DEVELOPMENT OF AN IMMUNOTHERAPY TO TREAT PERSISTENT HEPATITIS C VIRUS INFECTION

A thesis submitted to the Faculty of Medicine, Nursing and Health Sciences, Monash University, in accordance with the requirements for the degree of Doctor of Philosophy

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Abstract

Hepatitis C Virus (HCV) infection causes liver disease, affecting approximately 200 million people worldwide. In most individuals acute HCV infection is asymptomatic prior to the development of persistent infection in approximately 80% of infected individuals leading to life-long infection. This results in active inflammation, fibrosis and cirrhosis of the liver, in some cases developing into hepatocellular carcinoma. In the remaining 20% of infected patients, spontaneous clearance of the virus is believed to be due to a broad, multi-specific CD4 and CD8 T cell response. Despite the advent of new direct acting antivirals which cure a high proportion of treated patients, many patients will not be cured or will not be treated due to the high cost of therapy. Therefore it is important to investigate immunotherapeutic strategies to combat persistent HCV infection.

This thesis focuses primarily on immunotherapeutic methods involving the use of dendritic cells (DC), natural immune sentinels, to illicit an HCV specific immune response. Chapter 3 investigated the anti-HCV immune responses of 6 persistently infected HCV positive patients after administration of autologous monocyte derived DC pulsed with lipopeptides which represent HLA-A2 restricted CTL epitopes. The results of this study showed that the patients developed transient HCV specific responses including responses to HCV peptides not contained within the vaccine.

Chapters 4 investigated the use of RNA to endogenously express entire HCV proteins in dendritic cells, using a murine DC cell line as a surrogate to assess different transfection protocols, before application in human monocyte derived DC. Ultimately, although effective protocols for the transfection of the murine cell line were developed, these were inefficient in human monocyte derived DC, highlighting the difficulty in working with these cells. However, the findings of this chapter led to the development of the hypothesis

that cells expressing HCV antigens, which are made necrotic, would be powerful immunogens.

In chapter 5 the technology explored in chapter 4 was used to transfect the murine DC cell line with an RNA construct encoding the HCV NS3 protein. These cells were then made necrotic and injected into mice. This led to high, specific responses to NS3, highlighting the effective adjuvant activity of necrotic cells.

In order to apply this strategy in humans, an appropriate vector to express HCV proteins in monocyte derived DC was required. Consequently, in chapter 6 an effective method involving the pre-treatment of DC with the detergent, polybrene, prior to adenoviral transduction was found to be effective and resulted in high transfection efficiencies. Further experiments in Chapter 6 developed a GMP-like protocol to generate recombinant adenoviruses for large scale applications.

Finally in chapter 7, the results of a dose escalation study of HCV antigen- positive, monocyte derived necrotic DC, administered to persistently infected HCV positive patients is presented. Minimal transient HCV-specific immune responses were observed throughout the monitoring period.

In summary this thesis presents work resulting in the development of an effective and efficient method of transducing monocyte derived DC with a recombinant adenovirus using lower multiplicities of infection than reported previously by others, the development of a method for the manufacture and purification of recombinant adenoviruses for clinical scale production to GMP standard, and the results from two first in man studies utilising monocyte derived DC for the treatment of HCV.

General Declaration

This work was conducted at the Burnet Institute in Melbourne towards a Doctor of Philosophy through the Department of Microbiology, School of Biomedical Sciences, Faculty of Medicine, Nursing and Health Sciences at Monash University.

I declare and certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in the submission for any other degree or diploma in any university or other tertiary institution without the prior approval of Monash University and where applicable, any partner institution responsible for the joint-award of this degree.

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List of Publications

The following manuscripts were derived in whole or in part from experiments and data derived from this PhD thesis.

Kathryn L. Jones, Lorena E. Brown, Emily M.Y. Eriksson, Rose A. Ffrench**, Philippe A. Latour**, Bruce E. Loveland, Dominic M. Wall, Stuart K Roberts, David C. Jackson, Eric J. Gowans (2008). *Human dendritic cells pulsed with specific lipopeptides stimulate autologous antigen-specific $CD8^+$ T-cells without the addition of exogenous maturation factors.* J Viral Hepatitis 15: 761-72.

Gowans EJ, Roberts S, Jones K, Dinatale I, **Latour PA**, Chua B, Eriksson EMY, Chin R, Li S, Wall DM, Sparrow RL, Moloney J, Loudovaris M, Ffrench R, Prince HM, Hart D, Zeng W, Torresi J, Brown LE, Jackson DC (2010). *A phase I clinical trial of dendritic cell immunotherapy in HCV-infected individuals.* J Hepatology. 53(4): 599-607.

Li S, Roberts S, Plebanski M, Gouillou M, Spelman T, **Latour P**, Jackson D, Brown L, Sparrow R, Prince M, Hart D, Loveland B, Gowans EJ (2012). *Induction of multifunctional T cells in a Phase 1 clinical trial of dendritic cell immunotherapy in hepatitis C virus infected individuals*. PLoS One. 2012; 7(8):e39368. Epub 2012 Aug 14.

Philippe A Latour , Branka Grubor-Bauk, Shuo Li, Joseph Torresi, Paula Lewis, Stuart Roberts, Bruce E Loveland and Eric J Gowans (2014). *Immunotherapy with necrotic HCV antigen-positive dendritic cells is safe and does not induce any adverse events in HCV patients*. In preparation.

List of Abbreviations

Ad5 – Adenovirus Serotype 5

ALT - Aminotransferase

APC – Antigen presenting cells

bDNA – branched chain DNA

BH3 – Bcl-2 homology 3

BSE – Bovine spongiform encephalopathy

cDC – conventional dendritic cell

CAR – Coxsackie adenovirus receptor

CFSE - carboxyfluorescein succinimidyl ester

CMI – Cell mediated immunity

CTL – Cytotoxic T lymphocyte

CTLA-4 – Cytotoxic T lymphocyte Antigen 4

DAA – Direct acting antiviral

DAMP – Damage associated molecular pattern

DC - Dendritic cell

DC2.4 – Dendritic cell 2.4 (murine cell line)

DU – Dose unit

EIA – Enzyme immunoassay

ELIspot – Enzyme linked immunospot

EMCV - Encephalomyocarditis virus

ER – Endoplasmic reticulum

FCS – Foetal calf serum

GFP – Green fluorescent protein

GM-CSF – Granulocyte macrophage colony stimulating factor

GMP – Good manufacturing practice

gt1 – Genotype 1

HBsAg – Hepatitis B surface antigen

HCC – Hepatocellular carcinoma

HCV – Hepatitis C virus

HEK – Human embryonic kidney

HMGB1 – High mobility group box 1

HREC – Human research ethics committee

HRP – Horse radish peroxidase

HSA – Human serum albumin

HSP – Heat shock protein

Huh8 – Human hepatoma 8

HVR – Hyper variable region

ID - Intradermal

iDC – immature dendritic cell

IFN - Interferon

IFN-α - Interferon alpha

IFN-γ – Interferon gamma

IL-1 – Interleukin 1

IL-4 – Interleukin 4

IL-10 – Interleukin 10

IL28B – Interleukin 28 B

IRES – Internal ribosome entry site

IV - Intravenous

JFH1 – Japanese fulminant hepatitis virus 1

LB – Luria broth

LCMV - Lymphocytic choriomeningitis virus

LDLR – Low density lipoprotein receptor

mAb – Monoclonal antibody

Mcl-1 – Myeloid cell factor 1

MCS – Multi cloning site

mDC – Mature dendritic cell

MHC – Major histocompatibility Ccomplex

MLTU – Major late transcription unit

MLR – Mixed lymphocyte reaction

Mo-DC – Monocyte derived dendritic cell

MOI – Multiplicity of infection

mRNA – Messenger RNA

Mut - Mutant

NFW – Nuclease free water

ORF - Open reading frame

OxMan-PEI – Oxidised mannan polyethylenimine

pAb – Polyclonal antibody

PAMP – Pathogen associated molecular pattern

PBMC – Peripheral blood mononuclear cells

PCD – Programmed cell death

PD-1 – Programmed cell death 1

pDC – Plasmacytoid dendritic cell

PD-L1 – Programmed death ligand 1

peg-IFN-α – Pegylated interferon alpha

PEI - Polyethylenimine

PHA - Phytohaemagglutinin

PCR – Polymerase chain reaction

RecAd – Recombinant adenovirus

RT – Room temperature

SFU – Spot forming units

SR-BI – Scavenger receptor class B type 1

SVR – Sustained virological response

TCR – T cell receptor

TLR – Toll-like receptor

TNFα – Tumor necrosis factor alpha

TP – Terminal protein

TRAIL – TNF-related apoptosis inducing ligand

UTR – untranslated region

WT – wild type

Chapter 1 – Literature Review

1.1 Introduction

Hepatitis C Virus (HCV) infection causes liver disease, affecting approximately 200 million people worldwide. Discovered in 1989, HCV has since been accepted as a major cause of chronic liver disease and is the leading single indication for liver transplantation in developed countries. Hepatitis C virus infection in most individuals has an asymptomatic progression prior to the development of persistent infection and liver disease; 20% of infected individuals develop an acute, self-limited acute infection (Gerlach et al., 2003), however in the remaining 80% of cases persistent, life-long infection occurs. This results in active inflammation, fibrosis and eventually cirrhosis. Persistent HCV infection is also strongly associated with the development of hepatocellular carcinoma. The mechanism behind the development of chronicity is still unclear, but it is likely to be multi-factorial. However, an important feature in those patients who resolve the infection includes a strong, multi-specific T cell response to several of the HCV proteins, whereas patients who develop persistent infection have a weak, limited response.

Until recently, the standard of care for HCV infection involves the administration of ribavirin and pegylated interferon-α (peg-IFN-α), which is accompanied by a number of adverse side effects. Furthermore, approximately only 50% of cases are responsive to therapy (Cornberg et al., 2002); for the remaining patients, treatment options remain limited despite the introduction of expensive direct acting antivirals (DAA).

In persistent HCV infection, the frequency of virus-specific T-lymphocytes is low. It has been suggested that a defect in T-cell priming by dendritic cells (DC) may contribute to the poor HCV-specific cell-mediated immune response (Auffermann-Gretzinger et al., 2001, Bain et al., 2001).

1.2 Hepatitis C Virus

HCV was first identified in 1989 from the serum of a patient with post-transfusion non-A, non-B hepatitis. The serum was immuno-screened against an expression library (made from random-primed complementary DNA) constructed from plasma containing the virus (Choo et al., 1989). The virus however was not visualized, and low viral titres in serum and liver tissue precluded biochemical characterization of native viral products. Perhaps most importantly, it was not possible to culture HCV efficiently *in vitro*, impeding the elucidation of the viral lifecycle and the development of specific antiviral agents and preventive vaccines.

Six HCV genotypes have been generally recognised (2002, Colin et al., 2001, Penin et al., 2004, Simmonds et al., 2005) that differ in their nucleotide sequence by 30-35%, although an additional genotype was reported recently (Smith et al., 2014). Within each HCV genotype there are also a large number of subtypes (designated a, b, c, *etc*.) which differ in their nucleotide sequence by 20-25% (Simmonds et al., 2005). Humans are the only known natural host of HCV, but the virus is known to be able to infect chimpanzees. All genotypes currently identified are hepatotropic and pathogenic (Simmonds, 1995).

1.2.1 HCV Diagnosis and Current Therapy

Acute and persistent HCV infections are relatively asymptomatic, making diagnosis of infection difficult. Patients with a suspected case of acute HCV infection are tested for both anti-HCV antibodies by enzyme immunoassays (EIA) and HCV RNA with a sensitive technique such as an HCV RNA assay (e.g. PCR) with a lower limit of detection of 50 IU/mL or less (Chevaliez and Pawlotsky, 2007). Four profiles exist depending on the presence or absence of either marker (antibodies and HCV-RNA) (Chevaliez and Pawlotsky, 2007).

1.) The presence of HCV RNA in the absence of anti-HCV antibodies, which may be indicative of acute HCV infection. The later appearance of anti-HCV antibodies (seroconversion) can confirm this; however, this profile may also be indicative of a persistent infection.

2.) The presence of HCV RNA and anti-HCV antibodies at the time of diagnosis. It is difficult to distinguish between persistent and acute Hepatitis C, and this can only be ascertained from case history.

3.) The absence of HCV RNA with detectable anti-HCV antibodies. The HCV RNA can be temporarily undetectable due to transient partial control of viral replication before infection becomes chronic. Therefore, patients with this profile need to be retested after a few weeks. However, if the HCV RNA is negative, this pattern generally suggests that the patient has recovered from a past HCV infection.

4.) The absence of both HCV RNA and anti-HCV antibodies, suggests no current or prior HCV infection in the patient.

Patients with detectable HCV RNA are considered for peg-IFN-α and ribavirin combination therapy NIH (2002) using peg-IFN-α and an oral nucleoside broad-spectrum antiviral known as Ribavirin (Katayama et al., 2001, Okuse et al., 2005). This therapy results in sustained biochemical and virological response in 40-50% of patients infected with HCV genotype 1 (Cornberg et al., 2002), and up to 80% of those infected with HCV genotypes 2 and 3 (Cornberg et al., 2002). As a proportion of patients spontaneously clear the infection in the acute phase, the current strategy involves monitoring without treatment for 3-6 months, measuring viral load (by RT-PCR) and alanine aminotransferase (ALT) levels (an indicator of liver damage), and treating those with persistent viremia (Alberti et al., 2002).

The treatment of patients with chronic hepatitis C is highly dependent on a number of factors including the severity of liver disease, the possibility of treatment having no effect, and the patient's willingness to be treated. The HCV genotype determines the duration of treatment, the dose of ribavirin, and also the virological monitoring procedure and therefore must be determined before treatment (Hadziyannis et al., 2004).

There is no standard treatment for patients with acute Hepatitis C. The asymptomatic nature of the disease and difficulty in the identification of ideal candidates for treatment makes it difficult to enlist candidates for clinical trials. Potential candidates who are found show high risk behaviour such as intravenous drug use (Chevaliez and Pawlotsky, 2007). Hence, there remains a lack of comparative studies regarding the appropriate regimen for treatment.

Interferon-alpha (IFN-α) was first shown to have beneficial effects in patients with chronic non-A, non-B infection in 1986 (Hoofnagle et al., 1986), well before HCV was identified as the major cause of non-B, non-B hepatitis. The role of IFN became clear after the development of assays for HCV detection in the serum, as it became evident that IFN-α therapy led to a rapid decline in the levels of HCV RNA in serum and the sustained loss of HCV RNA from the serum and liver hallmarked long- term responses (Lau et al., 1998). Studies utilising monotherapy with IFN-α have shown very limited responses, with a maximum response rate of about 20% (Di Bisceglie and Hoofnagle, 2002). The introduction of peg-IFN and the broad-spectrum antiviral Ribavirin, increased the proportion of patients achieving a sustained virological response (SVR); which is defined as a loss of detectable HCV RNA during treatment and sustained absence for 24 weeks after completing therapy (Feld and Hoofnagle, 2005), increasing to 54-56% in selected populations (Hadziyannis et al., 2004, Manns et al., 2001, Fried et al., 2002). Peg-IFN is believed to act like endogenously produced IFN in that it induces IFN-stimulated genes which establish a non-virus specific antiviral state within cells, however its effects are more pronounced due to the higher concentrations achieved in conjunction with the improved pharmacokinetic profile given to it by virtue of the covalently linked polyethylene glycol (PEG) (Glue et al., 2000). The role of Ribavirin is less clear but it is believed to act in 3 possible ways which include direct inhibition of HCV replication (by inhibition of HCV RNA polymerase) (Maag et al., 2001), disruption of RNA synthesis by competing with inosine monophosphate dehydrogenase which is essential for viral RNA synthesis (Lau et al., 2002), and finally as a viral mutagen, causing a higher frequency of

mutations which pushes the virus towards producing defective viral particles (Crotty et al., 2001). Combination therapy with peg-IFN-α and Ribavirin is the standard of care for persistent HCV infection for most genotypes, however the advent of new DAA has provided further options for those infected with genotype 1 (Wilby et al., 2012).

There are three classes of response to combination therapy. These are defined as Non responders who retain a high viral load throughout treatment, however some patients experience a slight drop in viral load while on treatment, Relapsers who attain undetectable levels of HCV RNA during treatment however relapse upon the discontinuation of treatment, and then finally those who attain SVR hallmarked by a drop in HCV RNA to undetectable levels that are maintained after treatment.

It is not yet possible to predict which category individual patients will fall into; however a range of both host and viral factors may determine patient outcome. These factors include sex (females faring better), age, degree of liver fibrosis, weight, ethnic origin, co-morbidities such as alcohol abuse, renal disease and HIV infection; HCV genotype (Genotypes 2 and 3 having much better outcomes), viral load, quasispecies diversity and disease progression (acute versus persistent) all playing a role in determining the likelihood of attaining SVR (Feld and Hoofnagle, 2005).

Recently a polymorphism in the gene encoding IL28B was identified as a predictor for peg-IFN-α and ribavirin treatment-induced viral clearance. The polymorphism residing 3kb upstream of the Il28B gene, encoding IFN-λ-3, on chromosome 19, rs12979860, was

found to be strongly associated with SVR in all patient groups examined (Ge et al., 2009). The genotype CC was associated with SVR in approximately 79% of patients regardless of ethnicity, compared with 38% for the TC allele and 26% for the TT allele (Ge et al., 2009). The polymorphism also partially explains the reduced response to therapy in certain ethnic populations, for example those of African descent have generally been associated with poor outcomes following treatment, and it was found that this polymorphism occurs at a very low frequency in this population, whereas those of Asian or European descent have a very high frequency of the C allele, and consequently better documented outcomes following treatment (Ge et al., 2009).

Although peg-IFN-α and ribavirin combination therapy has long been the standard of care for treatment of HCV, the recent development of two direct-acting antiviral agents approved for use by the American Food and Drug Administration are set to change the standard of care in certain patients. The NS4/4A protease inhibitors telaprevir (Incivek; Vertex Pharmaceuticals Cambridge, MA, USA) and boceprevir (Victrelis; Merck & Co Whitehouse Station, NJ, USA) have been indicated for use in genotype 1 infected patients (Welsch et al., 2012). These drugs act to directly inhibit the HCV NS3/4A protease, unlike the non-specific antiviral activity of peg-IFN-α and ribavirin. Due to the nature of HCV and its error prone replication, neither of these drugs is therefore suitable for use as a monotherapy (Wilby et al., 2012). Triple therapy consisting of peg-IFN-α, ribavirin and telaprevir or boceprevir results in SVR in approximately 80% of patients in selected groups (Welsch *et al.,* 2012). Telaprevir and Boceprevir are the most advanced DAAs currently available however numerous other classes of drugs are undergoing

clinical trials including other forms of NS3/4A protease inhibitors, Nucleoside and non-nucleoside NS5B polymerase inhibitors, NS5A inhibitors, New Interferons and indirect inhibitors with unknown mechanisms of action (Vermehren and Sarrazin, 2011). All these have varying degrees of success however what is apparent is that none are suitable as a monotherapy to treat persistent HCV infection, and are also presenting additional side effects some of which are life threatening (Vermehren and Sarrazin, 2011), and resulting in the generation of new resistant variants of HCV. In fact it is becoming increasingly apparent that a cure may only ever be obtained through the use of a combination of these compounds.

1.2.2 Hepatitis C Virus Molecular Biology

Hepatitis C virus is an enveloped positive stranded RNA virus, primarily infecting the liver. The virus belongs to the genus *Hepacivirus*, in the *Flaviviridae* family which also includes the classical *flaviviruses*, animal *pestiviruses* and the GB viruses. The 9.6 kb genome consists of a 5'-untranslated region (UTR) which includes an Internal Ribosome Entry Site (IRES), a single open reading frame (ORF) which encodes a polyprotein of approximately 3000 amino acids forming 10 viral gene products (Thimme et al., 2006), and finally a 3'-UTR as can be seen in Figure 1.1. The viral gene products are separated according to their function, into structural and non-structural proteins. The structural

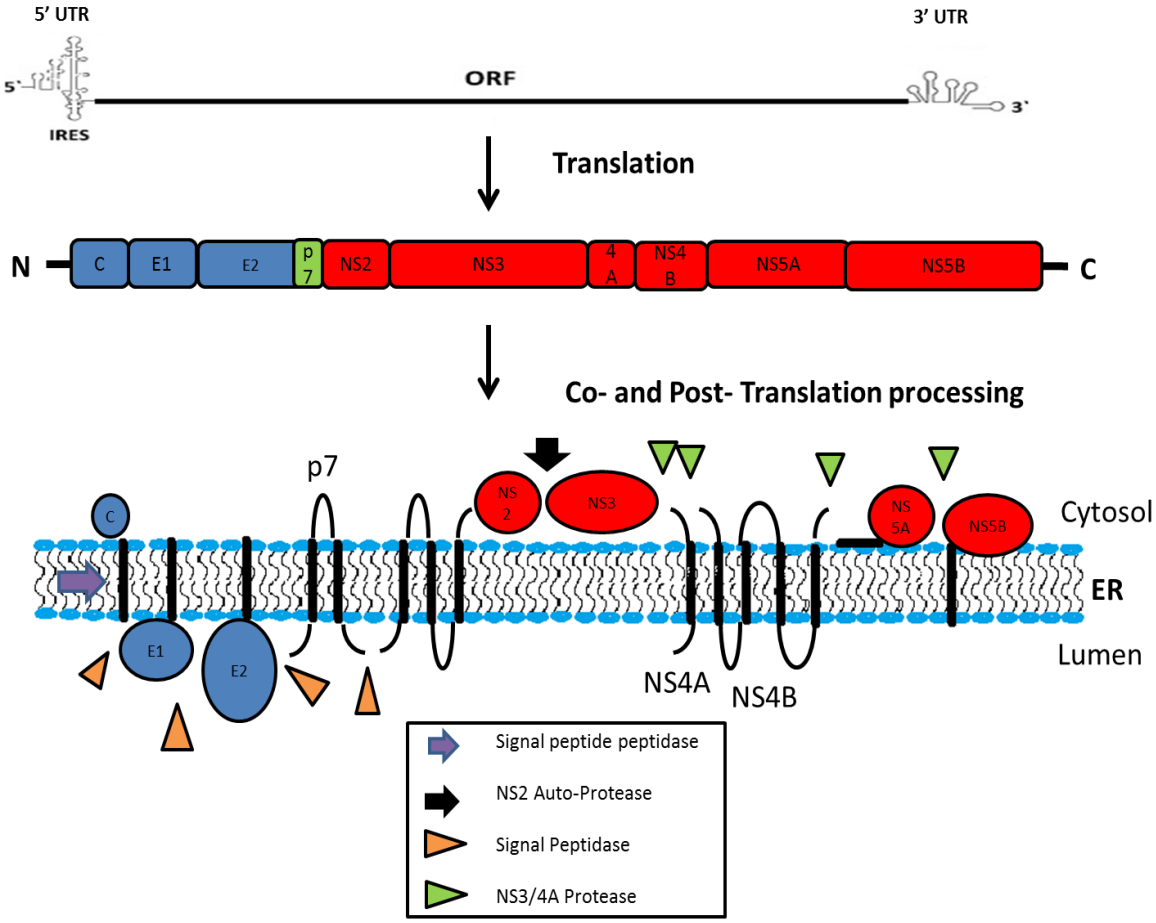

Figure 1.1. HCV genome translation and processing. The HCV genome (top panel) contains a 9.6kb open reading frame (ORF) flanked by 5' and 3'un-translated regions (UTR). The Internal Ribosome Entry Site (IRES) is located within the 5' UTR. The polyprotein generated from translation produces a ~3000aa polypeptide containing the structural proteins Core, E1 and E2 (in blue), p7 (in green), and the non-structural proteins NS2, NS3, NS4A, NS5A and NS5B (in red). The polyprotein then undergoes co- and post-translational processing (as indicated in the bottom panel) to form the mature viral proteins. Modelled on a figure from (Atoom et al., 2014).

proteins which form the viral particle are the nucleocapsid (known as Core) and the envelope glycoproteins E1 and E2. The non-structural proteins include p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B.

The 5′-UTR is highly conserved among different HCV isolates. This region is composed of four highly ordered domains numbered I to IV of which domains I and II are essential for HCV RNA replication and synthesis (Friebe et al., 2001). This section of the genome contains the HCV IRES element that is required for the cap-independent translation of the viral RNA which is formed by domains II, III, IV and the first 24-40 nucleotides of Core. The ORF translation initiation codon is located in domain IV.

The 3′-UTR is composed of 3 main regions. The first is a short variable region, the second is a poly(U/UC) tract (average length of 80 nucleotides), and the third is designated the X-Tail and is an almost invariant 98 nucleotide RNA element (Kolykhalov et al., 1996, Tanaka et al., 1996). Conserved elements in the 3′-UTR are essential for replication and synthesis both *in vitro* (Friebe et al., 2001) and *in vivo* (Kolykhalov et al., 2000, Yanagi et al., 1999).

The virion structure of HCV has recently been described (Catanese et al., 2013). Core, E1, and E2 are believed to be the principal protein components of the virion. It is presumed that E1 and E2 are in a double-layer lipid envelope derived from the host cell, and this surrounds a nucleocapsid composed of the core protein around the genomic RNA.

1.2.2.1 HCV Core protein

The first structural protein encoded by the HCV ORF is the core protein. Core protein is an RNA-binding α-helical protein (Boulant et al., 2006, Moradpour et al., 2007) believed to form the viral nucleocapsid. The core protein is comprised of two domains: an N-terminal hydrophilic domain (domain 1) making up two-thirds of the protein, and a C-terminal hydrophobic domain (domain 2) which makes the other third of the protein (Blight et al., 2000). Domain 1 contains mainly basic residues with two hydrophobic regions and has similar characteristics to the capsid proteins of related pestiviruses and flaviviruses (Blight et al., 2000, Lohmann et al., 1999, Bartosch et al., 2003a). Domain 2 is required for proper folding of domain D1 and is critical for the membrane characteristics of core (Bartosch et al., 2003a, Lohmann et al., 1999). This domain is present in GB virus B but is absent in the pestiviruses and flaviviruses (Hsu et al., 2003, Lohmann et al., 1999).

The core protein can be found on membranes of the endoplasmic reticulum (ER), in membranous webs, and on the surface of lipid droplets (Blight et al., 2000, Zhong et al., 2005a). Association with lipid droplets, which is mediated by the Domain 2, ensures folding of the protein (Boulant et al., 2006), and furthermore may have a role during viral replication and/or virion morphogenesis (Moradpour et al., 2007). There is speculation that the interaction of core with lipid droplets might affect the metabolism of lipids, contributing to the development of liver steatosis (Lerat et al., 2002), which is often seen in HCV infection (especially in cases of genotype 3 infection) (Moradpour et al., 2007).

The mechanisms of HCV nucleocapsid assembly are unclear, with i*n vitro* and *de novo* synthesis experiments yielding irregular particles and nucleocapsid like particles (Klein et al., 2004, Kunkel et al., 2001). The core protein interacts with a variety of cellular proteins and appears to influence numerous host cell functions including cell signalling, apoptosis, carcinogenesis and lipid metabolism (McLauchlan, 2000, Ray and Ray, 2001, Tellinghuisen and Rice, 2002, Weiner et al., 1991). However, it is unclear if these interactions occur in the course of a normal infection or are experimental artefacts caused by ectopic expression or protein over-expression.

1.2.2.2 HCV proteins E1 and E2

The glycosylated envelope proteins E1 and E2 are believed to form the basis of the viral envelope (Carrere-Kremer et al., 2002) forming a non-covalent complex. The maturation and folding of HCV glycoprotein is a complicated process involving the chaperone machinery of the endoplasmic reticulum (ER), and is highly dependent on glycosylation and disulphide bond formation (Moradpour et al., 2007). Two hyper variable regions (HVR) have been identified in the E2 envelope glycoprotein sequence (Weiner et al., 1991). The first 27 amino acids of the E2 ectodomain form HVR1. The residues at each position and the final conformation are highly conserved amongst genotypes (Penin et al., 2001). Antibody selection of immune-escape variants seems to drive the variability of this region as it is likely to have a major function involved in viral entry (Bartosch et al., 2003b, Callens et al., 2005). HVR2, the second hyper variable region, modulates E2 receptor binding (Roccasecca et al., 2003). These glycoproteins play major roles at

different steps of the HCV life cycle, being essential for virus entry (Bartosch and Cosset, 2006, Cocquerel et al., 2006) and viral assembly (Wakita et al., 2005). As E1 and E2 are essential for virus entry, they are the target for the development of antiviral molecules that block HCV entry (Helle et al., 2006).

1.2.2.3 HCV p7 protein

p7 is thought to be an ion channel protein, part of a family of proteins known as viroporins, which have the function of enhancing membrane permeability to promote virus budding (Griffin et al., 2003). It is a hydrophobic membrane protein of 63 amino acids and comprises two hydrophobic a-helices, TM1 and TM2, and a basic loop located in the cytoplasm (Carrere-Kremer et al., 2002). p7 is not essential for HCV RNA replication *in vitro* (Lohmann et al., 1999), but it is essential for productive infection *in vivo* (Sakai et al., 2003) and has been shown to be essential for infectivity in chimpanzees (Sakai et al., 2003). p7 forms oligomers and has cation channel activity (Griffin et al., 2003), and has an important role in the maturation, assembly and release of infectious viral particles (Steinmann et al., 2007). This combined with evidence that p7 is sensitive to ion channel inhibitors such as amantadine (Griffin et al., 2003, Pavlovic et al., 2003, Premkumar et al., 2004) make it an attractive target for antiviral intervention.

1.2.2.4 HCV NS2 protein

NS2 is a transmembrane protein that is not essential for the formation of the replication complex (Blight et al., 2000, Lohmann et al., 1999) but is necessary for the complete replication cycle *in vitro* and *in vivo* (Kolykhalov et al., 2000, Pietschmann et al., 2006).

The function of mature NS2 has yet to be elucidated; however, the NS2-3 protease, formed by NS2 and the amino-terminus of NS3 is responsible for cleavage between NS2 and NS3. Cleavage of the NS2 N-terminus from p7 occurs via a signal peptidase within the ER (Carrere-Kremer et al., 2004, Carrere-Kremer et al., 2002). NS2 is found in association with membranes of the ER when expressed alone (Franck et al., 2005). Work conducted using artificial inter-genotypic HCV chimeras suggest that NS2 is also involved in virus assembly and release; however, the mechanism by which NS2 contributes to this process is unknown (Kalinina et al., 2002, Lindenbach et al., 2005, Pietschmann et al., 2006).

1.2.2.5 HCV proteins NS3 and NS4A

NS3 has a number of functions. The amino-terminal domain serine protease of NS3 is required for cleavage of the NS3 to NS5B proteins (Bartenschlager et al., 1993). The carboxyterminus consists of a helicase/ nucleoside triphosphatase (NTPase) domain (Kim et al., 1995). NS4A is a cofactor that activates the NS3 protease function by forming a heterodimer (Bartenschlager et al., 1995). NS3-4A protease activity also blocks the ability of the host cell to mount an innate antiviral response (Foy et al., 2003) by interfering with double-stranded RNA signalling pathways, by disrupting the RNA helicase retinoic acid-inducible gene I (RIG-I) pathway through proteolysis of an adaptor protein (MAVS/IPS-1/VISA/Cardif) involved in interferon regulatory factor-3 (IRF-3) activation (Meylan et al., 2005, Sen and Sarkar, 2005). Cleavage results in the dissociation from the mitochondrial membrane, and thus disrupt signalling of the antiviral immune response (Li et al., 2005c). NS3-4A also disrupts Toll-like receptor-3 (TLR-3)

signalling of IRF-3 activation (activated by extracellular double stranded RNA) by cleaving the TRIF adaptor protein (Li et al., 2005a).

1.2.2.6 HCV proteins NS4B, NS5A and NS5B.

NS4B is a hydrophobic protein targeted to the ER, and induces morphological changes to the ER to form a cytoplasmic vesicular structure known as the membranous web (Lundin et al., 2003, Liang and Heller, 2004). This structure contains the replication complex (Egger et al., 2002) including HCV NS5A, a phosphoprotein which binds RNA and plays an important role in viral replication (Huang et al., 2005) although the function is still unclear. NS5A has a potential role in the modulation of the IFN response to HCV (Tan and Katze, 2001) as it contains a region termed the interferon-α sensitivity-determining region (ISDR) which confers interferon resistance to the virus by interacting PKR (Gale et al., 1997). NS5B is the RNA-dependent RNA polymerase of HCV, and is the enzyme responsible for viral RNA transcription and replication (Behrens et al., 1996). The enzyme has no proof-reading mechanism, allowing the virus to mutate rapidly resulting in a 'quasispecies' within an infected individual. The virus is therefore able to evade neutralising antibody and cytotoxic T cell-mediated killing, which may lead to persistence.

1.2.3 HCV entry and replication

HCV infection is a highly active process with a viral half-life of only a few hours and production and clearance of an estimated 10^{12} virions per day in a given individual (Lindenbach et al., 2005, Zhong et al., 2005b). The life cycle of HCV first involves the

binding and internalisation of the virus, followed by release into the cytoplasm and uncoating of viral particles (Penna et al., 2001) (Figure 1.2). The binding of HCV is mediated by the envelope proteins interacting with cell surface molecules on hepatocytes. Currently, CD81 (a tetraspanin protein found on the surface of many cell types) (Pileri et al., 1998), the LDL receptor (LDLR) (Agnello et al., 1999) and glycosaminoglycans (GAGs), scavenger receptor class B type I (SR-BI) (Scarselli et al., 2002), Claudin-1 (Evans et al., 2007), and Occludin (Liu et al., 2009) are proposed to be the most likely candidates for HCV receptors (Figure 1.3). It has been suggested that LDLR and GAGs are responsible for initial attachment of viral particles to hepatocytes mediated by lipoproteins associated with HCV virions. Following this initial binding step, the virion then most likely interacts with SR-BI and CD81, while Claudin-1 and Occludin act later in the entry phase (Figure 1.3). More recently, other entry factors (putative receptors) have been identified (Martin and Uprichard, 2013, Sainz et al., 2012) and it is clear that HCV entry is complex. HCV enters the cell by clathrin-mediated endocytosis (Blanchard et al., 2006). This involves passage via a low pH endosomal compartment (Koutsoudakis et al., 2006, Tscherne et al., 2006) and endosomal membrane fusion. The envelope proteins of related viruses (Flaviviruses and Alphaviruses) have an internal fusion peptide that is exposed during low pH-mediated domain rearrangement and trimerization of the protein, and HCV is assumed to be similar (Moradpour et al., 2007). It is suggested that entry of all viruses in the *Flaviviridae* family, including HCV, may include a class II fusion step, however this has yet to be characterized.

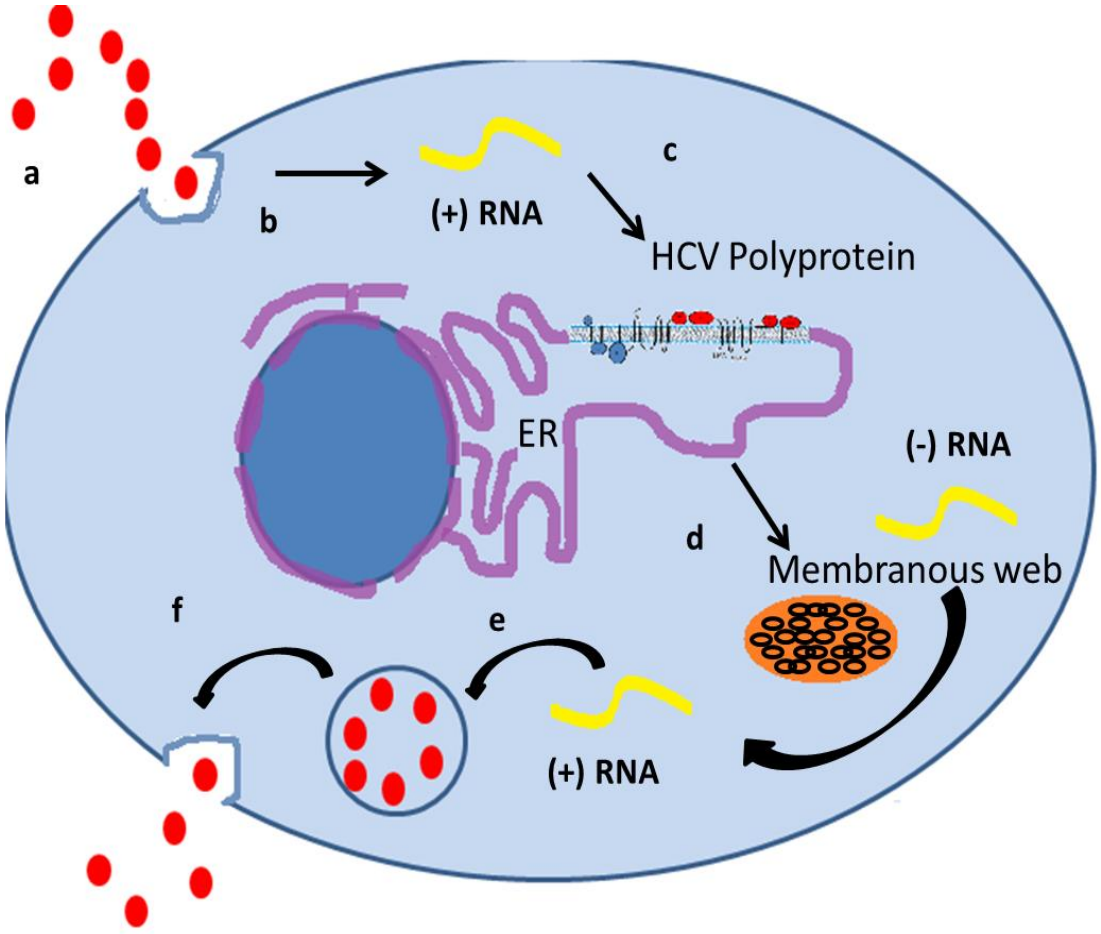

Figure 1.2. HCV life cycle. The HCV life cycle involves binding and internalisation of the virus (a). The viral RNA is then translated via the IRES element (b) and the polyprotein is processed (c); RNA replication then occurs (d) followed by packaging and assembly (e) and finally maturation and release of virus progeny. Modelled on a figure from (Moradpour et al., 2007).

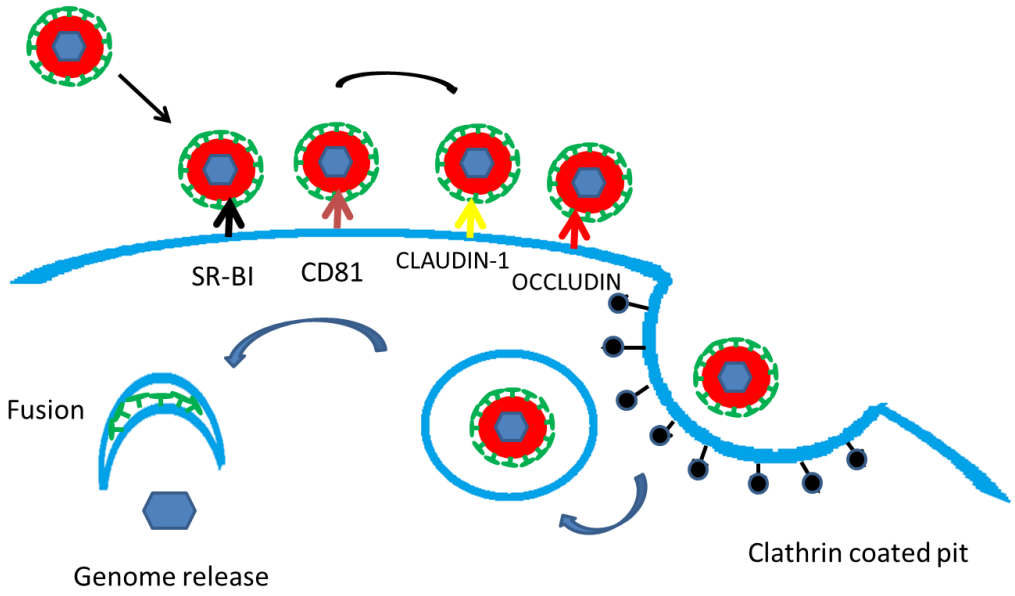

Figure 1.3. Model for the cellular uptake of HCV particles. Virus entry is mediated by the direct interaction of envelope glycoproteins with co-receptors in a consecutive manner. The HCV virion is bound to SR-BI and CD81, the virus is then transferred to tight junctions where it interacts with CLDN1 and Occludin. The virion then enters the cell via a clathrin-dependent process of endocytosis. The endosome containing the virus is acidified which fuses the envelope proteins to the membrane of the endosome, allowing for the release of the vial nucleocapsid. Modelled on a figure from (Burlone and Budkowska, 2009).

The translation of the viral RNA is mediated by the IRES, and the polyprotein precursor is processed by cellular and viral proteases into the mature structural and non-structural proteins. The structural proteins and the p7 polypeptide are processed by the ER signal peptidase. The non-structural proteins are processed by the two viral proteases, NS2–3 protease and NS3–4A serine protease.

The RNA is replicated in a specific membrane alteration named the membranous web. This was identified as the site of RNA replication in Huh-7 cells containing subgenomic HCV replicons (Gosert et al., 2003). As mentioned previously the membranous web is formed from the ER membranes, and this is mediated by NS4B (Egger et al., 2002) .

The final stages of the life cycle are the packaging and assembly of the viral particles, followed by the maturation and release. The late stages of the viral lifecycle have yet to be elucidated conclusively as the availability of an *in vitro* model has only recently become available (Moradpour et al., 2007). A number of elements including NS2, other non-structural proteins, and possibly unidentified RNA structures may be involved in these processes. The virions presumably form by budding into the ER, or an ER-derived compartment, and exit the cell via the secretory pathway (Moradpour et al., 2007).

1.2.4 HCV Immunity

Hepatitis C virus evades immune surveillance allowing the infection to progress to a chronic state in many individuals. The situation is complicated by the inability to appropriately diagnose the asymptomatic progression of the acute phase. The virus is

thought to infect not only hepatocytes, but also lymphoid cells and possibly dendritic cells (Bain et al., 2001). In persistently infected patients, not only is there a weak HCV-specific T cell response (Diepolder et al., 1997), there is also evidence of exhaustion of antigen specific CD4+ (Gerlach et al., 1999) and CD8+ T cells with a marked impairment in the function of these cells and the cytokine profiles they express (Boyer and Marcellin, 2000), thereby allowing chronic infection to develop. A strong multi-specific CD4+ and CD8+ T cell response to HCV proteins is an important factor in viral clearance, preventing the onset of persistent infection (Diepolder et al., 1997, Thimme et al., 2002, Gruner et al., 2000, Lechner et al., 2000) and studies in chimpanzees show this response is necessary to mediate protective immunity (Grakoui et al., 1993, Shoukry et al., 2003). In humans, CD4+ and CD8+ T cell responses to a number of HCV peptides correlate with recovery from acute infection (Cooper et al., 1999, Lamonaca et al., 1999, Day et al., 2002). In a study of recently infected needle stick patients (Thimme et al., 2001), IFN-γ production was important for clearance of the virus without associated liver damage, as it is capable of inhibiting viral replication (Guidotti and Chisari, 2001). Initially, CD8+ T cells were unable to produce IFN-γ, however at a later stage the cells regained their ability to produce IFN-γ and this correlated with a 5 log decrease in viremia. The results of this study were supported in chimpanzee studies by different groups (Bigger et al., 2001, Su et al., 2002a, Thimme et al., 2002). In contrast, persistently infected patients have weak oligo-/mono-specific CD4+ and CD8+ T cell responses to HCV proteins (Bowen and Walker, 2005, Lauer et al., 2004, Neumann-Haefelin et al., 2005). There are a number of hypotheses as to the cause of the limited T cell response resulting in chronic infection; these include (1) primary T cell failure and

(2) T cell exhaustion following the initial priming of T cells. As many patients are only diagnosed upon the development of chronicity, the exact cause of T cell dysfunction remains unclear due to the inability to detect a primary T cell failure. It is believed that primary T cell failure may be caused by dysfunction in antigen presentation (Bain et al., 2001, Lee et al., 2001, Sarobe et al., 2002). A high viral load has also been associated with T cell exhaustion in murine models of lymphocytic choriomeningitis virus (LCMV) (Moskophidis et al., 1993) and this may be the case with HCV. Other reasons for viral persistence include the immunosuppressive nature of HCV proteins, impairment of T cell priming and effector function due to defective antigen presentation, and the production of viral escape mutants. Selective pressure applied by the cellular and humoral immune responses favours selection of viruses with mutated epitopes (Neumann-Haefelin et al., 2005), caused by the lack of a proof reading mechanism in NS5B.

HCV can also disrupt the IFN response (Gale and Foy, 2005), with many of the proteins encoded by its genome capable of disrupting the IFN signalling pathway. The protease function of NS3/4a cleaves IPS-1/MAVS/VISA/Cardif and TRIF (Meylan et al., 2005, Breiman et al., 2005, Li et al., 2005b) which are adaptor proteins important for the production of IFN. Expression of the entire viral genome or the core protein is capable of suppressing IFN signal transduction (Heim et al., 1999, Melen et al., 2004). The ability of the virus to disrupt this signalling pathway suggests that the IFN response is important for the eradication of the virus.

Humoral immune responses are believed to be important in clearance of the virus. Antibodies to HCV, specifically to NS3, Core, NS4 and the envelope proteins (Orland et al., 2001) become detectable within 7-8 weeks after infection (Pawlotsky, 1999) although the range is highly variable (Ishii and Koziel, 2008). These antibodies only appear after increases in ALT and cellular immune responses (Heller and Rehermann, 2005) and their appearance is delayed further in persistently infected patients (Alter et al., 1989, Rahman et al., 2004). At this stage it is unclear if these naturally-acquired antibodies are capable of neutralizing HCV infectivity, as they are capable of neutralizing HCV *in vitro* (Farci et al., 1994) but fail to neutralize infection in humans and chimpanzees, and were unable to confer protective immunity against reinfection (Farci et al., 1992, Lai et al., 1994). Furthermore instances of spontaneous clearance have been reported in the absence of humoral immune responses (Bjoro et al., 1994, Post et al., 2004).

1.2.5 Hepatitis C Vaccine

There is currently no vaccine for Hepatitis C; the development of an effective vaccine had been hampered by the inability to culture the virus. Recently, a tissue culture system utilizing a specific strain of the virus was developed that infects the human hepatoma cell line, Huh7 (Wakita et al., 2005, Lindenbach et al., 2005, Zhong et al., 2005b). This allows the production of infectious virus and has overcome a great hurdle toward understanding various aspects of the HCV life cycle, and will pave the way for vaccine research for HCV. The ability of the virus to evade cytotoxic T cell responses by mutating regularly recognised epitopes has been problematic for the development of an effective vaccine, and treatment strategies. Several clinical trials reported using various

delivery systems including peptides (Firbas et al., 2010, Klade et al., 2008, Mine et al., 2004, Schlaphoff et al., 2007, Yutani et al., 2007), DNA based therapies (Alvarez-Lajonchere et al., 2009, Castellanos et al., 2010) and viral vectors (Habersetzer et al., 2011) with varying levels of success (for an in depth review see (Ip et al., 2012). However two methods which are of particular interest to this thesis include work being done by GlobeImmune and Okairòs reviewed in (Strickland et al., 2008). Briefly, GlobeImmune (Louisville, CO, USA) is carrying out work in which whole, heat-killed recombinant *Saccharomyces cerevisiae* yeast, genetically modified to express one or more HCV proteins, showed the eradication of tumours expressing HCV proteins in mice, and preliminary data of a phase 1b clinical trial shows a favourable safety profile with viral load reductions in six chronically infected patients. Okairòs (Rome, Italy) utilised an adenovirus and DNA combination-based therapy that expressed the entire NS region of HCV; the vaccine protected chimpanzees from acute and chronic infection after challenge with a heterologous virus (Strickland et al., 2008).

