Modulation of Innate Immune Responses by Hepatitis C Virus

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ABSTRACT

Hepatitis C virus (HCV) establishes a chronic infection in about 70% of infected individuals that is associated with the development of liver cirrhosis and hepatocellular carcinoma. The mechanisms by which HCV avoids clearance by the host immune response are not fully understood.

The first aim of this project was to determine whether immune cell subsets could become infected by HCV *in vitro*. None of the haematopoietic subsets analysed expressed all of the required entry factors, CD81, SR-BI, claudin-1 and occludin. Also, PBMCs were not susceptible to infection with HCVpp and HCVcc expressing glycoproteins of hepatotropic strains. Infection by a supposedly lymphotropic strain (SB) was found to be inefficient.

The second aim was to identify *in vitro* immunomodulatory effects of HCV on innate immune cells that may impact on the immune response activated in acute infection. Crosslinking of CD81 on NK cells by antibody was found to have a minor inhibitory effect on their activation via CD16, but CD81 crosslinking by viral particles had no detectable effect. In contrast to other viruses, HCVcc elicited very little interferon-α production by pDC. HCVcc also did not affect pDC or mDC responses to TLR ligation.

Systemic cytokine and chemokine responses were analysed in subjects with primary acute HCV infection and in HCV-infected patients undergoing liver transplantation (LT). Interestingly, induction of systemic type I and type III interferon was not observed in either group. Marked perturbations in systemic cytokine and chemokine levels were detected in uninfected LT patients, precluding use of HCV-infected LT patients to study the innate immune response activated in response to acute viral replication.

Together, these results suggest that HCV may principally evade innate immune cell responses by avoidance rather than impairment strategies.

LIST OF ABBREVIATIONS

aa	Amino acid
Ab	Antibody
ADAR	RNA-specific adenosine deaminase
ADCC	Antibody-dependent cellular cytotoxicity
ALT	Alanine transferase
APC	Allophycocyanin
APC	Antigen presenting cell
BAT	HLA-B-associated Transcript
BCR	B cell receptor
BDCA	Blood dendritic cell antigen
bp	Base pair
BVDV	Bovine viral diarrhoea virus
CAR	Coxsackievirus and adenovirus receptor
CARDIF	CARD adaptor inducing IFN–β
CHC	Chronic hepatitis C
CHV	Canine hepacivirus
CLDN	Claudin
CLR	C-type lectin receptor
CMV	Cytomegalovirus
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
CVB	Coxsackievirus B
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule 3- grabbing integrin
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DV	Dengue virus
dsRNA	Double-stranded RNA
E2e	E2 ectodomain
EBV	Epstein-Barr virus
EDTA	Ethylene-diamino-tetra-acetate
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid

EI	Exposed infected
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EU	Exposed uninfected
FACS	Fluorescence activated cell sorting
FASL	Fas ligand
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage-colony stimulating factor
gt	Genotype
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCV-LP	HCV-like particle
HCVcc	Cell culture-derived HCV
HCVpp	HCV pseudoparticle
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HS	Heparan sulphate
HSC	Hematopoietic stem cell
HSV	Herpes simplex virus
HVR	Hypervariable region
HTLV	Human T cell leukemia virus
ICAM	Intercellular adhesion molecule
IDU	Injecting drug user
I-E2	Intracellular E2
IFN	Interferon
IL	Interleukin
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene

ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
IU	International unit
JAK	Janus kinase
KIR	Killer immunoglobulin-like receptor
ko	Knock-out
KSHV	Kaposi's sarcoma-associated herpes virus
L-SIGN	Liver/lymph node-specific intercellular adhesion molecule 3-grabbing integrin
LB	Luria-Bertani
LCMV	Lymphotropic choriomeningitis virus
LD	Lipid droplet
LDL	Low density lipoprotein
LDL-R	Low density lipoprotein receptor
LEL	Large extracellular loop
LPS	Lipopolysaccharide
LT	Liver transplantation
LTR	Long terminal repeat
LVP	Lipo-viro-particle
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MBP	Mannose-binding protein
MDA5	Melanoma differentiation-associated gene 5
mDC	Myeloid dendritic cell
MDDC	Monocyte-derived dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MICA/B	MHC class I-related chain A/B
MLV	Murine leukemia virus
MPCC	Micro-patterned co-cultures
NANBH	Non-A, non-B hepatitis
NEAA	Non-essential amino acids
NF-κB	Nuclear factor-κB
NK	Natural killer
NKT	Natural killer T cell
NLR	Nod-like receptor

NS	Non-structural
OAS	Oligoadenylate synthetase
OCLN	Occludin
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PB	Pacific blue
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PFA	Paraformaldehyde
PHH	Primary human hepatocyte
PRR	Pattern recognition receptor
RANTES	Regulated on activation normal T-cell-expressed and secreted
RdRP	RNA-dependent RNA polymerase
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor
RSV	Respiratory syncytial virus
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sE2	Soluble E2
SEL	Small extracellular loop
SR-BI	Scavenger receptor class B type I
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription
TBK	TANK-binding kinase
TCID	Tissue culture infectious dose
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TM	Transmembrane
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell

TRIF	Toll-IL-1 receptor domain-containing adaptor-inducing IFN-β
ULBP	UL-16-binding protein
uPA	Urokinase-type plasminogen activator
UTR	Untranslated region
VLDL	Very low density lipoprotein
VLPs	Virus-like particles
VSV	Vesicular stomatitis virus

1 INTRODUCTION

1.1 Virology of HCV

1.1.1 History of HCV

In the mid 1970s, Harvey J. Alter's group noticed that in most post-transfusion hepatitis cases the causative agent could not be identified as Hepatitis A or B, leading to the name of non-A, non-B hepatitis (NANBH) [1]. It soon became clear that NANBH lead to liver cirrhosis and hepatocellular carcinoma [2,3]. This extreme outcome prompted extensive research in the next decade and finally, in 1989, the etiologic agent of NANBH was found to be hepatitis C virus (HCV). Two articles were published in *Science*, showing cloning of the HCV genome and development of a sensitive assay to detect HCV antibodies [4,5].

Further milestones in the HCV field were the establishment of an infectious clone that lead to acute infection in chimpanzees in 1997 [6,7]; the development of a replicon system in 1999 [8], enabling studies of HCV replication; the production of the pseudoparticle system aiding research in the field of HCV entry in 2003 [9,10]; and eventually the development of cell culture-derived HCV (HCVcc) in 2005 [11,12].

HCV is classified as a member of the genus of *Hepacivirus* together with GB virus B (GBV-B) and canine hepacivirus (CHV) [13] in the family *Flaviviridae,* which also include the genera flaviviruses [such as dengue virus (DV) and yellow fever virus] and pestiviruses [such as bovine viral diarrhoea virus (BVDV) and GBV-A] [14].

1.1.2 HCV variability

One of the problems for HCV vaccine development and treatment strategies is the great variability of the virus. There are 6 genotypes (gt) and multiple subtypes [15]. The former have a sequence variability of 31-33%, whilst between the subtypes the sequence diversity is 20-25%. The most conserved regions in the genome are the 3' and 5' untranslated regions

(UTR), followed by core, and the least conserved is E2 [16]. The six gt are distinctly distributed across the globe [17-19]. Gt 1a and 1b are the most prevalent in Europe, America, Australia, New Zealand, Japan and China, whereas gt 5 predominates in sub-Saharan Africa and gt 4 is almost exclusively found in Egypt and is the predominating gt there. Gt 2 has the highest prevalence in Japan, but is present in all other parts of the world. Similarly, gt 3 has the highest occurrence in South East Asia, but is also found around the world. Finally, gt 6 is confined to China and South East Asia and occurs there amongst other gt. The six gt behave very differently in terms of treatment outcome and propensity to cause HCV-associated diseases such as liver steatosis and fibrosis, amongst many other factors.

HCV exhibits further heterogeneity, as it exists as a quasispecies within the host, with a sequence diversity of 1-5% between the different members of the quasispecies [17]. The underlying cause of this genetic diversity is the lack of a proofreading mechanism for the RNA polymerase leading to the generation of multiple mutations during virus replication, which can pose a problem for treatment regimes or can lead to evasion of adaptive immune responses.

1.1.3 HCV genome and proteins

HCV is an enveloped virus with a diameter of 40-50nm and contains a 9.6 kilo base (kb) positive-sense single-stranded RNA (+ssRNA) genome that encodes the 10 HCV genes in one open reading frame (ORF) flanked by a 5'UTR and 3'UTR [20] (Figure 1-1).

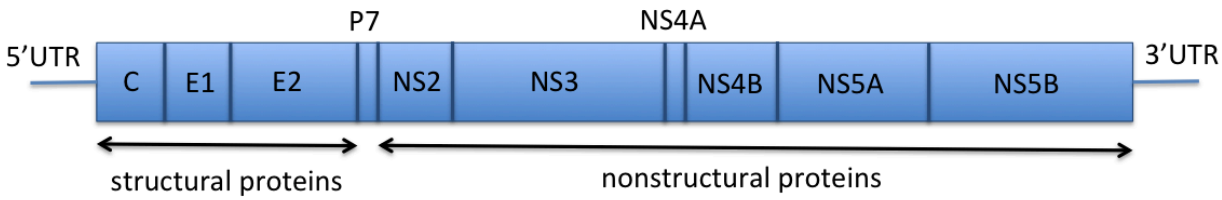

Figure 1-1 Schematic representation of the HCV genome

The 9.6kb positive strand RNA genome of HCV encodes a polyprotein about 3000 amino acids. This polyprotein is cleaved by host proteases into structural proteins (core, E1 and E2) and p7, and by viral proteases into non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B).

In the 5'UTR there is an internal ribosome entry site (IRES), where translation into a single polyprotein of approximately 3000 amino acids is initiated. The N-terminal part of the polyprotein contains several signal sequences that ensure delivery to the endoplasmic reticulum (ER), where it is further processed into 10 polypeptides. The structural proteins (core, E1 and E2) and p7 are cleaved by host signal peptidases in the ER, and the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B are released from the polyprotein by the two HCV proteases NS2-3 and NS3-4A. All HCV proteins are found in close association with ER membranes, and two proteins, core and NS5A, have been shown to colocalise with lipid droplets (LDs) [21], the intracellular storage sites for cholesterol esters and triglycerides [22].

Core is an RNA-binding protein that forms the viral capsid [23]. It contains two domains, the N-terminal D1 and the C-terminal D2 domain. The D1 domain is hydrophilic and is related to the capsid proteins of other *Flaviviridae*. The D2 domain is hydrophobic and is responsible for the association with lipid droplets and the ER. The translocation from the ER to LDs is thought to depend on a further cleavage in the C-terminal region mediated by a host signal peptide peptidase, which results in the mature form of core protein [24].

E1and E2 are the viral envelope glycoproteins that mediate viral attachment and entry into host cells. They are both type I transmembrane (TM) proteins with large N-terminal ectodomains and almost no cytoplasmic tail, are heavily glycosylated and are stabilised by several disulfide bonds [25,26]. The C-termini of the core and E1 proteins contain signal sequences in their TM domains, which mediate translocation of E1 and E2 into the ER lumen [27], forming two hairpin structures spanning the membrane. After cleavage at the core-E1, E1-E2 and E2-p7 junctions through a host signal peptidase in the ER, the C-termini are reoriented towards the cytosol. After cleavage and correct orientation, the glycoproteins E1 and E2 accumulate within the ER owing to the presence of ER retention signals in the TM domains [28,29]. Correct folding of E1 and E2 is aided by the ER chaperone calnexin [30], although another

report suggests that only E1 requires the chaperone activity and E1 is essential for correct folding of E2 [31]. N-linked glycans are also indispensable for proper folding of E1 and E2 [25]. E1 and E2 are mainly retained on ER membranes until the HCV particle is assembled and are incorporated into the virion membrane as virus particles bud into the ER lumen. While E1E2 assemble as non-covalent heterodimers and were thought to be present as such on mature virions [32], a recent report indicates that during maturation E1E2 form covalent heterodimers, which is how they are found on mature virions [33]. E2 contains three hypervariable regions (HVR), of which HVR1 is the best characterised. It seems to be important for evasion of the host antibody response, as agammaglobulinemic patients show hardly any sequence changes in this region over time [34] and deletion of HVR1 results in a highly attenuated virus in the chimpanzee [35].

p7 is a transmembrane protein with two transmembrane domains and belongs to the class of "viroporins" [36], which form pores by oligomerisation and let ions and small molecules through membranes. p7 has been shown to be necessary for infection in the chimpanzee model [37] and a recent study using the HCVcc model system showed that p7 prevents endosomal acidification and is necessary for production of virus [38].

NS2 forms part of the NS2-3 protease that is responsible for cleavage at the NS2-NS3 junction, but its function as a protease in its mature form remains unknown [39]. It is a dimeric cysteine protease and has recently been shown to be essential for virus assembly by providing a scaffold for both structural and non-structural proteins [40,41]. NS3 has an N-terminal protease domain and a C-terminal helicase domain [39]. NS4A is a cofactor required to potentiate the action of the NS3 protease, forming the NS3-4A protease, which is responsible for the cleavage of the polyprotein downstream of NS3. The interaction between NS3 and NS4A is noncovalent and tethers NS3, which does not have a TM domain, to the membrane.

NS4B is a highly hydrophobic TM protein that is thought to be involved in mediating alterations of the ER membrane to generate a "membranous web", where HCV replication

takes place. NS4B has a nucleotide-binding motif and is indispensable for RNA replication [42-44]. NS5A contains an amphipathic alpha-helix that serves as membrane anchor and three other domains [39]. Domain I has an RNA-binding site and may be responsible for delivering RNA to core on lipid droplets. Domains II and III are less well characterised but domain II has been proposed to inhibit the interferon (IFN) response [45]. NS5A interacts with several host factors, and hyper-phosphorylation of NS5A can inhibit this interaction [39]. NS5B is the RNA-dependent RNA polymerase (RdRP) that can initiate *de novo* RNA synthesis *in vitro* and, similar to other polymerases, has a structure containing palm, finger and thumb [39]. NS5B, and all the other non-structural proteins, are candidates for drug targets.

1.1.4 HCV life cycle

1.1.4.1 Attachment and entry

HCV attaches to the surface of host cells, is taken up by clathrin-mediated endocytosis [46] and the virion contents are then transferred to the cytosol by a process involving endosomal acidification [47], which induces conformational rearrangement of HCV glycoproteins and fusion of the virion and endocytic membranes [48]. Entry is a complex process involving sequential interactions of the viral E2 glycoprotein with multiple host cell receptors (Figure 1-2). Some receptors serve as primary attachment factors, but have no real role in HCV entry, while others are specific entry factors.

Interactions between the HCV glycoproteins and heparan sulphate (HS), a glycosaminoglycan (GAG), contribute to primary binding of the virion to host cells [49]. In the serum HCV associates with low-density-lipoprotein (LDL) and very low-density-lipoproteins (VLDL), and the LDL receptor (LDL-R) has also been suggested as a capture molecule [50]. The C-type lectins DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing integrin) and L-SIGN (liver/lymph node-specific intercellular adhesion molecule 3-grabbing integrin) have also been shown to function as capture receptors and mediate transinfection [51-53]. After

this initial engagement, interaction with specific HCV entry factors occurs. There are four receptors that are absolutely required for HCV entry into hepatocytes. These are CD81 [54], the scavenger receptor B type 1 (SR-BI) [55], occludin (OCLN) [56,57] and claudin-1 (CLDN-1)[58].

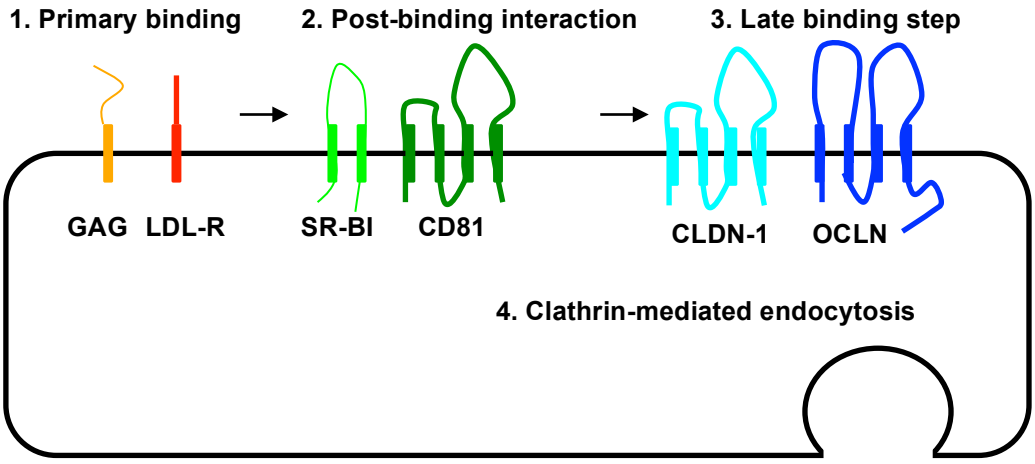

Figure 1-2 HCV entry requires several receptors

HCV entry is a multi-step process involving 1) binding to primary receptors, 2) binding to specific early entry receptors, 3) binding to specific late entry receptors and finally 4) clathrin-mediated endocytosis.

The tetraspanin CD81 was the first HCV receptor to be discovered [54]. Tetraspanins are involved in the organisation networks at the cell membrane but do not have any intrinsic enzymatic activity and are thought to act primarily as adaptor proteins. CD81 has a small and a large extracellular loop (SEL and LEL), and the LEL is responsible for binding to E2 [54]. The CD81-binding site is made up of several residues on both domain I and III of E2 [26,59,60].

The next receptor discovered was SR-BI, which is a scavenger receptor involved in lipid metabolism [55] and binds to HVR1 on E2. Its natural ligands include oxidised LDL and high density lipoprotein (HDL), from which it selectively mediates uptake of cholesterol [61] but it has also been shown to be involved in the uptake of bacteria [62]. Surprisingly, whereas oxidised LDL inhibits HCV infection [63], HDL has been shown to enhance HCV infection [64].

The two tight junction proteins, CLDN-1 and OCLN, were only recently discovered as the missing two HCV receptors [56,57,58]. Ectopic expression of CLDN-1 was shown to confer susceptibility to HCV infection in non-permissive 293T cells [58] and it has been shown that

CLDN-1 can also be substituted by CLDN-6 or CLDN -9 [65,66]. Ploss *et al.* showed that OCLN expression rendered murine cell lines (that had previously been transfected with human CD81, SR-BI and CLDN-1) susceptible to HCV infection [56]. Furthermore, this study showed that only CD81 and OCLN needed to be of human origin for infection to occur, although murine SR-BI and CLDN-1 functioned less well than their human counterparts in mediating HCV entry.

CD81 and SR-BI are thought to be involved in an early stage of the entry process [67,68], whereas CLDN-1 and OCLN seem to be involved in late steps of HCV entry [58,69], suggesting similarities with Coxsackievirus B (CVB) entry into cells. CVB binds its primary receptor (decay accelerating factor) on the luminal cell surface of an epithelial cell, then migrates to the tight junctions, where it interacts with the tight junction protein coxsackievirus and adenovirus receptor (CAR) and OCLN, followed by uptake into the cell [70].

The actual fusion process has still not been deciphered and there have been reports suggesting either E1 or E2 as the class II fusion proteins [71,26]. Reports also showed that mutation of the proposed class II fusion peptide in E1 did not affect fusion [72,73], but that certain residues rather than a fusion domain on E1 might be important for fusion [73]. It seems HCV fusion might be a complex process involving both E1 and E2 in the fusion process, while the actual fusion peptide might be on E2 [26,73,74].

HCV might also be able to travel directly from infected cells to neighbouring cells, bypassing the normal entry process. A study by Witteveldt *et al.* showed that CD81 is not needed for cell-to-cell transmission [75], which may help explain why neutralising antibodies are not able to eliminate HCV from the system.

1.1.4.2 Replication

After HCV entry and translation of the 10 proteins, NS4B forms the membranous web, which contains ER membranes and LDs [76]. Within this structure the replication complex is formed, which comprises many NS proteins and HCV RNA. The viral genome is amplified by the

action of NS5B, which first creates a negative strand template from which more positive strand RNA genomes are transcribed. All NS proteins play a role (some yet to be elucidated) in the replication process (with the exception of NS2) and various host factors have also been implicated in this process. Cyclophilin A and B, for example, have been shown to enhance RNA replication [77,78]. Similarly, miR-122, a highly expressed microRNA in the liver, has been shown to be very important for HCV replication [79], making the host environment within the cell an important factor for successful propagation of the virus.

1.1.4.3 Assembly and release

As the HCVcc culture system, which enables study of particle assembly, was only developed relatively recently, this is probably the least well-understood part of the HCV life cycle. Nearly all the HCV proteins (with the exception of NS4A and NS5B) have been implicated in the formation of infectious virions. Moreover, HCV is associated with (V)LDL *in vivo*, and it is not fully understood how this association comes about.

In a recent review, Jones and McLauchlan suggest a three-step process of HCV virion assembly [80]. According to this model, the initial phase of assembly occurs on LDs and is mediated by the action of core and NS5A, which might be involved in trafficking the HCV RNA from the RC to the LD. In a second step, other HCV proteins, such as p7, NS2, NS3 and NS4B, contribute to the assembly process, although the mechanisms, by which they mediate their activity, still remain elusive. In the third step, maturation and release of viral particles is closely coupled to the (V)LDL synthesis pathway. After assembly of lipo-viro-particles (LVPs) on ER membranes they leave the cell via the exocytic pathway and are released non-cytopathically. One factor complicating the understanding of this process is that hepatocellular carcinoma cell lines have an altered lipid metabolism and thus do not reflect what is happening in the infected liver. It will be interesting to see exactly how particle assembly proceeds to produce the LVPs observed *in vivo*.

1.2 Models used to study HCV

Until recently, it was not possible to grow HCV *in vitro* and the lack of an undemanding animal model made research on this virus very difficult. Initial attempts to study the virus used plasma-derived HCV from patients [81,82]. When it became clear that plasma-derived HCV could not simply be grown to high titres *in vitro*, alternative models were developed. This chapter focuses on soluble E2 (sE2) [54] or E2 ectodomain (E2e) [26], HCV-like particles (HCV-LPs) [83], HCV pseudoparticles (HCVpp) [9,10], replicons [8], HCVcc [11,12] and several HCV infection systems including liver cell lines and animal models. In addition to all the models used, several molecular clones have been made over the past two decades. Some important examples are summarised in Table 1-1.

HCV isolate	Genotype	Reference
H77	1a	Kolykhalov *et al.* (1997)[6]; Yanagi *et al.* (1997)[7]
Con1	1b	Lohmann *et al.* (1999)[8]
HC-J6(CH)	2a	Yanagi *et al.* (1999)[84]
JFH-1	2a	Kato *et al.* (2001)[85]

Table 1-1 Examples of HCV molecular clones

1.2.1 sE2 and E2e

sE2 is a truncated version of E2 that lacks the TM domain and is produced by transfecting HEK 293T cells and collecting cell-associated sE2 protein or supernatants containing secreted sE2. There are considerable differences between the intracellular and secreted forms of sE2, one example being that intracellular sE2 (I-E2) binds CD81 better than extracellular sE2 [86]. The use of sE2 led to the discovery of two HCV receptors, CD81 [54] and SR-BI [55], and other binding partners, i.e. HS proteoglycans[49] and DC-SIGN/L-SIGN [53]. In addition to hepatocytes, sE2 was shown to bind to human T and B cell lines and peripheral blood mononuclear cells (PBMCs) [87,88]. Disadvantages of using sE2 include the fact that it can only be used for analysis of binding to receptors and antibodies; also E1E2 is expressed as

heterodimer on the virion and hence the binding properties of sE2 may not reflect those of E2 on the virion surface. The crystal structure of E2 has not yet been solved, but Krey *et al.* recently proposed a model for the tertiary organisation of E2 [26]. In this study they used a new construct, which contains full-length E1 to ensure proper folding of E2e in the ER, and E2 lacking its transmembrane domain (Figure 1-3). The construct was transfected into insect cells and the resulting glycosylation pattern made E2 more uniform and easier to study in crystallography studies. Their model features domain I, domain II (containing the putative fusion loop), a linker, domain III and a stem region.

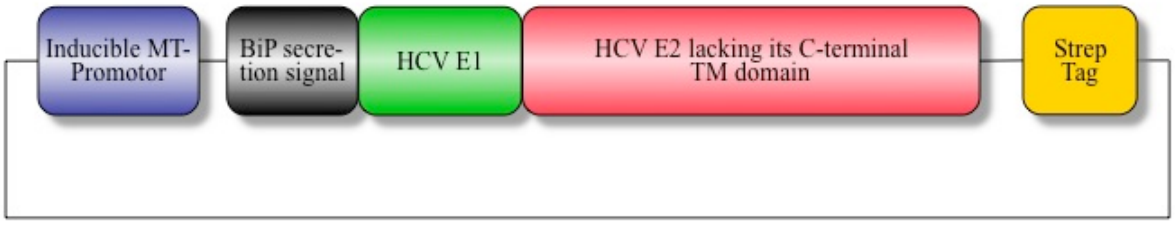

Figure 1-3 Construct for production of E2e

The construct contains an inducible metallothionein (MT) promoter, a binding immunoglobulin protein (BiP) secretion signal, HCV E1, HCV E2e and a Strep tag. The presence of E1 enables correct folding of E2, which is then secreted into the supernatant.

1.2.2 HCV-LPs

HCV-LPs are produced in insect cell cultures using a baculovirus expression system [83]. In insect cells, HCV structural proteins assemble into HCV-LPs, with a diameter of 40-60nm and biophysical properties similar to those of native HCV particles. Using this model, the asialoglycoprotein receptor was identified as a putative HCV receptor [89]. These were the first particles that enabled study of interactions with the E1E2 heterodimer, but disadvantages of this model are that the glycosylation pattern in insect cells does not mimic the mammalian pattern; also, the particles are assembled intracellularly, which makes preparation and purification challenging; and the particles can only be used for attachment studies as they lack a reporter gene to enable evaluation of entry into host cells.

1.2.3 Replicons

Subgenomic replicons only contain the genes for non-structural proteins [8], whereas full-length replicons contain all genes [90,91]. In the replicon systems, RNA is *in vitro* transcribed from the replicon construct and transfected into Huh7 cells. Once the RNA is translated, the successfully transfected cells can be selected by addition of G418. This system enables study of HCV replication, translation and drug testing, but is not useful for study of attachment/entry or assembly/release, as no mature virions are released from the cell.

1.2.4 HCVpp

In 2003 two groups published the development of a robust HCV pseudoparticle system [9,10], enabling study of the HCV entry process. HCVpp have retrovirus (derived from murine leukemia virus (MLV) [9]) or lentivirus (a subclass of retroviruses, derived from human immunodeficiency virus (HIV) [10]) cores and carry the HCV glycoproteins E1 and E2 on the surface and a reporter RNA gene, such as green fluorescent protein (GFP) (Figure 1-4) or luciferase. They are produced by co-transfection of HEK 293T cells with three plasmids, one encoding the glycoproteins, one encoding the lentiviral or retroviral core and polymerase and one encoding the reporter gene flanked by long terminal repeats (LTRs). It was initially thought that pseudotyped retroviruses assemble at the plasma membrane, but subsequent studies showed that the site of virion assembly depended on the glycoprotein rather than the retroviral core [92]. For HCVpp, this process is proposed to occur in late endosomes [92,93]. HCVpp are infectious in both primary hepatocytes and liver cell lines and can be used to study HCV entry, tropism and neutralising antibodies [9,10]. The disadvantage of this system is that it can only be used to evaluate the early entry steps of the HCV life cycle.

1.2.5 HCVcc

In 2005, two different groups described systems for achieving complete replication of HCV in cell culture [11,12]. In these systems there were not only high levels of replication intracellularly,

but also high infectious titres of HCV virions were produced. HCVcc was shown to be infectious in Huh7 cells, chimpanzees [11,12] and urokinase-type plasminogen activator $(uPA)^{+/+}$-transgenic severe combined immunodeficiency (SCID) mice [94]. Moreover, HCVcc was also neutralised by patient-derived antibodies, was sensitive to IFN-α and other HCV-specific drugs, and infection was inhibited by addition of anti-E2 and anti-CD81 antibodies [11,12]. Originally, JFH-1 [11] and two JFH-1 chimeras (J6/JFH-1 and H77/JFH-1) [11,12] were used for production of HCVcc, but only gt 2a viruses (JFH-1 and J6/JFH-1) yielded high infectious titres. Several attempts have been made to construct HCVcc from other gt [95,96] or JFH-1 chimeras containing the structural proteins from other strains [97], but none of these have resulted in similarly high-titre virus. The HCVcc culture system has many advantages, but it is still questionable whether HCVcc represents "real HCV", as HCVcc differs in density compared to clinical isolates due to its differential association with lipoproteins.

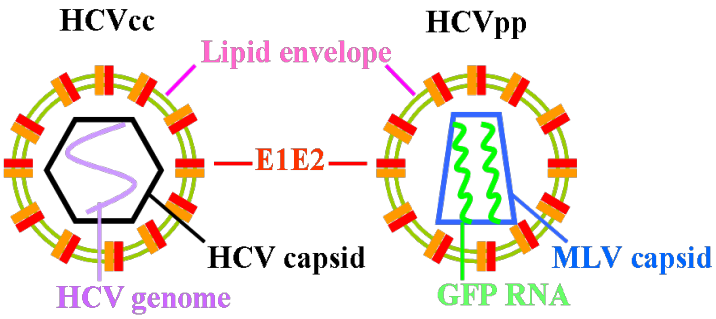

Figure 1-4 Diagram of components in HCVcc and HCVpp

HCVcc particles composed of a positive strand HCV RNA genome, a HCV capsid and a lipid envelope containing E1E2. HCVpp contain two copies of positive strand GFP RNA with an attached reversed transcriptase originating from MLV, a MLV capsid and a lipid envelope containing E1E2.

1.2.6 HCV infection systems

1.2.6.1 *In vitro* infection systems

HCV primarily infects hepatocytes in the liver, so any *in vitro* cell infection system should ideally mimic or use the primary human hepatocyte (PHH) as it is found *in vivo*. Due to the

difficulties with maintaining PHHs in cell culture, dedifferentiated liver cell lines (derived from hepatocellular carcinomas) have most frequently been used to study HCV biology. In contrast to PHHs, these liver cell lines form thick layers, lack polarity and have other abnormalities [98]. Most studies with HCV have been carried out using the Huh7 cell line, or "cured" Huh7 cell lines, such as Huh7.5, Huh7.5.1 or Huh7-Lunet cells. Huh7.5 cells were made by treating replicon-infected Huh7 cells with IFN-α for a prolonged period and then selecting the surviving cells and testing them for HCV replicon permissiveness [99]. The Huh7.5 cell line has a defect in retinoic acid inducible gene I (RIG-I), a protein involved in detection of double-stranded RNA (dsRNA) motifs, which may be responsible for its high permissiveness [100]. More recently some attempts have been made to create immortalised human hepatocytes [98], which have the characteristics of healthy PHHs, and a PHH co-culture system has been developed by Ploss *et al.*, in which they used stromal cells to sustain the PHHs [micro-patterned co-cultures (MPCC)] [101]. In this system, MPCC can be cultured for up to two weeks, but the infection efficiency remains low.

1.2.6.2 *In vivo* infection systems

In 1997, it was published that chimpanzees could be inoculated intrahepatically with HCV RNA derived from the H77 isolate that produced HCV infection and disease progression [6,7]. The course of infection in chimpanzees is somewhat different to that observed in humans, for example, the clearance rates in chimpanzees are much higher than in humans [102]. This model has been invaluable for investigation of key questions in the HCV field, in particular for analysis of the immune response to HCV, though ethical concerns, limited availability and high maintenance costs have restricted the use of chimpanzees. Tree shrews might represent an alternative immunocompetent small animal model to study HCV infection [103].

A more recent development was the production of small animal models for the study of HCV infection, which involved the use of immunocompromised mice reconstituted with human liver [104,105,106]. The uPA$^{+/+}$-SCID mouse has become the most widely used mouse model. The

overexpression of the uPA transgene leads to extensive liver damage in this mouse [105]. The injured liver is then repopulated by human hepatocyte precursors, which are injected into the spleen and travel to the liver via the splenic and portal vein. The uPA$^{+/+}$-SCID mouse supports infection with clinical isolates [105] and HCVcc [94] and has been particularly useful for evaluation of the efficacy of neutralising antibodies and antiviral drugs [107]. The main problem with this mouse model is that it does not contain a functional immune system. A recent study by Washburn *et al.* reported transplantation of human hematopoietic stem cells (HSCs) into a similar mouse model [108], which enables study of the HCV-specific immune response.

A common problem with all of these mouse models is that their generation is challenging due to the need for transplanting human hepatocytes and HSCs. Very recently a humanised mouse model was reported that expresses human HCV entry factors and supports HCV replication of several genotypes [109].

1.3 Pathogenesis of HCV infection

1.3.1 Transmission

HCV is mainly transmitted via the parenteral route. Transmission can occur by transfusion of unscreened blood or blood products or by cross-contaminated equipment, such as inadequately sterilised non-disposable medical equipment and during procedures such as tattooing, acupuncture, piercing or injecting drug use [110]. In the developed world, it is now common procedure to screen blood products for HCV and to use disposable or sterilised medical equipment, so HCV is mainly transmitted by sharing of injecting devices amongst injecting drug users (IDUs) [111]. In the developing world, inadequate health standards are still the main cause of HCV infection. Two noteworthy cases of HCV transmission are the infection of >2500 Irish women by contaminated anti-D immunoglobulin [112] and infection of 10-20% of the Egyptian population during mass treatment with contaminated parenteral anti-schistosomal therapy [113], leading to very high prevalence of gt 4a in this country.

1.3.2 Course of infection

The early course of HCV infection has been studied using the chimpanzee model and also in post-transfusion and post-needle-stick accident cases [19]. After infection, HCV RNA levels can be detected in the serum after 1 to 2 weeks. RNA levels rise rapidly reaching their peak at 10^5 to 10^7 international units (IU)/ml at approximately 6 weeks post infection. Serum alanine aminotransferase (ALT) levels start rising with a corresponding lag phase, indicating liver damage. At their peak, they are about 10 times greater than normal. Only a third of acutely infected patients develop clinical symptoms and jaundice. After a period of acute infection (about 12 weeks), HCV RNA and ALT levels drop and the virus is either cleared or persists and establishes chronic infection.

Chronic infection is described as the persistence of HCV RNA for at least 6 months after infection. The early stages of chronic infection are similar to those of self-limiting acute infections with respect to HCV RNA and ALT levels. During the transition of acute to chronic infection, HCV RNA and ALT levels can fluctuate markedly, but they stabilise once chronic infection is established. In a minority of chronic cases, spontaneous loss of HCV RNA has been documented in the absence of therapy. Chronic hepatitis C infection is not very symptomatic, the most common symptom being fatigue, but liver ache, nausea, and poor appetite do occur in some patients. The chronicity rate of hepatitis C ranges from 55% to 85%, depending on the study population.

1.3.3 Tropism

The primary site of infection is the liver, where HCV replicates in hepatocytes [114], consistent with the observation that hepatocytes express all four of the main HCV receptors, CD81, SR-BI, CDLN-1 and OCLN [115,116]. *In vitro*, a few hepatoma cell lines are susceptible to HCV, such as Huh7 (and its clones), Hep3B and PLC/PRF/5 [117]. HCV has also been detected in extrahepatic compartments, such as PBMCs [118] and the central nervous system (CNS) [119-121], but it is unclear whether productive infection can take place in these cells.

There is some controversy in the literature as to whether HCV can infect PBMCs. While some studies have not been able to detect any HCV RNA in PBMCs [122], several PBMC subsets have been reported to be associated with HCV RNA or proteins *in vivo*, such as neutrophils [123], dendritic cells (DCs) [124], monocyte-derived DCs (MDDCs) [125], T cells and B cells [126], although the levels of RNA or protein reported are very low. While the presence of HCV RNA or protein does not necessarily indicate productive infection of these cells, as this could simply be due to uptake of viral particles or parts of cells with on-going HCV replication, several studies demonstrate upregulation of HCV RNA upon mitogenic stimulation [126]. Furthermore, the use of the thermostable rTth enzyme and strand-specific real-time PCR circumvent the problem of false priming of the negative RNA strand [122,127].

Also, several reports show *de novo* infection with plasma-derived HCV of monocytes, MDDCs and macrophages [128-130] and T cells [131] and the establishment of B cell lines from HCV-infected patients that continuously release HCV [132,133]. One of these B cell lines (SB), isolated from the spleen of a HCV-infected patient with a monocytoid B-cell lymphoma, has been used to generate SB-HCV [132]. These SB cells continuously release SB-HCV, which can be used to study infection of leukocytes. SB-HCV can infect PBMCs and Raji cells *in vitro* and has been used for several studies to evaluate direct effects of HCV on T cells [132,134,135].

Some studies have used HCVpp and HCVcc model systems to study HCV tropism. Firstly, there are a few papers in the literature examining infection of hematopoietic cell lines with retroviral [9,117] and lentiviral HCVpp [136] and one study where lentiviral HCVpp were used to infect PBMCs [136], which all showed that PBMCs could not be infected by HCVpp. However one report in the literature showed that vesicular stomatitis virus (VSV) pseudotyped with HCV glycoproteins could enter MDDC, but not plasmacytoid DCs (pDCs), through interaction with lectins [137]. Furthermore, there are two more recent studies that used the HCVcc system. Marukian *et al.* showed that HCVcc did not infect PBMC, MDDCs or macrophages [138] and Murakami *et al.* showed that HCVcc did not infect T- or B-cell lines [139].

1.3.4 HCV-associated diseases

1.3.4.1 Hepatic diseases

HCV infection is the main cause of chronic liver diseases. About 20% of the affected subjects develop chronic liver inflammation that can progress to fibrosis, liver cirrhosis and hepatocellular carcinoma [140], which leads to the need for a liver transplant (LT). Since HCV is non-cytopathic virus, liver damage is immune-mediated. The time from initial infection with HCV to the development of liver cirrhosis is estimated to be around 30 years on average [141]. High age at infection, alcohol consumption, male sex and high body mass index are important factors contributing to fibrosis progression. Once liver cirrhosis has developed, the disease can advance to hepatic decompensation with portal hypertension and, in some cases, the additional complication of hepatocellular carcinoma develops [142].

HCV is the most common indicator (40-50%) for liver transplantation [143]. In the UK alone, there were 668 liver transplantations from deceased donors in the NHS financial year 2010/2011 [144]. HCV recurrence in HCV+ transplant patients is practically universal, as treatment for HCV is stopped in patients with end-stage liver disease due to severe side affects and mortality [145]. Reinfection occurs during reperfusion of the liver graft and attempts to prevent this by administration of hepatitis C antibody enriched immune globulin prior to transplantation have been unsuccessful [146]. Reperfusion of the graft lowers viral titres in serum, probably because the uninfected graft removes the virus from the circulation, and viral titres reach pre-transplant levels at around 3 days post transplantation [143]. Due to the immunosuppressive treatment regime used to protect the host from graft rejection (normally a calcineurin inhibitor and gradually declining doses of corticosteroids), viral titres continue to increase thereafter and reach higher levels than prior transplant. HCV recurrence is thus accelerated in the new graft, leading to cirrhosis in 20% of patients in 5 years and 50% of patients in 10 years [147].

1.3.4.2 Extra-hepatic manifestations

Apart from the liver-specific complications, HCV infection also manifests itself in extrahepatic diseases [148]. Associations include lymphoproliferative disorders, skin diseases and many autoimmune disorders, the most common one being mixed cryoglubulinemia. The link between HCV and disorders involving B cells is interesting. Firstly, B cell lines from HCV-infected patients with B cell lymphomas have been established that continuously release HCV [133,132], indicating a possible link between HCV infection of B cells and lymphoma. Furthermore Machida *et al.* found that HCV can induce mutations in immunoglobulins and proto-oncogenes and that this mechanism involves binding of E2 to CD81 [149,150]. Further studies have proposed that after engagement of CD81 with HCV E2 protein B cells may be polyclonally activated [151] and protected from Fas-mediated apoptosis [152]. Furthermore, it has been shown that mice, which express the full HCV genome in their B cells, develop B cell lymphomas [153].

1.3.5 Treatment

The current treatment for HCV consists of pegylated IFN-α (peg-IFN-α), which has a longer half-life than normal IFN-α, and ribavirin [154]. With the two-component therapy individuals infected with gt 2 and 3 achieve the highest rate (more than 75%) of sustained virologic response (SVR), whereas individuals infected with gt 1 and 4 viruses respond less well, with up to 50% and 55% showing a SVR, respectively [155,156]. Unfortunately, the therapy has considerable side effects, mainly induced by IFN-α, such as fatigue, depression and flu-like symptoms. These adverse effects, together with the fact that there are still many non-responders, necessitate the development of other drugs. Currently the use of peg-IFN-λ is being explored [157], as IFN-λ is supposed to have similar effects as IFN-α, but with a milder and more long-lasting course of action [158].

There are several approaches for drug development, including the use of virus-specific or host-specific targets. The latter approach is to inhibit (or activate) host factors, such as HCV receptors or other molecules involved in the virus life cycle. A promising study showed that inhibition of CD81 by prophylactic anti-CD81 antibody administration inhibited HCV infection in the uPA$^{+/+}$-SCID mouse [159]. This could be especially important in the liver transplant setting to prevent reinfection of the graft. On the virus side, the most common drug targets are the RNA polymerase NS5B and the protease NS3-4A [154]. There are many different drugs at various stages of clinical trials in the pipeline, and two of them, Boceprevir and Telaprevir, both NS3 inhibitors, have just been approved in the therapy of HCV [160-163], making way for a triple therapy in the treatment of HCV infection.

1.4 The immune response to HCV

Antiviral immunity involves the coordination between the innate and the adaptive immune system. The innate immune system typically plays a key role in control of viral replication and dissemination at the early stage of infection. It also activates the adaptive immune system, tailoring the nature of the adaptive response to provide optimal pathogen control.

1.4.1 Innate immunity

The innate immune response is the first line of defence against invading microorganisms and innate immune responses activated in early infection may be critical in determining the subsequent disease outcome. The classic characteristics of the innate immune system are that it recognises pathogen patterns, responds swiftly and possesses no memory. While almost every cell in the body has a certain set of pattern recognition receptors (PRRs) that enable it to respond appropriately to pathogens in the vicinity (extracellular or intracellular), there are also specialised cells of the innate immune system that are capable of detecting and responding to pathogens in additional ways. These include granulocytes (neutrophils, basophils and eosinophils), mast cells, monocytes, macrophages, DCs, natural killer (NK)

cells and natural killer T (NKT) cells. These cells specialise in different effector functions, such as phagocytosis, antigen presentation, antibody-dependent cell-mediated cytotoxicity (ADCC), secretion of soluble factors and killing of infected cells. In this introduction, PRRs and type I IFN induction, DCs and NK cells and their role in HCV infection will be discussed in more detail.

1.4.1.1 Pattern recognition receptors and type I interferon induction

Pathogen-associated molecular patterns (PAMP) are components of foreign microorganisms that are recognised by PRRs. There are secreted PRRs such as complement proteins or mannose-binding protein (MBP) that serve to opsonise microorganisms or infected cells [164]. There are also membrane-spanning PRRs, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and cytosolic receptors, including RIG-I-like receptors (RLRs) and nod-like receptors (NLRs), and engagement of these PRRs results in the activation of signalling pathways that lead to transcription of type I IFN and proinflammatory cytokines.

Type I interferons (the best-characterised types of which are the IFN-α subtypes and IFN-β) are important cytokines in viral infections, having a direct anti-viral effect on virus-infected cells, including HCV-infected cells [165,166], and also modulating the activation of innate and adaptive immune cell subsets. Type III interferons are IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). Like IFN-α, IFN-λ can also inhibit HCV replication *in vitro* [158,167,168]. Furthermore, IL-28B has received a lot of attention in HCV infection lately. In genome-wide association studies two SNPs upstream of the *IL-28B* gene have been shown to be predictors of both spontaneous clearance and SVR after treatment [169,170].

Upon binding of type I (and III) IFN to its receptor (IFNAR), a JAK-STAT signalling pathway is initiated, which results in transcription of many ISGs, which have antiviral and immunomodulatory functions [171]. Many of these, including protein kinase R (PKR), the RNA-specific adenosine deaminase (ADAR-1), RnaseL, 2'-5' oligoadenylate synthetase (2-5 OAS),

MxA and p56, have been shown to have anti-HCV activity and certain polymorphisms in these genes have been shown to be associated with disease outcome [172].

HCV has a +ssRNA genome, and dsRNA replicative intermediates are formed during the virus replication process. Both ssRNA and dsRNA have the capacity to act as PAMP. The role of RLR- and TLR-mediated triggering of type I IFN production following recognition of viral components is discussed below.

RIG-I and melanoma differentiation-associated gene 5 (MDA5) are both members of the RLR family, which have been shown to recognise dsRNA and 5' triphosphate uncapped RNA ends in the cytosol. Interestingly, RIG-I and MDA5 have different viral genome ligands. MDA5 has been shown to recognise picornaviruses, whereas RIG-I has been shown to recognise orthomyxoviruses, rhabdoviruses and flaviviruses [173]. Recent studies show that HCV RNA is also recognised by RIG-I and that this interaction initiates an antiviral state in both murine and human cells [100,174]. These studies identified the 3'UTR and to a lesser extent the 5'UTR as the PAMP for RIG-I.

RIG-I and MDA5 signal through CARD adaptor inducing IFN-β (CARDIF), also known as IPS-1, MAVS or VISA, which activates TANK-binding kinase 1 (TBK-1), leading to phosphorylation of interferon regulatory factor 3 (IRF3) and its translocation to the nucleus [166], where IFN-β transcription is initiated. CARDIF also interacts with TNF receptor associated factor 6 (TRAF6), leading to transcription of inflammatory cytokines via nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways.

In contrast to the cytosolic RLRs, TLRs reside on membranes, with their pattern recognition domain oriented away from the cytosol. Four TLRs are important for the recognition of nucleic acids: TLR9 recognises CpG-DNA, whereas TLR3 recognises dsRNA and TLR7 and TLR8 recognise ssRNA. In contrast to other TLRs, which are expressed on the plasma membrane, these TLRs reside on intracellular membranes. They are found on membranes of the ER in an inactive state and are recruited to endosomes/lysosomes upon inflammation

(caused for example by viral infection) [164]. Endolysosomes are the final compartments of the endocytic pathway, where endocytosed or phagocytosed material (including viruses) can be degraded. In cells where autophagy occurs, endolysosomes can also fuse with autophagosomes, which are double-membraned vesicles that contain cellular (and viral) components from within the cell. Once in the endosomal pathway, TLRs reach their mature state and can be activated by interaction with viral nucleic acids.

TLR3 recognises dsRNA, including both the genome of dsRNA viruses, such as reoviruses, and the dsRNA replicative intermediates of ssRNA viruses, such as respiratory syncytial virus (RSV), encephalomyocarditis virus and West Nile virus [175]. Once TLR3 has bound dsRNA, it signals through the adaptor molecule TIR domain-containing adaptor inducing IFN-β (TRIF). TRIF then interacts with TRAF6 leading to transcription of proinflammatory cytokines via NF-κB and MAPK pathways, and with TBK-1, which in results in IFN-β transcription by IRF3. RIG-I and TLR3 therefore share a common signalling pathway.

