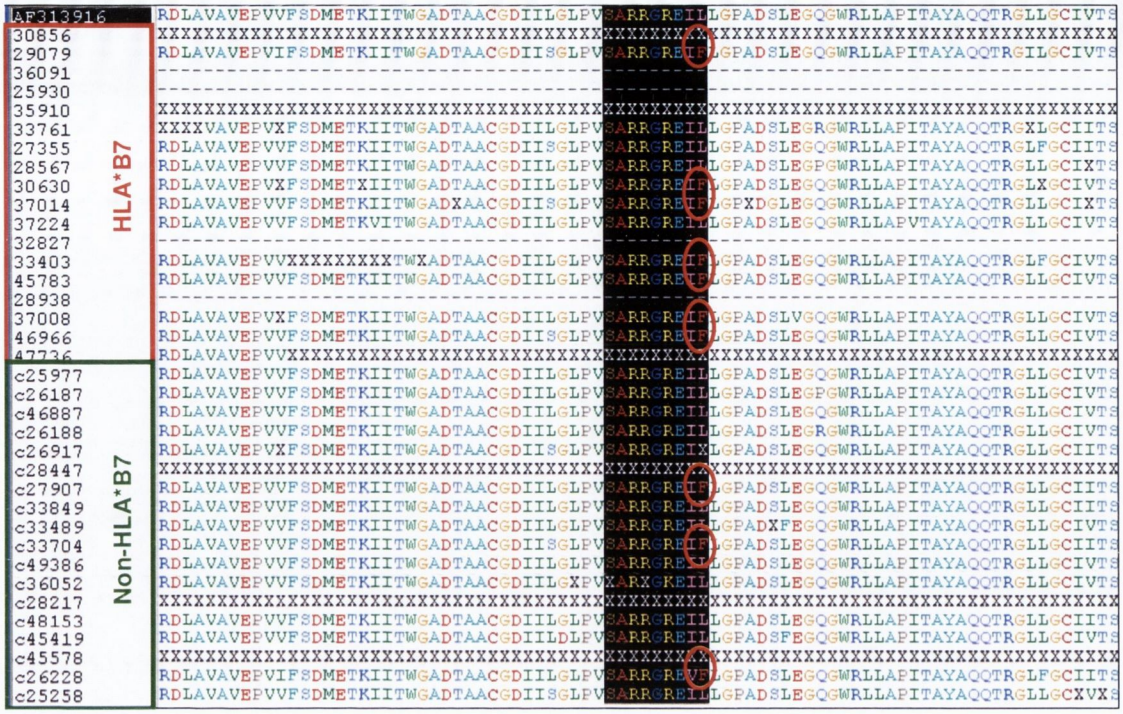



**Upper panel represents highly conserved epitope. Lower panel shows poorly sequenced region and mutation (arginine-R to lysine-K in both groups) towards consensus HCV sequence.** Sequences alignment is taken from BioEdit software package (donor sequence is on the top – AF313916; patients samples are in red box; control samples are in green box).