Vaccine research in the context of HCV had been hampered due to the lack of suitable small animal models. The only complete animal model available is the chimpanzee allowing for the study of the complete HCV life cycle (Bukh, 2012), however being an endangered species research using these animals is expensive and increasingly difficult (Altevogt et al., 2012). Developments in the humanisation of mice have improved our understanding of the HCV life cycle. The chimeric mouse model, uPA-SCID, are T- and B-cell deficient mice (severe combined immunodeficiency – SCID) also carrying a urokinase-type plasminogen activator (uPA) transgene which causes degeneration of their

livers, were grafted with human hepatocytes allowing infection with HCV (Mercer et al., 2001). The FRG ($Fah^{-/-}Rag2^{-/-}IL2rg^{-/-}$) mouse model has a genetic alteration which degrades the mouse liver, however this can be blocked by administration of an oral drug (Bissig et al., 2010). The mice can be engrafted with human hepatocytes by ceasing administration of the drug and injecting with hepatocytes. This eliminates the problem of mortality as found in the uPA-SCID mouse model (Bukh, 2012). The grafted FRG mice are then able to be infected with HCV from both in vivo (Mercer et al., 2001; , Meuleman et al., 2005; Bissig et al., 2010) and in vitro (Lindenbach et al., 2006; Hiraga et al., 2007; Bissig et al., 2010) sources. The limiting factor to both these models is these mice cannot be used in studies of the adaptive immune response to HCV, and therefore cannot be used as challenge models for vaccine studies (Bukh, 2012). To overcome this, genetic manipulation of mice to enable infection by HCV has been trialled. CD81 and Occludin have been identified as the minimal receptors required to make mouse cells permissive to HCV (Dorner et al., 2011). These immunocompetent fully inbred mice expressing these two human receptors have enabled the analysis of humoral responses and are able to be used as a challenge model (Dorner et al., 2011). More recently a mouse challenge model where HCV proteins were expressed in the livers of immunocompetent inbred mice via hydrodynamic injection of a plasmid encoding HCV NS3/4A was developed (Yu et al., 2014). The plasmid also encoded secreted alkaline phosphatase (SEAP) which was able to be detected in the serum of mice. When used as a challenge model, reduction in serum levels of SEAP correlated with clearance of NS3/4A positive cells in the liver (Yu et al., 2014).

The development of suitable small animal models will aide in the development of vaccine strategies for HCV, however due to the complex nature of the natural progression of HCV, and the multi-factorial mechanisms behind its clearance and development into persistence, a suitable animal model which matches the chimpanzee may yet be a long way off.

1.3 Dendritic Cells

Dendritic cells (DC) were first described in 1973 (Steinman and Cohn, 1973) and are found in virtually all organs of the body (Janeway et al., 2001). They are the major antigen presenting cells of the immune system. Depending on their developmental origin, cytokine activators, surface antigens, and functional capacity, DC are subdivided into two major distinct populations, referred to as conventional (cDC) and plasmacytoid DC (pDC) (Shortman and Naik, 2007). cDC develop from myeloid progenitors in the bone marrow, whereas pDC develops from lymphoid precursors (Banchereau et al., 2000). DCs found in the steady state in lymphoid and non-lymphoid tissues have a phenotype which is dependent upon their environment; and the observed phenotype is dependent on the organ from which the DC is derived. DCs which are not found in the steady state, and are instead induced after infection and inflammation, include monocyte derived DCs, TNF-producing and inducible nitric oxide synthase expressing DCs (Geissmann et al., 1998, Naik et al., 2006, Randolph et al., 1999, Serbina et al., 2003). Peripheral blood monocytes are able to develop into DC's when cultured with granulocyte/macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) *in vitro* (Sallusto and Lanzavecchia, 1994). cDCs are believed to play a role in the enhancement of the innate

immune response (Ardavin, 2003) and are the only cells capable of efficiently activating naïve T cells. Plasmacytoid DC are recognized by their ability to secrete large amounts of type I interferons (Diebold et al., 2003).

Dendritic cells exist in the periphery where they express an immature phenotype, and are found under most epithelial surfaces and in most solid organs (Janeway et al., 2001). In the absence of ongoing inflammatory responses, immature DC (iDC) constantly monitor peripheral tissues, lymph and secondary lymphoid organs. They sample the surrounding environment by macropinocytosis, receptor mediated endocytosis and phagocytosis (Sabatte et al., 2007). The extraordinary capacity of iDC to capture antigens is related not only to their high endocytic capacity but also to the fact that they are strategically localized at anatomic sites frequently exposed to antigens such as skin, mucosal surfaces and spleen (Guermonprez et al., 2002). An example of immature DC (iDC) is the Langerhan cells of the skin. These cells regularly take up antigens via phagocytosis and endocytosis via the macrophage mannose receptor and Fc receptors (Lanzavecchia, 1996) or take up extracellular antigens via macropinocytosis (Janeway et al., 2001). These immature cells are incapable of stimulating naïve T cells due to low levels of MHC expression and B7 co-stimulatory molecules.

After the uptake of antigen, iDC lose their ability to phagocytose antigen and simultaneously gain the ability to migrate to regional lymph nodes. The uptake of antigen initiates maturation of the DC, which occurs during migration, resulting in up regulation of MHC molecules for antigen presentation, adhesion molecules such as ICAM-1,

ICAM-2, LFA-1 and LFA-3 to facilitate interaction with T-cells and co-stimulatory molecules B7.1 and B7.2 (also known as CD80 and CD86, respectively) which are essential for T-cell activation. mDC secrete chemotactic chemokines such as DC-CK, IL-8, MIP-1α and MIP-1γ which strongly attract naïve T cells (Heufler et al., 1992, Caux et al., 1994, Mohamadzadeh et al., 1996).

1.3.1 Antigen Presentation

T cells are incapable of producing immune responses to antigens without the process of antigen presentation. Antigen presentation requires antigenic peptides to be coupled with specialised proteins known as the Major Histocompatibility complexes (MHC). Intracellular antigens, such as those produced by viruses replicating within a host cell are presented to T cells via MHC Class I. Proteins associated with pathogens, as well as endogenous self proteins and tumour associated antigens are digested by cellular proteasomes. The peptides produced by this enzyme are transported to the endoplasmic reticulum where it is coupled to MHC Class I, which is then transported to the cell surface.

The presentation of antigen by dendritic cells to T cells is achieved by the MHC Class II: peptide complex. Extracellular antigen is taken up into intracellular vesicles. Here, acidification activates proteases which degrade the antigen into peptide fragments (Janeway et al., 2001). The vesicle then fuses with vesicles containing MHC class II where the peptides are bound to the complex (Janeway et al., 2001). The bound peptide is then transported to the cell surface to be presented to the T cell receptor (TCR). However,

in order to allow activation of the naïve T cell, co-stimulation is essential. This function is performed by the co-stimulatory molecules on the DC binding to CD28 on the T cell surface, in order to effectively stimulate the proliferation of the antigen specific T cell subset. These co-stimulation signals must come from the same cell presenting antigen (DC). Failure to do so results in anergy, in which the naïve T cell becomes tolerant to activation by the specific antigen even upon presentation by an APC. This is the mechanism enlisted in the deletion of auto-reactive T cell subsets produced in the thymus during development and in peripheral tolerance.

Upon activation of naïve T cells by engagement of the T cell receptor (TCR) and CD28, expression of cytotoxic T lymphocyte antigen-4 (CTLA-4, CD152) is up-regulated. This molecule is a negative regulator and has 20 times greater affinity for the B7 ligand than that of CD28, preferentially binding to it and thereby controlling the T cell response.

1.3.2 Cross Presentation

The manner in which an antigen is acquired greatly influences the efficiency and pathway of antigen presentation, and ultimately the quality of the resulting immune response. Experimental evidence indicates that effective CD4+ and CD8+ T cell responses are induced when endogenous antigens are processed by mDC, resulting in high levels of immunogenic peptides produced and presented in a Class I or II restricted manner over an extended period of time via MHC (Schuler et al., 2003). DC also internalise exogenous antigen, and antigens from dead or dying cells, and process these in an endosomal compartment or cytosol for presentation in a MHC Class I and II restricted manner

(Albert et al., 1998). This presentation of exogenous antigen via MHC Class I has been termed “cross presentation” because these exogenous antigens, which are not expected to gain access to the DC cytoplasm in a classical manner, have “crossed over” to the endogenous antigen pathway. The formation of MHC: peptide complexes and the immunogenicity of antigens is enhanced when a protein is taken up as part of a dying cell (Albert et al., 1998, Inaba et al., 1998). It is unclear as to whether apoptotic or necrotic cells are required to induce cross-presentation (den Haan and Bevan, 2001). Apoptotic cells occur naturally in the body, and whether they are able to induce an immune response is a contentious issue, as they are believed to primarily induce tolerance. However, in the context of virally infected cells, a large number of apoptotic cells are produced which cannot be efficiently cleared. This allows for secondary necrosis to occur in some cells, and it is these necrotic cells which elicit an immune response. It has also been shown that apoptotic cells are not capable of maturing DC as effectively as necrotic cells (Sauter et al., 2000), and this is important as it is believed that DC, not macrophages, are the main contributors to cross presentation, (Albert et al., 1998, den Haan et al., 2000). It is also important to note, that although iDC are capable of phagocytosing both apoptotic and necrotic cells, it is only necrotic cells which selectively induce the maturation of the DC (Sauter et al., 2000), therefore necrotic cells are the perfect vehicle in which to allow presentation of antigens via the MHC Class I and II pathways.

1.3.3 Dendritic Cells and HCV

DC are likely to be important in the immune response towards HCV. CD8+ T cells are able to be primed by presentation of HCV antigens by DC (Accapezzato et al., 2004, Barth et al., 2005) It has been suggested that HCV infects DC *in vivo* (Bain et al., 2001) although evidence for this remains poor. The functional capacity of DC derived from persistently infected patients remains contentious as a number of studies have presented evidence for and against impaired DC function. Studies which examined the allostimulatory capacity of blood derived DC primarily report an apparent defect of these cells to induce T cell responses when compared with healthy individuals (Anthony et al., 2004, Kanto et al., 1999a, Murakami et al., 2004, Szabo and Dolganiuc, 2005, Tsubouchi et al., 2004, Ulsenheimer et al., 2005, Wertheimer et al., 2004). However, other studies argued that no defect was observed (Goutagny et al., 2004, Longman et al., 2005). Blood derived DC are scarce, and are difficult to expand *in vitro,* and therefore are not suitable for use in therapeutic applications. Procedures which can utilise DC derived from progenitor cells such as monocyte derived DC are much more attractive for use in therapeutic studies. As with blood derived DC there are some groups who report no impairment in moDC derived from persistently infected patients compared with healthy donors (Longman et al., 2004, Piccioli et al., 2005). Others report mo-DC from persistently infected patients are defective in their alloreactivity, and their ability to mature (Auffermann-Gretzinger et al., 2001, Bain et al., 2001, Kanto et al., 1999b) One group found that moDCs from persistently infected patients were less mature than those derived from healthy patients, showing lower levels of HLA-DR, CD80, CD86 and CD40 (MacDonald et al., 2007). This group also found that at physiological ratios of DC to T

cells, moDC from persistently infected patients indeed have an impairment, however when this ratio was raised this defect was non evident (MacDonald et al., 2007). It was also found that moDCs derived from persistently infected patients were just as competent at generating cytokines and chemokines in response to the physiological stimulus CD40 ligand (MacDonald et al., 2007).

Importantly, although these studies utilised cells derived from infected individuals, they do not account for the presence of the virus in the environmental milieu, and presume that any affect that HCV has on these cells is not dependent on the continued presence of the virus. In fact, DC derived from uninfected individuals, were shown to be defective in their alloreactive capacity upon expression of viral proteins such as core and E1 (Sarobe et al., 2002). NS3 can impair DC function *in vitro* causing elevated levels of IL-10 and decreased levels of IL-12, and an impairment in mixed lymphocyte reaction (MLR) (Dolganiuc et al., 2004). However the role of HCV in DC impairment remains unclear, as other groups have found no impairment in DC derived from HCV infected patients (Longman et al., 2004).

1.3.4 Dendritic Cell Immunotherapy

Dendritic cell immunotherapy involves the use of DC to initiate a disease-specific T cell response in the patient. The procedure involves the use of progenitor cells such as monocytes and $CD34^+$ stem cells, as they allow the production of a large number of DC. The use of pDC or cDC is not viable as these cells are scarce and are difficult to isolate in large numbers from the blood (Banchereau et al., 2001). DC immunotherapy involves the

ex vivo culture of monocyte derived-DC (MoDC) or $CD34^+$ stem cell derived DC from the patient into iDC. These are then pulsed with specific peptides, proteins, cell lysate or nucleic acid and mDC generated. The mDC are then transfused back into the patient where the mDC migrate to regional lymph nodes to interact with naïve and/or memory T cells, either boosting the patient's existing immune response or by generating immunity *de novo*. Trials involving DC immunotherapy have to date mainly involved the treatment of solid tumours including malignant melanoma, colorectal cancer, myeloma and prostate cancer, however DC immunotherapy has been used to treat viral infections such as HIV and HBV (Lu et al., 2004, Chen et al., 2005). Studies in animals, injected with HCV antigen-pulsed DC, were shown to elicit a protective immune response against challenge with recombinant vaccinia viruses (Racanelli et al., 2004, Yu et al., 2007, Kuzushita et al., 2006) or tumour cells expressing a homologous HCV protein (Encke et al., 2005).

A disadvantage of DC immunotherapy is that there is relatively little known as to the appropriate route/site of administration, and the dosage and frequency of doses required (Onaitis et al., 2002). Trials, in which DC immunotherapy has been utilised, vary in all these aspects. The trafficking of DCs from the site of injection is also poorly understood. In mice, DC seem to remain at the site of injection, whereas in humans, radio-labelling of both iDC and mDC seem to show migration to regional lymph nodes suggesting interaction with T cells (Prince et al., 2008).

1.4 Adenovirus

Adenovirus vectors have become a very popular tool for gene transfer into mammalian cells, becoming the vector of choice since 1990 (Nadeau and Kamen, 2003). Adenoviruses were discovered in 1953, in studies of cultures from human adenoids and virus isolation from respiratory secretions (Rowe et al., 1953, Hilleman and Werner, 1954). Over 52 different serotypes exist in humans, accounting for 5–10% of upper respiratory infections in children, with many infections occurring in adults as well. Some species are known to cause conjunctivitis, tonsillitis, ear infections, croup, gastroenteritis, bronchiolitis or pneumonia, viral meningitis or encephalitis, and in rare cases cystitis (urinary tract infection). These viruses belong to the family *Adenoviridae* which infect various species of animals, including humans. Large scale DNA sequencing has led to the formation of four genera in this family, Aviadenovirus containing avian viruses, Mastadenovirus, containing viruses that infect mammalian hosts; including humans, Atadenovirus contains some bovine adenovirus and sheep adenovirus (Leppard et al., 2008). The fourth genus, Siadenovirus, contains a frog and a turkey adenovirus.

Six human adenovirus species exist classified as A through to F, all of which belong in the genus Mastadenovirus. These viruses were initially classified on the basis of their virion hemagglutination characteristics, genome nucleotide composition, and oncogenicity in rodents (Leppard et al., 2008).

Adenoviruses are well known in terms of their double-stranded genomes, virion structure, and method of replication (Danthinne and Imperiale, 2000). Adenoviruses are naturally

benign viruses of the upper respiratory tract. Adenoviruses have numerous advantages as a vector including their ability to replicate at high titres in complementing cell lines; from which they are easily purified, and their ability to infect a wide variety of dividing or non-dividing cells (Danthinne and Imperiale, 2000). The strains most commonly used to construct recombinant viruses include Ad2 and Ad5. These are well characterized; and can accommodate up to 10 kb foreign genetic material (Bett et al., 1993). The adenovirus genome rarely integrates into the host chromosome, making it suitable for applications requiring transient gene expression. The use of hybrid adenovirus/retrovirus or adenovirus/adeno-associated virus vectors allows the integration of transgenes into the genome and long-term gene expression.

1.4.1 Adenovirus Molecular Biology

The adenovirus is a non-enveloped virus approximately 80-110 nm in diameter with icosahedral symmetry, composed of a nucleocapsid, and a double stranded linear DNA genome with a terminal protein attached covalently to the 5' terminus. The DNA which has a length of approximately 37 kb is wrapped in a histone like protein and has inverted terminal repeats of 50-200bp, which act as origins of replication.

As adenovirus is a non-enveloped virus, no host proteins are found in the virion, as all components of the particle are encoded by the virus. The structural proteins of the particle are based on the decreasing apparent size on polyacrylamide gels, and are identified by roman numerals (Figure 1.4) (Russell, 2009). The nucleocapsid shell comprises of 252 capsomers, of which the majority (240 capsomers) are the hexons

(trimers of the hexon polypeptide-protein II). The remaining twelve capsomers are known as pentons. These form the vertices of the shell and consist of two components, the pentonbase (a pentamer of the penton polypeptide protein III), and fiber (a trimer of the fiberpolypeptide protein IV) which projects outward from each vertex of the particle (Russell, 2009). It has a long shaft and a distal knob domain. Both components of the penton play specific roles in attachment and internalization of the virus.

The viral core comprises the genome in complex with three proteins: VII, V and mu (a processed form of polypeptide X). Proteins IIIa, VI, VIII, and IX, play roles in the stabilisation of the capsid, some of which bridge the shell and the core. Protein IX trimers occupy crevices on the external faces of the shell increasing the stability of the virus. Finally, the particle carries a virus-coded, sequence-specific protease of 23kDa that is essential both for the maturation of newly formed particles to an infectious state and for particle un-coating after infection.

All adenovirus genomes are linear, double-stranded DNA molecules with a high level of conservation of genome organization between human adenoviruses. The genomes have a virally encoded terminal protein (TP) covalently attached to each genome 5’ ends. Also at each end of the linear genome is an inverted terminal repeat sequence varying in length from 40 to 200 bp. The proteins encoded by the genes are designated E1A, E1B, E2, E3, E4, IX, IVa2, E2 and E3 (which are divided into regions A and B), and the major late transcription unit (MLTU) which is divided into five regions (L1-L5). Each of these has

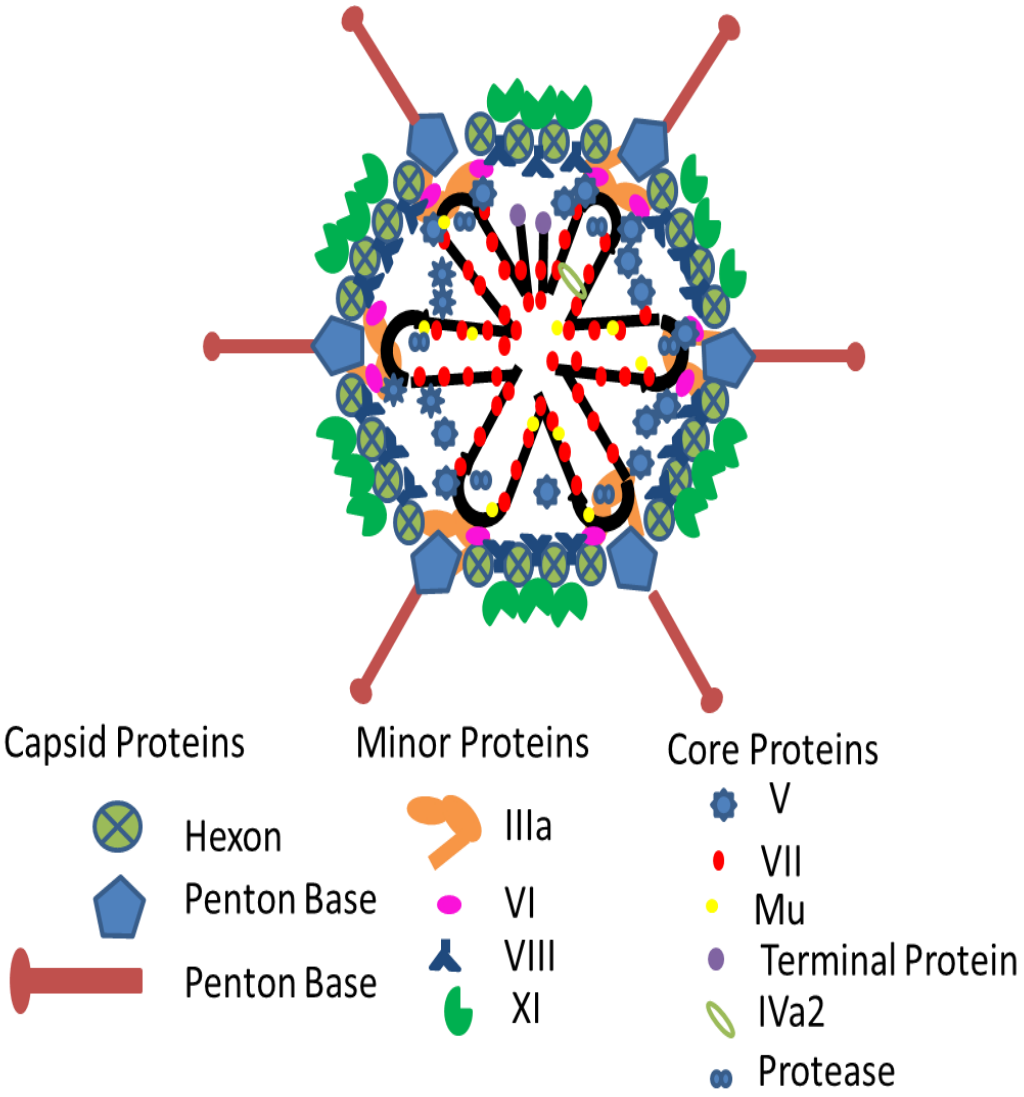

Figure 1.4. Structure of adenovirus. A schematic depiction of the structure of Adenovirus based on cryo-electron microscopy and crystallography. Schematic modelled on a figure from (Russell, 2009).

distinct polyadenylation sites. Two small noncoding RNAs are expressed from the VA genes (Figure 1.4). Within each gene/region there are typically multiple proteins encoded.

1.4.2 Adenovirus replication cycle

Human adenovirus primarily infects epithelial cells with primary attachment of the virion being mediated by the knob domains of the penton fiber protein. For Human Adenovirus species A, C, D, E and F, the coxsackie adenovirus receptor (CAR) seems to be the initial cellular receptor. CAR is a cell surface protein belonging to the immunoglobulin superfamily, and is present on a variety of cell types mediating cell to cell interactions and at tight junctions and other types of intercellular contact. Adenoviruses of species B utilise the cell surface protein CD46, present on most nucleated cells, that is involved in regulating complement activation.

Following primary attachment via the receptor of choice, secondary interactions occur between integrins (heterodimic cell adhesion molecules) on the cell surface including αVβ3, αVβ5, αMβ2, and α5β1 and the penton base (Bai et al., 1993, Davison et al., 1997, Huang et al., 1995, Mathias et al., 1994, Stewart et al., 1997, Wickham et al., 1994, Wickham et al., 1993). These interactions lead to an irreversible interaction of the capsid with the cell surface (Persson et al., 1985) resulting in receptor-mediated endocytosis (Maxfield and McGraw, 2004). This process has been shown to be a clathrin dependent process requiring the presence of free cholesterol in the plasma membrane through studies using the dynamin K44A mutant which only allows clathrin mediated endocytosis at a specific temperature (Chen et al., 1991, Damke et al., 1994, FitzGerald et al., 1983,

van der Bliek and Meyerowitz, 1991, Wang et al., 1998, Worgall et al., 2000). Upon internalization, the vesicle acidifies which causes conformational changes in the virion proteins causing the loss of several proteins from the particle vertices, including protein VI which is thought to cause endosomal lysis and therefore allow the remnant viral particle to enter the cytoplasm (Greber et al., 1993). Once the virion is released from the vesicle it is rapidly transported to the nucleus via microtubules which also results in further loss of viral proteins. To accomplish intracellular translocation, the adenovirus capsid interacts with cytosolic dynein, molecular motors which drive capsid motility rapidly along microtubules through the cytosol (Kelkar et al., 2004, Leopold et al., 2000, Mabit et al., 2002, Suomalainen et al., 2001, Suomalainen et al., 1999). Studies in which cytoplasmic dynein-mediated motility was inhibited have shown the accumulation of adenovirus at the nucleus is prevented (Leopold et al., 2000, Suomalainen et al., 2001, Suomalainen et al., 1999). Once the viral genome reaches the nucleus, it binds to the nuclear membrane via nuclear protein CAN/nup214 (Trotman et al., 2001) and enters via nuclear pores where it is then used as a template for transcription.

The genome is used as template for transcription by host RNA polymerase II. The entire length of the viral genome is transcribed; however, not all the regions of the genome are expressed at the same time during infection. Instead, gene expression is tightly regulated by a combination of host and viral proteins which act as specific activators. Initially upon infection, the E1A gene is the only gene to be transcribed and if protein production from these mRNAs is prevented by metabolic inhibitors then further gene expression is inhibited. This indicates that E1A proteins are important for the activation of viral gene

expression. The action of this protein, together with host transcription factors that E1A activates indirectly, leads to activation of the remaining early gene promoters. Among the proteins produced from these genes are those required for DNA replication. Other early proteins modulate the host environment including the host response to infection by blocking pro-apoptotic signalling (activated early in the infectious process) and the adaptive immune response by preventing the export mature MHC class I antigens to the cell surface, and interferon production. The host cell stress and DNA damage sensor, p53, is inactivated and targeted for degradation, and the double-stranded DNA break repair pathway is blocked. Viral proteins also have effects on the host gene expression apparatus, blocking the export of host cell mRNA from the nucleus during the late phase of infection, instead favouring the export of viral mRNA.

The packaging of viral progeny is mediated in the nucleus with progeny genomes being generated within prominent replication centres in the nucleus. The proteins necessary to package this DNA are produced in the cytoplasm and transported to the nucleus. Immature adenovirus particles are assembled with the aid of scaffolding proteins prior to the insertion of the DNA genome. Maturation cleavage then occurs which is essential for the particles to acquire infectivity. Severe cytopathic effect is observed in immortalised cell lines, with loss of cytoskeleton leading to rounding and detachment from the monolayer within 1–2 days. However, the release of virus particles is rather inefficient, with considerable amounts of virus remaining cell associated. One of the E3 gene products that are produced late in infection facilitates loss of cell viability and particle

release (Tollefson et al., 1996). In its absence, although cytopathic effect occurs normally, cell death is delayed and virus release is even less effective.

In vivo, epithelial cell sheets are targeted by adenovirus where individual cells are linked by tight junctions that define distinct apical and basolateral membrane surfaces, as CAR is expressed primarily on the latter. When virus first enters the body and encounters the apical surface of an epithelium, it cannot access the receptors necessary to initiate infection; therefore the initial round of infection must depend on a break in epithelial integrity exposing the basolateral surface.

1.4.3 Adenovirus as a gene delivery vector

Human adenoviruses are used as potential gene delivery vectors for human cells as they have been shown to have widespread applications. Adenovirus vectors have been shown to infect a range of human cell types. It is also easy to produce high viral titres in complementing cell lines. They provide tumour-specific cell killing for cancer therapy, or long-term persistence and expression of a delivered gene for gene therapy approaches.

Early work focused on human adenovirus 5 because of its ease of use in the laboratory and its ability to deliver genes into a variety of cell types (dividing or non-dividing), and its mild natural disease course. However, to convert human adenovirus 5 into a passive gene delivery vector, genome modifications are required to render it replication-defective and to create sufficient capacity for the insertion of foreign DNA. Usually the E3 region is deleted, as its functions are dispensable for growth in cell culture, and the E1A and

E1B genes are also removed which renders the vector replication defective. This deficiency can be complemented by growth in various cell lines. Vectors from which all the viral genes have been stripped from the genome to leave only the replication origins and packaging sequence from the termini provide the best performance among adenovirus vectors for long-term gene delivery. However, it is more difficult to complement their growth in culture and hence much lower yields of vector particles are achieved.

Adenoviral vectors have been the focus of intense research for their suitability as a vaccine delivery vehicle. They have been used in a number of trials involving various solid-tumours, genetic disorders such as haemophilia A and B, rheumatoid arthritis, and also viral infections such as HIV. Adenoviral based vaccines came under scrutiny following a failed HIV vaccine clinical trial directed by Merck (2007). Known as the STEP clinical trial, interim analysis indicated that the vaccine did not work. The vaccine failed to reduce the acquisition of HIV infection, nor the viral load in those who became infected. It was also discovered that the vaccine made some individuals more susceptible to infection with HIV. However, although the vaccine itself was not responsible for the increased acquisition of HIV infection, it seemed to particularly increase the risk of infection in males who were both uncircumcised and had pre-existing antibodies specific for Ad5 (which occurs in approximately 50% of the population). Other similarly designed trials for HIV have since been cancelled as they were similar to the STEP trial and additional basic immunological responses required to combat HIV infection were still unavailable.

Despite the failure in the treatment of HIV, Adenovirus vectors have shown acceptable safety profiles and efficacy in other trials, showing that they are still a viable candidate as the basis for vaccine approaches. In the context of HCV, adenoviral vectors have been well tolerated in man (Barnes et al., 2012; Swadling et al., 2014), however due to the pre-existing immunity in humans to Ad5, these studies introduced the use of rare serotypes such as Ad6 (Barnes et al., 2012) or non-human adenoviruses such as Chimpanzee Ad3 (Barnes et al., 2012; Swadling et al., 2014). These vectors were capable of priming T cell responses against HCV proteins, generating CD4 and CD8 T-cell subsets which were able to be sustained for a year following boosts with heterologous adenoviral vectors (Barnes et al., 2012).

1.5 Cell Death

Homeostasis is maintained in multicellular organisms by a balance of cell proliferation and cell death (Broker et al., 2005). Disorders in either process lead to many pathological conditions including but not limited to disturbed embryogenesis, neurodegenerative diseases, and the development of cancer (Kono and Rock, 2008). This fine equilibrium is controlled by a process known as Programmed Cell Death (PCD) and has been primarily associated with apoptosis (Broker et al., 2005). Apoptosis is the result of a molecular cascade in response to death signals provided by one of two pathways. The first being the release of cytochrome-c from mitochondria, and secondly the activation of death receptors in response to ligand binding (Ashkenazi and Dixit, 1998, Hengartner, 2000). Triggering of either pathway results in the activation of a family of cysteine proteases

known as caspases which execute the cells' fate in a programmed fashion giving rise to the distinct morphological changes associated with apoptosis which include shrinkage of the cell, condensation of chromatin, followed by disintegration of the cell into smaller fragments (known as apoptotic bodies) which allow the cell to be more readily removed via phagocytosis (Kerr et al., 1972). Cells which undergo apoptosis generally show distinct morphological features including rounding and shrinkage, retraction of pseudopodes, reduction of cellular and nuclear volume, nuclear fragmentation, minor modification of cytoplasmic organelles and plasma membrane blebbing (Kroemer et al., 2009). Apoptosis is often associated with either no response or an anti-inflammatory response (Kono and Rock, 2008).

Necrosis on the other hand is a much more chaotic method of cell death which generally results from extracellular stresses imposed on the cell, and is characterized by cellular oedema and disruption of the plasma membrane, resulting in a pro-inflammatory response (Hirsch et al., 1997). Necrosis is characterised morphologically by cytoplasmic swelling, rupture of the plasma membrane, swelling of cytoplasmic organelles, and moderate chromatin condensation (Kroemer et al., 2009). It is this feature, the disruption of the plasma membrane, facilitating the release of intracellular contents into the extracellular environment, which allows necrotic cells to induce a pro-inflammatory response. Necrosis had long been considered to be relevant in circumstances of accidental or uncontrolled cell death; however there is evidence to suggest that some forms of necrosis may be finely regulated by signal transduction pathways and catabolic mechanisms (Kroemer et al., 2009). Death domain receptors such as TNFR1, Fas/CD95

and TRAIL-R and Toll-Like Receptors (TLR), such as TLR3 and TLR4 (Holler et al., 2000); have been shown to induce necrosis even in the presence of caspase inhibitors, suggesting a pathway to cell death which is "programmed" like apoptosis however is independent of it (Kroemer et al., 2009).

Cell death is an increasingly complex area of research with recommendations for the classification of cell death nomenclature to include various forms of cell death such as apoptosis, necrosis, necroptosis (programmed necrosis as mentioned above), autophagic cell death, cornification and various forms of cell catabolism including Wallerian degeneration, pyroptosis, pyronecrosis and entosis (Kroemer et al., 2009). For the purposes of this thesis the term "apoptosis" will refer to the method of cell death induced by programmed internal pathways (such as caspases) and "necrosis" will refer to catastrophic cell death from an external source resulting in the rupture of the cell membrane.

1.5.1 Dying Cells and the Immune System – Stranger vs Danger

The immune system has evolved to protect the host, protect it from infection, and protect it from damage. It is clear that immune responses can be generated to molecules which are not derived from microbial sources, and these include molecules from the host itself, generated under completely sterile conditions (Rock et al., 2011). Mechanisms have evolved to detect the death of cells, and these allow the body to discern between cell death caused by normal physiological processes, and death caused by other external factors. This ability to distinguish between the two allows the immune system to combat

invading pathogens or injurious agents which can cause disease. Cells which have died via normal physiological processes are engulfed and cleared by both professional and non-professional phagocytes (Kono and Rock, 2008). On the other hand, cells which have died via non physiological processes such as severe physical or chemical trauma induce inflammatory responses (Kono and Rock, 2008).

The immune system has also learned to discriminate between "self" and non-self" with the ability to respond to not only entire micro-organisms, but also to molecules which are seen as foreign (Kono and Rock 2008). Early work through the use of microbially derived adjuvants showed that these molecules were immunostimulatory, generating robust immune responses when mixed with an antigen of choice (Dresser, 1961). In 1989 it was proposed that the immune system does not respond to all foreign antigens, but only to those which are associated with infection (Janeway, 1989). This is known as the "stranger" model as seen in Figure 1.5. It was postulated that the discrimination between infectious and non-infectious molecules was made by APC through the recognition of pathogen-associated molecular patterns (PAMPs). These PAMPs work by activating the innate immune system through macrophages, Natural killer Cells and DC. However, although largely correct, this model does not explain immune responses generated to disease states which do not produce PAMPs and yet induce a response from the immune system, such as certain viral infections and tumours. These disease states appear to have no source of PAMPs to induce such an adjuvant effect, and yet; depending on the setting, the immune system is still capable of mounting an immune response towards these disease states. Consequently, dead and dying cells are avidly taken up by APC and it has

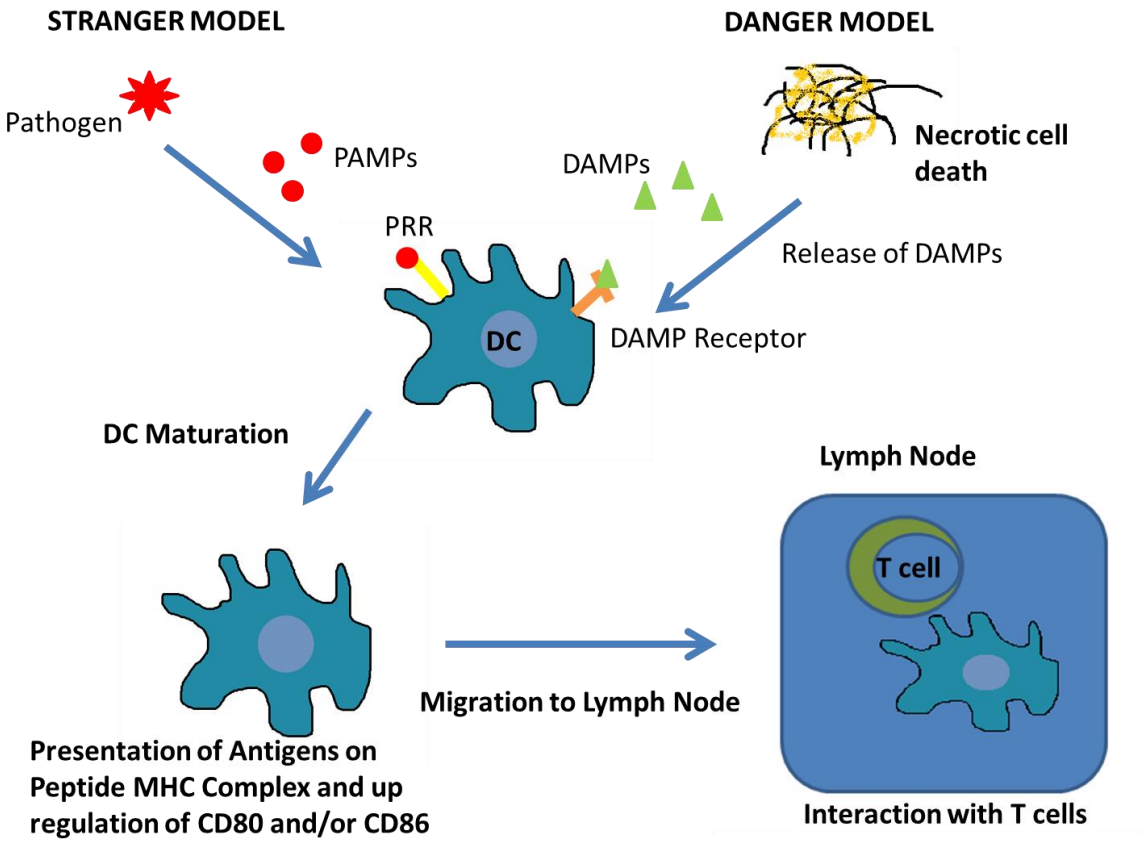

Figure 1.5. The Stranger vs Danger Model. In the stranger model, DC recognise specific pathogen associated molecular patterns (PAMPs) on invading pathogens, which cause them to mature and migrate to regional lymph nodes to interact with naïve T cells and generate an immune response. In the Danger model, the maturation of DC is through recognition of Danger associated Molecular Patterns (DAMPs) released from necrotic cells, which as in the stranger model, migrate to regional lymph nodes to interact with naïve T cells. Modelled on a figure from (Kono and Rock 2008).

been proposed that signals supplied by these cells alert the immune system to danger and elicit an immune response. It has been shown that dead tumour cells are a source of tumour associated antigens (Kotera et al., 2001) and that injury or death of tumour cells provides a potent adjuvant like effect (Shi et al., 2000).

The danger hypothesis on the other hand proposes that the immune system has also evolved to respond to non-physiological cell death (Matzinger, 1994). In this model, the immune response reacts to potential threats such as infection, however the initial signal to induce the response is provided by the abnormal death of cells and the release of molecules with endogenous adjuvant activity (Rock at al., 2011). These pre-existing endogenous adjuvants contained within intracellular stores of all viable cells are known as damage associated molecular patterns (DAMPs). Although it is unclear whether these DAMPS contain specific molecular patterns which induce inflammatory immune responses, the acronym is used to highlight the similarities in function to PAMPs (Janeway, 1989). The immune system is capable of distinguishing pathogens via PAMPs in the "stranger model" (Figure 1.5) where DC mature upon interaction of the particular PAMP with the PAMP receptor, migrate to regional lymph nodes and present the antigen to T cells via the classical peptide-MHC complex pathway. The other arm is the danger model (Figure 1.5), which has the same endpoint in lymph nodes; however the initial maturation signal for the DC is supplied by interaction of DAMPs released by necrotic cells.

1.5.2 Danger Associated Molecular Patterns.

Therefore in the stranger vs danger model, the crucial event which occurs is the release of DAMPs from necrotic cells. As mentioned previously, DAMPs are endogenous proteins present in viable cells, which are usually hidden from the immune system (Figure 1.6). As cells undergo physiological stress and become necrotic, they lose the integrity of their plasma membrane, thereby allowing these usually hidden pro-inflammatory molecules to enter the extracellular environment and engage the immune system. These DAMPs have been detected in the cytosol of live cells, as the cytosol of healthy cells show adjuvant activity, indicating the presence of intracellular DAMPs (Gallucci et al., 1999, Shi et al., 2000). The classification of DAMPs is still an area of ongoing work. In order to classify a particular protein as a DAMP a number of criteria must be met; firstly the DAMP should be a highly purified molecule, secondly its biological activity has to be shown to be independent of PAMPs (which are known to bind tightly to host proteins), thirdly the DAMP should be active in physiological concentrations, and finally blockade of the DAMP should inhibit its biological activity (Kono and Rock, 2008). Numerous DAMPs have been identified however the most widely studied are uric acid, heat shock proteins (HSP) and high-mobility Group Box 1 protein (HMGB1) (Kono and Rock, 2008). Other molecules identified with DAMP properties include Galectins, S100 proteins, double stranded DNA, adenosine and ATP, thioredoxin, cathelicidins, defensins and N-formylated peptides (Kono and Rock, 2008).

Shi and colleagues were the first to identify uric acid as a potential DAMP (Shi et al., 2003). In this study the cytosol of cells were subjected to chromatographic separation and

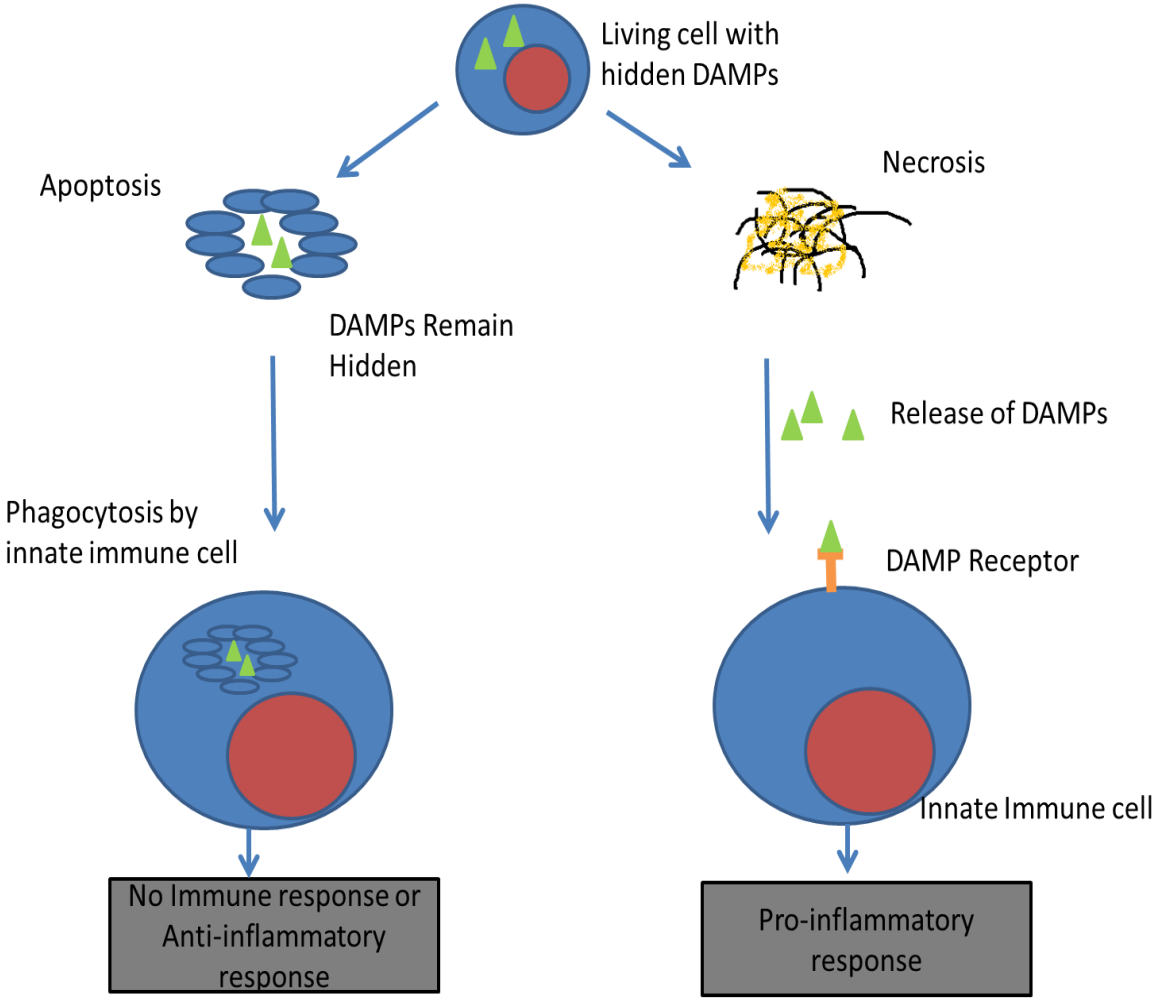

Figure 1.6. The immune response to apoptotic vs necrotic cells. All living cells contain DAMPs which are hidden to the immune system. Upon cell death caused by apoptosis, the cell undergoes fragmentation and blebbing; however the DAMPs remain hidden within the cell fragments. These fragments are engulfed by phagocytic cells of the innate immune system which either have no response or an anti-inflammatory response. On the other head cells which have undergone necrosis rupture their cell membranes releasing DAMPs. These DAMPs are recognised by DAMP receptors on cells of the innate immune system which go on to induce a pro-inflammatory response. Modelled on a figure from (Kono and Rock, 2008).

the fractions screened for adjuvant activity, through which one of the molecules identified was uric acid. It was initially surprising to find that uric acid was a DAMP with biological activity as it is produced in all cells during the catabolism of purines from DNA and RNA, and it is normally present in humans at approximately 60µg/dl (Rock et al., 2011). However, it is thought that the immune system senses uric acid after it has undergone a chemical phase change into monosodium urate microcrystals, which results when dying cells release their pools of uric acid into interstitial tissues (Shi et al., 2003). Later work from Shi and colleagues also showed that enzymatic depletion of uric acid decreased its adjuvant activity (Shi et al., 2006).

Heat shock proteins are capable of promoting immune responses to bound and admixed antigenic peptides in mice (Udono and Srivastava, 1993, Feng et al., 2003). HSPs are also capable of maturing DC *ex vivo* (Basu et al., 2000) and stimulating APCs to migrate to lymphoid organs *in vivo* (Binder et al., 2000). However recently the role of HSPs as a DAMP, especially the most studied; HSP70, has come under scrutiny. Work proclaiming HSP70 to have danger signal properties, which was very attractive as it was purported to be both an antigen carrier and have potent adjuvant activity, utilised a recombinant HSP70 which was only active when endotoxin levels were high (Asea et al., 2000). In fact, it has been reported by some that the literature shows that HSP70 in fact has more immunosuppressive properties as opposed to immunostimulatory (Stocki and Dickinson, 2012). However, a recent study showed that HSP70 expressed from a DNA vaccine had immunostimulatory properties, thus removing any doubts related to the purity of recombinant HSP70 used as an adjuvant (Garrod et al., 2014).

HMGB1 is an intracellular DNA-binding protein which stabilises nucleosomes, assists with the bending of DNA and regulates transcription. HMGB1 was identified as a pro-inflammatory molecule released from necrotic cells and increased antibody responses and induced protection from tumour challenges (Lotze and Tracey, 2005, Rovere-Querini et al., 2004, Scaffidi et al., 2002). Supernatants from necrotic fibroblasts were utilised in tumour immunization, however the supernatant from HMGB1 deficient cells or the introduction of neutralizing antibody specific for HMGB1 resulted in a reduction in the adjuvant activity by up to 50% (Rovere-Querini et al., 2004).