TLR3 might be important for sensing HCV and establishing an antiviral state in hepatocytes. Wang *et al.* demonstrated that TLR3 is expressed in primary human hepatocytes but not in Huh7 cells [176]. Furthermore partial containment of HCV replication was achieved upon exogenous TLR3 expression in Huh7 cells, arguing for a role for TLR3 in HCV recognition. As dsRNA only exists as a replicative intermediate within the cytoplasm of HCV-infected cells, this suggests that autophagy may be involved in delivery of HCV RNA to TLR3-containing endolysosomes in Huh7 cells. Apart from hepatocytes, TLR3 is also expressed in myeloid DCs (mDCs) and Kupffer cells (hepatic macrophages) in the liver [177]. TLR3 might be engaged in these cells after phagocytosis of HCV-infected hepatocytes or parts of these cells.

Another candidate for HCV nucleic acid recognition is TLR7/8, which recognises G/U-rich ssRNA [146]. TLR7/8 has been shown to detect several other ssRNA viruses, including VSV, influenza virus, HIV-1 and DV [178-181]. In humans, TLR7 is expressed by pDCs and mDCs and

TLR8 by monocytes and mDCs [182], and TLR7 is expressed by hepatocytes [183]. TLR7/8 and TLR9 (and all other TLRs apart from TLR3) signal through MyD88, a key molecule that relays the signal to several pathways. MyD88 interacts with members of the IRAK family and with TRAF6, which in turn directly or indirectly result in activation of NF-κB and IRF7, which mediate transcription of proinflammatory cytokines and type I IFN, respectively [166]. Interestingly, TLR7 has recently also been shown to recognise HCV sequences [184]. In this study, the authors showed that full-length HCV RNA and certain HCV RNA oligonucleotides (in the 3' and 5' regions) but not HCVcc could trigger IFN-α production in pDCs. As HCV is primarily a hepatotropic virus, a type I IFN response might be localised mainly to the liver. Several studies in the chimpanzee show that changes in intrahepatic ISGs occur as early as 2 days after infection [185-187]. Furthermore, studies showed that resolving infections are characterised by high hepatic ISG expression [186,188]. Furthermore, Takahashi *et al.* showed that pDCs could produce high IFN-α levels after contact with infected hepatocyte cell lines, but not after contact with virions [189]. This IFN-α production was TLR7-dependent and necessitated cell contact and active viral replication, suggesting a novel cell-to-cell RNA transfer mechanism.

1.4.1.2 Dendritic cells

DCs are professional antigen presenting cells (APCs) that provide an important link between the innate and adaptive immune response [190]. Upon activation, they upregulate costimulatory molecules and can mediate antiviral activity by secretion of type I IFN and proinflammatory cytokines, which have both direct and indirect effects. Once activated, DCs migrate to the lymph nodes, where they are able to activate naive T cells (DCs are the only type of APC capable of doing this) and thereby initiate the adaptive immune response.

There are multiple types of DCs. Langerhans cells and interstitial DCs are long-lived cells that populate the skin and other peripheral tissues, respectively. Three further types of DCs

circulate as immature cells in the blood: Blood dendritic cell antigen (BDCA)2+BDCA4+CD123^{high} pDCs and the conventional DCs (cDC), BDCA1+CD11c+CD123^{low} mDCs and BDCA3+DNGR-1+ DCs [191-193]. All DC types are present at a very low frequency in peripheral blood: 0.2-0.5% for pDCs, 0.5-1% for mDCs and even lower frequencies for BDCA3+DCs. Although peripheral blood DC subsets have some overlapping roles, their primary functions differ.

pDCs make all types of type I (besides IFN-κ) and type III IFN [194] and 95% of the total type I IFN produced by PBMCs is produced by pDCs [195]. Due to their constitutive expression of IRF7 pDCs are specialised for production of large amounts of IFN-α, 100-1000 times more than that produced by other cell types [196] and importantly, do not themselves need to be infected for the production of IFN-α to be triggered [197]. pDCs have also been shown to present antigens to CD4 T cells [198] and to cross-present antigens to CD8 T cells [199], but their APC activity is less potent than that of conventional DC subsets. pDCs express TLR7 and TLR9 [200], the former of which is able to recognise HCV RNA [184]. Interestingly, pDCs do not or only produce very small amounts of IFN-α after they contact HCVcc [189,201-203], but produce IFN-α when directly transfected with HCV RNA or after contact with HCV-infected Huh7.5.1 cells [189]. Notably, recent reports indicate that acute HCV infection in humans and chimpanzees is not associated with an increase in systemic levels of IFN-α [204,183], suggesting that the interaction of pDCs with HCV virions in the blood does not result in IFN-α production *in vivo*.

cDCs take up exogenous antigens and prime naive CD4 and CD8 T cells by cognate interaction of antigen on major histocompatibility complex (MHC) class II and class I molecules with the T cell receptor (TCR), respectively. The process of presenting exogenous antigens on MHC class I molecules is referred to as cross-presentation and this process is mainly carried out by BDCA3+DCs [192]. mDCs are typically involved in classical antigen presentation to CD4 T cells, via MHC class II. They also produce IL-12, IL-15, IL-18 and

some type I IFN, which activate NK cells, and promote differentiation of naive CD4 T cells into T helper 1 (Th1) cells and naive CD8 T cells into cytotoxic T lymphocytes (CTLs) [190, 205]. mDCs express TLR3 and TLR7/8 [205], both of which have the potential to recognise HCV RNA. MDDCs are the *in vitro* equivalent of mDCs and are generated by culturing monocytes with GM-CSF and IL-4. Like pDCs, MDDCs are not activated in response to HCVcc *in vitro*. MDDCs do not produce IFN-β and IL-6 and do not mature in response to HCVcc exposure [206]. As seen with pDCs [189], MDDCs were able to mature and produce IFN-β when incubated with HCVcc-infected Huh7.5 cells [206]. This activation was shown to be dependent on TLR3 and to involve the uptake of HCV dsRNA, presumably by phagocytosis of apoptotic vesicles released from infected cells.

1.4.1.3 Natural killer cells

NK cells were originally described as large granular lymphocytes, able to spontaneously kill tumour cells without the need for priming, contrary to what was known about T cells [207]. Today it is known that in addition to killing tumour cells, NK cells also play an important role in combatting infections, including many virus infections, and have important regulatory roles, e.g. mediating 'DC editing', a process where NK cells eliminate immature DCs in lymph nodes to ensure that antigen is presented to T cells by mature DCs [208,209]. NK cells mediate these diverse functions via cell lysis and production of cytokines and chemokines.

NK cells can kill cells by exocytosis of cytotoxic granules containing perforin, a pore-forming molecule, granzymes, a family of serine proteases, and granulysin, a cytolytic protein [210]. All these molecules can activate apoptosis pathways and lead to the death of the target cell. NK cells can also kill cells by engagement of death receptors, including TNF-related apoptosis-inducing factor (TRAIL) and Fas ligand (FasL), with their corresponding ligands, TRAIL receptors and Fas on the target cell. Interestingly, higher expression of TRAIL also correlated with disease resolution in HCV infection [211].

The other main effector function of NK cells is secretion of cytokines and chemokines, especially IFN-γ and TNF-α. These two cytokines influence other cell types, including Th cells, CTLs, B cells and macrophages and create a Th1 environment, essential for the elimination of viruses. In addition, IFN-γ also has a direct antiviral effect on infected cells. This antiviral effect of IFN-γ has also been demonstrated in HCV infection. Studies using both the HCVcc and the replicon model showed that NK-derived IFN-γ inhibited HCV replication [212-214].

It was formerly thought that activation of NK effector functions would be triggered by any cell not expressing self MHC, a mechanism of action originally described as the "missing self" hypothesis [215]. It has now become clear that NK functions are tightly regulated by signals originating from both activating and inhibitory receptors, arguing for more flexibility in effector function than was previously thought.

Inhibitory receptors on NK cells include inhibitory killer cell immunoglobulin-like receptors (KIRs), and NKG2A [216]. Inhibitory KIRs have either two or three extracellular immunoglobulin domains (called 2D or 3D) and have a long (L) intracellular domain [217]. Examples include KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1, KIR3DL2 and KIR3DL3. Inhibitory KIRs engage different HLA class I alleles (HLA-A, B and C) on the target cell and upon recognition of a sufficient quantity of "self" (not allogeneic MHC) submit an inhibitory signal to prevent activation of NK cells [218]. Another important inhibitory receptor is NKG2A. Its ligand is the non-classical MHC molecule HLA-E. CD94/NKG2A binds to the nonclassic MHC molecule HLA-E, which presents the leader peptide of MHC class I molecules on the cell surface.

The finding that certain HLA-C and KIR alleles are associated with enhanced spontaneous clearance of HCV and SVR after treatment, suggests that NK cells may play an important role in HCV infection [219-222]. In all but one study, the protective effect was attributed to expression of KIR2DL3 in combination with its ligand HLA-C1. Interestingly, KIR2DL2 is a stronger

ligand for HLA-C1 than KIR2DL3 [223], raising the possibility that expression of KIR2DL3 could result in less overall inhibition of NK cells, making them more capable of responding to HCV-infected cells. Furthermore, lower expression of NKG2A correlated with disease resolution in a single-source exposure study [224].

Since a lot of viruses and tumour cells downregulate MHC class I expression to avoid detection by CTLs, NK cells are alerted to respond to these cells by release of the inhibitory signals they would normally receive from healthy cells. However, loss of inhibition alone is not sufficient to render NK cells cytotoxic; they also need to be stimulated via activating receptors.

Activating receptors include CD16, the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46, activating KIRs, NKG2C, NKG2D, NKG2E and many others [216]. Activating receptors are triggered following recognition of ligands that are preferentially expressed on infected or transformed target cells. CD16 is a low affinity Fcγ receptor (FcγR) III that binds to the Fc piece of IgG molecules. Once CD16 is crosslinked by interaction with multiple ligands, e.g. on the surface of a virus-infected cell, it becomes a mediator of ADCC. It is the most potent activating NK cell receptor and is able to induce cytotoxicity without the need for engagement of other activating receptors in resting NK cells [216]. It has been shown that resolvers in a single-source exposure expressed more CD16 than progressors [224].

The NCRs are also important mediators of cytotoxicity. The ligands for NCRs are not fully defined yet, but it was shown that NKp44 and NKp46 can bind to the hemagglutinins of influenza and Sendai viruses [225-228], whereas NKp30 can bind to HCMV protein pp65 [229], HLA-B-associated Transcript 3 (BAT-3) expressed on DCs [230] and B7-H6 [231]. Although many more ligands need to be identified, it seems to be a common pattern that NCRs bind to HS [232,233]. NCRs, especially NKp30, seem to play a major role in HCV infection. In a study using IDUs, it was shown that exposed uninfected (EU) individuals had higher levels of NKp30 on their NK cells than exposed infected (EI) individuals at a pre-infection time point

[234]. Furthermore, it was shown that high NKp30 expression on NK cells correlated with more inhibition of HCVcc replication in infected liver cell lines. It remains to be determined how NCRs recognise HCV-infected cells.

NKG2D can bind to proteins that are upregulated in stress situations, such as MHC class I chain-related gene A (MICA), MICB [235] and UL16-binding protein (ULBP) [236] and NKG2C and NKG2E bind to HLA-E. In acute HCV infection, individuals expressed more NKG2D compared to healthy controls [237] and it seems that NKG2C and NKG2D might also be upregulated in CHC infection [278,287].

All of the activating receptors mentioned above apart from NKG2D (which uses DAP10 as an adaptor molecule) signal through immune receptor tyrosine-based activating (ITAM)-containing adaptor molecules, such as FcεRIγ, CD3ζ and DAP12 [238]. Downstream, both ITAM-dependent and ITAM-independent pathways result in activation of many signalling pathways, including MAPK and NF-κB pathways, which lead to triggering of NK cell effector functions.

Resting NK cells, as opposed to cytokine-stimulated cells, are poorly cytotoxic and require stimulation via CD16 or via several activating receptors in order to reach their full cytotoxic potential [239]. NK cells become more cytotoxic upon stimulation by cytokines, including type I IFN, interleukin (IL)-2, IL-12, IL-15 and IL-18. Many of these cytokines are produced by DCs, and DCs play a large role in activating NK cells and vice versa [240].

In humans, NK cells make up 5-20% of cells in peripheral blood and can be divided into two populations, the $CD56^{dim}$ cells and the $CD56^{bright}$ cells. Approximately 90% of NK cells in peripheral blood are $CD56^{dim}$ cells [241]. These cells express KIRs and CD16, are cytotoxic and also produce IFN-γ upon interaction with altered cells. The other ~10% are the $CD56^{bright}$ cells, which are thought to be a more immature NK population. They produce high levels of IFN-γ and other cytokines and mediate important immunoregulatory effects. In contrast to peripheral blood, NK cells make up 50% of the liver's lymphocyte population and may

therefore be very important in containing the replication of hepatotropic viruses including HCV [242].

Despite the proposed role for NK cells in HCV infection, *in vitro* studies showing that NK cells can mediate control of HCV replication remain scarce. This might be due to the fact that there is no autologous culture system available for HCV, necessitating the use of hepatocyte cell lines, which HCVcc and HCV replicons are typically cultured in. One report showed that NK cells could be activated by cells containing HCV replicons and exhibited increased cytotoxicity against these cells compared to uninfected Huh7 cells [243]. Studies by the Ho group did not find specific cytolytic activity directed against HCV replicon-containing cells, but did find that IFN-γ originating from NK cells inhibited HCV replication in the replicon system and the HCVcc system [212,213]. Furthermore, it was shown that high NKp30 expression on NK cells correlated with more inhibition of HCVcc replication in infected liver cell lines [234]. In conclusion, there is some evidence from these studies that NK cells play a role in HCV eradication, possibly via IFN-γ, NKp30 or cytotoxicity, but more *in vitro* studies are needed to address the ability of NK cells to contain HCV replication, the mechanisms by which virus control is effected and the receptor/ligand interactions that regulate NK cell interactions with HCV-infected cells.

1.4.2 Adaptive immunity

The adaptive immune system consists of T and B lymphocytes, which typically reach their peak action 1-2 weeks after infection with a pathogen. In contrast to most cells of the innate immune system, T and B lymphocytes are able to mount an antigen-specific immune response, generating a memory repertoire capable of mediating a more rapid and effective response in a secondary infection. In HCV infection, HCV-specific antibodies typically start to be detected between 4 and 14 weeks post infection [244,245], and HCV-specific T cell responses after 5 to 9 weeks [246], showing a very delayed adaptive immune response.

1.4.2.1 T cells

T cells comprise an essential part of the adaptive immune system and are crucial to the elimination of viral infections. They are specifically activated via the TCR, which, together with its co-receptor (CD4 or CD8), binds to a complex consisting of a MHC class I or II molecule and a peptide. Upon specific interaction with DCs, naive T cells become activated and differentiate into T helper (Th) cells, CTLs, regulatory T cells (Tregs) and other T cell subsets. CTLs kill cells infected with intracellular pathogens, whereas Tregs suppress immune responses. Th cells provide help to B cells by cognate interaction and cytokine secretion, and to other cell types by cytokine secretion, which leads to activation of these cells. Th cell responses are classically divided into Th1 and Th2. The development of a Th1 response is crucial for elimination of viral infections. Upon infection with viruses, PAMPs bind to TLRs on DCs. In response, DCs become activated and start producing high amounts of IL-12, IL-15, type I and III IFN and many chemokines. Many of these analytes activate NK cells and direct T helper cell differentiation down the Th1 pathway. The main effector Th1 cytokine is IFN-γ. Secretion of IFN-γ results in activation of CTLs, NK cells and macrophages and promotes further Th1 development. IFN-γ has also been shown to directly inhibit HCV replication [212-214] and genetic studies have also shown that a SNP in the IFN-γ gene might have a beneficial outcome on both clearance and treatment-induced SVR [247]. Once activated, Th1 effector cells have to be recruited to the liver, which happens via the interaction of chemokines with their corresponding receptors [248]. Th1 cells preferentially express CXCR3 and CCR5, which bind to Th1-associated chemokines. CXCR3-associated chemokines are CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC) and CCR5-associated chemokines are CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES).

A Th2 response is important for clearance of several helminthic infections, but also mediates allergy and atopy [249]. Characteristics of a Th2 response include IL-4, IL-5, IL-9 and IL-13 secretion, elevation of IgE levels, eosinophilia, and increased mast cell and basophil numbers

[250]. Th2 cytokines can also directly suppress a Th1 response (and vice versa). Apart from these several more Th phenotypes are known today, such as Th3, Th9 and Th17. Th3 is another phenotype that can secrete the immunosuppressive cytokines IL-10 and TGF-β. Th9 is similar to Th2, but is characterised by high IL-9 production. Finally Th17 cells secrete inflammatory cytokines such as IL-17 and IL-21.

In HCV infection, strong multi-specific CD4 and CD8 T cell responses are associated with viral clearance and resolution of disease, whereas chronic disease is associated with weak and narrow T cell responses or a short-lived T cell response [187,251-256]. The importance of T cells in the control of HCV infection was also demonstrated in chimpanzee studies, where depletion of CD4 or CD8 T cells before inoculation with HCV prevented clearance and recovery [257,258]. Furthermore, a vigorous Th1 response has been shown to be crucial to eliminate the virus during the acute phase of infection [259,260], but once infection has become chronic, a strong Th1 response can also result in non-specific recruitment of Th1 cells, CTLs and macrophages to the liver, which can mediate inflammation and hepatocellular damage [261,262,263]. As for Th1 cytokines, chemokines also have a dual role; while they are beneficial in order to combat the virus, they also induce hepatic inflammation, which can ultimately lead to fibrosis and cirrhosis. It has been shown that HCV-specific CTLs do not only kill HCV-infected cells but can also lyse uninfected bystander cells [264], thus contributing to liver immunopathology in chronic infection.

The existence of associations between particular HLA alleles and control of HCV replication supports the role of T cells in HCV infection. Possession of the HLA-B*27 allele correlated with HCV clearance in a single-source exposure setting [265], and it was shown that HLA-B*27 presented an immunodominant HCV CD8 T cell epitope. In another study in a single-source exposure setting several HLA alleles, including HLA-A*03, HLA-B*27 and HLA-Cw*01 have also been implicated in HCV clearance [266,267]. A number of class II alleles, including

HLA-DRB1* and -DQB1* alleles have been shown to be protective in respect to spontaneous clearance and SVR after therapy in a cohort of patients infected with gt 3a [234].

In summary, while both CD4 and CD8 T cell responses have a beneficial role in combatting HCV infection, CTLs (together with other immune cell subsets) can also mediate liver damage and inflammation.

1.4.2.2 B cells

B lymphocytes form the second main component of the adaptive immune system. Each B cell expresses a unique B cell receptor on its surface. Naive B cells differentiate into memory B cells and plasma cells upon activation with antigen and provision of help by DCs and CD4 T cells. Plasma cells have the unique ability to produce high amounts of antibodies capable of neutralising and opsonising pathogens. Neutralising antibodies contribute to clearance of viral infections and can also confer protective immunity upon secondary infection. They can aggregate virions, prevent attachment of the virus to its receptor(s), inhibit fusion or can interfere with uncoating or early replication steps [268].

In contrast to the widely accepted role of CD4 and CD8 T cell responses in control of HCV infection, the role of neutralising antibodies is not quite as clear. *In vivo*, neutralising antibodies were first described in the chimpanzee model, where pre-incubation of HCV with either serum from a chronic HCV patient or serum raised against E2 HVR1 protected the chimpanzees against infection with HCV of the same isolate [269,270]. On the other hand, chimpanzees that had cleared one HCV infection were not protected against reinfection with homologous or heterologous HCV [271,272], suggesting that neutralising antibodies are not always elicited following HCV infection and/or that escape mechanisms may exist.

In humans, the study of neutralising antibodies *in vitro* was hampered for a long time by the lack of a cell culture system for HCV. The development of HCVpp and then HCVcc systems has now allowed the study of several aspects of neutralising antibodies in HCV infection. Using these neutralisation assays, some studies did not identify a correlation between the

presence of neutralising antibodies and clearance of infection [244,245], whereas other studies show that neutralising antibodies correlate with viral clearance or control of viral replication [273,274]. In summary, there is evidence for some degree of participation of neutralising antibodies in the clearance of HCV. It is likely that the humoral response aids the cellular immune response in combatting infection.

1.5 Immune evasion strategies

The high rate of persistence of HCV infection indicates that in many cases the immune response activated *in vivo* is not sufficient to clear the virus. HCV is just one of many viruses that are capable of persisting in their hosts. Other examples of clinically important viruses include HIV, and herpesviruses such as human cytomegalovirus (HCMV), Epstein Barr virus (EBV) and herpes simplex virus (HSV). Persistent viruses have evolved many strategies to avoid clearance by the immune response, including escape from recognition, sequestration in immunologically privileged sites, block of antigen presentation, antigenic variation, cytokine evasion and the inhibition of apoptosis [275]. In this chapter, viral evasion strategies, especially the ones used by HCV, will be described.

1.5.1 Impairment of type I IFN induction and activity

Many viruses, including influenza, vaccinia and HSV, use multiple mechanisms to evade recognition and IFN induction by the host innate immune system [276]. The same is true for HCV. A key player in this process is the NS3-4A protease, which inhibits IFN-β production by interruption of both the TLR3-TRIF-TBK-1-IRF3 pathway and the RIG-I-CARDIF-TBK-1-IRF3 pathway. It was initially discovered that NS3-4A could interfere with the RIG-I signalling pathway and inhibit induction of IFN-β in infected cells [277]. Shortly after this CARDIF was found to be the adaptor molecule involved in the RIG-I signalling pathway [278], and several groups showed that CARDIF was the target molecule that was cleaved by the

NS3-4A protease [278-280]. Another study showed that NS3-4A not only inhibits IFN-β induction, but also the induction of other chemokines [281]. Furthermore, NS3-4A was also shown to cleave TRIF [282], therefore interfering with both the TLR3 and the RIG-I signalling pathways, although some authors do not agree that NS3-4A cleaves TRIF [283]. Proteolysis of adaptor molecules such as TRIF and CARDIF is a strategy that several viruses employ to eliminate antiviral signalling. It has also been shown that CARDIF is cleaved by the proteases of hepatitis A virus (HAV), GB virus B, rhinovirus and coxsackievirus, while TRIF has been shown to be cleaved by the coxsackievirus protease [284-287]. HCV might also be able to inhibit RIG-I signalling via NS2 [288] and core. Core has been implicated in binding to the complement receptor gC1qR [289], which has been shown to inhibit RIG-I signalling [290].

The moderate response rate of HCV to IFN therapy indicates that HCV might also be able to resist the actions of IFN *in vivo*. Indeed, HCV has been reported to interfere with the induction of ISGs by IFN. Several proteins have been implicated in this process, including core, E2 and NS5A. HCV core (and the full-length virus) interferes with the JAK-STAT pathway [291-293], and core activates SOCS-3, an inhibitor of the JAK-STAT signalling pathway [293,294]. NS5A has been shown to bind PKR and 2-5 OAS and inhibit their activity [295,296,297], although other studies could not confirm this [298,299]. Also, transgenic expression of NS5A impairs induction of PKR, 2-5 OAS and IFN-β in mice infected with LCMV [300]. E2 has also been shown to interfere with PKR activity [301]. In addition to its action on multiple ISGs, NS5A also induces IL-8, a chemokine that counteracts the action of IFN [302], which correlates with elevated IL-8 levels in HCV-infected patients [303].

In summary, HCV interferes with the interferon pathway at multiple stages, by inhibiting IFN induction and by counteracting the effects of IFN.

1.5.2 Evasion and subversion of responses mediated by innate immune cells

1.5.2.1 Subversion of DC responses

HCV has been suggested to exploit DCs and to subvert DC functions via several mechanisms, including direct infection of DCs, use of DCs to disseminate itself in the body without being degraded and interference with DC functions. Many viruses can directly infect DCs, which can impact on their functions. Both positive and negative strand HCV RNA has been found in the DCs of patients with chronic infection [124,129,304]. Also, reports indicate that HCVpp, HCV-LPs and HCVcc can be taken up by DCs *in vitro* [137,305,306]. Despite these findings, there is no evidence for robust HCV replication in DCs.

Viruses can also exploit DCs to spread to other parts of the body. HIV is especially well known for this strategy. HIV binds to DC-SIGN via its glycoprotein gp120 and is internalised into non-lysosomal compartments [307]. It can then be transported to other parts of the body within the DC and be transferred to its target cell, the CD4 T cell, in a process called trans-infection. Interestingly, HCV also binds DC-SIGN and DCs have been implicated in trans-infection of liver cell lines [51,52]. HCV E2 also binds to DC-SIGNR (also known as L-SIGN), which is expressed on liver sinusoidal endothelial cells (LSECs). Furthermore, reports show that HCV-LP are taken up by DCs by DC-SIGN and can reside within the DC for more than 24h [308], providing a mechanism similar to HIV, where the virus might be taken up by DCs or LSECs and transmitted to hepatocytes.

There are conflicting results in the literature about whether DC functions are impaired in chronic HCV infection: some reports do show impaired DC function in chronic HCV infection [309-322], whereas other reports do not [323-327]. For pDCs, reduced numbers in blood [317,318,328] and liver [322] have been observed, whereas others have correlated the reduced number of pDCs in blood to intrahepatic enrichment [329]. Also, less type I IFN production [309,310,318], reduced HLA-DR expression [310], less CD4 T cell activation [310], decreased maturation [328],

higher levels of the regulatory receptor BDCA2 [322] and differential chemotaxis[328] have been reported. For mDCs (or MDDCs), reduced numbers [317,328], impaired allostimulatory capacity [315,318,319], reduced IL-1β [316], IL-6 [316], IL-12 [317,321,330], IL-15 [331] and TNF-α production [316,321], increased IL-10 production [317], reduced activation and maturation marker expression [315,316,320] and reduced MICA/B levels [332] have been reported. Since the findings between these studies differ and a lot of studies cannot detect phenotypically or functionally impaired DCs, these data should be viewed with caution. Furthermore, a general immunosuppression as is known in other persistent infections (e.g. HIV or LCMV clone 13) is not seen in patients with CHC infection, so the impairment in DCs (if present) is not sufficiently severe to impact greatly on the overall immune competence.

To gain insight into the mechanisms that may underlie the abnormalities in DC functions observed in chronically infected individuals, *in vitro* studies have been carried out to address the effect of HCV proteins and more recently HCVcc on DCs. The core, NS3 and E2 proteins of HCV have all been shown to induce defects in DC functions [289,329,318], but the validity of these studies remains to be confirmed with whole virus particles at physiological concentrations. Three recent reports (published during the course of my DPhil studies) used HCVcc to address HCV-induced DC impairment. Two of these studies showed that pre-treatment of DCs with HCVcc reduced IFN-α production in response to subsequent CpG stimulation [202,203]. The third study showed that HCVcc inhibited the ability of PolyI:C-activated mDCs and TLR7 ligand-stimulated pDCs to activate naive CD4 T cells, and inhibited upregulation of CD40 expression on pDCs in response to TLR7 stimulation [333].

1.5.2.2 Evasion of NK responses

Many human viruses, including HCMV, Kaposi's sarcoma-associated herpes virus (KSHV), HSV and HIV, have evolved mechanisms to avoid NK recognition or to impair NK effector function [334]. The best-studied example of the former is HCMV. HCMV expresses four

proteins that interfere with the MHC class I biosynthetic pathway: U2, U3, U6 and U11. By their action MHC class I molecules are retained in the ER, loading of peptides onto MHC class I is inhibited and MHC class I is targeted for proteasomal degradation. However to prevent NK recognition of infected cells expressing low levels of MHC class I, HCMV possesses a MHC class I homologue (UL18) that inhibits NK activation by binding to the inhibitory NK receptor LIR-1. Furthermore UL40 preserves HLA-E expression on the cell surface by loading its own leader peptide and UL16 retains MICB and ULBPs intracellularly, avoiding detection by NKG2D.

Whether HCV possesses strategies for avoiding NK cell recognition of the cells it infects is not well understood. Expression of the HCV core protein (and a full-length construct) in liver cell lines and primary hepatocytes was found to lead to upregulation of MHC class I and impaired natural killer cell cytotoxicity towards HepG2 cells [335]. Furthermore, a T cell epitope (HCV core 35-44) was shown to stabilise HLA-E expression and inhibit cytolysis mediated by NK cells [336]. Both these mechanisms together could tip the balance of ligands in favour of NK inhibition, but the role that these mechanisms play in impairing NK recognition has yet to be validated using the HCVcc system.

More work has been done to address whether NK functions are impaired during HCV infection and several studies indicate that NK cells might be dysregulated in chronic HCV infection. Firstly, it was shown that individuals with chronic HCV infection had fewer total NK cells in peripheral blood than healthy controls [337-342]. Furthermore, a skewing of the NK subsets towards fewer $CD56^{dim}$ and more $CD56^{bright}$ NK cells has been shown [337-339,341,342].

There is more controversy in the literature concerning the phenotype and function of NK cells in chronically HCV-infected subjects. Different studies report enhanced, decreased or unchanged NK cell function and expression of NK receptors and cytokines [338-340,343-350]. Several reports indicate that the expression of CD94/NKG2A is upregulated on NK cells from individuals with CHC infection [341,346,347]. The cytokine production profile of CHC patients

also seems to be dysregulated. Upon stimulation, heightened IL-10 [346,348] / TGF-β [346] and decreased IFN-γ [340,342,350] / TNF-α [340] production have been reported. Interestingly, IFN-γ expression by NK cells seems to be heightened in acute infection [237]. Similarly, in acute HCV infection, it has been demonstrated that NK cells are more cytotoxic than NK cells from healthy controls [237]. On the other hand, it is not very clear how cytotoxicity is affected in chronic hepatitis C (CHC) infection. Two earlier reports showed impaired cytolytic NK function in CHC infection [343,345], more recent reports show unchanged cytolytic activity [338,340,342,344]. For some NK receptors such as NCRs, NKG2C and NKG2D and NK markers such as TRAIL and CD107a increased expression has even been reported [340,348-350]. The conflicting results of different studies may be due to differences in timepoint of sampling (with regard to virus or ALT levels and stage of disease), experimental design, PBMC preservation methods and the use of small study numbers.

If the NK cell impairments observed in some studies of CHC infection are real, then these impaired functions could result from several causes. HCV may have a direct inhibitory effect on NK cells. Several reports suggest that CD81 crosslinking by anti-CD81 antibody or immobilised sE2 can downregulate NK activation [351-353], but the same was not shown when whole virus (HCVcc) was used [354], suggesting that this probably does not occur *in vivo*. Much work has also been done with non-structural and core proteins but the need for these proteins to be expressed intracellularly makes the system artificial, as it is yet not clear whether HCV proteins are expressed in immune cells and if they are, whether protein concentrations within the cells ever reach the level observed for liver cells. Decreased NK cell function could also be mediated indirectly through effects of HCV on other cell types. NK cells are activated by DCs and it has been shown that DCs may be impaired in many ways (see 1.5.2.1) in CHC infection, which may lead to less NK activation.

1.5.3 Evasion of the adaptive immune response

1.5.3.1 Evasion of T cell responses

Viruses may evade T cell responses by several mechanisms, including direct modulation of T cell numbers/functions by infection or interaction with viral proteins, evasion of the T cell response by mutation, downregulation of MHC or induction of regulatory T cells, and impairment of other cell types that influence T cell activity, such as DCs and other APCs. T cells may over time become exhausted/anergic in persistent infections. There is some evidence for many of these mechanisms in HCV infection.

Subjects who progress to chronic HCV infection show few and weak HCV-specific T cell responses. Some of these patients never mount an appropriate T cell response, but more often, they mount a vigorous T cell response, able to transiently control the virus, but then undergo loss of these T cell responses, resulting in viral persistence [252]. The mechanisms for both primary T cell failure and the loss of HCV-specific T cell activity are not fully understood.

As discussed in 1.5.2.1, impairment of DCs (and other APCs) could result in less efficient T cell priming. It has also been shown that in HCV infection Tregs (CD4+CD25+FoxP3+ cells) are induced, which are able to suppress CD8 T cells [355-357]. A similar CD8 subset has also been described [358] and it has been shown that, while not able to control HCV replication, HCV-specific IL-10-producing CD8 T cells have a beneficial role in the prevention of liver injury [359].

Another possible mechanism is T cell exhaustion in the face of high antigenic load, as has been shown for LCMV [360]. In this model, HCV-specific T cells would gradually lose their effector functions and then undergo apoptosis and be deleted. There is evidence that HCV-specific T cells display a more immature phenotype, are less cytotoxic and show impaired proliferation [187,251-255]. This effect might be due to exhaustion or may be NS5A-mediated, as CTL responses to NS3-expressing hepatocytes are impaired in NS5A-transgenic mice [300].

The observed impairment of the T cell response could also result from direct infection of T cells by HCV. As for B cells, it was shown that SB-HCV can infect T cells and also can affect IFN-γ signalling, proliferation and the induction of apoptosis [134,135]. Interestingly, one study demonstrated HCV core expression upregulated anergy-related genes [361].

Mutational escape is another T cell evasion strategy that HCV employs. In both chimpanzees and humans it has been shown that HCV mutates to alter CD8 epitopes [362,363].

In summary, there is some evidence for possible means of T cell evasion, but a better understanding of the mechanisms leading to the suboptimal activity of this important arm of the immune response is needed.

1.5.3.2 Evasion of the humoral response

In the acute phase of HCV infection, seroconversion occurs very late (4 and 14 weeks post infection [244,245]), giving the virus the possibility to spread unimpaired. Despite the fact that patients with CHC infection have cross-neutralising antibodies (antibodies that can neutralise both homologous and heterologous strains), these antibodies are not able to control HCV infection, indicating that HCV has evolved ways to escape from antibody-mediated neutralisation. Several factors might contribute to this escape.

Firstly, HCV constantly mutates and changes its glycoprotein sequence, therefore rendering neutralising antibodies unable to bind to the altered epitopes. HCV also exists as a quasispecies, changing the composition of viral variants constantly over time. Indeed a study showed that the immune response lags behind the rapidly mutating virus, with the current neutralising antibodies only able to neutralise viral variants from an earlier timepoint [364].

Interestingly, non-neutralising antibodies have also been shown to interfere with neutralising antibodies by masking the epitopes they bind to [365]. HCV may also shield its epitopes by close association with lipoproteins and by the heavy glycosylation pattern present on its glycoproteins. Studies showed that removal of glycans from E2 made the virus more susceptible to neutralisation by antibodies [366,367]. HCV can associate with VLDL, LDL and

HDL *in vivo*. It has been shown that HDL can enhance infection of hepatoma cells with HCVpp and that this enhancement was dependent on interaction of SR-BI with HDL and HVR1 on E2 [368]. This interaction also led to escape from neutralising antibodies.

Recently there have been several studies on cell-to-cell transmission of HCV [75,369,370]. This is a mechanism of viral transfer that enables the virus to hide from the antibodies, which has been shown to be employed by human T cell leukemia virus 1 (HTLV-1) and HIV [371]. It has been shown that cell-to-cell transfer makes the HCV resistant to antibody neutralisation [370].

Finally, *in vitro* studies have shown that engagement of sE2 with CD81 can activate naive B lymphocytes [151] and induce hypermutation of the immunoglobulin gene in B cells [149,150,372], which could alter the immunoglobulin sequence, possibly helping the virus to escape. Furthermore, chronic B cell activation could contribute to some of the pathologies associated with chronic HCV infection, e.g. mixed cryoglobulinemia and B cell lymphoma.

1.5.4 The liver as tolerogenic organ

A further complication in HCV infection is that the liver is a tolerogenic environment, as the induction of immunity against harmless antigens, coming through the portal vein from the gut, has to be prevented. Hepatic cells, including Kupffer cells and DCs, secrete immunosuppressive cytokines, such as IL-10 and transforming growth factor beta (TGF-β) [373]. The combination of this tolerogenic environment and innate inhibitory mechanisms that HCV possesses make the liver the ideal place for HCV to establish a persistent infection.

1.6 Rationale and Aims

HCV infection is a global health problem affecting about 170 million people worldwide. Severe complications are associated with the disease, such as liver cirrhosis and hepatocellular carcinoma. Therefore, there is a need for both a prophylactic vaccine and also new therapies. The rational design of novel approaches to control HCV infection would be facilitated by a better understanding of the strategies the virus uses to evade clearance by the

host immune response, and of abnormalities in immune functions induced during HCV infection.

Many viruses modulate aspects of the host immune response to promote their survival *in vivo*. Innate responses are commonly targeted, as they constitute the first line of defence during infection and also play a critical role in the induction and maintenance of adaptive responses. The main goal of my project was to identify how the activation state and functions of two important innate cell types, DCs and NK cells, may be modulated by HCV, addressing whether the activation of these cells was triggered by HCV or not, and the potential for the virus to modulate their response to other stimuli. Much of my work was carried out *in vitro*, and made use of the HCVcc system, which had been developed 2 years before I began my studies [11,12]. I also wanted to study the innate response activated during acute HCV infection *in vivo* in the context of acute HCV replication. The aims of my work were as follows:

1. To identify the potential for HCV to infect PBMC subsets

One of the mechanisms by which viruses can modulate the host immune response is by infecting immune system cell subsets, which can lead to the destruction of these cells (either as a direct consequence of virus infection or by rendering them targets for the host immune response), or can result in their functions being impaired. To determine whether this may be one of the mechanisms that HCV uses to evade immune control, and also to gain insight into whether haematopoietic cells may represent a reservoir for HCV, which would have implications for settings such as liver transplantation and the development of entry inhibitors, I carried out *in vitro* studies to assess the potential for HCV to infect PBMC subsets, aiming to:

A. Characterise the expression of known HCV attachment/entry receptors on PBMC subsets and complete the partial picture of receptor expression available when I began my work

B. Resolve the controversy in the literature about the capacity of HCVpp to gain entry into PBMC subsets

C. Explore the ability of HCVcc to infect PBMC subsets *in vitro*, working with both HCVcc derived from a classic hepatotropic virus (JFH-1) and a newly-generated chimeric virus (SB/JHF-1) expressing the structural proteins from a reportedly lymphotropic virus, SB [132]

2. To explore modulation of NK cell responses by HCV *in vitro*

As reviewed in 1.4.1.3, there is increasing evidence to suggest that the NK cell response may be one of the determinants of the outcome of HCV infection. However, relatively little is known about the receptor-ligand interactions that regulate the triggering of NK effector activity on contact with HCV-infected cells, or strategies that the virus employs to evade control by this arm of the immune response.

NK cells express high levels of CD81, and studies published prior to the start of my DPhil work had shown that cross-linking of CD81 by antibody or arrays of immobilised sE2 derived from the H77 virus inhibits NK cell activation in response to stimuli including CD16 cross-linking [351-353]. As CD81 is one of the HCV attachment/entry receptors and is known to be engaged by the E2 glycoprotein [54], this raised the hypothesis that NK cell responses may be impaired during HCV infection by virion-mediated cross-linking of CD81. To gain insight into the potential for virions to impair NK cell responses via interaction with CD81 *in vivo,* I carried out studies to:

A. Address whether NK cell responses were inhibited by exposure to HCVpp, HCVcc or plasma-derived HCV *in vitro*

B. Determine whether arrays of immobilised E2 derived from different genotypes could inhibit NK cell responses via CD81 cross-linking

3. To determine whether HCV modulates the activation and functions of DCs *in vitro*

Dendritic cells have been described as the sentinels of the immune system: they are adapted to recognise the presence of infection and trigger a cascade of innate and adaptive responses. Given their central role in the host immune response, many viruses have evolved strategies for subverting their functions. I focused on two DC subsets, pDCs, which produce high levels of type I IFNs and inflammatory chemokines during the acute phase of many virus infections to contain virus replication and attract other cell types, and mDCs, a more conventional DC subset that stimulate the activation of T and NK cell responses. I aimed to:

A. Address whether exposure to HCV particles stimulated the activation of these DC subsets *in vitro*, and if so, which functions were triggered
B. Determine whether exposure to HCV particles modulated the response of these DC subsets to other activation signals
C. Explore the mechanisms underlying effects observed in A and B above

4. To gain insight into the innate response activated during acute HCV infection *in vivo*

Although *in vitro* systems can be used to explore putative interactions that may occur between a virus and components of the host immune system, studies in humans or animal models are required to determine the relevance of *in vitro* observations to *in vivo* events. Relatively few studies have addressed the nature of the innate response activated during acute HCV infection *in vivo*, and there is only a fragmentary picture of the magnitude and kinetics of activation of cellular innate subsets including DCs and NK cells and the production of innate cytokines/chemokines [185-188]. One of the factors that hampers the study of immune responses during acute HCV infection in humans is the difficulty in identifying infected individuals sufficiently early after virus acquisition to address events taking place at this time. I thus aimed to:

A. Extend studies by our laboratory, employing unique samples collected from plasma donors who acquired HCV infection during the sampling timecourse to study changes in circulating cytokine and chemokine levels during acute HCV infection
B. Evaluate the utility of the LT setting as a model to explore the innate responses activated in the context of acute HCV replication *in vivo* (and if appropriate, to characterise the activation of cellular innate subsets in these patients).
C. Relate cytokine and chemokine levels to different outcomes within the LT cohort

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Plasticware and other laboratory supplies

Product	Catalogue No.	Supplier
12-well plates	353225	BD Biosciences, Oxford, UK
13 mm Coverslips	ML-579-13	Western Lab Supplies, Aldershot, UK
15 ml Falcon tubes	352096	BD Biosciences, Oxford, UK
24-well plates	353047	BD Biosciences, Oxford, UK
48-well plates	353078	BD Biosciences, Oxford, UK
50 ml Falcon tubes	352070	BD Biosciences, Oxford, UK
6-well plates	353224	BD Biosciences, Oxford, UK
96-well flat bottom plates	353936	BD Biosciences, Oxford, UK
96-well round bottom plates	353227	BD Biosciences, Oxford, UK
Cell culture flasks T150	Z707511	Sigma Aldrich, Poole, UK
Cell culture flasks T25	Z707538	Sigma Aldrich, Poole, UK
Cell culture flasks T75	Z707503	Sigma Aldrich, Poole, UK
Cell Strainers 100 μm	352360	BD Biosciences, Oxford, UK
Cell Strainers 40 μm	352340	BD Biosciences, Oxford, UK
Cluster Tubes	AB-0672	Abgene, Surrey, UK
Culture dishes 100mm, Corning CellBIND	168381A	Sigma Aldrich, Poole, UK
Cuvette, 0.4cm gap	165-2081	BioRad Laboratories Ltd,Hemel Hempstead, UK

Eppendorf Tubes 0.5 ml	96.4625.9.01	Treff Ag, Degersheim, Switzerland
Eppendorf Tubes 1.5 ml	96.8160.9.02	Treff Ag, Degersheim, Switzerland
FACS tubes 5 ml	352054	BD Biosciences, Oxford, UK
Finntips 300 µl	9401253	Thermo Scientific, Basingstoke, UK
Haemocytometer	N/A	Weber Scientific, International, Lancing, UK
Hybond ECL nitrocellulose membrane	RPN303D	Amersham Biosciences, Little Chalfont, UK
Immuno 96-well Nunc plates	M0661	Sigma Aldrich, Poole, UK
Internal Thread 1.8 ml cryovials	377267	Life Technologies, Paisley, UK
LD MACS Columns	130-042-901	Miltenyi Biotech, Bisely, UK
LS MACS Columns	130-042-401	Miltenyi Biotech, Bisely, UK
Magnetic 24-well plate		Chemicell, Berlin, Germany
MicroAmp® Fast Optical 96-Well plate	4346906	Applied Biosystems, Warrington, UK
MicroAmp® Optical Adhesive Film	4360954	Applied Biosystems, Warrington, UK
Pasteur Pipettes	475-068	Jencons Scientific, Leighton Buzzard, UK
PCR Reaction Tubes	2441/3004/10	Merck BDH Ltd., Lutterworth, UK
Pipettes 10 ml	SS114	Appleton Woods, Birmingham, UK
Pipettes 2 ml	SS105	Appleton Woods, Birmingham, UK
Pipettes 25 ml	SS116	Appleton Woods, Birmingham, UK
Pipettes 5 ml	SS109	Appleton Woods, Birmingham, UK
Reagent reservoirs, Costar 100mL	4870	Sigma Aldrich, Poole, UK
Reagent reservoirs, Costar 50mL	4872	Sigma Aldrich, Poole, UK
StrepTactin Superflow columns	2-1207-550	IBA, Goettingen, Germany

FACS: Fluorescence-activated cell sorting, ECL: Enhanced chemiluminescence, MACS: Magnetic cell sorting, PCR: Polymerase chain

Superdex200 columns	17-5175-01	GE Healthcare, Uppsala, Sweden
Syringe filter 0.22 µm	GPWP02500	Millipore, Watford, UK
Syringe filter 0.45 µm	HPWP02500	Millipore, Watford, UK
Syringes 10 ml	309604	BD Biosciences, Oxford, UK
Syringes 20 ml	309661	BD Biosciences, Oxford, UK
Syringes 3ml	309585	BD Biosciences, Oxford, UK
Syringes 60 ml	309653	BD Biosciences, Oxford, UK
Tips 10 µl	GPS-10GS	Anachem Ltd., Luton, UK
Tips 10 µl, filtered	GP-L10F	Anachem Ltd., Luton, UK
Tips 1000 µl	GPS-1000S	Anachem Ltd., Luton, UK
Tips 1000 µl, filtered	GP-L1000F	Anachem Ltd., Luton, UK
Tips 200 µl, filtered	GP-L200F	Anachem Ltd., Luton, UK
Tips 250 µl	GPS-250S	Anachem Ltd., Luton, UK
Universal Tubes	275/0460/04	BDH Lab Supplies, Poole, UK

2.1.2 Chemicals and biological reagents

Product	Catalogue No.	Supplier
10% ready gels	161-1155	BioRad Laboratories, Hemel Hempstead, UK
1kb Plus DNA Ladder	10787-018	Invitrogen, Paisley, UK
Acetic acid solution	34254	Sigma Aldrich, Poole, UK
Agarose, analytical grade	A9539	Sigma Aldrich, Poole, UK
Ampicillin	A5354	Sigma Aldrich, Poole, UK
Annexin V-APC	550474	BD Biosciences, Oxford, UK
ATP (Adenosine 5′-triphosphate disodium)	A6559	Sigma Aldrich, Poole, UK
Blue/Orange 6x loading dye	G190A	Promega, Southampton, UK
$CaCl_2$ (Calcium chloride)	499609	Promega, Southampton, UK
D-Glutathione, reduced	G4251	Sigma Aldrich, Poole, UK
EGTA (Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid)	E0396	Sigma Aldrich, Poole, UK
Ethanol	652261	Sigma Aldrich, Poole, UK
Ethanol, absolute	E7023	Sigma Aldrich, Poole, UK
Ethidium bromide	E1510	Sigma Aldrich, Poole, UK
FluidMAG-DP beads	4103-1	Chemicell, Berlin, Germany
Glycerol	101186M	BDH Lab Supplies, Poole, UK
Glycine	15709-017	Invitrogen, Paisley, UK
Heparin 1000 IU/ml ampoule	N/A	Leo Laboratories, Princes Risborough, UK
HEPES	102415K	BDH Lab Supplies, Poole, UK
Isopropanol	59080	Sigma Aldrich, Poole, UK