## 5.2 HLA*B7 restricted epitopes

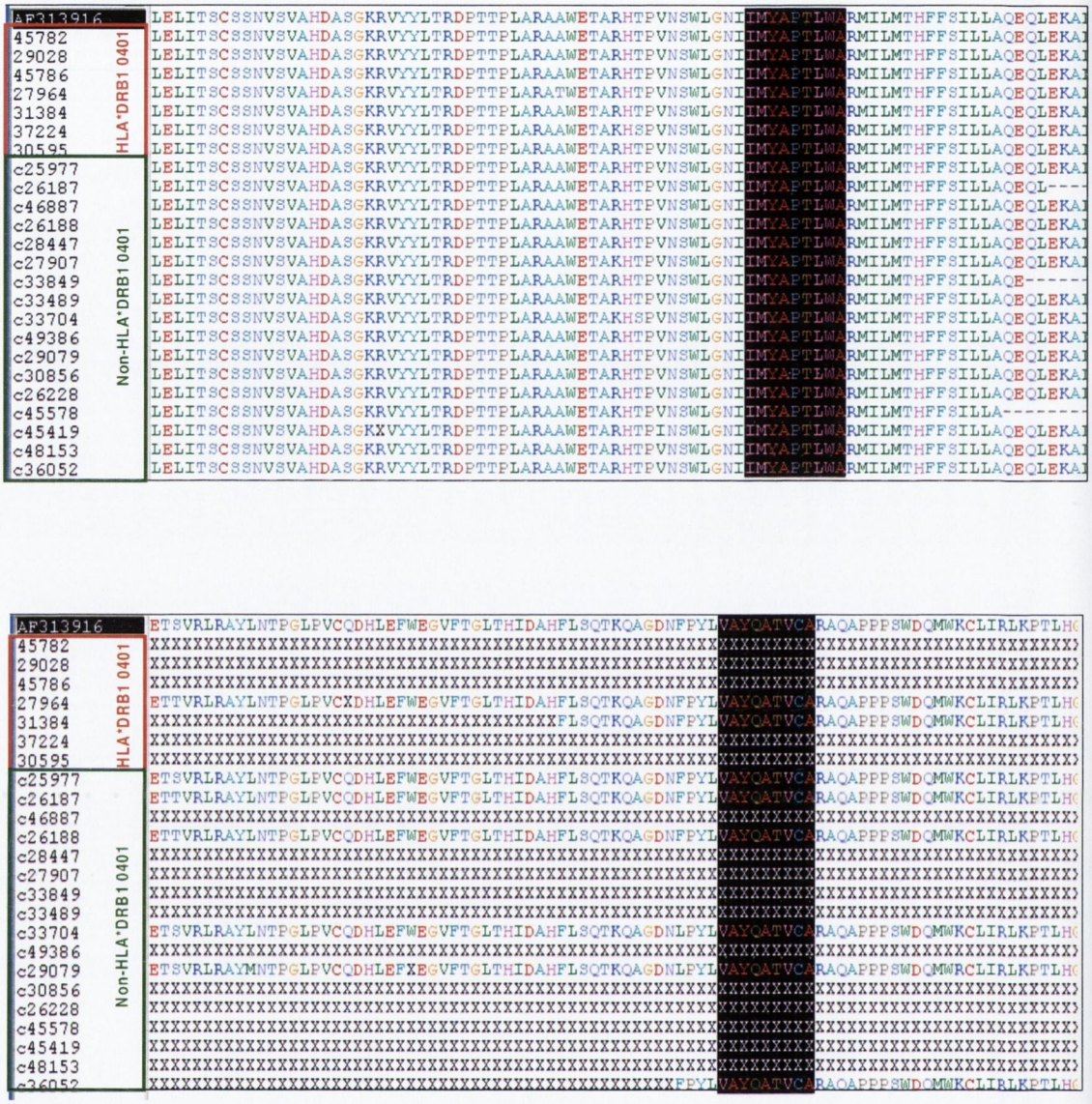



**Upper panel represents highly conserved epitope. Lower panel shows lack of sequencing data.**




**This panel shows substitution of leucine (L) to phenylalanine (F) in patient and control group.** This substitution is aligned with consensus HCV sequence. Sequences alignment is taken from BioEdit software package (donor sequence is on the top – AF313916; patients samples are in red box; control samples are in green box).

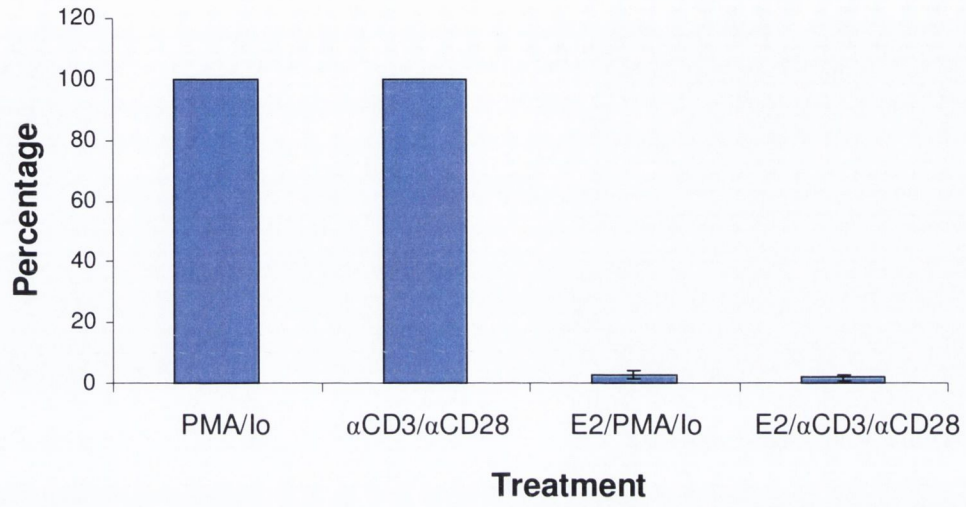
## 5.3 HLA*DRB1 0401 restricted epitopes




**Upper panel represents highly conserved epitope. Lower panel shows lack of sequencing data.** Sequences alignment is taken from BioEdit software package (donor sequence is on the top – AF313916; patients samples are in red box; control samples are in green box).