1.5.3 DAMP receptors.

The main receptors for DAMPs are largely unknown (Kono and Rock, 2008). Some TLRs (TLR 2 and TLR4) have been implicated (Liu-Bryan et al., 2005, Ohashi et al., 2000, Park et al., 2004, Vabulas et al., 2002) however the presence of microbial contaminants cannot be ruled out in these studies (Kono and Rock, 2008). Pattern recognition receptors often associated with PAMPs may also be implicated in the recognition of DAMPs. These include TLRs, C-type lectins, RNA-sensing RIG-like helicases (RLHs), RIG-I and MDA5, and the DNA sensors, DAI and AIM2 (Schroder and Tschopp, 2010). Due to the large range of proteins which seem to act as DAMPS, it is likely there is not one receptor; but a range of receptors which result in the pro-inflammatory response associated with necrotic cell death.

It is clear from the literature that there are many more DAMPs to be discovered, however it is not yet clear which DAMPs are the most important, and whether all DAMPs show similar biological activity (Kono and Rock, 2008).

1.5.4 Dead Cells and the Immune Response

During injury, the body produces redness, heat and swelling which are hallmarks of the inflammatory response. When cells die *in vivo,* the innate immune system induces this inflammatory response whilst also alerting the adaptive immune response (Majno et al., 1960). In fact the inflammatory response to cell death is stereotypical, allowing the time of tissue injury to be determined upon pathological examination (Kono and Rock, 2008). During this inflammatory response, pro-inflammatory mediators act on the local vasculature causing the arterioles to dilate and venules to leak fluid. This allows the recruitment of leukocytes and the flow of protein-rich plasma from the blood into the affected tissue (Kono and Rock). The main pro-inflammatory mediators which have been linked to the sterile immune response associated with necrotic cells are Interleukin-1 (IL-1) and Tumour Necrosis Factor alpha (TNF-α). It has long been known that IL-1 can be produced from cells lysed experimentally (Rock et al., 2011); therefore the rupture of cellular membranes and the release of IL-1 during necrosis is theoretically plausible. It has been shown that necrotic IL-1α deficient DCs were unable to trigger a response when injected into mice whereas necrotic IL-1 α competent DC were capable of eliciting a response (Eigenbrod et al., 2008). However it has been shown that live DC are capable of making IL-1 which can also induce inflammation (Kono et al., 2010). It has been shown, that the IL-1 which drives the sterile immune response is not derived from the necrotic

cells themselves, but from cells in the host which recognise cell death (Kono et al., 2011). It is believed that macrophages and DC are the primary cells involved in the recognition and clearance of dead and dying cells, therefore it makes sense that these would be the cells producing the IL-1 which drives inflammation. It has been found that macrophages, and to a lesser degree DC, play a major role in the production of IL-1 associated with the inflammatory response, however there seems to be an as-yet unidentified bone marrow derived cell which contributes to the IL-1 response (Kono et al., 2010).

1.5.5 Cell Death and HCV

The exact role of cell death in HCV infection is as yet undefined (Fischer et al., 2007). It is known that a large degree of apoptosis occurs in the liver (Calabrese et al., 2000) of persistently infected HCV patients, and apoptotic hepatocytes can account for between 0.54 and 20% of hepatocytes, largely independent of HCV genotype (Fischer et al., 2007). Histologically, the close physical proximity of infiltrating lymphocytes and apoptotic hepatocytes has led to the suggestion that HCV-induced apoptosis is mediated by immune cells (Calabrese et al., 2000), and that apoptosis is a major contributor to liver fibrogenesis (Fischer et al., 2007). HCV replication also sensitizes host cells to TNF-related apoptosis inducing ligand (TRAIL) induced apoptosis (Deng et al., 2012). HCV infection has also been shown to disrupt the mitotic cell cycle of hepatocytes, which is suggested to result in apoptosis and therefore may contribute to the multi-factorial progression to hepatocellular carcinoma through the development of apoptosis-resistant clonal populations of cells (Kannan et al., 2011). Examination of levels of apoptotic markers in HCV infected patients showed that HCV infection modulates the apoptotic

machinery through the course of infection (Zekri et al., 2011). The infection of Huh7.5 cell cultures with HCV was also shown to have maximal production of cell-death related genes at the point of peak viral production, inducing apoptosis in infected cells. These cell-death genes were also found to be regulated by HCV (following *in vitro* infection) in liver tissue from a cohort of HCV-infected liver transplant patients with rapidly progressive fibrosis (Walters et al., 2009). Others have also found that HCV-induced apoptosis has a direct link to the incidence of liver injury (Lim et al., 2014) and may contribute to the pathogenesis of HCV (Lan et al., 2008).

Components of the HCV virion have been shown to have both pro- and anti-apoptotic properties. The core protein has been shown to inhibit TNFα and CD95-ligand induced apoptosis (Ruggieri et al., 1997), achieved by the prevention of release of cytochrome-c from mitochondria (Machida et al., 2001). Berg and colleagues have shown the core protein is capable of inducing a caspase-independent form of cell death which is similar to apoptosis, which was inhibited in part by the presence of the non-structural proteins of HCV (Berg et al., 2009). They speculated that the non-structural proteins have an anti-apoptotic effect which allows prolonged survival of the cell and the induction of apoptosis-like death by the core protein facilitates viral release (Berg et al., 2009). The core protein contains a Bcl-2 Homology 3 (BH3) domain which is capable of regulating apoptosis through specific interaction with the human myeloid cell factor 1 (Mcl-1), which contributes to apoptosis during HCV infection (Mohd-Ismail et al., 2009).

The structural proteins E1 and E2 have been shown to have primarily anti-apoptotic effects. In a hepatoma cell line, apoptosis was dependent on the presence of the C-terminal transmembrane domain of E1 (Ciccaglione et al., 2003, Ciccaglione et al., 2004). The mechanism of action for E2 was through the inhibition of cytochrome-c release from mitochondria in both a hepatoma cell line and transgenic mice (Kamegaya et al., 2005, Lee et al., 2005, Lee et al., 2007).

The role of the HCV non-structural proteins in the induction of apoptosis remains unclear. NS3 has been shown to induce caspase 8-dependent apoptosis in hepatocytes (Prikhod'ko et al., 2004) and DC (Siavoshian et al., 2005) but the method of action remains unclear. NS5A has been shown to have sequence homology to bcl-2, an integral apoptotic protein, and can thereby inhibit apoptosis in hepatoma cell lines (Chung et al., 2003). NS5B has demonstrated a pro-apoptotic effect in DC (Siavoshian et al., 2005).

In the context of development of hepatocellular carcinoma HCV has been shown to trigger mechanisms which induce cell survival and proliferation. HCV infection resulted in an increase in expression of signal transducer and activator of transcription 3 (STAT3), a signal transduction protein found to be activated in a number of human cancers which targets proteins which regulate cell survival such as Bcl-2, mcl-1, Fas, cyclin D1, cyclin E1, and p21 (Ogata et al., 2006). This made the hepatocytes specifically resistant to apoptosis and accelerated proliferation (Ogata et al., 2006).

The role of apoptosis in HCV infection remains unclear; however it is unclear if the HCV virion is capable of modulating apoptosis in order to allow the infected cell to persist. The literature concentrates on asking the question, why does HCV persist in spite of apparent apoptosis within the infected liver? It is possible that apoptosis is induced by the replication of HCV specifically to allow persistence, and is the underlying cause of the hallmarks of hepatitis, fibrosis and cirrhosis of the liver, eventually developing into hepatocellular carcinoma. However, the virus has evolved mechanisms to directly control apoptosis to prevent the induction of cellular necrosis, which would, according to the mechanisms discussed earlier, allow both the innate and adaptive immune system to focus on the viral infection.

1.6 Concluding Remarks and Aims of Thesis

People with persistent HCV infection who have failed standard combination therapy have few therapeutic options available despite the introduction of DAA. Initially, DC immunotherapy; which involves the administration of autologous DC primed with an antigen of interest, was an attractive option. However, the use of cell therapy utilizing the adjuvant effect of necrotic cells now presents itself as an attractive candidate of priming DC and other APC. I **hypothesize** that the use of necrotic autologous cells expressing HCV proteins can result in an HCV specific immune response. The **specific aims** of this thesis are:

1. To assess the immune response in patients receiving DC immunotherapy.
2. To test necrotic cells as HCV antigen carriers.
3. To develop the necrotic cell therapy method for human application.

Chapter 2 - Materials and Methods

All procedures conducted in this thesis were approved by appropriate Ethics Committees governing the work.

2.1.1 Dendritic cell immunotherapy of persistent HCV infection

All *in vitro* assay work was performed by a combination of staff from the Centre for Blood Cell Therapies of the Peter MacCallum Cancer Centre, collaborators from Melbourne University, and the Hepatitis C Research Laboratory of the Burnet Institute (including myself). I performed all the *in vitro* ELIspot assays.

2.1.2 Patients.

The dendritic cell clinical trial was reviewed and approved by the Human Research Ethics Committee (HREC) of the Alfred Hospital, Melbourne, with informed consent obtained from each patient in writing.

Potential patients were screened by Dr Stuart Roberts of the Alfred Hepatitis Clinic. The inclusion criteria for the study were; patients infected with a genotype 1 virus for ≥6 months, who had previously failed interferon-based therapy. The patients had to be aged between 18-65 years, have tissue type HLA A2, and chronic hepatitis. The exclusion criteria were; alternative causes of liver disease, co-infection with HIV, HBV or HDV, alcoholic liver disease, histologically proven bridging fibrosis or cirrhosis, decompensated liver disease, impaired coagulation, previous variceal haemorrhage,

hepatocellular carcinoma, pregnant or lactating women and subjects with a history of autoimmune disease.

Patients were determined to be HLA A2-positive by flow cytometry and confirmed by DNA sequencing performed by the Australian Red Cross Blood Transfusion Service.

2.1.3 Description of clean room process

2.1.3.1 Day -1

Leukocytes were collected by trained staff of the Peter MacCallum Cancer Centre apheresis unit. The apheresis was performed using a Gambro instrument equipped with a COBE Spectra leukocyte collection set. The product was generated by passing the patient's blood volume through the instrument to an equivalent of 2 blood volumes, with an ACD-A: whole blood ratio of 1:12. The final volume of the product was usually around 200ml with a red cell packed cell volume of <5%.

2.1.3.2 Day 0 – Monocyte isolation

Upon receipt of the apheresis product, operators within the cleanroom weighed the apheresis bag to determine its approximate volume, and then attached the bag to the Cytomate apparatus (Miltenyi Biotec) to wash the cells. Aliquots of the samples were then taken to determine the total number of mononuclear cells, calculated using the CELL-DYN 1200 apparatus, and the proportion of $CD14^+$ monocytes determined using flow cytometry. Following this, the bag was injected with 7.5ml of CD14 microbeads and placed on the CliniMACS instrument (MiltenyiBiotec) to isolate clinical grade $CD14^+$

monocytes. The cells were then cultured in Vuelife Telflon culture bags (CellGenix, Freiburg, Germany) in CellGro serum free medium (CellGenix, Freiburg, Germany) containing 1000U/ml of GM-CSF (Berlex, Montville, NJ, USA) and 1000U/ml IL-4 (Cellgenix) for 4 days at 37°C.

2.1.3.3 Day 4

On day 4, the immature DC were washed using the CYTOMATE apparatus and placed in fresh CellGro medium containing GM-CSF and IL-4. In order to pulse and mature the cells, 6μM of lipopeptides and 1μg/ml prostaglandin E_2 were added to the culture and incubated for 48 h at 37°C.

The lipopeptides used have been described previously (Chua et al., 2003, Jackson et al., 2004, Jones et al., 2008) and were chosen to represent HLA A2-restricted CTL epitopes, which are associated with clearance of acute phase HCV infection, linked to a universal $CD4^+$ T helper cell epitope (KLIPNASLIENCTKAEL) and the lipid moiety, Pam2Cys. The peptides, termed CTL1-6 are shown in table 2.1.

2.1.3.4 Day 6

On day 6, the matured cells were washed using the CYTOMATE, and 1 x 10^7 cells resuspended in 1ml sodium chloride injection BP containing 10% human serum albumin (HSA)(CSL Ltd, Melbourne) for intradermal (ID) infusion and 1-5 x10^7 cells resuspended in 100ml of the same diluent for intravenous (IV) infusion. Any remaining

Table 2.1 6 HLA-A2 restricted CTL epitopes

Designation	Peptide	Sequence
CTL1	HCV core 132	DLMGYIPLV
CTL2	HCV core 35	YLLPRRGPRL
CTL3	HCV core 177	FLLALLSCLTV
CTL4	HCV NS3 1406	KLVALGINAV
CTL5	HCV NS4B 1807	LLFNILGGWV
CTL6	HCV NS4B 1851	ILAGYGAGV

cells were resuspended in freezing medium (5% glucose, 80% HSA, 10% DMSO) in 1.5ml aliquots containing 2 x 10^7 cells, prior to cryopreservation in liquid nitrogen. Flow cytometry was performed on the cells to determine the level of maturity and a Gram stain was also performed on the product to detect any bacterial contamination.

The initial dose of DC used fresh cells and additional doses used the cryopreserved cells. These were thawed on appropriate days by warming vials of cells in a 37°C water bath, pelleted by centrifugation at 400g for 7 min, then prepared for infusion in the same manner as described above.

The cells were then transported to the Alfred Hospital where Dr Stuart Roberts administered the ID infusion and IV injection to the patient. The ID infusion was injected into the groin region and the IV infusion was given over a period of 30 min. Each patient remained under observation for approximately 18 h as an in-patient. Patients who received >1 dose of DC were infused at 2 weekly intervals.

2.1.4 Monitoring program

Following treatment, the patients were monitored for a total period of 4 months, with blood samples taken from each patient on days 1-3, weekly for 1 month then every two weeks for a period of 3 months, with viral load and ALT levels assessed at each timepoint. Blood was also collected at specific timepoints for collection of PBMC for ELIspot analysis. The viral load was determined by a commercial branched-chain DNA (bDNA) assay by VIDRL.

2.1.5 Peripheral Blood Mononuclear Cell Separation

Peripheral blood specimens were collected in 9 ml lithium heparin vacutainer tubes (Becton Dickinson) by trained staff of the Alfred Hospital Liver clinic.

For every 15ml of blood an equal volume of RPMI (R-) (Invitrogen) was mixed by swirling into a 50 ml Falcon centrifuge tube. Ficoll-paque solution was then under laid beneath the 30 ml of diluted blood using a syringe and cannula. Tubes were placed in a centrifuge with aerosol containment, and centrifuged at 400g for 30min at 22^{o}C.

The cloudy mononuclear cell interface was aspirated using a syringe/canula. The cells were then transferred into a separate fresh sterile 50 ml tube (Falcon) and made up to a volume of 45 ml using RPMI to wash cells. The cells were then centrifuged at 400 g for 8 min at room temperature. The supernatant was discarded and the cells from each tube were pooled into a 50ml centrifuge tube and resuspended in 50 ml of RPMI. The cells were then enumerated using a Neubauer Haemocytometer and 2x10^{7} cells for use in the ELIspot assay were washed one final time in 45ml of RPMI.

2.1.6 IFN-γ ELIspot preparation

The capture mAb (1-DIK) (Mabtech) was diluted to 5 μg/ml final in D-PBS (Invitrogen), 100 μl of diluted antibody was added per well, and this was incubated at 4^{o}C overnight.

The capture antibody was removed and the wells washed 6 times (200ul/well) with D-PBS. The plate was then blocked using RPMI containing 10% FCS. The plates were then

incubated at room temperature (RT) for a minimum of 1 h, or stored at 4°C until cells were ready.

The cells for the ELIspot (section 2.1.5) were then resuspended in RPMI containing 10% FCS and penicillin/streptomycin, with the final cell concentration adjusted to either 4 $x10^6$ PBMC/ml or $2x10^6$ cells/ml.

100µg/ml stocks of HCV-specific peptide pools representing the complete HCV polyprotein (NIH AIDS Research and Reference Reagent Program), as well as the CTL epitopes and Th epitope, Influenza A virus HLA A2-restricted peptide epitope (GILGFVFTL) (Flu matrix peptide), CEF peptides (Mabtech), Phytohaemagglutinin (PHA) (Sigma-Aldrich) and anti-CD3 (Mabtech) were diluted to 4 µg/ml.

The blocking buffer was removed from the wells. Each cell and antigen combination was set up in triplicate. 50µl/well of the PBMC suspension was added to appropriate wells followed by 50µl of the appropriate antigen to result in a final cell number of either 100,000 or 200,000 PBMC per well, and a final antigen concentration of 2µg/ml. The plates were then incubated at 37^oC for 20-24 h.

Following this incubation period, the cells and medium were removed from the plate and wells were washed 6 times with 200µl D-PBS. The detector mAb (7-B6-1) (Mabtech)

was diluted 1:1000 to 1 μg/ml in D-PBS, 100 μl/well added and incubated for 2h at room temperature. The wells were then washed 6 times with D-PBS.

Streptavidin-alkaline phosphatase (Sigma), at a concentration of 1 μg/ml in D-PBS, was added to the wells and incubated for 1 h at room temperature. Following this incubation period, the Streptavidin-alkaline phosphatase was removed from the plate and the wells were washed 6 times with 200μl D-PBS.

In order to visualise spots, 100 μl/well of BCIP/NBT solution (Sigma) was added and incubated at RT until spots developed (usually 15-20 min). Colour development was stopped by washing the wells twice with 200μl of H_2O. The membranes were then allowed to dry overnight at room temperature. The AID ELIspot reader was then used to photograph and enumerate spots.

2.2 Expression of HCV proteins in DC

The premise for this section of the thesis was to screen a number of protocols for RNA transfection in a mouse dendritic cell line, DC2.4. The initial work for this required the construction of a RNA construct encoding GFP.

2.2.1 Construction of pcDNA3.1 EMCV IRES GFP

In order to create an EMCV IRES GFP construct, a pcEMCV IRES Core/TOPO plasmid construct (made by Dr. Wesley Black) was utilised. To facilitate the studies, it was

necessary to remove the Core gene from the pcEMCV IRES Core/TOPO plasmid, and replace it with the GFP gene derived from a pFBM GFP-TK construct (supplied by Dr John Martyn).

2.2.2 Design of primers

Specific oligonucleotide primers (Table 2.2) were designed with specific restriction enzyme sites to clone the GFP gene into the EMCV IRES/TOPO backbone and produced by Invitrogen. The underlined nucleotides represent the specific annealing nucleotides and the highlighted portions represent the engineered restriction sites. The sense primer contained a BspEI site and the antisense primer contained an XbaI restriction site.

2.2.3 Polymerase Chain Reaction (PCR) amplification of GFP

The PCR was performed using Phusion High Fidelity DNA Polymerase (Finnzymes, New England Biolabs) in a 50µl reaction volume containing 1X PCR Buffer [containing 20mM Tris-HCl (pH 8.4), 50mM KCl], 1µl of Phusion enzyme, 1.5mM $MgCl_2$, 0.2mM dNTPs mixture, 200mM of the respective oligonucleotide primer and 2µl (500ng) of template DNA. Thermocycler conditions were: an initial denaturation at 98°C for 30 sec; then 30 PCR cycles consisting of denaturation at 98°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min.

PCR products were visualised on a 1% TAE agarose gel. Fragments were purified using the Roche High Pure PCR Product Kit (Roche Applied Science) according to the manufacturer's instructions.

Table 2.2 GFP specific primers used for cloning into EMCV IRES/TOPO.

Product	Primer	Sequence
GFP forward	sense	5'- TTTA**TCCGGA**ATG AGC AAG GGC GAG GAA CTG TTC -3'
GFP reverse	antisense	5'- AGAG**TCTAGA** CTA CTT GTA CAG CTC GTC CAT GCC -3'

2.2.4 Restriction enzyme digestion

Double restriction enzyme digests were set up as per the manufacturer's protocol. Briefly, 1µg of DNA was added to a 1.5ml conical tube (Eppendorf) along with 1µl of each enzyme (1000 U) (BspEI and XbaI, Promega), 2µl of 10x Buffer D (Promega). The reaction mixture was adjusted to a total of 20µl with Nuclease free water (NFW) (Promega) and incubated for 3 h at 37°C.

2.2.5 Ligation

Ligations were set up according to the manufacturer's protocol using a T4 DNA ligase kit (Invitrogen). Briefly, the pcEMCV IRES/TOPO digested vector was incubated with a 3 molar excess of digested GFP insert in a reaction mix containing 1 unit of enzyme in the ligation buffer, the reaction mixture made up to 20µl with NFW, and incubated at 4°C overnight.

2.2.6 Transformation

The ligation product was then transformed into *E. coli* (JM109 strain) (Promega) using heat shock. 50µl of bacterial suspension was thawed on ice. 2µl of ligation mixture was added to the bacteria, the tube was placed in a heat block at 42°C for 50 sec, then placed immediately on ice and incubated for 5 min. 950µl of SOC medium was then added and the cells were resuscitated for 1 h at 37°C with shaking. The cells were plated onto Luria Broth (LB) agar plates containing 30µg/ml ampicillin and incubated at 37°C in an incubator, with the plates inverted, for 16 h.

2.2.7 Colony screening

Colonies were samples using a sterile pipette tip and inoculated into 50µl sterile H_2O and 1ml of LB containing ampicillin (for miniprep and glycerol stock). The water was then heated to 100°C for 5 min, and then placed on ice for 5 min. 5µl of this template DNA was then added to a PCR.

PCRs were set up as described previously (section 2.2.3), however a Taq DNA polymerase kit (Invitrogen Life Technologies Inc.) was used according to the manufacturer's protocol. In this case thermocycler conditions were an initial denaturation at 94°C for 30 sec; then 30 cycles consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 68°C for 1 min.

PCR products were visualised on a 1% TAE agarose gel. Fragments were purified using the Roche High Pure PCR Product Purification Kit (Roche Applied Science) according to the manufacturer's instructions. Briefly, the reaction mix was mixed with a binding buffer (proprietary formula) and placed into a column containing glass fibre fleece. The column was then centrifuged at max speed for 60 sec and the flow through discarded. The column was then washed twice with wash buffer, and finally the DNA was eluted using elution buffer.

A positive colony was then cultured and DNA prepared using the Qiagen Midiprep Kit as per the manufacturer's protocol. Essentially, *E. coli* from a 200 ml culture were pelleted at 6000g for 15 min, and then resuspended in buffer P1. This was then followed by the

addition of buffer P2 causing lysis of the cells. The addition of buffer N3 precipitated the cellular debris and genomic DNA, which was centrifuged at 15,000g for 30 min. The supernatant was then passed through a Midiprep column (Qiagen) by gravity flow, to allow the plasmid DNA to bind to the column matrix. The column was washed twice with wash buffer and the DNA eluted from the column using the elution buffer. The DNA was then precipitated using isopropanol (0.7 volume), followed by a 70% ethanol wash. The DNA pellet was dried for 15 min and then re-dissolved in 200µl of TE buffer.

2.2.8 Sequencing

The inserts were fully sequenced at the Micromon DNA Sequencing Facility - Monash University, Department of Microbiology, Clayton, VIC and analysed using Chromas Lite software. PCRs were performed according to the manufacturer's instructions (Applied Biosystems Inc.). Briefly, the reaction mixtures were assembled by adding 200ng of template DNA (of construct to be sequenced) to 0.2ml microcentrifuge tubes along with CMV sense sequencing primer or BGH antisense primers (provided by TOPO cloning kit), sequencing buffer and 1µl of BigDye Terminator. The reaction was then placed into a thermocycler and subjected to an initial denaturation at 96°C for 60 sec; then 30 PCR cycles consisting of denaturation at 96°C for 20 sec, annealing at 50°C for 15 sec and extension at 60°C for 4 min. The reactions were then pelleted using ethanol precipitation; the pellets were dried and send to Micromon.

2.2.9 Linearisation of DNA

Midiprep DNA was used and linearised using XbaI restriction enzyme (Promega) and purified using Roche High Pure PCR Product Kit (Roche Applied Science). This linearised DNA was then used as a template to produce *in vitro* transcribed RNA.

2.2.10 *In vitro* synthesis of RNA

The MEGAscript T7 Kit (Ambion) was used for the *in vitro* transcription reaction to produce mRNA. 1μg of the linearised DNA plasmid was used in the kit according to the manufacturer's instructions. After 2h incubation, 1μl TURBO DNase (Ambion) was added and the reaction incubated for 15min at 37°C. The entire reaction volume was then placed into the Poly(A) Tailing Kit (Ambion) according to the manufacturer's instructions. The polyadenylated RNA was purified using MegaClear (Ambion) and examined on a 1% agarose gel. RNA aliquots were stored at -80°C.

2.3 Transfection of DC2.4 with RNA

2.3.1 Cell Preparation

The day before transfection, DC2.4 and/or Huh8 cells were seeded into 24 well plates (Costar) at a concentration of approx $1.2x10^5$ cells/ml (1ml/well) to result in a 60-80% confluent culture. The cells in each well were then washed with 1ml OptiMEM (Invitrogen).

2.3.2 Transfection protocols

2.3.2.1 Lipofectin

Lipofectin (Invitrogen)-RNA complexes were prepared according to the manufacturer's protocol, either with or without Phosphate buffer (40mM final concentration). Briefly, 100µl of OptiMEM or OptiMEM+Phosphate buffer was placed in each of two polystyrene tubes. A volume of lipofectin was added to one tube and mixed (Lipofectin: RNA ratio of 4:1) by vortexing. This was then incubated at RT for 30 min. In the second tube, the required amount of RNA was added and mixed, and left at RT until the lipofectin was ready. Following the 30 min incubation the diluted RNA and lipofectin were mixed together and incubated at RT for a further 10 min to allow the complexes to form. Thereafter, 800µl of warm OptiMEM was added to the lipid/RNA complex and the entire volume (1ml) was then added to the washed cell monolayer and incubated at 37°C for 5 h. The transfection medium was then replaced with growth medium and incubated for a further 48 h at 37°C.

2.3.2.2 Lipofectamine 2000

Lipofectamine2000 (Invitrogen)-RNA complexes were prepared according to the manufacturer's protocol, either with or without Phosphate buffer (40mM final concentration). Briefly, 100µl of OptiMEM or OptiMEM+Phosphate buffer were placed in each of two polystyrene tubes. A volume of Lipofectamine 2000 was added to one tube, mixed by vortexing and incubated at RT for 5 min. RNA was then added, mixed and incubated at RT for 20 min to allow the complexes to form. Following this incubation step, the entire volume of the transfection mixture was added to the cells and

incubated at 37°C for either 1 h or 5 h. The transfection mixture was then removed and replaced with fresh growth medium and the cultures incubated at 37°C for 48 h.

2.3.2.3 DMRIE-C

DMRIE-C (Invitrogen)-RNA complexes were prepared according to the manufacturer's protocol, either with or without phosphate buffer (40mM final concentration). Briefly, 300µl of OptiMEM or OptiMEM+phosphate buffer and 3µl of DMRIE-C were mixed by pipetting. 1 or 3µg of RNA was then added and mixed. The RNA-Lipid mixture was then added to OptiMEM-washed cells and incubated for 4 h at 37°C, when the transfection medium was replaced with growth medium, and the cells were then incubated for 48 h at 37°C.

2.3.2.4 Transmessenger

Experiments carried out using Transmessenger (Qiagen) were performed according to the manufacturer's protocol. Briefly, on the day of transfection, RNA and Enhancer R reagent were diluted and mixed into buffer EC-R, made up to a total volume of 100µl. This was then allowed to stand at RT for 5min. Following this, Transmessenger reagent was added to the mixture and allowed to stand for 10min at RT. 900µl of warm growth media was then added to the complexes the total 1ml volume added to washed cells and incubated at 37°C for 3 h. The transfection mixture was then removed and replaced with fresh growth medium. The cultures were incubated for a further 48 h at 37°C.

2.3.2.5 TransIT

Experiments carried out using Trans IT (Mirus) were performed according to the manufacturer's protocol. Briefly, 50µl of OptiMEM serum free medium were added to a 1.5ml conical tube, the RNA added and mixed thoroughly by pipetting. The mRNA Boost Reagent was added immediately and mixed, followed by the TransIT component and mixed by pipetting. The reaction was then incubated for 5 min at RT. The complexes were then added to cells in growth media drop by drop, with gentle rocking of the tissue culture plate to ensure even mixing of the complex over the cells, without disrupting the cell monolayer. The cells were then incubated for 48 h at 37°C.

2.3.2.6 Electroporation

DC2.4 were harvested and washed once with PBS. The cells were resuspended in OptiMEM at a concentration of 5 x 10^6 cells/ml. 5 µg of RNA was electroporated into the cells with a Genepulser II apparatus (Bio-Rad). The parameters for electroporation are shown in Table 2.3. Immediately after electroporation, the cells were transferred to warm growth medium and cultured for 48 h at 37°C.

2.3.2.7 Polyethylenimine

Polyethylenimine (PEI) was also used to transfect RNA into DC2.4 using a protocol adapted from established protocols used in the laboratory of Associate Professor Johnson Mak (Burnet Institute). RNA was added to 100µl of RPMI/DMEM without FCS or antibiotic. A series of volumes of 1mg/ml PEI was then added to each tube as in

Table 2.3 Conditions to electroporate DC2.4 cells with EMCV IRES GFP RNA.

Condition	1	2	3	4	5	6	7	8	9	10
Cuvette size (cm)	0.2	0.2	0.2	0.2	0.2	0.2	0.4	0.4	0.4	0.4
Voltage (V)	320	320	300	300	300	300	300	300	300	300
Capacitance (μF)	125	125	125	125	125	125	125	125	125	125
% Modulation	0	0	0	100	0	100	0	100	0	100
Number of Bursts	1	2	1	1	2	2	1	1	2	2

Table 2.4, vortexed briefly and the reaction then incubated for 10 min at RT. The complexes were then added to cells drop by drop and incubated at 37°C for 48 h.

2.3.2.8 Oxidised-Mannan-PEI

Oxidised-Mannan-PEI (OxMan-PEI) was also used to transfect RNA into DC2.4 cells using a protocol adapted from established protocols used in the laboratory of Dr Geoff Pietersz (Burnet Institute). Briefly, 1.7µg of EMCV IRES GFP RNA was mixed with varying amounts of OxMan-PEI for 30 min at RT to achieve complexes of varying ratios of OxMan-PEI: RNA. After this incubation period, the complexes were added to cultures of DC2.4 cells (200,000 cells per well of a 24 well late). The cultures were then cultured at 37°C for 48 h to allow for gene expression.

2.2.3 Cell preparation for GFP analysis

Following the 48 h incubation period, the cells were washed with PBS and removed from the wells with trypsin and transferred to FACS tubes (5ml polypropylene tubes, Falcon). The cells were then centrifuged for 5 min at 400g, resuspended in 200µl of FACS buffer (PBS, 0.5% BSA, 1% EDTA) and analysed on a BD flow cytometer for GFP expression.

2.4 HCV protein expression in mo-DC

2.4.1 Generation of Monocyte Derived DC

PBMC were isolated from whole blood as described previously (section 2.1.5). PBMC were then resuspended in MACS buffer (PBS, 2mM EDTA, 1% BSA) at a concentration of $2.5x10^8$ cells/ml. CD14 Microbeads were added then to the cells at 20µl per $1x10^7$

Table 2.4 Volumes and PEI and amounts of RNA transfected onto DC2.4 cells.

Tube #	1	2	3	4	5	6	7	8
Amount of RNA	1µg	1µg	1µg	1µg	2µg	2µg	2µg	2µg
Volume of PEI (µl)	1	2	3	4	2	4	6	8

cells. This mixture was incubated at 4°C for 15 min and then washed with MACS buffer. The cells were then applied to an LS+ MACS column (Miltenyi) in a magnetic field supplied by a MACS magnet. CD14 negative cells were allowed to pass through the column to waste. The column was then removed from the magnet, and CD14 positive cells eluted from the column by applying 10 ml of MACS buffer using the plunger supplied in the kit. The CD14 positive monocytes were then washed with MACS buffer and resuspended in Aim-V media (Invitrogen) at concentration of $1x10^6$ cells per ml, with 1000U/ml of GM-CSF (Berlex) and IL-4 (R&D Systems). The cells were then incubated for 4 days at 37°C to obtain immature monocyte derived DC.

2.4.2 Adenovirus Vectors

Adenovirus constructs were made using the Adeasy kit. Components of the Adeasy kit were supplied as a kind gift by Associate Professor Torresi from Melbourne University. This included the pShuttle-CMV bacterial plasmid and Adeasier cells (electrocompetent BJ5183 cells containing the pAd5 backbone). Adenovirus 5 constructs encoding GFP (Ad5-GFP) and HCV Core, E1, E2 and GFP (Ad5-C-E1-E2-GFP) were also gifts from Associate Professor Torresi.

2.4.3 Creation of mutNS3/4A and cloning into pcDNA3.1 vector.

Fusion PCR was used to create a protease deficient (C1125A) mutant of the HCV NS3/NS4A protein [described previously (Foy *et al*, 2003)]. A PCR was prepared containing NS3 sense oligonucleotide primer (N1) and antisense mutation

oligonucleotide primer (M2) (Table 2.5), and cDNA of a genotype 1b HCV isolate (Trowbridge and Gowans., 1998; GenBank accession number AJ000009). A separate PCR was prepared containing sense mutation oligonucleotide primer (M1) and antisense NS4A oligonucleotide primer (N2). Both reactions were amplified using Phusion DNA Polymerase High Fidelity using the same PCR conditions described in section 2.2.3. The fragments were purified using the Qiaex II Gel extraction kit (Qiagen) according to the manufacturer's instructions. The PCR product was diluted to 500ng/µl and 1µl transferred to a new PCR with sense and antisense NS3/NS4A oligonucleotide primers (as per table 2.5). The PCR was performed using Phusion polymerase PCR conditions. The fragment was then cloned into pCDNA3.1 (+) using specific restriction enzymes and ligation protocols as described above (2.2.4 and 2.2.5). The ligation mixture was then transformed into JM109 and positive colonies screened as described previously (sections 2.2.6 and 2.2.7). The plasmid was purified using the Maxi Prep Plasmid Purification Kit (Qiagen) and the resultant plasmid DNA stored at-20°C (referred to as pCDNA3.1 mut NS3/A). A pCDNA3.1 wt NS3/4A vector was constructed in a similar fashion but omitting the fusion PCR step, and instead using the NS3 forward and NS4A reverse primers.

2.4.4 Cloning of HCV proteins into pShuttle Vector.

The first step to inserting HCV genes into the adenovirus 5 DNA backbone required cloning into the pShuttle-CMV vector (a gift from Associate Professor Joe Torresi).

Table 2.5 Oligonucleotide primers to construct protease deficient NS3/4A.

Primer Name	Product	Primer	Sequence
N1	NS3 forward	sense	5’-CCGCTCGAGATGGCACCCATCACGGCTTACTC -3’
N2	NS4A reverse	antisense	5’-CTAGTCTAGATTAGCACTCTTCCATCTCATCGAACTC-3’
M1	Mutation forward	sense	5’ –CTTGACACCATGCACC**GC**CGGCAGCTCGGAC-3’
M2	Mutation reverse	antisense	5’ –GTCCGAGCTGCCG**GC**GGTGCATGGTGTCAAG-3’

2.4.4.1 Oligonucleotide Primer design

Specific oligonucleotide primers were designed to allow cloning of HCV genes into the multi-cloning site (MCS) of the pShuttle-CMV vector. The primers are outlined in table 2.6. The Core forward primer was designed to include a Bgl II restriction enzyme site, and the E2 reverse primer and NS3 forward primers were designed with a Sal I restriction enzyme site. The NS4A reverse primer was designed with a Hind III restriction enzyme site. All restriction enzyme sites are highlighted in blue and allow for the insertion of the genes independently into the pShuttle-CMV vector.

2.4.4.2 PCR of HCV genes

Amplification of HCV specific DNA was performed using Phusion High Fidelity DNA polymerase according to the manufacturer's protocol as described previously in section 2.2.3. The DNA sequences encoding HCV proteins Core, E1 and E2 were amplified from a plasmid containing a genotype 1b isolate using the Core forward and E2 reverse primers as outlined in table 2.6. The NS3/4A sequence was amplified from the pCDNA 3.1-WT NS3/4A and pCDNA3.1 mut NS3/4A as described above (section 2.3.2). PCR products were purified using MinElute gel extraction kit (Qiagen) according to the manufacturer's instructions.

2.4.4.3 Digestion of HCV gene fragments

PCR products and the pShuttle-CMV vector were digested using the restriction enzymes and buffers as outlined in table 2.7. Briefly, 20µl reactions were set up using the appropriate DNA at 100µg/ml, each enzyme at 1000U/ml and corresponding buffers at

Table 2.6 Primers for insertion of HCV sequences into pShuttle vector

Primer Name	Product	Primer	Sequence
C1	Core forward	sense	5'- CGAAGATCTATGAGCACGAATCCTAAACC-3'
E1	E2 reverse	antisense	5'- CCGACGTCGACGGCCTCAGCTTGGGCTATCAGC -3'
N3	NS3 forward	sense	5'-ACGCGTCGACGCACCCATCACGGCTTACTC-3'
N4	NS4a reverse	antisense	5'- CCCAAGCTTTTAGCACTCTTCCATCTCATCGAACTC-3'

Table 2.7 DNA enzyme and buffer combinations for cloning into pShuttle-CMV vector.

DNA	Enzymes used	Buffer (1X concentration)
Core E1 E2	Bgl II, Sal I	D (6mM Tris-HCl, 6 mm MgCl2, 150mM NaCl, 1 mM DTT, pH 7.9)
WT NS3/4A	Sal I, Hind III	C (10mM Tris-HCl, 10 mm MgCl2, 50mM NaCl, 1 mM DTT, pH 7.9)
Mut NS3/4A	Sal I, Hind III	C
pShuttle-CMV	Bgl II, Hind III	B (6mM Tris-HCl, 6 mm MgCl2, 50mM NaCl, 1 mM DTT, pH 7.9)

1X concentration. The reactions were incubated at 37°C overnight and the DNA purified using Qiaex II gel extraction kit according to the manufacturer's protocol.

2.4.4.4 Ligation of fragments into pShuttle

Purified restriction enzyme-digested DNA products were ligated into the restriction enzyme-digested pShuttle-CMV vector using a 3M excess of each insert (Core E1 E2 and NS3/4A) by T4 DNA ligase (as per section 2.2.5). Following overnight incubation, the ligated DNA was transformed into *E. coli* as described previously (2.2.6). Colonies were screened as described previously (2.2.7) using the Core forward and NS4A reverse primers described in table 2.6. Positive colonies were cultured and the plasmid was purified using the Midi Prep Plasmid Purification Kit (Qiagen) and stored at -20°C. Constructs were also sequenced as described previously (2.2.8).

2.4.5 Recombination in Adeasier cells.

In order to insert the HCV genes into the adenovirus DNA backbone, the pShuttle-CMV constructs were linearized as described previously (section 2.2.4) using the restriction enzyme, Pme I, (New England Biosciences) and Buffer 1. The linearized DNA was purified and 10µl electroporated into Adeasier cells using the Genepulser II apparatus. The parameters for the electroporation were 1.5kV, 200 Ohm and 25uF in 2mm electroporation cuvettes. The electroporated cells were then plated onto LB agar plates containing Kanamycin and incubated overnight at 37°C while inverted. Colonies were then screened by PCR as described previously (section 2.2.7) and by Pac I digestion.

DNA preparations of positive colonies were made using the Midi Prep plasmid purification kit (Qiagen).

2.4.6 Amplification of Adenovirus in complementing cell line.

In order to amplify the defective adenovirus vector it is necessary to use the Human Embryonic Kidney 293T (HEK-293T) cell line which provides the adenovirus proteins E1 and E3 *in trans*. The pAd5 DNA was linearized with PacI (New England Biosciences) and this was then transfected into HEK293T using Lipofectamine 2000 as described in section 2.3.2.2 using 4µg of DNA and 20µl of Lipofectamine 2000 reagent per reaction. The transfection complex was added to the cells and these were incubated for 7 days at 37°C to allow virus growth.

2.4.7 Adenovirus passage

The recombinant adenovirus was passaged in HEK293T cells to generate virus stocks. Transfected cells were harvested by gentle tapping and the cell suspension transferred to a 50ml tube then centrifuged at 300g for 10 min. The supernatant was discarded and the cell pellet resuspended in a minimal volume of PBS. The cell suspension was then freeze thawed 3 times in a dry ice-ethanol bath to lyse the cells and release the virus. The cell lysate was then centrifuged at 1000g for 10 min; the supernatant containing the virus was removed and clarified by passing the lysate through a 0.45µm filter (Sartorius). The clarified lysate was aliquoted and stored at -80°C. In order to scale up the growth of the virus, 50% of the lysate derived from the transfection was diluted in 10ml of growth medium and plated onto a fresh monolayer of HEK293T. This was then cultured for 24-

48 h to allow production of virus. Infected cells were centrifuged, and subjected to the same freeze thaw procedure described above; this constitutes one passage. The virus can be passaged up to 5 times before the generation of replication competent adenovirus or loss of the gene insert.

2.4.8 Titration of Adenovirus

Adenovirus preparations were titrated using a fluorescence-based assay adapted from the commercially-available Adeno-X rapid titration kit, which allows enumeration of virus infected cells by detecting expression of the adenoviral protein hexon. The commercial kit uses a histological stain; however this was adapted to a fluorescence format. Serial dilutions of adenovirus preparations were added to HEK293T grown in the wells of a 24 well plate (150,000 cells per well, 100µl of viral dilution) in duplicate. The plates were then incubated at 37°C for 48 h to allow viral growth. The growth medium was then removed from the wells and the cells allowed to air dry for 5min. The cells were then fixed by the addition of ice cold 100% methanol for 10min at -20°C. The methanol was then removed and replaced with PBS/2% FCS containing an anti-hexon antibody conjugated to FITC (Millipore). The plates were then incubated for 1 h at 37°C and the wells washed three times with PBS/FCS solution.

Virus infected cells were enumerated by virtue of their green fluorescence using a fluorescence microscope (Olympus IX51). A dilution which allowed easily defined, isolated infected cells was chosen and 5 fields per well were scored in two wells. The

average of both wells of the dilution was used to calculate the titre of virus (infectious units per ml IFU/ml) using the formula found in the Adeno-X rapid titration manual.

$$\text{IFU/ml} = \frac{\text{(No. of infected cells) x (fields per well)}}{\text{(Dilution) x (volume of virus suspension)}}$$

For titration of adenovirus constructs which express GFP, virus titres were calculated as above although infected cells were enumerated by virtue of their endogenous GFP expression.

2.4.9 Purification of Adenovirus

Virus preparations were purified using the commercially available kit, Virabind (Cellbio), according to the manufacturer's instructions. Briefly, the viral lysates were passed through the Virabind column by gravity flow to allow binding of the adenoviral particles. The column was then washed 3 times with wash buffer. Purified adenovirus was then eluted using the Virabind elution buffer (25mM Tris, pH 7.5, 2.5mM $MgCl_2$, 1mM NaCl). Glycerol was added to the purified adenovirus to a final concentration of 10% and aliquots of the preparations were then stored at -80°C.

2.4.10 Transduction of cells with Adenovirus

2.4.10.1 Transduction of HEK293T

HEK293T cells were resuspended in growth media (DMEM/10%FCS) and dispensed into an appropriate vessel. Titrated virus was then added to culture to achieve the desired multiplicity of infection (MOI). The cells and virus were then incubated at 37°C for 48-72h to allow gene expression.

2.4.10.2 Transduction of Monocyte Derived DC

Mo-DC generated using the method described in section 2.3.1 were resuspended in AIM-V media containing 2µg/ml of polybrene (Santa Cruz Biotechnology) at a concentration of $1x10^6$ cells/ml. The cells were then incubated for 24 h at 37°C. The pre-treated mo-DC were then resuspended in fresh AIM-V containing 2µg/ml of polybrene and adenovirus was then added to the cells at an MOI of 100. The cells were then incubated at 37°C for 48 h to allow gene expression.

2.4.11 Detection of adenovirus infected cells

2.4.11.1 Flow cytometry

In HEK293T and mo-DC cultures which were transduced with Adenovirus constructs encoding GFP, expression was determined using flow cytometry. Transduced cells were harvested, washed 3 times in PBS, and then fixed for 20min with 4% paraformaldehyde solution (Polysciences Inc,). The fixed cells were then analysed on a FACS Calibur (Becton Dickinson), with 20,000 events, based on Forward and Side scatter, recorded. Collected data were then analysed using CellQuest software.

2.4.11.2 Western blot analysis

2.4.11.2.1 SDS-PAGE sample preparation

Cells were resuspended in Laemmeli buffer (BioRad) containing 5% 2-mercaptoethanol. Samples were boiled for 5 min and then quenched for 5 min on ice.

2.4.11.2.2 SDS-PAGE

Cell lysates were electrophoresed in 10% SDS-PAGE gels for 1-1.5 h at 150V. 8µl of Benchmark protein marker was loaded to allow visualisation of protein separation and protein transfer. The gels were then transferred to PVDF (Millipore) membrane using a Trans-Blot SD semi-dry transfer cell (BioRad) for 1 h at 15V. The membrane was then blocked for 2 h at RT with PBS/5% skim milk/0.05%Tween.

The membranes were then probed overnight with either goat polyclonal anti-NS3 (Virostat), or goat poly clonal anti-Core antibody (Virostat). The blots were then washed three times in PBS-T and probed with a rabbit anti-goat horseradish peroxidase (HRP) (Dako) for 1 h at RT. The membranes were then washed 3 times with PBS-T and once with water before being immersed in ECL substrate solution (Amersham, GE Healthcare) for 5 min. The membranes were then exposed to ECL Film (Amersham, GE Healthcare) for 1-5 min.

2.5 Necrotic cell therapy

2.5.1 Transfection and induction of necrosis in DC2.4 cells, and vaccination of mice.

DC2.4 cells were transfected with TransIT and an EMCV-IRES-Core and EMCV-IRES-NS3/4A as described in section 2.3.2.5. Forty-eight hours later, the cells were harvested and washed with PBS. The cells were then resuspended in PBS and heated at 63°C for 30min on a heat block to induce necrosis. Cell necrosis was determined by Trypan Blue inclusion. Necrotic cells were then adjusted to the appropriate dose, restricted to a volume of 100µl per injection. Each mouse (C57BL/6) received two injections of the pre-determined dose 1 week apart, subcutaneously in the nape of the neck. The mice were then culled 2 weeks post final injection by cervical dislocation for spleen removal.

2.5.2 Mouse splenocyte preparation

The mice were killed by cervical dislocation and the spleens removed. The spleens were pressed through a 0.45µm mesh and released cells washed through with growth media (RPMI supplemented with 10% FCS, HEPES buffer, L-glutamine, penicillin/Streptomycin, and 2-mercaptoethanol). The cells were then centrifuged at 1500rpm for 5 min to pellet the cells, resuspended in 1ml of ACK lysis buffer (Invitrogen) and incubated at RT for 1 min. The cells were then washed in growth media and resuspended at $2.5x10^6$ cells/ml for use in an IFN-γ ELIspot assay.