APC: Allophycocyanin

K_2HPO4 (Potassium phosphate dibasic)	P8281	Sigma Aldrich, Poole, UK
KCl (Potassium chloride)	P9541	Sigma Aldrich, Poole, UK
KH_2PO_4 (Potassium phosphate monobasic)	P9791	Sigma Aldrich, Poole, UK
KOH (Potassium hydroxide)	484016	Sigma Aldrich, Poole, UK
Laemmli sample buffer	161-0737	BioRad Laboratories, Hemel Hempstead, UK
Marvel	N/A	Premier International Food Ltd, Spalding, UK
MAX efficiency DH5α competent cells	18258-012	Invitrogen, Paisley, UK
Methanol	10158BG	BDH Lab Supplies, Poole, UK
$MgCl_2$ (Magnesium chloride)	M8266	Sigma Aldrich, Poole, UK
Novex® Sharp pre-stained protein standard	LC5800	Invitrogen, Paisley, UK
PCR primers and probes	custom	Applied Biosystems, Warrington, UK
PFA (Paraformaldehyde)	47629	Sigma Aldrich, Poole, UK
RNaseZap	AM9780	Applied Biosystems, Warrington, UK
Soluble CD40L (sCD40L)	PHP0024	Invitrogen, Paisley, UK
Streptavidin-FITC	554060	BD Biosciences, Oxford, UK
Streptavidin-PB	S-11222	Invitrogen, Paisley, UK
Sucrose	102744B	BDH Lab Supplies, Poole, UK
Trizma Base	T1503	Sigma Aldrich, Poole, UK
Tween-20	P7949	Sigma Aldrich, Poole, UK
Western blotting detection reagents	RPN2108	Amersham Biosciences, Little Chalfont, UK

FITC: Fluorescein isothiocyanate, PB: Pacific Blue

2.1.3 Buffers

Product	Composition/Catalogue No.	Supplier
Annexin V binding buffer, 10x	556454, dilute 1/10 in dH_2O	BD Biosciences, Oxford, UK
Coomassie Brilliant Blue R250 Stain	161-0436	BioRad Laboratories, Hemel Hempstead, UK
Coomassie destain buffer	400 methanol, 100ml acetic acid solution, 500ml dH_20	N/A
Cytofix/Cytoperm	554722	BD Biosciences, Oxford, UK
Cytomix	120mM KCl, 0.15mM $CaCl_2$, 10mM K_2HPO_4/KH_2PO_4 pH 7.6, 25mM HEPES, 2 mM EGTA, 5mM MgCl in dH_2O. Adjust pH to 7.6 with KOH, sterilly filter and store at 4°C. Before electroporation add 2 mM ATP and 5 mM glutathione	N/A
dH_2O (deionised water)	N/A	HIU media supplies, Oxford, UK
EB buffer	10 mM Tris-HCl (pH 7.5)	Qiagen, Crawley, UK
FACS buffer	1% FCS and 0.1% NaN_3 in PBS	N/A
Fixation buffer	4% PFA in PBS. Heat until PFA dissolves. Store at 4°C in dark	N/A
MACS buffer	0.5% FCS in PBS without Ca^{2+} and Mg^{2+}	N/A
Nuclease-free water	AM9938	Applied Biosystems, Warrington, UK
PBS (Phosphate buffered saline)	N/A	HIU media supplies, Oxford, UK
PBS 10x without Ca^{2+} and Mg^{2+}	14200-083	Invitrogen, Paisley, UK
PBS-T	0.05% Tween 20 in PBS	N/A
Perm/Wash buffer	554723, dilute 1/10 in dH_2O	BD Biosciences, Oxford, UK
Transfer buffer	3.03 g Trizma Base, 14.4g Glycine, 200ml methanol, up to 1000ml dH_2O, store at 4°C	N/A

Tris/Glycine SDS running buffer, 10x	161-0732, dilute 1/10 in dH_2O	BioRad Laboratories,Hemel Hempstead, UK
NEBuffer 3 (10x)	B7003S	New England Biolabs, Hitchin, UK
NEBuffer 4 (10x)	B7004S	New England Biolabs, Hitchin, UK
Western Blotting buffer	5% Marvel in PBS, store at 4°C	N/A
Mung bean nuclease reaction buffer (10x)	B0250S	New England Biolabs, Hitchin, UK

2.1.4 Plasmids

Plasmid	Protein(s)	Supplier
pMLV-gagpol	MLV-gagpol	Francois-Loic Cosset, INSERM, Lyon, France
pMLV-GFP	MLV-GFP	Francois-Loic Cosset, INSERM, Lyon, France
pHCMV E1E2	H77.20 E1E2 UKNJFH-1 E1E2 UKN2B1.1 E1E2	Jonathan Ball, University of Nottingham, UK
pVSV-G	VSV-G	Jonathan Ball, University of Nottingham, UK
pUC19	AmpR (control for transformation efficiency)	Cat. No. 15364-011, Invitrogen, Paisley, UK
pJC-1	HCV(JC-1 (JFH-1/1-846 J6))	Ralf Bartenschlager, University of Heidelberg, Germany
pJFH-1	HCV (JFH-1)	Arvind Patel, University of Glasgow, UK
pJFH1/DGDD	HCV (Mutant JFH-1)	Giorgos Koutsoudakis, IDIBAPS, Barcelona, Spain
pJ6/JFH-1	HCV (JFH-1/ 34-842 J6)	Giorgos Koutsoudakis, IDIBAPS, Barcelona, Spain

SDS: Sodium dodecyl sulphate

2.1.5 Enzymes

Product	Catalogue No.	Supplier
MluI	R0198S	New England Biolabs, Hitchin, UK
Mung bean nuclease	M0250S	New England Biolabs, Hitchin, UK
Proteinase K	19131	Qiagen, Crawley, UK
RNase-free DNaseI	4716728001	Roche, Mannheim, Germany
XbaI	R0145S	New England Biolabs, Hitchin, UK
TaqMan FAST universal PCR master mix (2x)	4352042	Applied Biosystems, Warrington, UK
SYBR Green FAST master mix	4385616	Applied Biosystems, Warrington, UK

2.1.6 Kits

Product	Catalogue No.	Supplier
B cell isolation kit	130-046-901	Miltenyi Biotec, Bisley, UK
BDCA-4 cell isolation kit	130-090-532	Miltenyi Biotec, Bisley, UK
Bio-Plex 6-plex cytokine assay	custom	BioRad Laboratories, Hemel Hempstead, UK
Dneasy blood & tissue kit	69504	Qiagen, Crawley, UK
High-sensitivity human cytokine Lincoplex kit	HSCYTO-60SPMX13	Millipore, Watford, UK
IFN-α ELISA kit	41100	PBL InterferonSource, Piscataway, US
IL-18 ELISA kit	7620	MBL, Woburn, USA
IL-28B ELISA kit	ABIN414558	UCSN Life Sciences

ELISA: Enzyme-linked immunosorbent assay

Lightning-Link FITC antibody labeling kit	707-0010	Innova Biosciences, Cambridge, UK
Lightning-Link PE antibody labeling kit	703-0010	Innova Biosciences, Cambridge, UK
Monocyte isolation kit	130-091-153	Miltenyi Biotec, Bisley, UK
NK cell isolation kit, human	130-046-502	Miltenyi Biotec, Bisley, UK
Pan T cell isolation kit II	130-091-156	Miltenyi Biotec, Bisley, UK
Plasmid Maxi kit	12162	Qiagen, Crawley, UK
Qiaquick PCR purification kit	28104	Qiagen, Crawley, UK
Rneasy Mini kit	74104	Qiagen, Crawley, UK
Sigma CaPhos transfection kit	CAPHOS	Sigma Aldrich, Poole, UK
T7 RiboMAX™ Express large scale RNA production system	P1320	Promega, Southampton, UK
Taqman reverse-transcription reagents kit	N8080234	Applied Biosystems, Warrington, UK

2.1.7 Antibodies

2.1.7.1 Primary antibodies

Specificity	Fluorochrome	Species	Isotype	Clone	Cat. No	Company
BDCA-2	PE	mouse	IgG1	AC144	120000891	Miltenyi Biotec, Bisley, UK
CD107a	FITC	mouse	IgG1	H4A3	555800	BD Biosciences, Oxford, UK
CD11c	PE-Cy5	mouse	IgG1	B-ly6	551077	BD Biosciences, Oxford, UK
CD11c	APC-HiLyte 750	mouse	IgG1	B-ly6	custom	BD Biosciences, Oxford, UK

PE: Phycoerythrin

CD123	APC	mouse	IgG2a	AC145	130090901	Miltenyi Biotec, Bisley, UK
CD123	PE-Cy7	mouse	IgG1	6H6	25-1239	eBioscience, Hatfield, UK
CD14	PE-Cy7	mouse	IgG2a	M5E2	557742	BD Biosciences, Oxford, UK
CD14	PB	mouse	IgG2a	M5E2	558121	BD Biosciences, Oxford, UK
CD16	PB	mouse	IgG1	3G8	558122	BD Biosciences, Oxford, UK
CD16	unconjugated	mouse	IgG1	3G8	555404	BD Biosciences, Oxford, UK
CD19	APC	mouse	IgG1	HIB19	555415	BD Biosciences, Oxford, UK
CD19	PB	mouse	IgG1	LT19	MCA1940PB	AbD Serotec, Kidlington, UK
CD20	PB	mouse	IgG2b	2H7	MCA1710PB	AbD Serotec, Kidlington, UK
CD25	FITC	mouse	IgG1	M-A251	555431	BD Biosciences, Oxford, UK
CD3	PerCP	mouse	IgG1	SK7	347344	BD Biosciences, Oxford, UK
CD3	PE-Cy7	mouse	IgG1	SK7	557851	BD Biosciences, Oxford, UK
CD3	APC	mouse	IgG1	UCHT1	555335	BD Biosciences, Oxford, UK
CD3	PB	mouse	IgG1	UCHT1	558124	BD Biosciences, Oxford, UK
CD3	unconjugated	mouse	IgG1	SK7	347340	BD Biosciences, Oxford, UK
CD4	APC	mouse	IgG1	RPA-T4	555349	BD Biosciences, Oxford, UK
CD40	PE	mouse	IgG2a	LOB7/6	MCA1590PE	AbD Serotec, Kidlington, UK
CD56	APC	mouse	IgG1	B159	555518	BD Biosciences, Oxford, UK
CD56	PB	mouse	IgG1	B159	custom	ReaMetrix, San Carlos, UK
CD56	PE-Cy7	mouse	IgG1	B159	557747	BD Biosciences, Oxford, UK
CD69	APC-Cy7	mouse	IgG1	FN50	557756	BD Biosciences, Oxford, UK
CD69	FITC	mouse	IgG1	FN50	555530	BD Biosciences, Oxford, UK
CD69	PB	mouse	IgG1	FN50	310920	BioLegend, Cambridge, UK
CD8	PB	mouse	IgG1	RPA-T8	558207	BD Biosciences, Oxford, UK

PerCP: Peridinin chlorophyll protein complex

CD81	unconjugated	mouse	IgG1	JS-81	555675	BD Biosciences, Oxford, UK
CD81	FITC	mouse	IgG1	1.3.3.22	Sc-7637	Santa Cruz
CD83	APC	mouse	IgG1	HB15e	17-0839-42	eBioscience, Hatfield, UK
CD83	FITC	mouse	IgG1	HB15e	556910	BD Biosciences, Oxford, UK
CD86	Biotin	mouse	IgG1	B70/B7-2	555656	BD Biosciences, Oxford, UK
CLDN1	unconjugated	rat	IgG2a	421203	mAB4618	R&D Systems, Abingdon, UK
CLDN6	unconjugated	mouse	IgG2b	342927	MAB3656	R&D Systems, Abingdon, UK
DC-SIGN	PE	mouse	IgG2a	120612	FAB1621P	R&D Systems, Abingdon, UK
HCV E2	unconjugated	mouse	IgG1	ALP98	N/A	gift from A. Patel
HCV E2	unconjugated	mouse	IgG1	H53	N/A	gift from J. Dubuisson
HCV E2	unconjugated	mouse	IgG1	AP320	N/A	gift from A. Patel
HCV NS5A	unconjugated	mouse	IgG2a	9E10	N/A	gift from C. Rice
HLA-DR	PerCP	mouse	IgG2a	L243	347364	BD Biosciences, Oxford, UK
HLA-DR	PE-Cy7	mouse	IgG2a	L243	335795	BD Biosciences, Oxford, UK
IFN-α	FITC	mouse	IgG1	LT27:295	130-092-600	Miltenyi Biotech, Bisely, UK
IFN-γ	APC	mouse	IgG2a	B27	554702	BD Biosciences, Oxford, UK
IgG1	unconjugated	mouse	IgG1	MOPC-21	554121	BD Biosciences, Oxford, UK
IL-12	APC	mouse	IgG1	8.6	130-092-775	Miltenyi Biotech, Bisely, UK
LDL-R	Biotin	mouse	IgG	polyclonal	ab50296	Abcam, Cambridge, UK
MLV gag	unconjugated	rat	IgG	R142	N/A	gift from J. Dubuisson
NKp30	unconjugated	mouse	IgG1	P30-15	325202	BioLegend, Cambridge, UK
NKp46	unconjugated	mouse	IgG1	9E2/ NKp46	557911	BD Biosciences, Oxford, UK
OCLN	FITC	mouse	IgG1	OC-3F10	33-1511	Invitrogen, Paisley, UK
SR-B1	PE	mouse	IgG1	3D5	N/A	gift from A. Nicosia
TNF	PE	mouse	IgG1	MAb11	554513	BD Biosciences, Oxford, UK
TNF	PE-Cy7	mouse	IgG1	MAb11	557647	BD Biosciences, Oxford, UK

2.1.7.2 Secondary antibodies

Specificity	Fluorochrome	Species	Isotype	Clone	Cat. No	Company
mouse IgG	APC	goat	IgG	polyclonal	115-136-146	Jackson Immunoresearch, West Grove, USA
mouse IgG	AlexaFluor 488	donkey	IgG	polyclonal	A-21202	Invitrogen, Paisley, UK
mouse IgG	HRP	goat	IgG	polyclonal	ab6789	Abcam, Cambridge, UK
rat IgG	FITC	goat	IgG	polyclonal	A10528	CalTag, Buckingham, UK
rat IgG	HRP	goat	IgG	polyclonal	ab6845	Abcam, Cambridge, UK
rat IgG	APC	goat	IgG	polyclonal	112-136-143	Jackson Immunoresearch, West Grove, USA

2.1.8 Media

Product	Composition/Catalogue No.	Supplier
DMEM + GlutaMAX	32430100	Invitrogen, Paisley, UK
LB (Luria Bertani) medium	N/A	IAH supplies, Compton, UK
Opti-MEM I reduced serum medium	11058-021	Invitrogen, Paisley, UK
RPMI 1640+ GlutaMAX	61870044	Invitrogen, Paisley, UK
S.O.C. medium	15544-034	Invitrogen, Paisley, UK

HRP: Horseradish peroxidase, DMEM: Dulbecco's modified Eagle's medium, RPMI: Roswell park memorial institute, S.O.C: Super optimal broth with catabolite repression

2.1.9 Tissue culture reagents

Product	Catalogue No.	Supplier
CL097	tlrl-c97	InvivoGen, San Diego, USA
CpG-A ODN 2216	HC4037	Hycult Biotech, Uden, Netherlands
CpG-B ODN 2006	HC4039	Hycult Biotech, Uden, Netherlands
CpG-C ODN M362	tlrl-hodnc	InvivoGen, San Diego, USA
FCS (Fetal calf serum)	10106-169	Invitrogen, Paisley, UK
GolgiPlug	555029	BD Biosciences, Oxford, UK
GolgiStop	554724	BD Biosciences, Oxford, UK
Histopaque 1077	1077-1	Sigma Aldrich, Poole, UK
LPS (Lipopolysaccharide)	L2880	Sigma Aldrich, Poole, UK
NEAA	11140-035	Invitrogen, Paisley, UK
Pen/Strep (Penicillin/ Streptomycin) 100x	15140-114	Invitrogen, Paisley, UK
PolyI:C	31852-29-6	InvivoGen, San Diego, USA
Recombinant human sCD40L	PHP0024	Invitrogen, Paisley, UK
Saline solution, 0.9%	2F7124	Baxter Healthcare, Deerfield, USA
Trypan blue solution	15250-061	Invitrogen, Paisley, UK
TrypLE express	12605-010	Invitrogen, Paisley, UK
Trypsin-EDTA solution	R-001-100	Invitrogen, Paisley, UK

ODN: Oligodeoxynucleotides, FCS: Fetal calf serum, NEAA: Non-essential amino acids, EDTA: Ethylenediaminetetraacetic acid

2.1.10 Cytokines

Product	Catalogue No.	Supplier
Human recombinant GM-CSF	Gift from Dr John Tite	GlaxoSmithKline, Stevenage, UK
Human recombinant IL-12	219-IL	R&D Systems, Abingdon, UK
Human recombinant IL-15	247-IL	R&D Systems, Abingdon, UK
Human recombinant IL-18	B001-5	MBL, Woburn, USA
Human recombinant IL-2	799068	Roche, Lewis, UK
Human recombinant IL-3	203-IL/CF	R&D Systems, Abingdon, UK
Human recombinant IL-4	204-IL-025	R&D Systems, Abingdon, UK
Human recombinant IFN-α 2a	IMR-236	Imgenex, San Diego, USA

2.1.11 Cell lines

Cell Line	Origin	Supplier
HEK 293T	Human embryonic kidney	Arvind Patel, University of Glasgow, UK
HepG2	Human hepatocellular carcinoma	Arvind Patel, University of Glasgow, UK
Huh7	Human hepatocellular carcinoma	Arvind Patel, University of Glasgow, UK
Huh7.5	Human hepatocellular carcinoma	Charles Rice, Apath, New York, USA
K562	Human chronic myelogenous leukemia	Persephone Borrow, University of Oxford, UK
P815	Mouse mast cell	Persephone Borrow, University of Oxford, UK
Raji (MDA- / RIG-I kd)	Human Burkitt's lymphoma	Keigo Machida, University of Southern California, USA

2.2 Methods

2.2.1 Cell Biology

2.2.1.1 Culture conditions for cell lines and PBMCs

Hepatocyte cell lines (Huh7, Huh7.5 and HepG2 cells) and P815 cells were cultured in D10 [DMEM with Glutamax-1, supplemented with 10% foetal calf serum (FCS), 1μg/ml penicillin/streptomycin (Pen/Strep) and non-essential amino acids (NEAA)]. Raji and K562 cells were cultured in R10 (RPMI with Glutamax-1, supplemented with 10% FCS and 1μg/ml Pen/Strep). They were maintained at 37 °C in 5% CO_2 and subcultured, when they were confluent (approximately twice a week). Adherent cells were detached using 0.05% trypsin/ 0.02% EDTA solution or Tryple Express solution. PBMCs were cultured in R10, PBMC stimulation medium (R10 supplemented with 10U/ml IL-2 and 50ng/ml IL-4) or R10 supplemented with other cytokines; and MDDCs were cultured in MDDC medium (D10 supplemented with 50ng/ml IL-4 and 200ng/ml GM-CSF) at 37 °C in 5% CO_2.

2.2.1.2 Isolation of PBMCs

Blood samples were obtained from blood bags or cones purchased from the North London Blood Transfusion Service (Colindale, UK) or drawn with informed consent from anonymous healthy adult donors. Human PBMCs were isolated from heparinised blood using Histopaque density gradient centrifugation. Blood was diluted 1:1 with saline, layered onto 15ml of Histopaque in 50ml Falcon tubes and centrifuged at 2000rpm for 35min in a Sorvall RT7 centrifuge. PBMCs were harvested from the interface, washed twice in 50ml saline by centrifugation at 1500rpm for 5min, and resuspended in R10 medium. For all experiments apart from HCV receptor staining cells were used fresh; otherwise they were frozen in freezing medium [10% dimethylsulphoxide (DMSO) in FCS] and stored in liquid nitrogen until use.

2.2.1.3 Purification of PBMC subsets

T cells, NK cells, monocytes, or pDCs were purified from PBMCs using MACS microbeads cell isolation kits and LS MACS columns, following the manufacturer's instructions. All steps were carried out in MACS buffer (PBS without Ca^{2+} and Mg^{2+} and 0.5% FCS). PDCs were positively selected, whereas T cells, NK cells and monocytes were negatively selected.

T cells were isolated from total PBMCs with a pan T cell isolation kit II, which separates T cells from non-T cells by using a cocktail of biotin-conjugated antibodies against CD14, CD16, CD19, CD36, CD56, CD123 and glycophorin A and anti-biotin microbeads. After depletion, approximately 60% of the starting PBMCs were recovered in the negative fraction containing T cells. The purity of T cells was assessed by staining with an anti-CD3 antibody and analysis by flow cytometry. Approximately 90% of the live cells expressed CD3.

NK cells were isolated from total PBMCs with a NK cell isolation kit, which separates NK cells from non-NK cells by using a cocktail of biotin-conjugated antibodies against CD3, CD4, CD14, CD19, CD36, CD123 and glycophorin A and anti-biotin microbeads. After depletion, approximately 5% of the starting PBMCs were recovered in the negative fraction containing NK cells. The purity of NK cells was assessed by staining with an anti-CD3 and an anti-CD56 antibody and analysis by flow cytometry. Approximately 90% of the live cells were CD3-CD56+.

Monocytes were isolated from total PBMCs with a monocyte cell isolation kit, which separates monocytes from non-monocytes by using a cocktail of biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123 and glycophorin A, and anti-biotin microbeads. In addition, the cells were incubated with a Fc receptor (FcR) blocking reagent to minimise Fc receptor-mediated binding of monocytes to antibodies. After depletion, approximately 10% of the starting PBMCs were recovered in the negative fraction containing the monocytes. The purity of monocytes was assessed by staining with an anti-CD14 antibody and analysis by flow cytometry. Approximately 90% of the live cells expressed CD14.

PDCs were purified from total PBMCs with a BDCA4 isolation kit by using a FcR blocking reagent and anti-BDCA4 microbeads. The positive fraction was enriched for BDCA4+ pDCs. Approximately 0.3% of starting PBMCs were recovered in the positive fraction containing plasmacytoid DCs. The purity of pDCs was determined by staining cells with an anti-CD123 antibody and an anti-BDCA2 antibody and analysis by flow cytometry. 10-70% of the viable cells were BDCA2+CD123+.

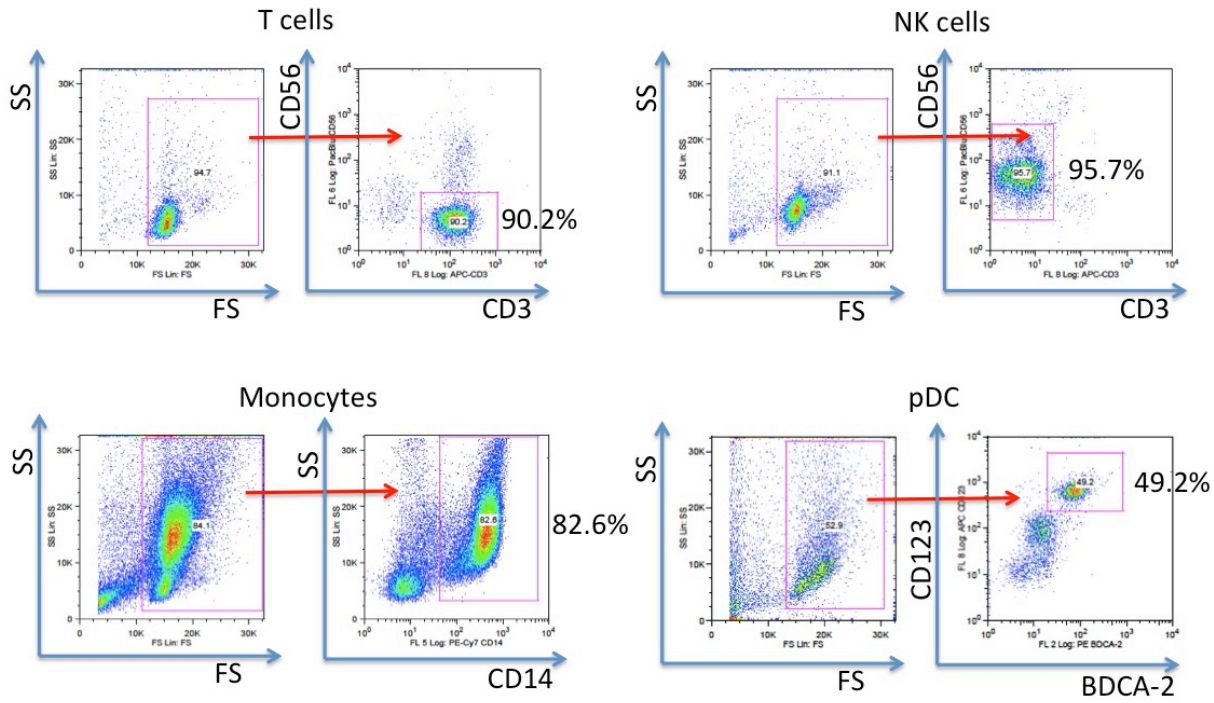

Figure 2-1 Purity of PBMC subsets

All PBMC subsets were purified using MACS beads and columns. After purification, cells were stained with anti-CD3 and anti-CD56 antibodies (T cells and NK cells), an anti-CD14 antibody (monocytes), or anti-BDCA2 and anti-CD123 antibodies (pDCs) and analysed by flow cytometry. Gates were placed on viable cells and purity assessed. SS= side scatter, FS= forward scatter

2.2.1.4 Generation of MDDC

MDDCs were generated from CD14+ cells that were isolated from PBMCs by negative selection using a monocyte isolation kit as described in 2.2.1.3. CD14+ cells were cultured at 5 x 10^6 cells/well in 6-well plates in 3ml MDDC medium for 6 to 7 days, to allow monocytes to differentiate into MDDCs. On the third day of culture, the medium was replenished with

cytokines by replacing a quarter of the old medium with fresh MDDC medium containing four times more IL-4 and GM-CSF. 30-70% of the initial CD14+ cells were recovered on the day of harvest. The purity of MDDC was checked by staining with an anti-CD11c-PE-Cy5 antibody and analysis by flow cytometry. Approximately 90% of the viable cells expressed CD11c. Cells were used fresh for experiments between days 5 and 7.

2.2.1.5 Flow cytometry

Surface staining was performed by first washing the cells twice in FACS buffer (1% FCS and 0.1% NaN_3 in PBS) and then by incubation of cells with antibodies in FACS buffer for 15min at RT. After two more washes in FACS buffer, cells were fixed in fixation buffer (4% PFA in PBS). For intracellular staining, cells were first permeabilised in Cytofix/Cytoperm for 15min at RT, washed twice, then stained with antibodies in Perm/Wash buffer for 15min at RT, followed by two more washes in PBS and fixation. Cells were acquired on a Cyan ADP (DakoCytomation) and results analysed using FlowJo software version 8.8.7 (Tree Star Inc.).

2.2.1.6 Identification of PBMC subsets

PBMC subsets were identified by multiparameter flow cytometry as follows: T cells CD3+CD56-, NKT cells CD3+CD56+, NK cells CD3-CD56+, B cells CD19+, monocytes CD14+, pDC lineage-(CD3-CD14-CD16-CD19-CD20-CD56-)HLA-DR+CD11c-CD123+ and mDC lineage-HLA-DR+CD11c+CD123-. The antibodies used for identification of PBMC subsets were: anti-CD3-PE-Cy7 and anti-CD56-APC (T, NK and NKT cells), anti-CD19-APC (B cells), anti-CD14-PE-Cy7 (monocytes), anti-CD11c-PE-Cy5 (MDDCs), anti-CD123-PE-Cy7, anti-CD11c-APC-HiLyte750, anti-HLA-DR-PerCP, anti-CD3-PB, anti-CD14-PB, anti-CD16-PB, anti-CD19-PB, anti-CD20-PB and anti-CD56-PB (pDC and mDC).

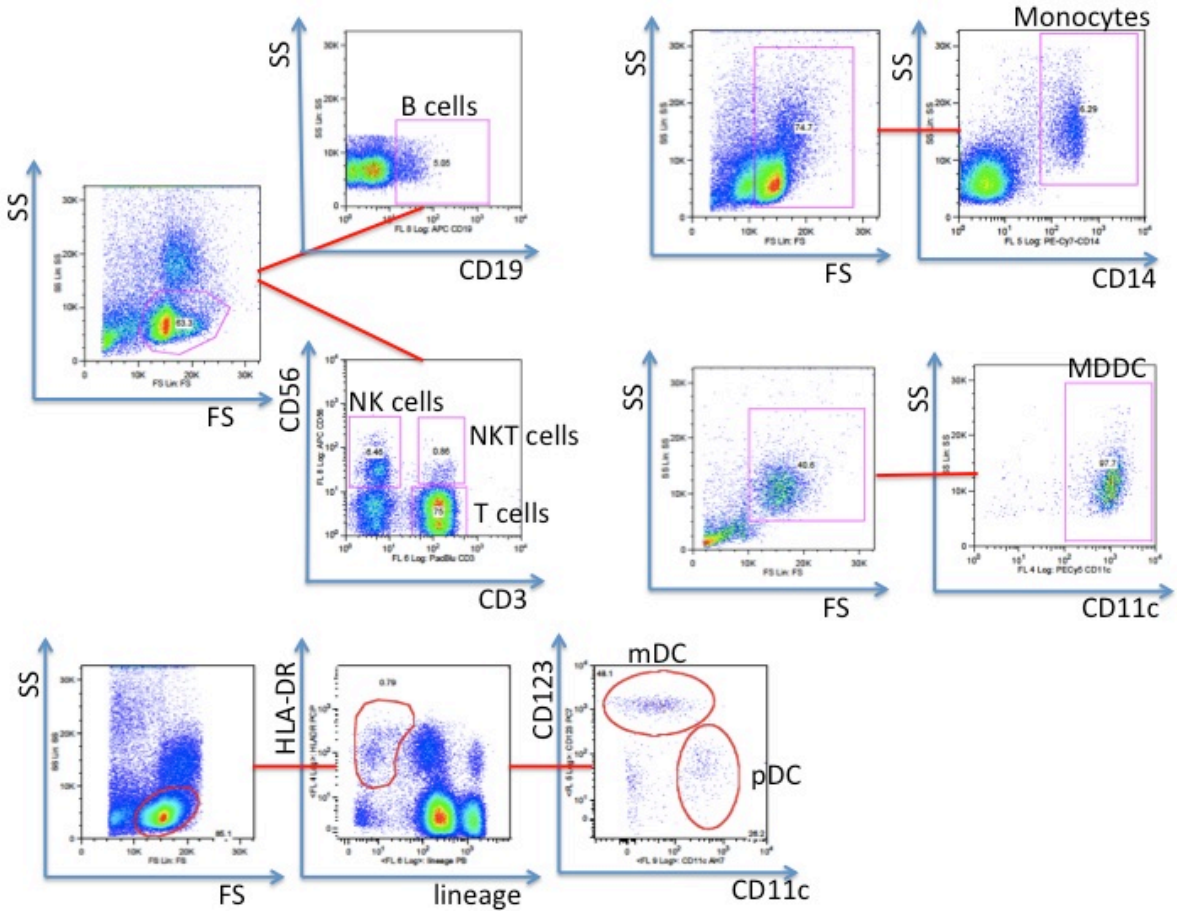

Figure 2-2 Identification of PBMC subsets by flow cytometry

Total PBMCs were stained with anti-CD3 and anti-CD56 antibodies (T cells, NK cells and NKT cells), anti-CD14 antibody (monocytes), anti-CD19 antibody (B cells), anti-CD11c-PE-Cy5 antibody (MDDCs), or lineage (anti-CD3-PB, anti-CD56-PB, anti-CD19-PB, anti-CD20-PB and anti-CD14-PB), anti-HLA-DR-PerCP, anti-CD123-PE-Cy7 and anti-CD11c-APC-HiLyte 750 antibodies (pDCs and mDCs) and analysed by flow cytometry. Gates were placed on viable cells or on the lymphocyte gate and further on respective cell populations. SS= side scatter, FS= forward scatter.

2.2.1.7 HCV Receptor staining

HCV receptor expression on hepatocyte cell lines and PBMC subsets was analysed by multiparameter flow cytometry. Hepatocyte cells lines were directly stained with anti-HCV receptor antibodies, whilst PBMCs were first stained with antibodies to allow identification of cell subsets as described in 2.2.1.6. HCV receptors were identified using the following antibodies: anti-CD81-FITC, anti-SR-BI, anti-claudin-1, anti-claudin-6, anti-occludin-FITC, anti-LDL-R-biotin and anti-DC-SIGN-PE. The anti-SR-BI antibody was directly conjugated to PE and the anti-claudin-6 antibody to FITC using a Lightning Link kit. The anti-claudin-1 antibody was identified using a secondary anti-rat IgG-FITC antibody and the anti-LDL-R

antibody by streptavidin-FITC. The proportion of cells expressing each receptor was then evaluated by flow cytometry.

2.2.1.8 T and NK cell activation assay using plate-bound antibodies

96-well Nunc-Immuno flat-bottomed plates were coated overnight (o/n) at 4°C with an anti-CD3 monoclonal antibody (mAb) (at concentrations between 0.004 and 1µg/ml) or anti-CD16 mAb (at concentrations between 0.016 and 4µg/ml) alone or with an anti-CD81 mAb (1µg/ml), IgG1 isotype control mAb (1µg/ml) or an anti-E2 mAb (1µg/ml). Antibodies were diluted in PBS to the desired concentration and added in a total volume of 100µl/well. After o/n incubation at 4˚C, wells were washed three times with PBS and blocked by incubation with R10 at 250µl/well for 1h. E2e was added to wells coated with anti-E2 mAb at 1µg/ml in 100µl PBS and incubated for 1h. After three washes, purified T or NK cells were added to the plate at 2-3x10^5 cells/well alone or together with HCVpp, HCVcc (JC-1, 2x10^5 TCID50/ml) or sHCV (~10^7 copies/ml) and incubated for 24h in a total volume of 200µl. Both HCVpp and HCVcc and the corresponding control preparations were sucrose cushion-purified as described in 2.2.4.3. HCVpp and sHCV were added at a 1/4 dilution in R10, HCVcc at a 1/2 dilution at 10^5 TCID50/ml. Cells were then washed and stained with the following antibodies: CD4-PE-Cy5, CD8-PB, CD69-APC-Cy7 and CD25-FITC (T cells) or CD3-PerCP, CD56-PB, CD69-APC-Cy7 and CD25-FITC (NK cells). The proportion of cells expressing the activation markers CD25 and CD69 was then evaluated by flow cytometry.

2.2.1.9 T and NK cell cytokine stimulation assay

96-well Nunc-Immuno flat-bottomed plates were coated with an anti-CD81 mAb or an IgG1 isotype control mAb at 10µg/ml in 100µl PBS o/n at 4°C. After washing three times with PBS and a blocking step (R10 at 250µl/well for 1h), cells were added at 5 x 10^5 PBMCs per well in 200µl R10 with or without the following cytokines: 1.6-200U/ml IL-2, 0.1-1000ng/ml IL-12,

0.1-100ng/ml IL-18, 0.1-100ng/ml IL-15 or 0.1-100µg/ml IFN-α 2a. The plates were incubated for 48h. Cells were then washed and stained with the following antibodies: CD3-PE-Cy7, CD56-APC and CD69- FITC. The proportion of cells expressing the activation markers CD25 and CD69 was then evaluated by flow cytometry.

2.2.1.10 NK degranulation assay following stimulation with P815 targets

P815 cells in D10 were added at $5x10^4$/well to a 96-well plate together with no antibody, an anti-CD16 mAb (at concentrations between 0.012 and 10µg/ml), an anti-NKp30 mAb or an anti-NKp46 mAb (both at concentrations between 0.024 and 20µg/ml) plus an IgG1 isotype control mAb or anti-CD81 mAb at 10µg/ml in a total volume of 200µl. After incubation for 30min, cells were washed three times in PBS and resuspended in 100µl D10. 100µl purified NK cells ($5x10^4$ cells) in R10 and 5µl anti-CD107a-FITC antibody were added to each well. After incubation for 1h, GolgiStop was added to all wells and cells were incubated for another 17h. Cells were then stained with anti-CD3-PerCP, anti-CD56-PB, permeabilised/fixed and stained with anti-IFN-γ-APC antibody. The proportion of NK cells expressing CD107a and IFN-γ expression was analysed by flow cytometry.

2.2.1.11 Evaluation of the effect of HCVcc on DC activation

2.2.1.11.1 MDDC

On day 6 of the MDDC culture, MDDCs were added to 96-well plates at $2x10^5$ cells/well and were incubated with either a 1/10 dilution of HCVcc (J6/JFH-1, 10^7 TCID50/ml) in 100µl DMEM (MOI 0.5) for 3h or were left untreated. The cells were then stimulated with various TLR ligands: CL097 (10µg/ml), LPS (10µg/ml) or Poly I:C (50µg/ml) in a total volume of 200µl DMEM. In those wells where cytokine production was to be analysed, GolgiPlug was added at 1µg/ml after 1h of stimulation. Wells, where upregulation of activation/maturation

markers was to be measured, were left untreated. Cells were removed after a total of 20h incubation and were then either permeabilised/fixed and stained with anti-IL-12-APC, anti-MIP-1β-FITC and anti-TNF-α-PE-Cy7 antibodies or were stained with anti-CD83-APC, anti-CD40-FITC, anti-CD86-Biotin, anti-HLA-DR-PECy7 antibodies and streptavidin-PB in a second step. DC activation was analysed by flow cytometry.

2.2.1.11.2 mDC and pDC (analysed within total PBMC)

Total PBMCs were added to the wells of a 96-well plate at 10^6 cells/well and were incubated with a 1/10 dilution of HCVcc (J6/JFH-1, 10^7 TCID50/ml) in 100µl R10 (MOI 0.1) for 3h or were left untreated. The cells were then stimulated with various TLR ligands: CL097 (10µg/ml), CpG-A, CpG-B and CpG-C (all at 10µg/ml), LPS (10µg/ml) or polyI:C (50µg/ml) in a total volume of 200µl R10. In those wells where cytokine production was to be analysed, GolgiPlug was added at 1µg/ml after 1h of stimulation. Wells, where upregulation of activation/maturation markers was to be measured, were left untreated. Cells were removed after a total of 3h (ideal for analysis of pDCs) and after a total of 20h (ideal for analysis of mDCs). pDCs and mDCs were identified as described above (2.2.1.7). In other experiments, PBMCs were incubated with different viruses [HIV (Bal, $4x10^6$ TCID50/ml), DV2 ($8x10^6$ PFU/ml), Influenza (PR8, $4x10^9$ PFU/ml and HCVcc (J6/JFH-1, $3x10^4$ TCID50/ml)] at different concentrations and their corresponding controls (containing no virus) with or without pre-stimulation with IFN-α at 10µg/ml for 2h, or with different amounts of HCV RNA complexed with 20µl DOTAP transfection reagent o/n. Cells were then either permeabilised/fixed and stained with anti-IL-12-APC, anti-IFN-α-FITC and anti-TNF-α-PE antibodies or were stained with anti-CD83-FITC, anti-CD40-FITC and anti-CD86-APC antibodies. DC activation was analysed by flow cytometry. Supernatants were also removed and frozen at -80°C for subsequent analysis of IFN-α levels.

2.2.1.11.3 Enriched pDC

pDCs were enriched from total PBMCs using a BDCA4 cell isolation kit. 2-5x10^4 cells/well were added to the wells of a 96-well plate and were incubated with a 1/5 dilution of HCVcc (J6/JFH-1, 10^7 TCID50/ml) in 50µl R10 (MOI of 2-5) for 3h or were left untreated. The cells were then stimulated with various TLR ligands: CL097, CpG-A or CpG-B (all at 10µg/ml) in a total volume of 100µl R10 supplemented with IL-3 (10ng/ml). Upregulation of activation markers was analysed by flow cytometry as described above and IFN-α levels in supernatants were analysed by ELISA.

2.2.1.11.4 Blocking experiments with enriched pDCs

In experiments where pDC receptors were blocked, 2-5x10^4 pDCs/well were added to the wells of a 96-well plate and were incubated with anti-CD81 antibody (20µg/ml), anti-BDCA2 antibody or a control IgG1 antibody (all 20µg/ml) for 1h, or cells were left untreated. To block interaction of HCVcc with DCIR, a soluble form of DCIR (sDCIR) was incubated with HCVcc at 1µg/ml for 1h. Cells were then washed, and undiluted HCVcc (J6/JFH-1, 3x10^4 TCID50/ml, MOI 0.1) was added for 3h. In experiments where E2 was blocked, HCVcc was preincubated with anti-E2 antibodies ALP98 and AP320 or control IgG1 antibody (all at 10µg/ml) for 1h prior to addition to the pDCs. After o/n incubation, supernatants were removed and frozen at -80°C for subsequent analysis of IFN-α levels.

2.2.2 Molecular Biology

2.2.2.1 Isolation of total RNA

RNA was isolated from cells or cleaned up after *in vitro* RNA transcription reaction using an RNeasy mini kit following the manufacturer's instructions. Briefly, cells were lysed or RNA transcripts resuspended in RLT buffer (containing β-Mercaptoethanol) and the cell lysates were homogenised using QiaShredder columns. Then one volume of 70% ethanol was added and the solution was loaded onto an RNeasy mini column. After spinning the column, the RNA bound to the silica in the column was washed once with RW1 buffer and twice with RPE buffer. RNA was eluted in 50µl RNase-free water and samples were stored at -80°C until use.

2.2.2.2 Isolation of total DNA

DNA was isolated from cells using a DNA Blood and Tissue kit following the manufacturer's instructions. Briefly, cells were resuspended in 200µl PBS and 20µl of proteinase K was added. 200µl AL buffer was then added and the solution was incubated at 56°C in order to allow cell lysis. 200µl ethanol was then added and the solution was loaded onto a DNeasy column. After spinning the column, the DNA bound to the silica in the column was washed once with AW1 buffer and once with AW2 buffer. DNA was eluted in 100µl EB buffer and samples were stored at -20°C until use.

2.2.2.3 Transformation and culture of transformed bacteria

Competent *Escherichia coli* DH5α were thawed on ice and 100µl aliquots of cells were prepared. 1µl (0.2-5µg) of the plasmid was added to the cells, and the cell suspension was mixed. To determine the transformation efficiency, 5µl (50 pg) of pUC19 plasmid (control) was added to one tube. The cells were incubated on ice for 30min and then heat-shocked for 45s at 42°C. The tubes were placed on ice for 2min and 0.9ml of S.O.C. medium was added

to cells, after which the tubes were transferred to a shaker and incubated for 1h. The cell suspension was then diluted 1/10 and 1/100 and the cells transformed with the control plasmid DNA were diluted 1:100 with S.O.C. medium. 100µl of this dilution was spread onto LB plates containing 100µg/ml ampicillin. Plates were then incubated o/n at 37°C.

The next day, a colony was picked from the plate and incubated in 5ml LB Amp (LB medium containing 100µg/ml ampicillin) in a shaker at 37°C for 8h. Then 100µl of this culture was diluted into 100ml LB Amp and incubated in a shaker at 37°C for another 12-16h. The next day, the bacterial cultures were pelleted and plasmid DNA was isolated.

2.2.2.4 Plasmid purification

Plasmids were purified using a Qiagen plasmid maxi kit following the manufacturer's instructions. Briefly, the pelleted bacteria were resuspended in 10ml P1 buffer containing RNase A and LyseBlue reagent and 10ml alkaline buffer P2 was added, resulting in lysis of the cells. Complete lysis of the cell suspension was checked by examining the suspension for a uniformly distributed blue colour. Then 10ml of chilled P3 neutralisation buffer were added and the cell lysate was transferred to a QIAfilter cartridge and incubated for 10min, allowing a precipitate to form containing genomic DNA, proteins and cell debris. During this incubation a QIAGEN-tip was equilibrated with 10ml QBT buffer. The clear lysate was then passed through the cartridge with a plunger and loaded onto the QIAGEN-tip, followed by two washes with buffer QC. DNA was then eluted with 15ml buffer QF and 10.5ml isopropanol was added to precipitate the DNA. The precipitate was then pelleted and the pellet transferred into a 1.5ml tube and washed three times with ethanol. The pellet was air-dried, resuspended in EB buffer and the DNA was quantified using a NanoDrop (Thermo Scientific). The DNA was stored at -20 °C until use.

2.2.2.5 Agarose gel electrophoresis of DNA

1% agarose gels were prepared by adding 1g of agarose to 100ml Tris-acetate-EDTA (TAE) buffer, heating the mixture in a microwave, cooling the solution to about 50°C and then adding 1µl ethidium bromide. This solution was poured into a gel tray with combs inserted and left to polymerize. The tray was then placed into an electrophoresis chamber containing TAE buffer and the combs were removed. 1µg of each DNA sample was loaded into the slots together with a 1 kb DNA ladder. Gels were run at 80V until samples had migrated out of the wells, then at 120V. DNA was visualized under UV light **(**260nm**)** and documented using an AlphaImager Gel Documentation System (Cell Biosystems).

2.2.2.6 SDS-PAGE

Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% ready gels together with protein molecular weight markers. Protein-containing samples were diluted 1:2 in reducing Laemmli sample buffer and denatured at 95°C for 4 minutes. The samples and markers were then loaded onto the gel and the gels were run at 80V until samples had migrated out of the wells, then at 120V. To visualise the protein bands, gels were stained with Coomassie brilliant blue stain solution for 1h at RT and destained in several changes of destain solution.

2.2.2.7 Western Blotting

Proteins were transferred from unstained gels to a nitrocellulose membrane using a semi-dry transfer method. Gels, filter papers and nitrocellulose membranes were equilibrated in transfer buffer for 10min on a shaker. Gels were then placed onto the membrane and sandwiched with thick filter paper. A current was passed through them at a constant 10V for 30min using a Trans-blot semi-dry (Bio-Rad).

Membranes were then blocked in Western blotting buffer (5% Marvel milk in PBS) for 1h at room temperature on a shaker. They were then washed twice with PBS-T and a primary

antibody was added. For detection of E2 proteins, ALP98 (2mg/ml) was diluted 1/1000 in Western blotting buffer, for MLV gag, concentrated supernatant from a hybridoma culture (clone R142) was added at 1/100. They were incubated with the membrane for 1h at RT on a shaker. After washing three times in PBS-T, bound antibodies were detected using a HRP-conjugated secondary anti-mouse or anti-rat IgG antibody at a 1:1000 dilution in Western blotting buffer. The secondary antibody was incubated with the membrane for 1h at RT on a shaker. After washing three times in PBS-T, the membrane was then treated with enhanced chemiluminescence (ECL) Western blotting detection reagents (solution 1 and 2 mixed at 1:1). Blots were sandwiched between two transparencies and exposed to a film for approximately 30s. The films were then developed using a Compact X4 X-ray film processor (Xograph).

2.2.2.8 Generation of E2e

E2e were produced from two clones, H77 (gt 1a) and 2b_2.8 (gt 2b), using Drosophila Schneider 2 (S2) cells stably transfected with the E1-E2ΔTM construct (Figure 1-3) as described previously [26].

Briefly, Drosophila S2 cells stably transfected with the E2e construct were cultured in spinner flasks at 28°C and induced with 4μM $CdCl_2$ at a density of $7x10^6$ cells/ml. After 8 days of culture, cells were pelleted and E2e was purified from the supernatant by affinity chromatography using a StrepTactin Superflow column, followed by gel filtration chromatography using a Superdex200 column. Pure protein was quantified using a NanoDrop and concentrated to approximately 1 mg/ml. The culture of cells and the induction with $CdCl_2$ were performed by the Krey group, the subsequent purification of the proteins was completed by me with help from the Krey group.