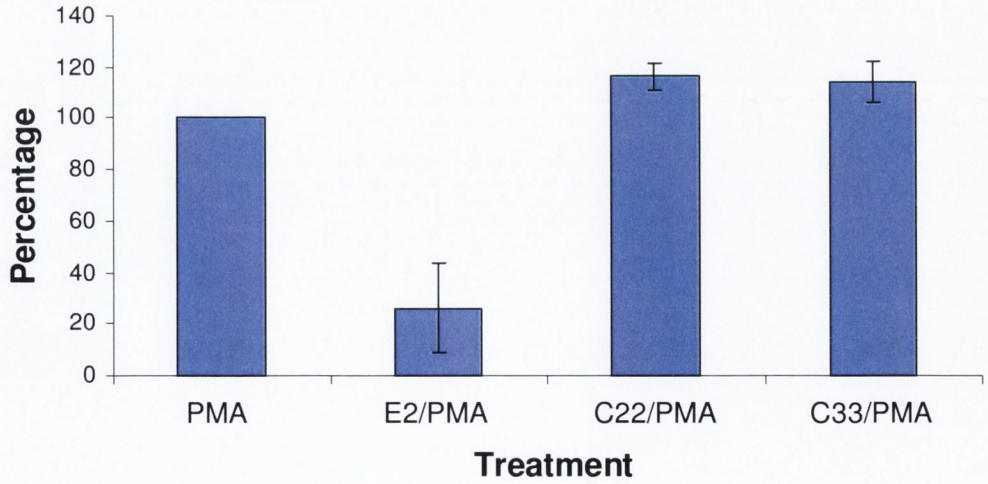
# Appendix 6

## 6.1 The envelope proteine (E2) of the HCV inhibits IL-2 secretion from PBMCs.



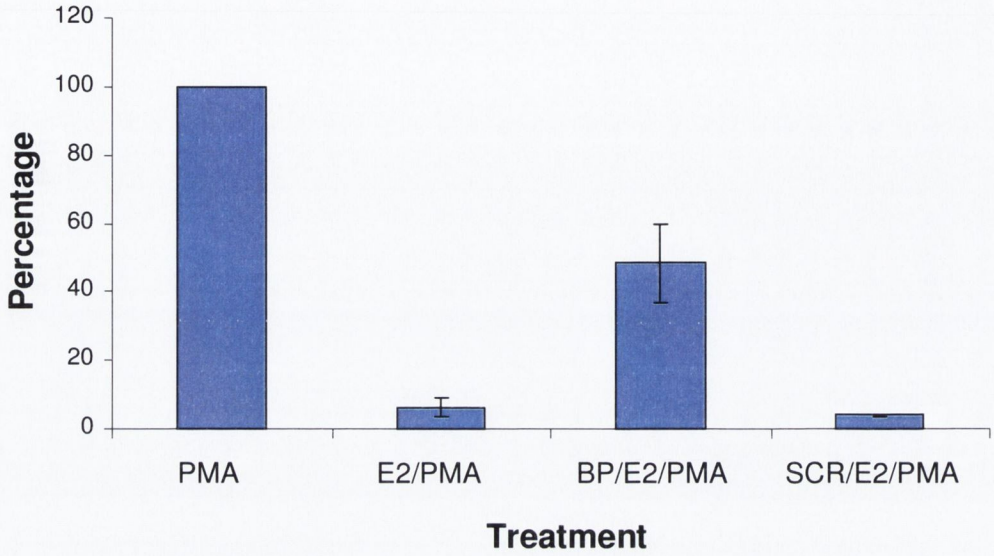

This graph indicates the mean percentage inhibition in IL-2 secretion in stimulated PBMCs, pretreated with E2, for three independent experiments.