2.5.3 Mouse IFN-γ ELIspot

Mouse IFN-g ELIspots were set as per section 2.1.6, with only minor alterations. ELIspot plates were pre-treated with 30µl of 70% ethanol for 1 min, and then washed 3 times with

PBS, prior to addition of the capture mAB (AN18) (Mabtech) used at 5 μg/ml final in PBS. Mouse splenocytes were used at a final cell number of 125,000 cells/ well. The detector mAb used was R4-6A2 (Mabtech) at a final concentration of 1 μg/ml.

2.5.4 Mouse challenge experiment

Splenocytes from healthy untreated C57BL/6 mice were pulsed for 1 h with an NS3 peptide pool or left untreated. The untreated cells were then stained with 0.2μM of carboxyfluorescein succinimidyl ester (CFSE), and the NS3 pulsed cells stained with 2μM of CFSE. These cells were then mixed at a ratio of 1:1 and a total of $1x10^7$ cells administered to mice which had previously been vaccinated with two injections of necrotic HCV antigen positive DC2.4 cells. The labelled cells were administered IV via the tail vein. The mice were sacrificed at either 5 h or 16 h post injection and their spleens removed. Splenocyte preparations were then made and these cells analysed by flow cytometry to detect the two CFSE labelled populations. The specific killing of NS3 pulsed; CFSE labelled cells was calculated using the following formulae:

Ratio = Percentage of $CFSE^{LO}$/ Percentage of $CFSE^{HI}$

Percentage of Specific Killing = [1 - (Ratio control animal/Ratio test animal)] x 100

2.6 Immunotherapy with necrotic HCV antigen positive dendritic cells.

Twelve patients chronically infected with hepatitis C genotype 1 (gt1) virus who had previously failed to clear infection after standard therapies were enrolled into the study.

Ten patients had been treated with pegylated IFN/Ribavirin (pIFN/Rb) alone while patient #2 was previously treated with pIFN/Rb and telaprevir, and patient #12 was treated with a combination of polymerase and protease inhibitors and had not previously been treated with pIFN/Rb. There were 10 males and 2 females aged between 45-77 years (median ~55); 7 were infected with gt1a, 1 with gt1b while the subtype was not confirmed in the remaining 4 patients. The study was approved by the Human Research Ethics Committee of The Alfred Hospital, Melbourne and registered with the Australian and New Zealand Clinical Trials Registry (ACTRN 12610000067077). All participants in the study provided informed consent in writing and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Chapter 3 – Dendritic Cell Immunotherapy of persistent HCV infection

3.1 Introduction

There are approximately 200 million HCV carriers worldwide. The current therapy (at the time this work was performed) consisting of a combination of pegylated IFN-alpha and ribavirin results in viral clearance rates of around 50%. The introduction of the new protease inhibitors, teleprevir (Incivek; Vertex Pharmaceuticals Cambridge, MA, USA) and boceprevir (Victrelis; Merck & Co Whitehouse Station, NJ, USA), is likely to increase these rates; however these are restricted to genotype 1 infected individuals (Welsch et al., 2012). The acute phase of infection is usually subclinical, however viral clearance is believed to be due to a diverse, sustained $CD4^+$ and $CD8^+$ T cell response (Bowen and Walker, 2005). In cases where a weak, transient cell mediated response is induced, the infection persists. The cause of this deficiency is unclear however the cause is likely to be caused by multiple factors, mediated by both the virus and the host.

The virus has evolved many mechanisms to ensure persistence. Some HCV proteins are immunosuppressive and block signalling pathways (Gale and Foy, 2005), and B and T cell epitopes induce selective pressure resulting in the generation of escape mutants (Kato et al., 1994, Erickson et al., 2001). There is a failure of the innate immune response due to a down regulation in NK cell activity caused by direct binding of the E2 protein via the CD81 receptor (Crotta et al., 2002, Tseng and Klimpel, 2002), and impairment in effector T cell function and in DC function which further amplifies the impairment in T cells by lack of priming (Wedemeyer et al., 2002, Gowans et al., 2004). T cell inhibition is further facilitated by upregulation of Programmed Death-1 (PD-1) a T cell inhibitory protein

(Radziewicz et al., 2007), and there is also evidence of an imbalance in the ratio of regulatory T cells and T effector cells (Li et al., 2007).

DC found in the blood of HCV infected patients have been reported to have a lower frequency compared with uninfected individuals and are impaired in their function (Auffermann-Gretzinger et al., 2001, Bain et al., 2001, Della Bella et al., 2007, Goutagny et al., 2004, Kanto et al., 1999b, Kanto et al., 2004, Murakami et al., 2004, Ulsenheimer et al., 2005). However there are studies which suggest otherwise (Longman et al., 2005, Piccioli et al., 2005). Regardless, DC are central to the development of antigen-specific $CD4^+$ and $CD8^+$ T cells, and are therefore necessary to elicit an effective immune response. DC immunotherapy has been proposed as a novel therapy to eliminate tumours and infectious agents by manipulating the cells *ex vivo*, then infusing these cells back into the patient. This is thought to boost the patient's existing immune response or generate immunity *de novo*. However, due to the scarcity of DC found in the blood, DC derived from $CD14^+$ monocytes or $CD34^+$ stem cells are used to attain the high numbers required for this procedure.

The aim of this section of this thesis was to examine the immune response of patients who had previously failed IFN-α based therapy after infusion with autologous monocyte-derived DC, loaded with HLA A2-restricted, HCV-specific CTL lipopeptides. This was to address the hypothesis that DC from HCV patients that are loaded and matured *ex vivo* with HCV lipopeptides, followed by autologous transfusion will be able to prime naïve T-cells and/or stimulate existing HCV-specific cell mediated immunity

3.2 Experimental design

The demographics of patients enrolled in the study are described in table 3.1. Eligible subjects included adults who had previously failed IFN-based therapy, aged 18–75 years with repeated serological evidence of HCV genotype 1 infection and/or quantifiable serum HCV RNA >600 IU/ml (Roche Cobas TaqMan HCV Test, Roche Diagnostics, Indianapolis, IN), compensated liver disease (Child–Pugh score <7) and histological findings consistent with chronic hepatitis C on a previous liver biopsy. Exclusion criteria included: non-HCV genotype 1 infection, hepatitis B virus and/or human immunodeficiency virus infection, history of decompensated liver disease, evidence of hepatocellular carcinoma, causes of chronic liver disease other than HCV, therapy with any systemic anti-viral, antineoplastic, or immunomodulatory agent within the preceding 6 months, pregnancy or breast feeding, neutrophil count <1500 cells/mm3, platelet count <90,000 cells/mm3, haemoglobin concentration <120 g/L in women or <130 g/L in men, serum creatinine level >1.5 times the ULN, history of autoimmune disease or any severe chronic or uncontrolled disease, current or recent drug or alcohol abuse, and unwillingness to provide informed consent. Patients with clinical and/or histologic evidence of cirrhosis were also excluded.

Patients 1 to 6 received injections according to an escalating dose schedule as shown in table 3.2. At each injection timepoint, the patient received 1 Dose Unit (DU) consisting of $1x10^7$ cells intradermally, simultaneously with the IV dose described in table 3.2, with multiple doses administered at two week intervals. In the initial design, patient 6 was to

receive 3 doses of 5DU, however due to a poor cell yield, he only received 2DU at each timepoint. In order to prevent merely repeating the schedule of patient 5, the patient received all injections by the ID route.

Blood samples were taken at two week intervals for up to twelve weeks post final injection. PBMC were isolated and assayed using IFN- γ ELIspot, as described in section 2.1.5 and 2.1.6.

3.3 Results.

3.3.1 IFN-γ responses of patients over course of treatment

3.3.1.1 Patient 1

Patient 1 received 1 dose unit (DU, consisting of $1x10^7$ cells) of peptide-pulsed autologous mo-DC and 1 DU administered ID, and was monitored for a period of 12 weeks post infusion. As shown in figure 3.1i, patient 1 had almost no baseline responses to any of the CTL peptides or HCV peptide pools. At 2 weeks post final infusion, elevated responses to CTL2, CTL4, Core and NS3 were observed although these were not significant (Figure 3.1ii). At 6 weeks post final infusion elevated responses to Core, NS4B and E2 were observed (Figure 3.1iii). As shown in Figure 3.1iv, the only

Table 3.1. Demographic and baseline disease characteristics of patients.

Characteristics	Patient No.					
	1	2	3	4	5	6
Sex (M/F)	F	M	M	M	M	M
Age (yr)	50	46	61	44	48	39
Caucasian (Yes/No)	Yes	Yes	Yes	Yes	Yes	Yes
HCV genotype	1a/b	1	1a/b	1a	1a/b	1b
HCV RNA titre ($\times 10^6$ IU/ml)	0.56	1.10	0.22	2.10	2.49	2.82
Alanine aminotransferase (U/L)	49	89	63	135	51	42
Metavir stage	1	0	2	1	2	2
Child–Pugh score	5	5	5	5	5	5
Haemoglobin (g/L)	139	153	167	166	155	150
Platelets ($\times 10^9$)	169	158	269	212	296	158
Lymphocytes ($\times 10^9$/L)	1.59	1.44	2.91	1.71	1.96	1.68

Table 3.2. Escalating Dose Schedule.

Patient #	Infusion 1	Infusion 2	Infusion 3
	IV dose units* (1 dose unit = 1 x 10^7 cells)		
1	1	-	-
2	1**	1	-
3	1	2	5
4	1	5	-
5	2	2	2
6	5***(2)	5***(2)	5***(2)

** Multiple doses were given at two week intervals.

*** This scheduled dose was not infused because the desired number of DC could not be generated. Instead, two dose units were infused at each timepoint by the intradermal route only.

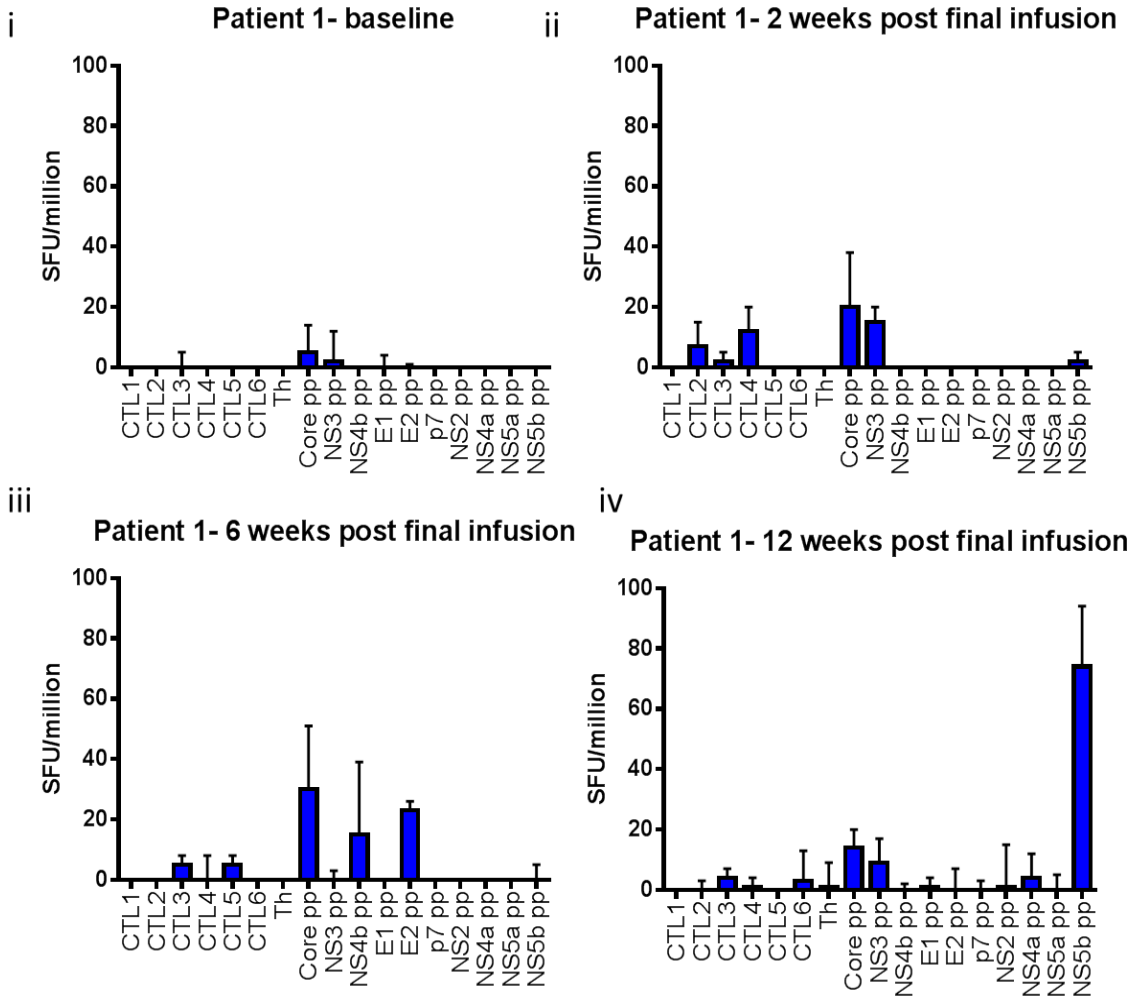

Figure 3.1. IFN-γ ELIspot results against specific peptides or peptide pools over the course of treatment for patient 1. Blood samples were taken at 2 weekly intervals over the course of the treatment up to 12 weeks post final infusion and ELIspot performed (i-iv) using PBMC isolated by ficoll-density gradient centrifugation and stimulated for 20-24h with 6 individual peptides representing the lipopeptides used in the DC immunotherapy, and 10 peptide pools spanning the entire HCV polyprotein. Blue bars represent spot forming units/million cells. Negative control responses were subtracted from each, with error bars representing +/- 1 standard deviation. These parameters also apply to the figures which follow in this section of the thesis.

significant response observed was to NS5B which was elevated to 74 Spot Forming Units (SFU) per million cells.

Although responses to individual HCV peptides were not sustained throughout the monitoring period, the total anti-HCV specific response was elevated throughout the course of treatment. As shown in figure 3.2, the total anti-HCV IFN-gamma response increased from 5 SFU/million at baseline, rose to 58 SFU/million at 2 weeks post infusion, 79 SFU/million at 4 weeks post infusion and peaked at 110 SFU/million at 12 weeks post infusion.

3.3.1.2 Patient 2

Patient 2 received two DU of autologous mo-DC pulsed with the HCV-lipopeptides 2 weeks apart, and was monitored for a period of 12 weeks post final infusion. Patient 2 showed a transient HCV-specific response, with responses to Core, CTL3 and NS5A observed at 2, 4 and 6 weeks post final infusion (Figure 3.3, iii, iv, v), with the strongest response of 320 SFU/million cells observed to NS5A 6 weeks post final infusion. However this response was not observed at 12 weeks post final infusion and all responses were reduced to below baseline levels (Figure 3.2 vi).

The total anti-HCV response showed a transient nature over the entire period. As shown in figure 3.4, Patient 2 had a total anti-HCV IFN-gamma response of 30 SFU/million at baseline that rose to 110 SFU/million prior to the second infusion and to 270 SFU/million at 2 weeks post final infusion. The response then fell to 70 SFU/million at 4 weeks post

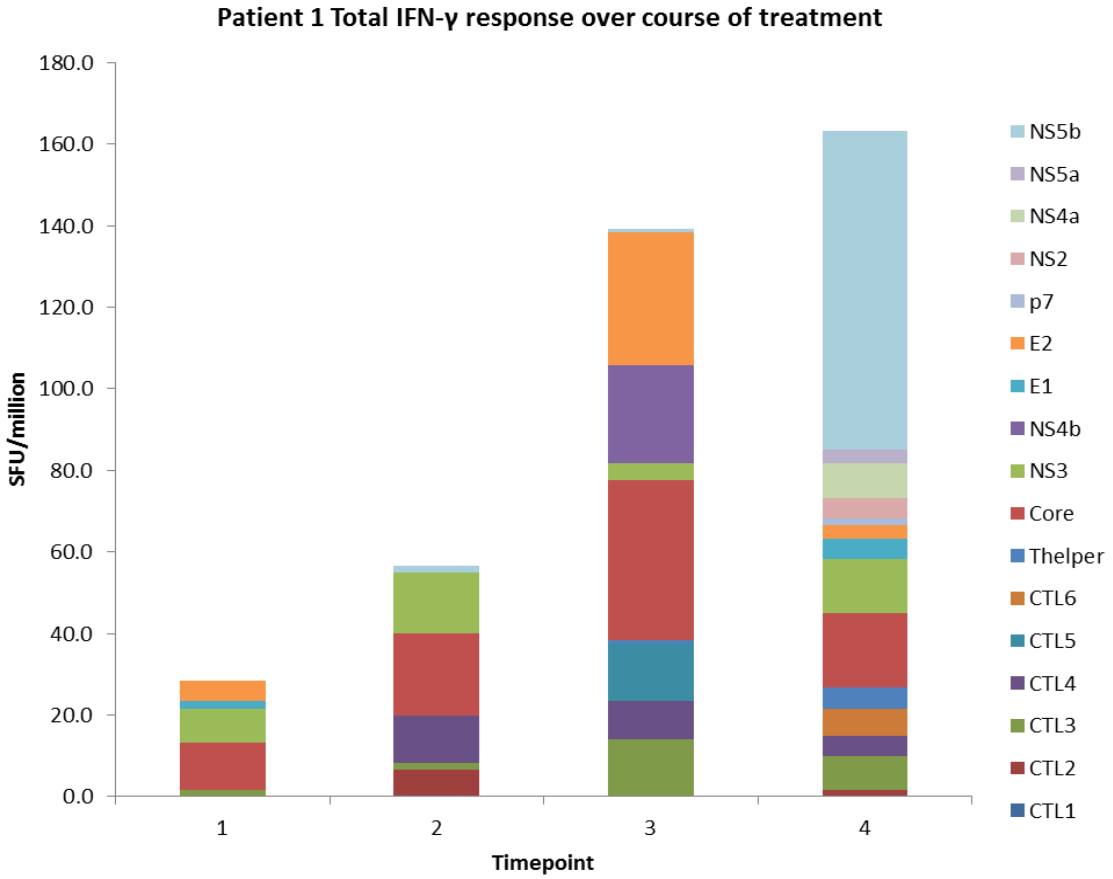

Figure 3.2 Summary of total IFN-γ ELIspot results over the course of treatment for patient 1. Timepoint 1 represents the Baseline, timepoint 2 represents 2 weeks post final injection, timepoint 3 represents 6 weeks and timepoint 4 represents 12 weeks. Blood samples were taken at specific intervals over the course of the treatment up to 12 weeks post final infusion and ELIspot performed using PBMC isolated by ficoll-density gradient centrifugation and stimulated for 20-24h with the 6 individual peptides representing the lipopeptides used in the DC immunotherapy and 10 peptide pools spanning the entire HCV polyprotein. Negative control responses were subtracted from each response and individual responses were totalled. Bars represent total spots/million cells for each timepoint with the individual colours representing the specific response to different peptide pools. These parameters also apply for the figures which follow in this section of the thesis.

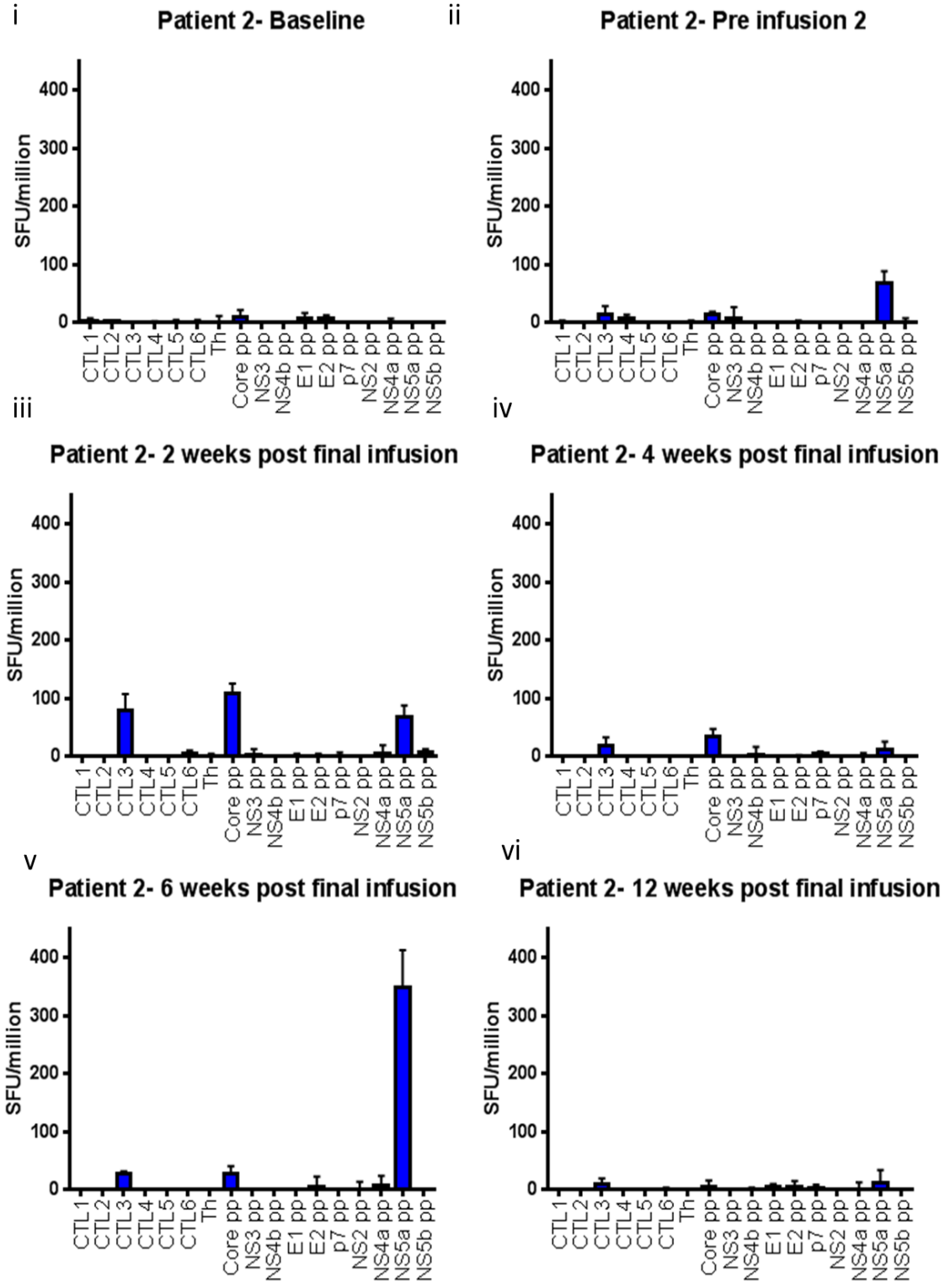

Figure 3.3. IFN-γ ELIspot results over the course of treatment for patient 2.

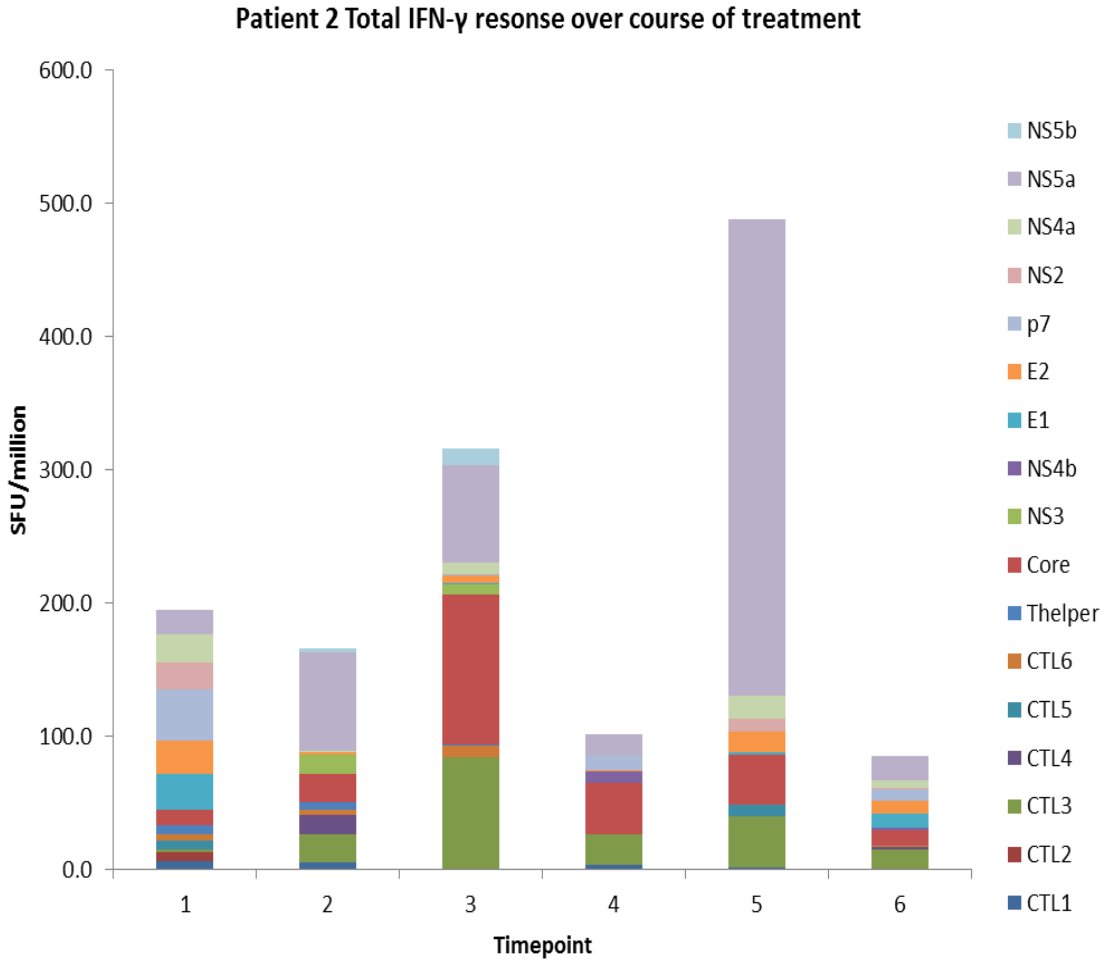

Figure 3.4 Summary of total IFN-γ ELIspot results over the course of treatment for patient 2. Timepoint 1 represents the Baseline, timepoint 2 represents pre injection 2, timepoint 3 represents 2 weeks post final injection, timepoint 4 represents 4 weeks, timepoint 5 represents 6 weeks and timepoint 6 represents 12 weeks.

final infusion and rose to 420 SFU/million at 6 weeks post final infusion. The total IFN-gamma response to HCV antigens was reduced to baseline levels at 12 weeks post final infusion.

3.3.1.3 Patient 3.

Patient 3 received an escalating dose schedule, with the first dose consisting of 1 DU, dose two consisting of 2 DU and dose 3 consisting of the highest dose used in the trial, viz. 5 DU. At baseline, this patient showed low responses to a range of HCV peptides including NS3, NS4B, E1, E2, p7, NS2, NS5A and NS5B and a higher response of 60 SFU/million to the Core peptide pool (Fig 3.5 i). The IFN-γ ELIspot performed prior to infusion 2 showed that this Core response remained constant, while a *de novo* response was seen to NS5A. These responses were further amplified prior to infusion 3 (Figure 3.5 iii) with the Core response elevated to 200SFU/million and the NS5A response reaching 130SFU/million. However, neither of these responses was sustained throughout the monitoring period. A response of 100SFU/million to NS5B was observed at 4 weeks post final infusion but this was not sustained either. All responses dropped to below baseline levels by the final week of monitoring (Fig 3.5 iv-vii).

At baseline, the total anti-HCV IFN-γ response was 110 SFU/million (Fig 3.6). Prior to the second infusion this response had risen to 150 SFU/million and prior to the third infusion to 400 SFU/million. These responses were not sustained as responses measured at 2, 6 and 12 weeks were observed to be below those of baseline although a slightly higher response of 150 SFU/million was observed at 4 weeks.

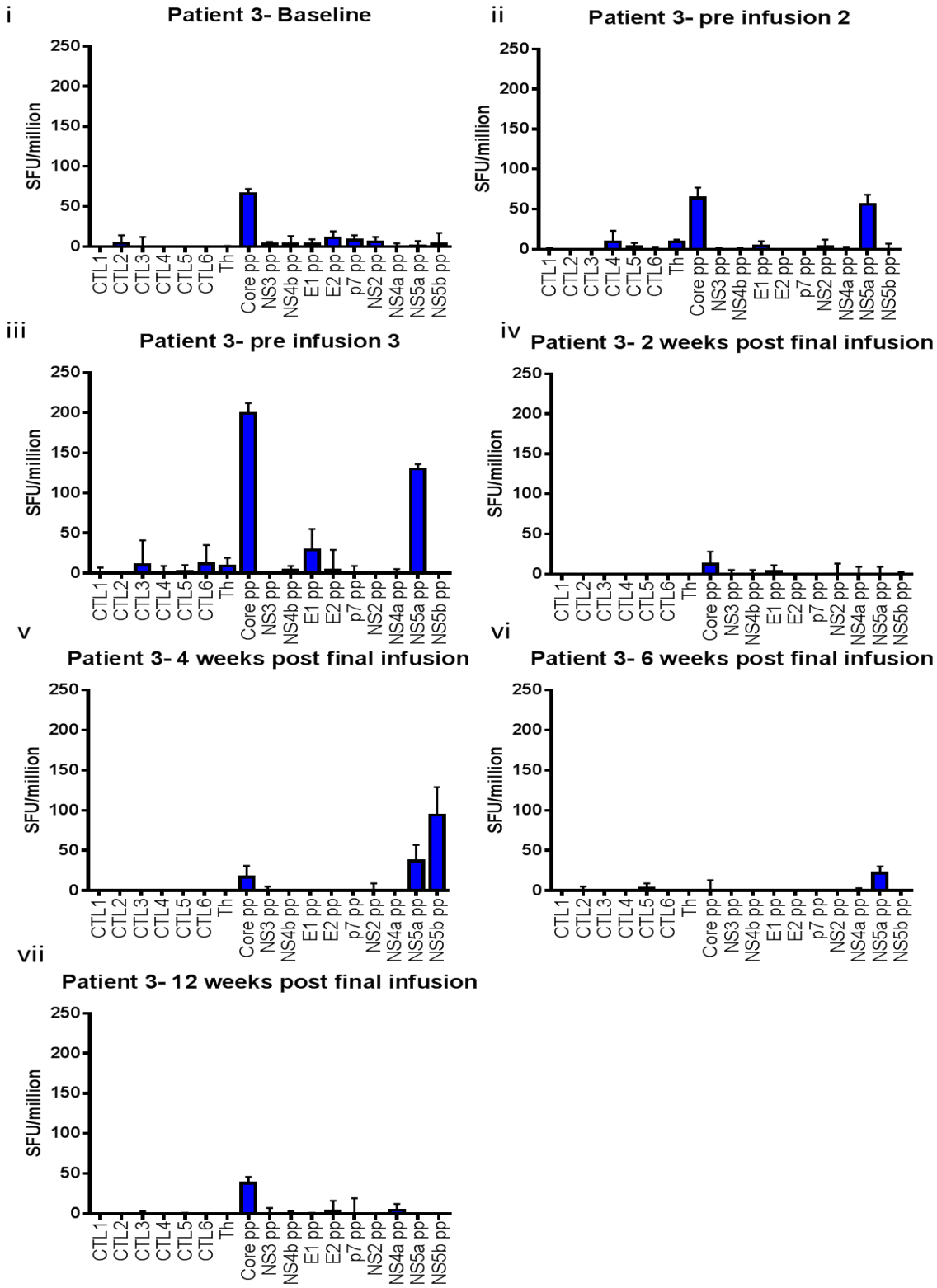

Figure 3.5. IFN-γ ELIspot results over course of treatment for patient 3.

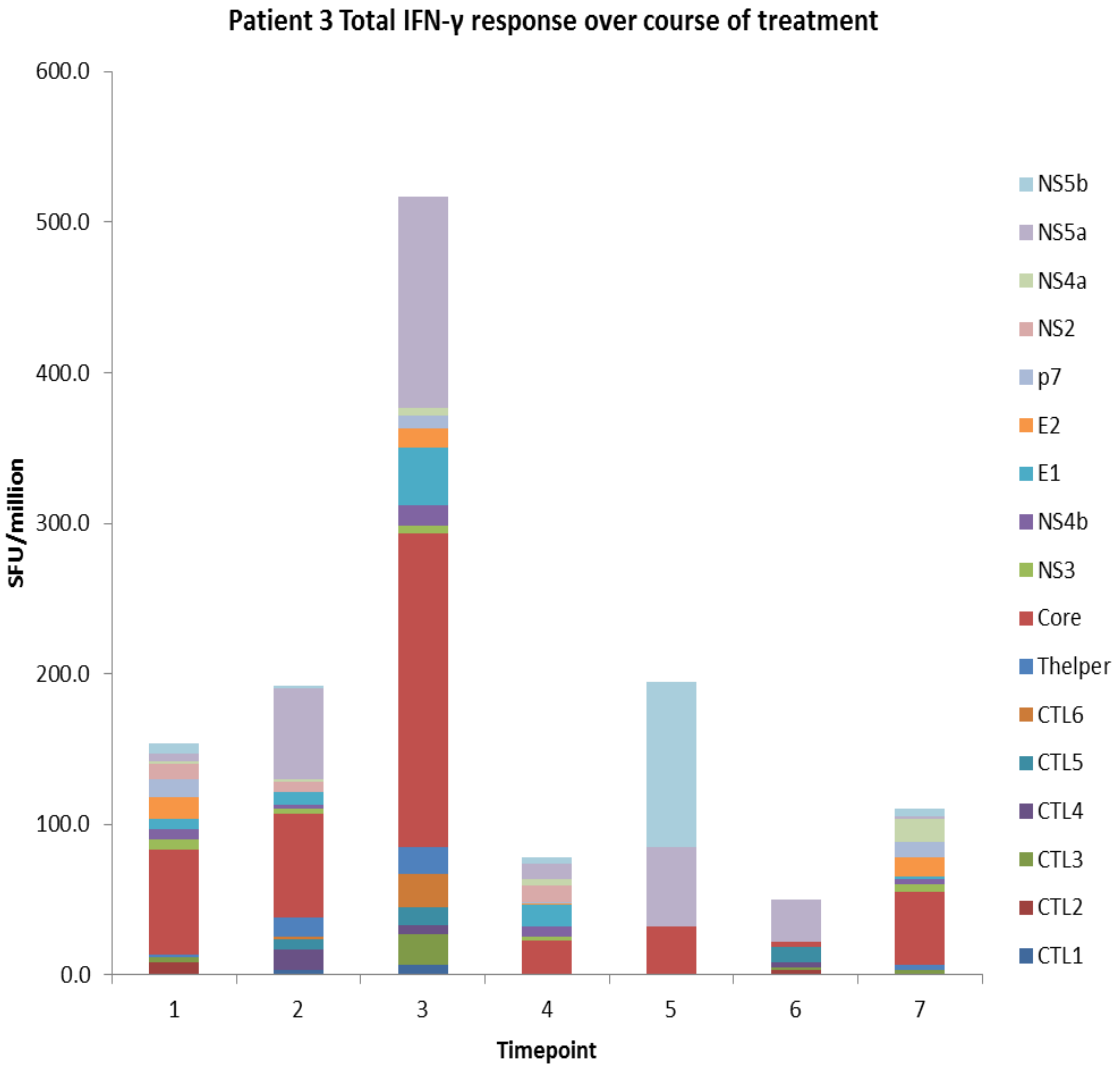

Figure 3.6 Summary of total IFN-γ ELIspot results over course of treatment for patient 3. Timepoint 1 represents the Baseline, timepoint 2 represents pre injection 2, timepoint 3 represents pre infusion 3, timepoint 4 represents 2 weeks, timepoint 5 represents 4 weeks, timepoint 6 represents 6 weeks and timepoint 7 represents 12 weeks.

3.3.1.4 Patient 4.

Patient 4 received two infusions; the first consisted of 1 DU and the second of 5 DU. At baseline, low responses to NS3, E2 and NS4A were observed (<20 SFU/million) as shown in figure 3.7i. Notable responses were not observed until 4 weeks post final infusion when responses to Core (43 SFU/million), NS4B (20 SFU/million), NS2 (10 SFU/million), E2 and NS5A (40SFU/million) were detected (Fig 3.7 iv). At 6 weeks post final infusion the responses to Core, NS3 and E2 were reduced to 10 SFU/million, 50 SFU/million and 20 SFU/million respectively (Fig 3.7 v). At 12 weeks post final infusion the only notable response was to the Core peptide pool with 30 SFU/million (figure 3.7 vi).

As shown in figure 3.8, at baseline the total anti-HCV response for patient 4 was 22 SFU/million. At 4 weeks post final infusion this rose to 150 SFU/million which was the peak for the period of monitoring. The total anti-HCV response was reduced to 90 and 50 SFU/million at 6 and 12 weeks post final infusion respectively.

3.3.1.5 Patient 5.

Patient 5 received 3 doses of 2DU by the IV route over the course of treatment. As shown in figure 3.9i, Patient 5 showed an uncharacteristically high baseline response, with detectable responses to CTL1, p7, NS2, and NS5B (<20 SFU/million). Higher responses were also observed to E2 and NS5A (45 and 50 SFU/million respectively). Prior to the second infusion, these responses were not detected as only the Core and E2 peptides produced any response, although these were below 20 SFU/million (Fig 3.9 ii). Prior to

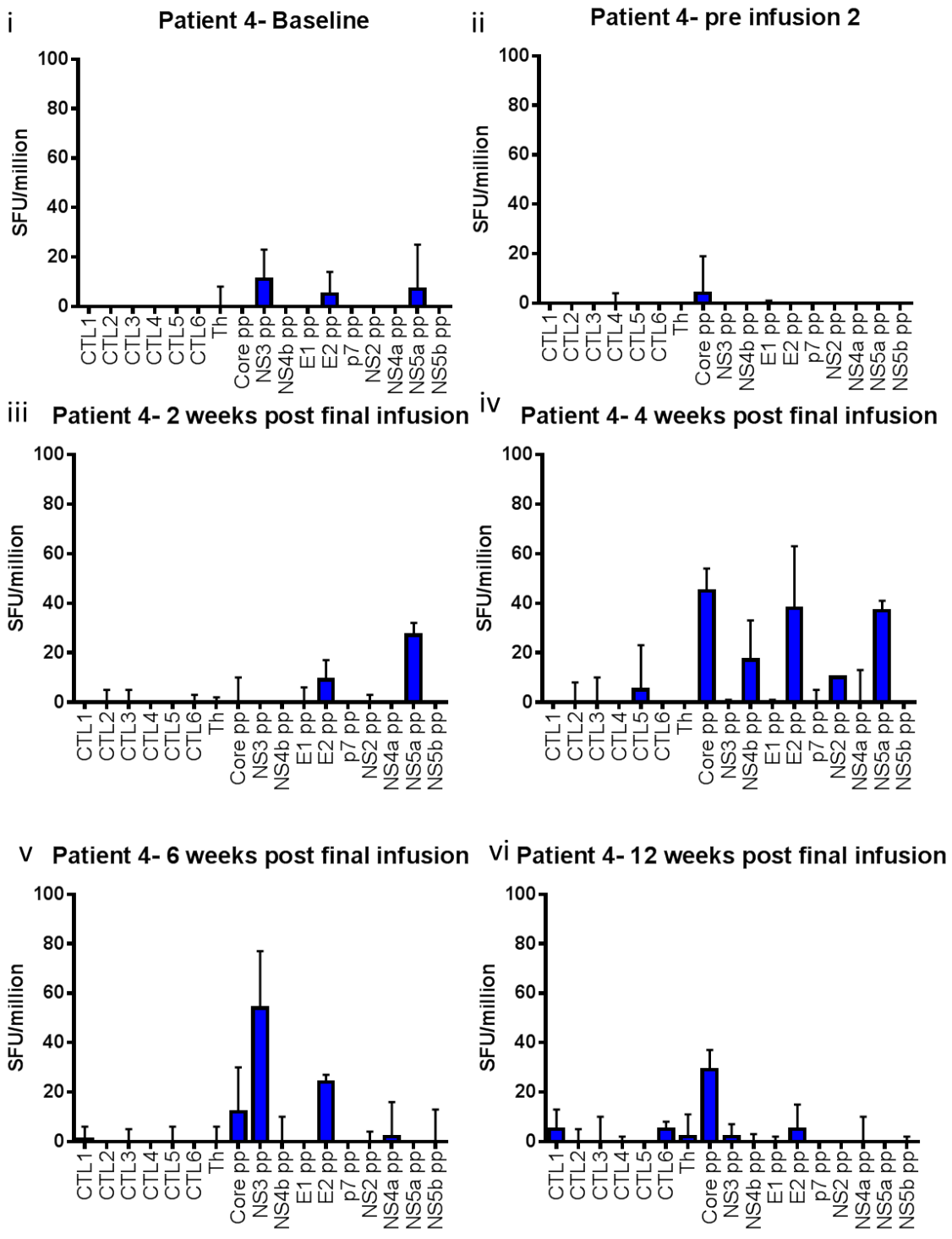

Figure 3.7. IFN-γ ELIspot results over course of treatment for patient 4.

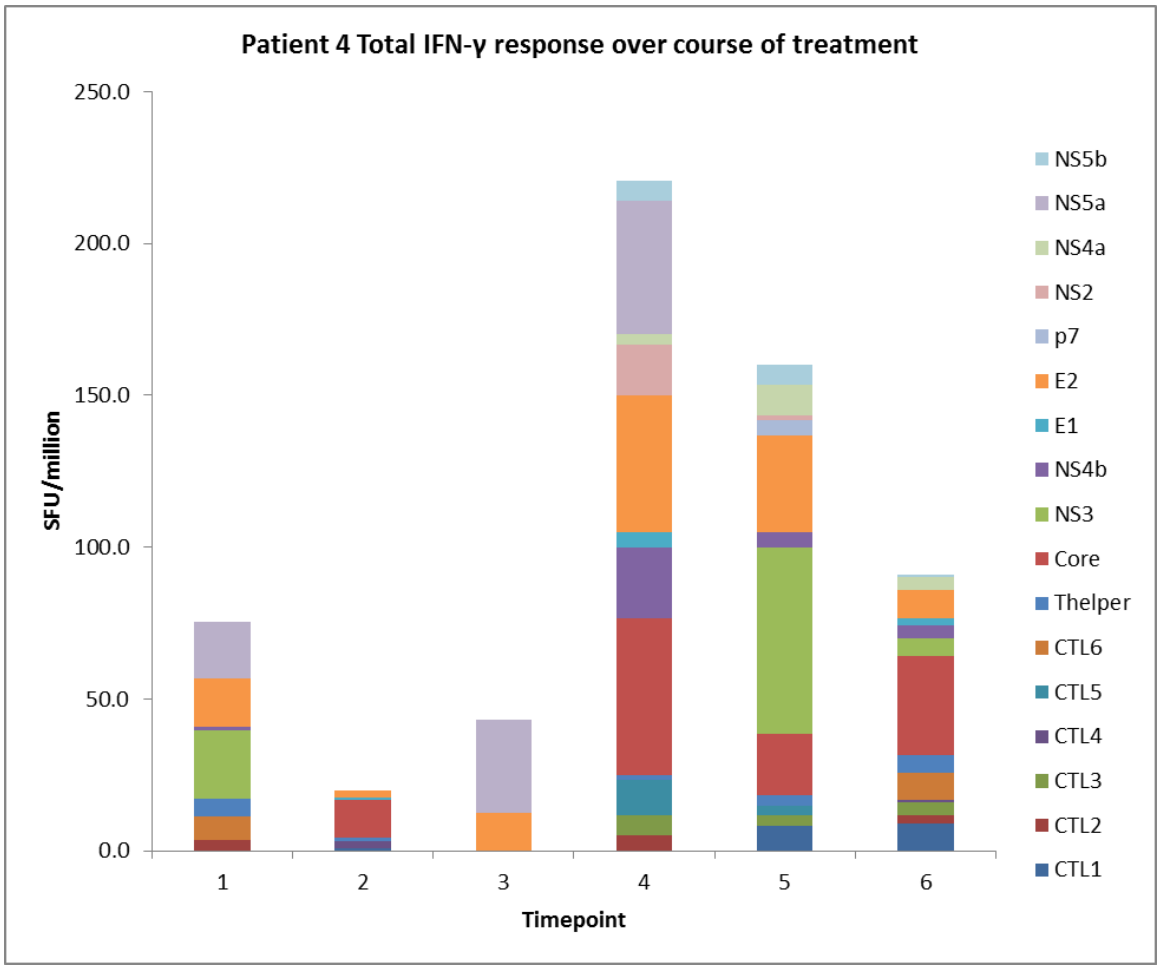

Figure 3.8 Summary of total IFN-γ ELIspot results over course of treatment for patient 4. Timepoint 1 represents the Baseline, timepoint 2 represents pre injection 2, timepoint 3 represents 2 weeks post final injection, timepoint 4 represents 4 weeks, timepoint 5 represents 6 weeks and timepoint 6 represents 12 weeks.

infusion 3 (Fig 3.9 iii) a response of 68 SFU/million to NS3 was noted, with lower responses (<20 SFU/million) to (Core, E2, and NS5B). As seen in figure 3.9 iv-vii, patient 5 failed to produce any notable responses over the remainder of the monitoring period.

This trend of high responses prior to the third infusion was also reflected in the total anti-HCV response for Patient 5 (Figure 3.10). The baseline response and the response prior to the third infusion were observed to be high (> 100 SFU/million) while total anti-HCV responses at all other timepoints were below 20 SFU/million.