2.2.3 HCVpp methods

2.2.3.1 Generation of HCVpp

Retroviral (MLV-based) HCVpp were generated as described previously [9]. Briefly, HEK 293T cells were cotransfected using a Sigma CaPhos transfection kit with a total of 19µg of DNA comprised of the following plasmids: pMLV-GFP (8µg) encoding the GFP reporter gene, pMLV-gagpol (8µg) encoding the MLV capsid and reverse transcriptase and pHCMV-E1E2 (3µg) encoding the HCV glycoproteins E1E2 from one of three different clones (H77, JFH-1 or 2b.1.1) [374]. To generate particles without viral envelope glycoproteins (NEpp), only pMLV-GFP and pMLV-gagpol were transfected. To generate particles pseudotyped with the VSV-G glycoprotein, cells were transfected with a VSV-G plasmid (pVSV-G) in addition to pMLV-GFP and pMLV-gagpol. After 40h of incubation the cell supernatants were harvested, centrifuged at 2000rpm for 10min in a Sorvall RT7 centrifuge and passed through a 0.45µm filter. Particles were frozen at -80°C until further use.

2.2.3.2 Infection of cells with HCVpp

For analysis by FACS

Huh7 and HepG2 cells were seeded at 10^5/well into a 12-well plate and incubated o/n. PBMCs and MDDCs (day 5 of culture) were seeded on the day of infection at $2x10^5$/well in a 96-well plate. HCVpp expressing E1E2 from H77, JFH-1 and UKN2.1.16, VSVpp or NEpp supernatants were diluted 1/2 in D10 and cells were infected with in a total of 300µl for 3h or left uninfected. The inoculum was then removed and the cells were incubated for three days in 2ml of D10 (hepatocyte cell lines), 200µl MDDC medium (MDDCs) or 200µl PBMC stimulation medium (PBMCs).

For analysis by q-PCR

Supernatants containing the pseudotyped particles were incubated with 300 U/ml DNase I for 1h at 37°C to remove excess plasmid DNA. Stocks were then purified on a sucrose cushion as described in 2.2.4.3. Huh7 and HepG2 cells were seeded at 10^5/well in a 96-well plate and incubated o/n. PBMCs and MDDCs (day 5 of culture) were seeded on the day of infection at $2x10^5$/well in a 96-well plate. Cells were then infected with 100µl purified HCVpp (E1E2 from JFH-1 and UKN2.1.16), VSVpp or NEpp in a total of for 3h or were left uninfected.

2.2.3.3 Analysis of HCVpp- infected cells

2.2.3.3.1 Flow cytometry

After incubating for three days with pseudoparticles, cells were harvested (trypsinising adherent cells) and were washed twice in PBS. GFP expression was then examined by flow cytometry.

2.2.3.3.2 Quantitative PCR

The inoculum was removed after the 3h infection period. Non-adherent cells were harvested and were washed three times in PBS. Adherent cell lines were washed three times in PBS, were then trypsinised, harvested and washed twice more. The cells were resuspended in 100µl PBS and genomic DNA was isolated as described in 2.2.2.2. Early reverse-transcribed viral DNA was detected by quantitative PCR. Primers hybridising to the U3 region of the LTR and primers hybridising to the albumin gene were used as described previously [46]:

U3	U3 fwd 5'-CCATCAGATGTTTCCAGGCT-3' U3 rev 5'-GCGACTCAGTCTATCGGAGG-3'
Albumin	Alb fwd 5'-GCTGTCATCTCTTGTGGGCTGT-3' Alb rev 5'-AAACTCATGGGAGCTGCTGGTT-3'

Absolute quantification was performed using an Applied Biosystems 7500 Fast Real-Time PCR system employing the following conditions:

Reaction Cycles:

Stage 1 (1 cycle)	95 °C for 20s
Stage 2 (40 cycles)	Hold at 95 °C for 3s Hold at 60 °C for 30s

Real-Time PCR Reaction Mix for MLV-GFP:

Component	**Volume**
10μM U3 fwd primer (final 500 nM)	1μl
10μM U3 rev primer (final 500 nM)	1μl
SYBR Green Fast universal mix (2x)	10μl
Nuclease-free water	3μl
DNA	5μl
Total Volume	**20μl**

Real-Time PCR Reaction Mix for Albumin:

Component	**Volume**
10μM albumin fwd primer (final 500 nM)	1μl
10μM albumin rev primer (final 500 nM)	1μl
SYBR Green Fast Universal Mix (2x)	10μl
Nuclease-free water	3μl
DNA	5μl
Total Volume	**20μl**

Using dissociation curve analysis, which displayed only one peak, the specificity of the amplified fragment was confirmed. A standard curve was made for quantification of the U3 LTR region of MLV using 10-fold serial dilutions of the MLV-GFP plasmid ranging from 10^2 to 10^5 copies. The standard curve for quantification of albumin was made using 5-fold serial dilutions ranging from 80 to 10^4 copies of albumin from an albumin standard. This standard was made by isolating DNA from 10^6 human PBMCs and then measuring DNA concentration to determine the actual number of cells corresponding to the amount of DNA isolated. The actual number of cells was calculated by relating the molecular weight of the human genome to the total weight of DNA in the sample. This value was multiplied by two to receive the albumin copy number in the sample. The slope of the standard curves (used to assess PCR efficiency) was consistently between -3.5 and -3.2, indicating very high efficiency (between

0.93 and 1.03). All samples were evaluated in triplicate. Copies of GFP per cell were calculated by dividing the mean copy number of U3 LTR DNA by the mean copy number of albumin DNA and multiplying by two.

2.2.4 HCVcc methods

2.2.4.1 HCVcc plasmids

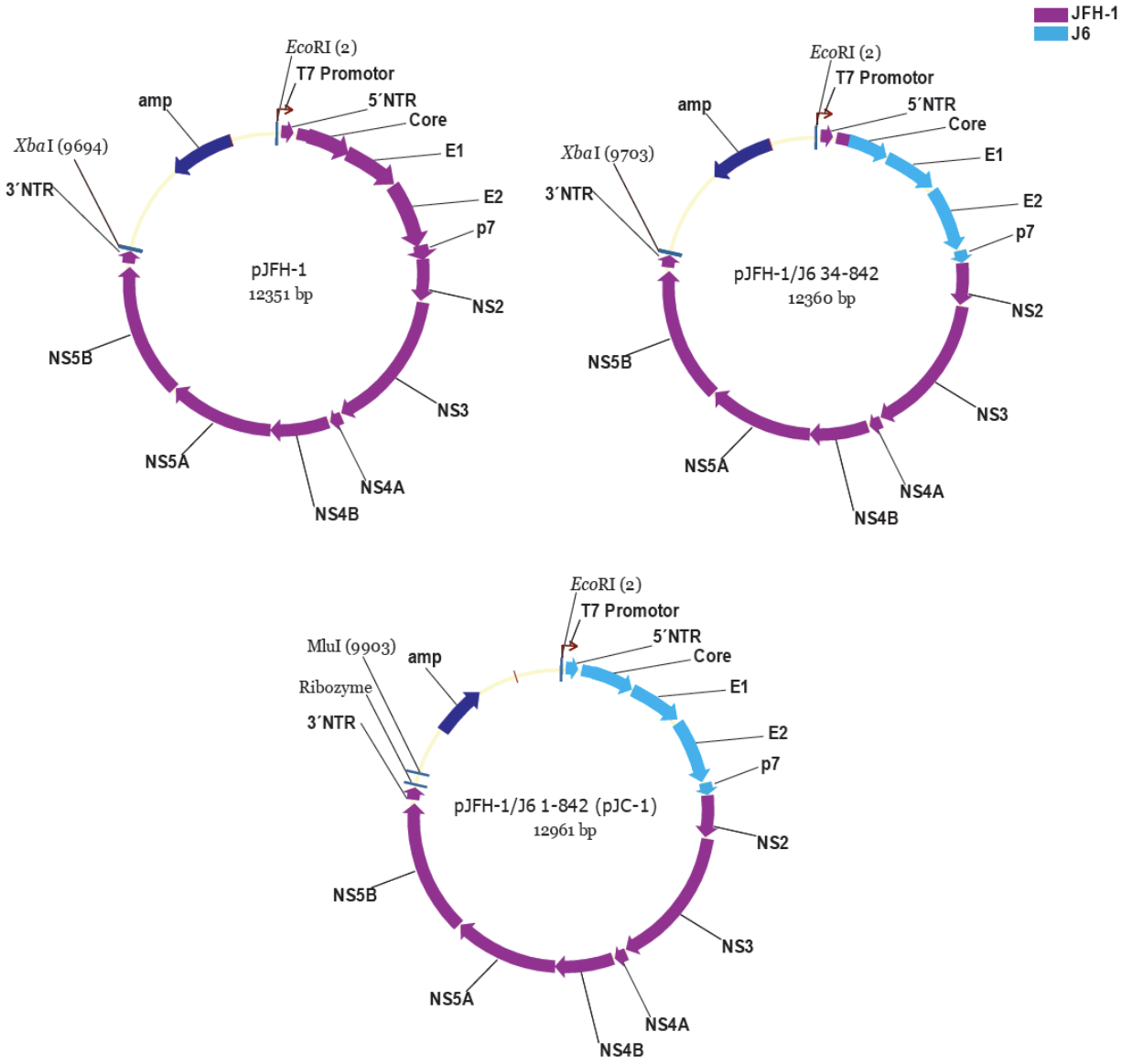

Figure 2-3 Plasmids for HCVcc production

The three different plasmids used in this project are depicted. The plasmids contain an ampicillin cassette, which enables selection of transformed bacteria, and a T7 promoter, driving the expression of the HCV genome. The restriction sites for linearisation of the plasmid together with the corresponding restriction enzyme are indicated.

2.2.4.2 Generation of HCVcc

2.2.4.2.1 Linearisation of HCVcc plasmids

16µg of plasmid (containing the HCV genome) was digested with XbaI (JFH-1 constructs) or MluI (JC-1) for 2h at 37°C using the following digestion mix:

Reagent	**Volume**
Plasmid	16µg (1-44µl)
NEBuffer 3 (10x) for MluI NEBuffer 4 (10x) for XbaI	5µl
XbaI or MluI (100U/µl)	1µl (100U)
Nuclease-free water	up to 50µl
Total Volume	**50µl**

Completion of digestion was checked by loading 2µl of the digest onto a 1% agarose gel and visualising the bands. In the case of the JC-1 plasmid, the DNA was then purified from the digestion reaction using a Qiaquick PCR purification kit according to the manufacturer's instructions and eluted in 50µl of nuclease-free water. JFH-1-based constructs (without the cloned ribozyme activity) were further processed by using mung bean nuclease to digest the overhangs for 30min at 30°C:

Reagent	**Volume**
Linearised plasmid solution	43µl
Mung bean nuclease buffer 10x	5µl
Mung bean nuclease (10U/ul)	2µl (20U)
Total volume	**50µl**

DNA was then purified from the digestion reaction using a Qiaquick PCR purification kit. Purified linearised plasmids were stored at –20°C until further use.

2.2.4.2.2 *In vitro* RNA transcription

In vitro RNA transcription was carried out using a T7 RiboMAX™ Express large scale RNA production system according to the following template:

Component	Sample reaction	Control reaction
RiboMAX Express T7 buffer 2x	10µl	10µl
Linear DNA template 1 µg	1-8µl	-
pGEM positive control template 1 µg	-	1µl
Nuclease-free water	0-7µl	7µl
Enzyme Mix T7 Express	2µl	2µl
Total volume	20µl	20µl

Components were gently mixed and incubated at 37°C for 30 minutes. After the transcription step, 2µl RQ1 RNase-Free DNase were added to remove the template DNA and the mixture was incubated at 37°C for 15 minutes. The transcribed RNA was then purified from the reaction mix using a RNeasy mini kit. The RNA concentration was measured on a Nanodrop and RNA was divided into 10µg aliquots and stored at -80°C.

2.2.4.2.3 Electroporation

Huh7.5 cells were grown to 80% confluency in T150 tissue culture flasks, then cells were trypsinised, washed and counted. 5-10 x 10^6 cells were transferred into a 50ml Falcon tube and washed once with 50ml PBS, and once with 50ml OptiMEM serum-free medium. The cells were then resuspended in 400µl Cytomix and 10µg *in vitro* transcribed HCV RNA was added. Cells were then transferred to a 0.4cm gap cuvette and were then pulsed once at 960µF and 270V using a BioRad Gene Pulser II electroporator. After the electroporation cells were immediately resuspended in 12ml of D10 medium, transferred to a T75 flask and cultured.

2.2.4.2.4 Harvest

Supernatant was harvested from cells on day 2 and 4 post-electroporation. After each harvest, supernatants were centrifuged at 2000rpm for 10min in a Sorvall RT7 centrifuge to remove cell debris and dead cells. On day 2, cells were trypsinised, washed and transferred into a T150 flask in fresh D10 medium. After the second harvest on day 4, the supernatants from days 2 and 4 were pooled and filtered through a 0.45µm syringe filter. They were stored at 4°C in Falcon tubes sealed with Parafilm for 2-3 weeks or at –80°C in cryotubes.

2.2.4.3 Purification of virus on a sucrose cushion

Filtered supernatants from HCVcc (or HCVpp) cultures or mock-infected cultures were concentrated by ultracentrifugation on a sucrose cushion. 10ml of supernatant were layered onto 1ml of 20% sucrose-PBS and spun at 1.05 x 10^5 x *g* for 3h at 4°C. After centrifugation, the supernatant was decanted and the resulting pellet was resuspended in 1ml D10 or PBS, resulting in a 10x more concentrated virus stock. Viruses were used within 2 weeks or stored at -80°C until use.

2.2.4.4 Determination of viral load using a clinical assay

Some samples were measured with a clinical assay to determine the genome copy number. COBAS Ampliprep Taqman HCV test was used for quantitation of viral load. Samples were measured by Richard Kirton at the John Radcliffe hospital. The assay measures IU, which was converted into copies using the recommended conversion factor (1IU/ml=2.7copies/ml).

2.2.4.5 Determination of viral titre (TCID50 assay)

Huh7.5 target cells were seeded at 10^4 cells/well into 96-well plates on the day before infection (or at least 4h prior to infection) in a total volume of 200µl D10. 40 wells were seeded per virus to be titrated. Serial ten-fold dilutions of the virus were made, from 10^{-1} to 10^{-7}. The medium was then removed from the cells and 5 wells were infected with each dilution of virus or left uninfected for 4h. The inoculum was then removed, replaced with 200µl D10, and the cells were incubated for 72h. After this period, the cells were washed twice with 200µl PBS/well and fixed with 100µl methanol (pre-cooled at -20°C) for at least 30 min at -20°C. The methanol was then discarded and the cells were stained and visualised under a fluorescent microscope as described in section 2.2.4.6.1. The TCID50 was then calculated by using the Reed & Muench method [375] by counting the positive wells in each row and using the following equation: Log10 (TCID50) = x + 0.5-(r/n), where x = dose of highest dilution (7), r = sum of negative wells, n = wells per dilution (5).

2.2.4.6 Analysis of infected cells

Infection of cells by HCVcc was examined using three different methods; Immunofluorescence, flow cytometry and reverse transcription- quantitative PCR (RT-q-PCR).

2.2.4.6.1 Immunofluorescence

Infected cells in 96-well plates were washed twice with 200µl PBS/ well and fixed with 100µl methanol (pre-cooled at -20°C) for at least 30 min at –20°C. The methanol was then discarded and the wells were incubated with 100µl 9E10 anti-NS5A antibody diluted 1/10000 in PBS-T (0.05%) for 1h at RT on a shaker. The cells were then washed twice with 200µl PBS/ well and incubated with 100µl donkey anti-mouse IgG- AlexaFluor 488 at 1/500 in PBS-T for 1h at RT on a shaker in the dark. The cells were then washed twice with 200µl PBS/ well and resuspended in 50µl dH20/well. Infected cells were then visualised under a fluorescent inverted microscope (Leica, model DMI4000).

2.2.4.6.2 Flow cytometry

Infected or uninfected (control) cells were trypsinised, washed in PBS and counted. $2x10^5$ cells were used for each stain. Cells were permeabilised in 100µl Cytofix/Cytoperm for 15min at RT, then washed in Perm/Wash buffer and stained with 100µl 9E10 anti-NS5A antibody at 1/10000 in Perm/Wash buffer for 15min at RT. Cells were washed again with Perm/Wash buffer and stained with 100µl secondary goat anti-mouse IgG-APC antibody at 1/200 in Perm/Wash buffer for 10min at RT. Finally cells were washed with PBS, fixed in 200µl fixation buffer and analysed by flow cytometry.

2.2.4.6.3 Reverse transcription-quantitative PCR (RT-q-PCR)

RT-q-PCR is a two-step protocol involving the reverse transcription of RNA into cDNA followed by a q-PCR reaction using relative quantification (RQ).

First-strand cDNA synthesis

Reverse transcription of RNA was carried out using a TaqMan reverse transcription reagents kit. In each reaction, 1µl of RNA was reverse-transcribed using the following reaction mix and reaction cycle:

cDNA synthesis reaction mix (1x):

Component	**Volume**
10x RT Buffer	2µl
$MgCL_2$	4.4µl
dNTPs	4µl
Random hexamers	1µl
RNase inhibitor	0.4µl
Multiscribe reverse transcriptase	0.5µl
Nuclease-free water	6.7µl
RNA	1µl
Total Volume	**20µl**

Reaction Cycle:

Stage 1	25 °C for 10 min
Stage 2	37 °C for 60 min
Stage 3	95 °C for 5 min
Stage 4	4 °C forever

Quantitative PCR

The relative quantity of viral RNA in infected or electroporated cells was determined by RQ. The cDNA obtained from the reverse transcription reaction was amplified using both HCV-specific and GAPDH-specific primers and probes. The HCV-specific probes were FAM-labelled and GAPDH-specific probes were VIC-labelled. The HCV probe and primer sequences were located in the 5' UTR of the viral genome as described previously [75].

Primers and probe for HCV RNA:

HCV fwd 5'-TCTGCGGAACCGGTGAGTAC-3'

HCV rev 5'-GCACTCGCAAGCGCCCTATC-3'

HCV probe 5'-AAAGGCCTTGTGGTACTG-3'

RQ was performed using the Applied Biosystems 7500 Fast Real-Time PCR System using the following conditions:

Real-Time PCR Reaction Mix for HCV RNA:

Component	**Volume**
18 μM HCV fwd primer (final 900 nM)	1μl
18 μM HCV rev primer (final 900 nM)	1μl
5 μM FAM HCV probe (final 250 nM)	1μl
TaqMan Fast universal mix (2x)	10μl
Nuclease-free water	5μl
cDNA	2μl
Total Volume	**20μl**

Real-Time PCR Reaction Mix for GAPDH RNA:

Component	**Volume**
GAPDH Probe and Primer Mix	1μl
TaqMan Fast Universal Mix (2x)	10μl
Nuclease-free water	7μl
cDNA	2μl
Total Volume	**20μl**

Reaction Cycles:

Stage 1 (1 cycle)	95°C for 20 s
Stage 2 (40 cycles)	Hold at 95°C for 3 s Hold at 60°C for 30 s

Each sample was run in triplicate. The HCV signals were normalised to the endogenous GAPDH control and the RQ analysis was performed using Applied Biosystems software SDS (version 2.1.1), according to the manufacturer's instructions.

2.2.4.7 Infection of different immune cell subsets and cell lines with HCVcc

Huh7.5 and HepG2 cell lines were plated at $5x10^4$ cells per well into 96-well plates the night before. Raji $^{MDA5/RIG\text{-}I\ kd}$, total PBMC or purified cell subsets were seeded on the day at 10^5 cells per well into 96-well plates in their medium. Total PBMC were cultured in PBMC

stimulation medium, T and NK/NKT cells were cultured in R10 plus 50U/ml IL-2, B cells were cultured R10 plus 50ng/ml IL-4 and 1μg/ml sCD40L and monocytes in MDDC medium to allow differentiation into MDDC. Cells were resuspended in 50μl of their medium and then infected with either 50μl SB/JFH-1 virus (10^4 IU/ml) or J6/JFH-1 virus ($3x10^4$ TCID50/ml or $2x10^6$ IU/ml) for 12h at 37°C. After the 12h cells were washed five times in PBS and then either analysed by flow cytometry (2.2.4.6.2) or by RT-q-PCR (2.2.4.6.3).

2.2.4.8 Magnetofection of cells with HCVcc

MDDCs were plated at $5x10^5$ cells per well into 12-well plates in 800μl MDDC medium. Duplicate wells were set up for each time point to be analysed. Prior to addition of virus or control supernatant, cells were pelleted. Sucrose cushion-purified virus ($5x10^5$ TCID50/ml) or control supernatant was preincubated with FluidMag beads (370μg/ml) for 2min at RT. 200μl virus-bead or mock-bead complexes were then added to the target cells in 800μl MDDC medium. The plate was transferred onto a 12-magnet plate and kept under the magnetic field for 2min at RT. After magnetic adsorption, the medium was removed and replaced with fresh medium and the plates were incubated further until the indicated time point. Supernatants were then removed and centrifuged at full speed (13000rpm) in a benchtop centrifuge to remove cells and cell debris and were stored at -80°C until further use.

2.2.4.9 HCVcc binding/entry assay

After magnetic adsorption the medium was removed and replaced with fresh medium. Cells were incubated for different lengths of time and were then either washed twice with PBS and lysed in 350μl RLT buffer for total RNA extraction, or washed three times with MDDC medium and then incubated with 50μg/ml PK in 200μl MDDC medium at 4°C for 1 h. Cells were then washed twice more in PBS before being lysed in 350μl RLT buffer for total RNA extraction. Lysed cells were stored at -20°C prior to RNA extraction.

2.2.5 Measurement of cytokines and chemokines

2.2.5.1 Serum and plasma samples

For analysis of changes in plasma cytokine levels in primary acute HCV infection, sequential samples obtained by plasmapheresis from 12 US plasma donors who acquired acute HCV infection (SeraCare life sciences and Zeptometrix) were studied. Viral loads were measured by Mike Busch using a Roche Amplicor-HCV Monitor assay (sensitivity 600 IU/ml) or were supplied by Zeptometrix. As negative controls, sequential samples from three uninfected donors were used.

Sequential serum samples from 13 HCV-infected and 4 uninfected patients undergoing LT were obtained from Xavier Forns at the liver transplant unit at the hospital clinic of Barcelona. Written informed consent was obtained from all patients included in this study and ethical approval was obtained from the investigation and ethical committee of the hospital clinic of Barcelona. All patients underwent standard immunosuppression protocols consisting of prednisone and cyclosporine or tacrolimus. In patients who did not tolerate the cyclosporine/tacrolimus therapy well, mycophenolate mofetil was used as a substitute. Viral loads were measured using a Roche Cobas Amplicor-HCV Monitor assay (sensitivity 25IU/ml) by Jakub Dragun.

To remove debris all serum and plasma samples were spun at full speed (13000rpm) in a benchtop centrifuge and the supernatant was used for subsequent analysis of cytokine/chemokine levels.

2.2.5.2 Cell culture supernatants

Collected supernatants from pDC and MDDC experiments were centrifuged in order to pellet cell debris and then diluted at least 1/2 in sample buffer for subsequent analysis of cytokine/chemokine levels.

2.2.5.3 ELISA

Levels of IFN-α, IL-18 and IL-28B were determined by sandwich ELISA according to the manufacturer's instructions. Samples were run in duplicate, and concentrations calculated using the kit's standard curve. Where the values fell beneath half of the lower limit of detection (LLD) for the corresponding analyte, half of the LLD value was used.

Briefly, 96-well plates were used that had been precoated with the capture antibody. Samples were diluted at least 1/2 in sample buffer and 100µl of diluted sample were added to the wells, allowing the analyte to bind to the capture antibody. After incubating, wells were washed and a detection antibody was added that bound to another epitope on the analyte. Following another incubation, the wells were washed again and a secondary HRP-coupled antibody was then added that bound to the detection antibody. After this incubation, wells were washed and a TMB substrate was added for a maximum of 20 minutes to allow a colour reaction. Finally stop solution was added and plates were read on a Spectramax 250 ELISA plate reader (Molecular Devices) using Softmax Pro (version 5.3).

2.2.5.4 Luminex

Thirteen cytokines and chemokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IFN-γ, GM-CSF and TNF-α) were measured using a high-sensitivity human cytokine Lincoplex kit. Six additional factors were measured by Bio-Plex cytokine assay: MIG, RANTES, IP-10, MIP-1α, MIP-1β and MCP-1. Each sample was assayed in duplicate and cytokine standards supplied by the manufacturer were run on each plate, following the manufacturer's instructions. Where the values fell beneath half of the LLD for the corresponding analyte, half of the LLD value was used.

Briefly, mixtures of antibody-coated beads (pre-labelled with different concentrations of two fluorophores) were added to the diluted samples, standards and controls, allowing the capture antibodies to bind specifically to the analytes. After removal of the liquid by a vacuum

device, a mixture of biotin-conjugated detection antibodies (directed to distinct epitopes on the analytes) was added to the wells, followed by addition of Streptavidin-PE. After a washing step, beads were resuspended in assay buffer and acquired using a Luminex-100 system (Millipore) and cytokine/chemokine levels determined using Bio-Plex manager software, version 4.1 (Bio-Rad).

2.2.6 Statistics

To test for differences between mean values for multiple groups, one-way ANOVA (using Tukey's correction for multiple pairwise comparisons) was used. To test between differences between mean values for two groups, a Mann Whitney test was used. Corrections for comparisons of multiple analytes were not performed, as the study numbers were small. Statistical analyses were performed using Prism software, version 5 (Mozilla Labs).

3 POTENTIAL FOR HCV TO INFECT PBMC

3.1 Introduction

The primary site of HCV infection is the liver, where the virus replicates in hepatocytes [114]. HCV has also been detected in PBMCs [118], but there is some controversy in the literature as to whether HCV can productively infect PBMCs. If HCV does indeed infect PBMCs, this could impact on the survival and functions of the infected cells, constituting a mechanism by which HCV could modulate the host immune response. The aims of the work in this chapter were to examine HCV receptor expression on PBMC subsets and to evaluate the potential for HCV to infect PBMCs *in vitro* using HCVpp and HCVcc model systems.

To date, there are four HCV receptors that have been shown to be necessary for HCV infection. These are CD81, SR-BI, CLDN-1 and OCLN [54-58], which are expressed on hepatocytes and liver cell lines such as Huh7 [115,116,117]. Apart from these, there are also several HCV attachment factors, including GAGs, the LDL-R and DC-SIGN/L-SIGN.

At the beginning of this project, information was only available on the pattern of expression of CD81 and SR-BI on PBMC subsets. CD81 is expressed universally on all PBMC subsets, whereas SR-BI is only expressed on monocytes and DCs [88], although a more recent report indicates that SR-BI might also be expressed on B cells at low levels [376]. CLDN-1 was only discovered as a HCV co-receptor shortly before this project was started and it was reported that neither CLDN-1 RNA nor protein could be detected in PBMCs [65,66,138]. Shortly thereafter, CLDN-6 and CLDN-9 were shown to act as additional co-receptors for HCV, which can substitute CLDN-1 [65,66]. Low CLDN-6 RNA levels and higher CLDN-9 RNA levels have been reported in PBMCs [65,138], but expression of CLDN-6 and 9 proteins has not been reported in PBMCs. OCLN is the most recent receptor to be discovered as an entry factor for HCV [56]. OCLN is present on a variety of tissues and cell types [377], but expression on PBMCs has not been documented. My first aim was to examine the expression of the four principal

HCV receptors, and also the LDL-R and DC-SIGN/L-SIGN on different PBMC subsets, to give a comprehensive picture of the potential for HCV to attach to and enter these cells.

As receptor expression is not always predictive of virus tropism, I also wanted to investigate the potential for HCV infection of PBMC subsets more directly. In support of the idea that HCV can infect PBMCs, studies performed using cells from CHC patients have found that PBMC subsets including neutrophils [123], DCs [124], MDDCs [125], T cells and B cells [126] have been reported to be associated with HCV RNA or proteins *in vivo* and it has been shown that HCV RNA levels can be upregulated in T cells, B cells and total PBMCs upon mitogen stimulation [126]. Other studies have addressed the ability of HCV to infect PBMC subsets or lymphoid cell lines *in vitro*. Following *de novo* infection of monocytes, monocyte-derived DCs and macrophages [128-130] and T cells [131] with HCV-containing plasma/serum both positive and negative strand RNA could be detected, and expression of HCV proteins E2 and NS5A was also observed in T cells [131]. However infection levels in these studies were low; furthermore the relative ability of HCV to infect different PBMC subsets was not extensively explored, prompting the need for further studies to verify the ability of HCV to infect and replicate within PBMC subtypes.

The HCVpp and HCVcc model systems can also be used to study HCV tropism. HCVpp are pseudotyped viruses expressing E1E2 on either MLV or lentiviral (HIV) cores. MLV vectors do not transduce non-dividing cells, whereas lentiviral vectors can. After reverse transcription has taken place, the pre-integration complex needs to be transported to the nucleus. In MLV, this transport requires the breakdown of the nuclear membrane, which only happens during active cell division [378], while HIV possesses a nuclear import mechanism, which does not depend on cell division [379]. Studies carried out to address infection of hematopoietic cell lines with retroviral [9,117] and of hematopoietic cell lines and PBMCs with lentiviral HCVpp [9,136] showed that these cells are not susceptible to efficient transduction with HCVpp,. I thus planned to use HCVpp to study the potential of the HCV glycoproteins to mediate virion

entry into different PBMC subsets. MLV-based HCVpp were used, and to circumvent the problem of these particles being unable to transduce non-dividing cells, a sensitive q-PCR method to detect reverse transcription after viral entry was employed as the readout for particle entry [46].

Furthermore, the ability of HCVcc not only to enter but also to replicate in PBMCs was addressed. First a hepatotropic virus (J6/JFH-1) was used for experiments. During my project two studies were published that also used the JFH-1-based HCVcc system to infect PBMC subsets [138] and T- or B-cell lines [139], the results of which were compared to my findings in the discussion.

The idea that there may be variants of HCV that exhibit differences in cellular tropism is supported by the fact that distinct variants have been found in liver/serum, PBMC fractions and the CNS [120,380-382]. Furthermore, the establishment of B cell lines releasing lymphotropic HCV strains has been reported [133,132]. One of these B cell lines (SB), isolated from the spleen of a HCV-infected patient with a monocytoid B-cell lymphoma, was used to generate a virus called SB-HCV [132]. SB-HCV can infect PBMCs and Raji cells *in vitro* and has been used in studies evaluating the affect of HCV on T cells [132,134,135]. More recently, this virus was cloned and a chimeric virus has been made containing the core-NS2 region of SB-HCV and the NS3-NS5 region of JFH-1 was also generated (Machida *et al.*, unpublished data). This chimeric SB/JFH-1 virus supposedly infects Raji cells and activated T and B cells and is thought to use CD86 rather than CLDN-1 as receptor (Machida *et al.*, unpublished data). I obtained the chimeric SB/JFH-1 virus from Dr. Machida, and planned to explore its tropism for PBMC subsets more fully (focusing on infection of innate immune cells too), and also to define the receptors used for entry into innate immune cells.

3.2 HCV receptor expression on PBMC subsets

Several attachment and entry factors are thought to play a role in HCV entry. In order to gain more understanding about which of these factors are expressed on which immune cell subsets, expression of HCV entry factors and other binding partners (CD81, SR-BI, CLDN-1, CLDN-6, DC-SIGN/L-SIGN, LDL-R and OCLN) was analysed by flow cytometry on liver cell lines, MDDCs and PBMC subsets, including T cells, NK cells, NKT cells, B cells, monocytes, pDCs and mDCs. MDDC were used for staining on day 7. Unfortunately, CLDN-9 expression could not be assessed due to the lack of a suitable antibody and efforts to obtain an antibody that could be used for surface CLDN-9 staining were hampered by the lack of a positive control cell line. HCV receptor expression was first examined on two liver cell lines: Huh7 cells, which are susceptible to HCV infection and express all known receptors and attachment factors except DC-SIGN/L-SIGN, and HepG2, which are not susceptible to HCV infection because they lack CD81 expression. As expected, Huh7 cells expressed CD81, SR-BI, CLDN-1, CLDN-6, OCLN and LDL-R, and HepG2 cells expressed the same receptors apart from CD81 (Figure 3-1). PBMC subsets (as identified in Figure 2-2) and MDDC were also stained for HCV receptor expression and the staining of one representative donor is shown in Figure 3-2.

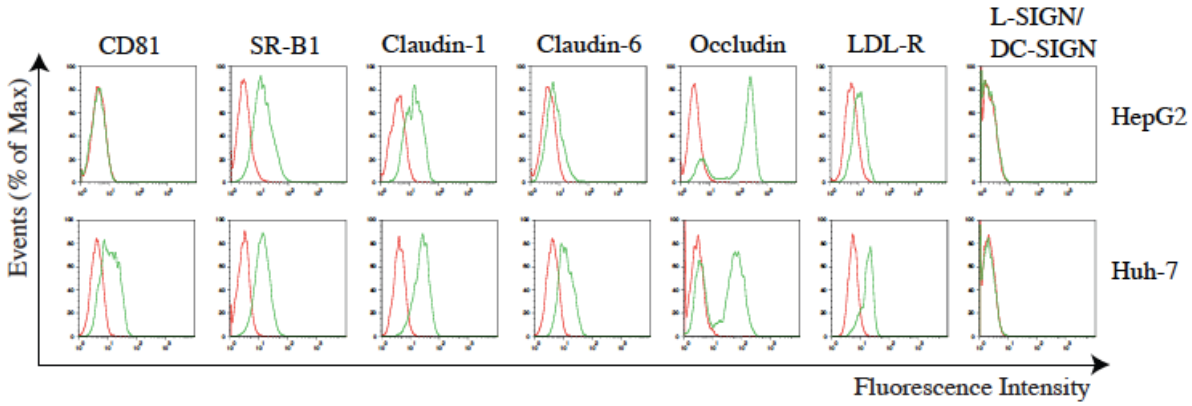

Figure 3-1 HCV receptor expression on the liver cell lines HepG2 and Huh7

Cells were stained with antibodies to CD81, SR-BI, CLDN-1 and -6, OCLN, LDL-R and DC-SIGN/L-SIGN and were analysed by flow cytometry. The red lines show the background fluorescence of either unstained cells, where directly conjugated primary antibodies were used, or of cells stained only with the secondary antibody (CLDN-1 and LDL-R), where two-step staining was performed. The green lines show staining with the indicated anti-receptor antibody.

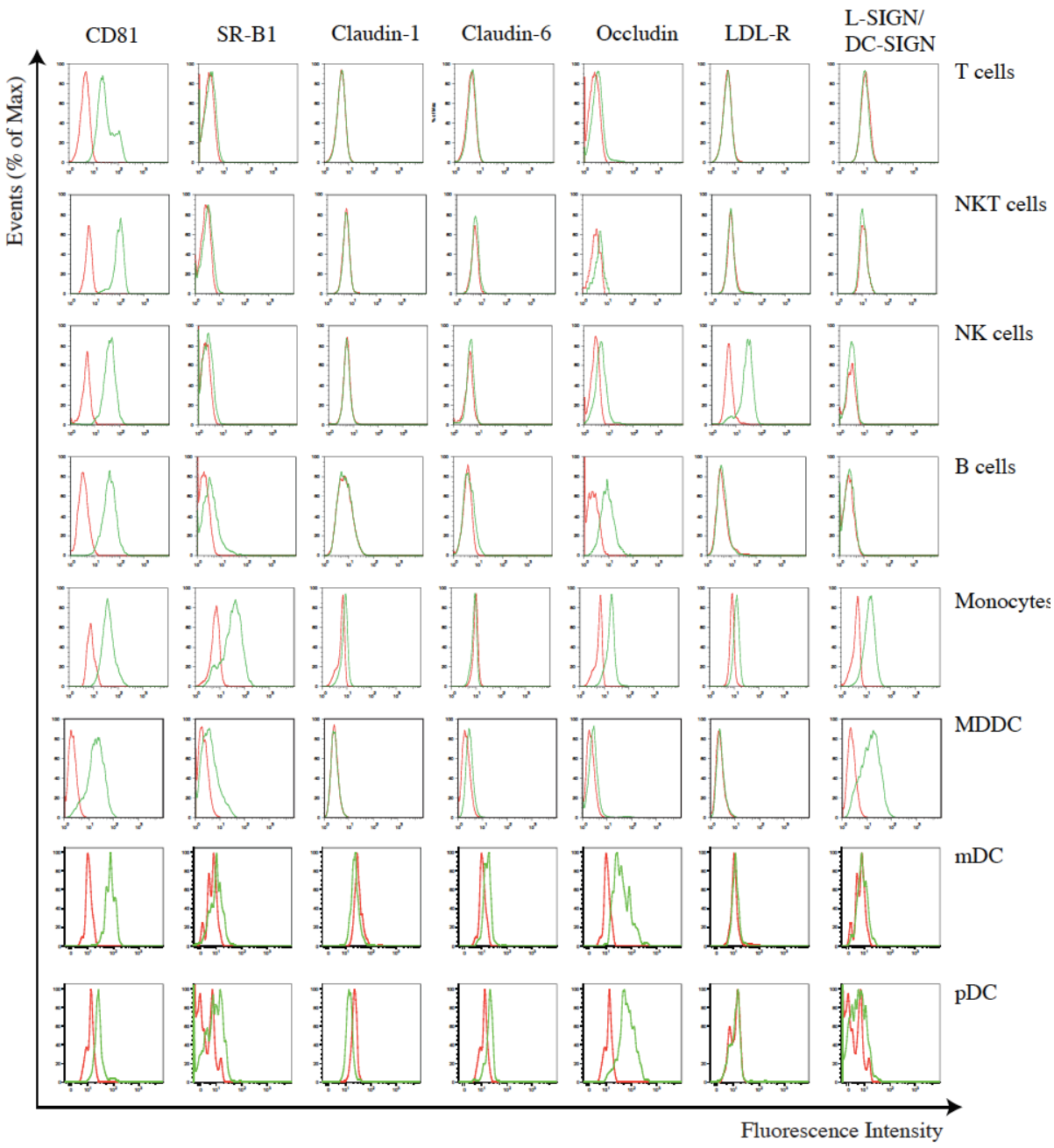

Figure 3-2 HCV receptor expression on PBMC subsets and MDDC

PBMC and MDDC were stained with antibodies to CD81, SR-BI, CLDN-1 and -6, OCLN, LDL-R and DC-SIGN/L-SIGN, and were analysed by flow cytometry. PBMC subsets were identified as described in Figure 2-2. The red lines show the background fluorescence of either unstained cells, where directly conjugated primary antibodies were used, or of cells stained only with the secondary antibody (CLDN-1 and LDL-R), where two-step staining was performed. The green lines show staining with the indicated anti-receptor antibody. Staining was performed on at least 3 donors and results from one representative donor are shown.

In this donor and other subjects, CD81 was found to be expressed on all subsets, and CLDN-1 and CLDN-6 were not reproducibly detected on any of them. SR-BI was detected on all DC subsets, monocytes and B cells. OCLN was detected on all populations except MDDC,

although expression on T, NKT and NK cells was very low. DC-SIGN/L-SIGN was found only on MDDC and monocytes. Finally, LDL-R was detected mainly on NK cells, but also on monocytes. Also the intensity of staining with a given anti-receptor antibody varied considerably between the different subsets, indicating differences in the amount of antibody-accessible surface receptor expression. CD81 was found at high levels on NKT cells, at intermediate levels on T cells, NK cells, B cells, mDCs and MDDCs, at low levels on monocytes and at very low levels on pDCs. SR-BI was found at intermediate levels on monocytes, at low levels on MDDCs, and at very low levels on B cells, pDCs and mDCs. DC-SIGN was found at intermediate levels on MDDC, at low levels on monocytes and possibly at very low levels on pDCs and mDCs. LDL-R was found at intermediate levels on NK cells and at very low levels on monocytes. Finally, OCLN was found at intermediate levels on mDCs and pDCs, at low levels on B cells and monocytes and at very low levels on T cells, NKT cells and NK cells. A summary of all the results is given in Table 3-1. Notably, none of the subsets expressed all the four of the factors required for entry (CD81, SR-BI, CLDN-1/-6 and OCLN), although CLDN-9 expression was not examined due to the lack of a suitable antibody.

	T	NK	NKT	B	Mono	pDC	mDC	MDDC
CD81	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
SR-BI	**-**	**-**	**-**	**YES**	**YES**	**YES**	**YES**	**YES**
DC-SIGN	**-**	**-**	**-**	**-**	**YES**	**(YES)**	**(YES)**	**YES**
CLDN-1	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
CLDN-6	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
LDL-R	**-**	**YES**	**-**	**-**	**(YES)**	**-**	**-**	**-**
OCLN	**(YES)**	**YES**	**(YES)**	**YES**	**YES**	**YES**	**YES**	**-**

Table 3-1 Summary of HCV receptor expression on PBMC subsets and MDDC

PBMC and MDDC from at least 3 donors were stained with antibodies to CD81, SR-BI, CLDN-1, C CLDN-6, LDL-R, OCLN and DC-SIGN/L-SIGN and analysed by flow cytometry.

3.3 Infection of PBMC with HCVpp

Previous reports indicated that lentiviral HCVpp could not infect PBMCs [136,138]. These studies used expression of a reporter gene (GFP or luciferase) as the readout for infection. Notably infection with pseudoparticles expressing glycoproteins derived from viruses known to infect all cell types (such as VSV) resulted in much lower reporter gene expression than in the dividing cell lines. Thus, although lentiviral vectors are able to transduce non-dividing cells, this process might be more ineffective, reducing the sensitivity with which infection can be detected. While MLV-based pseudoparticles cannot transport the pre-integration complex across the nuclear membrane of non-dividing cells, entry and reverse transcription should occur normally after infection with these particles. Hence, in this project a quantitative PCR approach was used to evaluate the early reverse transcription event after infection with retroviral pseudoparticles as previously described [46].

Pseudoparticles bearing no viral envelope glycoproteins (NE) or bearing viral glycoproteins from VSV or two strains of HCV, JFH-1 (gt 2a) and 2b.1.1 (gt 2b) were produced in 293T cells as described in section 2.2.3.1, after which the supernatants from the transfected 293T cells were purified and concentrated on a sucrose cushion. First, the content of capsid in all the pseudoparticle preparations was assessed by Western blot, to give an estimate of the relative particle content of the different preparations. As seen in Figure 3-3, the content of capsid protein varied between the preparations.

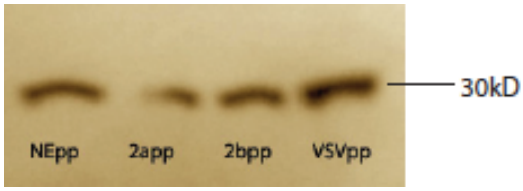

Figure 3-3 Content of MLV gag in different pseudoparticle preparations

The MLV gag content of the four pseudoparticle preparations was assessed by Western blotting. Gag expression in equal quantities of NEpp (no viral envelope proteins), 2app and 2bpp (HCVpp pseudotyped with E1E2 from JFH-1 and 2b1.1 respectively) and VSVpp (pseudotyped with VSV-G envelope protein) preparations is shown.

The VSVpp seemed to contain most capsid, followed by NEpp and the two HCVpp preparations. Since the content of the JFH-1 HCVpp preparation was low, I decided not to dilute out the other preparations but to instead take these differences into account in the interpretation of the results.

HCVpp entry into different cell types was then assessed. In dividing cells, such as the liver cell lines HepG2 and Huh7, infection could be assessed by analysing GFP expression after 3 days. As shown in Figure 3-4, the positive control VSVpp transduced a high proportion of both Huh7 cells and HepG2 cells. Huh7 cells were also transduced by HCVpp. Despite the fact that there was a higher particle content in the negative control NEpp preparation, the percentage of cells transduced by both the HCV-JFH-1pp and HCV-2b1.1pp was higher than that transduced by the NEpp, indicating that the HCV glycoproteins were mediating attachment and entry into Huh7 cells. Conversely, they did not mediate infection of HepG2 cells, which do not express CD81. As expected, entry of VSVpp and HCVpp into PBMCs could not be detected reading out GFP expression.

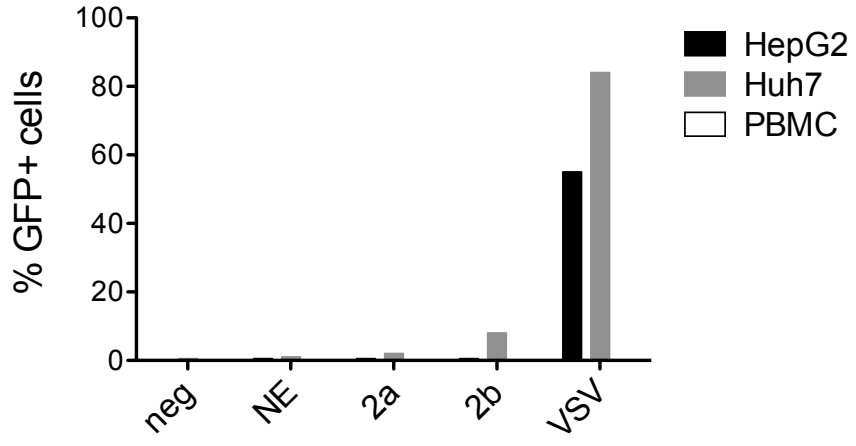

Figure 3-4 GFP expression in hepatocyte lines and PBMCs after infection with HCVpp

Huh7 cells, HepG2 cells and PBMCs were infected with VSVpp (VSV), HCVpp from two gt JFH-1 (2a) and 2b1.1 (2b), or control particles not containing viral glycoproteins (NE) for 3h, or were left uninfected (neg). The inoculum was then removed and cells were incubated for 3 days and analysed for GFP expression by flow cytometry. The percentage of GFP-expressing Huh7 cells (grey), HepG2 cells (black) and PBMCs (white) is depicted. The results represent the mean from one experiment performed in triplicate.

Therefore, a sensitive quantitative PCR approach was used to look at entry of HCVpp and VSVpp into PBMCs [46]. This method detects entry of pseudoparticles into cells by measuring the GFP DNA product produced by reverse transcription immediately after particle entry into cells but does not require transcription of GFP RNA and expression of protein, which only occur following DNA transport into the nucleus and proviral integration. To remove contaminating DNA, HCVpp preparations were first treated with DNase and then purified by ultracentrifugation on a sucrose cushion. To look at the very early post-entry reverse transcription events, cells were only infected for 3h with DNase-treated pseudoparticles before DNA was extracted for q-PCR analysis.

As shown in Figure 3-5, VSVpp entered Huh7 cells most efficiently and also entered HepG2 cells, albeit less efficiently. Interestingly, although NEpp entered Huh7 cells considerably less efficiently than VSVpp, they were taken up to some extent by these cells, whereas entry of NEpp into HepG2 cells was below detectable levels, suggesting that Huh7 cells support particle entry more readily. The uptake of VSVpp into PBMCs was very low but nonetheless was sufficiently higher than the uptake of NEpp into PBMCs to provide evidence of some level of glycoprotein-mediated particle entry. There was some evidence of HCV glycoprotein-mediated particle entry into Huh7 cells, as HCVpp entered these cells slightly more efficiently than NEpp. However, uptake of pseudoparticles into PBMCs was extremely inefficient (with fewer copies of GFP being detected per cell in HCVpp-treated PBMCs than HCVpp-treated HepG2 cells).