## 6.2 Pre-treatment of HuT78 cells with HCV envelope protein E2 but not core peptide C22 and NS3 peptide C33 inhibited PMA-stimulated Il-2 secretion.



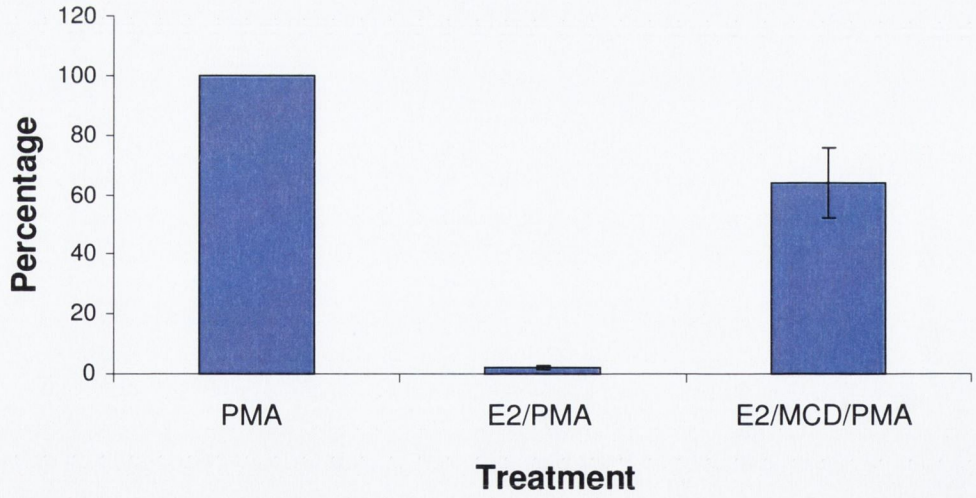

This graph indicates the mean percentage IL-2 secretion in stimulated HuT 78 cells, pretreated with E2 or C22 or C33, for three independent experiments.

**6.3 A blocking peptide (BP) which can abolish E2 binding to CD81 can rescue IL-2 secretion.**



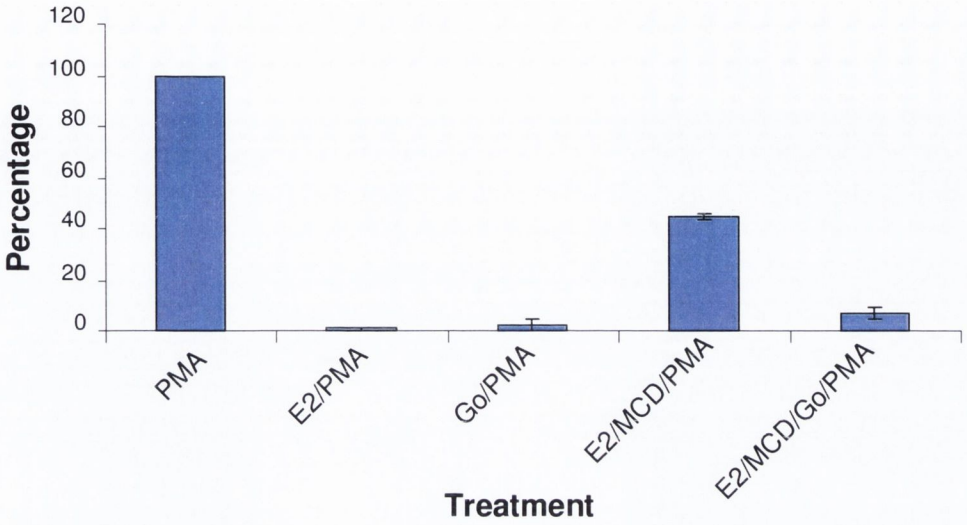

This graph indicates the mean percentage recovery in IL-2 secretion in HuT 78 cells in the presence of BP for the three independent experiments.

**6.4 MCD treatment annuls the inhibitory effect of E2 on IL-2 secretion.**




This graph indicates the mean percentage recovery in IL-2 secretion in HuT 78 cells in the presence of MCD for the three independent experiments.

**6.5 PKC-β selective inhibitor Go6976 prevents the MCD induced recovery of IL-2 secretion.**




This graph represents the mean percentage IL-2 secretion in the presence of Go6976 inhibitor for the three independent experiments.