3.3.1.6 Patient 6.

Initially patient 6 was to receive 3 doses of 5 DU by the IV route. However, due to a low cell yield, only 3 doses of 2DU were infused but all doses were administered via the ID route. As shown in figure 3.11 i, patient 6 also had relatively high responses evident at baseline, with responses to Core, E2 and NS5A noted (39, 29 and 44 SFU/million respectively. Prior to infusion 2 (figure 3.11 ii), a high response of 135 SFU/million to CTL4 was noted, with lower responses to CTL5, Core and E2 of 13, 18 and 13 SFU/million respectively. A response of 33 SFU/million was observed to the Th epitope. A much broader range of responses was observed prior to the third infusion and 2 weeks post final infusion, with responses detected to CTL2, CTL3, CTL4, CTL 5, Th, Core, NS3, NS4B, E2, p7, NS2, NS4A, NS5A and NS5B (Fig 3.11 iii and iv). The most notable responses were to CTL4, Core, NS4B and E2 prior to the third infusion with responses of 57, 37, 27 and 39 SFU/million and 37, 60, 25, and 22 SFU/million 2 weeks post final

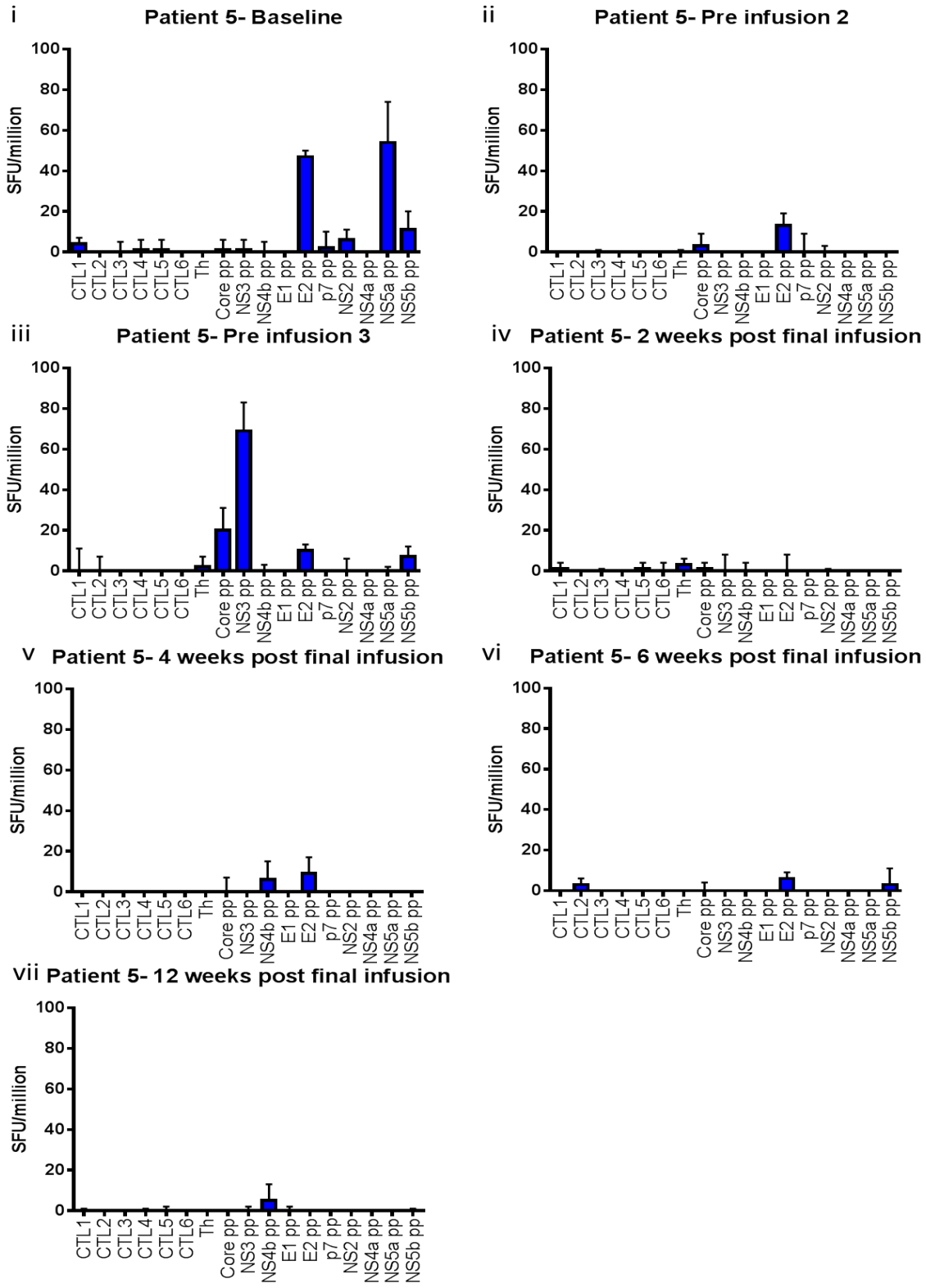

Figure 3.9. IFN-γ ELIspot results over course of treatment for patient 5.

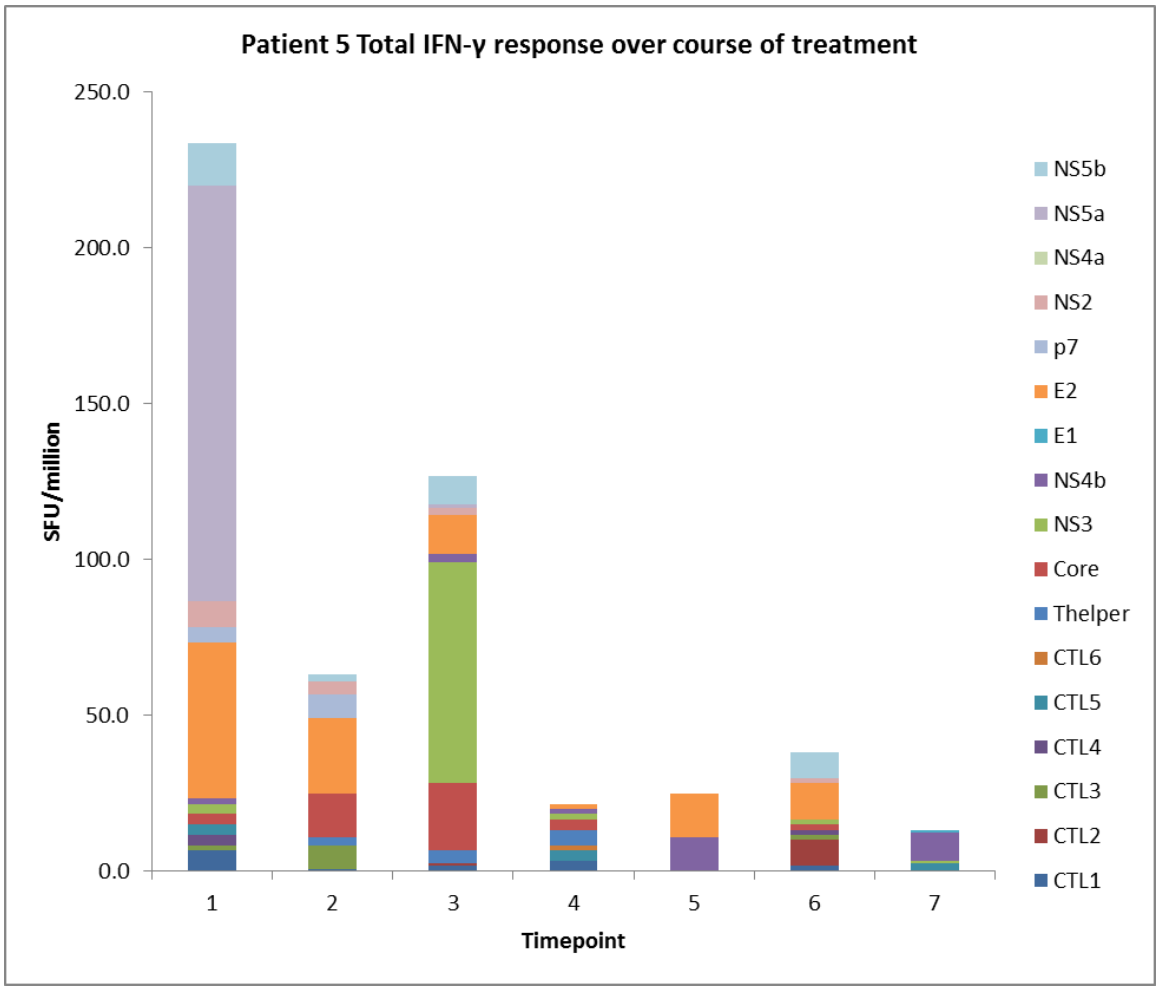

Figure 3.10 Summary of total IFN-γ ELIspot results over course of treatment for patient 5. Timepoint 1 represents the Baseline, timepoint 2 represents pre injection 2, timepoint 3 represents pre infusion 3, timepoint 4 represents 2 weeks, timepoint 5 represents 4 weeks, timepoint 6 represents 6 weeks and timepoint 7 represents 12 weeks.

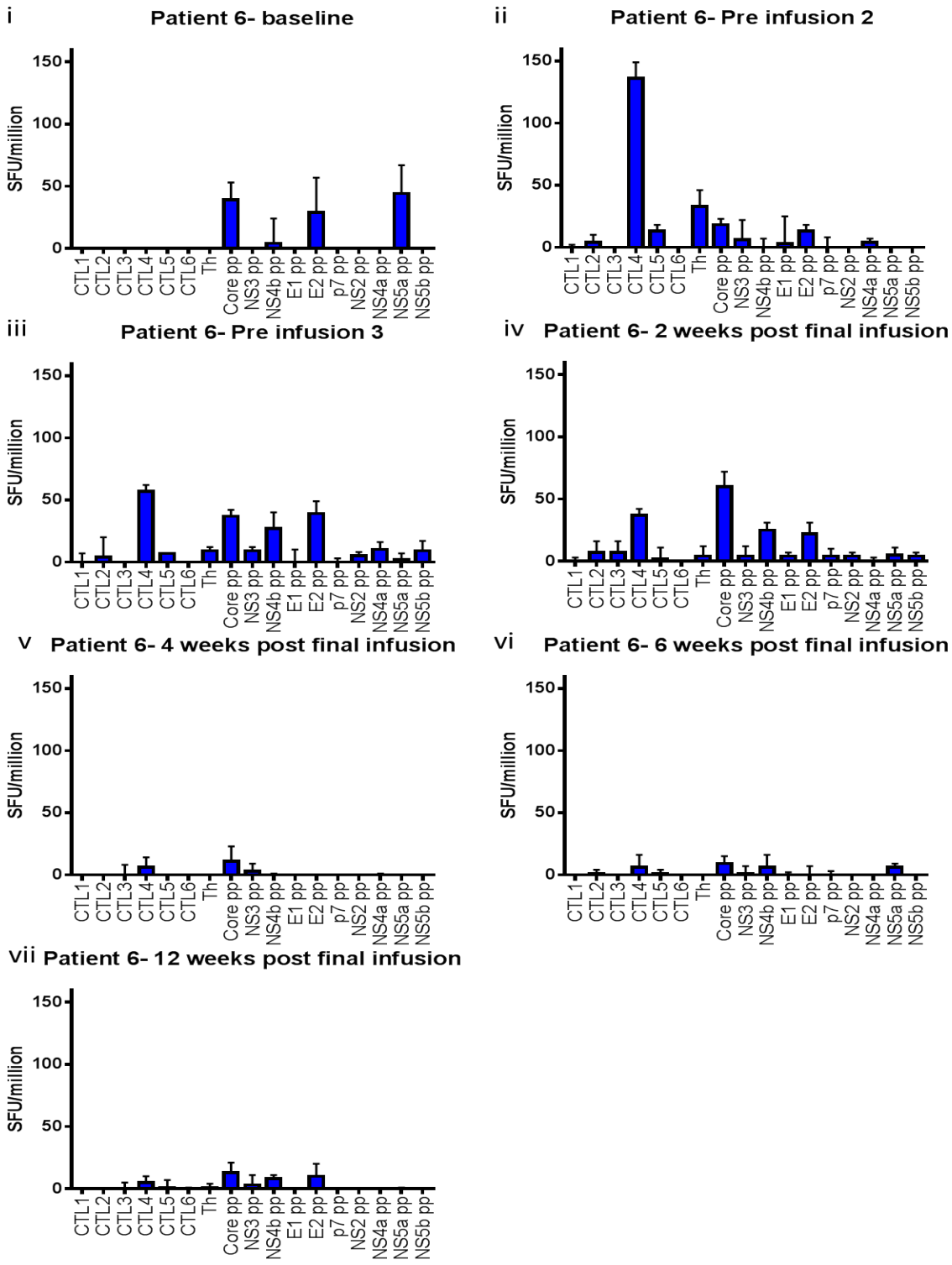

Figure 3.11. IFN-γ ELIspot results over course of treatment for patient 6.

infusion. These responses were transient however and were not sustained throughout the monitoring period (Fig 3.11 v-vii).

As seen in figure 3.12 the total anti-HCV response for patient 6 at baseline was 110 SFU/million, rising to 225 SFU/million prior to infusion 2. This was reduced to 215 SFU/million prior to infusion 3 and further reduced to 190 SFU/million at two weeks post final infusion. For the remainder of the monitoring period the total anti-HCV response was reduced to below 40 SFU/million, well below the baseline level.

3.3.1.7 Patient responses to an influenza A virus conserved peptide.

In order to ensure that changes in IFN-γ responses were not simply due to variations in the ELIspot, at each time point, patient responses to an influenza virus peptide (Flu matrix peptide) were tested. Flu Matrix Peptide was selected as was believed the majority of the Australian population would have been exposed to Influenza virus either via vaccination or infection. Two other positive controls were included in the assays (Cytomegalovirus, Epstein-Barr virus and Influenza virus peptide pool (CEF) and Phytohaemagglutinin) however these responses were too high to be measured. As shown in figure 3.13 the responses to the Flu peptide showed some variation over the entire monitoring period essentially for each patient. However, these variations were generally lower than those observed in the anti-HCV responses presented in the previous figures.

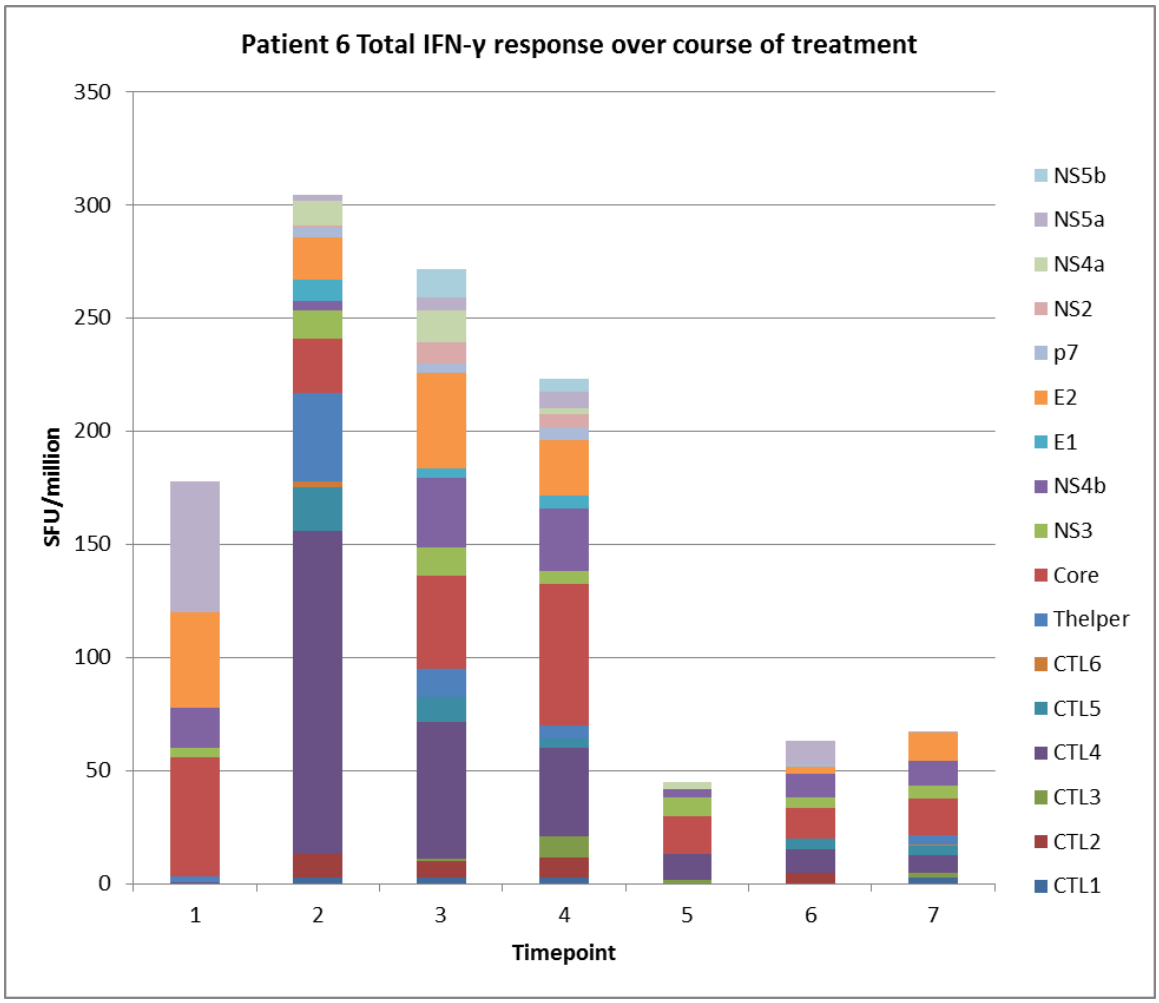

Figure 3.12 Summary of total IFN-γ ELIspot results over course of treatment for patient 6. Timepoint 1 represents the Baseline, timepoint 2 represents pre injection 2, timepoint 3 represents pre infusion 3, timepoint 4 represents 2 weeks, timepoint 5 represents 4 weeks, timepoint 6 represents 6 weeks and timepoint 7 represents 12 weeks.

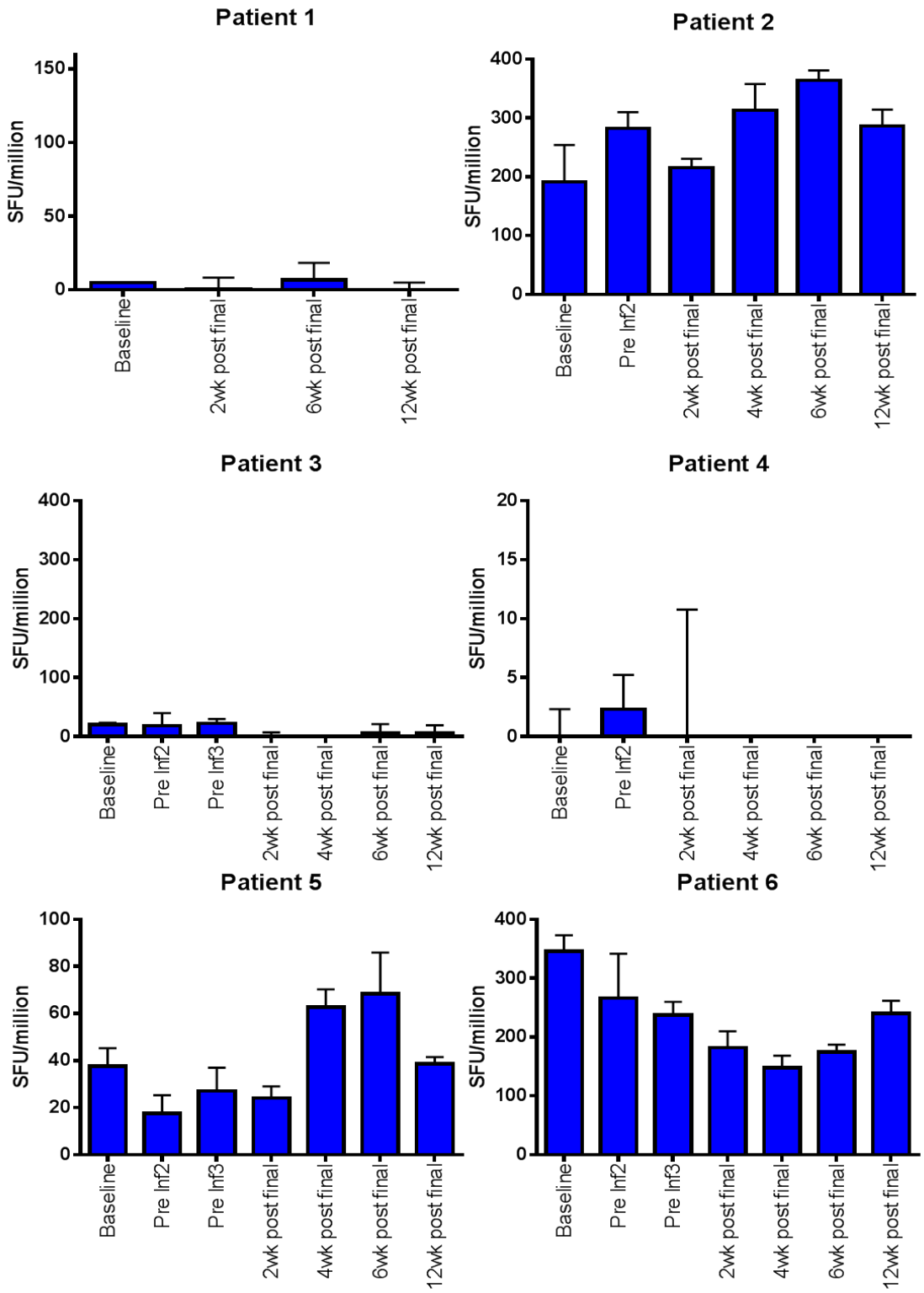

Figure 3.13 IFN-γ ELIspot results to Influenza virus (flu) matrix peptide over course of treatment for each patient (1-6).

3.3.2 Patient Clinical outcomes

3.3.2.1 Patient ALT, viral load, and sequencing results

In addition to IFN-γ ELIspot responses, changes in the viral load and ALT levels were assessed to determine the efficacy of the treatment. These tests were performed by the Alfred Hospital pathology services as part of the patient's standard of care and analysed by Associate Professor Stuart Roberts. As shown in figure 3.14 no significant changes were observed in either the viral load or ALT levels in any of the patients over the entire monitoring period.

The CTL peptide epitopes used in the vaccine were chosen based on a high degree of conservation and because responses to these epitopes were detected in patients who recovered from acute HCV infection (Ward et al., 2002). However it was not clear if any induced T cell responses would recognise epitopes expressed by the circulating virus. Accordingly, three regions of the viral genome viz. core, NS3, NS4B, were sequenced in the baseline or close to baseline serum sample from each patient (work done by Joe Torresi and Ruth Chin of Melbourne University). Although the NS4B region from three patients was unable to be amplified, RT-PCR amplification followed by sequencing of the product showed that only three epitopes differed from the sequence of the epitopes included in the cellular vaccine (Table 3.3). Of these mutations, the mutation in the core 132 epitope in patient #3 was located in an anchor residue suggesting that T cell responses to the epitope included in the vaccine would not recognise virus-infected cells. However, the other two mutations were not located in anchor residues. Consequently, the

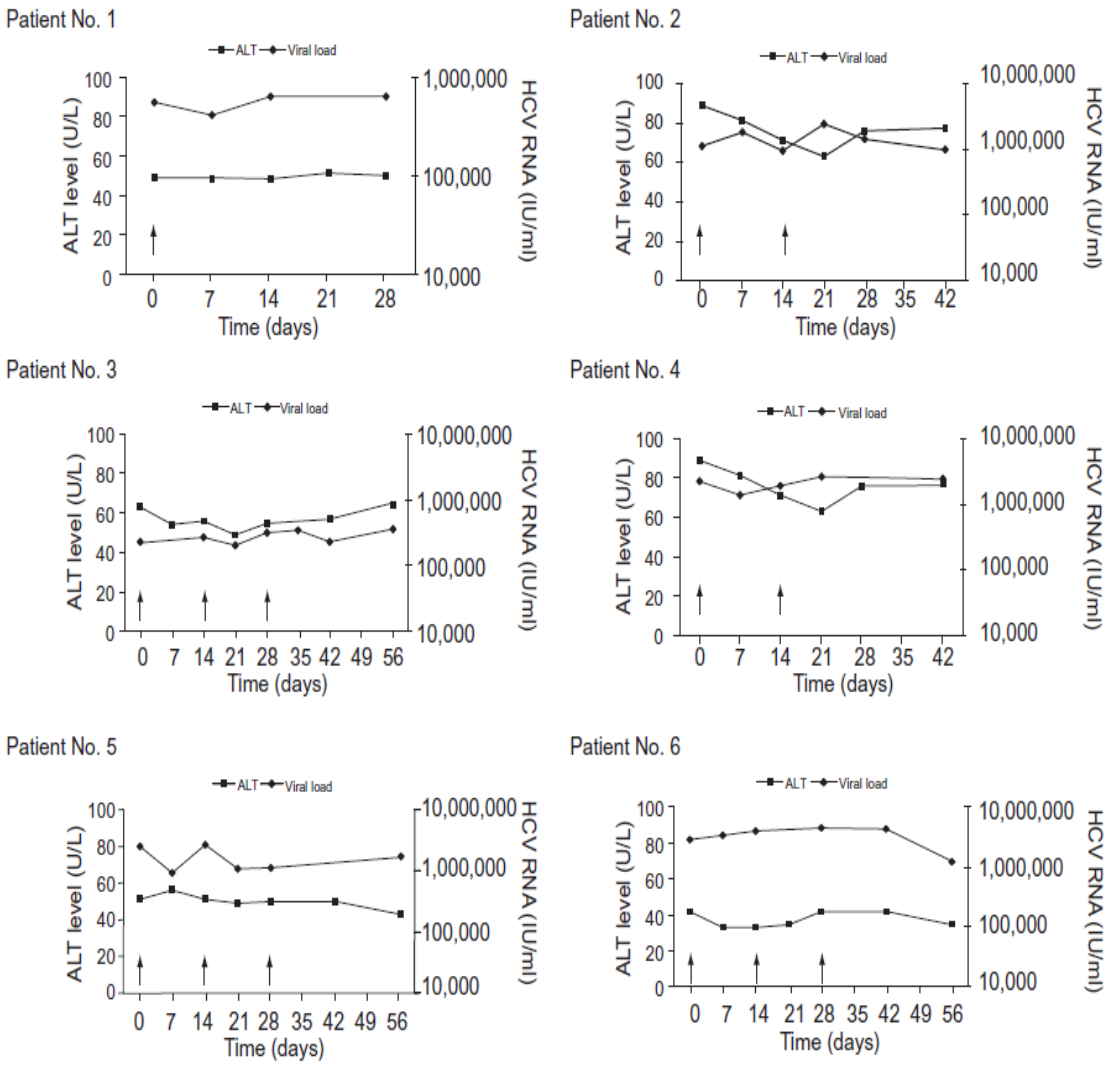

Figure 3.14. Viral load and liver function analyses. Serum HCV RNA (diamonds) and ALT levels (squares) in individual patients during and after injections of autologous HCV lipopeptide-primed dendritic cells (arrows). The results were analyzed by Associate Professor Stuart Roberts.

Table 3.3. Summary of baseline RNA sequences. (Data kindly provided by Ruth Chin and Joseph Torresi of Melbourne University).

Patient #	1	2	3	4	5	6
CTL1, core 132 DLMGYIPLV	No change	No change	D**I**MGYIPLV	No change	No change	No change
CTL2 core 35 YLLPRRGPRL	No change	No change	No change	No change	No change	No change
CTL3 core 177 FLLALLSCLTV	No change	No change	No change	No change	No change	No change
CTL4 NS3 1406 KLVALGINAV	KLVALG**V**NAV	No change	KLVALG**V**NAV	No change	No change	NP
CTL5 NS4B 1807 LLFNILGGWV	NP**	NP	No change	No change	No change	NP
CTL6 NS4B 1851 ILAGYGAGV	NP	NP	No change	No change	No change	NP

* Amino acids shown in bold represent mutations from the peptide epitope contained in the vaccine.

** NP, no product from PCR.

inability of the vaccine to reduce the viral load is probably not related to discordant sequences in the epitopes included in the vaccine and those in the circulating virus.

3.4 Discussion.

The exact cause of immune dysfunction during HCV infection has yet to be characterized, although it is believed to be multi-factorial. The virus has developed many mechanisms which allow it to persist. Along with immunosuppressive proteins which block signaling pathways (Gale and Foy, 2005), persistence of HCV is also thought to be due to primary T cell failure and T cell exhaustion. However primary T cell failure is difficult to discern as many patients are diagnosed late in infection after persistent infection is established. Primary T cell failure may be caused by a dysfunction in antigen presentation (Bain et al., 2001, Lee et al., 2001, Sarobe et al., 2002), and the cause of T cell exhaustion is unclear; however a high viral load has been associated with T cell exhaustion in chronic virus models (Moskophidis et al., 1993) with T cell inhibition further facilitated by upregulation of PD-1 and TIM-3 during persistent HCV infection (Radziewicz et al., 2007).

Nevertheless, it may be possible to overcome these factors. Prior to this study, DC immunotherapy had not been used to treat HCV infection but had been used in cancer trials. The primary aim of this part of this thesis was to assess the immune response of persistently infected patients to DC Immunotherapy, in the hope that the level of HCV-specific cell mediated immunity could be increased to result in a reduction in the HCV viral load.

Initially this study was designed as a dose escalation trial to mimic the schedule of cancer trials using DC immunotherapy. However, these did not describe the optimal dose or the optimal route of infusion. Due to the natural variability in the number of $CD14^+$ monocytes in individuals it was not always possible to obtain the proposed cell dose. The difficulty in achieving the required cell yield reflects the difficulty in transferring laboratory scale conditions to Good Manufacturing Practice (GMP)/clinical scale conditions. However, alteration of the proposed protocol for patient 6, who received all injections I.D, resulted in a broader immune response when compared to the other patients. This may indicate the benefit of administering mo-DC solely via the I.D route. Furthermore, the duration and specificity of the immune response observed in this study failed to correlate with the number or size of the doses received by the patients.

This study showed that DC immunotherapy was able to elicit a specific immune response, as highlighted by the breadth of responses to HCV proteins seen in each of the six patients. It is important to note that the patients generally responded to the Core protein, and although the responses were not sustained they responses appeared early and were amongst the strongest and broadest responses present in 5 out of 6 patients. It is possible that these responses may have been sustained if patients had received a boost with a core-containing vaccine. Responses were also observed to proteins not contained in the cellular vaccine (such as E2, NS5A and NS5B). It is possible that this was caused by the targeted lysis of HCV-infected hepatocytes the contents of which were phagocytosed by resident APC, to result in epitope spreading through the cross

presentation of HCV proteins present in the lysed cells but not in the cellular vaccine. This hypothesis is addressed in detail in Chapter 6 of this thesis.

Responses to individual proteins were shown to be transient and were not sustained throughout the entire monitoring period in each patient. It is believed that a robust multi-specific sustained response to CD4 and CD8 T cell epitopes is required for viral clearance, and although responses to individual HCV proteins were not sustained there was evidence of increased total anti-HCV immune responses in some patients. For example the total anti-HCV response increased in patient #1 at each time point over the monitoring period. The total anti-HCV response was also increased in other patients at specific timepoints but none was able to break HCV tolerance and therefore no significant reduction in viral load was observed (Fig 3.14). No changes in ALT levels were observed, however this was expected, as previous studies showed that HCV clearance is likely to be non-cytolytic, as the HCV viral load in acute phase patients was decreased by several logs co-incident with T cell responses without associated increases in ALT (Bowen and Walker, 2005, Thimme et al., 2001). However, this is contradictory to the evidence for epitope spreading which was observed. It is possible that this was a consequence of the downstream effect of IFN-γ release, viz. by interferon stimulated genes (ISGs) and the increased activity of p53 (Fensterl and Sen, 2009, de Veer et al., 2001, Takaoka et al., 2003, Moiseeva et al., 2006), which promotes apoptosis in virus-infected cells, thereby providing a mechanism for epitope spreading. However, the number of cells lysed during this treatment was not sufficiently high to produce a significant change in ALT levels.

In theory DC immunotherapy is more likely to succeed in the treatment of viral infections such as HCV compared with solid tumors, due to the ease of which organs such as the liver can be infiltrated by effector T cells. Although the DC immunotherapy was capable of eliciting anti-HCV immune responses, these were not sustained, nor were they sufficient to affect a change in viral load. When this study was designed, the optimal route of DC administration was not clear, and future studies using DC therapy should infuse the cells solely via the intradermal route. Furthermore, the CTL epitopes used in this trial, although selected due to the high degree of conservation between genotypes, may differ to those contained in the analogous proteins of the infecting virus. It is also possible that the number of epitopes in any vaccine required to clear the virus is greater than six. Thus, further studies should use whole proteins to increase the number of epitopes and to ensure the presentation of known and unknown CD4 and CD8 T cell epitopes, and therefore remove the requirement for the patient to be HLA-A2 positive.

Chapter 4 – Expression of HCV proteins in DC.

4.1 Introduction

In the previous chapter, DC immunotherapy involving the use of DC, primed and matured using HCV-specific lipopeptides, was proposed as a novel method to induce significant immune responses to HCV to mediate HCV clearance. However the use of these HLA-restricted lipopeptides is likely to be ineffective because i) the number of epitopes required may be higher than 6, ii) they may be required to be tailored to the patient, leading to increased cost. Instead, the expression of entire proteins in DC may allow broader immune responses to be raised as endogenously expressed entire proteins will result in the presentation of all CD4 and CD8 T cell epitopes, including as yet undefined epitopes.

Consequently, it is proposed that priming the DC with RNA will allow an effective method of priming DC whilst avoiding HLA restrictions. The use of RNA has a number of benefits, including ease of preparation, no potential for insertional mutagenesis (as is the case with DNA), and it will allow complete proteins to be expressed, rather than the pre-selected restricted epitopes present in the lipopeptide-based technology. Most mRNA molecules in mammalian cells contain a 7m-G cap which is recognised by the ribosome, and which is necessary for effective translation of the encoded protein. However, the cap analogue for the *in vitro* transcription of RNA is not only expensive, but also reduces the yield of RNA by ≥10 fold (Qiagen). Furthermore, as the proportion of capped mRNA varies in different *in vitro* transcribed RNA preparations, this is likely to prevent its approval for clinical use by regulatory authorities.

In contrast, RNA molecules transcribed from DNA which encodes a gene whose expression is controlled by an Internal Ribosome Entry Site (IRES) provide a consistent, reliable method of producing the protein of interest. The use of mRNA expression vectors utilizing IRES technology represents a practical, inexpensive means to generate a GMP product for therapeutic use.

The literature revealed that there is no generally established protocol for the transfection of DC, as numerous papers report different techniques to load DC with antigens from a variety of different sources (see table 4.1). Transfection with RNA presents a useful technology whereby a protein of interest can be expressed in DC using simple technology. The use of cationic lipid-RNA complexes, electroporation, and simple incubation has been described as sufficient to successfully transfect DC (Table 4.1). However no protocol stands out as the gold standard which is why the use of RNA is being investigated in this thesis.

4.1.1 IRES mediated translation

IRES elements were first discovered in 1988 within poliovirus and encephalomyocarditis virus (EMCV) RNA (Jang et al., 1988, Pelletier and Sonenberg, 1988). IRES elements are regions of RNA molecules which are able to utilise eukaryotic ribosomes to allow translation initiation. Approximately 90% of eukaryotic mRNAs are translated from the first AUG codon which is located downstream of the 5'cap structure. However, some proteins are expressed from RNA as a result of the specialised IRES RNA sequences, which are responsible for 5'cap-independent translation. IRES elements are typically

Table 4.1. Outline of the various methods used to transfect RNA in different types of DC.

Cell type	Transfection method	Reference
Murine DC2.4	Electroporation	(Racanelli and Manigold, 2007)
Human Mo-DC	DOTAP	(Su et al., 2002b, Zeis et al., 2003)
Human Mo-DC	Lipofectin	(Weissman et al., 2000, Ni et al., 2002)
Human mo-DC	Simple incubation	(Heiser et al., 2002, Heiser et al., 2000, Van Tendeloo et al., 2001)
Human Mo-DC	Electroporation	(Abdel-Wahab et al., 2005, Bonehill et al., 2004, Ponsaerts et al., 2002, Sæbøe-Larssen et al., 2002, Van Tendeloo et al., 2001)
Human Mo-DC	DMRIE-C	(Bergant et al., 2006, Van Tendeloo et al., 2001)
Human CD34+ derived DC	Lipofectamine	(Mockey et al., 2006)
CD34+ derived DC CD34+ derived DC	Passive Pulsing DMRIE-C	Van Tendeloo et al (2001) Van Tendeloo et al (2001)
CD34+ derived DC	Electroporation	(Ueno et al., 2004, Van Tendeloo et al., 2001)
Human precursor derived DC	DMRIE-C	(Nair and Boczkowski, 2002)
Murine DC	Transmessenger	(Yu et al., 2007)
Murine DC	Electroporation	(Yu et al., 2007)
Murine DC	DOTAP	(Ashley et al., 1997, Bacci et al., 2002, Koido et al., 2000)

several hundred nucleotides in length, and fold into specific structures which bind many of the same proteins that are used to initiate normal cap-dependent translation. Different IRES elements require different subsets of initiation factors, although, all bypass the need for a 5' cap and the translation initiation factor which recognizes it, the cap binding complex eIF-4E.

The EMCV and polioviruses produce proteases that cleave the cellular translation factor eIF-4G, making it unable to bind to eIF-4E (Jang et al., 1988, Pelletier and Sonenberg, 1988). As a result, the virus shuts down the great majority of cell-specific translation, and diverts the translation machinery to the viral IRES which is recognized by the cleaved eIF-4G.

IRES elements also occur naturally in mammalian cells, with the selective activation of cellular IRESs occurring at certain points in the cell life cycle. For example, during the M phase of the cell cycle, 5' cap dependent translation of mRNA is significantly reduced due to dephosphorylation of eIF-4E, which lowers its affinity for the 5' cap (Alberts et al., 2002). Therefore IRES-mediated translation is increased as IRES elements are immune to this effect. IRES-mediated translation is also important in eukaryotic cell apoptosis (Spriggs et al., 2005). Caspase activation during apoptosis reduces translation initiation using the cap-dependant pathway, and instead several proteins required for apoptosis are mediated by IRES-containing mRNAs which continue to be synthesized throughout the process (Spriggs et al., 2005). IRES elements allow a high rate of translation despite a lower overall capacity of the cell for protein synthesis.

The EMCV IRES is an RNA element which has gained great deal of popularity in experimental and pharmaceutical applications after its commercialisation in 1990 (Bochkov and Palmenberg, 2006). The inclusion of this IRES in the vector pCITE-1 allowed easy linkage of exogenous cistrons to allow cap-independent translation of proteins in eukaryotic cells or cell free extracts (Bochkov and Palmenberg, 2006).

4.2. Experimental plan.

An EMCV IRES-GFP was used to initiate expression of GFP and this construct was then used to examine protocols to optimise delivery of RNA to DC. Previous studies showed that the efficiency of cationic lipid-based transfection reagents can be enhanced by the presence of a phosphate buffer during the lipid-nucleic acid complex formation (Kariko et al., 1998, Weissman et al., 2000), and consequently this was investigated in the setting of their use with RNA. Due to the high cost of GM-CSF and IL-4 required to generate DC from human monocytes, a mouse DC line, DC2.4, was used before the technology was transferred to mo-DC.

4.3 Results.

4.3.1 Construction of the EMCV IRES-GFP construct.

An EMCV IRES-GFP RNA construct was generated as described in section 2.2.1 and *in vitro* transcribed mRNA synthesized using a commercial kit (section 2.2.10). The *in vitro* transcribed RNA was transfected into the human hepatoma 8 (Huh8), a JFH1 cured cell line (Blight et al., 2000). This was to confirm protein expression and also to assess whether RNA was stable with or without polyadenylation (section 2.2.10). The cells were

transfected using DMRIE-C and either 1µg or 3µg of RNA. GFP expression was assessed using flow cytometry. As shown in Figure 4.1, only polyadenylated RNA was capable of protein expression in this cell line.

4.3.2 Transfection of DC2.4 cells using Lipofectin

DC2.4 cells were transfected using Lipofectin and a range of conditions, and GFP expression examined using flow cytometry to determine the efficacy. Cells were transfected with either 1µg or 3µg of GFP EMCV IRES polyadenylated RNA, with or without the addition of a phosphate buffer (see section 2.3.2). The RNA-lipid complexes were then added to the cells for either 1 or 5 h. As shown in figure 4.2 maximum expression was observed after transfection with 3µg of RNA for 1 h without phosphate buffer. However, the proportion of positive cells was low as only 9% showed GFP expression (Fig 4.2). The presence of the phosphate buffer during complex formation seemed to reduce the level of expression (burgundy and cyan bars). Transfection with 1µg of RNA resulted in <4% GFP positive cells regardless of the complex-cell contact time or the presence of the phosphate buffer. The GeoMean Fluorescence of the cells was also examined and, as shown in figure 4.2b, the cells which expressed high levels of GFP, with a GeoMean Fluorescence of 35, were exposed for 5 h to the 3µg RNA-Lipofectin complex containing phosphate buffer. The DC2.4 cells which showed the highest percentage of cells expressing GFP had GeoMean Fluorescence of 22 and 20. The transfection efficiency and corresponding GeoMean of fluorescence was inadequate for application into human mo-DC, therefore alternative strategies needed to be investigated.

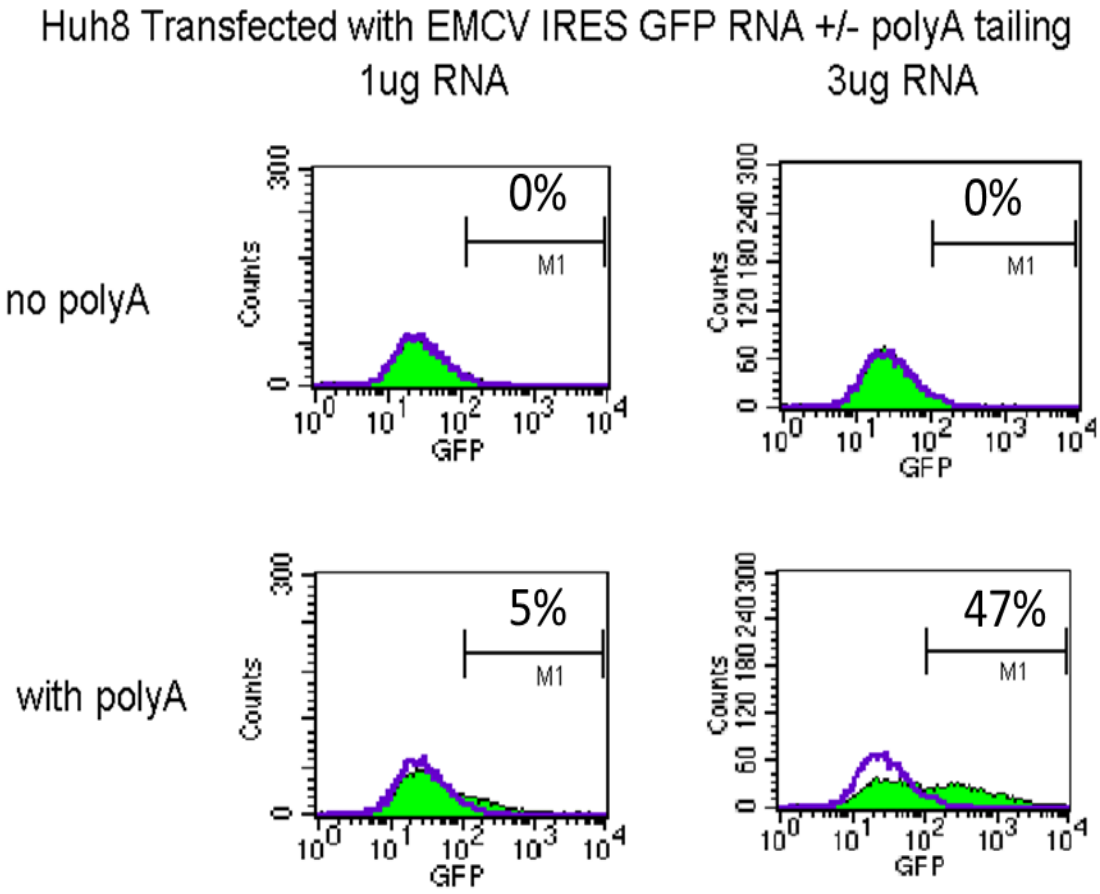

Figure 4.1 GFP expression in Huh8 cells after transfection of EMCV IRES-GFP RNA. EMCV IRES-GFP RNA was prepared with and without polyadenylation and transfected into Huh8 using DMRIE-C and either 1, or 3µg of RNA per transfection. GFP expression was detected by flow cytometry 48 h post transfection. Histograms in purple indicate the mock transfected control, and the green histograms reflect GFP expression in transfected cells.

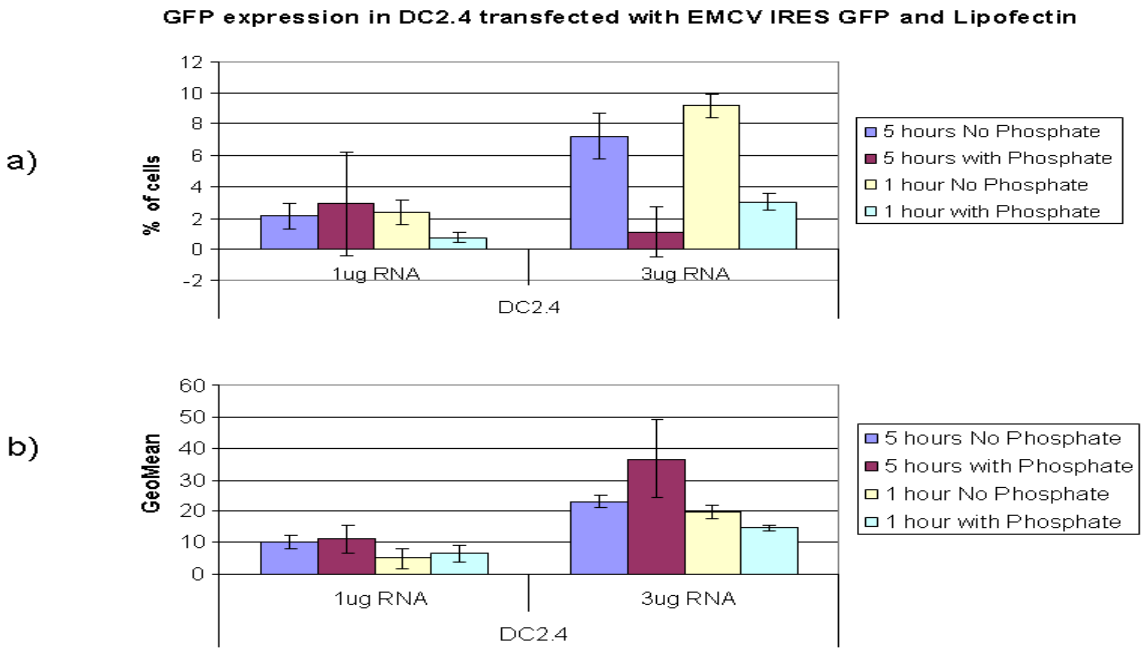

Figure 4.2. GFP expression in DC2.4 cells following transfection with EMCV IRES GFP RNA using Lipofectin transfection reagent. DC2.4 cells were transfected with 1 or 3 µg of RNA with or without the presence of a phosphate buffer during the RNA-lipid complex formation period. Final complexes were incubated on cells for 1 or 5 h prior to change of media and the cells were incubated for 48 h before GFP expression was examined by flow cytometry. Panel a) shows the percentage of cells expressing GFP, and panel b) shows the GeoMean fluorescence of cells expressing GFP. Data are derived from two independent experiments and the error bars represent +/- 1 standard deviation.

4.3.3 Transfection of DC2.4 cells using Lipofectamine 2000.

DC2.4 cells were transfected using Lipofectamine 2000 as described in section 2.3.2.2. As shown in figure 4.3a, DC2.4 cells transfected with 1µg of EMCV IRES GFP RNA for 5 h without phosphate buffer showed that only 2.5% of cells were GFP positive with a GeoMean Fluorescence of 19 (Figure 4.3b) whereas 1µg of RNA incubated for 5 h with phosphate buffer produced <2% GFP-positive cells with a GeoMean Fluorescence of 5. Addition of the 1µg RNA complexes to DC2.4 cells for 1 h with or without phosphate buffer during complex formation produced 2.5% and 4% of cells expressing GFP with GeoMean Fluorescences of 48 and 12 respectively. The highest level of expression was observed in cells which were transfected with 3µg of RNA-Lipofectamine 2000 complex and incubated for 1 h without the phosphate buffer, as 18% of these cells were GFP positive with a GeoMean Fluorescence of 62. Transfection of 3µg RNA for 1 h with phosphate buffer produced 12% of GFP positive cells with a GeoMean Fluorescence of 25. Transfection of the cells with complexes for 5 h showed that <1% expressed GFP. This highlighted that in the case of Lipofectamine 2000, a shorter duration with more RNA was more effective for transfecting DC2.4 cells.