Given the low levels of entry of even the positive control VSVpp into PBMCs, the HCVpp system was not suitable for evaluating the potential for HCV to infect PBMC subsets. Further experiments were thus carried out using the HCVcc system.

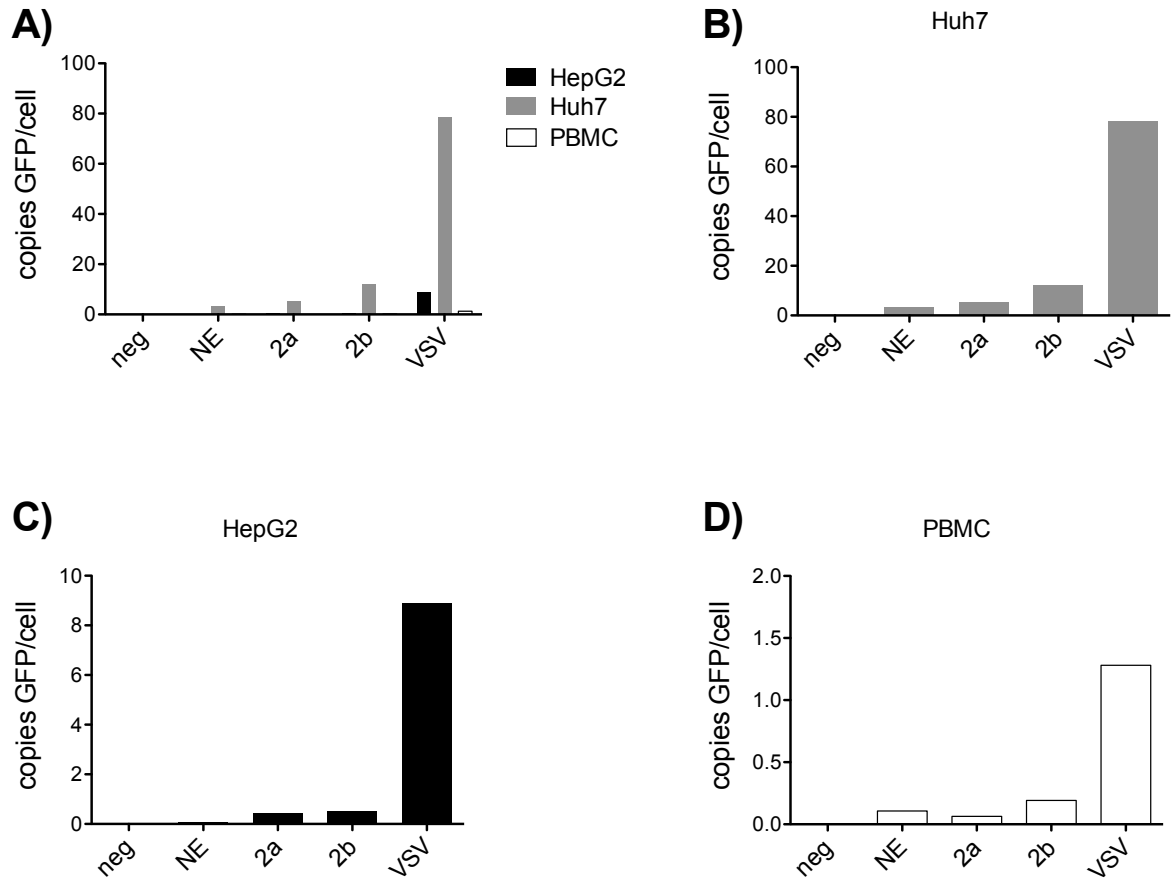

Figure 3-5 Reverse transcription of GFP RNA in hepatocyte lines and PBMCs after infection with HCVpp

Huh7 cells, HepG2 cells and PBMCs were infected with VSVpp (VSV), HCVpp from two gt JFH-1 (2a) and 2b1.1 (2b), or control particles not containing viral glycoproteins (NE) for 3h, or were left uninfected (neg). The inoculum was then removed and cells were washed and trypsinised. Genomic DNA was isolated from these cells and subjected to quantitative PCR analysis in triplicate. Levels of GFP and albumin were quantified and GFP levels were normalised against albumin DNA copies to give copies of GFP per cell. The copies of GFP per cell in A) all cell types, B) Huh7 cells, C) HepG2 cells and D) PBMCs are depicted.

3.4 Infection of PBMC with HCVcc

Another approach for evaluating the potential for HCV to infect PBMCs involves the use of the HCVcc system. The ability of HCVcc bearing glycoproteins from a hepatotropic strain (J6/JFH-1) to infect PBMCs was evaluated and infection of PBMCs with HCVcc bearing glycoproteins form a putatively lymphotropic strain (SB/JFH-1) was also assessed.

3.4.1 Infections with J6/JFH-1

The J6/JFH-1 chimeric virus was produced by electroporation of Huh7.5 cells with *in vitro* transcribed RNA from the J6/JFH-1 plasmid, a map of which is shown in Figure 2-3. Expression of NS5A in the transfected cells was evaluated on day 2 post-electroporation by immunofluorescence and FACS staining (Figure 3-6).

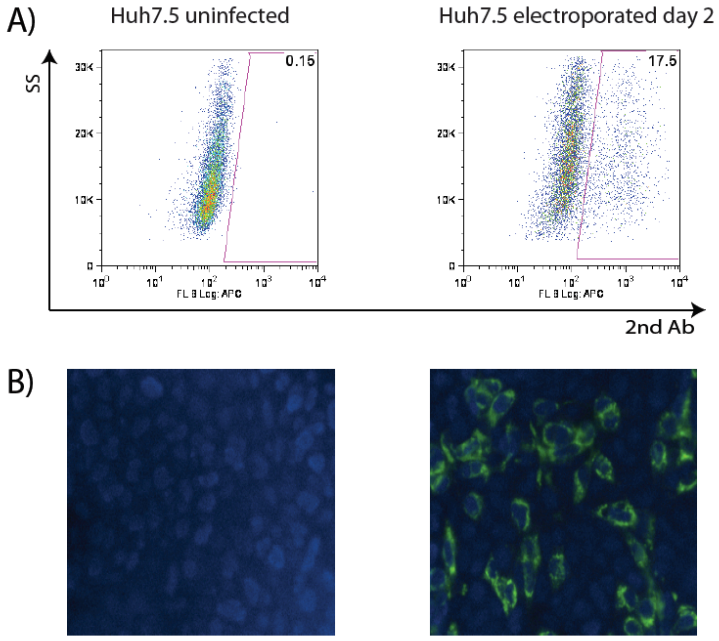

Figure 3-6 Expression of NS5A protein in Huh7.5 cells electroporated with J6/JFH-1 RNA

Huh7.5 cells were electroporated with *in vitro* transcribed RNA and left for two days. Cells were then permeabilised, stained with an anti-NS5A antibody and analysed by flow cytometry (A) or immunofluorescence (B). In B, the blue staining represents the nuclei as stained by DAPI, and the green is NS5A staining.

J6/JFH-1 chimeric virus was harvested from the supernatant of transfected cells and concentrated and purified by ultracentrifugation on a sucrose cushion. Virus prepared in this way was used to infect HepG2, Huh7.5, total PBMCs (stimulated with IL-2 and IL-4 to enhance *in vitro* survival) and MDDCs. Cells were infected for 2 days before the inoculum was removed and NS5A expression was analysed by FACS staining on day 2, day 4, day 6 and day 9. As seen in Figure 3-7, an increase in the percentage of NS5A+ Huh7.5 cells but this was not seen with any of the other cell types.

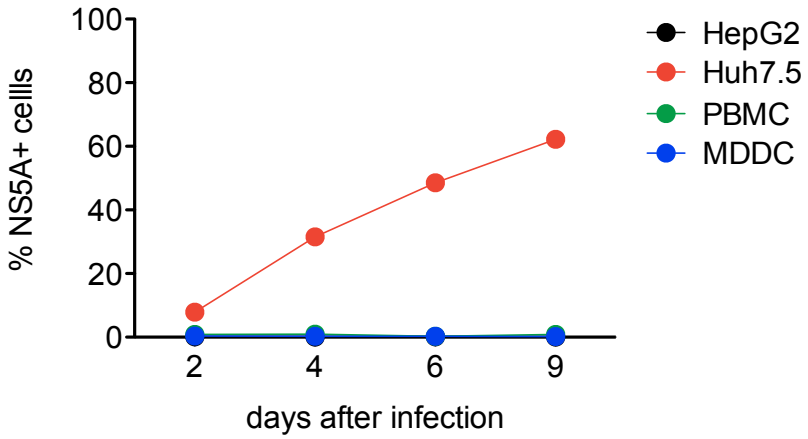

Figure 3-7 HCV NS5A expression in HepG2 and Huh7.5 cells, PBMCs and MDDCs after infection with HCVcc (J6/JFH-1)

HepG2 and Huh7.5 cells, PBMCs and MDDCs were infected with J6/JFH-1 virus at a MOI of 0.1. On the indicated days, cells were permeabilised and stained with NS5A antibody 9E10 and a secondary anti-mouse IgG-APC antibody. Live cells were gated on and the percentage of cells expressing NS5A was determined. Results are representative of infection rates typically observed.

Since staining for the NS5A protein revealed no sign of on-going HCV replication in stimulated PBMCs or MDDCs, a RT-q-PCR approach was used to measure HCV RNA. If any RNA replication should occur in these cells, then one would expect an increase in HCV RNA over time. HCV has been shown to be associated with many PBMC subsets *in vivo*, so in order to determine in which immune cell subsets low-level RNA replication was most able to occur *in vitro*, PBMCs were sorted into four subsets by MACS bead purification: T cells, B cells, NK/NKT cells and monocytes. To prolong survival, T cells and NK/NKT cells were cultured in R10 + 50U/ml IL-2, B cells were cultured in R10 + 1μg/ml sCD40L + 50ng/ml IL-4 and monocytes were cultured in D10 + 50ng/ml IL-4 + 200ng/ml GM-CSF and were left to differentiate into MDDC. HepG2 and Huh7.5 cells and the four PBMC subsets were infected at a MOI of 0.01 for 1 day and then, after several washes, were cultured for 5 more days. RNA was extracted on days 0, 1, 2, 4 and 6 and viability of all cells was closely monitored. All PBMC subsets remained healthy over the 6-day time frame studied. In Huh7.5 cells, a time-dependent increase in HCV RNA was seen over the 6 days studied (Figure 3-8).

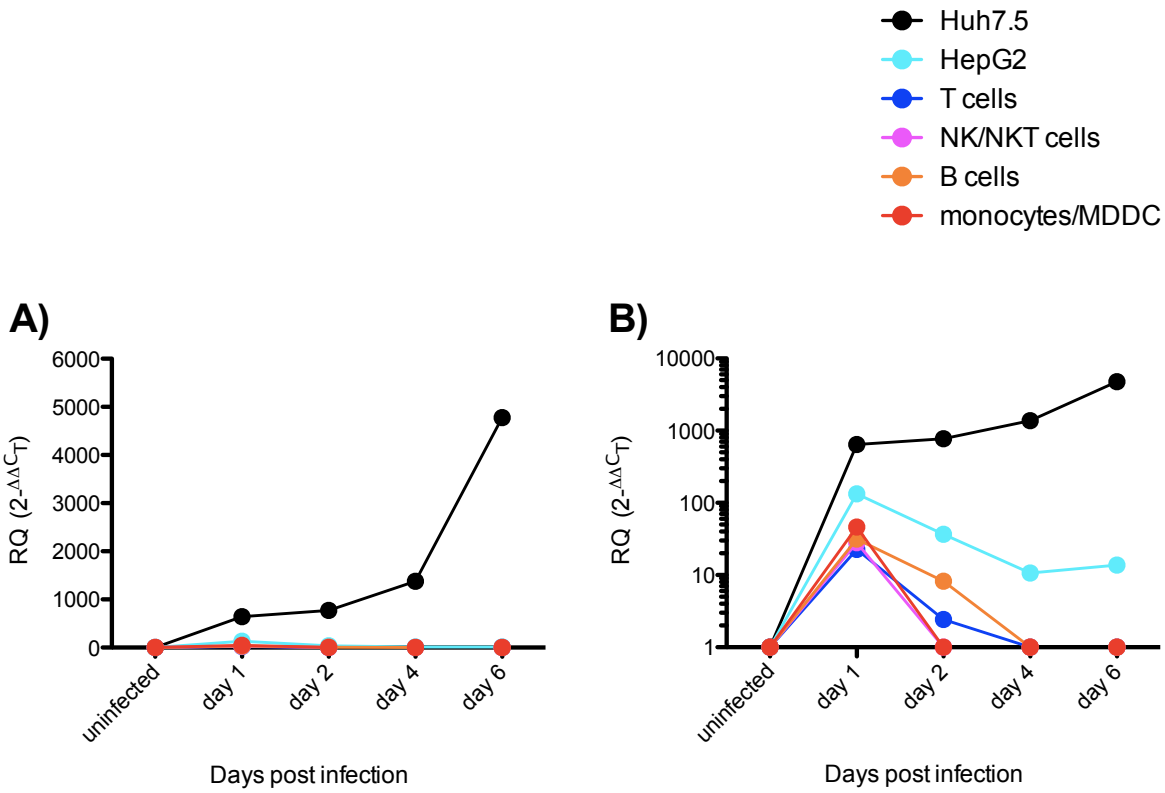

Figure 3-8 HCV RNA expression in Huh7.5 and HepG2 cells and PBMC subsets after infection with HCVcc (J6/JFH-1)

HepG2 and Huh7.5 cells, PBMC subsets were infected with J6/JFH-1 virus at a MOI of 0.01. On the indicated days RNA was isolated and subjected to RT-q-PCR. Levels of both GAPDH and HCV RNA were measured in triplicate and RQ was carried out using the uninfected sample of each cell type as an internal calibrator. RQ is plotted on a linear scale (A) and on a logarithmic scale (B).

Figure 3-8A, which depicts the increase of HCV RNA on a linear scale, shows clearly that no significant amount of RNA is associated with any of the other cell types. Figure 3-8B, which depicts the same results on a logarithmic scale, shows that there were low levels of HCV RNA associated with HepG2 cells and all the PBMC subsets after the initial infection period, but that these levels decline over time, indicating that the HCV strain J6/JFH-1 does not replicate in PBMCs.

3.4.2 Infections with SB/JFH-1

A recently constructed chimeric virus (SB/JFH-1) has been shown to infect B cell lines and primary B cells (unpublished data, Keigo Machida, presented at the international HCV conference in Yokohama 2010). According to the authors, this virus combines the

lymphotropic properties of the SB glycoproteins with the high replication of the JFH-1 virus in cell culture. Furthermore, it is reported to use CD86 rather than CLDN-1 as a receptor to enter lymphoid cells. A special Raji cell line, which has RIG-I and MDA-5 knocked down (Raji $^{\text{RIG-I/MDA-5 kd}}$), was used to propagate this virus.

Raji $^{\text{RIG-I/MDA-5 kd}}$ cells were electroporated with RNA *in vitro* transcribed from the linearised SB/JFH-1 plasmid and stained for NS5A expression 2 days post electroporation, but surprisingly no NS5A protein could be detected in the cells. The experiment was repeated several times, but NS5A was never detected. I then obtained some SB/JFH-1 virus from our collaborators to perform experiments. After trying to titrate the virus on Raji $^{\text{RIG-I/MDA-5 kd}}$ cells to determine the TCID50, it became clear that even the Raji $^{\text{RIG-I/MDA-5 kd}}$ cells that were infected with the most concentrated preparation of the virus did not express any NS5A protein after a 3-day infection period. To see whether there was any virus in the preparations, the titre of the virus was measured by a clinical HCV assay. The titre of the virus as measured by the clinical testing lab was only 1.6×10^4 copies/ml, which was 100-1000 lower than the titre of the J6/JFH-1 dilution typically used to infect cells. The infectious titre of HCV preparations is 100-1000 times lower than the RNA copy count [12,383,202]. The quantity of infectious virus in the preparation was thus probably extremely low.

In a next step, Raji $^{\text{RIG-I/MDA-5 kd}}$ cells (and HepG2 and Huh7.5 cells) were infected for a total of 6 days (with 2 days of contact) with undiluted SB/JFH-1 virus and in parallel with J6/JFH-1 virus at an MOI of 0.01 (corresponding to 100 times more copies/ml than in the SB/JFH-1 preparation). HCV RNA levels were then quantitated by RT-q-PCR. As expected HepG2 cells did not show any signs of infection with either virus (Figure 3-9). Huh7.5 cells had very high levels of associated J6/JFH-1 RNA and no SB/JFH-1 RNA. Interestingly, quantitation of HCV RNA in the infected Raji $^{\text{RIG-I/MDA-5 kd}}$ cells revealed that there were low levels of J6/JFH-1 RNA and high levels of SB/JFH-1 RNA associated with these cells. These

results indicated that SB/JFH-1 might, as reported, preferentially infect cells of the lymphoid lineage.

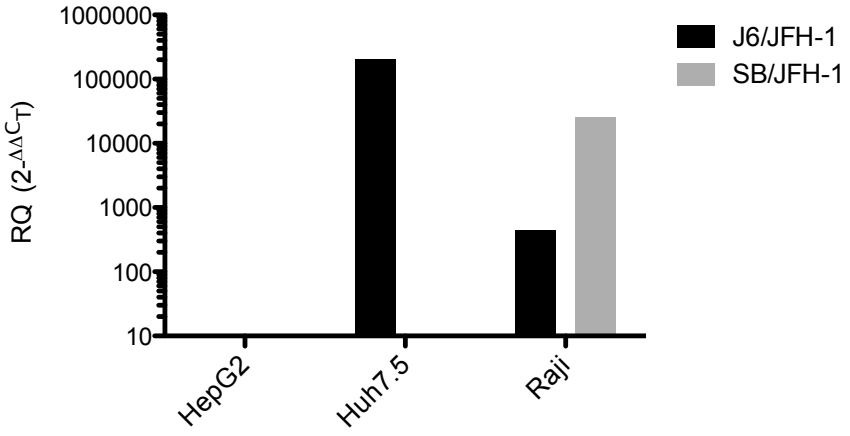

Figure 3-9 Expression of HCV RNA in HepG2, Huh7.5 and Raji$^{RIG\text{-}I/MDA\text{-}5\ kd}$ cells after infection with HCVcc (J6/JFH-1 and SB/JFH-1)

HepG2, Huh7.5 and Raji$^{RIG\text{-}I/MDA\text{-}5\ kd}$ cells were infected with J6/JFH-1 virus at an MOI of 0.01 ($1.6x10^6$ copies/ml) or with undiluted SB/JFH-1 ($1.6x10^4$ copies/ml). At the indicated days RNA was isolated and subjected to RT-q-PCR. Levels of both GAPDH and HCV RNA were measured in triplicate and RQ was carried out using the uninfected sample of each cell type as an internal calibrator.

An experiment using sorted PBMC subsets similar to that performed with the J6/JFH-1 virus (as in Figure 3-8) was then carried out. No SB/JFH-1 RNA could be detected in any of the infected cells including the Raji $^{RIG\text{-}I/MDA\text{-}5\ kd}$ cells. Unfortunately, there was not enough SB/JFH-1 virus to perform infections with undiluted virus, so the virus had to be diluted ten-fold in order to have enough SB/JFH-1 virus for all the cell types and timepoints. Also the contact time of the virus with the cells in this experiment was shorter (o/n as opposed to 2 days) than the contact time of the experiment with the cell lines (Figure 3-9).

In conclusion, although there was a significant amount of SB/JFH-1 RNA associated with Raji $^{RIG\text{-}I/MDA\text{-}5\ kd}$ cells after a long infection with undiluted virus (Figure 3-9), the virus titre was very low and further dilution of this virus resulted in an absent RNA signal, when PBMC subsets were infected with diluted SB/JFH-1 virus. I was thus unable to answer the question whether this virus can productively infect cells of the hematopoietic lineage.

3.5 Discussion

Many viruses that persist in their hosts are able to infect cells of the immune system, sometimes in addition to other cell types that constitute the principal sites of virus replication [275]. This may have deleterious consequences for the host immune response, as the infected cells may be destroyed by the virus or by antiviral effector mechanisms, or their functions might be impaired or modulated. Infection of immune cells may be one mechanism by which HCV modulates the host immune response, as HCV has been found to be associated with several PBMC subsets *in vivo* [123-126].

In this chapter, the potential for HCV to infect PBMCs was thus assessed. To find out which PBMC subsets might be most susceptible to HCV infection, surface expression of all known HCV entry and attachment factors (apart from CLDN-9) was evaluated on T cells, B cells, NK cells, NKT cells, monocytes, mDCs, pDCs and MDDCs. To date, four HCV receptors have been shown to be required for HCV infection, CD81, SR-BI, CLDN-1 and OCLN [54-58]. Staining of these receptors showed that none of the PBMC subsets analysed expressed all four known required entry factors. Specifically CLDN-1 expression could not be detected in any of the subsets, in line with other studies that could not or only detect very low levels of CLDN-1 RNA [138] or protein [384]. Since two studies showed that in addition to CLDN-1, CLDN-6 and CLDN-9 could also mediate HCV infection [65,66], expression of these factors was also of interest to analyse. No CLDN-6 expression could be detected on any of the cells analysed, although previous reports indicated that low levels of CLDN-6 RNA could be detected in PBMCs [65,138]. Unfortunately, no antibody known to be suitable for flow cytometry was available for CLDN-9, and furthermore a cell line expressing CLDN-9 (Bel7402) could not be obtained to test other staining protocols. This was very unfortunate, since two studies indicated that CLDN-9 RNA could also be detected at higher levels in PBMCs than both CLDN-1 and CLDN-6 [65,138]. If CLDN-9 protein were indeed present on PBMCs, then this would possibly make subsets like monocytes, DCs and possibly B cells susceptible to HCV

entry, as these cells expressed the three other HCV receptors. As reported by other studies [88,138,384], CD81 was expressed universally on all PBMC subsets and MDDCs, and OCLN was also found on all subsets (although sometimes only a fraction of cells seemed to express the receptor). As CD81 and OCLN were present on all cell subsets isolated *ex vivo*, SR-BI expression is the other factor likely to determine HCV entry into PBMC subsets. I found that SR-BI was expressed on monocytes and MDDC at considerable levels and only at very low levels on B cells, mDCs and pDCs. Likewise, other studies have shown that SR-BI (RNA and protein) is expressed in monocytes, DCs and MDDCs [88,138,384], whereas SR-BI (as opposed to SR-BII) RNA was not detected in B cells in one study [138], but protein expression could be detected in another study at low levels [376].

Together, my results and those of published studies addressing HCV receptor expression on PBMC subsets suggest that the PBMC types, into which HCV is likely to gain entry using the usual receptors, are monocytes, DCs and B cells. Although HCV has been found to be associated with these cell types *in vivo*, HCV has also been found to be associated with T cells, which only express CD81 and OCLN, raising the possibility of the existence of HCV variants that use alternative receptors to those used by the hepatotropic strains.

A system that can be used to assess entry (rather than later steps in the HCV life cycle) is the HCVpp system [9,10]. Stimulated PBMCs (and HepG2 and Huh7.5 cells) were infected with MLV-based HCVpp, VSVpp and NEpp and reverse transcription of the reporter gene was measured by q-PCR. Unfortunately, even entry of VSVpp into PBMCs was so inefficient compared to the cell lines that this system could not be used to study the ability of HCV E1E2 to mediate entry into PBMCs. Furthermore, contrary to previous publications, I found that the capsid content varied considerably between the pp preparations, complicating interpretation of the results. Several other studies have shown that HCVpp pseudotyped with certain glycoproteins do not infect cell lines of the hematopoietic lineage [9,117,136,138] but the results are less clear for primary blood cells. In the two studies where lentiviral HCVpp were

used to infect PBMCs, reporter gene expression in PBMCs infected with the positive control particles (MLVpp or VSVpp) was substantially lower than in cell lines [136,138], so these results cannot be interpreted with confidence, although it seems unlikely that HCVpp would infect PBMCs but not their cell line equivalents.

Even if certain PBMC subsets do express the four principal HCV entry factors (CD81, SR-BI, OCLN and possibly CLDN-9) and could therefore support HCV entry, there could also be additional blocks to HCV infection. Additional factors can negatively influence HCV entry, replication or translation. It was shown that expression of EWI-2wint on permissive host cells inhibited HCV entry by blocking the interaction between CD81 and E1E2 [385]. Furthermore, presence or absence of certain microRNAs can block or facilitate viral replication. For example, microRNA-122 (miR-122), a microRNA specifically expressed in the liver, stimulates both HCV replication and translation [79,386]. HCVcc provides a system by which HCV replication in PBMC subsets can be explored *in vitro*. In support of the theory that a replication/translation factor might be lacking in PBMCs, experiments performed by Marukian *et al.*, which were published while my work was in progress, showed that HCVcc (JC-1) did not productively infect any PBMC subsets and that even when JC-1 RNA was transfected into monocyte-derived macrophages or MDDCs, RNA titres did not rise within these cells in the 72h period studied [138]. The results from this chapter with the J6/JFH-1 virus point to a similar conclusion. Although some J6/JFH-1 RNA was associated with different PBMC subsets directly after the infection period, the levels declined over time, presumably as the virus was degraded. The levels and kinetics of HCV RNA expression in HCVcc-exposed HepG2 cells, a liver cell line that lacks CD81 expression and is not susceptible to HCV infection, showed a similar pattern to those of the different PBMC subsets, arguing for association by binding rather than infection. PBMCs express high levels of CD81 and varying amounts of some of the other receptors that can bind to virus particles. Furthermore, MDDCs

express DC-SIGN and monocytes and NK cells express LDL-R, two further receptors known to bind to and capture HCV [50,51].

In summary, my results and those of others show that PBMCs (and lymphoid cell lines) cannot be infected by several HCV strains that were selected on the basis of their ability to infect hepatocyte cell lines, e.g. JFH-1. However the possibility remains that within an infected patient there might be lymphotropic variants. In support of this, variants with different sequences have been found in PBMCs as compared to liver or serum [380-382] and HCV has been grown in B cell lines established from patients with CHC infection [132,133]. One of these lymphotropic viruses, SB-HCV, has recently been cloned and a chimeric virus (SB/JFH-1) has been made (Machida *et al*, unpublished data). The SB/JFH-1 virus I received from Keigo Machida was of very low titre ($1.6x10^4$ copies/ml) and infection of Raji $^{RIG\text{-}I/MDA\text{-}5\ kd}$ cells did not result in any NS5A expression, although a lot of associated RNA could be found in the cells after prolonged exposure to undiluted virus and a 6 day infection period. The same infection experiments were done with this virus as with the J6/JFH-1 virus, but unfortunately the dilution of the virus resulted in undetectable levels of HCV RNA not only in all the PBMC subsets but also in the Raji $^{RIG\text{-}I/MDA\text{-}5\ kd}$ cells, leaving the question, as to whether productive infection of PBMCs can occur using this virus, unanswered. Had time allowed, I would have tried to make more of this virus and the experiment would have been repeated using more virus and longer contact times. However production of sufficient virus to perform experiments of this type would probably have been difficult, as SB and its chimeras can only be produced to low titres (Dr. Machida, personal communication). Furthermore I was unable to detect any NS5A protein in either the electroporated or the infected Raji $^{RIG\text{-}I/MDA\text{-}5\ kd}$ cells, making the prospects of amplifying viral stocks successfully seem unlikely. Whether this virus can really be used as a good system to study HCV infection of PBMCs is thus questionable. To address HCV infection of PBMC subsets in future it is important to try to characterise possibly lymphotropic variants better. For this, more viruses associated with

PBMC subsets *in vivo* should be amplified, cloned and cultured in hematopoietic cell lines. Since good replication of HCVcc in cell culture has only been achieved using a JFH-1 backbone, efforts could be directed towards making chimeras that contain the structural proteins from the newly cloned viruses and the non-structural proteins from JFH-1.

In conclusion, better characterisation of the ability of HCV to infect PBMC subsets would be beneficial in several scenarios, such as design of antiviral drugs that block entry or in an LT setting, where PBMC might represent an additional reservoir. Even if HCV does not productively infect immune cell subsets, it may mediate immunomodulatory effects through binding of viral components to host cell receptors, or via indirect mechanisms, e.g. stimulating secretion of soluble factors. To address this, immunomodulatory effects of HCV, HCV glycoproteins or HCV-infected cells on NK cells and DCs were explored *in vitro* in the following chapters.

4 MODULATION OF NK CELL RESPONSES BY HCV *IN VITRO*

In the previous chapter, the hypothesis that HCV glycoproteins enable viral entry into PBMCs was addressed, which could potentially result in targeting of these cells for destruction by the host immune response or impairment if their responses by viral proteins. It has also been suggested that HCV proteins might modulate the function of immune cells. Here the hypothesis that viral particles make contact with immune cells via their glycoproteins and hence modulate the functions of these cells was addressed. This chapter focuses on immunomodulatory effects of HCV on NK cells and chapter 5 on the effects of HCV on DCs.

4.1 Introduction

NK cells play an important role in many viral infections [387]. They can combat viral replication directly by mediating lysis of infected cells via exocytosis of granules containing perforin and granzymeB or via engagement of death receptors such as TRAIL or FasL with ligands on the target cell, or by producing effector cytokines, such as IFN-γ and TNF-α [210] and mediate immunoregulatory functions. Their triggering to mediate effector functions on contact with virus-infected cells is controlled by the balance of signals received through activating and inhibitory receptors that interact with ligands on the target cell. Factors that promote NK cell activation include upregulation of stress molecules, alterations in HLA class I expression and expression of viral glycoproteins on the target cell [225-229]. The activation state and functional properties of NK cells are regulated by cytokines, including type I IFNs, IL-2, IL-12, IL-15 and IL-18 [241].

There is evidence from both studies with HCV-infected subjects and *in vitro* studies that NK cells may contribute to control of HCV infection. It was found that expression of KIR2DL3 in combination with its ligand HLA-C1 was associated with enhanced spontaneous clearance of

HCV and SVR after treatment [219-222]. Furthermore, in a study of a single-source exposure, it was shown that resolvers had higher NK cell numbers and expressed more CD16 and less NKG2A/C/E on their NK cells than non-resolvers [224] and also showed heightened TRAIL expression on IFN-α-stimulated cells [211]. Also, in a study using IDUs, it was shown that EU individuals had a higher proportion of cells expressing NKp30 than EI individuals at a pre-infection time point [234]. Studies using *in vitro* systems showed that NK cells exhibited increased cytotoxicity towards HCV-infected cells [243] and that NK-derived IFN-γ and high NKp30 expression inhibited HCV replication in liver cell lines [234,212,213].

If NK cells were important in mediating clearance of HCV, then impairment of their effector functions would allow HCV to cause a persistent infection. While it seems that in acute HCV infection NK cells have a more activated phenotype, produce more IFN-γ and are more cytotoxic than those of healthy controls [237,350], there is some evidence that NK cells might be impaired in CHC infection. Most consistently, reduced NK cell numbers and skewing of the NK subsets towards fewer $CD56^{dim}$ and more $CD56^{bright}$ NK cells have been reported [337-342], but there is on-going controversy in the literature concerning the impairment of phenotype and function of NK cells in chronically HCV-infected subjects (as reviewed in 1.5.2.2).

It was reported by two groups that engagement of antibody-mediated crosslinking of CD81 on NK cells inhibited their response to activating stimuli including CD16 [352,353]. Furthermore it was shown that this inhibition could also be mimicked by CD81 crosslinking via sE2. Likewise, crosslinking of CD81 on T cells by immobilised sE2 was also shown to mimic the effects of CD81 signalling in these cells [388], although in T cells binding of E2 to CD81 provided a costimulatory signal for human T cells. The costimulatory effect of CD81 signalling on T cells (and B cells) is well documented in the literature [151,388-391].

In this chapter, I set out to determine whether E2 expressed on whole virus particles would inhibit NK cell activation in response to CD16 crosslinking and other signals in the same way as arrays of immobilised E2 had been reported to do. During the project, two publications

examining the role of HCVcc on NK cell function were published [354,214], the results from which are discussed and compared to my findings at the end of this chapter.

4.2 Interaction of HCV particles with NK cells

As mentioned above, reports suggested that CD81 crosslinking by anti-CD81 antibody or immobilised sE2 could costimulate T cell responses [388] and downregulate NK activation [352,353]. Hence, I set out to assess whether E2 expressed on viral particles induces immunomodulation via CD81 crosslinking on NK cells, using T cells in parallel as a control system, as the functions of CD81 on T cells have been more extensively characterised than CD81 functions on NK cells.

4.2.1 Effect of antibody-mediated CD81 crosslinking on T cell activation

Before proceeding to assess the effects of HCV E2-mediated crosslinking of CD81 on NK and T cells, initial experiments were performed, in which CD81 was crosslinked using an anti-CD81 mAb, to optimise the assay system and provide a positive control for further experiments. The anti-CD81 mAb used was the JS-81 clone, which was shown to costimulate T cell responses and downregulate NK cell activation. This antibody is also able to neutralise binding of E2 to CD81 [54], suggesting that it binds to CD81 at the same or an overlapping epitope as E2 and hence may function as a surrogate for E2.

Purified T cells were incubated on plates coated with different concentrations of anti-CD3 mAb, alone or together with anti-CD81 mAb or an isotype-matched control IgG1 mAb. T cell activation was assessed 24h later by measuring the percentage of cells that had upregulated the early activation marker CD69 by flow cytometry. The results showed that T cells were activated by the anti-CD3 mAb in a dose-dependent manner, and that their activation was slightly (but not significantly) enhanced by addition of the control IgG1 mAb and was more strongly costimulated by addition of the anti-CD81 mAb (Figure 4-1).

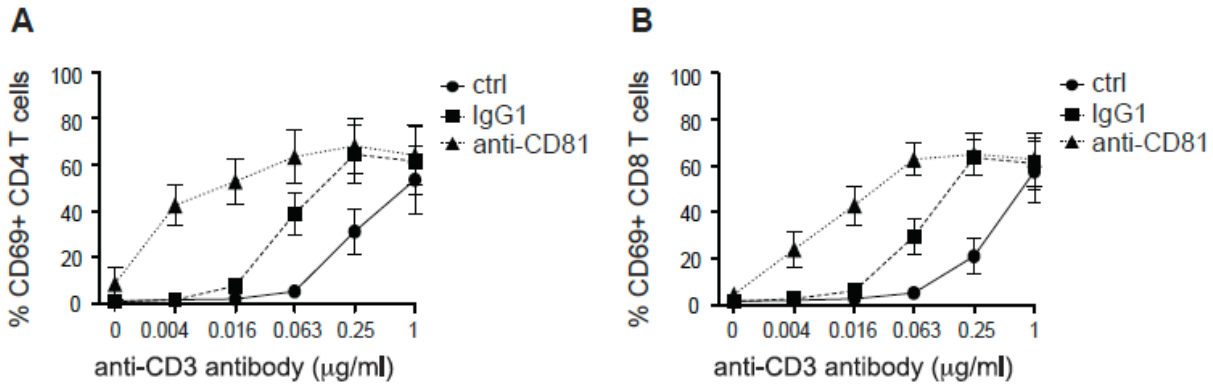

Figure 4-1 CD81 crosslinking by anti-CD81 antibody costimulates T cell activation

A) CD4 T cell and B) CD8 T cell activation (percentage of cells expressing CD69) following stimulation of purified T cells in plates coated with the indicated concentrations of anti-CD3 mAb alone (circles), or in combination with anti-CD81 mAb (triangles) or a control IgG1 mAb (squares) at 1µg/ml. The data shown are an average of results from 5 donors obtained in 3 independent experiments and the error bars indicate the standard error of the mean (SEM).

For CD4 T cells, the anti-CD81 mAb significantly enhanced T cell activation as compared to the control IgG1 mAb at the two lowest concentrations of anti-CD3 mAb ($p<0.0001$ for both 0.004 and 0.016µg/ml). For CD8 T cells, the anti-CD81 mAb significantly enhanced T cell activation as compared to the control IgG1 mAb at the three lowest concentrations of anti-CD3 mAb (($p<0.05$ for 0.004µg/ml, $p<0.0001$ for 0.016µg/ml and $p<0.001$ for 0.063µg/ml). CD25 upregulation was also assessed in this and all following experiments with T cells, and results were similar (data not shown).

Overall, these results show that antibody-mediated crosslinking of CD81 results in costimulation of both CD4 and CD8 T cell activation and are hence in agreement with the findings reported in previous studies [393-396].

4.2.2 Effect of antibody-mediated CD81 crosslinking on NK cell activation

Further experiments assessed the effect of antibody-mediated crosslinking of CD81 on NK cell activation in response to CD16 stimulation. Purified NK cells were incubated on plates coated with different concentrations of anti-CD16 mAb, alone or together with anti-CD81 mAb or an isotype-matched control IgG1 antibody, and NK cell activation was measured by assessing upregulation of the early activation marker CD69 24h later. The results showed that

the NK cells were activated in a dose-dependent manner by the anti-CD16 mAb and that their activation was slightly (but not significantly) enhanced by the control IgG1 mAb (Figure 4-2).

There was a significant inhibitory effect of the anti-CD81 mAb compared to the control IgG1 mAb at the concentration of 0.25μg/ml anti-CD16 mAb ($p<0.05$), but overall the inhibitory effect of the anti-CD81 mAb was a lot less pronounced than its costimulatory effect on T cells. The results were also in agreement with the results from prior publications [352,353].

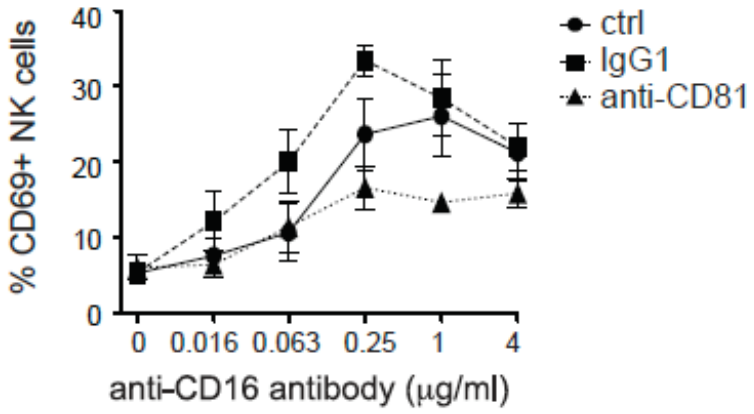

Figure 4-2 CD81 crosslinking by anti-CD81 antibody slightly inhibits NK cell activation

NK cell activation (percentage of cells expressing CD69) following stimulation of purified NK cells in plates coated with the indicated concentrations of anti-CD16 mAb alone (circles), or in combination with anti-CD81 mAb (triangles) or a control IgG1 mAb (squares) at 1μg/ml. The findings shown are an average of results from 4 donors in 2 independent experiments and the error bars indicate the SEM.

Interpretation of the results from the previous experiment was complicated by the fact that the control IgG1 mAb enhanced NK cell activation slightly (perhaps via interaction with CD16). Further experiments were thus performed using P815 cells, a more physiological system than the plate-bound antibody system. P815 is a mouse mast cell line that expresses Fc receptors on its surface and therefore displays bound antibodies with the Fab fragment oriented outwards, allowing specific interaction of the antibody with antigen on the NK cell (in addition to providing additional stimuli via the cell-to-cell contact). Purified NK cells were

incubated with P815 cells precoated with different concentrations of anti-CD16 mAb, alone or together with anti-CD81 mAb or an isotype-matched control IgG1 antibody at 10μg/ml. NK cell activation was assessed by measuring upregulation of CD69 expression, degranulation (CD107a expression) and IFN-γ production after an o/n incubation. Using this system, the anti-CD16 mAb was again found to stimulate NK cell activation in a dose-dependent fashion, and this was not enhanced by the control IgG1 mAb. Notably, the effect of CD81 crosslinking on NK activation in response to stimulation via CD16 was hardly evident in this system and was not statistically significant in any of the read-outs (Figure 4-3).

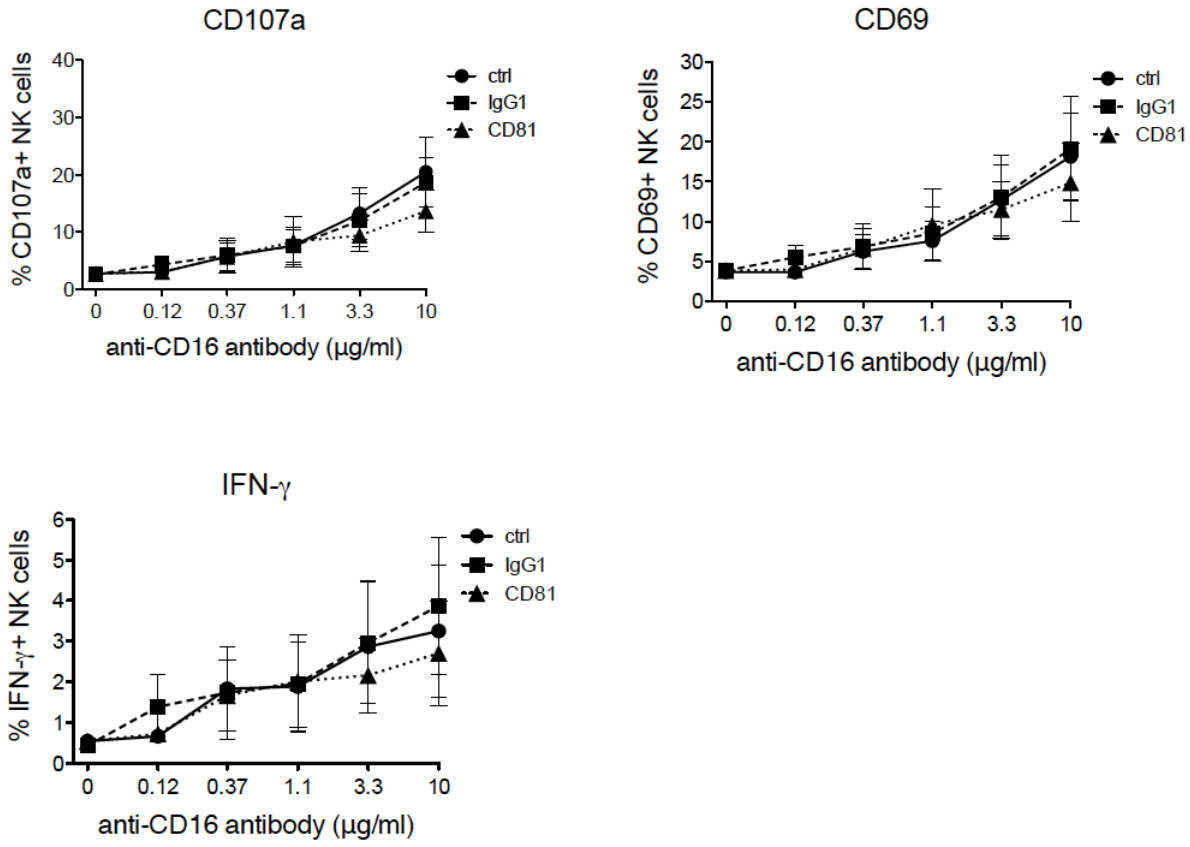

Figure 4-3 CD81 crosslinking does not significantly modulate NK cell activation in response to CD16 in the P815 system

NK cell degranulation (the percentage of NK cells expressing CD107a), activation (the percentage of NK cells expressing CD69) and cytokine production (the percentage of NK cells expressing IFN-γ) after incubation with P815 cells which had been pre-incubated with the indicated concentrations of anti-CD16 mAb only (circles), or in combination with anti-CD81 mAb (squares) or IgG1 control mAb (triangles) at 10μg/ml. The findings shown are an average of results from 5 donors in 2 independent experiments and the error bars indicate the SEM.

Previous reports also showed that IL-2, IL-12 and IL-15-mediated NK cell activation, cytotoxicity and IFN-γ production could be inhibited by antibody-mediated CD81 crosslinking [352,353]. To see whether CD81 crosslinking mediated a more marked inhibition of NK cells in response to cytokine stimulation, total PBMCs were added to plates coated with an anti-CD81 mAb or an isotype-matched control IgG1 mAb and stimulated with increasing concentrations of IL-2, IL-12, IL-15, IL-18 or IFN-α, and the activation of NK cells within the PBMC population was measured 24h later by assessing CD69 expression on NK cells. NK cells exhibited a dose-dependent activation in response to each of the cytokines that was slightly but not significantly increased by the control IgG1 mAb (Figure 4-4). Contrary to the slightly inhibitory effect of the anti-CD81 mAb on NK cell activation through CD16, no effect of CD81 crosslinking could be detected here.

It is unknown whether CD81 crosslinking can modulate NK responses to other activating receptors. As NKp30 and NKp46 use the same signalling subunits and downstream signalling pathways as CD16, the effect of CD81 crosslinking on activation through these receptors was also analysed. Unless they are stimulated via CD16, resting purified NK cells require stimulation via at least two activating receptors to become cytotoxic [216]. To provide extra stimulation for the NK cells, in experiments where responses to NCR stimulation were evaluated, total PBMCs (rather than purified NK cells) were added to plates or P815 cells precoated with antibodies directed towards the NCRs. As shown in Figure 4-5, a moderate response could be observed to anti-NKp30 and anti-NKp46 antibody stimulation. Thus, the effect of CD81 crosslinking on NK cell activation via NKp30 and NKp46 was addressed using P815 cells coated with different concentrations of antibodies to the NCRs NKp30 and NKp46 alone or together with anti-CD81 mAb or an isotype-matched control IgG1 mAb. In contrast to the response observed to CD16 stimulation, the response elicited to NCR stimulation was much less pronounced, with expression of the degranulation marker CD107a

not reaching more than 10-20% at the highest concentration of anti-NCR antibodies used (Figure 4-6). No specific inhibitory effect of CD81 crosslinking on NK activation was observed, although the low level of response made it difficult to discriminate between different responses.

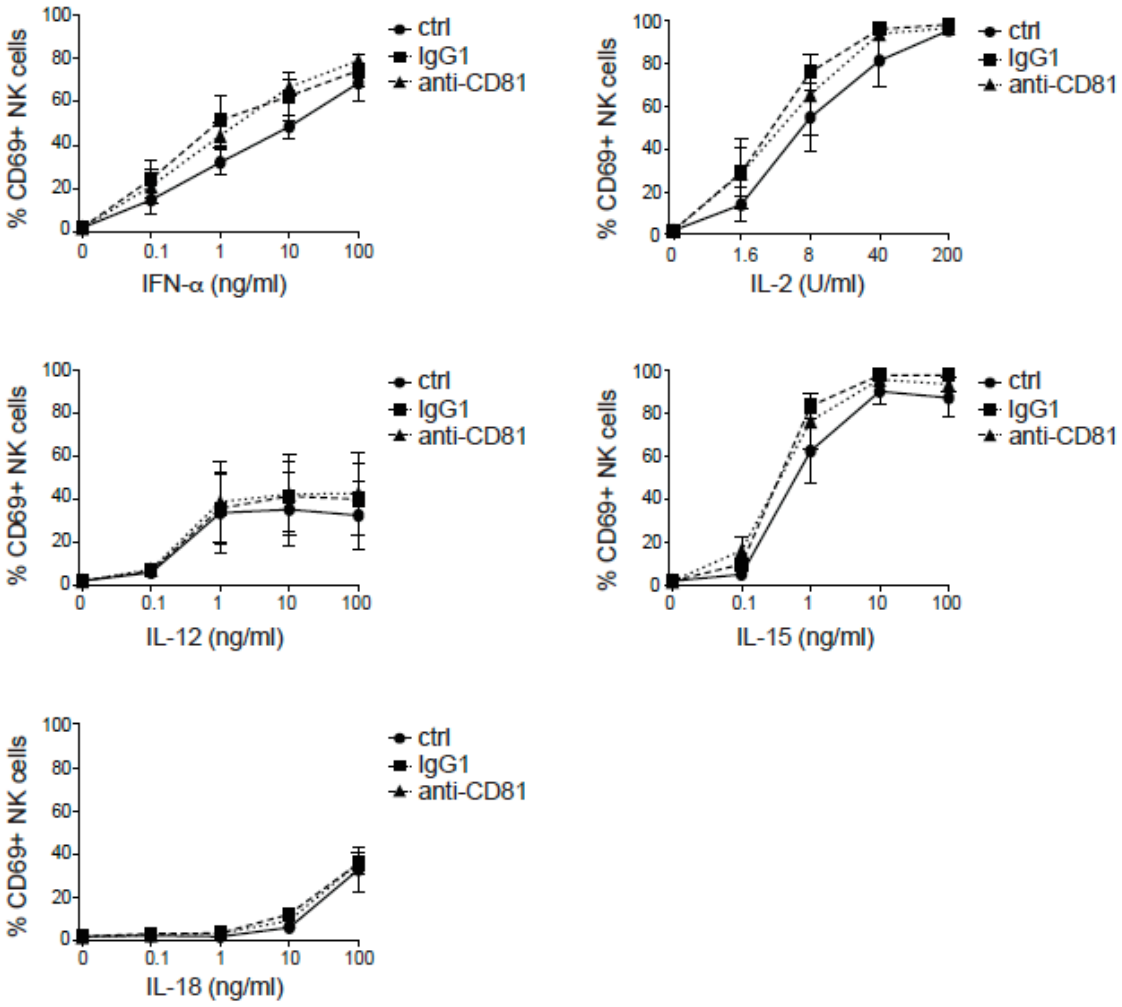

Figure 4-4 CD81 crosslinking does not modulate NK cell activation in response to stimulation with IFN-α, IL-2, IL-12, IL-15 and IL-18

NK cell activation (percentage of cells expressing CD69) following stimulation of total PBMCs in plates coated with the indicated concentrations of an anti-CD16 mAb alone (circles), or in combination with anti-CD81 mAb (triangles) or a control IgG1 mAb (squares) at 10μg/ml. The findings shown are an average of results from 3 donors in 2 independent experiments and the error bars indicate the SEM.

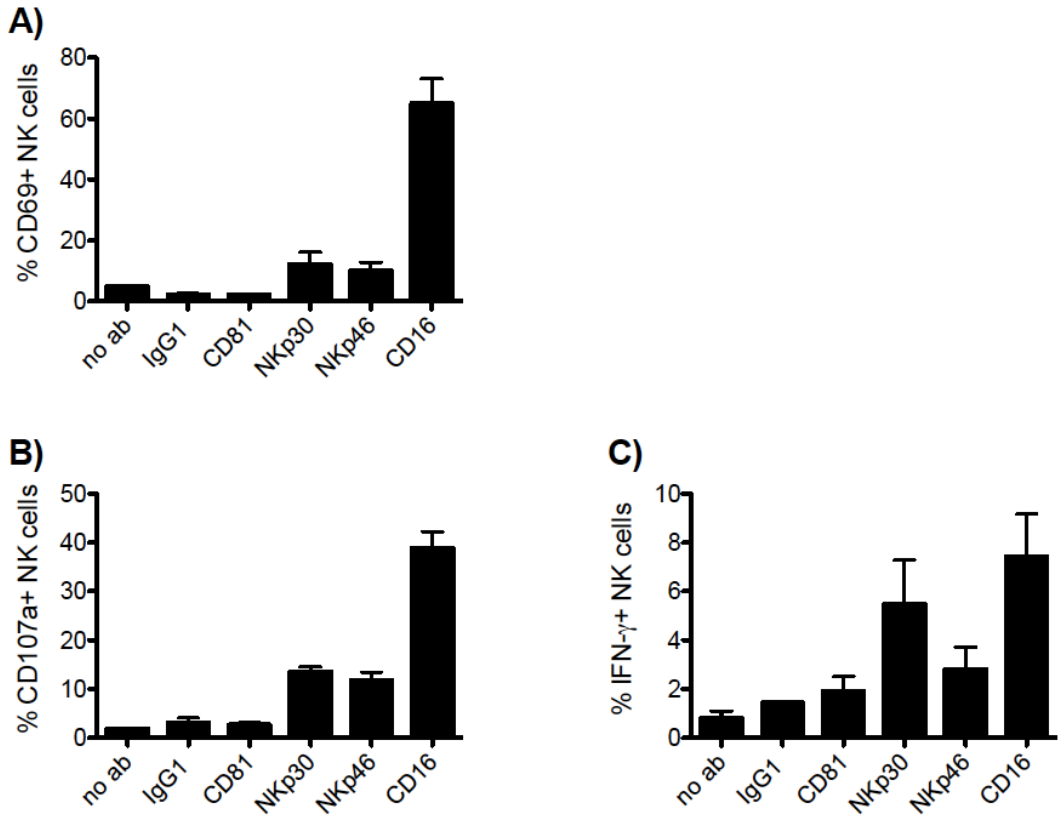

Figure 4-5 NK cell activation in response to stimulation via different activating NK receptors

A) NK cell activation (percentage of cells expressing CD69) following stimulation of total PBMCs in plates coated with the indicated antibodies at 10μg/ml o/n. B) NK degranulation (the percentage of NK cells expressing CD107a) and C) cytokine production (the percentage of NK cells expressing IFN-γ) after incubation with P815 cells which had been pre-incubated with the indicated antibodies at 10μg/ml o/n. The findings shown are an average of results from 2 donors and the error bars indicate the SEM.

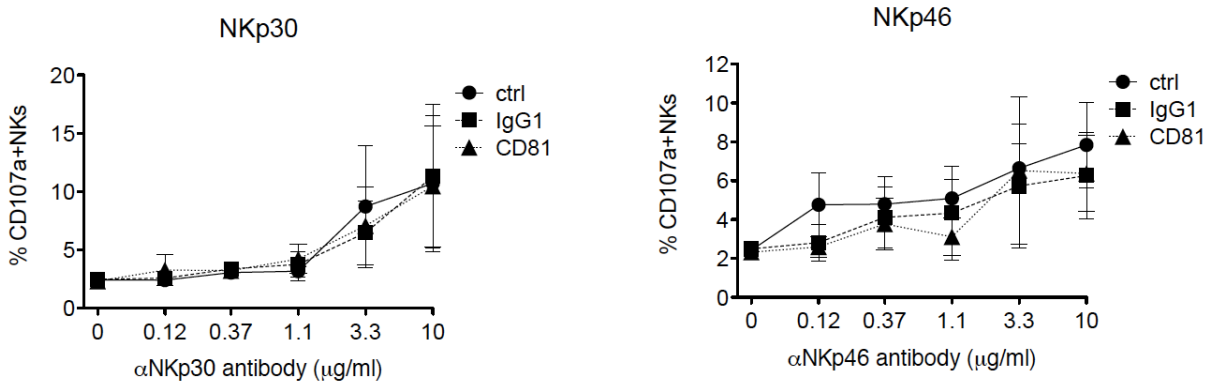

Figure 4-6 CD81 crosslinking does not modulate NK cell activation in response to NCR stimulation in the P815 system

NK degranulation (the percentage of NK cells expressing CD107a) after incubation with P815 cells which had been pre-incubated with the indicated concentrations of anti-NKp30 or anti-NKp46 mAbs alone (circles), or in combination with anti-CD81 mAb (triangles) or a control IgG1 mAb (squares) at 1μg/ml. The findings shown are an average of results from 2 donors in two independent experiments and the error bars indicate the SEM.

In summary, although the costimulatory effect of antibody-mediated crosslinking of CD81 on T cell activation through CD3 was readily reproduced in the system used here, the inhibitory effect of antibody-mediated crosslinking of CD81 on NK cell activation through CD16 proved much more difficult to confirm, with a very modest inhibitory effect being observed in the plate-bound CD16 antibody stimulation system, whilst CD81 crosslinking did not modulate activation through CD16 in the P815 system and also did not modulate NK cell activation by cytokines or NCRs.

4.2.3 Effect of whole virus particles on T and NK cell activation

Having established systems for evaluating the effects of CD81 crosslinking on T cell activation through CD3 and NK cell activation through CD16, the next step was to find out whether the effects of CD81 crosslinking by antibodies could also be mimicked by HCV particles. In a physiological situation one could envisage a T or NK cell being activated through interaction with an appropriate target cell, whilst viral particles simultaneously interact with CD81 on the cell surface. The effect of E2 as a part of viral particles on T and NK cell responses was assessed using HCVpp, HCVcc and plasma-derived HCV.

First, retroviral particles pseudotyped with E1E2 glycoproteins of different HCV clones (H77, JFH-1 and 2b_1.1) or not pseudotyped with any viral envelope proteins (NEpp) were used. Purified T and NK cells were added to plates coated with different concentrations of anti-CD3 mAb or anti-CD16 mAb, respectively. Pseudoparticle preparations, which had been purified on a sucrose cushion, were added to the cells at a 1/4 dilution or cells were just left in media as a control. As seen in Figure 4-7, no costimulatory effect of the HCVpp could be observed on T cells. In this and all following figures, data for CD8 T cells is shown, but similar results were obtained with CD4 T cells. For NK cells, a slightly costimulatory effect could be observed when HCVpp were added, but this effect was also observed after addition of NEpp, so was probably a non-specific effect mediated by stimulation via components of the pseudoparticle preparations rather than being a glycoprotein-mediated effect.

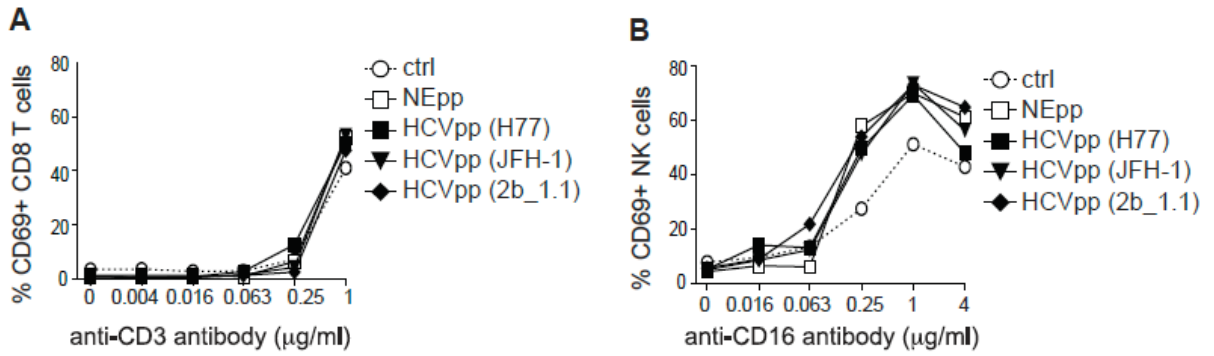

Figure 4-7 HCVpp do not influence T cell activation through CD3 or NK cell activation through CD16

A) CD8 T cell or B) NK cell activation (percentage of cells expressing CD69) following stimulation of purified T or NK cells in plates coated with the indicated concentrations of anti-CD3 or anti-CD16 mAb in the presence of medium only (open circles), or with pseudoparticles without viral glycoproteins (NEpp, open squares) or HCV pseudoparticles containing glycoproteins from three different clones (H77 filled squares, JFH-1 filled triangles and 2b_1.1 filled diamonds). Results shown are representative of findings made in two independent experiments using two different donors.

The effects of HCVcc on T and NK cell responses were then evaluated. Purified T and NK cells were added to plates coated with different concentrations of anti-CD3 mAb or anti-CD16 mAb, respectively. JC-1 (10^5 TCID50/ml, MOI 0.1), a control preparation or media only was added to the cells. Figure 4-8 shows that HCVcc did not costimulate CD4 (not shown) or CD8 T cell responses to CD3 or inhibit NK cell responses to CD16.

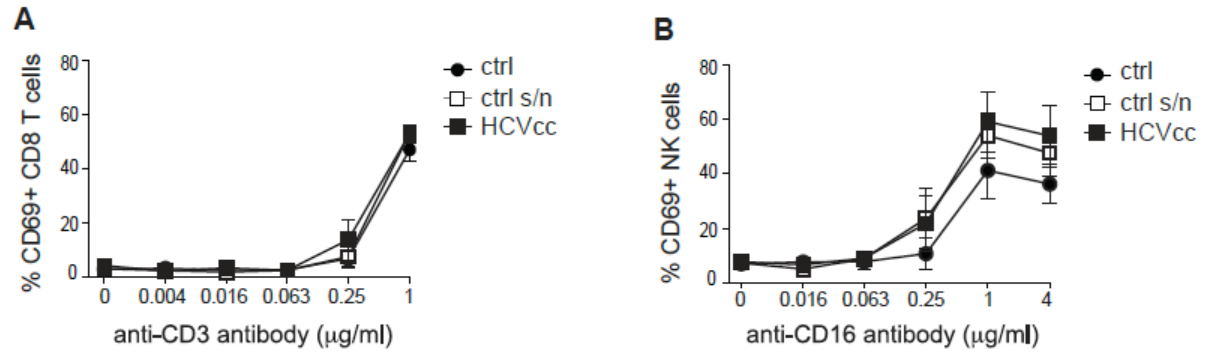

Figure 4-8 HCVcc does not influence T cell activation through CD3 or NK cell activation through CD16

A) CD8 T cell or B) NK cell activation (percentage of cells expressing CD69) following stimulation of purified T or NK cells in plates coated with the indicated concentrations of a anti-CD3 or anti-CD16 mAb in the presence of medium only (open circles), or with control supernatant (open squares) or HCVcc JC-1 (final concentration 10^5 TCID50/ml, filled squares). The findings shown are an average of results from 3 donors in 3 independent experiments and the error bars indicate the SEM.

In a final series of experiments, the effects of HCV-containing plasma from acutely infected and HCV seronegative plasma donors on T and NK cells were examined. To control for non-specific effects of the addition of plasma, pre-infection and post-infection plasma samples from the same plasma donor were used in parallel. The post-infection timepoint was chosen to have a very high HCV RNA titre and to be before seroconversion had taken place. As shown in Figure 4-9, the addition of plasma from both HCV+ and HCV- timepoints had very little effect on CD8 T cell responses to CD3 crosslinking (or on CD4 T cell responses, not shown).

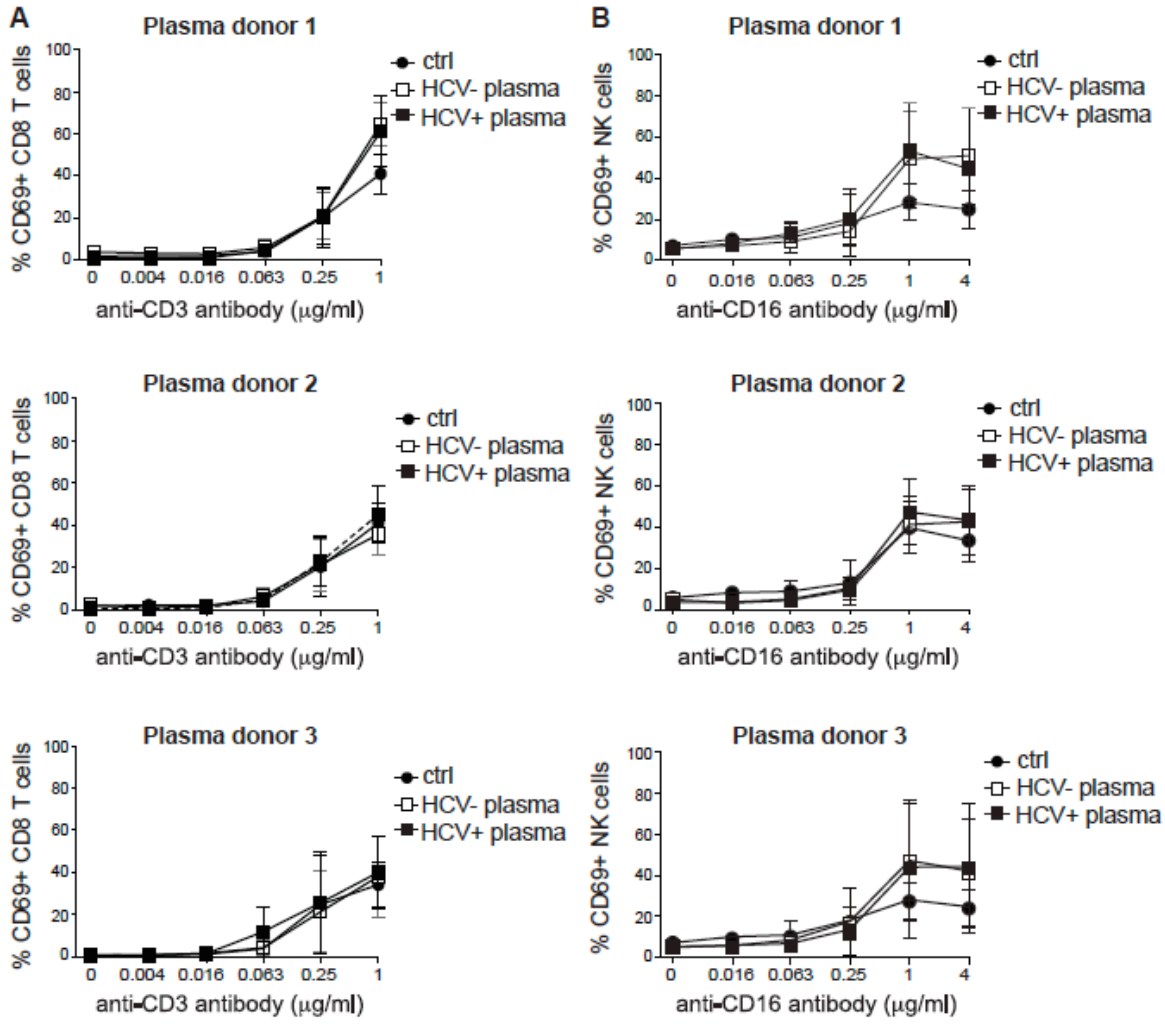

Figure 4-9 Plasma HCV does not influence T cell activation through CD3 or NK cell activation through CD16

A) CD8 T cell or B) NK cell activation (percentage of cells expressing CD69) following stimulation of purified T or NK cells in plates coated with the indicated concentrations of anti-CD3 or anti-CD16 mAb in the presence of medium only (open circles), or with HCV- plasma (open squares) or HCV+ plasma (final concentration 10^7copies/ml, filled squares) from the same donor. The findings shown are an average of results from 3 donors in 3 independent experiments and the error bars indicate the SEM.

Plasma from both HCV- and HCV+ timepoints (particularly for donors 1 and 2) had a slightly costimulatory effect on NK cell activation in all plasma donors, but no specific effect of the HCV+ plasma was apparent.

In summary, in this system, neither HCVpp, HCVcc, nor plasma-derived HCV replicated the effect of the anti-CD81 antibody on T or NK cells.

4.2.4 Effect of E2e on T and NK cell activation

As HCV glycoprotein-expressing particles were not found to have any significant effect on T or NK cell responses, I wanted to check whether, as previous reports had suggested, CD81 crosslinking by immobilised sE2 (derived from the H77 clone) could costimulate T cell responses [388] and downregulate NK activation [352,353].

The sE2 used in prior studies was purified from intracellular fractions of CHO cells transfected with a sE2 construct. The group and others had previously shown that I-E2 bound CD81 better than secreted E2 due to the more complex glycosylation pattern on secreted E2 [86,392], and showed that CD81 binding to E2 was independent of glycosylation, as deglycosylated E2 bound CD81 in the same way as the I-E2 [392]. From these data, it seemed that a simple glycosylation pattern favoured CD81 binding, so I made use of a novel system recently described in the literature [26], the E2e construct. This contains full-length E1 to ensure proper folding of E2e in the ER, and $E2_{715}$ lacking its transmembrane domain (Figure 1-3). E2e is produced in insect cells, which makes the E2 glycosylation pattern more uniform and simple. Two E2 ectodomains (H77 and 2b_2.8) were produced for use in these experiments, as it is known that other E2 proteins bind CD81 less well than H77 E2 [393]. Figure 4-10 shows the protein fractions of the two-step purification process (affinity chromatography using strep tag columns followed by gel filtration chromatography) resulting in pure monomeric fractions of E2.

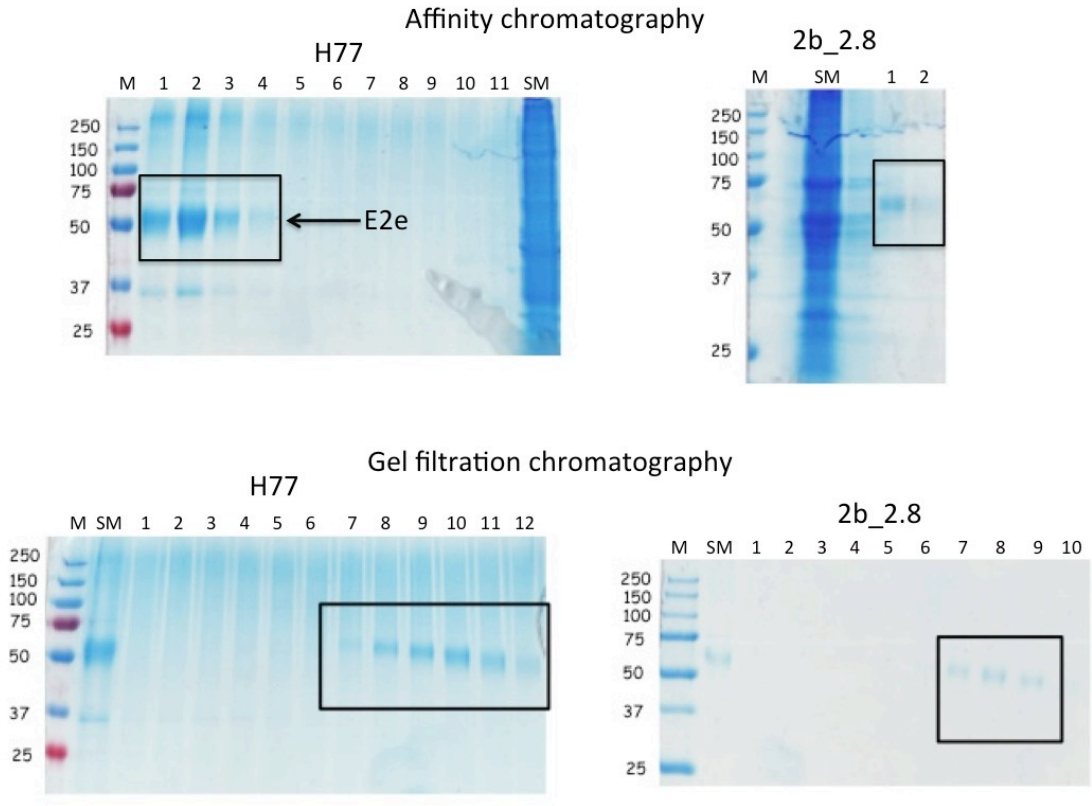

Figure 4-10 E2e purification

Stably transfected S2 cells were induced to produce E2e (H77 and 2b_2.8) for 8 days. E2e was purified from the supernatant by affinity chromatography using columns binding to the strep tag, followed by gel filtration chromatography. The different fractions collected during the purification process and a protein marker were loaded onto protein gels. The protein marker (M) and the starting material (SM) are indicated and the different fractions collected are numbered from 1 onwards. The monomeric fractions of E2e pooled for experimental use are indicated by the black rectangles. E2e was quantified using a NanoDrop and concentrated to approximately 1 mg/ml.

In the previous publications the authors used the anti-E2 mAb 291A2 to immobilise E2 on the plate for CD81 crosslinking. Attempts to obtain this antibody were unsuccessful and it was not clear, which epitope on E2 it bound to. In the experiments carried out here, E2e was presented by several antibodies that bind to different regions on E2 but do not interact with the CD81-binding site. Three antibodies were used to immobilise H77 E2e (H53, AP320 and ALP98) and two antibodies were used to immobilise 2b_2.8 E2e (AP320 and ALP98), as the H53 antibody is specific for H77. The aim was to immobilise E2e in different orientations, in at least one of which it would be able to bind and crosslink CD81 on T and NK cells. Plates were coated with one of the anti-E2 antibodies together with different concentrations of anti-CD3 mAb or anti-CD16 mAb, respectively, then E2e was added. As controls, only the anti-

E2 antibody was added to plates or no E2 antibody and no E2 were added. After 2h the plates were washed and purified T and NK cells were added. T and NK cell activation was read out 24h later by evaluation of CD69 expression. A dose-dependent response of the T cells and NK cells was seen to the CD3 or CD16 stimulation and this response was slightly enhanced by the anti-E2 mAb (Figure 4-11). Surprisingly, no effect of E2e on T cell and NK cell activation could be detected compared to the antibody-only control, no matter how E2e was presented to the cells, which is in contrast to findings presented in previous reports [388,352,353].

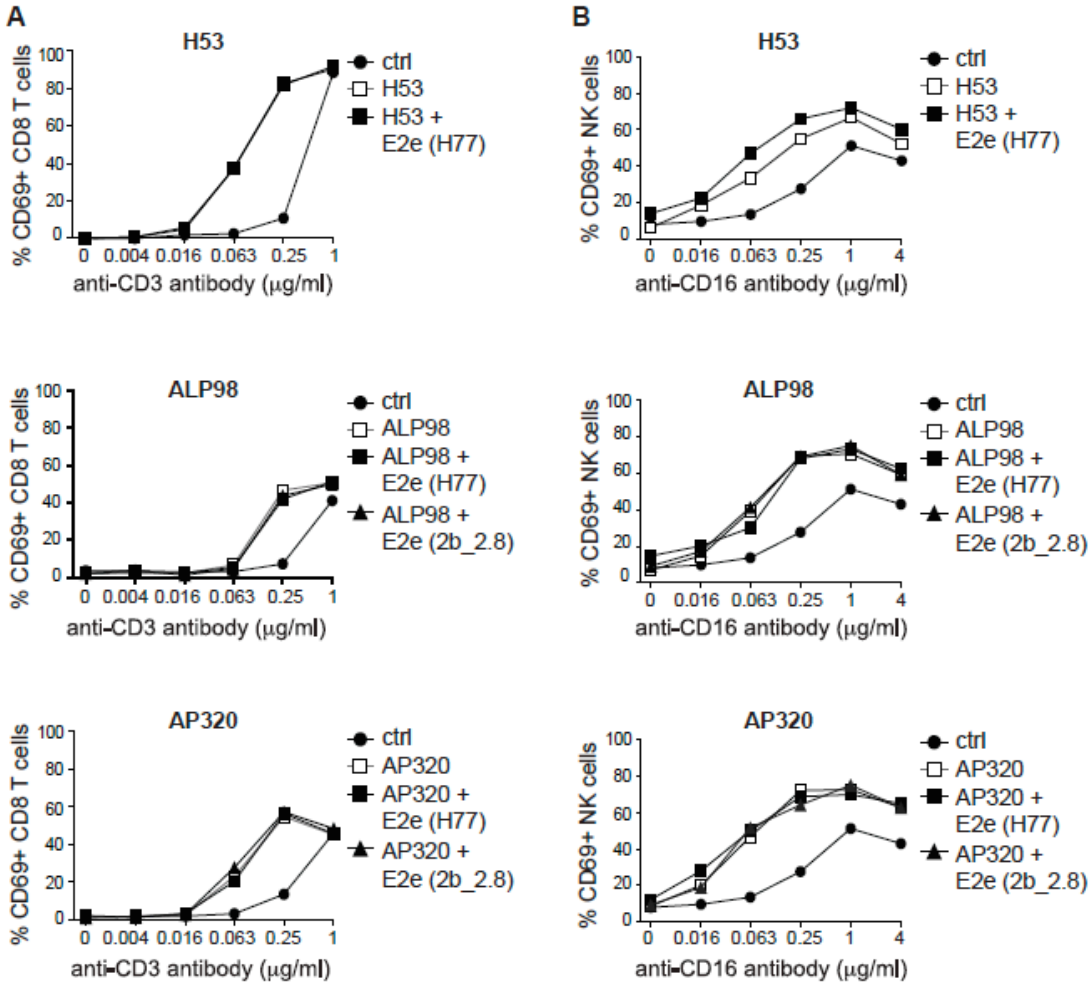

Figure 4-11 Immobilised E2e does not influence T cell activation through CD3 or NK cell activation through CD16

A) CD8 T cell or B) NK cell activation (percentage of cells expressing CD69) following stimulation of purified T or NK cells in plates coated with the indicated concentrations of anti-CD3 or anti-CD16 mAb alone (circles), or in combination with an anti-E2 mAb (open squares, H53, ALP98 or AP320), or sE2 (H77 or 2b_2.8) captured on an anti-E2 antibody (filled squares and triangles). Results shown are representative of findings made in at least two experiments with different donors.

4.3 Discussion

NK cells play an important role in containment of viral replication in many viral infections [387]. The role of NK cells in HCV infection *in vivo* has been demonstrated by documentation of the beneficial effect of certain NK receptors on the outcome of HCV infection [211,219-222,224], and the effectiveness of NK-derived IFN−γ and NKp30 in control of HCV replication has also been demonstrated *in vitro* [212-214,234]. Impairment of NK responses in HCV infection would be thus expected to be detrimental to the outcome of infection. If NK cells were inhibited during chronic HCV infection, as some (but not all) reports suggest, it would be of interest to understand the mechanisms involved. One mechanism proposed to contribute to NK impairment in CHC infection was the inhibitory effect of E2-mediated crosslinking of CD81 on NK responses [351-353], whereas CD81 crosslinking on T cell responses costimulates their activation in response to CD3 signalling [351,388,394]. Antibody-mediated crosslinking of CD81 on NK cells was shown to inhibit their activation in response to CD16 crosslinking and cytokines, and this inhibitory effect was reported to be mimicked by CD81 crosslinking by arrays of sE2 [393,351-353]. However, this is not a very physiological system, as *in vivo* E2 is expressed as a heterodimer with E1, and the E2 displayed on virions is likely to be very differently arrayed to that displayed on a flat plastic surface coated with a high concentration of E2. So, in this chapter I set out to address whether the inhibitory effects of sE2 on NK responses were also mediated by whole virus particles.

Initial experiments confirmed the costimulatory effect of antibody crosslinking of CD81 on T cells on their activation in response to CD3 crosslinking and the inhibitory effect on NK cells in response to CD16 crosslinking. In both CD4 and CD8 T cells CD81 crosslinking by antibody led to strong costimulation of activation in response to CD3 stimulation. Interestingly, the control antibody also had a slight costimulatory effect on T cell activation, which might reflect non-specific binding of T cells to the antibody, making it easier for the anti-CD3 antibody to crosslink CD3 on T cells. Alternatively it could also be an Fc-receptor

mediated effect, as there is some evidence that T cells can upregulate FcRs during T cell activation [395], which would then have to react to mouse IgG1 antibodies. In this respect, it has been shown that a human FcR (FcγRIIa) could induce cytokine release after interaction with a mouse monoclonal IgG1 antibody (OKT3) [396].

The inhibitory effect of CD81 crosslinking on NK activation was much less pronounced. I first analysed the effect of CD81 crosslinking on CD16-mediated activation using the plate-bound antibody system and observed a lower level of upregulation of the activation markers CD25 and CD69 when NK cells were stimulated with anti-CD16 antibody in the presence of anti-CD81 antibody as compared to a control mAb. However interpretation of results from these experiments were complicated by the fact that the control antibody had a stimulatory effect on the NK response to CD16 crosslinking, which may have been due to interaction of the Fc piece with receptors on NK cells.

Next the P815 system was used, where antibodies are bound to P815 cells via their Fc pieces orienting the Fab fragment towards the NK cells. In addition to CD69 upregulation, NK cell degranulation and IFN-γ production were examined, as in previous publications the effects of CD81 crosslinking on the functional activation of NK cells were found to be more marked than the effects on phenotypic activation. Using this system, although there was a slight trend towards inhibition by the anti-CD81 antibody, the effect was less pronounced and not statistically significant. Reasons for this discrepancy might lie in the fact that the density of anti-CD81 antibody displayed on P815 cells may have been lower than that in the plate-bound system (although the antibody had been added at higher concentrations) or that additional stimuli provided by the P815 cells may have lessened the inhibitory effect of the anti-CD81 antibody. As NKp30 and NKp46 also use the same signalling subunits and downstream signalling pathway as CD16, the effect of CD81 crosslinking on activation through these receptors was also analysed. Although there was a response detected to stimulation to either

NCR, these responses were quite low, which made it hard to detect an inhibitory effect of the anti-CD81 antibody.

In addition to the effect of CD81 crosslinking on CD16-mediated NK activation, the same authors also showed inhibition of cytokine-mediated NK activation via IL-2, IL-12 and IL-15 [352,353]. In my experiments I did not see any inhibition of NK activation through IFN-α, IL-2, IL-12, IL-15 or IL-18 caused by the anti-CD81 antibody. The differences in these findings might be due to several reasons. Contrary to the publications, where only NK cell production of IFN-γ was used as readout, I examined NK activation. Furthermore, the authors used only one concentration of the cytokines in all but one experiment, which was very high. In my experiments, similarly high concentrations of cytokines were all well above the levels needed to saturate CD69 expression on NK cells, so inhibitory effects of the CD81 antibody may have been difficult to observe. In this set of experiments I also used total PBMCs, whereas the other authors used purified or cultured purified NK cells.

In summary, it is possible that CD81 crosslinking has an inhibitory effect on NK cell activation via diverse stimuli including CD16 crosslinking and cytokines, but in my hands the inhibitory effects proved difficult to reproduce and were a lot less pronounced than the general picture projected from the two previous publications. Differences between my results and the published results might be due to different readouts and the use of cultured NK cells rather than purified NK cells. Also, in these publications the authors did not use all the controls I did and used just use one concentration of stimulating antibody or cytokine, which might have lead to an overestimation of the significance of the results. It would have been of interest to do more experiments to compare my results to those in the publications, but due to time constraints this was not possible.

Given that the inhibitory effects of antibody-mediated crosslinking of CD81 on NK cells could not be fully reproduced, in further experiments carried out to address whether HCV particles were capable of modulating immune responses via interaction with CD81, I

examined both NK cell and T cell responses (where more robust effects of antibody-mediated CD81 crosslinking had been observed) in parallel. The system used in the previous reports used to demonstrate the effects of sE2 interaction with CD81 on T and NK cell responses is quite artificial. Plates are first coated with anti-E2 antibodies and then E2 is added to the plates. The idea is that E2 is presented in a conformation where it will interact with CD81, as the coating antibody binds to a non-CD81-binding epitope, but in actual fact, both the antibody and E2 can bind the plate in any conformation. In my experiments I deployed as physiological a setting as possible adding viral particles (HCVcc and sHCV) to cells at similar concentrations to those found in blood and also tested the effects of HCVpp. The virus particles were not immobilised but were added in solution to reproduce contact of cells and viral particles, as they might occur *in vivo* in blood. I found that neither HCVpp, HCVcc nor plasma-derived HCV modulated T cell activation through CD3 or NK cell activation through CD16. These results are in agreement with observations in a report published during my D.Phil project, which also showed that HCVcc did not have the same inhibitory effect as the anti-CD81 antibody on NK cell activation and IFN-γ production in response to CD16 activation or on K562 killing, even when very high concentrations of HCVcc were used [354]. Another paper published subsequently by the Wack group showed that immobilised but not soluble HCVcc could inhibit IL-12-mediated IFN-γ production by NK cells [214]. However the *in vivo* relevance of these results is highly questionable, as there is unlikely to be a situation *in vivo* where large numbers of viral particles are immobilised on a surface in a fashion similar to that deployed *in vitro* in this publication. Interestingly, a very recent study examined the effects of prolonged exposure (5 days) of NK cells to serum-derived HCV and HCVpp and found increased upregulation of activating receptors, increased cytokine production and increased cytotoxicity after this exposure [397], suggesting that prolonged contact with particles might actually enhance NK activity, in line with what is shown in the literature for activation of NK cells in acute infection [237,350].

A final series of experiments were carried out to determine what effect CD81 crosslinking via immobilised arrays of E2 had on T and NK cell responses in the system I was using. Notably, the original reports of sE2-mediated immunomodulation had all used preparations of sE2 derived from H77, which is known to bind CD81 better than other HCV proteins [393], so I was interested to compare E2 proteins from H77 and another HCV isolate side by side. Although it is not quite clear which sE2 construct ($E2_{661}$ or $E2_{715)}$ was used in said publications, sE2 is typically produced by transfection mammalian cells with a construct ($E2_{661}$) that has an uneven numbers of cysteines and is not co-expressed with E1, resulting in many misfolded and aggregated proteins. Instead, I used a novel system for E2 production developed by the Rey group, which uses a construct with even numbers of cysteines ($E2_{715}$) and where E1 was co-expressed in a membrane-bound form (Figure 1-3) and is produced in insect cells. Using this system, I was able to generate monomeric E2e proteins from two HCV clones, H77 and 2b_2.8, the CD81 binding of which was demonstrated by the Rey group. E2e was presented to the T and NK cells by three different antibodies, each binding to a different epitope on E2 that did not interfere with CD81 binding (also demonstrated by the Rey group). Somewhat surprisingly, the published effect of CD81 crosslinking by immobilised sE2 on T or NK cell responses could not be reproduced using this system. Efforts to obtain the anti-E2 mAb and the E2 preparation used in previous publications were unsuccessful, so I was unable to perform a direct comparison with these reagents. However my results suggest that any immunomodulatory effects of E2-mediated CD81 crosslinking may be highly dependent on features of the system to capture E2 and/or features of the E2 protein preparations used in the studies where these effects were seen [391,351-353], all of which used the same E2 and anti-E2 mAb.

In summary, although the E2 glycoprotein of HCV is able to bind CD81, a protein that has a variety of roles in the host immune response including costimulation of T and B cell responses [151,351,388-391,398] and (possibly less potent) downregulation of NK cell responses [351-

[353], my results and those of others [354] suggest that it is highly unlikely that E2-CD81 interactions mediate significant immunomodulatory effects during HCV *in vivo*. Indeed, if this were the case, the clinical picture of HCV infection may be very different, with hyperactivated T cells and inhibited NK cells, which is not observed to that extent. There is some evidence that NK cells might be impaired in CHC infection, but findings differ between the studies and many have reported unchanged or even heightened functional or phenotypic activation of NK cells. The most common findings reported are reduced NK numbers in blood and a shift from the $CD56^{dim}$ subset to the $CD56^{bright}$ subset [337-342], representing a more immature phenotype less capable of performing cytotoxicity on target cells. If there is indeed some impairment in NK functions in CHC infection, this is probably not due to E2-CD81 interactions, but probably due to indirect effects. In a chronically HCV-infected patient, there is increased influx of NK cells (and other immune cells) to the liver, owing to production of chemokines in the infected liver. As the liver is a tolerogenic organ, where more immunosuppressive cytokines such as IL.10 and TGF-β are produced and intrahepatic lymphocytes are generally hyporesponsive, increased contact with this organ could influence the phenotype of NK cells, making them less responsive to stimulation.

5 IMMUNOMODULATION OF DENDRITIC CELLS BY HCV *IN VITRO*

5.1 Introduction

DCs have key effector and immunoregulatory roles in the host immune system and provide an important link between the innate and adaptive immune response [190]. They express multiple PRRs that enable them to detect the presence of infection and pathogen components. Upon activation, they produce soluble factors including type I interferon, proinflammatory cytokines and chemokines that have direct antiviral activity and mediate immunoregulatory effects. They also undergo a maturation process, during which their antigen uptake capacity declines and costimulatory molecules are upregulated and they migrate to the lymph nodes, where they are able to activate naive T cells and thereby initiate an adaptive immune response.

In this chapter the effects of HCV on two major subtypes of DCs were addressed: pDCs and mDCs (and MDDCs, which are an *in vitro* cultured homologues of mDCs). BDCA1+CD11c+CD123^{low} mDCs are typically involved in classical antigen presentation to CD4 T cells via MHC class II. They produce cytokines, including IL-12, IL-15, IL-18 and some type I IFN, which activate NK cells and promote differentiation of naive CD4 T cells into Th1 cells and naive CD8 T cells into CTLs [190, 205]. The main function of BDCA2+BDCA4+CD123^{high} pDCs is secretion of high levels of IFNs that mediate direct antiviral activity and help to activate immune cells involved both in innate and adaptive immunity, including DC subsets, NK cells, T cells and B cells. pDCs make all types of type I IFN (except IFN-κ) and type III IFN [194]. They are specialised for production of large amounts of IFN-α, 100-1000 times more than that produced by other cell types [196]. Unlike many other cell types, pDCs do not themselves need to be infected for the production of IFN-α to be

triggered [197], as shown by several studies that demonstrated that pDCs can produce IFN-α following exposure to inactivated viruses including HSV and influenza virus [179,399]. Furthermore, several studies show that after pDC depletion no IFN-α is made in response to TLR7 or TLR9 ligation [180], showing that pDCs are the major IFN-α-producing cells within the total PBMC population.

pDCs and mDCs express distinct arrays of PRRs, with which they recognise pathogens, for example pDCs express TLR7 and TLR9 [200], whereas mDCs express TLR2, TLR3, TLR4, TLR7 and TLR8 [205]. Four of these TLRs are important for the recognition of nucleic acids: TLR9 recognises CpG-DNA, whereas TLR3 recognises dsRNA and TLR7 and TLR8 recognise ssRNA. In contrast to other TLRs, which are expressed on the plasma membrane, these TLRs reside on intracellular membranes, where they can interact with endocytosed material or with components from cytosol via autophagy.

The roles played by DCs in HCV infection are not well understood and their importance in determining the outcome of acute HCV infection (viral clearance versus persistence) has not been investigated. Only very few studies have addressed the phenotype and functions of DCs during acute HCV infection [400-402]. While one study found normal or heightened numbers of circulating mDCs in the blood of patients with acute HCV infection [402], decreased [400] or normal [401] numbers have been reported for pDCs. Both of the latter studies found that pDCs did not have an activated pDC phenotype in acute HCV infection, but their findings differed in respect to the ability of pDC to produce IFN-α following stimulation with TLR9 agonists; one study suggesting a normal IFN-α response and the other a decreased IFN-α response. As reviewed in 1.5.2.1, there is also some evidence that DC functions might be impaired in CHC infection, although there are conflicting results in the literature. Some reports do show impaired DC function [309-322], whereas other reports do not [323-327] and the details of the abnormalities observed differ between the studies. The most commonly reported fact is that

circulating numbers of both mDCs and pDCs are reduced, possibly owing to increased recruitment to lymph nodes or the liver.

One of the mechanisms suggested to explain the DC abnormalities seen in chronic HCV infection is direct impairment of DC functions by HCV proteins. Several HCV proteins, including core, NS3 and E2, have been implicated affecting DC functions *in vitro* [289,329,318]. In these studies high concentrations of proteins were typically used, either added exogenously or expressed endogenously using adenoviral vectors. Since DCs are unlikely to come into contact with high amounts of NS proteins and are not susceptible to high-level HCV replication, the *in vivo* relevance of the results from such studies is questionable and more *in vitro* studies are needed to address the effects of exposure of DCs to whole virus particles at physiological concentrations. In this project, I thus set out to evaluate the effect of HCVcc on pDCs, mDCs and MDDCs and their responses to subsequent TLR stimulation.

During my project other studies were published that also used HCVcc to address HCV-induced DC impairment and examined DC activation following exposure to HCV or HCV-infected cells. The results from these studies are discussed and compared to my findings at the end of this chapter.

5.2 Interaction of HCV with dendritic cells

5.2.1 Interaction of HCV with MDDCs

To assess the effects of HCVcc on DCs, I first chose to perform experiments using MDDCs, as these cells resemble peripheral blood mDCs and can be generated in reasonably large quantities. Also, these cells can be obtained at a high purity, making it easier to interpret the data without other contaminating cell types.

Magnetofection was used to synchronise contact of HCVcc with MDDCs, and then the quantities of virus bound to and taken up into cells was assessed over time. Magnetofection is a method that involves mixing virus with magnetic beads (with which virions interact), then

adding the cells that have been placed on magnetic plates. The magnet draws the beads (and virions) onto the cells, synchronising virus-cell contact.

The first question that was addressed was the efficiency of uptake of HCVcc by MDDC. At 0h, 1h and 3h post-magnetofection MDDCs were treated with proteinase K to remove virus that had not entered the cells, or were left untreated to assess the total amount of virus (bound and entered). HCV RNA was then quantified in relation to GAPDH RNA using RT-q-PCR. Figure 5-1 shows that in the untreated cells the total amount of bound or internalised virus stayed constant for the first hour of incubation and then declined by 3h. In the proteinase K-treated cells, hardly any internalised virus was detectable right after magnetofection, but after 1h about half of the virus could be detected intracellularly, indicating that virus was indeed entering MDDC, and that this occurred quickly and relatively efficiently. Interestingly, levels of intracellular viral RNA declined to baseline after 3h, indicating that virus taken up by MDDCs is subsequently degraded.

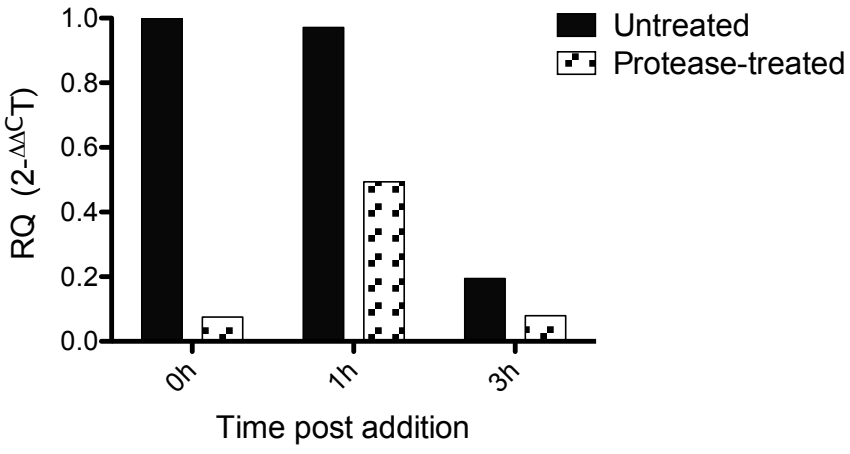

Figure 5-1 Uptake of HCVcc by MDDC following magnetofection

MDDCs were magnetofected with HCVcc at a MOI of 0.2 for 2min. After magnetofection (0h) and at 1h and 3h cells were either left untreated or were treated with proteinase K, then RNA was isolated and subjected to RT-q-PCR. Levels of both GAPDH and HCV RNA were measured in triplicate and RQ was carried out using the 0h timepoint of the untreated cells as an internal calibrator. The experiment was carried out once using two donors. Data from one representative donor are shown.

Next, upregulation of activation markers on MDDCs and cytokine and chemokine production were assessed at different timepoints after magnetofection. CD40, CD80, CD86 and HLA-DR expression was analysed by flow cytometry at 5h and 20h post-infection in cells treated with the virus-bead mixture, with a control-bead mixture (beads mixed with a control preparation) and in untreated cells. No upregulation of any of the markers examined could be seen, indicating that exposure to the beads ± HCVcc did not lead to activation or maturation (Figure 5-2).