4.3.4 Transfection of DC2.4 using DMRIE-C

More effective transfection strategies were still required in light of the poor performance of Lipofectin and Lipofectamine 2000. To address this, DC2.4 and Huh8 cells were transfected with EMCV IRES GFP RNA using DMRIE-C with or without the presence of phosphate buffer during RNA-DMRIE-C complex formation. Huh8 Cells were used as

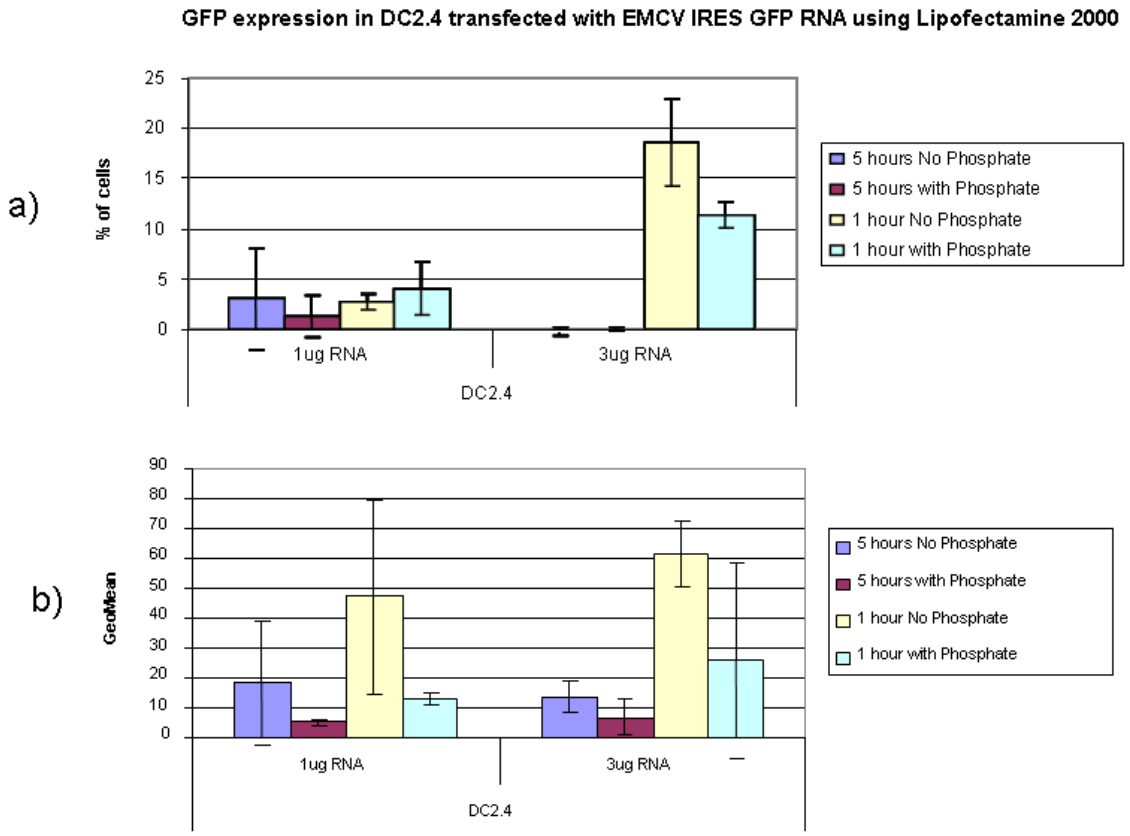

Figure 4.3. GFP expression in DC2.4 cells following transfection with EMCV IRES GFP RNA using Lipofectamine2000 transfection reagent. DC2.4 cells were transfected with 1 or 3 µg of RNA with or without the presence of phosphate buffer during the RNA-lipid complex formation period. Final complexes were incubated on cells for 1 or 5 h prior to change of media and the cells were incubated for 48 h before GFP expression was examined by flow cytometry. Panel a) shows the percentage of cells expressing GFP, and panel b) shows the GeoMean fluorescence of cells expressing GFP. The data were derived from two independent experiments and the error bars represent +/- 1 standard deviation.

a control as they allow the expression of HCV proteins. These reactions were performed using either 1µg or 3µg of RNA and the complexes added to the cells for 4 h, prior to washing, and the addition of fresh growth medium. Shorter time periods were not used with this reagent as previous work in our laboratory showed no expression for incubation periods shorter than 4 hours. The cells were then incubated for 48 h to allow gene expression and examined by flow cytometry. As shown in figure 4.4a almost no expression was observed in DC2.4 cells under any conditions whereas 55% and 45% of Huh8 cells were GFP positive (Fig 4.4a). Transfection with 1µg of RNA showed reduced expression, 15% and 34% in Huh8 cells, with and without phosphate buffer respectively. These results also reflected the GeoMean Fluorescence of the cells (Figure 4.4b). The use of 3µg of RNA with phosphate gave the greatest GeoMean Fluorescence of 162, 3µg RNA without phosphate produced a GeoMean Fluorescence of 120, while 1µg of RNA showed even lower expression.

4.3.5 Transfection of DC2.4 cells using TransIT

As the above results were still unsatisfactory, additional strategies were investigated. Consequently, DC2.4 cells were transfected with EMCV IRES GFP RNA using the Trans IT transfection reagent kit. The reagents were titrated according to the manufacturer's instructions using 3µg of RNA as standard. The cells were harvested and examined for GFP expression by flow cytometry. As shown in Figure 4.5a, 0, 1, 2, 3µl of the TransIT reagent (x axis) was used in conjunction with 0, 0.5, 1, or 3 µl of the Boost reagent (blue, burgundy, beige, and cyan bars respectively). In general, an increase in the proportion of

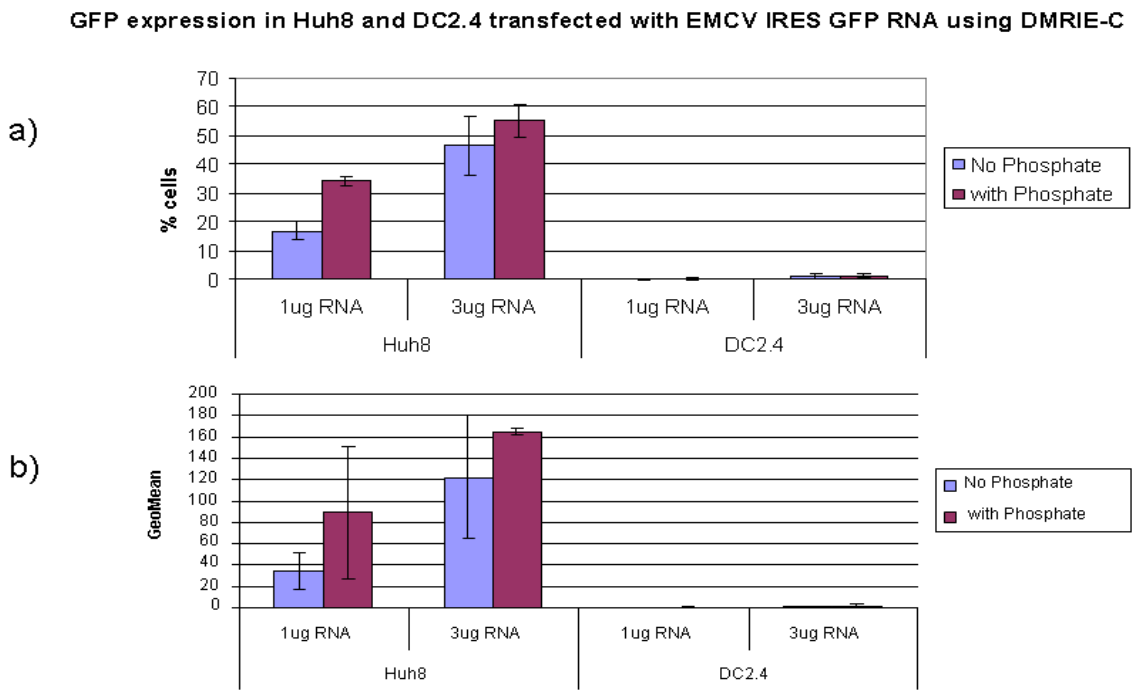

Figure 4.4. GFP expression in Huh8 and DC2.4 cells following transfection with EMCV IRES GFP RNA using DMRIE-C transfection reagent. Cells were transfected with 1 or 3µg of RNA with or without phosphate buffer during the RNA-lipid complex formation period. Final complexes were incubated on cells for 4 h prior to change of media and the cells were incubated for 48 h before GFP expression was examined by flow cytometry. Panel a) shows the percentage of cells expressing GFP, and panel b) shows the GeoMean Fluorescence of cells expressing GFP. The data were derived from two independent experiments and the error bars represent +/- 1 standard deviation.

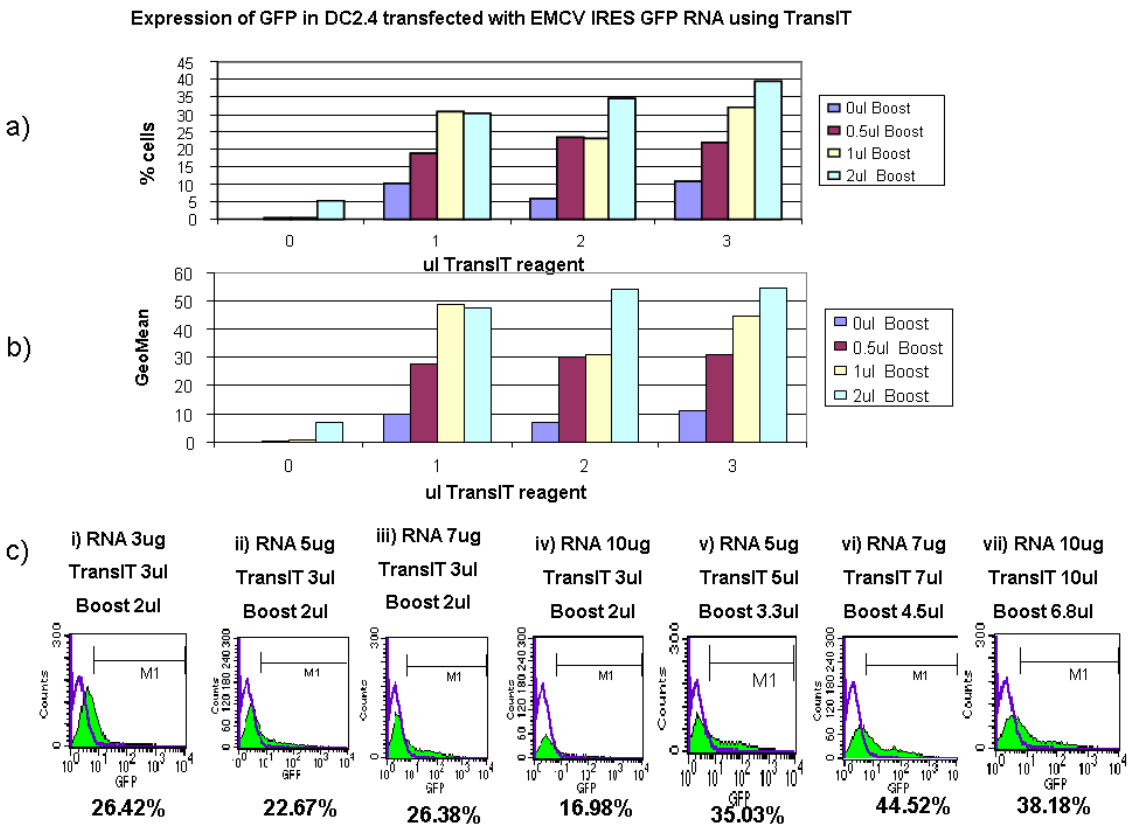

Figure 4.5 GFP expression in DC2.4 cells following transfection of EMCV IRES GFP RNA using TransIT transfection reagent. Cells were transfected with either 1 or 3µg of RNA using different combinations of TransIT and Boost components of the transfection reagent. Panel a) shows the percentage of cells expressing GFP and panel b) shows the GeoMean Fluorescence of GFP expressing cells. The volume of TransIT reagent used is shown on the x-axis. Flow cytometry histograms in c) i) to iv) show the results of DC2.4 cells transfected using 3, 5, 7 and 10µg of RNA respectively with the same volume of TransIT and Boost reagent (3µl and 2µl), histograms v-vii) show DC2.4 transfected with 5, 7 and 10µg of RNA with the concentration of TransIT and Boost reagents increased to generate consistent ratios of RNA: Reagent. Purple histograms denote the mock transfected controls and the green histograms show specific GFP expression.

the Boost reagent resulted in an increase in GFP expression (Fig 4.5a). The highest level of GFP expression was observed in DC2.4 cells transfected using 3µl of TransIT and 2µl of Boost, with 40% of cells showing GFP expression. The fluorescence intensity of these cells was also measured, and as shown in Figure 4.4b the GeoMean Fluorescence of GFP expression in these cells followed the same pattern as that shown in Fig 4.4a, i.e. higher readings with more RNA and longer duration of RNA complex exposure. Huh8 cells transfected with phosphate buffer present in the mixture showed higher GeoMean Fluorescence readings compared with those transfected without phosphate buffer.

In an independent experiment, the optimal conditions of 3µl of TransIT and 2µl of Boost, determined above, were examined to determine if the process could be scaled up by a) increasing the amount of RNA whilst keeping the same amount of transfection reagent, or b) increasing the RNA and reagents at the same ratio, with a clinical trial in mind. However, in this experiment, the optimal conditions noted previously (3µg RNA, 3µl transit and 2µl of Boost) showed a reduced level of expression as only 26.42% of cells showed GFP expression (Figure 4.5c i) and attempts to scale up the reaction had no effect (Figure 4.5c ii-iv respectively). However, when the proportion of transfection reagents was increased at the same ratio as the RNA, a change in GFP expression was observed. GFP expression was increased to 35% using 5µg RNA, 5µl of TransIT and 3.3µl of Boost (Figure 4.5c v), with maximal expression of 44.52% observed using 7µg of RNA, 7µl TransIT, and 4.5µl of Boost. 10µg of RNA (with 10µl of TransIT and 6.8µl of Boost) produced an expression level of 38.18%. However this level of transfection reagent/RNA produced cytotoxic effects (data not shown) and consequently, the highest level of GFP

expression (44.52%) was achieved with 7µg of RNA, 7µl of TransIT and 4.5µl of Boost reagent (Figure 4.5c vi).

4.3.6 Transfection using Transmessenger

The transmessenger transfection reagent was used to transfect EMCV IRES GFP RNA into DC2.4 cells. Initially reagents in the kit were titrated according to the manufacturer's instructions, by using known amounts of RNA, and varying the ratio of RNA to Transmessenger until optimal transfection conditions were determined. Initially, DC2.4 cells were transfected using 1µg or 3µg of RNA and ratios of 1:2, 1:4 and 1:8 were tested (as shown in figures 4.6a and 4.6b). Transfection with 3µg of RNA and 6µl and 12µl of Transmessenger (1:2, 1:4 repectively), did not increase the level of GFP expression significantly. However, 24µl of Transmessenger (ratio 1:8) increased transfection efficiency to 26%, and these cells were also the brightest observed, with a GeoMean Fluorescence of 45 (Figure 4.6b). In an independent experiment, the conditions optimised by titration were used and compared to conditions reported by Yu et al (Yu et al., 2007); 3µg and 5µg of RNA were tested using RNA:Transmessenger ratios of 1:6 and 1:8. As shown in Figure 4.6c i and ii, 3µg of RNA resulted in transfection efficiencies of 18.26 % (ratio 1:6) and 39.84% (ratio 1:8). Increasing the amount of RNA to 5µg increased GFP expression to 49.17% (ratio 1:6) and 76.61% (ratio 1:8) (figure 4.6 c iii and iv) which was greater than that observed by Yu et al (50%). In this latter panel, an almost complete shift of the population towards the right indicates that a majority of the cells expressed GFP. This shows that using 5µg of RNA with an RNA: Transmesenger ratio of 1:8 represents the optimum conditions using this reagent.

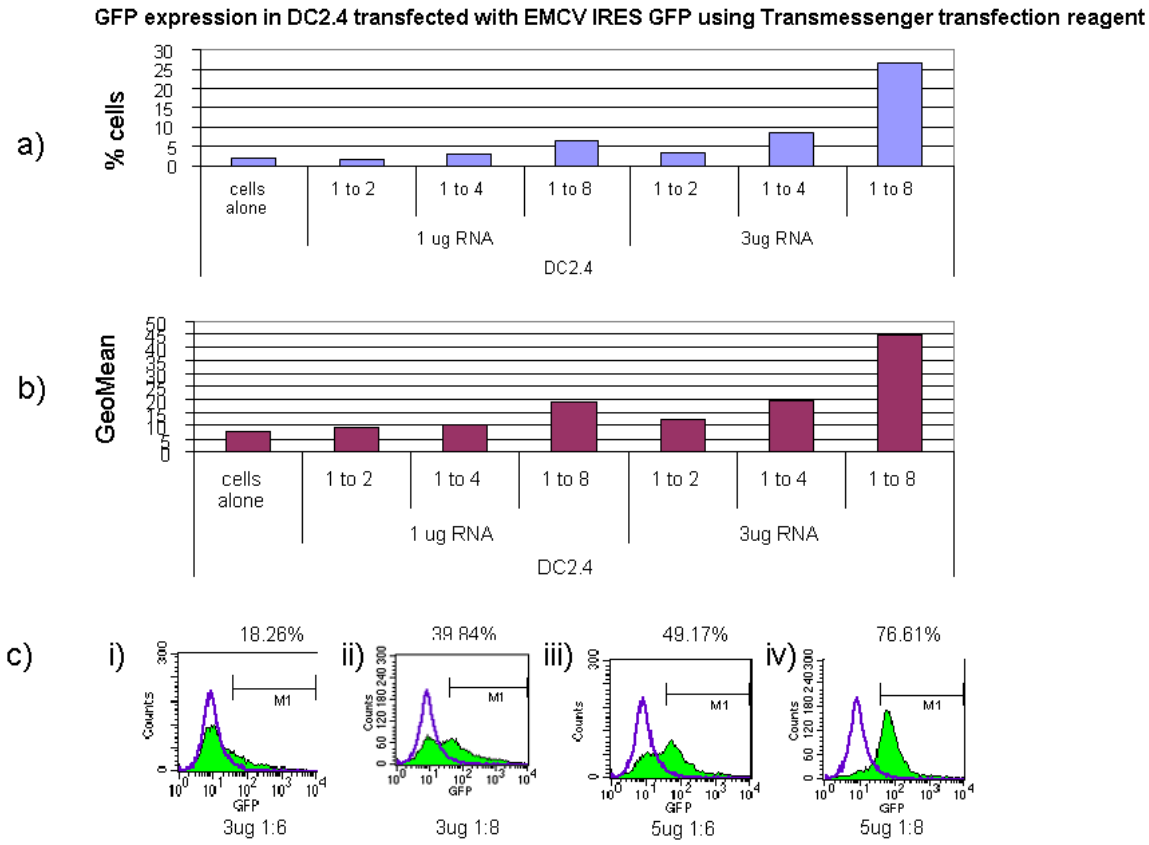

Figure 4.6 GFP expression in DC2.4 cells following transfection of EMCV IRES GFP RNA using Transmessenger transfection reagent. Cells were transfected with either 1 or 3 µg of RNA using different ratios of Transmessenger reagent and RNA. Panel a) shows the percentage of cells expressing GFP and panel b) shows the GeoMean fluorescence of GFP expressing cells. Flow cytometry histograms in c) i) and ii) show the results of DC2.4 cells transfected using 3µg of RNA with a transmessenger ratio of 1:6 and 1:8 respectively. Histograms in c) iii) and iv) show the results of DC2.4 cells transfected with 5µg of RNA and a transmessenger ration of 1:6 and 1:8 respectively. Purple histograms denote the mock transfected controls and the green histograms show specific GFP expression

4.3.7 Transfection using Polyethylenimine (PEI), Oxidized Mannan-PEI, and Electroporation.

RNA transfection is not limited to lipid-based transfection reagents. Polyethylenimine (PEI), Oxidised-Mannan-PEI and electroporation have been reported to effectively deliver RNA into cell lines. DC 2.4 cells were transfected with EMCV-IRES-GFP RNA using PEI, Oxidized Mannan-PEI, and electroporation; however these protocols failed to show GFP expression. Transfection using PEI also proved to be cytotoxic.

Transfection studies in DC2.4 highlighted the difficulty posed in the transfection of DC (albeit a cell line), with the majority of the reagents utilized in this study showing sub optimal performance. Two candidate reagents, TransIT and Transmessenger, were the only reagents which were observed to have the best performance were selected for further testing in human mo-DC.

4.3.8 Expression of HCV proteins in DC2.4 cells

EMCV IRES driven RNA constructs encoding the HCV proteins Core (genotype 1b) or NS3/4A (genotype 1b) were generated in the laboratory by Dr. Wesley Black. These constructs were used to determine if HCV proteins could be expressed in the DC2.4 cell line. DC2.4 cells were transfected with either RNA construct using TransIT. After 48 h to allow gene expression, the cells were harvested and lysed as described (chapter 2.4.11.2) and the samples analysed on a 10% SDS PAGE,

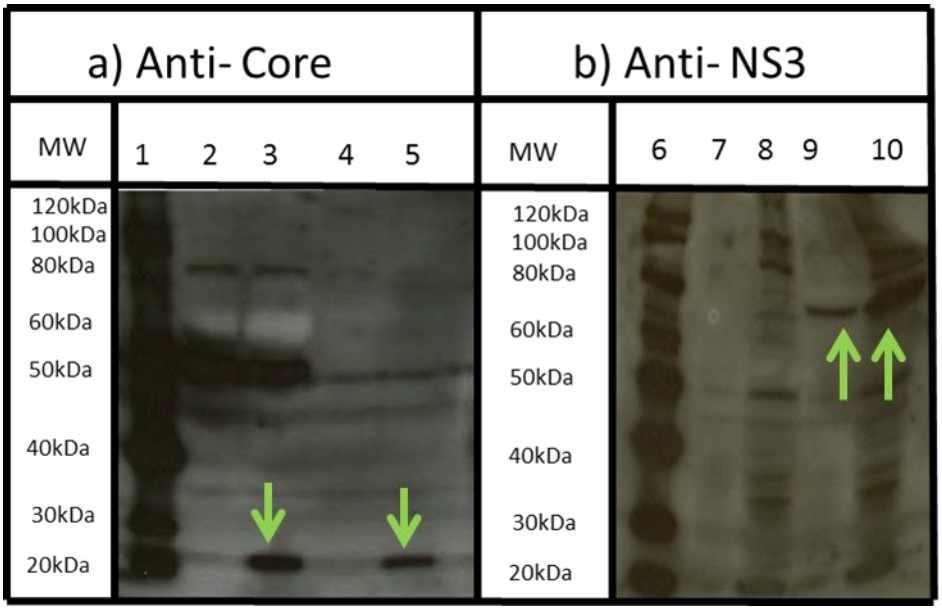

Figure 4.7. Western blot analysis of DC2.4 cells transfected with EMCV IRES Core RNA or EMCV IRES NS3/4A using TransIT. DC2.4 cells were transfected with either EMCV-IRES Core or EMCV IRES NS3/4A RNA. Panel (a) shows transfected DC2.4 lysates probed with anti-core antibody. Lane 1 is the Magicmark XP molecular weight ladder, lanes 2 and 4 contain lysates from mock transfected DC2.4 from separate transfections, while lanes 3 and 5 contain lysates from EMCV IRES Core RNA transfected DC2.4 lysates. Panel (b) shows transfected DC2.4 lysates probed with an anti-NS3 antibody. Lane 6 is the Magicmark XP molecular weight ladder. Lane 7 and 8 are mock transfected DC2.4 lysates from 20,000 and 200,000 cell input respectively. Lanes 9 and 10 contain lysates derived from EMCV IRES NS3/4A RNA transfected DC2.4 with a cell input of 20,000 and 200,000 respectively.

transferred to a PVDF membrane and probed with HCV specific antibodies. As shown in figure 4.7, only cells which were transfected with EMCV IRES Core (lanes 3 and 5) expressed the Core protein at 20kDa, and those transfected with the EMCV IRES NS3/4A construct expressed the 70kDa NS3 protein (lanes 9 and 10), while mock transfected cells showed no expression of either protein (lanes 1, 4, 7 and 8).

4.3.9 Transfection of RNA into Human mo-DC

The optimal transfection reagents for transfection of EMCV IRES GFP RNA into DC2.4 were Transmessenger and TransIT. These two reagents were then used to transfect EMCV IRES GFP RNA into human monocyte derived DC. Monocytes were isolated from human PBMC and cultured with GM-CSF and IL-4 for 4 days to differentiate into immature DC (section 2.4.1). The mo-DC were then transfected with RNA, and examined 48 h later for GFP expression. However, neither reagent was capable of transfecting mo-DC as shown in Figure 4.8.

4.4 Discussion.

There are many published protocols for the transfection of human mo-DC however no method is considered to be the gold standard (reviewed in section 4.1.1). The aim of this part of this thesis was to investigate different methods of RNA transfection into DCs with the ultimate goal of determining whether the transfection of RNA was a viable method of expressing proteins in mo-DC. In order to screen reagents quickly and relatively inexpensively, a murine DC cell line known as DC2.4 was utilized in the unavailability of

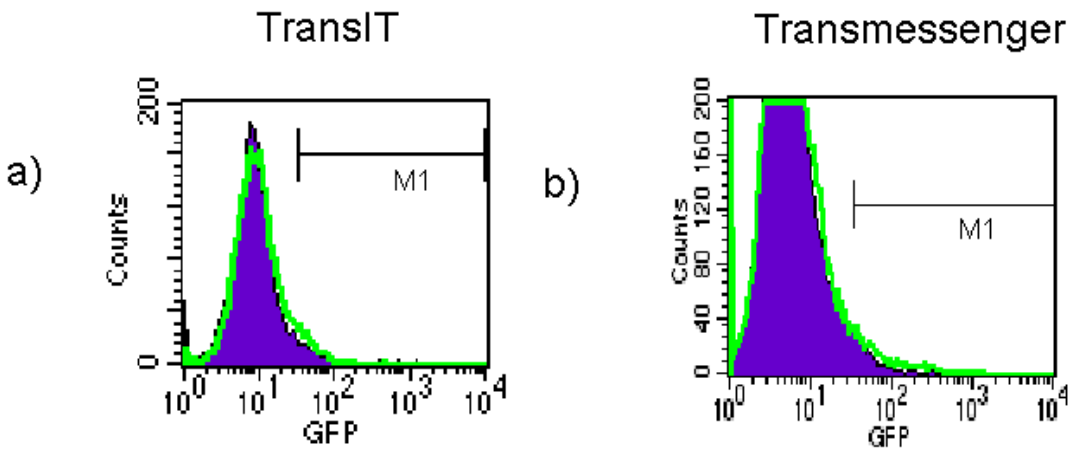

Figure 4.8. Human monocyte derived DC transfected with EMCV-IRES GFP RNA using TransIT and Transmessenger. Mo-DC were transfected with EMCV-IRES GFP RNA using (a) TransIT and (b) Transmessenger. The purple histograms denote the mock transfected controls and the green histograms show specific GFP expression.

any human DC cell line. The costs involved in maintaining a cell line is far lower and the process more reliable than the isolation and culture of $CD14^+$ monocyte derived DC. The DC2.4 cells resemble immature DC, and are capable of phagocytosis and antigen uptake (Bao et al., 2007, Shen et al., 1997). These cells are also capable of maturation and antigen presentation like normal murine DC hence they were deemed suitable for use as a DC model for RNA transfection.

Electroporation was reported to be a highly successful method to transfect DCs as noted in table 4.1, with transfection efficiencies often above 60% in various human DC of different origins ($CD14^+$ monocyte derived, $CD34^+$ stem cell derived) and also murine DC including DC2.4 cells. In this study, electroporation was found to be ineffective to transfect RNA into the DC2.4 cells (section 4.3.7). As with RNA transfection in general, protocols describing the transfection of RNA using electroporation into DC vary greatly, with the required RNA type and amount, the media used during the electroporation process, and the apparatus differing between studies. Reagents such as PEI and oxidized-Mannan-PEI also failed to produce any detectable levels of GFP expression in this study. At least for PEI, this was presumably due to the observed toxic effect on cultures.

Cationic lipid-based reagents were shown to be the best candidates for RNA transfection as they do not necessitate the use of specific expensive equipment, are generally reproducible, can transfect various nucleic acids, and at least for DNA, have high transfection efficiency. The action of lipid-based transfection reagents can be enhanced by use of a phosphate buffer during the initial complex formation of RNA to the cationic

lipid. Popular transfection reagents such as Lipofectin, Lipofectamine 2000 and DMRIE-C were tested in this study. The use of a phosphate buffer gave mixed results dependent on the reagent used, however in all conditions assessed the transfection efficiencies remained low (below 20%) in DC2.4 cells.

The reagents Transmessenger and TransIT are RNA-specific transfection reagents, and as such were expected to generate favorable results, however alternatives were tested prior due to delays in supply and shipment of both reagents. Although the Transmessenger transfection reagent was capable of achieving a transfection efficiency of 76%, these cells appeared dim in terms of GFP expression, especially when compared with cells transfected with TransIT which showed a greater number of cells expressing a higher GFP intensity. It is more likely that cells transfected with this reagent would be able to induce a greater immune response by virtue of their higher level of protein expression. Transfection of DC2.4 using this reagent was also capable of expressing HCV Core and NS3/4a derived from IRES driven constructs. Therefore this method could potentially be used to examine the effectiveness of DC transfected with IRES RNA constructs, but this protocol was unable to transfect human monocyte derived DC and hence it's use will be restricted to a mouse model. This highlights the difficulty of translating protocols to human models and the need to investigate other methods of expressing entire proteins in mo-DC which are capable of being produced at a GMP level.

Chapter 5 - Mouse Model for Necrotic Cell Therapy

5.1 Introduction

Cell death is an event which does not go unnoticed by the immune system and mechanisms to detect the death of cells have evolved to allow the body to discern between cell death caused by normal physiological processes, and death caused by other factors. This ability to distinguish between the two allows the immune system to combat invading pathogens or other agents which can cause disease. Cells which have died via normal physiological processes, such as apoptosis, are engulfed and cleared by phagocytes. On the other hand, cells which have died via non physiological processes such as severe physical or chemical trauma induce inflammatory responses (Kono and Rock, 2008).

Apoptosis is an important physiological process involved in homeostasis, mediating the clearance of old and damaged cells. Apoptosis primarily induces a non-inflammatory immune response, however if prolonged apoptosis occurs (e.g. due to lack of cell clearance by scavenger cells) the cell undergoes secondary necrosis, which then results in a pro-inflammatory immune response. Apoptosis also plays a role in the clearance of virus infected cells and a number of viruses have developed strategies to control apoptosis in order to benefit virus propagation, for example the blockade of anti-infective apoptotic pathways by adenoviral and herpes virus proteins (Benedict et al., 2003, Bertin et al., 1997, Griffith et al., 1995, Karp et al., 1996).

The literature has highlighted pro- and anti-apoptotic functions of a number of HCV viral proteins, particularly the Core protein (reviewed in section 1.5.4). Although the role of apoptosis and cell death during HCV infection and associated liver disease is not clear, it is possible that it is a physiological process which is controlled by HCV. Cell death is inhibited or initiated depending on the cell types in which particular HCV proteins are expressed, at least in *in vitro* experiments using plasmids for over expression (reviewed in section 1.5.4). When the ability of the virus to modulate other cellular metabolic processes such as intracellular trafficking is taken into account, it becomes apparent that the virus can induce apoptosis whilst remaining hidden from the adaptive immune system.

It is possible that HCV uses the induction and modulation of apoptosis not only to control viral propagation and release, but also to control and inhibit the adaptive immune response through the infection of lymphoid cells and by preventing the progression of secondary necrosis in cells, which ultimately would result in a pro-inflammatory response directed against HCV viral proteins. Necrosis on the other hand is a much more chaotic form of cell death and is usually associated with physical and chemical trauma, and results in an inflammatory response through the release of DAMPs.

Therefore it is the hypothesis of this section of the thesis that HCV proteins delivered in necrotic cells will induce a robust anti-HCV specific immune response. Prior to studying the efficacy of HCV antigen-positive necrotic cells in a clinical setting it was necessary to show that the method had the capacity to elicit an HCV specific immune response.

Therefore I designed experiments to prove the principle of the technology in an animal model viz. to elicit HCV-specific immune responses by vaccination with HCV antigen-positive necrotic cells. The RNA technology developed in chapter 4, involving transfection of the murine DC2.4 cell line with RNA encoding HCV proteins was utilized.

5.2 Experimental plan

C57BL/6 mice were used to determine if necrotic, antigen loaded cells could elicit an HCV-specific immune response. Two species of mRNA encoding the HCV Core and NS3/4A proteins respectively in which translation initiation was controlled by the EMCV IRES were used to transfect the C57BL/6 derived dendritic cell line, DC2.4. These antigen-loaded cells were then made necrotic by heat treatment and administered to the mice. The mice were then sacrificed 2 weeks post final injection and their IFN-γ response to HCV-specific peptide pools measured by ELIspot.

Eight groups of 3 C57BL/6 mice received two injections of cells I.D in the nape of the neck. Mice received either $1x10^5$ or $1x10^6$ cells per injection, which consisted of live or necrotic DC2.4 cells which were either HCV antigen-positive or -negative. The antigen positive cells consisted of 50% Core transfected cells and 50%NS3/4A transfected cells.

Therefore the eight groups received injections of cells as follows:

1. $1x10^5$ Antigen negative live DC2.4 cells.
2. $1x10^6$ Antigen negative live DC2.4 cells
3. $1x10^5$ Antigen negative necrotic DC2.4 cells
4. $1x10^6$ Antigen negative necrotic DC2.4 cells
5. $1x10^5$ Antigen positive live DC2.4 cells
6. $1x10^6$ Antigen positive live DC2.4 cells
7. $1x10^5$ Antigen positive necrotic DC2.4 cells
8. $1x10^6$ Antigen positive necrotic DC2.4 cells

Transfection of the DC2.4 cells was performed in the Burnet Institute, Melbourne. On the days of injection, the cells were harvested and transported live on wet ice to the Basil Hetzel Research Institute in Woodville South, South Australia. The cell viabilities were calculated, concentrations were adjusted, the cells made necrotic, then transported to the animal house of the Women's and Children's Hospital, North Adelaide where the mice were subsequently injected by Dr Branka Grubor-Bauk. I performed the ELIspots at the Basil Hetzel Research Institute. Ethics approval for mouse related work using this technology had expired in Melbourne, which is the reason cells were transported to Adelaide for subsequent injection into mice.

5.3 Results

5.3.1 HCV antigen delivery via Necrotic Cells

The RNA technology developed in Chapter 4 of this thesis was used to express HCV proteins in the murine cell line DC2.4, after transfection with RNA as described in section 2.3.2.4 of this thesis. Transfected cells were tested and did indicate the expression of Core and NS3 in the DC2.4 cell line, however the quality of the Western Blots were very low, and due to low cell yields samples could not be retested. The transfected cells were then made 100% necrotic and injected into the mice as described in section 2.5; the mice received two I.D injections one week apart and were then sacrificed two weeks post final injection, the spleens removed and the response of the splenocytes to the HCV Core, NS3 and NS4A peptide pools was examined by IFN-γ ELIspot. No responses were observed against Core or NS4A (data not shown). However, robust responses (> 2000 SFC/10^6 cells) were observed against the NS3 peptide pool in mice which received antigen-positive necrotic cells (Figure 5.1). Mice which received the lower dose ($1x10^5$ cells) showed a higher response. It was noted however, that the control groups (Group 1 and 2) which received $1x10^5$ and $1x10^6$ live, antigen-negative cells displayed relatively high background responses (>100 SFC/10^6 cells). The reason for this anomaly could not be determined and the experiment was repeated.

Consistent with the above data, no responses were observed to the Core or NS4A peptide pools by IFN-γ ELIspot (Figure 5.2a and 5.2b). However, although the number of SFCs was lower against the NS3 peptide pool than the responses described above, a similar

trend to that noted in Figure 5.1 was apparent, as mice which received the necrotic antigen-loaded cells showed robust responses to the NS3 peptide pool compared to the control groups (Figure 5.3). The mice in group 7 generated responses of 500 SFC/10^6 splenocytes and those in group 8, 900 SFC/10^6 splenocytes. Although these responses were noticeably lower than those noted above, the lack of responses in the control groups enhanced the validity of this data. The results were analysed using an Unpaired two-tailed t test, and found to have P values <.05 for group 7 and <.01 for group 8. Therefore the administration of necrotic antigen loaded cells was able to elicit an HCV-specific immune response.

5.3.3 Maturation status of DC2.4 transfected with RNA.

The maturation status of the DC2.4 cells was determined by flow cytometry after transfection of the RNA to determine if the viable antigen-loaded DC2.4 were mature and thus able to induce an immune response, whereas cells with an immature phenotype would be expected to have a tolerogenic effect. Although no maturation agents were included in the cell culture medium to specifically mature the DC2.4, it was found that the levels of CD80 and CD86, two markers of DC maturation, were high in non-transfected cells, and showed no change after transfection with either the Core mRNA or the NS3/4A mRNA (Figure 5.4).

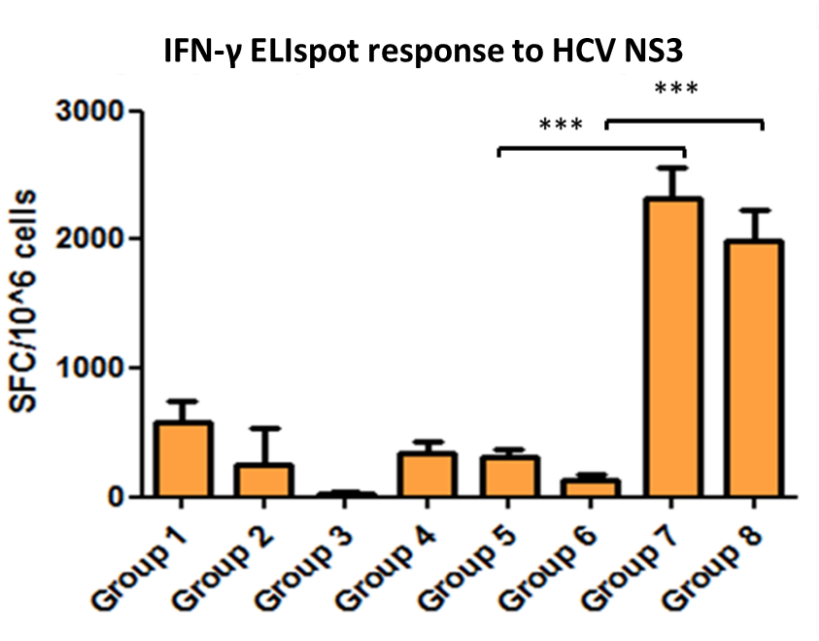

Figure 5.1 Mouse Splenocyte IFN-g ELIspots to NS3 from mice treated with HCV antigen loaded necrotic DC2.4 cells. Eight groups consisting of 3 C57BL/6 mice received two injections of cells I.D in the nape of the neck. Mice received either $1x10^5$ (Groups 1, 3 5 and 7) or $1x10^6$ cells (Groups 2, 4, 6 and 8) per injection, which consisted of live (Groups 1,2,5 and 6) or necrotic (Groups 3,4, 7 and 8) DC2.4 cells which were either antigen positive (Groups 1-4) or antigen negative (Groups 5-8). Antigen positive cells consisted of 50% EMCV-IRES Core transfected cells and 50% NS3/4A transfected cells. The mice were then culled and spleens removed, Splenocytes were then harvested and analysed by IFN-γ ELIspot. The histograms represent the average results depicted as Sport Forming Cells per 10^6 cells in each group of mice, with error bars representing +/- 1 standard deviation. All these parameters apply for the following figures in this section of the thesis. The response to the NS3 peptide pool is shown here. *** ($P<0.001$) $P = 0.0001$, Unpaired two tailed t test.

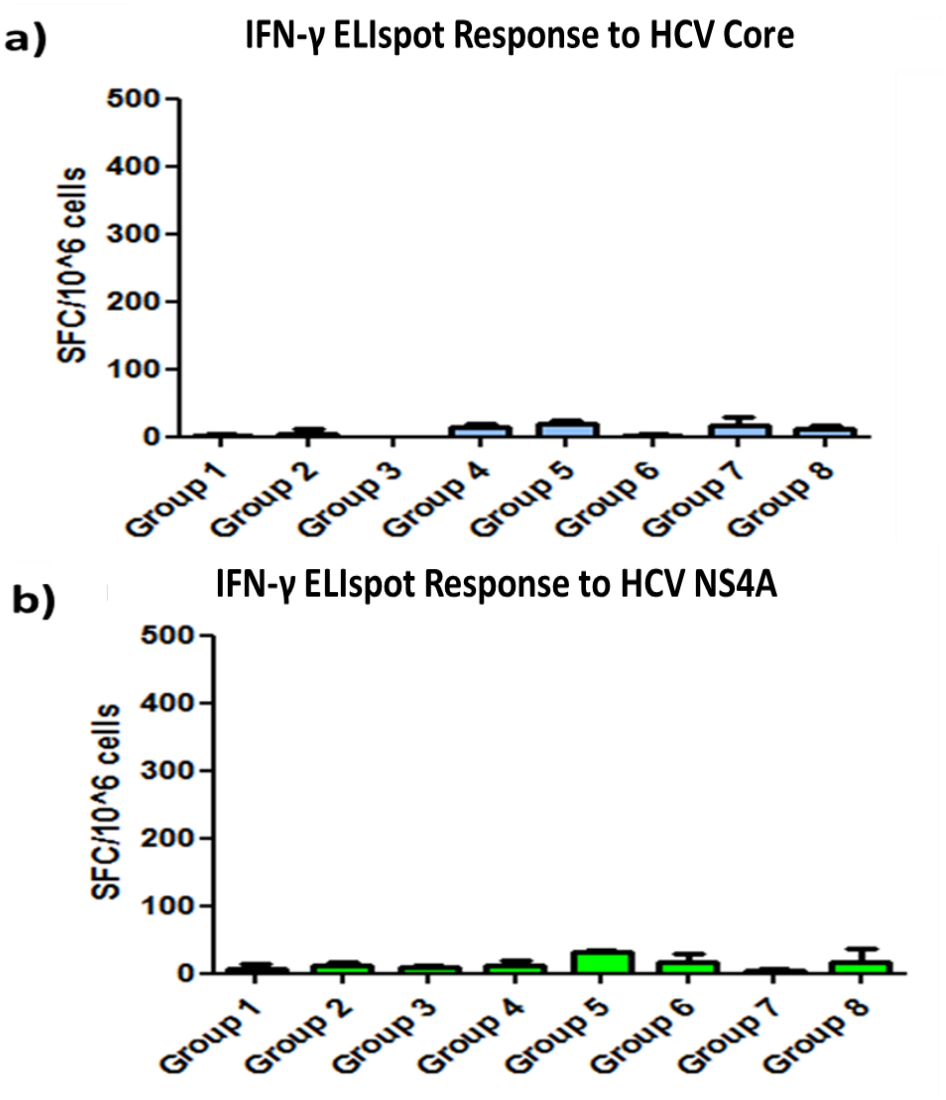

Figure 5.2 Mouse Splenocyte IFN-g ELIspots to Core and NS4A from mice treated with HCV antigen loaded necrotic DC2.4 cells. The IFN-g ELIspot response to Core peptide pool (a), and NS4A peptide pool in experiment 2.

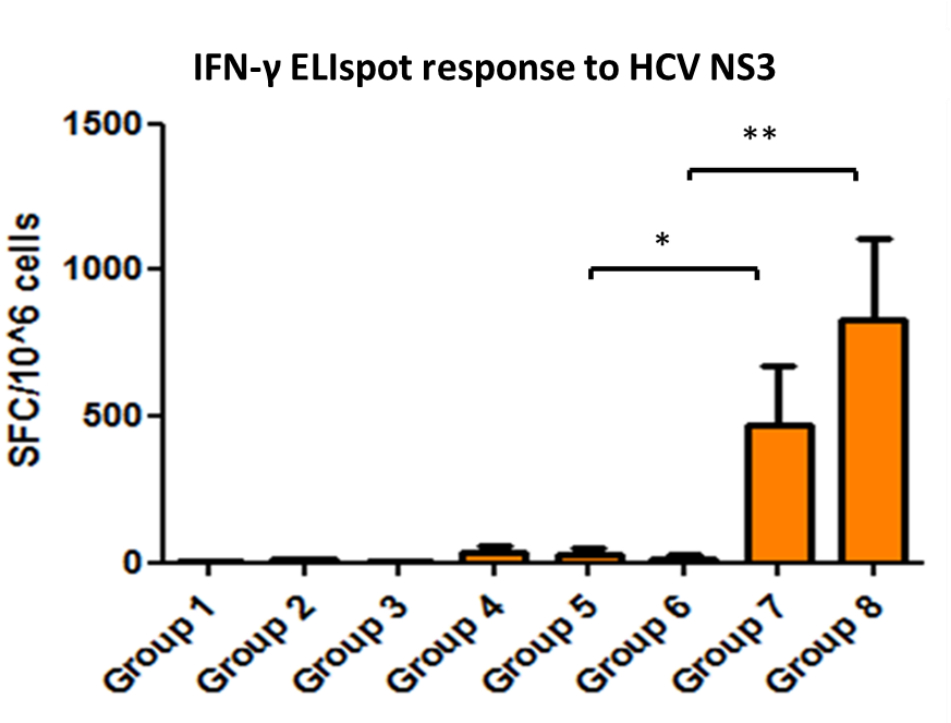

Figure 5.3 Mouse Splenocyte IFN-g ELIspots to NS3 from mice treated with HCV antigen loaded necrotic DC2.4 cells. * (P<.05) P= 0.0174, ** (P<0.01) P = 0.0076, Unpaired two tailed t test.

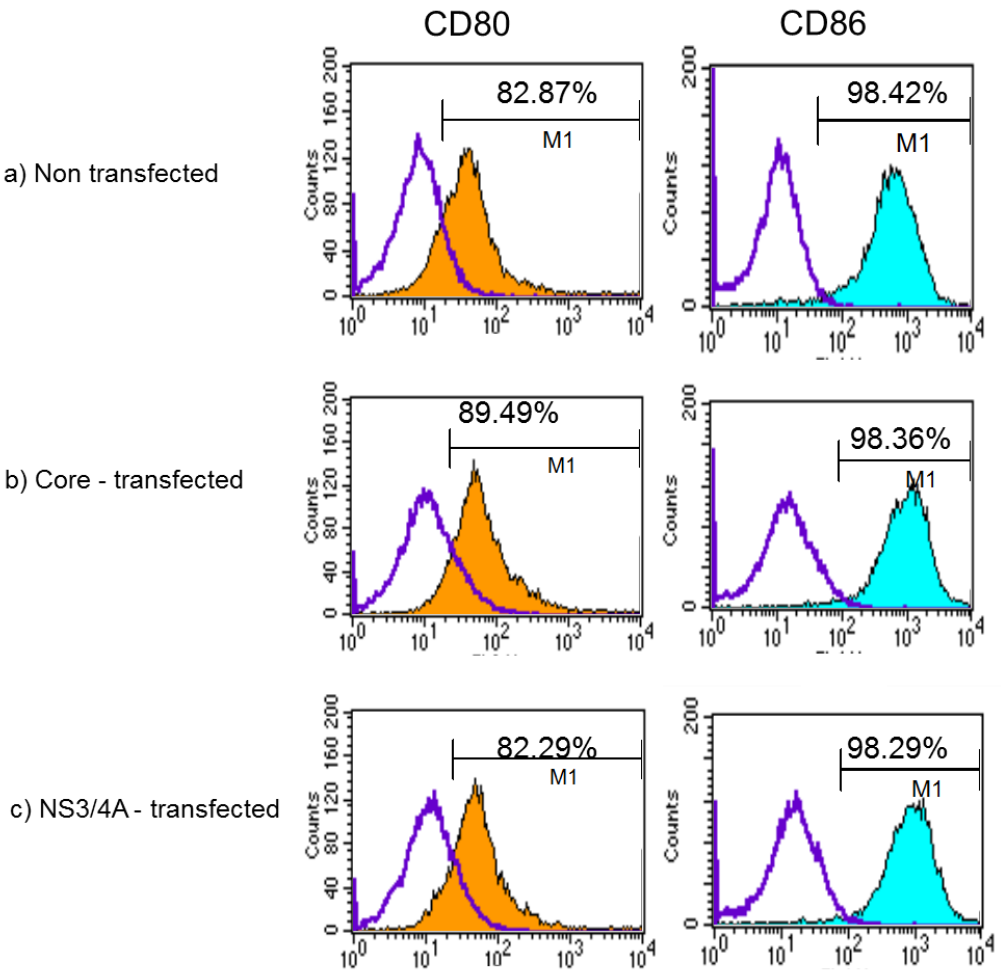

Figure 5.4. Maturation level of DC2.4 following RNA transfection. The expression of CD80 and CD86 on DC2.4 cells which were (a) non transfected, (b) transfected with EMCV-IRES Core RNA, or (c) EMCV-IRES NS3/4A RNA was determined by flow cytometry. Purple histograms show DC2.4 stained with isotype controls, while orange histograms show CD80 expression and blue histograms show CD86 expression.

5.3.4 Specific *in vivo* killing of NS3 pulsed splenocytes following vaccination with HCV antigen-loaded necrotic cells.

The administration of necrotic, antigen-loaded DC2.4 cells was capable of eliciting a robust immune response to NS3; therefore I wished to determine if this response could provide protection. In order to investigate this, a challenge model (Jackson et al., 2005) was used, utilising NS3 peptide pulsed- and unpulsed- splenocytes from healthy naïve C57/BL6 mice that were labelled with different concentrations of carboxyfluorescein succinimidyl ester (CFSE). These cells were injected via the intravenous route into mice which had been previously vaccinated with necrotic HCV antigen positive DC2.4 cells. The mice were sacrificed 5 h or 16 h post challenge and the numbers of specific CFSE-labelled populations present in the spleen were determined by flow cytometry. It was expected that the NS3 pulsed splenocytes (high CFSE) would be cleared more rapidly than the unpulsed splenocytes (low CFSE) in mice which were vaccinated with the NS3/4A-positive, necrotic DC2.4 cells. In order to calculate the percentage of specific killing, the ratio of $CFSE^{LO}$ and $CFSE^{HI}$ (NS3 pulsed) cells was calculated in each animal and compared with the ratio observed in a control tube containing an equal number of each group of cells (as per the formula in Section 2.5.4). Therefore the unpulsed $CFSE^{LO}$ cells were used as the internal control for each animal. At 5 h post challenge, 5 and 7% of NS3 pulsed cells were killed in mouse 1 and 2 respectively (Figure 5.5). At 16 h post challenge, mice 3, 4 and 5 showed an increase in specific NS3 peptide-pulsed cell killing to 10, 8 and 14% respectively. This experiment was initially designed to examine specific *in vivo* killing in 5 mice at each time point, but due to loss of cells during CFSE labelling

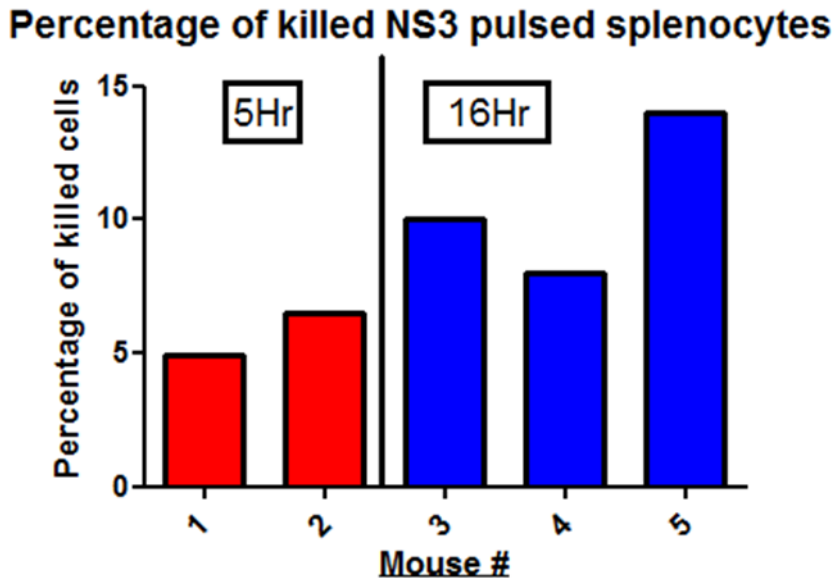

Figure 5.5. Specific *in vivo* killing of NS3 peptide pulsed splenocytes in mice vaccinated with HCV antigen positive necrotic cells. Mice were injected with $1x10^6$ antigen positive necrotic cells on two occasions, 2 weeks apart, then injected with unpulsed and NS3 peptide pool pulsed splenocytes stained with 0.2uM and 2uM of CFSE respectively, with the specific level of killing determined at 5 and 16 hours post challenge by using Flow Cytometry to detect and enumerate the labelled cell populations.

the numbers were reduced in each group, with the data representing individual mice, which accounts for the lack of error bars. Due to time and financial constraints the experiment was not able to be reproduced.

5.4 Discussion

Clearance of HCV infection is believed to be associated with broad multi-specific CD4 and CD8 T cell responses which occur spontaneously in a proportion of acute phase patients. However in a majority of cases, infection persists leading to a plethora of complications including fibrosis and cirrhosis of the liver, hepatocellular carcinoma, B cell lymphoma and various autoimmune diseases. The exact mechanism of persistence in HCV infection has yet to be characterized, although it is probably multi-factorial. As a result of the blockade of signalling and molecular trafficking pathways (Gale and Foy, 2005) and homeostatic functions such apoptosis, the failure of T cells through T cell exhaustion associated with up regulated PD-1 and TIM-3 to clear HCV antigen-positive hepatocytes (McMahan et al., 2010, Radziewicz et al., 2007), and dysfunction in antigen presentation (Bain et al., 2001, Lee et al., 2001, Sarobe et al., 2002), infection with the virus results in persistence in a high proportion of individuals.

Cell necrosis, as reviewed in section 1.5.3 of this thesis, is a chaotic form of cell death which induces pro-inflammatory responses. I hypothesised that if HCV antigens were presented in necrotic cells, this would increase HCV-specific immune responses as a result of cross presentation, as strategies to target DC directly with an immunogen or vector are still relatively ineffective. This hypothesis was tested in a mouse model vaccinated with the DC2.4 cell line expressing the HCV proteins Core and NS3/4A. Mice

which received the necrotic, antigen-positive cells developed a robust response to NS3 (Figure 5.1 and 5.3), but no response was detected to Core or NS4A (Figure 5.2). The response to NS3 can be attributed solely to the fact that the cells were necrotic, as the response to vaccination with viable, antigen-positive, mature DC2.4 cells was poor (Figure 5.3). The lack of response to NS4A may be due to the limited size of the protein that contains very few epitopes (Ward et al., 2002) or because the response was driven towards the large immunodominant epitope-rich NS3 protein.

IFN-γ ELIspot responses to the Core protein in mouse models have been shown previously (Murata et al., 2003, Torbati et al., 2012), but no significant responses were observed in the studies reported above. The reasons for this are unclear, and will require further work beyond the scope of this thesis, however I propose two possibilities. First, similar to the immune response to NS4A, the immune response may have been driven primarily by NS3 although responses to Core have previously been shown even when co-expressed with multiple immunodominant HCV proteins (Bruna-Romero et al., 1997, Makimura et al., 1996). Furthermore, the cells containing Core protein were transfected independently of those containing NS3/4A, and although the two cell populations were mixed, it is unlikely that an immune response would favour NS3 over Core due to immunodominance.

The second possibility is the immunosuppressive nature of the Core protein which is capable of inhibiting IFN signalling (Lin et al., 2006) and impair *in vitro* priming of specific T cell responses (Zimmermann et al., 2008). Recent work showed that Core,

when co-expressed with Hepatitis B surface Antigen (HBsAg), after co-administration of DNA vaccines, was capable of suppressing the response to HBsAg, which was interpreted to mean that the Core protein is able to inhibit the initiation of an immune response (Zhu et al., 2012). However, if the Core protein suppressed the initiation of the immune response it would be expected that the response to all proteins would be affected, and this clearly was not the case. Therefore the reasons for the lack of Core response remain unclear.

Mice vaccinated with necrotic, antigen-positive cells were capable of generating robust immune responses to NS3 protein, and although not conclusive, these mice were also capable of clearing NS3 peptide-pulsed splenocytes when challenged two weeks later (Figure 5.5), as 5-7% of NS3 pulsed splenocytes were killed 5 hours post challenge, and 10-14% were killed 16 hours post challenge when compared to unpulsed splenocytes. Due to time constraints and funding, this experiment was not able to be repeated. Future work should increase the number of mice tested in each group, and an unvaccinated control group of mice should also be included. The use of a single immunodominant peptide epitope to pulse the splenocytes prior to CFSE labelling and subsequent injection would not only show greater killing, but also highlight the specificity of the killing.

The immune response observed in the mice vaccinated with the necrotic, antigen-positive DC2.4 cells is presumably the result of cross presentation by APC, which phagocytosed the necrotic cells and processed and presented the viral proteins via MHC Class I. However to elucidate the mechanism behind the generation of this immune response, and

to prove this to be due to cross presentation, experiments utilising different strains of mice, and /or cell lines are required. Although any increased immunogenicity is likely to result from increased cross presentation of HCV antigens released from necrotic cells, it is important to demonstrate this. Thus, to elucidate the mechanism by which this vaccine increases immune responses, Clec9A-/- and Batf3-/- knockout mice, available from WEHI through collaboration with AI Lahoud should be vaccinated. Clec9A on DC was recently shown to recognize necrotic cells by binding to exposed actin filaments (Zhang et al., 2012), and Batf3 KO mice have no CD8α-positive conventional DCs and consequently are defective in antigen cross-presentation and cannot develop effective cytotoxic T lymphocyte (CTL) responses.

Nevertheless, the necrotic cell strategy presents an exciting and novel method of generating HCV-specific immune responses.

Chapter 6 - Human Mo-DC transduced with an adenoviral vector for use in immunotherapy for persistent HCV infection

6.1 Introduction.

The induction of immune responses by T cells is impossible without prior stimulation by APC such as DC. DC are strategically located at various sites, such as the skin, mucosal surfaces and the spleen throughout the body where they are most likely to encounter foreign antigens. DC are the most potent and efficient inducers of immune responses despite their relatively low frequency (Figdor et al., 2004). Their ability to prime naive T cells with different antigens from various sources, combined with their ability to be produced to a large scale *in vitro* has made them the focus of much research as the primary vehicle for vaccine delivery (as reviewed in section 1.3.4).

In the context of persistent HCV infection, the administration of HCV antigen-loaded, mature DC is capable of inducing HCV specific responses (shown in Chapter 3). However the source of antigen is likely to determine the breadth of the response achieved, as expression of entire proteins, rather than individual defined epitopes, is more likely to induce a broad response capable of clearing the virus. Initially it was thought that the use of mRNA was a viable, cost effective method to express entire HCV proteins in mo-DC, however the protocol proved difficult to replicate in human mo-DC in spite of the extensive literature (see section 4.1.1). It was therefore necessary to investigate other methods of expressing entire proteins in mo-DC that would be functional, reproducible,

and able to be scaled up to clinical scale and achieve GMP quality. One such method is the use of an adenovirus vector.

Adenoviral vectors have been the focus of intense research for their suitability as vaccine delivery vehicles, as reviewed in section 1.4. They have been used in a number of trials to treat various solid tumours, genetic disorders such as haemophilia A and B, rheumatoid arthritis, and also viral infections such as HIV. Adenoviral vectors are capable of transducing mo-DC via a CAR-independent manner (Adams et al., 2009) and are a viable method to express entire proteins in mo-DC for use as a therapeutic tool.

The use of Adenoviral vectors necessitates the use of FCS to support the growth of the cell line in which the vector is produced. However in Australia, the use of products derived from bovine sources to manufacture vaccines is actively discouraged in order to eliminate the risk of Bovine Spongiform Encephalopathy (BSE), a progressive neurodegenerative disease of cattle which is transmissible to humans (Therapeutic Goods Administration, Australia). Thus the aim of this section of the thesis was to investigate methods of producing adenovirus of GMP quality for use in a cellular vaccine to treat persistent HCV infection.

6.2 Experimental Plan

Four recombinant adenoviruses were used in this thesis (see table 6.1). Initial work with a recombinant adenovirus type 5 encoding GFP (Ad5-GFP), a kind gift from Associate Professor Joe Torresi, was used to determine the multiplicity of infection (MOI) required

Table 6.1. Description of Adenovirus constructs used in this thesis and their source.

Construct	Proteins Encoded	Source
Ad5-GFP	GFP	Associate Professor Joe Torresi
Ad5-C-E1-E2+GFP	HCV Core, E1, E2 and GFP.	Associate Professor Joe Torresi
Ad5-C-E1-E2-mutNS3/4A	HCV Core, E1, E2, mutant NS3, and NS4A.	This thesis (P Latour).
Ad5-E1-E2-optNS3	HCV E1, E2 and a codon optimised NS3.	This thesis (P Latour).

to transduce mo-DC efficiently, and to investigate the ability of various serum-free culture media to generate GMP-quality adenovirus stocks. A recombinant adenovirus type 5 encoding HCV Core – E1 – E2 + GFP (Ad5-C-E1-E2-GFP), another kind gift from Associate Professor Joe Torresi was used to further investigate the conditions required to effectively and efficiently transduce mo-DC.

The Adeasy system was used to generate two adenovirus vectors. The first encoded the HCV proteins Core, E1, E2 and a mutant NS3/4A (Ad5-C-E1-E2-mutNS3/4A) which contained a mutation (C1125A) that ablates the protease activity of the NS3/4A complex, as described in section 2.4.3. As this construct encoded 5 complete HCV proteins containing numerous identified and unidentified epitopes it was initially the prime candidate to test for suitability in a therapeutic strategy. However, as the thesis progressed, it became clear that this construct would not be suitable as the size of the insert (4.4kb) made the virus difficult to propagate, therefore a third construct was made encoding HCV E1, E2 and a codon optimised NS3 (Ad5-E1-E2-optNS3), which had an insert size of 3.5kb.

6.3 Results

6.3.1 Adenovirus transduction of mo-DC

Initially an Ad5-GFP vector was used to assess the MOI required to transduce mo-DC. Immature mo-DC, generated *in vitro* with GM-CSF and IL-4 (section 2.4.1), were transduced with an MOI of 50 or 100 and cultured for 72 h to allow GFP expression. The total percentage of GFP-positive cells at a given MOI differed from donor to donor,

however an MOI of 100, yielded expression levels > 45% in mo-DC from all donors (Figure 6.1).

An adenovirus vector encoding Core, E1, E2 and GFP (Ad5-C-E1-E2-GFP), was then used to determine if an Ad5 construct encoding HCV proteins was equally capable of transducing mo-DC. However, mo-DC transduced with an MOI of 100 showed very little GFP expression (Figure 6.2). The reasons are still unclear but it is possible that the problem is linked to the size of the insert. Consequently, strategies to increase the transduction efficacy were examined. The use of polybrene has been reported to increase the transduction efficiency by adenovirus in certain cell types (Lehmusvaara et al., 2005), and this was investigated using the Ad5-C-E1-E2-GFP. Mo-DC were pre-treated with 4µg/ml of polybrene for 2, 24 or 72 h. These cells were then transduced with an MOI of 100, and the cells cultured for 48 h to allow for GFP expression. Mo-DC which were pre-treated with polybrene for 2h, 24 h and 72h showed higher levels of GFP expression with the greatest level of GFP expression occurring 72 h after Ad5 transduction (Figure 6.3). This experiment showed that DC were capable of being transduced when pre-treated with polybrene, therefore allowing the expression of encoded proteins in a large proportion of the cells.

6.3.2 Generating Adenovirus to GMP quality.

Mo-DC are able to be manufactured to a GMP level (as reported in chapter 3), and as the above data showed that it was possible to transduce mo-DC efficiently with a RecAd, it was important to show that adenovirus can also be manufactured to a GMP level in an

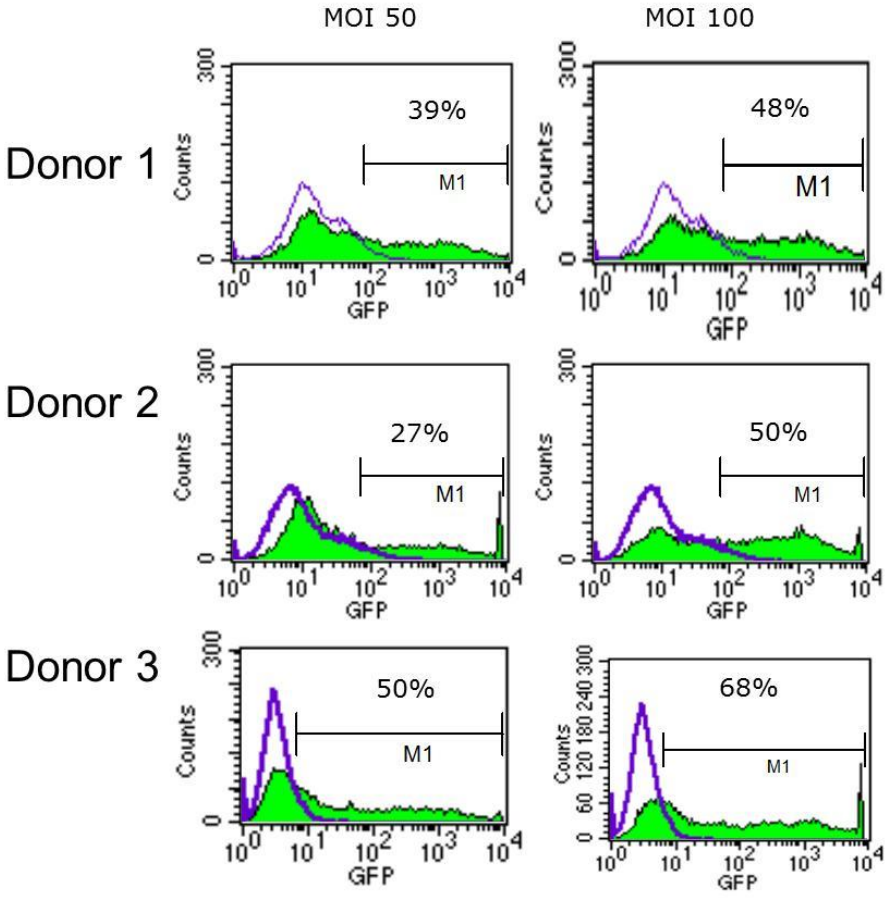

Figure 6.1. Recombinant adenovirus transduction of human mo-DC. GFP expression in monocyte derived DC from three separate donors at 72 h post transduction with Ad5-GFP at an MOI of 50 or 100. Purple histograms show the uninfected controls and the green histograms show specific GFP expression.

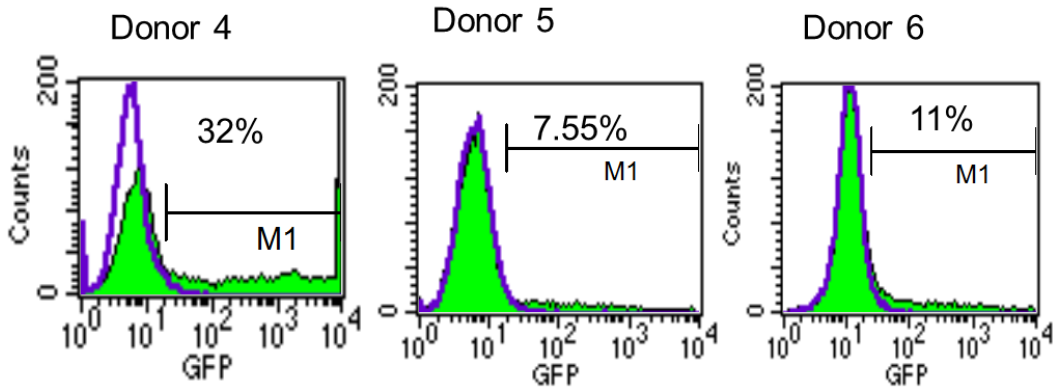

Figure 6.2. Recombinant adenovirus transduction of human mo-DC. GFP expression in monocyte derived DC from three separate donors at 72 h post transduction with Ad5-Core-E1-E2-GFP at an MOI of 100 in three separate donors. Purple histograms show the uninfected controls and the green histograms show specific GFP expression.

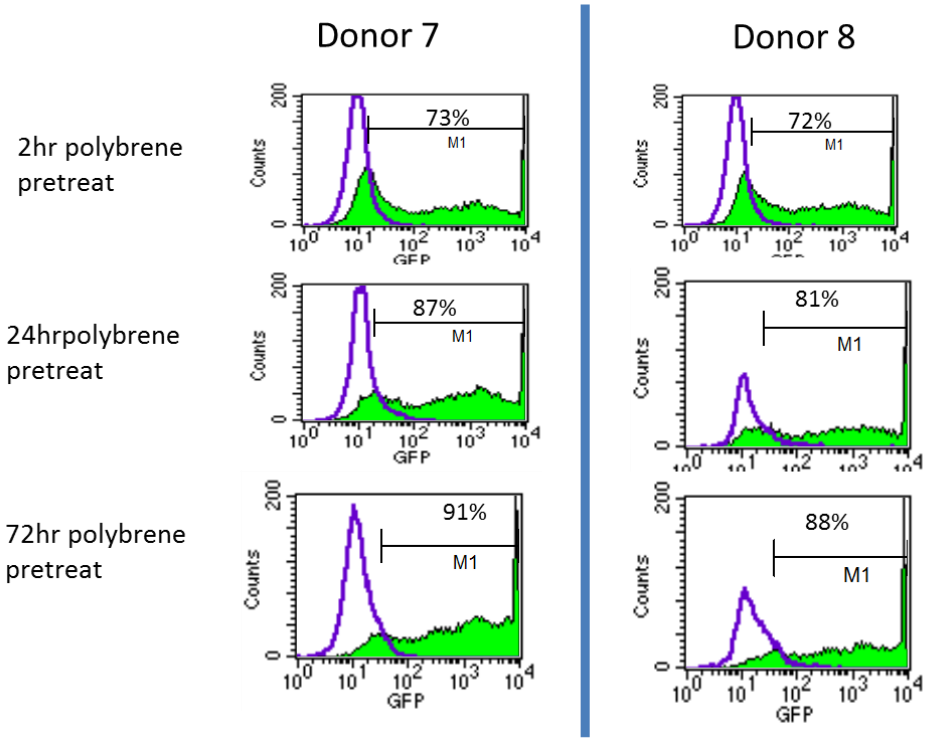

Figure 6.3. Recombinant adenovirus transduction of polybrene treated human mo-DC. Monocyte derived DC from two separate donors were pre-treated with 4 µg/ml polybrene for 2, 24 and 72 h prior to transduction with Ad5-Core-E1-E2-GFP at an MOI of 100. GFP expression was examined 48 h post transduction. Purple histograms show the uninfected controls and the green histograms show specific GFP expression.

academic as opposed to commercial setting. Therefore the aim of this section of this thesis was to assess the feasibility of producing adenovirus vectors in a completely serum free system to warrant use in a clinical setting.

6.3.2.1 Growth of HEK293 in serum free media

The use of bovine-derived products such as FCS is standard practice in cell culture. However therapeutic and prophylactic vaccines must be safe for use in humans, and the inclusion of bovine material in any step of the manufacture of a product intended for use in humans may constitute a risk. Furthermore, the cell line used to produce adenovirus vectors (HEK293T) contains DNA encoding SV40 T antigen, which therefore excludes it from use in a clinical grade manufacturing process. Therefore to permit the use of the adenovirus system in mo-DC therapy I wished to investigate various serum-free formulations which would allow the generation of adenovirus vectors without the need of FCS.

An HEK293 cell line (ATCC), which lacks the T antigen, was used to assess the suitability of 3 serum free formulations, AIM-V, SFM II, and CD293 (all supplied by Invitrogen), to grow and amplify adenovirus. These formulations were selected as they are reputed by the manufacturer to be suitable for the maintenance of HEK cell lines. The HEK293 cell line was first grown in parallel in each of the three media to determine which best supported the growth of the cells. Aim-V was shown to be unsuitable as very little cell division was observed over 13 days (Figure 6.4). SFM II showed the best growth as $>4x10^7$ cells accumulated after 13 days, compared with $2.8x10^7$ cells that were

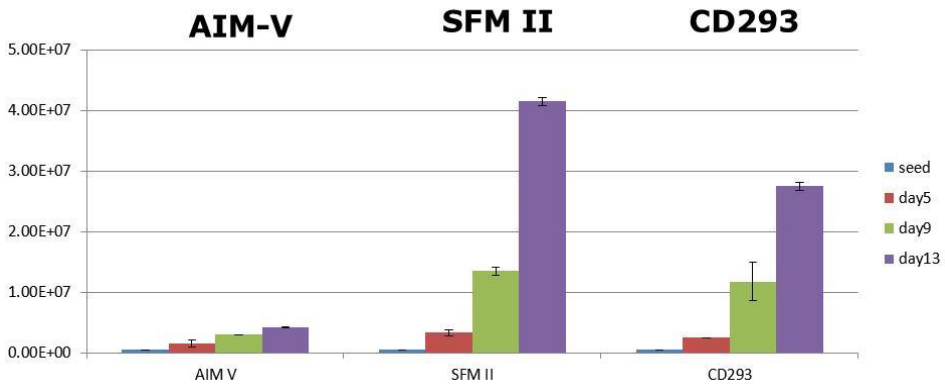

Figure 6.4. Comparison of the growth kinetics of HEK293 cells cultured in serum free formulations. AIM-V, SFM II and CD293 media were compared. The cultures were initially seeded at $1x10^6$ cells, with two flasks per growth medium. The cells were monitored over 13 days, and counted at 5, 9 and 13 days post seed. Each histogram depicts cell counts derived from duplicate cultures for each media formulation. Error bars represent +/- 1 standard deviation.

cultured in the CD293 media in the same time frame. In conclusion, as SFM II was shown to be the most conducive to cell propagation, it was selected for use in additional experiments.

6.3.2.2 Adenovirus propagation in serum free media.

In order to initiate a GMP quality virus stock, the recombinant adenovirus DNA must first be transfected into the cell line. In normal laboratory practice, HEK293T cells are grown in DMEM supplemented with 10% FCS, and recombinant viral DNA is transfected using Lipofectamine 2000 (as per section 2.3.2.2) to initiate the production of adenovirus. However, the product insert for the optimal growth medium (SFM II) to be used with HEK293, details specifically that Lipofectamine 2000 transfection can be inhibited by the SFM II culture medium, although multiple washes of cells with Opti-MEM (Invitrogen) can ablate this effect and restore transfection efficiency. However in my hands, Lipofectamine 2000 was unable to transfect HE293 cells with the recombinant Ad-GFP DNA despite using this protocol. DMEM itself is a serum-free salt solution, but is incapable of supporting the cell growth without supplementation with a protein source such as FCS, although it does allow the transfection of adenoviral DNA.

Therefore I wished to investigate if the presence of a protein source could enhance the transfection efficiency of adenoviral DNA into cells cultured in SFM II media, compared with DMEM. HEK293 cells were grown in DMEM and SFM II media with or without supplementation by FCS, or Human AB serum, a human-derived protein source certified for clinical use. These cultures were then transfected with a recombinant Ad5-GFP DNA

(as per section 2.4.10), and GFP expression assessed at 48 h to determine initial transfection efficiency, as longer periods would result in virus spread and alter the observed transfection efficiency. Cultures grown in DMEM supplemented with either FCS or Human AB serum showed transfection efficiencies of 16 and 10% respectively (Figure 6.5) while cultures grown in SFM II, regardless of supplementation showed no GFP expression (denoted as <2% expression). Although human AB serum, as a human source of serum would be more suitable for clinical studies; it was not as effective at supporting adenoviral propagation as FCS. Therefore in the interest of project progression, a screened source of FCS was used in the initial transfection of HEK293. Virus harvested from this initial transfection was then passaged into HEK293 grown in serum free media.

6.3.2.3 Construction of Adenovirus vectors.

The Adeasy system was used to construct a recombinant adenovirus encoding Core, E1, E2, and NS3/4A of HCV genotype 1b. Two variants of this virus were made, one containing the wildtype NS3/4A, and the other containing the protease-deficient mutNS3/4A (Section 2.4.3). The viral genes were cloned into the pShuttle-CMV vector and this was recombined with the adenoviral backbone in Adeasier cells. The adenoviral DNA containing the HCV genes was transfected into HEK293T cells grown in DMEM supplemented with FCS, and the virus subsequently passaged and harvested as described previously (sections 2.4.5, 2.4.6, and 2.3.7). The Adeasy system was also used to generate an adenovirus encoding E1, E2 and a codon optimised NS3 of HCV genotype

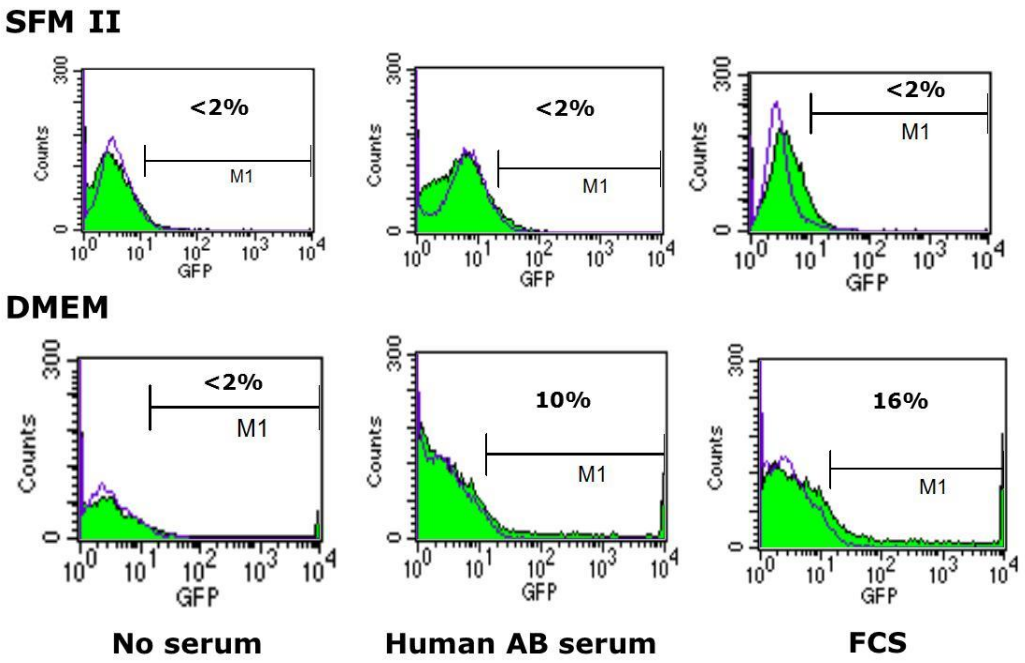

Figure 6.5 Efficiency of transfection of Adenovirus-GFP DNA into HEK293 cells cultured in DMEM or SFM II media supplemented with No serum, Human AB serum or FCS. HEK293 cells were cultured in either SFM II or DMEM media alone, or supplemented with Human AB serum or FCS, then transfected with 4µg of recombinant Ad5-GFP DNA and cultured for 48 h to allow gene expression. GFP expression was then examined by flow cytometry. The purple histograms show the mock transfected controls and the green histograms show specific GFP expression.

1b. The initial pShuttle-E1-E2-optNS3 vector was generated by Dr Wenbo Yu of the Basil Hetzel Research Institute in Adelaide.

Infection of HEK293T cells with Ad5-C-E1-E2-WTNS3/4A or Ad5-C-E1-E2-mutNS3/4A for 48h at an MOI of 2 resulted in expression of the HCV proteins as determined by western blot. A band of approximately 100kDa was observed when probed with an anti-Core or an anti-E2 antibody (figure 6.6). It is possible that this represents a polyprotein corresponding to Core-E1-E2. A larger band was also observed at approximately 120kDa, which may represent a glycosylated form of the polyprotein observed at 100kDa. The putative cleavage sites of C-E1-E2 appeared to remain intact in these constructs, however as cleavage of the HCV structural proteins is dependent on cellular proteases, it is possible that these proteases are either lacking in this cell line, which is unlikely, or it may be an effect of active adenovirus replication. It is likely that the lack of cleavage in this cell line may result from a defect in the constructs because HEK293T cells, infected with the alternative construct, Ad5-E1-E2-optNS3 at an MOI of 2 after 48 h, showed specific cleaved proteins of the expected sizes (Figure 6.7) that were not detected in uninfected cells. When lysates were probed with a pooled human serum derived from patients with chronic HCV infection, a band at approximately 67kDa was observed corresponding to the predicted size of NS3. NS3 and E2 - including possible glycosylated forms of a higher molecular weight, were detected using specific antibodies, and multiple HCV specific bands detected when probed using a pooled human serum from persistently infected patients.

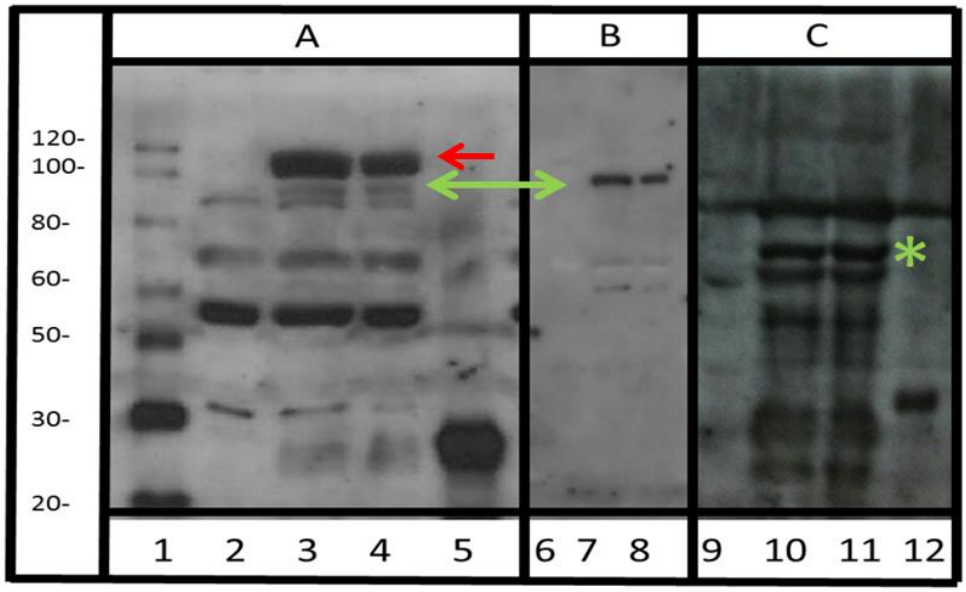

Figure 6.6 Western blot analysis of recombinant adenovirus infected cells. HEK293T cells were infected with Ad5-CoreE1E2wtNS3/4A or Ad5-CoreE1E2mutNS3/4A at an MOI of 2 for 48 h. Each panel shows replicate samples probed with (A) anti-core antibody, (B) anti-E2 antibody, (C) pooled human serum from persistently infected patients. Lane 1 contains the Magic mark XP protein ladder, lanes 2, 6 and 9 contain uninfected HEK293T, lanes 3, 7 and 10 contain HEK293T infected with Ad5-CoreE1E2wtNS3/4A and lanes 4, 8 and 11 contain HEK293T infected with Ad5-CoreE1E2mutNS3/4A. Lane 5 contains purified recombinant core protein and lane 12 contains purified recombinant NS3 protein used as positive controls. A green arrow marks the position of a putative HCV polyprotein of 100kDa detected in panels A and B with the anti-core and anti-E2 sera respectively and an asterix in panel C highlights a 67kDa protein detected using the pooled human serum. A larger band of approximately 120kDa can be observed in Lane 3 and 4 (red arrow).

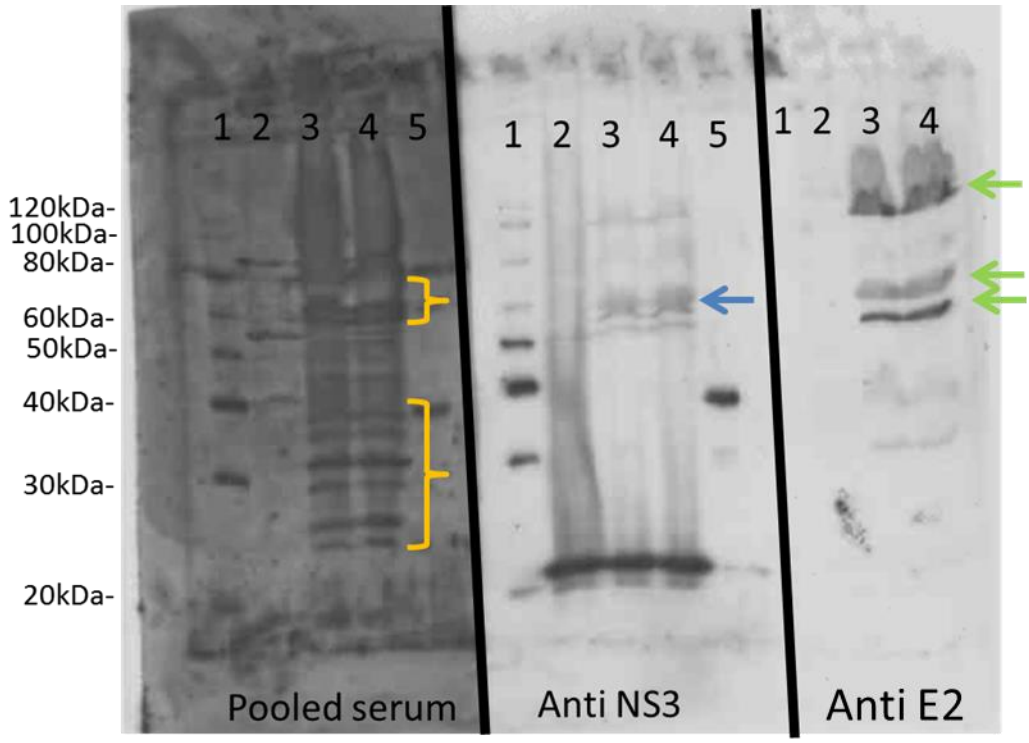

Figure 6.7 Western blot analysis of recombinant adenovirus infected HEK293T cells. HEK293T cells were infected with Ad5-E1-E2-NS3 at an MOI of 2 for 48 h. Lane 1 contains the Magic mark XP protein ladder, lane 2 contains lysate from uninfected HEK293T, lanes 3 and 4 contain lysates from HEK293T infected with Ad5-E1-E2-NS3, Lane 5 contains a purified recombinant NS3 as a positive control. Multiple bands specific to adenovirus infected lysates were observed when probed with a pooled human serum from persistently infected patients (yellow brackets). Bands corresponding to the putative size of NS3 were detected (blue arrow) and multiple bands for E2 (green arrows), with larger molecular weight bands possibly corresponding to glycosylated forms of E2.

6.3.2.4 Titration of Adenovirus vectors

The above recombinant adenovirus encoding Core E1, E2 and NS3/4A was made using the Adeasy system and does not contain the gene for GFP, as GFP cannot be used in constructs designed for clinical use. Consequently, an alternative method was necessary to determine the titre of the virus. Although plaque assays are the industry standard for the titration of adenovirus, these assays are not only lengthy (over two weeks), but the results vary considerably between users (Bewig and Schmidt, 2000). A commercial kit, Adeno-X Rapid Titration kit (Cellbio), which uses immunostaining to detect the adenoviral protein hexon in infected monolayers of HEK293T cells, was modified to detect the number of infected cells to then permit the viral titres to be calculated. I modified the protocol supplied online with this kit, and substituted the antibodies provided (mouse anti-hexon antibody and a rat anti mouse-HRP conjugate) with a single FITC-conjugated antibody against the hexon protein to allow direct detection and titration. A representative image of an infected HEK293T monolayer after immune-staining of the hexon protein is shown in Figure 6.8.

6.3.3 Manufacture of recombinant adenovirus in serum free media.

Initially, the recombinant adenoviral construct Ad5-Core-E1-E2-mutNS3/4A was the prime candidate for amplification in serum free media for use in a clinical setting. However, despite numerous attempts, amplification of Ad5-C-E1-E2-mutNS3/4A in serum free media failed. I believe that this may be due to the poor transfection efficiency in HEK293 cells (despite culture in DMEM supplemented with FCS), which I attribute to

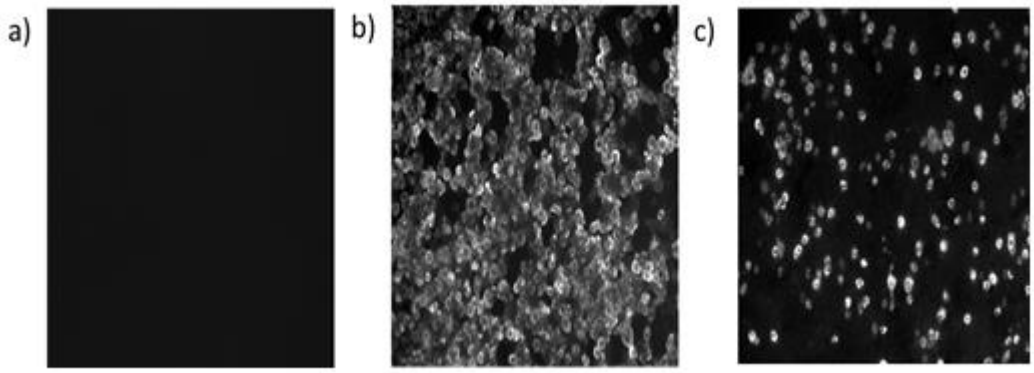

Figure 6.8 Immunostaining of recombinant adenovirus in HEK cells. HEK293T monolayers depicting uninfected cells (a) and monolayers infected with two dilutions; (b) 10^-2 dilution (c) and 10^-3 dilution, of an Ad5-CoreE1E2mutNS3/4A virus preparation and cultured for 48 h to allow for virus production. The monolayers were then fixed using ice cold methanol, then stained with an anti-hexon FITC conjugated antibody. The image was captured using a fluorescence microscope (Olympus IX51) at 100 x magnification.

the size of the construct, containing a much larger insert than the initial Ad5-GFP construct used in initial experiments (4.4kb compared to 0.5kb).

For this reason, it was decided to use the recombinant Ad5-E1-E2-optNS3. This smaller construct was successfully transfected into the HEK293 cell line grown in DMEM supplemented with a FCS derived from a BSE-free environment (Hyclone), and the virus produced from this transfection was passaged in HEK293 grown in SFM II media, which after 4 passages in HEK293 produced a final lysate of high titre. The final viral lysate was then purified using a commercial kit (section 2.4.8) and titrated to produce a GMP-compliant product.

6.3.4 Transduction of mo-DC using recombinant Ad5-E1-E2-optNS3.

Mo-DC from two healthy donors were treated with polybrene as described above then transduced with Ad5-E1-E2-optNS3 (at an MOI of 100) and HCV protein expression assessed by western blot 48 h later. When lysates were probed with pooled serum from HCV-infected individuals, specific bands were detected in the adenovirus transduced lysates but not in control lysates (figure 6.9).

6.4 Discussion

Adenoviral vectors are one of the most efficient methods to transfer genes into human cells. The main advantages of adenoviral vectors include the fact that they are replication defective coupled with their ability to replicate to high titres in complementing cell lines such as HEK293, and the subsequent high titres attained. Adenoviruses are also able to

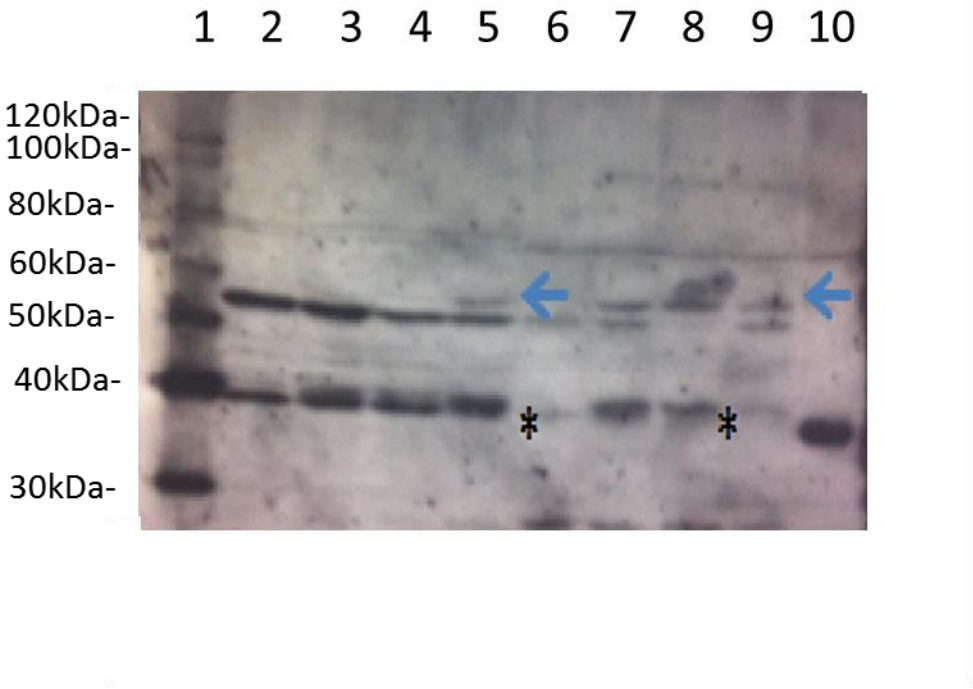

Figure 6.9. Western blot analysis of recombinant adenovirus infected Mo-DC. Polybrene treated Mo-DC were infected with Ad5-E1-E2-NS3 at an MOI of 100 and examined 48 h later. Lane 1 contains the Magic mark XP protein ladder, lanes 2 to 5 contain lysates from Mo-Dc derived from one donor, with lane 2 containing uninfected Mo-DC, and lanes 3 to 5 containing lysates from Mo-DC infected with 3 different Ad5-E1-E2-NS3 clones. Lanes 6 to 9 contain lysates from Mo-DC derived from a different donor, with lane 6 containing uninfected Mo-DC, and lanes 7 to 9 containing lysates from Mo-DC infected with 3 different Ad5-E1-E2-NS3 clones. The arrows and asterixes show 60kDa and 40kDa proteins respectively which appear specific to virus-infected cells. Lane 10 contains purified recombinant NS3 protein. The blot was probed with pooled human serum from persistently infected patients.

infect a range of dividing and non-dividing cells. Extensive knowledge of Adenovirus molecular biology especially that of Adenovirus type 5, has resulted in this serotype becoming the major vector used in many gene therapy and vaccine applications (Liu et al., 2010).