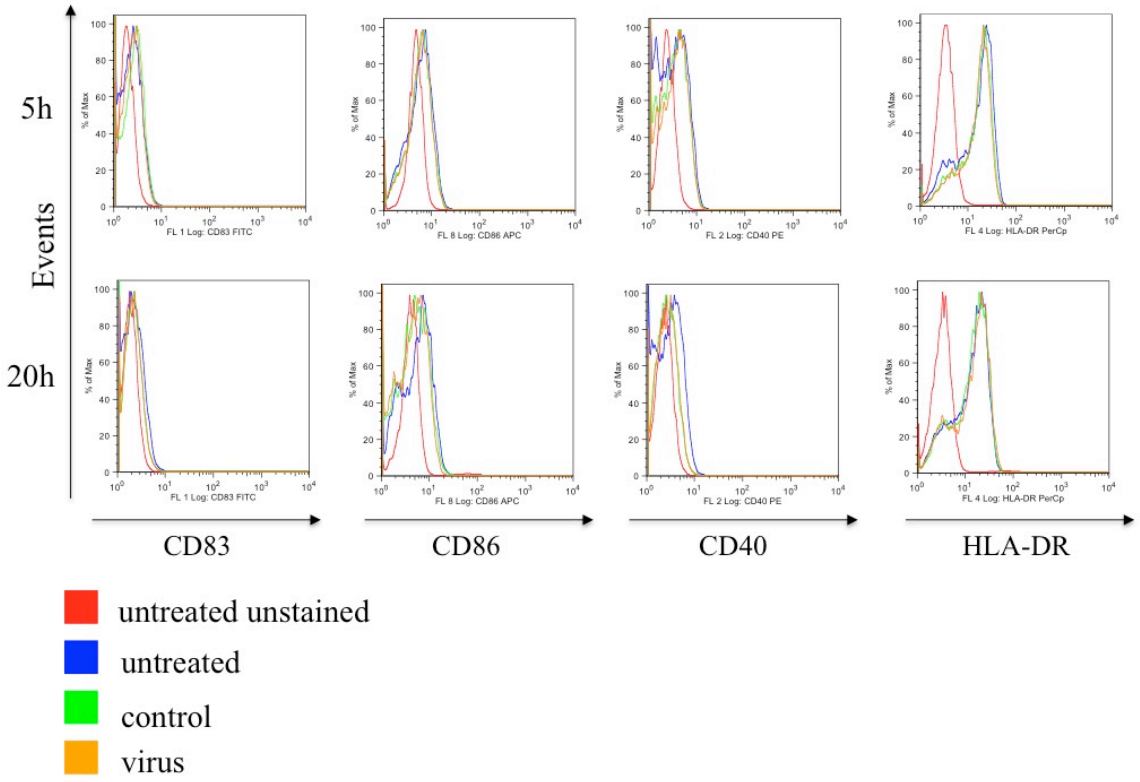

Figure 5-2 MDDC are not activated by contact with HCVcc

MDDCs were magnetofected with HCVcc at a MOI of 0.2 or with a control preparation for 2min or left untreated. At 5h and 20h cells were stained with anti-CD83, anti-CD86, anti-CD40 and anti-HLA-DR antibodies or were left unstained. Histograms are shown of unstained cells (red) or staining performed on untreated (blue), control-treated (green) or virus-treated (orange) cells. Results are representative of findings made with MDDCs from two different donors.

Supernatants of control or virus-treated MDDCs were also collected at various timepoints post-infection for analysis of chemokines and cytokines by Luminex or ELISA. No upregulation of GM-CSF, IFN-α, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-12, IL-13, MIG and RANTES was seen in either control or virus-treated cell supernatants but other analytes, such

as IL-6, IL-7, IL-8, IL-10, IL-18, IP-10, MCP-1, MIP-1α, MIP-1β and TNF- α, were produced over the timecourse of 20h (Figure 5-3).

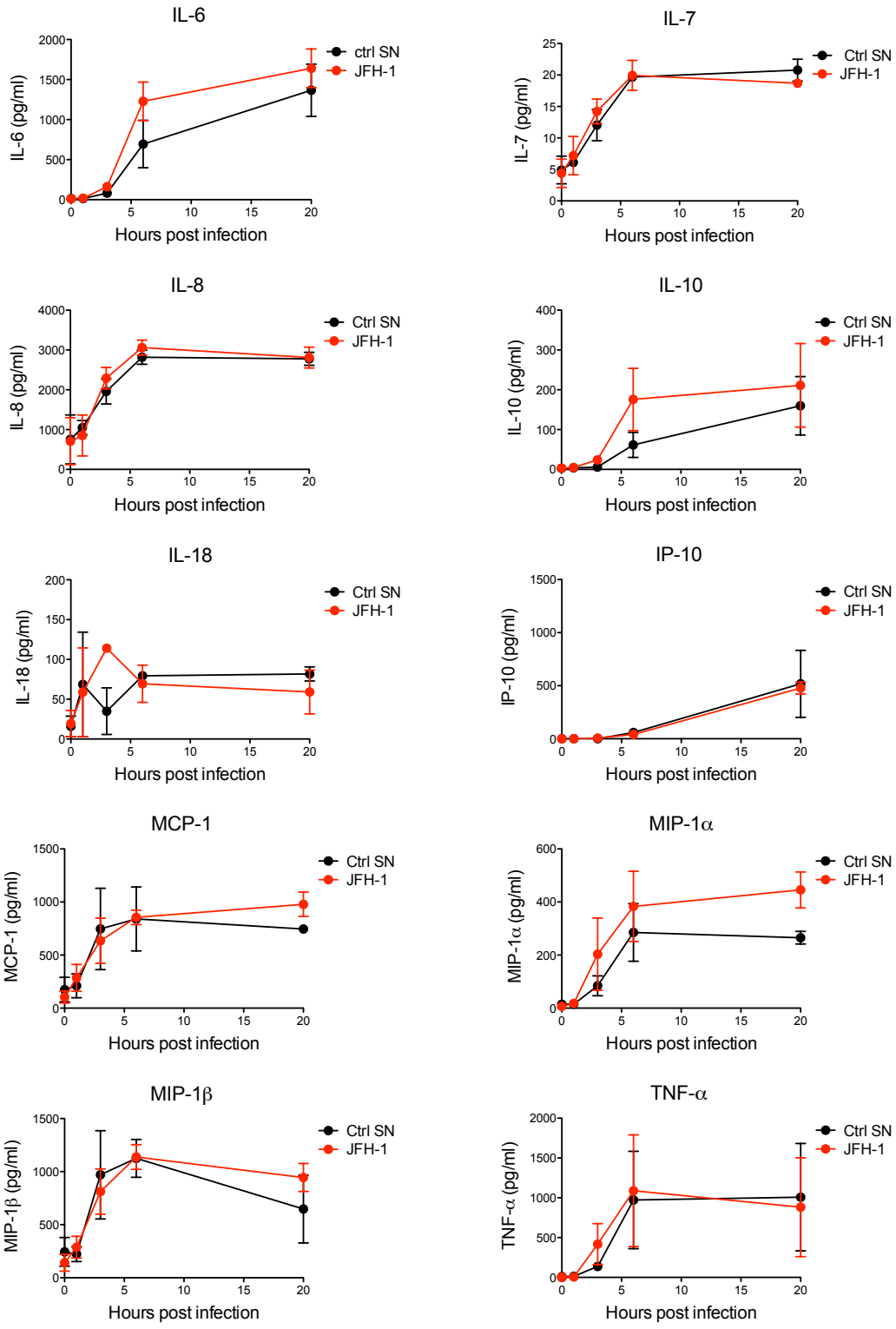

Figure 5-3 Cytokine/chemokine production by MDDCs after magnetoinfection with HCVcc

Levels of 10 cytokines and chemokines are shown in supernatants collected at indicated timepoints post magnetofection from MDDCs treated with control SN+ beads (ctrl SN, black) or with HCVcc JFH-1 + beads (JFH-1, red). Results are shown as the mean of data from 2 donors and the error bars indicate the SEM.

Although there was a trend to higher levels of cytokines and chemokines being produced by the cells treated with the virus-bead mixture, all analytes were also produced by cells magnetofected with the control-bead mixture. Either the increase in these analytes indicated a steady-state production by these MDDCs, or the beads may have had a stimulatory effect, although no upregulation of activation and maturation markers was seen in the bead-treated MDDCs.

A next set of experiments addressed whether HCVcc could modulate the response of MDDCs to TLR ligation. In these and all subsequent experiments virus was added to the DCs without magnetofection, to eliminate any confounding effects of the magnetofection process. MDDCs were incubated with either a 1/10 dilution of HCVcc (J6/JFH-1, 10^6 TCID50/ml final concentration) in 100μl DMEM (MOI 0.5) for 3h or were left untreated. Cells were then stimulated with various TLR ligands (adding CL097 (10μg/ml), LPS (10μg/ml) or Poly I:C (50μg/ml) in a total volume of 100μl DMEM) and upregulation of activation/maturation markers and cytokine production were assessed by flow cytometry after a total of 20h incubation. In the cells that were pre-treated with media only, LPS induced the greatest phenotypic activation of MDDC, while PolyI:C and CL097 induced a more modest MDDC activation, with only CD83 being induced above the levels of the unstimulated cells (Figure 5-4A). To test for differences between mean values for multiple groups in this graph and all following graphs of this chapter, a one-way ANOVA (using Tukey's correction for multiple pairwise comparisons) was used. Pre-treatment of MDDC with HCVcc did not induce any MDDC maturation and did not modulate activation in response to any of the TLR ligands either. Intracellular cytokine staining (ICS) was also performed to examine cytokine/chemokine production by MDDCs. Stimulation with both LPS and CL097 resulted in robust production of IL-12, MIP-1β and TNF-α, while PolyI:C treatment and no stimulation did not result in upregulation of any of the analytes (Figure 5-4B).

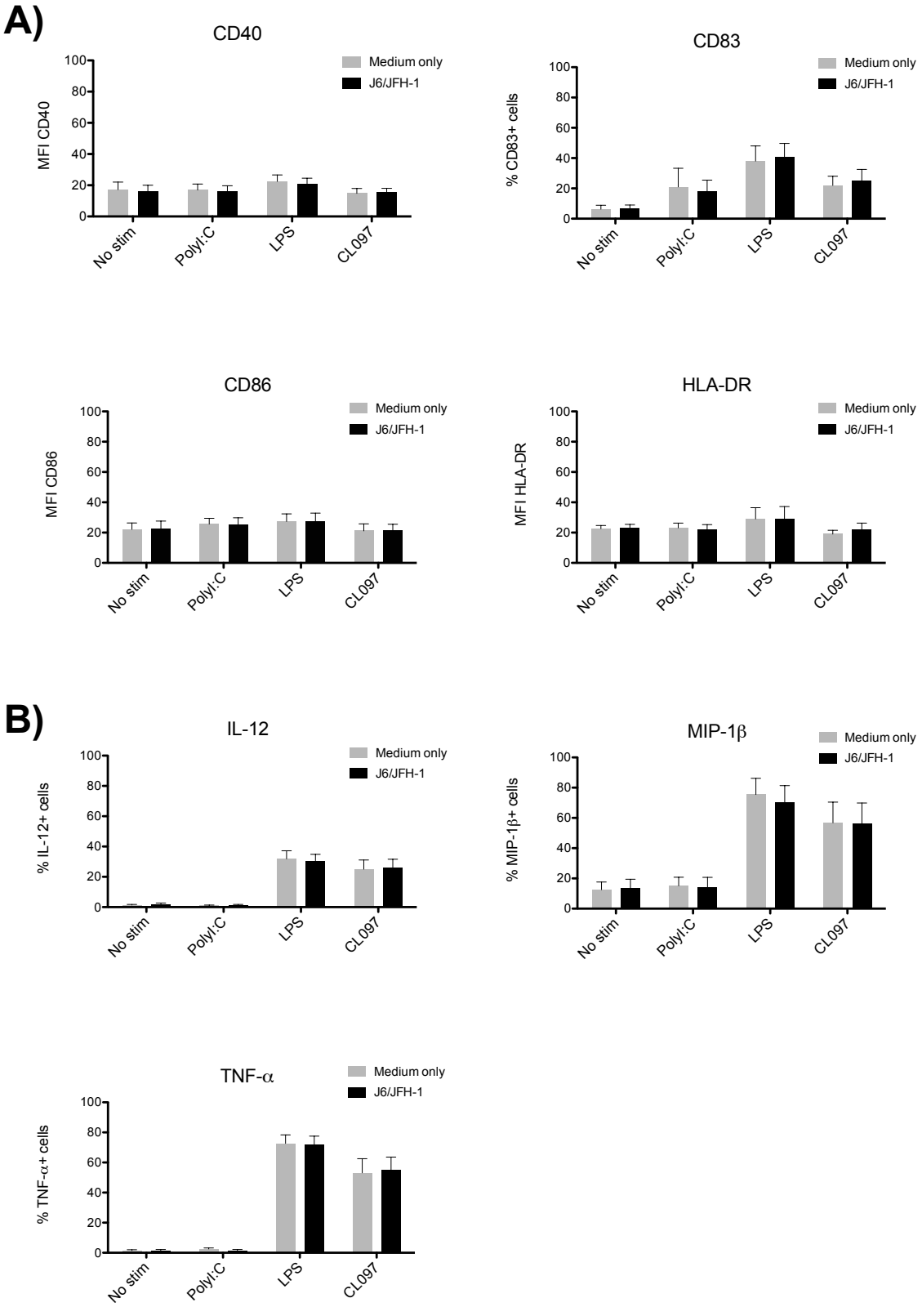

Figure 5-4 HCVcc does not alter MDDC activation in response to TLR ligation

A) MDDC activation (percentage of cells expressing CD83 or MFI of CD40, CD86 and HLA-DR staining) or B) MDDC cytokine/chemokine production (percentage of cells expressing IL-12, MIP-1β or TNF-α) following stimulation with PolyI:C (50μg/ml), LPS (10μg/ml) or CL097 (10μg/ml) for 20h, after pre-treatment of MDDC with HCVcc (MOI 0.5, black) or medium only (grey) for 3h. The findings shown are an average of results from 3 donors tested in 2 independent experiments and the error bars indicate the SEM.

Again, HCVcc alone did not elicit any cytokine/chemokine production and did not modulate the TLR-stimulated response in MDDCs.

In summary, there was no evidence that HCVcc induced any phenotypic or functional activation of MDDCs and HCVcc also did not modulate MDDC responses to TLR ligation.

5.2.2 Interaction of HCV with peripheral blood DCs

5.2.2.1 Interaction of HCV with mDCs

To address the effects of HCVcc on mDC activation and functions, experiments similar to those carried out with MDDCs were performed with mDCs. Rather than using purified or enriched mDCs, total PBMCs were used for these experiments, to mimic the *in vivo* situation where mDCs circulate as part of the total PBMC population. Total PBMCs were incubated with either a 1/10 dilution of HCVcc (J6/JFH-1, 10^6 TCID50/ml final concentration) in 100µl R10 (MOI 0.1) for 3h or were left untreated. Cells were then stimulated with various TLR ligands (adding CL097 (10µg/ml), LPS (10µg/ml) or Poly I:C (50µg/ml) in a total volume of 100µl R10) and upregulation of activation/maturation markers on and cytokine production by mDCs was measured by flow cytometry after a total of 20h. In the PBMCs that were pre-treated with medium only, mDCs upregulated all activation markers (CD40, CD83 and CD86) in response to PolyI:C and LPS treatment as compared to unstimulated cells. CL097 treatment proved less stimulatory, only inducing heightened CD40 expression (Figure 5-5A). Conversely, the highest cytokine response (IL-12 and TNF-α) was induced by CL097, followed by LPS, while PolyI:C only induced a TNF- α response (Figure 5-5B). The mDC responses did not fully replicate the responses seen in MDDCs, which might have been due to the additional stimuli received by the mDCs from other cell types in the PBMC preparation. Pre-incubation with HCVcc did not modulate mDC responses to TLR ligation, but there was a trend for higher mDC activation marker upregulation in HCVcc-treated cells as compared to untreated cells. However none of these comparisons were statistically significant.

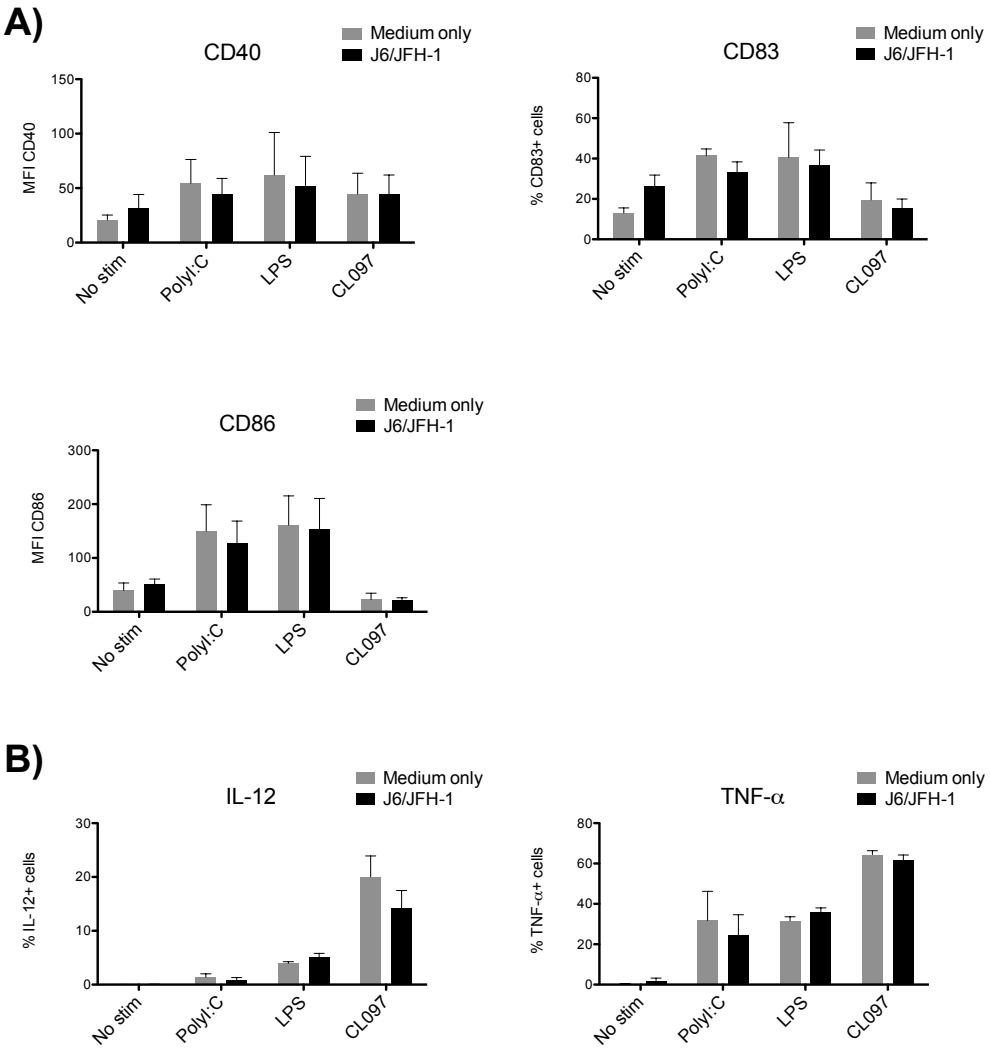

Figure 5-5 Influence of HCVcc on activation marker upregulation on mDCs in response to TLR ligation

A) Activation of mDCs (percentage of mDCs expressing CD83 and MFI of CD40 and CD86 staining) and B) production of cytokines (percentage of mDCs expressing IL-12 and TNF-α) after a 20h incubation with no stimulant (no stim) or the indicated TLR ligands, following pre-treatment of total PBMC with HCVcc (black) or medium only (grey) for 3h. The findings shown are an average of results from 3 donors tested in 2 independent experiments and the error bars indicate the SEM.

5.2.2.2 Interaction of HCV with pDCs

Further experiments addressed the effects of HCVcc on pDC activation and functions. pDCs are the professional IFN-α-producing cells and constitute the predominant source of production of systemic IFN-α in many virus infections. Hence the ability of HCV to induce IFN-α production by pDCs *in vitro* was addressed and the pDC-stimulatory capacity of HCV was compared to that of other viruses.

In order to compare the ability of HCV to stimulate IFN-α production by pDCs to that of other RNA viruses, CL097 (10μg/ml), influenza (MOI 10), HIV (MOI 0.1), DV (MOI 0.1) and HCVcc J6/JFH-1 (MOI 0.01) and control preparations were added to total PBMCs overnight, then supernatant IFN-α levels were quantitated by ELISA. Analysis of IFN-α levels in the supernatant of stimulated PBMC populations could thus be used to gain insight into IFN-α production by pDCs. As shown in Figure 5-6, the unstimulated PBMCs and PBMCs stimulated with all the control preparations did not produce any IFN-α, whereas CL097 and especially influenza induced production of high levels of IFN-α response. HIV also elicited high levels of IFN-α production by cells from some but not all of the donors tested. However HCV and another member of the *Flaviviridae* family, DV, failed to elicit any IFN-α production.

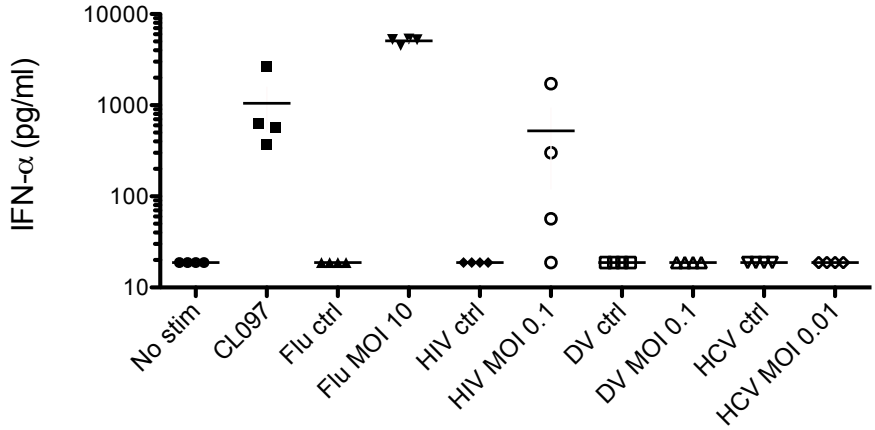

Figure 5-6 IFN-α production following stimulation of PBMCs with different viruses

IFN-α production after stimulation with CL097 (10μg/ml) or the indicated viruses and controls or no stimulation. Results are shown as the mean from values using PBMCs from four donors (individual symbols).

As the titres of the virus preparations used in this experiment differed and the MOI at which HCV had been tested was relatively low, in a next step, the viruses were tested at a series of different dilutions to enable better comparison of their pDC-stimulatory capacity. PBMCs were pre-stimulated with a suboptimal dose of IFN-α (as IFN-α is known to enhance its own production) to see whether this treatment would enable detection of IFN-α production in

response to low concentrations of virus that would not otherwise induce a response. In the cells that were not pre-treated with IFN-α, influenza virus stimulated a sub-maximal but nonetheless robust IFN-α response at a MOI of 0.01, and HIV elicited detectable levels of IFN-α production at a MOI of 0.1, whereas both DV and HCV did not stimulate IFN-α production at the highest MOIs tested (1 and 0.01, respectively) (Figure 5-7). Pre-stimulation with IFN-α did not elicit IFN-α production from PBMCs that were exposed to medium only, but it did enhance IFN-α production in response to low-dose viral stimulation. Influenza virus stimulated an IFN-α response from IFN-α-pre-treated pDCs at a MOI of 0.000001 and HIV at a MOI of 0.001. Interestingly, after IFN-α pre-stimulation, very low levels of IFN-α production were elicited by both DV (MOI 0.1) and HCV (MOI 0.01).

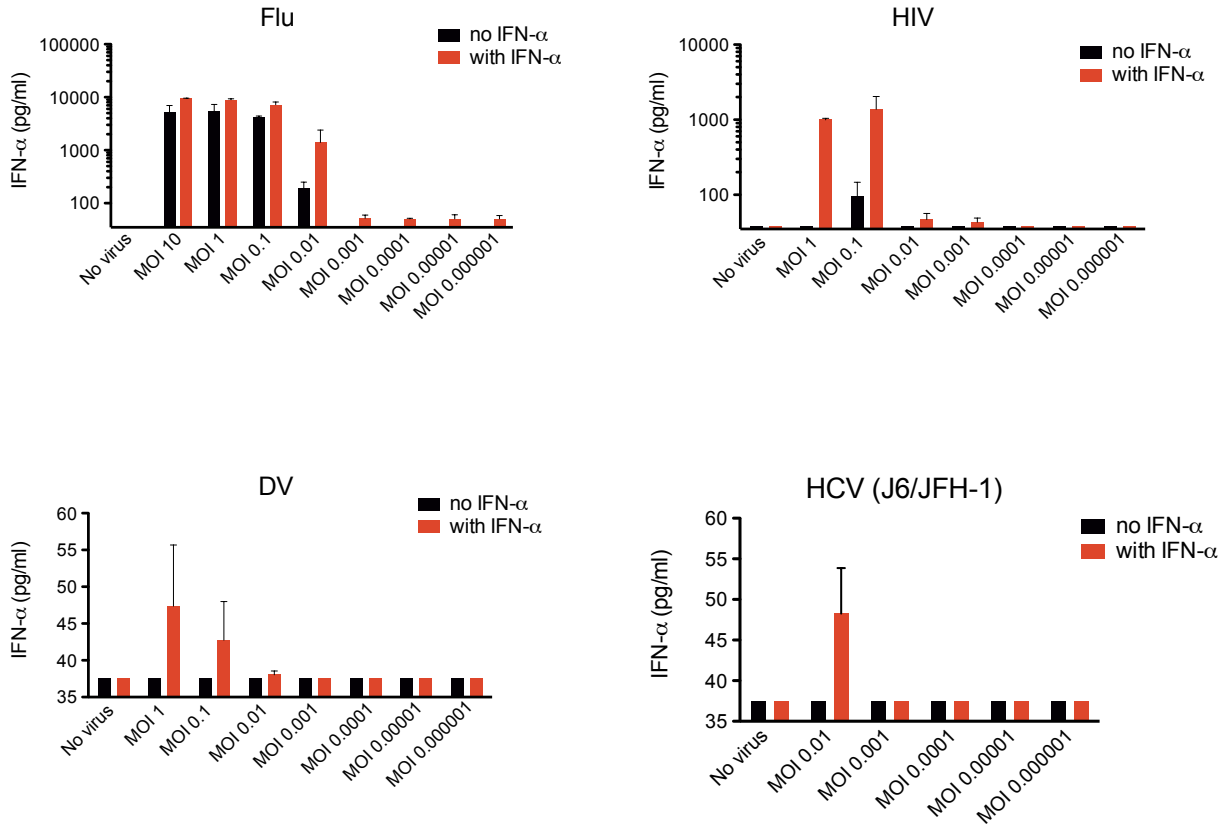

Figure 5-7 IFN-α production stimulated by different viruses from IFN-α-pre-treated or untreated PBMCs

IFN-α production by PBMCs pre-treated for 2h with IFN-α at 10μg/ml or medium in response to overnight stimulation with the indicated viruses or medium alone (no virus). Results are shown as the mean of values from PBMCs of two donors tested in one experiment and the error bars indicate the SEM.

It is possible that the ratio of defective to infectious particles in the four virus preparations used here may have differed. Nonetheless the fact that HCV and DV elicited detectable levels of IFN-α production from PBMCs only at MOIs considerably higher than the minimum MOIs, at which HIV and influenza viruses elicited IFN-α production, coupled with the low levels of IFN-α production stimulated by HCV and DV suggest that the latter viruses are very ineffective stimulators of IFN-α production by pDCs.

Since the HCVcc preparation used for the previous experiments had a relatively low titre, further experiments were performed in which total PBMCs were stimulated with high-titre HCVcc (10^7 TCID50/ml) at an MOI of 0.1, or enriched pDC were stimulated with high-titre HCVcc at a MOI of 2-5 or plasma-derived HCV at a final concentration of 10^7copies/ml overnight, to determine whether higher concentrations of virus were capable of eliciting IFN-α production by pDCs not pre-treated with IFN-α at 10μg/ml. No IFN-α was induced in total PBMCs stimulated with HCVcc at a MOI of 0.1 or in plasma-derived HCV-stimulated enriched pDCs (Figure 5-8), but low levels of IFN-α production were observed when enriched pDCs were stimulated with HCVcc at high MOI.

There may be several reasons for the poor IFN-α production elicited by HCVcc. Firstly virion uptake into pDCs might not be as efficient as that of viruses like HIV and influenza, which can both enter pDCs via specific receptors [403,404]. Secondly, HCV RNA might fail to ligate TLR7. Finally, HCV might exert inhibitory effects on IFN-α production, possibly via engagement of its glycoproteins with inhibitory receptors on pDCs, such as BDCA2 or DCIR.

To determine whether HCV RNA can be recognised (via TLR7) and elicit a robust IFN-α response when it is delivered into PBMCs, *in vitro* transcribed HCV RNA was transfected into PBMCs at different concentrations. The PBMCs were then incubated overnight, and IFN-α levels in the supernatant were quantitated by ELISA. Neither HCV RNA nor the transfection reagent DOTAP stimulated an IFN-α response when added to PBMCs alone,

whereas HCV RNA transfected into the cells stimulated robust production of IFN-α in a dose-dependent manner (Figure 5-9), indicating that a sufficient amount of intracellular HCV RNA can trigger strong production of IFN-α.

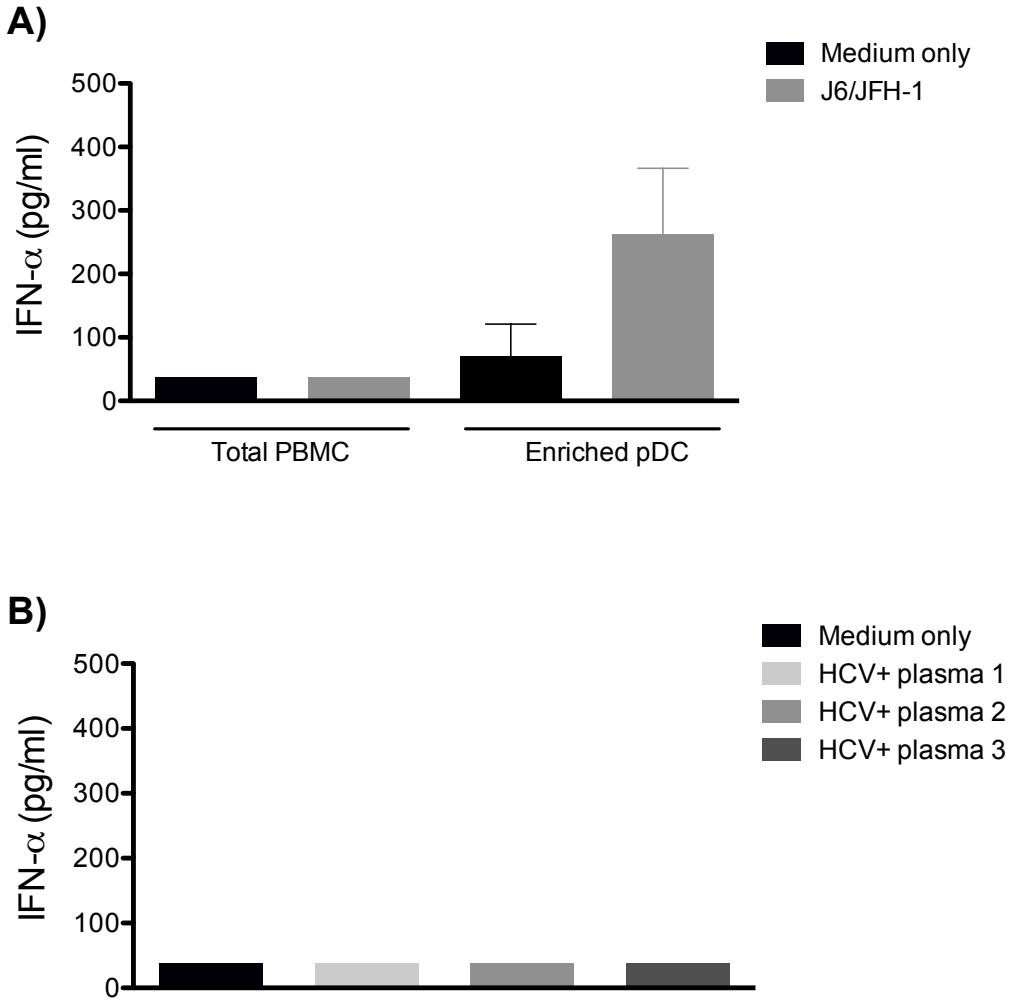

Figure 5-8 IFN-α production in pDCs in response to high-titre HCVcc and plasma-derived HCV

IFN-α production after stimulation with A) high-titre HCVcc in total PBMCs (MOI 0.1) and enriched pDCs (MOI 2-5) and B) plasma-derived HCV (from acutely infected patients pre-seroconversion) in enriched pDCs (final concentration 10^7 copies/ml). Results are shown as mean of values from at least five donors tested in least two independent experiments and the error bars indicate the SEM.

I did not show that the IFN-α production in these experiments was made by pDCs and was triggered by TLR7 ligation, however this seems likely as the capacity of HCV RNA to trigger IFN-α production via TLR7 has been shown in pDCs previously [184,189].

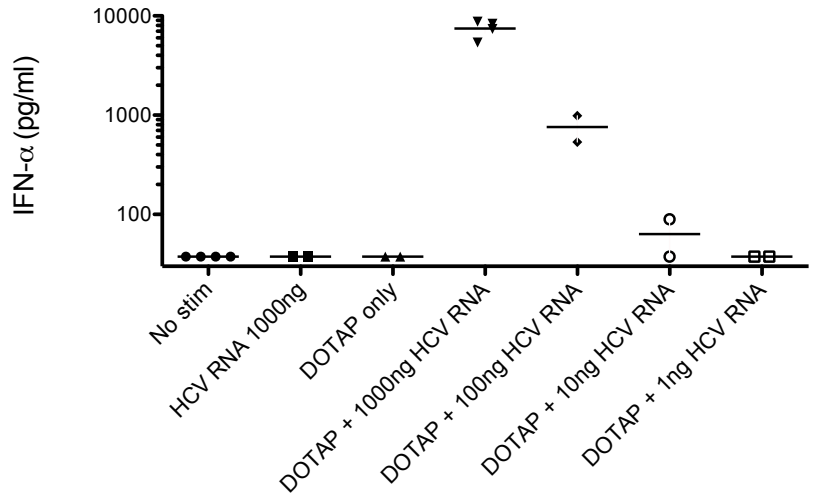

Figure 5-9 HCV RNA induces IFN-α production when transfected into PBMCs

PBMCs were treated with HCV RNA, with the transfection reagent DOTAP, or with DOTAP plus the indicated concentrations HCV RNA. Cells were incubated o/n, and supernatant IFN-α levels were quantitated by ELISA. Each symbol represents data from one donor, and the horizontal line shows the mean.

To address whether the HCV glycoproteins interact with inhibitory receptors on pDCs, downregulating IFN-α production that would otherwise be triggered following particle uptake, experiments were carried out to address whether HCVcc would elicit IFN-α production by pDCs if glycoprotein-inhibitory receptor interactions were blocked. HCV particles were pre-treated with murine anti-E2 antibodies or alternatively, pDC inhibitory receptors BDCA2 and DCIR were blocked by either adding a BDCA2-blocking antibody to purified pDCs or pre-incubating HCVcc particles with soluble DCIR. HCVcc (used at a MOI of 0.1) still did not elicit IFN-α production by enriched pDCs when potential interactions between the inhibitory receptors and HCV E2 were blocked (Figure 5-10). Taken together, these results do not support a role for inhibition mediated via E2 interaction with receptors on pDCs in suppressing pDC production of IFN-α on exposure to HCVcc.

To gain further insight into whether HCVcc can mediate immunomodulatory effects on pDCs, a series of experiments were carried out to address the effects of HCVcc on pDC responses (in either total PBMCs or enriched pDCs) to TLR ligation.

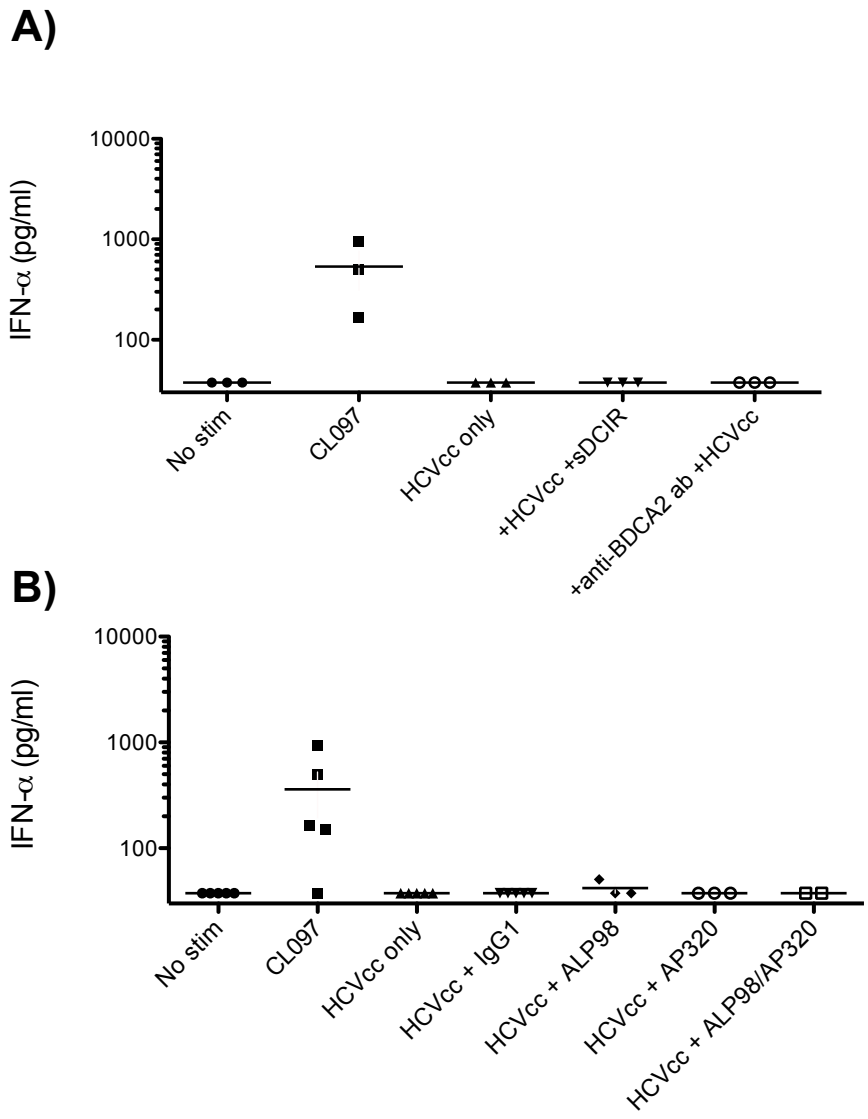

Figure 5-10 Blocking E2 or inhibitory receptors on pDCs does not induce IFN-α production in response to HCVcc

Enriched pDCs were stimulated with A) HCVcc after pre-incubation of pDCs with anti-BDCA2 or IgG1 antibodies or pre-incubation of HCVcc with sDCIR or B) HCVcc alone or preincubated with anti-E2 antibodies (ALP98 and AP320) or IgG1 control antibodies. Cells were incubated o/n, and supernatant IFN-α levels were quantitated by ELISA. Each symbol represents data from one donor, and the horizontal line shows the mean.

PBMCs were incubated with either a 1/10 dilution of HCVcc (J6/JFH-1, 10^7 TCID50/ml) in 100μl R10 (MOI 0.1) for 3h or were left untreated. Cells were then stimulated with various TLR ligands (adding CL097 (10μg/ml) or CpG-A, B and C (10μg/ml) in a total volume of 100μl R10) and upregulation of activation/maturation markers on and cytokine production by pDCs was measured by flow cytometry after a total of 3h or 20h. In the PBMCs that were pre-treated with medium only, all of the TLR ligands stimulated pDC activation/maturation

(as assessed by upregulation of CD40 and CD83 expression) compared to unstimulated cells (Figure 5-11A). HCVcc on its own did not stimulate the upregulation of activation/maturation markers and furthermore did not inhibit upregulation of these markers in response to TLR7 and TLR9 ligation. Similar results were obtained when cytokine production by pDCs was assessed by intracellular cytokine staining (ICS). All TLR ligands elicited TNF-α production in a high proportion of pDCs (Figure 5-11B). CL097 and CpG-A stimulated a robust IFN-α response, whereas CpG-B did not and CpG-C induced only a low IFN-α response by pDCs. HCVcc did not induce any cytokine production by pDCs, and also did not modulate pDC responses to TLR ligation.

Since there were some concerns that the transport inhibitors added to the cells during the ICS assays might interfere with a potential inhibitory mechanism, supernatant IFN-α levels were also measured by ELISA in the absence of transport inhibitors. No reduction in IFN-α production in response to TLR7 and TLR9 stimulation was observed in cells that had been pre-treated with HCVcc (Figure 5-12).

pDCs only make up about 0.1% of PBMC and hence the HCV/pDC ratio in the preceding experiments would not have been very high. In order to use higher HCVcc to pDC ratios, and also to exclude influences of other cell subsets on pDCs, further experiments were performed using enriched pDC populations. Enriched pDCs were pre-treated with HCVcc at a MOI of 2-5 (1/10 dilution of HCVcc (J6/JFH-1, 10^7 TCID50/ml)) in 100µl R10 (MOI 0.1) for 3h or were left untreated. Cells were then stimulated with various TLR ligands (adding CL097 (10µg/ml) or CpG-A and B (10µg/ml) in a total volume of 100µl R10). Upregulation of activation/maturation markers on pDCs was measured by flow cytometry, and supernatant IFN-α levels were measured by ELISA. In the pDCs pre-treated with medium only, all TLR ligands stimulated upregulation of CD40 and CD83 and production of IFN-α (Figure 5-13).

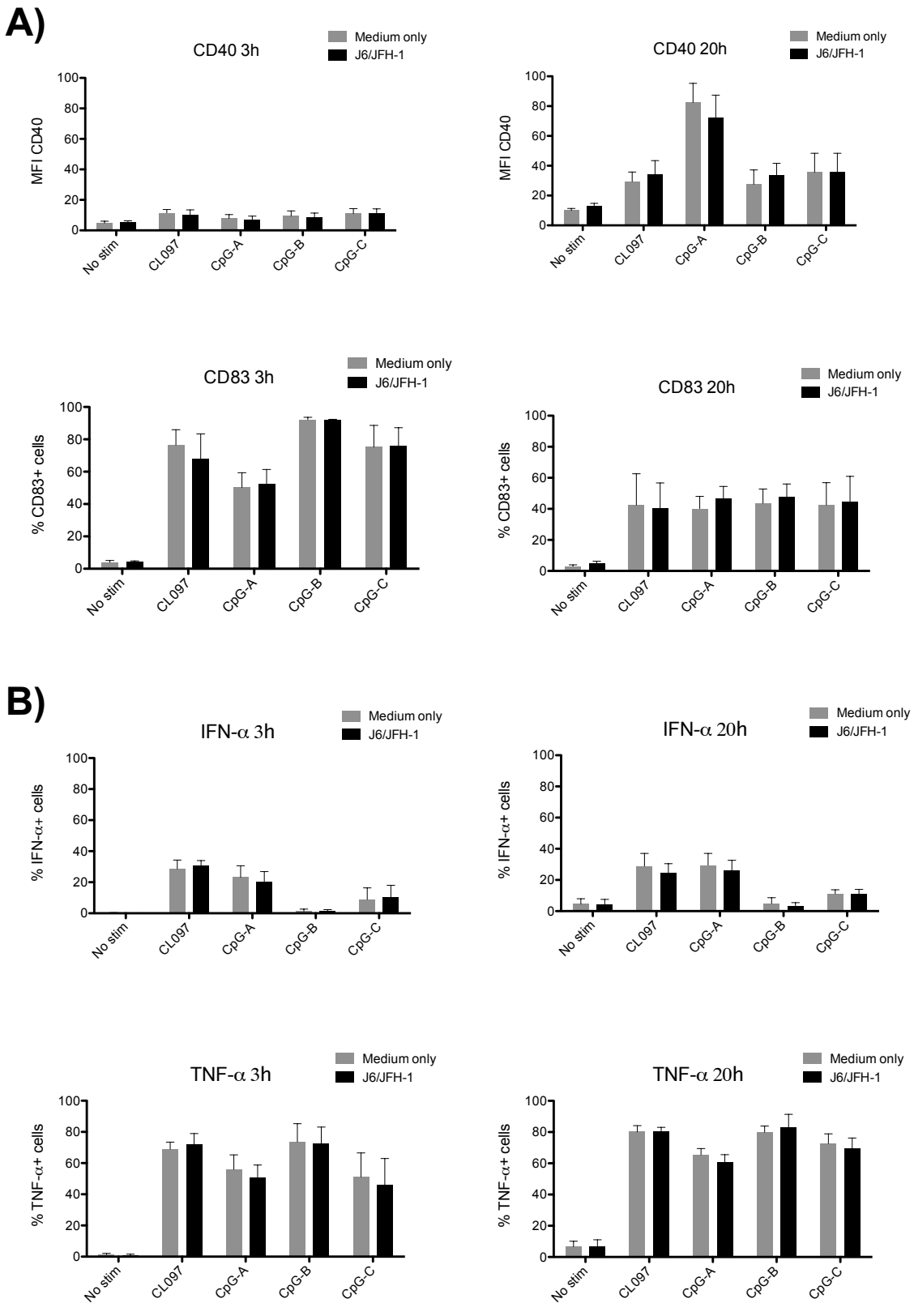

Figure 5-11 Influence of HCVcc on activation marker upregulation in response to TLR ligation in plasmacytoid dendritic cells

A) Activation of pDCs (percentage of pDCs expressing CD83 and MFI of CD40 staining) and B) production of cytokines (percentage of mDCs expressing IFN-α and TNF-α) after a 3h or 20h incubation with no stimulant (no stim) or the indicated TLR ligands, following pre-treatment of total PBMC with HCVcc (black) or medium only (grey) for 3h. The findings shown are the mean of results from 5 donors tested in 2 independent experiments and the error bars indicate the SEM.

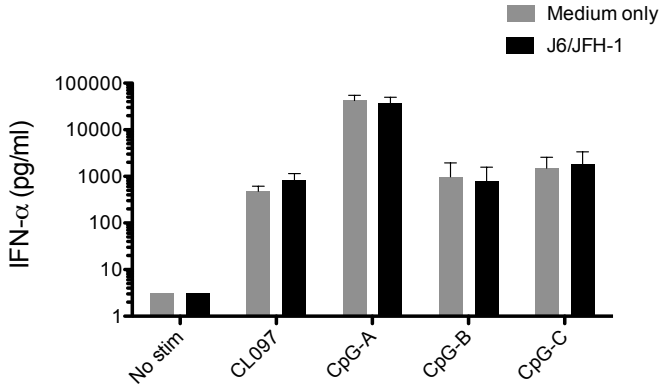

Figure 5-12 Influence of HCVcc on IFN-α production by PBMCs following TLR ligation

IFN-α levels in supernatant after overnight incubation with no stimulant (no stim) or the indicated TLR ligands, following incubation of total PBMC with HCVcc (black) or medium only (grey). Results are shown as the mean of values from five donors tested in two independent experiments and the error bars indicate the SEM.