The technology behind DC immunotherapy is capable of being scaled to GMP level, and used to not only treat HCV infection (as in Chapter 3), but also other pathologies. However, although the technology has major limitations, it nevertheless has the capacity to efficiently and consistently express endogenous proteins in monocyte derived DC. It was therefore the aim of this section of this thesis to assess the feasibility of producing adenovirus vectors in a completely serum free system to warrant use in a clinical setting, and combining this with a method to express HCV proteins in monocyte derived DC.

Methods to generate mo-DC infected with an adenovirus vector generated at a GMP level using serum free media will not only benefit the production of a HCV therapeutic vaccine but also other disease states. Although clinical trials which used adenovirus vectors have been described (Catanzaro et al., 2006, Chuang et al., 2013, Kim et al., 2012, Peters et al., 2013) the exact methods of adenovirus production were unreported, but presumably used animal-derived serum at least in the initial steps of virus production, viz. transduction of HEK293 cells with DNA. In this thesis, the production of adenoviral vectors was limited by two factors: the first was the growth media used in the initial transfection with recombinant adenoviral DNA. Transfection of HEK293 grown in DMEM supplemented with FCS proved superior to those grown in DMEM supplemented with human AB

serum. The use of animal derived serum in the initial transfection was the only method which generated sufficient virus for subsequent passage in cells grown in serum free media (SFM II), and allow the generation of high titre recombinant adenovirus stocks. Therefore it was determined that the best strategy was to initiate the culture by transfecting cells grown in DMEM supplemented with screened bovine-derived serum (screened for endotoxin, various viral agents, bovine haemoglobin, cytopathogenic agents and haemadsorbing agents), and for subsequent viral passages to be performed in cells grown in serum-free medium. This still constitutes acceptable conditions for manufacture of a GMP-compliant product.

The second factor was the manufacture of GMP-grade adenovirus that was affected by the transgene cloned into the adenoviral vector. Although the adenovirus genome can accept inserts up to 7kb, it was found in this study that smaller inserts (Core-E-1E2-mutNS3/4A, 4.4kb) severely inhibited virus production, especially when using HEK293 cells in serum free conditions (section 5.3.3). This effect was also noted when the virus was grown in HEK293T cells in DMEM supplemented with FCS, but not to the same degree.

It was necessary to devise a method to consistently transduce human mo-DC to result in foreign protein expression. Initial work using an Ad5-GFP vector was not transferrable to Ad5 constructs encoding larger more complex transgenes. The reasons for this are unknown, and require further investigation beyond this thesis. Although the exact mechanism of adenovirus entry into mo-DC has yet to be fully characterised, the poor

transduction efficiency was overcome through the use of the detergent polybrene. Mo-DC, pre-treated with polybrene-containing growth medium for 24 hours, then infected with a RecAd at an MOI of 100 permitted efficient transduction with complex constructs (Figure 6.3). The MOI used to transduce DC varies greatly in the literature; total virus particles were used by one group to determine MOI (Philpott et al., 2004), whilst others used infectious units (Adams et al., 2009, Morelli and Thomson, 2003) as did this thesis. The most efficient MOI was 100 when used in combination with polybrene pre-treatment, however it is unknown whether this same procedure could be used to transduce DC from other sources, for example naturally occurring blood DC and skin DC, or CD34 stem cell derived DC. A recombinant adenovirus encoding the HCV proteins E1, E2 and NS3 was capable of transducing mo-DC to allow expression of the proteins in the DC.

The concept of using mo-DC transduced with adenovirus to treat HCV infection has been suggested for both prophylactic and therapeutic uses (Li et al., 2006), although no clinical trials to investigate the use of autologous mo-DC transduced with adenovirus have been reported. In pre-clinical models, direct vaccination with adenovirus vectors was able to induce strong CD4 and CD8 T cell response to the protein encoded by the transgene, however these responses were significantly reduced in patients with pre-existing immunity to Ad5 (Casimiro et al., 2004, Fitzgerald et al., 2003, Roberts et al., 2006).

Although infusion of a large number of antigen-specific, autologous *in vitro*-produced mo-DC to patients should allow increased presentation to T cells, little is known of the fate of DC once administered to the patient (Prince et al., 2008). Although a broad CD4

and CD8 T cell response, including the production of CD4 T helper cells, is required to clear the HCV infection, as HCV is non-cytolytic, this is not conducive to the production of CD4 T helper cells, and this is likely to inhibit the production of a robust response which can be mounted against the virus.

HCV proteins have been reported to have both pro- and anti-apoptotic properties (Fischer et al., 2007, Nguyen et al., 2006), and it was suggested that cell death is purposefully controlled in order to allow the virus to evade eradication. Necrotic cells are also recognised as effective immunogens, providing an adjuvant-like effect (Gallucci et al., 1999, Kono et al., 2010, Shi et al., 2000, Shi and Rock, 2002), and not only induce immune responses initiated by APC such as macrophages, but also by DC through cross presentation.

Therefore, an important question raised as a consequence asks; if autologous cells which express HCV antigen are induced to become necrotic, can they elicit an HCV specific immune response? I propose that mo-DC transduced with an adenovirus encoding HCV proteins that are induced to become necrotic, could act as the perfect vehicle to deliver HCV antigens for presentation to the immune system to produce a specific anti-HCV response. This hypothesis is examined in Chapter 7 of this thesis.

Chapter 7 - Necrotic cell immunotherapy for persistent HCV infection

7.1 Introduction.

The interaction between antigen loaded DC and naïve T cells is the centre point of basic immune responses, an event which occurs mainly in lymph nodes. Therefore measurement of this interaction has been used to determine the outcomes of immunotherapies, however the reality is that most MoDC delivered by intradermal or subcutaneous injection remain at the injection site (Morse et al., 1999), with only ~5% detected in draining lymph nodes (Zitvogel and Tursz, 2005). A study in chimpanzees detected MoDC in draining lymph nodes after subcutaneous injection but not in inguinal lymph nodes after intravenous injection (Barratt-Boyes et al., 1997).

After infection with HCV, the induction of broadly cross reactive CD4+ and CD8+ T cell responses is believed to be the major requirement to achieve viral clearance, and although a number of studies suggest various forms of DC impairment and defects in HCV positive patients, others report a normal capacity of DC from HCV-infected individuals to stimulate CD4 T cells (Fan et al., 2007). Furthermore, Mo-DC from HCV infected patients and healthy donors were phenotypically and functionally indistinguishable (Barnes et al., 2008) therefore providing the rationale for DC-based immunotherapy for the treatment of HCV infection.

In chapter 3, six patients who had previously failed IFN-based therapy were treated with autologous monocyte-derived dendritic cells pulsed with 6 HCV-specific peptides

representing HLA A2.1-restricted CTL epitopes in a dose escalation regimen. The vaccine resulted in an increase in HCV-specific cell mediated immunity in all patients, with responses to peptides not only contained in the vaccine, but also to peptides derived from antigens which were not, however these responses were transient with no effect on the viral load. It became apparent that although DC immunotherapy was safe, additional work would be required to produce sustained cell mediated responses.

As noted in Chapter 6, initial studies towards this goal were based on the premise of expressing entire HCV proteins within DC to allow the presentation of all CD4+ and CD8+ T cell epitopes, including unidentified epitopes. This was achieved using the Adenovirus based technology. However the ability of DC to take up extracellular antigens and present them to naïve CD8 T cells through cross presentation represented an attractive option. As noted in chapter 5, studies in mice showed that HCV proteins presented in necrotic cells were capable of inducing high HCV-specific immune responses, and although it has not been substantiated that this was due to cross presentation, this is the most likely mechanism. Necrotic cells are a rich source of DAMPs, which include heat shock proteins (HSP), uric acid, high mobility group box 1 protein (HMGB1), ectopic nucleic acids and extracellular ATP, and act as natural adjuvants to activate innate immune responses as a prelude to adaptive immune responses (Rock et al., 2011).

The ultimate goal of these studies was to develop an immunotherapy which allowed DC to present all possible HCV specific epitopes to naïve T cells in order to elicit an HCV

specific immune response capable of clearing the virus. Therefore combining the technology of DC loaded with HCV proteins via Adenovirus-polybrene mediated transduction and antigen delivery via necrotic cells appeared to be a useful strategy to achieve this goal. The hypothesis for this section of the thesis was that HCV antigen-positive MoDC which were made necrotic, when injected into the dermis would be phagocytosed by fully functional resident APC, including Langerhans cells and DC, resulting in cross presentation of the HCV antigens and migration of the antigen-loaded DC to the lymph nodes resulting in an HCV specific cell mediated response.

Therefore the aim of this section of the thesis was to examine the immune response generated by necrotic cell immunotherapy. The results presented in this section of the thesis are the results obtained from a Phase I clinical trial conducted by the Gowans' HCV laboratory based at the Burnet Institute in Melbourne. The manufacture of the recombinant adenovirus construct (described in Chapter 6), the manufacture of the necrotic cell vaccine and the ELIspot results of the clinical trial were completed exclusively by me. Patients were enrolled, injected and monitored by Dr Stuart Roberts and staff of the Gastroenterology Clinic of the Alfred Hospital in Melbourne.

7.2 Experimental Plan

Patients enrolled in this study were selected by Dr Stuart Roberts of the Gastroenterology Clinic at the Alfred Hospital. Patient demographic can be seen in table 7.1. In a departure from the previous clinical trial with peptide pulsed mo-DC, all patients received a 4 week induction of Interferon-α in order to reduce the viral load and circulating viral proteins,

Table 7.1 Baseline characteristics of the patient cohort.

Patient	Age at enrolment (years)	Gender	Ethnicity	HCV genotype	HCV viral load (log10 IU/ml)	IL28B genotype	Fibroscan (kPa)	Prior treatment	Treatment response	ALT level (U/L)
1	58	M	Caucasian	1a	5.99	CT	5.7	Peg/RBV	Relapser	109
2	45	M	Caucasian	1a	6.35	TT	20.9	Peg/RBV/TVR	Relapser	235
3	48	M	Caucasian	1	5.41	ND	15.2	Peg/RBV	Null	134
4	67	F	Caucasian	1	6.81	CC	16.0	Peg/RBV	Relapser	64
5	58	F	Caucasian	1a	6.57	CC	6.7	Peg/RBV	Relapser	63
6	54	M	Caucasian	1	6.05	CT	6.8	Peg/RBV	Relapser	58
7	49	M	Caucasian	1a	6.19	ND	6.2	Peg/RBV	Relapser	56
8	54	M	Caucasian	1b	5.23	CT	17.1	Peg/RBV	Relapser	175
9	54	M	Caucasian	1	5.85	CT	10.8	Peg/RBV	Null	27
10	77	M	Caucasian	1a	6.38	ND	ND	Peg/RBV	Relapser	123
11	56	M	Caucasian	1a	6.25	TT	7.4	Peg/RBV	Relapser	98
12	50	M	Caucasian	1a	5.95	ND	9.2	HCV Pol + PI	Non-responder	119

some of which are immunosuppressive, as it was thought that this was likely to increase the probability of inducing an effective immune response. The day after their final dose of Interferon- α, hallmarking 4 weeks since commencement of interferon, patients 1 to 12 received the first injections of necrotic adenovirus-transduced autologous moDC, and were given subsequent injections according to an escalating dose schedule as shown in table 7.2. At each injection timepoint, the dose was separated into two injections and the patient received these intradermally in the deltoid. Patients receiving multiple doses were administered these 1 week apart.

Blood samples were taken at Baseline, 2 weeks, 6 weeks and 12 weeks post final injection. PBMC were isolated and assayed using IFN- γ ELIspot, as described in sections 2.1.5 and 2.1.6.

7.3 Results

Patients 1 to 3 received intradermal injections consisting of $1x10^4$ cells. Patient 1 received 1 dose, patient 2 received 2 doses and patient 3 received 3 doses. As shown in Figure 7.1a, patient 1 had no detectable responses to E2 and NS3 at baseline, but showed a low response of 11 SFU/10^6 cells to E1. Low level responses were detected to E2 at 2 weeks post final injection and to NS3 at 2 and 6 weeks post final injection. No responses were detected at 12 weeks post final injection. Patient 2 had a similar response profile with a low level response to E1 at baseline, with no detectable response throughout the remainder of the time points (Figure 7.1b). A low response of (11 SFU/10^6 cells) to E2 was detected at baseline and at 2 weeks post final injection, and then fell below

Table 7.2. The escalating dose schedule in 12 persistently infected HCV patients treated with autologous antigen loaded necrotic DC.

Patient Number	Cell Dose	Dose 1	Dose 2	Dose 3
1	10^4 Cells	•		
2	10^4 Cells	•	•	
3	10^4 Cells	•	•	•
4	10^5 Cells	•		
5	10^5 Cells	•	•	
6	10^5 Cells	•	•	•
7	10^6 Cells	•		
8	10^6 Cells	•	•	
9	10^6 Cells	•	•	•
10	10^7 Cells	•		
11	10^7 Cells	•	•	
12	10^7 Cells	•	•	•

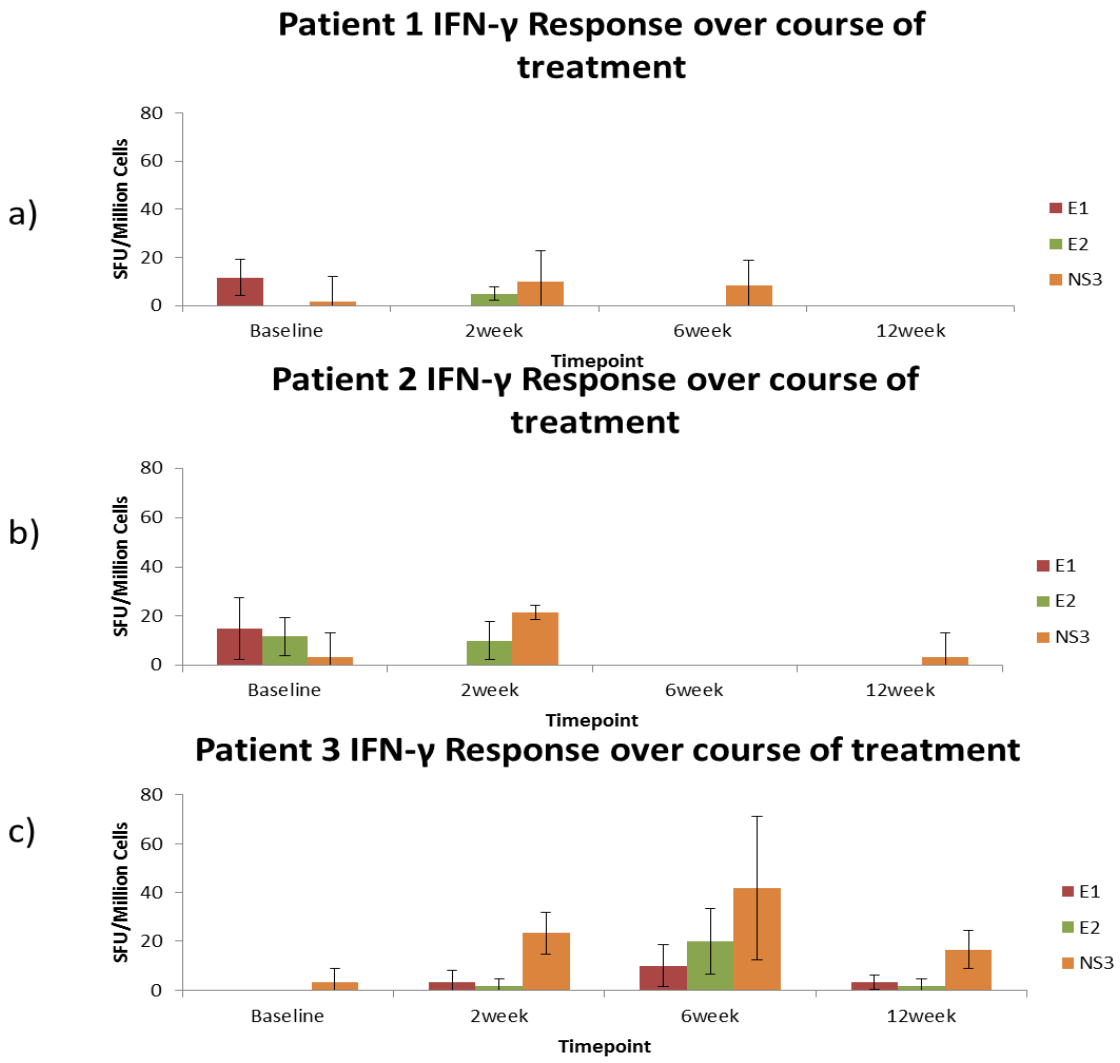

Figure 7.1. IFN-γ ELIspot results against HCV peptide pools over the course of treatment. IFN-γ ELIspot results against HCV peptide pools over the course of treatment for patient 1 (a) patient 2 (b) and patient 3 (c). Blood samples were taken at Baseline, 2, 6 and 12 weeks post final injection ELIspot performed using PBMC isolated by ficoll-density gradient centrifugation and stimulated for 20-24h with peptide pools spanning the entire HCV proteins E1 (Red), E2 (Green) and NS3 (Orange). Bars represent spot forming units/million cells. Negative control responses were subtracted from each, with error bars representing +/- 1 standard deviation.

detectable levels for the remainder of the treatment. An NS3 response was detected (22 SFU/10^6 cells) at 2 weeks post final injection, no response was detected at 6 weeks and a low level response detected at 12 weeks post final injection. Patient 3 showed no responses at baseline (Figure 7.1c). Low level responses of 3, 10 and 3 SFU/10^6 cells to E1 were detected at 2, 6 and 12 weeks respectively. A response to E2 peaked at 6 weeks post final injection with a response of 20 SFU/10^6 cells detected while a NS3 response of 23 SFU/10^6 cells was detected at 2 weeks, that increased to 41 SFU/10^6 cells at 6 weeks but fell 16 SFU/10^6 cells at 12 weeks post final injection.

Patients 4 to 6 received intradermal injections consisting of $1x10^5$ cells. Patient 4 received 1 dose, patient 5 received 2 doses and patient 6 received 3 doses. Patient 4 showed baseline responses to E1, E2 and NS3 of 10, 12 and 20 SFU/10^6 cells (Figure 7.2a), with E1 and NS3 rising to 30 and 33 SFU/10^6 cells respectively. These responses dropped at 6 weeks while NS3 responses of 45 SFU/10^6 cells were detected at 12 weeks post final injection. Patient 5 also had uncharacteristically high baseline responses to all three peptide pools (figure 7.2b), although the responses at the subsequent time points were lower and of no significant value. Patient 6 showed no responses at baseline and these did not change throughout the course of the treatment with the exception of responses of 3 and 9 SFU/10^6 cells to E1 and NS3 at 2 weeks post final injection, and 12 SFU/10^6 cells to E2 at 12 weeks post final injection (Figure 7.2c).

Patients 7 to 9 received intradermal injections consisting of $1x10^6$ cells. Patient 7 received 1 dose, patient 8 received 2 doses and patient 9 received 3 doses. The only notable response from Patient 7 was to NS3 at 13 SFU/10^6 cells at 6 weeks post final

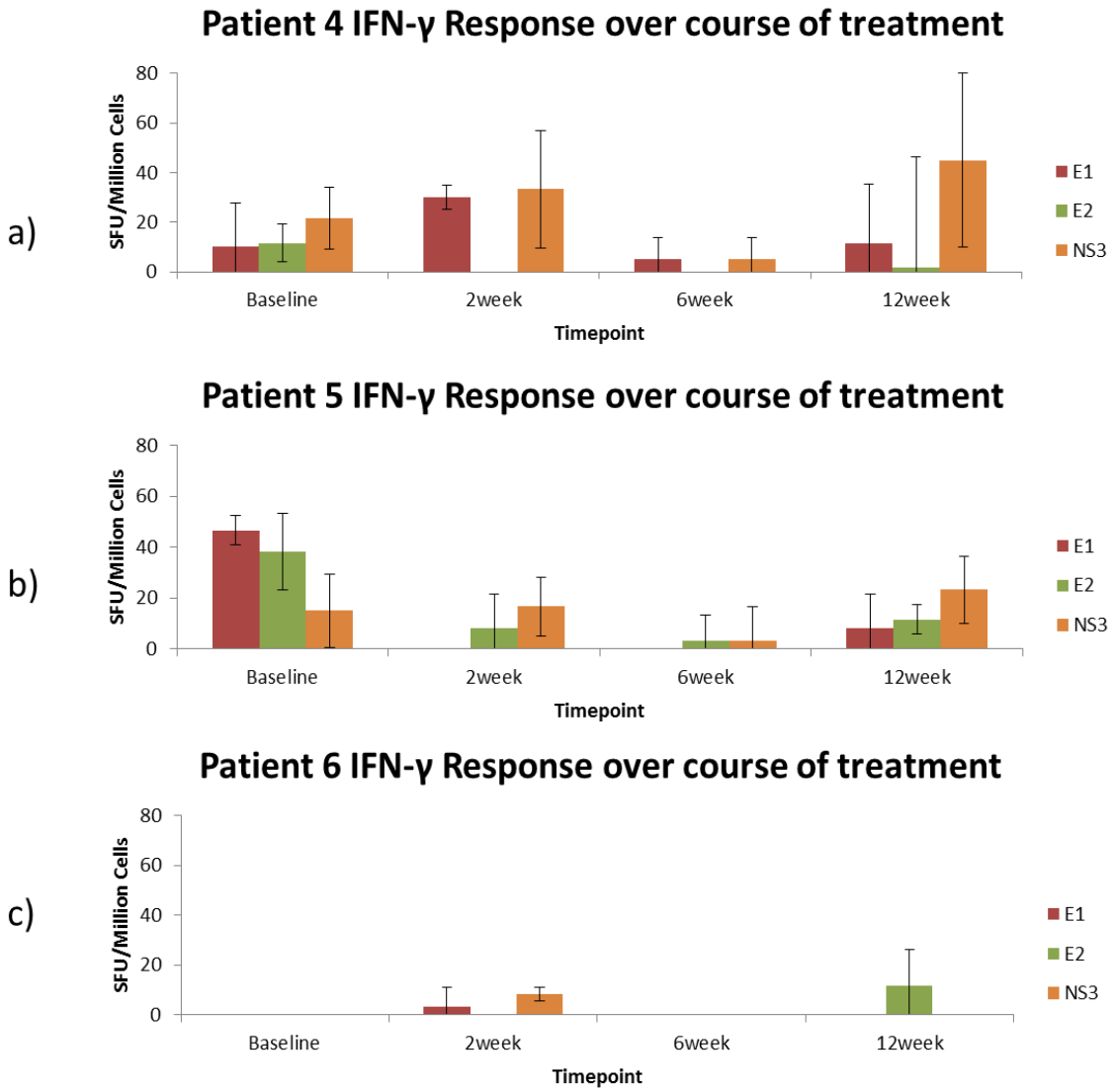

Figure 7.2. IFN-γ ELIspot results against HCV peptide pools over the course of treatment. IFN-γ ELIspot results against HCV peptide pools over the course of treatment for patient 4 (a) patient 5 (b) and patient 6 (c). Blood samples were taken at Baseline, 2, 6 and 12 weeks post final injection ELIspot performed using PBMC isolated by ficoll-density gradient centrifugation and stimulated for 20-24h with peptide pools spanning the entire HCV proteins E1 (Red), E2 (Green) and NS3 (Orange). Bars represent spot forming units/million cells. Negative control responses were subtracted from each, with error bars representing +/- 1 standard deviation.

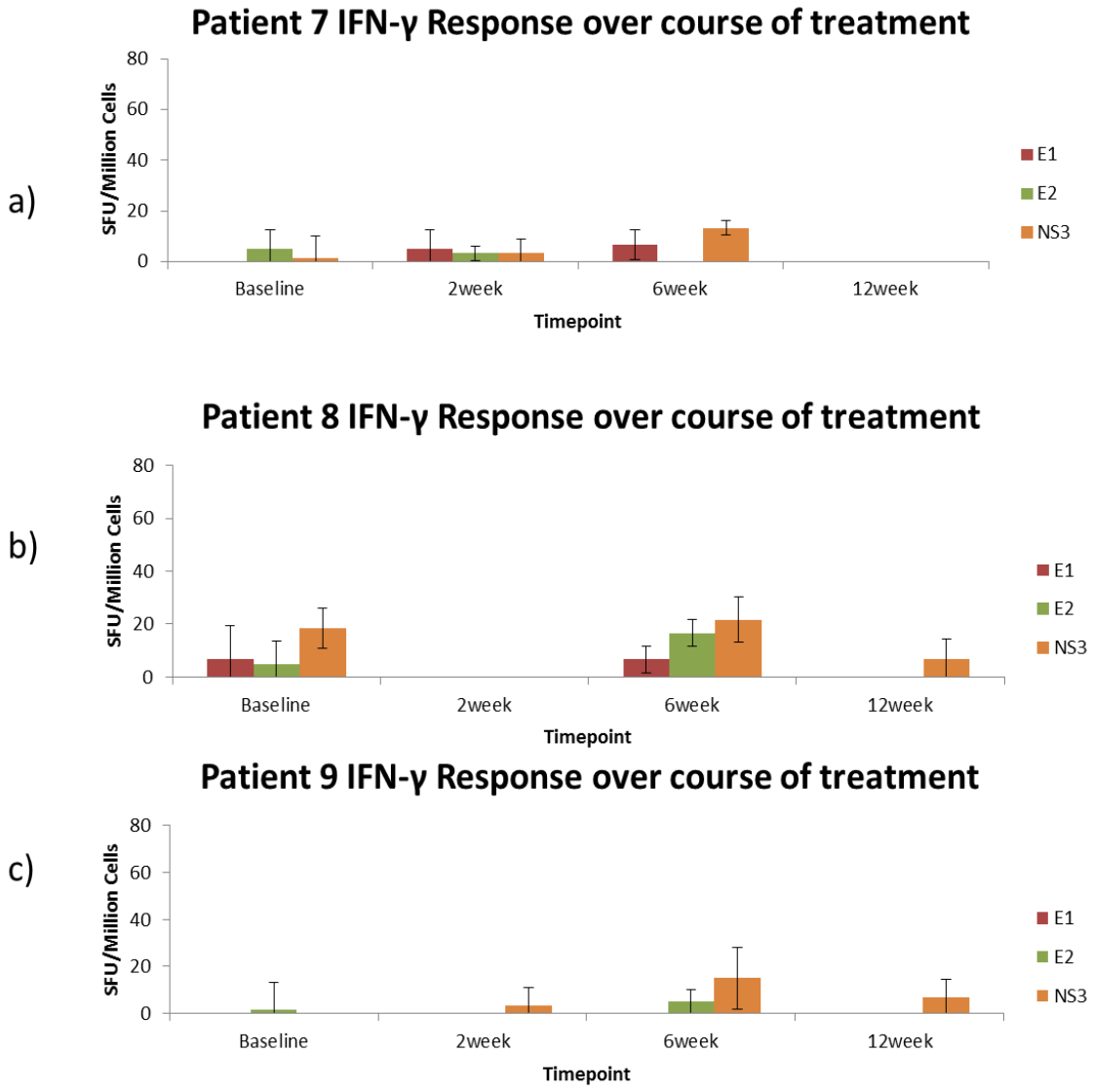

Figure 7.3. IFN-γ ELIspot results against HCV peptide pools over the course of treatment. IFN-γ ELIspot results against HCV peptide pools over the course of treatment for patient 7 (a) patient 8 (b) and patient 9 (c). Blood samples were taken at Baseline, 2, 6 and 12 weeks post final injection ELIspot performed using PBMC isolated by ficoll-density gradient centrifugation and stimulated for 20-24h with peptide pools spanning the entire HCV proteins E1 (Red), E2 (Green) and NS3 (Orange). Bars represent spot forming units/million cells. Negative control responses were subtracted from each, with error bars representing +/- 1 standard deviation.

injection (Figure 7.3a). Patient 8 displayed baseline responses of 6, 5 and 18 SFU/10^6 cells to E1, E2 and NS3 respectively. No responses were detected at 2 weeks post final injection, while E2 and NS3 responses rose to 16 and 21 SFU/10^6 cells respectively at 6 weeks but fell to below baseline levels by 12 weeks post final injection (Figure 7.3b). Patient 9 showed no responses to E1 throughout the entire period of monitoring (Figure 7.3c). Very low responses to E2 were observed at baseline (1 SFU/10^6 cells) and 6 weeks post final injection (5 SFU/10^6). Low responses to NS3 were detected at 2, 6 and 12 weeks post final injection with readings of 3, 15 and 7 SFU/10^6 cells.

Patients 10 to 12 received intradermal injections consisting of $1x10^7$ cells. Patient 10 received 1 dose, patient 11 received 2 doses and patient 12 received 3 doses. Patient 10 had baseline responses above 20 SFU/10^6 cells for all three peptide pools, but responses observed at later time points failed to peak above this threshold (Figure 7.4a). Patient 11 also displayed relatively high baseline responses of 8, 6 and 30 SFU/10^6 cells to E1, E2 and NS3 respectively, however unlike patient 10, patient 11 showed increases in these responses over the course of treatment. Responses to E1 were detected at 25 and 11 SFU/10^6 cells at 6 and 12 weeks post final injection (Figure 7.4b). Similar responses to E2 were detected at 20 and 13 SFU/10^6 cells at 6 and 12 weeks post final injection, while the responses to NS3 increased to 61 SFU/10^6 cells at 6 weeks, dropping to just 57 spot forming cells per million at 12 weeks. Patient 12 also displayed high baseline responses to E1 and NS3, and consequently responses at later time points were unremarkable

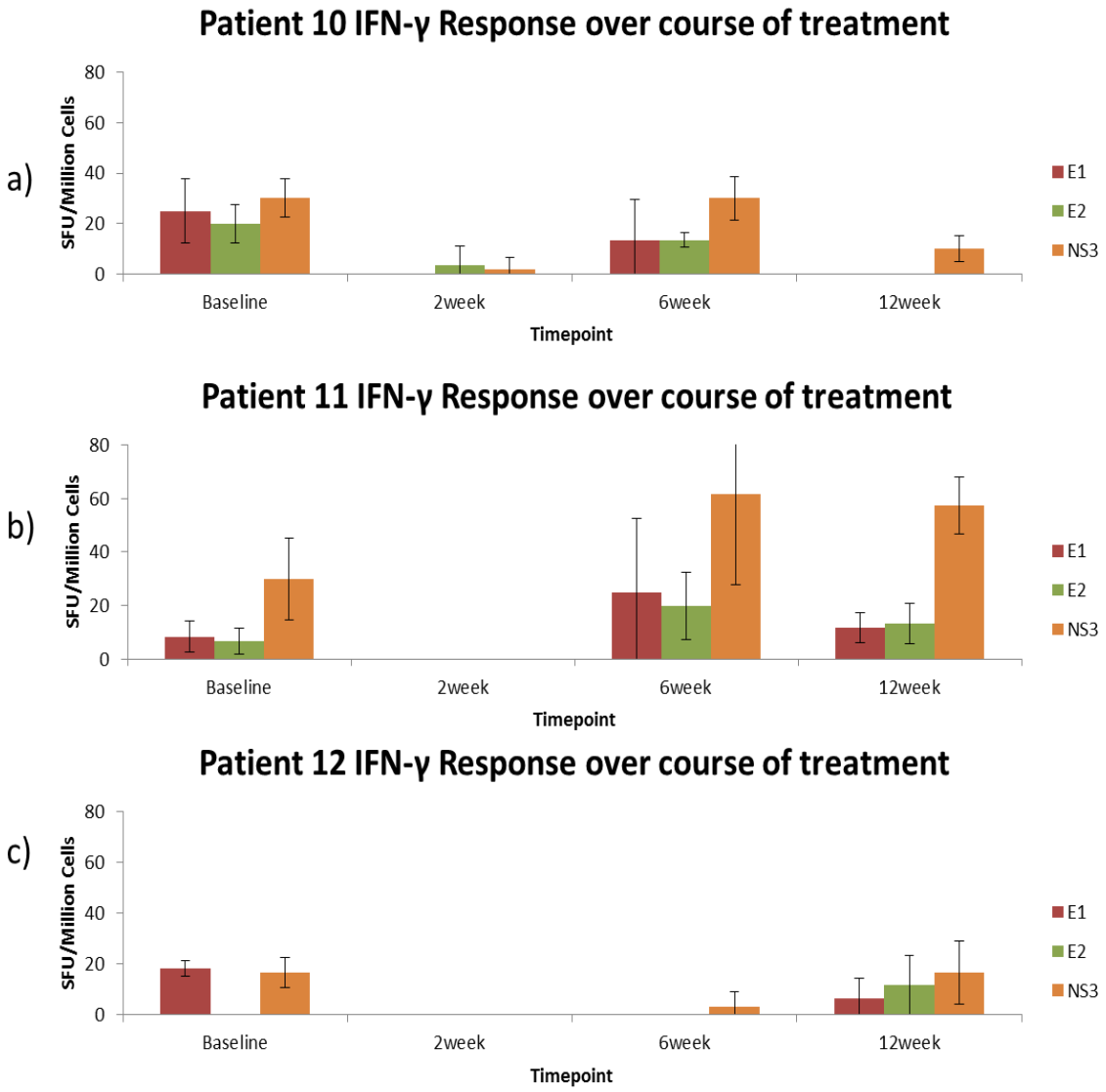

Figure 7.4. IFN-γ ELIspot results against HCV peptide pools over the course of treatment. IFN-γ ELIspot results against HCV peptide pools over the course of treatment for patient 10 (a) patient 11 (b) and patient 12 (c). Blood samples were taken at Baseline, 2, 6 and 12 weeks post final injection ELIspot performed using PBMC isolated by ficoll-density gradient centrifugation and stimulated for 20-24h with peptide pools spanning the entire HCV proteins E1 (Red), E2 (Green) and NS3 (Orange). Bars represent spot forming units/million cells. Negative control responses were subtracted from each, with error bars representing +/- 1 standard deviation.

(Figure 7.4c). Responses to E2 remained at zero for most of the duration of the study except for a low response of 12 SFU/10^6 cells at 12 weeks.

7.4 Discussion

This study was designed to improve the immunogenicity of HCV proteins in a cellular immunotherapy strategy. Initially I perceived a deficiency in current dendritic cell-based immunotherapeutic approaches due to a lack of migration of therapeutic MoDC from the site of injection. Therefore it became apparent that perhaps therapeutic strategies should be targeting naturally occurring DC within the patient. It has been proposed that it may be possible to boost HCV-specific cell mediated immunity (CMI) in HCV patients, potentially eliminating the infection; or at least reducing the viral load, the cause of chronic disease, thereby providing long term improvement in the quality of life of patients and a reduced health burden to the community. However a boost in HCV specific CMI which has resulted in a sustained virological response has yet to be documented.

HCV immunotherapy strategies involving the use of some sort of adjuvant have yielded the most promising results. Yutani et al vaccinated twelve IFN-resistant patients with 4 peptides formulated in incomplete Freund's adjuvant. A clinical effect in 5 patients was indicated by reduced ALT levels, however increases in CMI and antibody titres had no effect on the viral load (Yutani et al., 2007). Vaccination with peptides representing cytotoxic T lymphocyte (CTL) epitopes formulated in poly-L-arginine as the adjuvant generated a transient reduction in the viral load in only 3 of 30 IFN resistant patients,

although all 30 showed nascent specific CMI (Klade et al., 2008). Most promising in light of this study is the use of necrotic *Saccharomyces cerevisiae*, designed to express a Core-NS3 fusion protein, which has been used in Phase I and II clinical trials (Habersetzer et al., 2009). Known as GI-5005, this product was administered to 54 patients in a phase I clinical trial. Six of the patients showed a reduction in the viral load of 0.75-1.4$_{log}$10. In the Phase II trial, 40 Yeast Units (1YU represents 10^7 yeast cells) were administered as 7 doses and appeared to reduce the viral load by 0.3$_{log}$10 per month over 3 months. There was no increased ALT associated with the therapy and ALT levels were reduced to normal in several patients.

Thus, it is evident that HCV specific CMI can be elicited by HCV infected patients when epitopes, provided as peptides or proteins, are presented to the immune system in conjunction with an adjuvant. As noted above, it is known that necrotic cells are highly immunogenic due to the expression and release DAMPs which act as natural adjuvants (Rock et al., 2011). Consequently, rather than depend on the migration of infused MoDC to lymph nodes as has been the classical approach for DC based immunotherapy, I hypothesised that HCV antigen-positive, necrotic MoDC injected into the dermis will be targeted by fully functional resident APC which will phagocytose the necrotic cells, resulting in cross presentation of HCV antigens followed by migration to lymph nodes for effective stimulation of naïve T cells.

This trial was designed as a dose escalation trial across a 1000-fold cell dose range, with a brief treatment period of only 1 to 3 weekly injections. No adverse events were

documented, although several patients reported some minor side effects including redness and swelling at the injection site, flu like symptoms and nausea that were similar to those reported by patients who were treated with viable dendritic cells (Chapter 3). Notably, these side effects were reported by the patients who received lower doses of the necrotic cells, and there was no evidence that the highest tolerable dose had been achieved.

Necrotic HCV positive DC administered in mice (via a DC cell line) generated robust HCV specific immune responses. However the responses in the patients were disappointingly low. No notably high responses were detected, and any responses detected were transient. No change in viral load was observed in any of the patients throughout the monitoring period. The reasons for this may be multi-factorial; firstly, the patients enrolled into the study had persistent HCV infection and had previously failed conventional therapy, and consequently represent an already difficult to treat cohort. This may explain why even patients who received 1 to 3 injections of the higher doses of the necrotic cells failed to respond to the therapy. Secondly, the injection regimen administered in this study consisting 1 to 3 weekly injections, while representing an effective strategy to prove safety, did not represent an effective immunization regimen. Intervals of 4-6 weeks between vaccine doses, given repeatedly, would be required to induce immunity capable of having an impact on established infection in IFN-non responders. In the previous dendritic cell based immunotherapy trial (Chapter 3) it was suggested that a sustained immunological response was unable to be produced due to the high viral load present in the systems of these persistently infected patients, which is why patients enrolled in this study were given a 4 week induction phase of IFN. Future studies

may necessitate the continuation of the IFN regimen throughout the treatment and monitoring period, to effectively examine the capability of mounting a sustainable HCV specific immunological response; however there is evidence of IFN having immunosuppressive properties (discussed in chapter 8). The results of this trial will now permit a further study to examine the efficacy of a high dose immunization regimen in therapy-experienced and -naïve patients.

Chapter 8 - General Discussion

The global prevalence of HCV has increased from 122 million to greater than 185 million between 1990 and 2005 (Mohd Hanafiah et al., 2013), while the major burden is due to sequelae from persistent HCV infection (Mohd Hanafiah et al., 2013). Approximately 75% of HCV-infected individuals develop a persistent infection resulting in chronic hepatitis. This leads to cirrhosis in approximately 20% of individuals, and eventually hepatocellular carcinoma (HCC) in 4-5% of these patients each year (Seeff, 2002). HCV infection is currently the leading cause for liver transplantation in Western countries (Burra et al., 2009).

The mechanisms resulting in the clearing immune response have not been determined empirically, however observations in patients who undergo an acute self-limited infection suggest that a broad, specific cell mediated immune response to several non-structural proteins including NS3 and NS5 is necessary (Bowen and Walker, 2005, Smyk-Pearson et al., 2006). Those who manage to clear the virus can be re-infected although the residual cell mediated immunity reduces the risk of re-infection, and subsequent infections are associated with a reduced viral load and a shorter duration of viremia (Grebely et al., 2006). Previously-infected injecting drug users show a reduced susceptibility to re-infection despite the higher risk of reinfection due to their lifestyle (Grebely at al., 2006). Recently it has been found that in previously-infected injecting drug users approximately half (of those tested) develop persistent infection despite a previous case of spontaneous clearance (Sacks-Davis et al., 2013). However in cases

where reinfection is evident, the spontaneous clearance occurs 4 times faster than in clearance following a primary infection (Sacks-Davis et al., 2013). The faster rate of clearance following reinfection is also supported by studies in chimpanzees (Grebely et al., 2012).

For the past 20 years the use of recombinant interferon (IFN) was the standard of care for HCV treatment, involving the co-administration of pegylated IFNα2b and the antiviral drug, ribavirin. However, in as many as 60% of patients, the therapy failed to clear the virus. Very little is known about the mechanisms involved in the clearance of HCV by IFN; however it is now clear that ongoing IFN signalling can in fact be detrimental to the immune response. There is mounting evidence that many of the immune dysfunctions associated with persistent virus infections are potentiated by increased IFN levels (Wilson and Brooks, 2013) and in addition, many studies have noted that upregulation of interferon-stimulated genes in persistent HCV infection prior to interferon therapy is associated with non-response to the therapy (reviewed in (Asselah et al., 2010).

The ultimate aim of this thesis was to develop an immunotherapy to treat persistent HCV infection. This was achieved through the investigation of dendritic cell-based technology (Chapter 3 and 7); encompassing HCV protein expression using adenovirus-based technology (Chapter 6) and necrotic cells as an antigen delivery vehicle (Chapters 5 and 7). The work presented in this thesis represents novel advances in technology such as the first-in- man treatment of persistent HCV using autologous monocyte-derived DC

(Chapter 3), a mouse model for necrotic cell based therapy in mice (Chapter 5), a method for the stable transduction of monocyte derived DC using adenovirus by pre-treatment using the detergent polybrene and a method for clinical trial scale production of recombinant adenovirus in serum free media (Chapter 6).

In the first clinical trial examined in this thesis, it was believed that the dendritic cell-based therapeutic vaccine technology developed utilising restricted HLA-A2 epitopes (Chapter 3) failed because, although it was capable of producing broad transient responses to multiple hepatitis C viral proteins; the vaccine was unable to break through the perceived barrier imposed by high viral titres, especially when utilising only a few (six) epitopes. These observations helped shape the protocol of the next trial which utilised necrotic cell therapy to treat persistent HCV. The use of whole proteins expressed intracellularly, presented in necrotic cells, was designed to take advantage of the patients' homeostatic mechanisms to recognise these cells, then detect the HCV antigens within the necrotic cell fragments to finally elicit an HCV-specific immune response. Patients enrolled in this trial were also given 4 weeks of IFN treatment prior to vaccination in an attempt to reduce the viral load and allow a nascent immune response the opportunity to clear the virus in a low viremic environment. However unlike the experiments conducted in mice (chapter 5), necrotic cell therapy in persistently infected humans showed very low, transient responses to HCV proteins (Chapter 7). It is possible that the low responses detected were the result of an escalating viral load following cessation of IFN treatment just prior to the vaccination. However recently it has been suggested that chronic levels of Type 1 interferon have a suppressive action on CD4 T

cells (Osokine et al., 2014). In particular, although CD4 T-cells primed in the midst of a persistent infection are able to help B cells, their ability to help CD8 T-cells is severely diminished (Osokine et al., 2014). This becomes an important factor when one takes into consideration that the clearance of HCV requires broad multi-specific CD4 and CD8 T cell responses, and cross presentation also requires help from CD4 T cells (Blachere et al., 2006). The results of the studies presented in this thesis suggest that IFN therapy may actually be counterproductive in attempts to eliminate the virus by immunotherapy. The mechanisms of the IFN signalling pathway are complex, recently it has been suggested that our understanding of the differences in the suppressive, antiviral and immune enhancing effects of IFN families are too poorly understood to be used effectively in the context of treatment of persistent viral infections (Wilson and Brooks, 2013). During the past twelve months, new DAA against HCV, such as Telaprevir and Boceprevir, have been introduced into the clinic. These are both NS3/4A protease inhibitors specific for genotype 1 viruses, with genotype 3 infected patients relying on the standard course of therapy. DAA could be used in place of IFN-ribavirin therapy in the context of immunotherapy to reduce the initial viral load, to allow the immunotherapeutic vaccine to act. However, recently it has been reported that use of DAA has led to the emergence of mutations leading to drug resistance (Andonov et al., 2013). These mutations show reduced susceptibility to DAA compared with the dominant virus population (Andonov et al., 2013). Furthermore evidence exists that some of these mutations exist even prior to treatment in some patients (Halfon and Locarnini, 2011, Lauck et al., 2012, Sarrazin and Zeuzem, 2010). Therefore the use of DAA could in fact accelerate the emergence of resistant mutants due to the added

immunological pressure. However, currently no trials using combination DAAs have reported DAA resistance as having an impact on clinical outcomes or chances of achieving SVR.

Alternatively, as expression of the inhibitory receptor programmed cell death-1 (PD-1) is associated with T cell exhaustion in persistent infection with HCV or HIV, strategies to inhibit PD-1 signalling may have the potential to reverse T cell function. Recently, antibodies to PD-1 were used to treat chimpanzees with persistent HCV infection; one animal showed a reduction in the viral load associated with loss of CD4 and CD8 T cell anergy, although withdrawal of the antibody therapy resulted in a rebound in the viral load (Fuller et al., 2013). It was suggested that anti-PD-1 therapy could be supported by therapeutic vaccination for optimum efficacy. As antibodies to PD-1 and its ligand PD-L1 have previously been used to treat cancer patients resulting in clinical responses in a proportion of patients (Brahmer et al., 2012, Topalian et al., 2012), it may now be possible to combine anti-PD-1/anti-PD-L1 therapy with therapeutic vaccination with HCV antigen-loaded DC or other therapeutic strategies, as a viable alternative to therapy with expensive DAA.

The main disadvantage of current HCV therapies apart from cost, including those utilising DAA, is that similar to IFN therapy, their efficacy is highly dependent upon the genotype of the infecting virus. Likewise, the methodologies described in this thesis also have associated constraints. The use of DC-based technology requires individualised treatment of the patient and DC culture is expensive. Future studies should aim to further

simplify the process. For example investigation into the presentation of HCV proteins in necrotic cells derived from cells which do not require extensive *in vitro* processing, such as peripheral blood mononuclear cells, could prove both cost effective and more immunologically relevant. Furthermore, in the context of an effective immunotherapeutic vaccine, the restriction imposed by HCV genotype is also a limiting factor. Ultimately a necrotic cell-based vaccine therapy utilising a cell source which was capable of being administered into patients regardless of MHC or HLA restrictions, covering a broader range of HCV genotypes would prove to be a much more promising candidate for a lasting therapeutic vaccine. Utilisation of a HLA negative cell line such as C1R, a HLA-A,B negative cell line used widely in functional studies of MHC Class I (Zemmour et al., 1992) may be one avenue of enquiry. An expandable cell line with a lack of HLA antigens would allow administration into most if not all patients. One concern with the use of allogeneic cells is the effect of allogeneic responses, however historically allogeneic responses to not pose problems in routine procedures such as blood transfusions. It should also be noted that allogeneic responses do not occur in pregnancy or in normal sexual relationships in which women are regularly exposed to different HLA antigens in sperm. However, to overcome potential adverse events associated with allogeneic cell therapy and the costs associated with autologous cell therapy, it may be possible to combine an effective DNA vaccine strategy, in which necrosis is induced in vaccine targeted cells (Gargett et al., 2014), with anti-PD-1/anti-PD-L1 therapy. The clinical trials described in this thesis have resulted in considerable expertise and experience to permit such a trial to proceed in the near future.

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