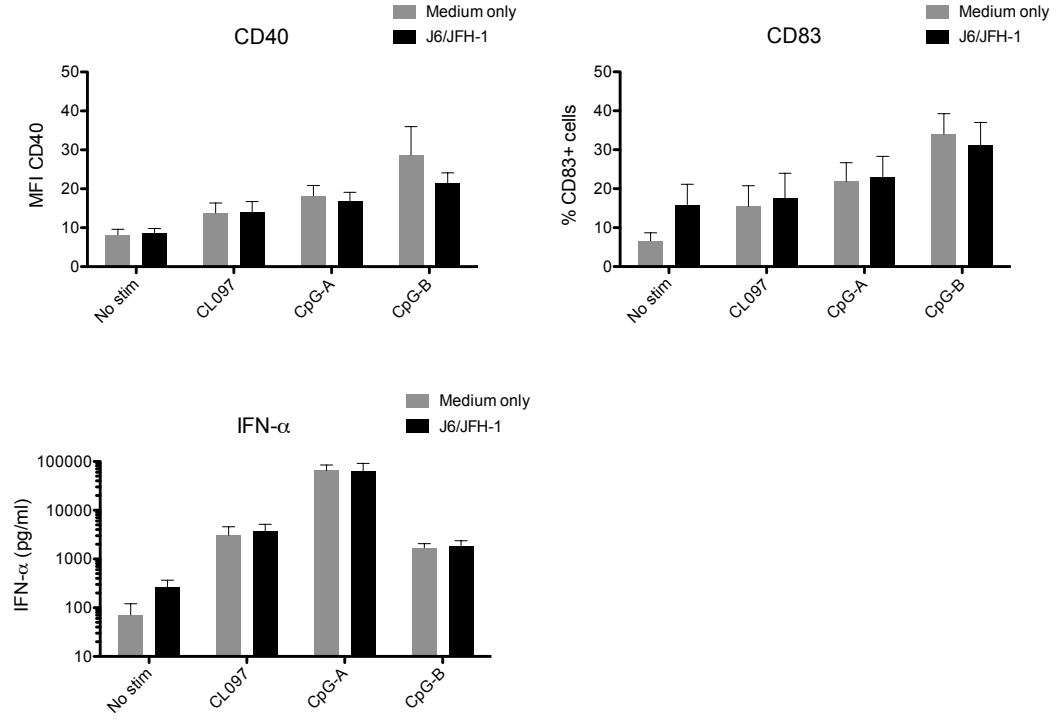

Figure 5-13 Influence of HCVcc on pDC activation in response to TLR ligation using enriched pDCs

Activation of pDCs (percentage of pDCs expressing CD83 and MFI of CD40 staining) and IFN-α levels in supernatant after overnight incubation with no stimulant (no stim) or the indicated TLR ligands, following incubation of enriched pDC with HCVcc (grey) or medium only (black) for 3h. Results are shown as the mean of values from nine donors tested in two independent experiments and the error bars indicate the SEM.

In summary these results showed that under the experimental conditions used no inhibitory effects of HCVcc on pDC activation/maturation or IFN-α production in response to TLR7 or TLR9 ligation could be detected.

5.3 Discussion

Dendritic cells mediate important innate effector functions and play a key role in the initiation of immune responses due to their unique ability to sense infections, produce antiviral factors and activate a variety of cell types, including NK cells, CD4 and CD8 T cells. It is therefore important to understand how DCs recognise HCV and whether HCV can subvert DC functions.

mDCs are highly important in viral infections. They take up viral antigens from the environment and following infection-associated signals become activated and migrate to the lymph nodes, where they present viral peptides to and activate naive CD4 and CD8 T cells. mDCs express several PRRs, including TLR3 and TLR7/8, which have the potential to recognise HCV RNA. This suggests that mDC may be activated during HCV infection following uptake of either HCV particles or parts of infected cells containing HCV RNA.

In the first part of this chapter interaction of mDCs with HCVcc particles was examined and although it was shown that HCVcc was taken up my MDDCs, HCVcc did not induce maturation or cytokine production in either mDCs or MDDCs. These results are in line with other studies that have shown that HCVcc could be taken up by MDDCs [306] or mDCs [384] but that this did not affect maturation or cytokine production in MDDCs or mDCs [202,206]. Another study showed that at high MOI HCVcc could induce some mDC maturation [333], which is also not in disagreement with my results, as I observed a trend for slightly higher CD40 and CD83 upregulation in HCVcc-treated cells compared to untreated cells. Consistent with the observation that MDDCs do not undergo phenotypic activation or induce cytokine production after contact with HCVcc, Ebihara *et al.* also showed that MDDC incubated with HCVcc did not activate autologous NK cells or allogeneic T cells more than non-treated MDDCs [206]. Yet another study showed that HCVcc induced downregulation of HLA-DR expression on MDDCs and induced IL-10 production in MDDC [417]. While I did not see any change in HLA-DR expression after prolonged contact of HCVcc with MDDC, I observed a trend for slightly

higher IL-10 production by MDDCs magnetofected with HCVcc than control-magnetofected cells. Taken together, my results (and those of others) suggest that HCV particles are very weak inducers of maturation and cytokine responses in mDCs. The failure of HCV particles to trigger mDC activation/maturation may be one of the factors that contributes to the relatively delayed activation of both innate [204] and adaptive [405] responses in acute HCV infection. Nonetheless, mDCs must become activated during HCV infection, as HCV-specific T cell responses are eventually induced. A recent study showed that MDDCs were able to mature and produce IFN-β when incubated with HCVcc-infected Huh7.5 cells (but not uninfected cells) [206]. MDDCs that had come into contact with HCVcc-infected Huh7.5 cells were also rendered good activators of NK cells and potent inducers of Th1 responses in T cells. In this study the authors proposed that phagocytosis of apoptotic bodies from infected Huh7.5 cells containing dsRNA replication intermediates lead to activation of MDDCs via TLR3 and type I IFN production. In contrast, uninfected cells that were induced to undergo apoptosis did not induce such a response. This may be the mechanism by which mDCs are activated to prime HCV-specific T cell responses in individuals infected with HCV.

It remains unclear why mDCs do not become activated following exposure to HCVcc, given that mDCs can take up HCVcc particles and express TLR7/8, which has been shown to recognise HCV RNA [184,189]. One possibility is that HCV may block mDC activation. In order to explore this possibility, the modulation of mDC (and MDDC) responses to TLR ligation by HCVcc was examined. I found that HCVcc did not modulate activation/maturation (CD40, CD83, CD86 and HLA-DR) or cytokine production (IL-12 and TNF-α) of mDCs and MDDCs in response to TLR3, TLR4 or TLR7/8 ligation. Similarly, Shiina *et al*. could not detect inhibition of TLR3 and TLR4-mediated maturation (CD80, CD86 and HLA-DR) and cytokine production (IL-6, IL-10 and TNF-α) or inhibition of CD4 T cell proliferation in mDCs and MDDCs by HCVcc [202]. Another study also showed that pre-incubation of mDC with HCVcc did not inhibit TLR3-induced maturation (CD40, CD83 and CD86) or cytokine

production (IL-6 and TNF-α), but slightly inhibited the ability to activate CD4 T cells [333]. While I did not perform experiments to address the ability of mDCs to activate T cells, my data and those of others give no reason to believe that HCV directly impairs T cell activation by mDCs, given that addition of HCVcc to mDCs did not lead to any downregulation of activation or cytokine production or impair their responses to other activation stimuli.

In conclusion, as mDCs (or MDDCs) did not or only marginally become activated in response to HCVcc particles in my and other studies [202,206,333], phagocytosis of apoptotic bodies from infected dying hepatocytes could account for activation of mDCs to stimulate HCV-specific T cell responses *in vivo*. The failure of HCV to trigger mDC activation directly may be one of the factors that delays the induction of HCV-specific T cell responses in infected individuals. The increase in IL-10 production triggered by HCVcc in mDCs observed in one study [417] (but not by others [202]), which my results lend some support to, could be another mechanism by which immune responses might be delayed or suppressed in HCV infection.

pDCs are professional type I IFN-producing cells and given the role of exogenous IFN-α in therapy and the inhibitory effect of type I IFN on HCV replication *in vitro*, pDCs could potentially play a very important antiviral effector role in HCV infection. pDCs express TLR7 and TLR9, the former of which is able to recognise HCV RNA [184,189]. However I found that HCV was only a weak inducer of IFN-α, eliciting only low levels of IFN-α production when added to pDCs from some donors at a high MOI (2-5). Other studies have also shown that pDCs do not or only produce very small amounts of IFN-α after they contact HCVcc [189,201-203] or plasma-derived HCV [201]. Furthermore, neither I nor others observed any maturation or activation in pDCs in response to stimulation with HCVcc [203,333]. To translate the infectious titre into copies/ml, the HCV RNA content of my HCVcc preparations was assessed by a clinical assay, which showed that the infectious titre was found to be around 100 times lower than the number of viral RNA copies/ml. This is in line with results from other publications which also show that RNA copies/ml are typically 100-1000 times higher than the

corresponding TCID50/ml or FFU/ml titre for HCVcc [12,383,202]. Hence the MOI of 2-5 required to induce an IFN-α response in pDCs would correspond to 200-500 viral particles per cell. Similarly, 100ng/ml of HCV RNA were needed to induce a good IFN-α response by transfection of pDCs, corresponding to about $2x10^{10}$ copies/ml. Others have also shown that high concentrations of HCV RNA are needed to induce a robust IFN-α response in pDCs by transfection [189]. HCV thus probably rarely reaches pDC-stimulatory titres *in vivo*. In patients with CHC infection, up to 10^{12} virions can be produced per day [406] but viral titres are typically around 10^5-10^7 copies/ml and reach slightly higher peak levels during acute infection [204]. I also showed that HCV+ plasma from seronegative subjects at the time of peak viremia acute infection did not induce IFN-α production by pDCs *in vitro*, providing further evidence that even maximal titres of patient-derived viruses did not elicit IFN-α production in pDCs. These findings might explain why no systemic IFN-α is seen in acute HCV infection *in vivo* either [188,204] (also explored in the next chapter).

In comparison to other viruses, including HIV and especially influenza virus, HCV and DV (both members of the *Flaviviridae* family) were very poor inducers of IFN-α. HIV could stimulate a robust IFN-α response at a MOI of 0.1 and influenza at a MOI of 0.01, whereas DV did not induce IFN-α production at the highest MOI tested (1) and HCV required a MOI of 2-5 to induce a weak response in some donors. Others also showed that for DV, high MOIs (3-10) are needed to induce an IFN-α response [407]. In agreement with these findings, another study where the ability of influenza, HIV-1 and HCV preparations normalised on the basis of copy number rather than infectious titre to allow direct comparison also showed that influenza was the most potent inducer of an IFN-α response followed by HIV-1 and HCV [203].

The mechanisms responsible for the very poor pDC-stimulatory capacity of HCV are of interest to understand. Different RNA viruses seem to be recognised by pDCs in distinctive ways. While some RNA viruses, such as influenza do not need to be in their infectious form

to elicit an IFN-α response (as UV-inactivated virus stimulates IFN-α production in the same way as live virus), other viruses such as VSV seem to require fusion and early RNA replication events to trigger an IFN-α response [408]. It was shown that VSV could stimulate an IFN-α response via autophagy-mediated transport of RNA replication intermediates to TLR7-containing lysosomes [408]. pDCs can sample the environment by pinocytosis or can take up molecules/particles by endocytosis. It has been shown that many viruses have to be taken up by endocytosis to elicit an IFN-α response in pDCs, e.g. HIV does not replicate efficiently in pDCs, but virions bind to its specific receptors on pDC, are endocytosed and are transported to TLR7-containing endosomes, where the viral RNA triggers IFN-α production by pDCs [409]. Since pDCs do not express all the entry factors and only express low levels of CD81 and SR-BI [384] (and shown in chapter 3), endocytosis of HCV by pDCs might not be very efficient. Nonetheless, Lambotin *et al*. showed that pDCs could take up HCVcc by mechanisms independent of the classic HCV entry factors, and furthermore reported that virions are transported into endosomes following uptake [384]. However this process might not be very efficient. Furthermore, HCV RNA might not be a very potent TLR7 agonist, as, even when HCV RNA was transfected directly into the pDCs, large quantities of RNA were needed to induce an IFN-α response. Another possibility is that HCVcc might inhibit IFN-α production by pDCs. One mechanism by which this might be mediated is via interaction with inhibitory receptors on pDCs, such as BDCA2 and DCIR, which when engaged can inhibit IFN-α production by pDCs in response to TLR ligation [410,411].

To explore this, experiments were performed to address whether blocking interaction of HCV glycoproteins with inhibitory receptors on pDCs would render them capable of producing IFN-α on exposure to HCVcc. However, blocking either HCV E2 or the inhibitory pDC receptors DCIR and BDCA2 did not lead to any IFN-α production in pDCs in response to HCVcc stimulation (at least not at the HCVcc concentration used (MOI 0.01)). It is possible that IFN-α production might have been observed if higher concentrations of HCVcc had been

used. Had time permitted, similar experiments could have been performed with antibodies to HCV E1 and blocking antibodies directed against other inhibitory receptors on pDCs, such as ILT7, NKp44 or FcεRI. Instead, further experiments were carried out to address whether HCVcc modulates pDC responses to TLR7 and TLR9 ligands.

I could not find any evidence that pre-treatment with HCVcc resulted in inhibition of either maturation or cytokine production in pDCs in response to either TLR7 or TLR9 ligation. In contrast, two studies showed that pre-treatment of pDCs with HCVcc reduced IFN-α production in response to subsequent stimulation with TLR9, but not TLR7 agonists [202,203]. Another study showed that HCVcc inhibited the ability of TLR7 ligand-stimulated pDCs to activate naive CD4 T cells and to upregulate CD40 expression (but not cytokine production), although TLR9-mediated responses were not tested here [333]. Whether HCVcc does inhibit pDC responses to TLR ligation, and if so, which TLR pathways are inhibited, thus remains unclear. It is difficult to understand how inhibition of TLR9 but not TLR7-stimulated pDC responses would be advantageous for HCV. Whilst this could occur fortuitously, it seems more likely that if HCV does inhibit pDC responses to TLR ligation, TLR7 would be targeted (alone or with TLR9). Reasons for the discrepancies between the results obtained by different investigators including myself might lie in aspects of the experimental design, including concentration of HCVcc and TLR ligands, and the nature of controls and virus-containing inoculum. It is possible that I may have observed inhibition of TLR-mediated IFN-α production had I exposed pDCs to lower concentrations of TLR ligands, as in my experimental setting the stimulation by TLR ligands may have overridden inhibitory signals. Indeed very high levels of IFN-α were observed after stimulation especially with CpG-A in my experiments. Had time allowed, it would have been of interest to use different concentrations of TLR ligands, to see whether any inhibitory effects might have been missed.

If HCVcc does mediate inhibition of TLR-stimulated IFN-α production, as data from some groups suggest, it could be that this inhibition is mediated by HCV engagement with

inhibitory receptors on pDCs, such as DCIR, signalling via which inhibits TLR9- (but not TLR7-) mediated IFN-α production [411] or BDCA2, signalling via which inhibits both TLR7 and TLR9-mediated IFN-α production by pDCs [410].

In conclusion, although the reasons for this remain unclear, both my results and those of other investigations show that pDCs are very poorly activated following exposure to HCV particles. However, another study found that pDCs produced IFN-α after contact with HCVcc-infected Huh7.5.1 cells [189]. The authors of this study showed that IFN-α production in response to infected cells was TLR7-dependent and required active replication and cell-to-cell contact, suggesting a mechanism whereby parts of infected cells are taken up by pDCs, and the replicating RNA is brought into contact with TLR7 and evokes an IFN-α response. This could be a mechanism by which pDCs could be triggered to produce IFN-α at least locally in the infected liver *in vivo*, which would lead to the induction of ISGs seen in the livers of infected chimpanzees [185,186], despite the fact that no increase is observed in circulating IFN-α levels in these animals [188], or as addressed in the next chapter during acute HCV replication in humans. Although some dysregulation of DC function has been observed in the context of CHC infection in some publications, no generalised immunosuppression accompanied by susceptibility to opportunistic infections is seen in CHC infection, leading to the conclusion that if inhibitory mechanisms are indeed present, these do not result in impairment of the capacity of the immune system to deal with other infections *in vivo*.

6 CHARACTERISATION OF THE CYTOKINE AND CHEMOKINE RESPONSE ACTIVATED DURING HCV INFECTION *IN VIVO*

6.1 Introduction

In the previous chapters mechanisms by which HCV may modulate innate responses of NK cells and DCs *in vitro* were examined. The goal of the work in this chapter was to gain insight into the nature of the innate responses that are actually activated during acute HCV infection *in vivo*, to determine the *in vivo* relevance of the *in vitro* findings.

There is relatively little known about the activation of innate responses in early acute HCV infection. Most studies examining acute HCV infection in humans have focused on later timepoints in acute infection and are typically cross-sectional rather than longitudinal. The chimpanzee model has been of great use to study the very early events in acute HCV infection and has the advantage that sequential and/or intrahepatic samples can be analysed. The systemic activation of innate immune responses has not been addressed extensively in this model. However several studies examined the intrahepatic mRNA expression profile in chimpanzees after inoculation with HCV and showed strong induction of ISGs (including MxA, 2-5 OAS and p56, upregulated as early as 2 days post infection), chemokines (IP-10, MCP-1, MIG, MIP-1β and RANTES) and many genes associated with antigen presentation (HLA alleles, proteasome components and peptide transporters) [185,186]. These findings suggest that interferon production is activated within the liver as viral replication is initiated there, and thus local induction of an inflammatory response. This may make an important contribution to the control of viral replication.

Certain cytokines, such as IFN (Type I, II and III), can have direct antiviral effects, whilst other cytokines/chemokines mediate antiviral activity indirectly by recruiting immune cells

and activating them to make an appropriate immune response towards the virus. However although local induction of cytokines and chemokines may be stimulated as HCV replication starts to occur in the liver, systemic immune activation may be impaired. A recent study from our laboratory, in which sequential plasma samples collected during the eclipse and viral expansion phases of acute HCV (and also HIV-1 and HBV) infection were analysed for cytokine and chemokine levels, showed that the systemic cytokine response in acute HCV (and HBV) infection was delayed relative to the increase in plasma viremia compared to the response observed in acute HIV-1 infection, and of a lower magnitude [204]. Modest increases were seen in circulating levels of a few analytes, including IL-10, IL-18, IP-10 and TNF-α but no induction of systemic IFN-α. Importantly, type III IFN expression was not examined in this study. Recent publications have shown that genetic variation in IL-28B (a type III IFN) is associated with enhanced spontaneous clearance and SVR after therapy [169,170], suggesting an important role for this cytokine in the control of HCV infection.

The first aim of the work in this chapter was to extend the acute HCV infection cytokine study by analysing levels of IL-28B in the plasma donor samples. Unfortunately, no PBMCs were available from the plasma donors analysed in this study, so it was not possible to look at cellular innate responses. It is very difficult to obtain samples from patients acutely infected with HCV, especially at very early timepoints, but since our group had good links to a liver transplant clinic in Barcelona, I decided to evaluate the utility of the LT setting as a model for events in acute HCV infection and to address the cytokine/chemokine response during the acute burst of viral replication in LT patients chronically infected with HCV. The LT setting has been suggested to be a model to study HCV pathogenesis [412], as the new liver invariably becomes infected with HCV following transplantation, potentially replicating events in primary acute infection.

After liver transplantation, viral titres drop in serum, presumably as the uninfected graft removes the virus from the circulation [143] (and possibly also because of the loss of blood

during transplantation). After a short period (at around 3 days post LT) viral titres have usually returned to pre-transplant levels. Due to the immunosuppressive treatment regime used to protect the host from graft rejection (normally a calcineurin inhibitor and gradually declining doses of corticosteroids), viral titres continue to increase thereafter and reach higher levels than prior to transplantation, usually 10 times higher than pre-transplant. When using these patients for the study of immune responses to HCV two confounding factors need to be considered: the immunosuppressive regime, which mainly affects T-cell mediated immunity, and the immune response activated in response to the new graft. To gain insight into the latter, changes in circulating cytokine/chemokine levels in non-HCV-infected LT patients were also studied.

6.2 Cytokine and chemokine upregulation during acute HCV replication

In order to understand HCV persistence and pathogenesis, it is important to describe the nature and timing of the early cytokine/chemokine response activated in HCV-infected individuals. I addressed this issue by firstly extending previous findings from our laboratory [204] on the systemic cytokine/chemokine response during primary acute HCV infection and furthermore by characterising the cytokine and chemokine response activated in a cohort of HCV-infected patients undergoing liver transplantation. In both cohorts, a time frame of 30 days post-infection or post-LT was chosen for this study and >20 analytes were examined. A high sensitivity Luminex kit was used to quantitate levels of GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13 and TNF-α, to get a general overview over the Th1/Th2 balance and pro-inflammatory cytokine upregulation. A few more selected cytokines, i.e. IFN-α, IL-18 and IL-28B, were also examined by ELISA, as these have been shown to be very important in HCV infection [158,167-170,413,414]. MCP-1, CXCR3-associated MIG (only in the LT cohort) and IP-10 and CCR5-associated MIP-1α, MIP-1β and RANTES

were also studied, as these chemokines have been correlated with disease progression and treatment outcome.

6.2.1 Cytokine and chemokine response during primary HCV infection

The first aim of the work in this chapter was to further characterise the systemic cytokine/chemokine response activated during primary acute HCV infection. In the study by Stacey *et al.* [204], no longitudinal control samples from uninfected plasma donors were analysed, so the first thing that was done was to analyse samples from 3 uninfected donors over a time course of approximately 30 days in order to be able to compare the fluctuations in cytokines and chemokines in subjects acutely infected with HCV to natural fluctuations. Furthermore, IL-28B levels were also measured in both the uninfected donors and 10 HCV-infected subjects.

The plasma donor panels originated from 10 donors who acquired HCV (and no other co-infections) over the timecourse studied and were all initially seronegative. All subjects showed an exponential elevation in plasma viral load that then remained at peak levels for the remainder of the 30-day post-LT study period (Figure 6-1). The viral loads of the 10 HCV+ plasma donors were measured by Mike Busch (BSRI, San Francisco). The sample timecourses from different subjects were then temporally aligned by Li Qin (University of Washington) to define a T_0, the timepoint, when viral load first reached detectable levels (600 copies/ml).

Temporal charges in circulating levels of >20 cytokines and chemokines had previously been quantitated in the 10 HCV+ plasma donors by Stacey *et al* [204]. I analysed levels of these cytokines and chemokines in sample timecourses of 3 uninfected plasma donors, and also measured the IL-28B levels in all 13 donors. I then re-analysed all the data comparing perturbations in the analyte levels in the HCV-infected subjects to those in the control subjects I had studied to identify infection-associated cytokine/chemokine responses.

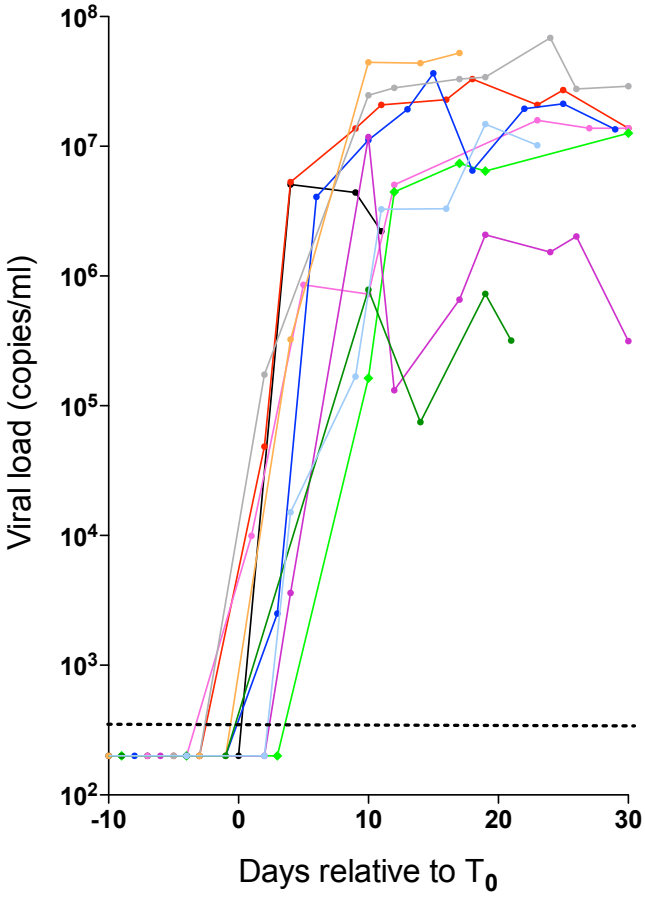

Figure 6-1 Viral loads in subjects with primary HCV infection

Viral loads were measured using a Roche Amplicor PCR assay (Mike Busch, BSRI). The horizontal dotted black line indicates the lower limit of detection (LLD) for the viral load (600 copies/ml). The sample time courses are aligned relative to a T_0 (calculated by Li Qin, University of Washington), which is defined as the time point when the viral load first reached detectable levels.

First, baseline values for each analyte were calculated in each subject. In the HCV-infected subjects, baseline analyte levels were calculated as the mean of analyte levels at all time-points thought to be prior to the acquisition of HCV infection. In many virus infections including HCV infection there is an eclipse phase before the virus reaches detectable levels in the circulation. In HCV infection, rapid viral amplification starts shortly after transmission, and the eclipse phase is thought to be relatively short. In chimpanzees HCV replication can be detected systemically as early as 2 days after intravenous inoculation [186], and also in human infection viremia becomes detectable within the first few days after inoculation [405]. I thus allowed for an eclipse period of three days and took the mean of analyte levels at all

timepoints (between 2 and 10) more than 3 days prior to T_0 as the subject-specific baseline value. In the uninfected plasma donors I calculated baseline analyte levels as the mean of values from the first two timepoints in the series of samples analysed. I then calculated the percentage of subjects in whom analyte levels were elevated by 2-fold or more over baseline in the 30 days post T_0, or in the uninfected subjects (Figure 6-2A).

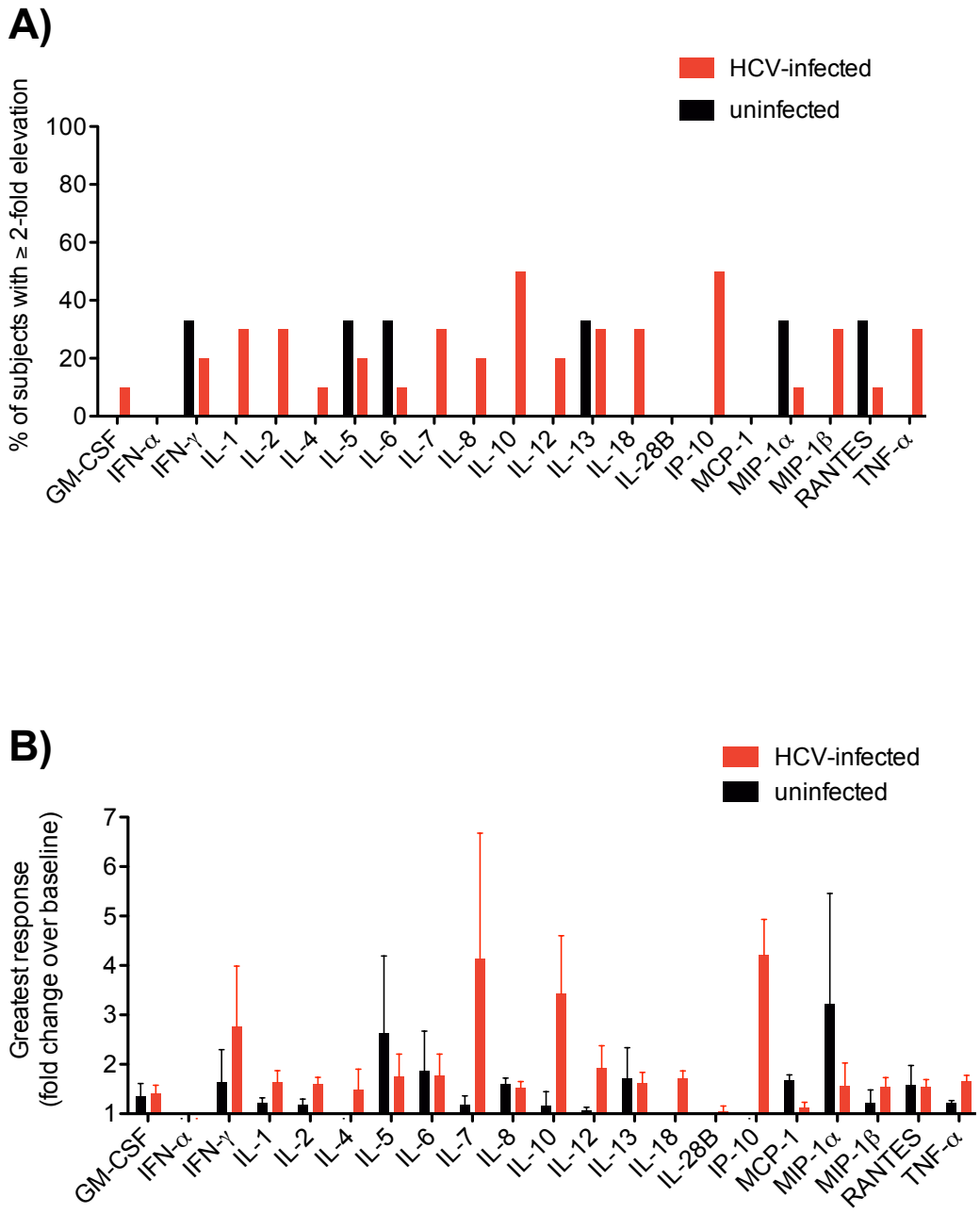

Figure 6-2 Chemokine and cytokine upregulation in primary HCV infection

Systemic levels of 21 analytes were analysed over a timecourse of 30 days in 3 uninfected plasma donors and 10 plasma donors, who became infected with HCV. A) Percentage of subjects with ≥2-fold elevation over baseline and B) greatest magnitude of response (as fold change over baseline) during the 30 day time frame. Results are plotted as mean and the error bars indicate the SEM.

In the uninfected plasma donors, perturbations were observed in only 6 of the 21 analytes measured, and when they did occur, were seen only in one of the three donors analysed. In the HCV-infected plasma donors perturbations were observed in 18 of the 21 analytes measured but also here most analytes were only upregulated in only a low proportion of subjects, with only 2 analytes (IL-10 and IP-10) being upregulated in at least 50% of the HCV-infected but not the uninfected donors. IL-1β, IL-2, IL-7, IL-18, MIP-1β and TNF-α were upregulated in at least 30% or more of the HCV+ but not the HCV- donors.

The magnitude of the response was then examined. The greatest elevation (fold change over baseline) within the 30-day post-infection period was measured and compared between the HCV- and the HCV+ group (Figure 6-2B). In this and all the following graphs statistical analysis was performed using a Mann-Whitney test. Ideally, a correction for multiple comparisons should have been performed, but this was not done due to the small sample sizes used. Analysis of the magnitude of the response in the plasma donors revealed significant differences in the highest elevation in two analytes, IL-18 ($p=0.014$) and IP-10 ($p=0.028$). There were also relatively low p values for IL-7 ($p=0.0769$), IL-10 ($p=0.1119$) and TNF-α ($p=0.1119$) and IL-2, IL-4 and IL-12 also showed p values smaller than 0.2. Some of the latter might have reached statistical significance if the group sizes had been larger. Notably, no systemic upregulation of either IFN-α or IL-28B was seen, indicating that the type I and III IFN response in acute HCV infection might remain local. In summary, these results show that neither the magnitude of the systemic cytokine/chemokine response nor the percentage of subjects upregulating a given analyte is very high during early acute HCV infection. Nevertheless, considering both the results in Figure 6-2A and the statistical analyses of the data in Figure 6-2B, there are at least 5 analytes that seemed to be specifically upregulated in acute HCV infection: IL-7, IL-10, IL-18, IP-10, TNF-α and possibly others (such as IL-2, IL-4 and IL-12). In Figure 6-3, the temporal changes in plasma viral load and circulating levels of 6 analytes (IL-10, IL-18, IP-10, TNF-α, IFN-α and IL-28B) in three representative

HCV-infected plasma donors are shown to illustrate the magnitude and dynamics of perturbations in these analytes in early acute infection.

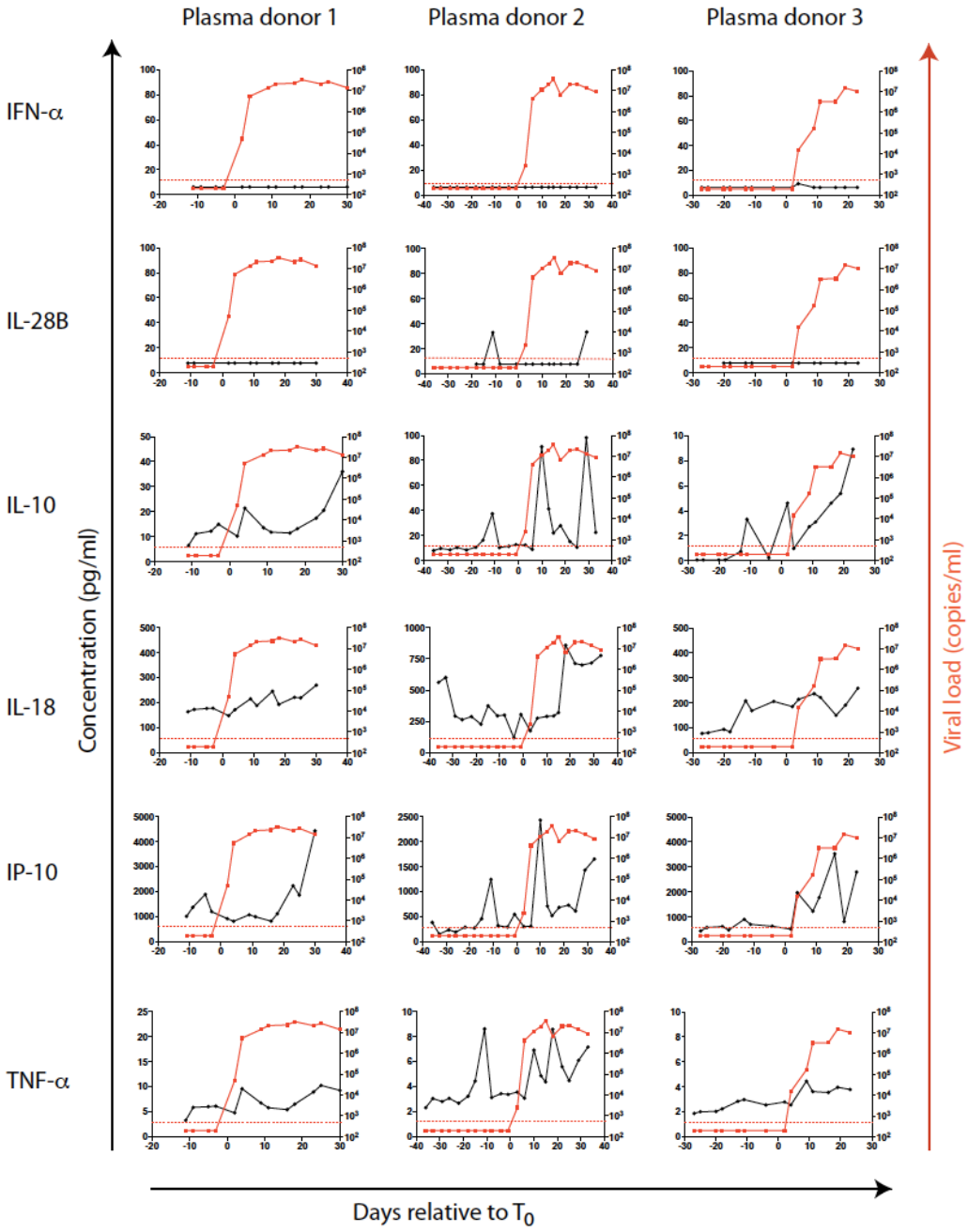

Figure 6-3 Cytokine/chemokine levels in individual subjects

The viral load (red) and systemic levels of 6 analytes (black) are depicted in 3 of the 10 plasma donors who became infected with HCV. The horizontal dotted red line indicates the LLD for the viral load (600 copies/ml).

To address what happens after the acute viral expansion phase of infection, cytokine/chemokine levels were analysed in two more HCV-infected plasma donors who

were followed up over a longer time course post-infection, but where very early responses were missed. The viral loads in these subjects follow a typical course of acute HCV infection, where viral loads start to decline after a 2-3 month period of infection and there are then transient secondary bursts of viral replication (Figure 6-4).

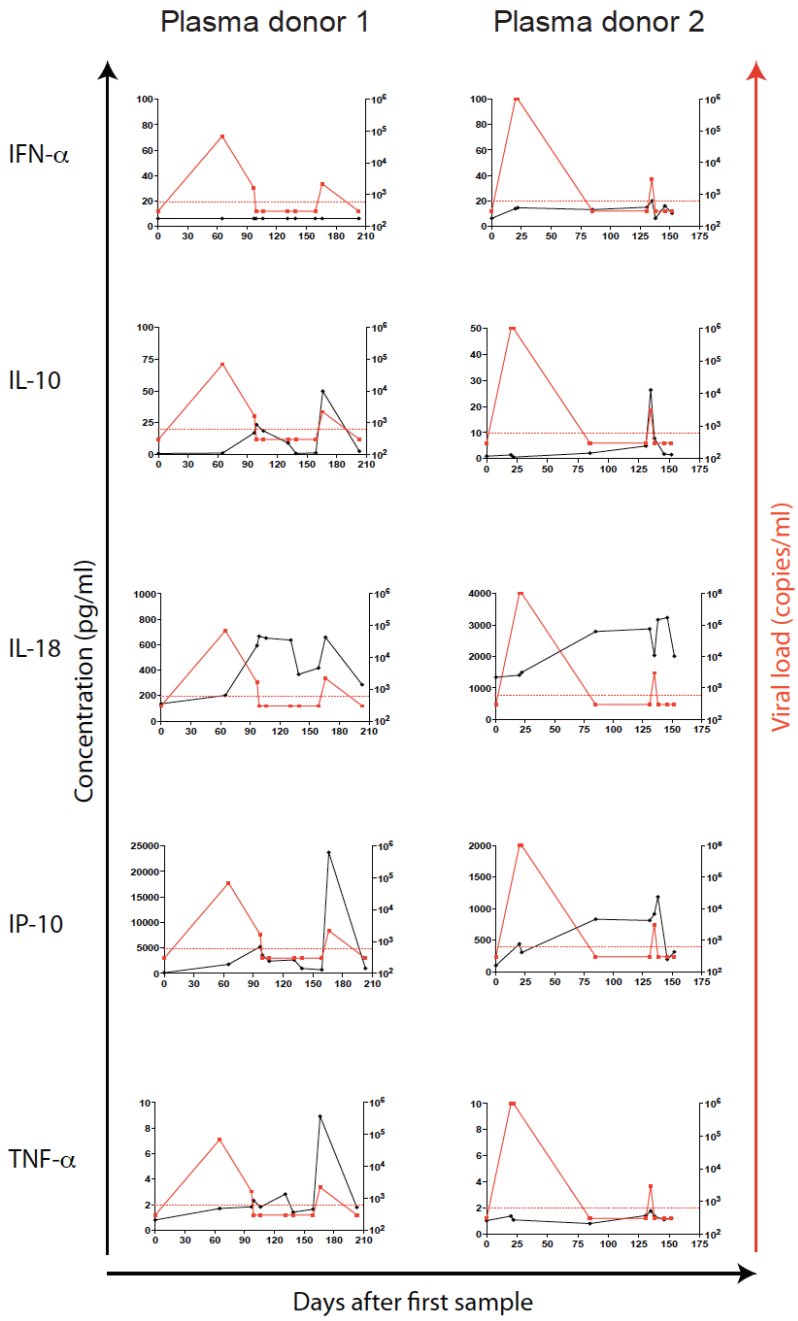

Figure 6-4 Induction of cytokine/chemokine response corresponds to secondary viral expansion in early HCV infection

Systemic levels of 20 analytes were analysed in two plasma donors who became infected with HCV. The viral load (red) and levels of systemic cytokines/chemokines (black) are shown. Time is plotted in days from the first sampling point, which in both subjects was prior to detection of any systemic signs of infection. The horizontal dotted red line indicates the LLD for the viral load assay (Roche Amplicor, 600 copies/ml, Zeptometrix).

No information is available on whether the infection was subsequently cleared or became chronic in these subjects. Regarding the cytokines and chemokines upregulated during these secondary phases of viral replication, patterns of analyte upregulation similar to those observed in the first 30 days of infection were seen, with elevations in analytes such as IL-10, IL-18, IP-10 and TNF-α coinciding with the secondary rises in viral titres. Chemokines such as MCP-1, MIP-1β and MIG, which were not significantly induced in the early 30-day time frame studied, were also found to be upregulated at these later timepoints, with the elevations again coinciding temporally with rises in viral titres (data not shown), indicating that a broader chemokine response is induced later in infection.

In summary, although some modest perturbations in systemic cytokine and chemokine levels were observed in subjects acutely infected with HCV that seemed to be specific to HCV infection differing from the fluctuations in circulating analyte levels observed in uninfected subjects, the responses were low and delayed relative to the increase in viremia as compared to the burst of innate cytokine and chemokine production observed in HIV infection [204].

6.2.2 Cytokine and chemokine responses in HCV-infected patients undergoing liver transplantation

I next went on to characterise the systemic cytokine/chemokine perturbations in a well-defined cohort of patients undergoing liver transplantation. 13 HCV-infected and 5 uninfected LT patients were analysed at the following timepoints: day 0 (pre-LT) and days 3,7,14 and 30 post-LT. The demographic and virological data are summarised in Table 6-1.

Feature	HCV- patients (n=5)	HCV+ patients (n=13)
Age (years)	46.2±18.47	55.08±10.62
Gender (male/female)	4/1	8/5
HCV genotype (1a/1b)	N/A	2/11

Table 6-1 Patient demographic and virological data

Data are presented as either the total number and ratio (gender, genotype) or the mean ± standard deviation (age)

In addition to comparing the cytokine and chemokine responses in the LT subjects to those in plasma donors, I also correlated virological and clinical findings (such as viral kinetics and fibrosis score) to the cytokine and chemokine levels present in the LT patients, to try to gain insight into HCV disease pathogenesis. Serum levels of 22 cytokines and chemokines (the same analytes as in 6.2.1 except MIG, which was analysed in addition) were analysed in 13 HCV-infected patients and 5 uninfected patients who underwent LT. Values from the pre-LT timepoint were taken as the baseline analyte levels in each subject.

Viral loads in the LT patients were measured by Jakub Dragun (Hospital clinic Barcelona) using a COBAS Amplicor HCV Test with a lower detection limit of 25 IU/ml. Figure 6-5 shows the viral loads in the 13 HCV+ patients studied.

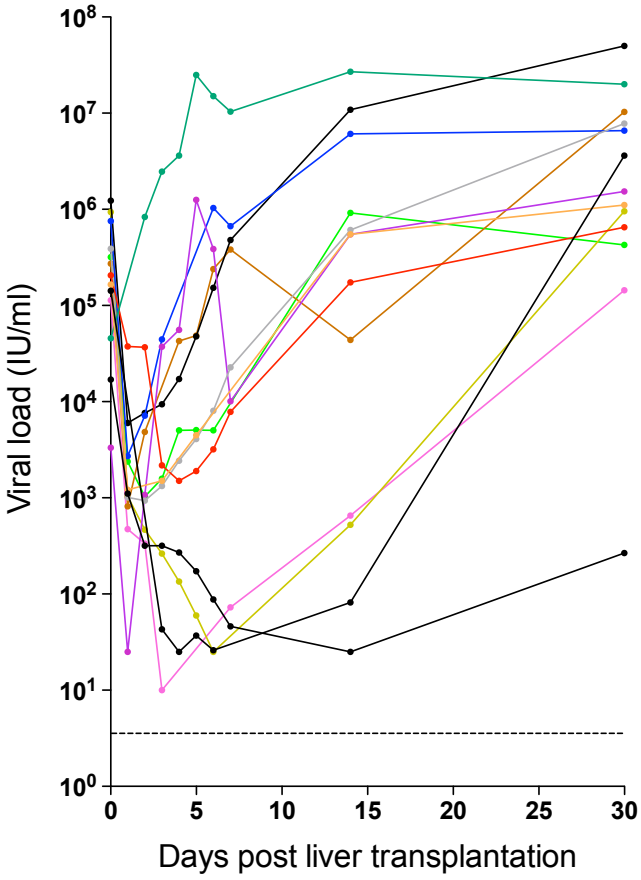

Figure 6-5 Viral loads in HCV-infected subjects undergoing liver transplantation

The viral titres in 13 HCV+ LT subjects were measured by Jakub Dragun using a COBAS Amplicor HCV test. The horizontal dotted black line indicates the LLD of the viral load assay (25 IU/ml).

Viral titres were around 10^4 to 10^6 IU/ml pre-transplant, and then declined about 2-4 logs, during which it is believed that the circulating virus is adsorbed by the new liver and infects it [143]. Viral titres subsequently rose, frequently to levels above the pre-transplant level, which is probably due both the immunosuppression that benefits viral replication and the capacity of the new liver to support better HCV replication than the old liver [147].

6.2.2.1 Comparison of cytokine/chemokine upregulations in uninfected and HCV-infected patients undergoing liver transplantation

The percentage of subjects with elevations in each analyte, defined as an increase of ≥2-fold over baseline in the 30 days post-LT, was initially addressed (Figure 6-6A). Contrary to what was observed in the HCV- plasma donors, almost all analytes (21/22) were upregulated in one or more of the HCV- LT patients, with 11/22 analytes being upregulated in more than 50% of subjects and more than 75% of subjects exhibiting elevations in IL-10, IP-10, MCP-1, MIG and TNF-α. The only analytes that were upregulated in a considerably higher proportion of HCV+ subjects compared to HCV- subjects were IFN-γ, IL-5, IL-12 and MIP-1β. Otherwise there were no major differences in the proportion of LT subjects who upregulated an analyte over time in the HCV- and the HCV+ groups. Liver transplantation itself is thus associated with upregulation of circulating levels of multiple cytokines and chemokines, with relatively few analytes being upregulated in a markedly higher proportion of HCV+ than HCV-subjects. Next the magnitude of the elevation was compared in the HCV+ and HCV- groups. The greatest elevation (fold change over baseline) within the 30-day time frame was analysed in each subject and mean values for the two groups are shown in Figure 6-6B. Not only the percentage of subjects showing an elevation but also the magnitude of elevation in almost all the analytes was markedly higher than in the plasma donor subjects, with increases in certain analytes reaching above 100-fold over baseline. Although most analytes were upregulated in both the uninfected and HCV+ LT patients, the magnitude of the elevation in the HCV+

group was considerably higher than in the uninfected ones. Statistical analysis revealed that the elevation in MIG (p=0.0385) was significantly greater in the HCV+ than in the HCV- LT patients, and relatively low p values were also seen for IFN-γ (p=0.111), IL-7 (p=0.1393), IL-8 (p=0.1676), IL-10 (p=0.1676) and MIP-1β (p=0.1676). As observed in the primary acute HCV subjects, type I and III IFN were not appreciably upregulated over baseline in the HCV+ LT patients.

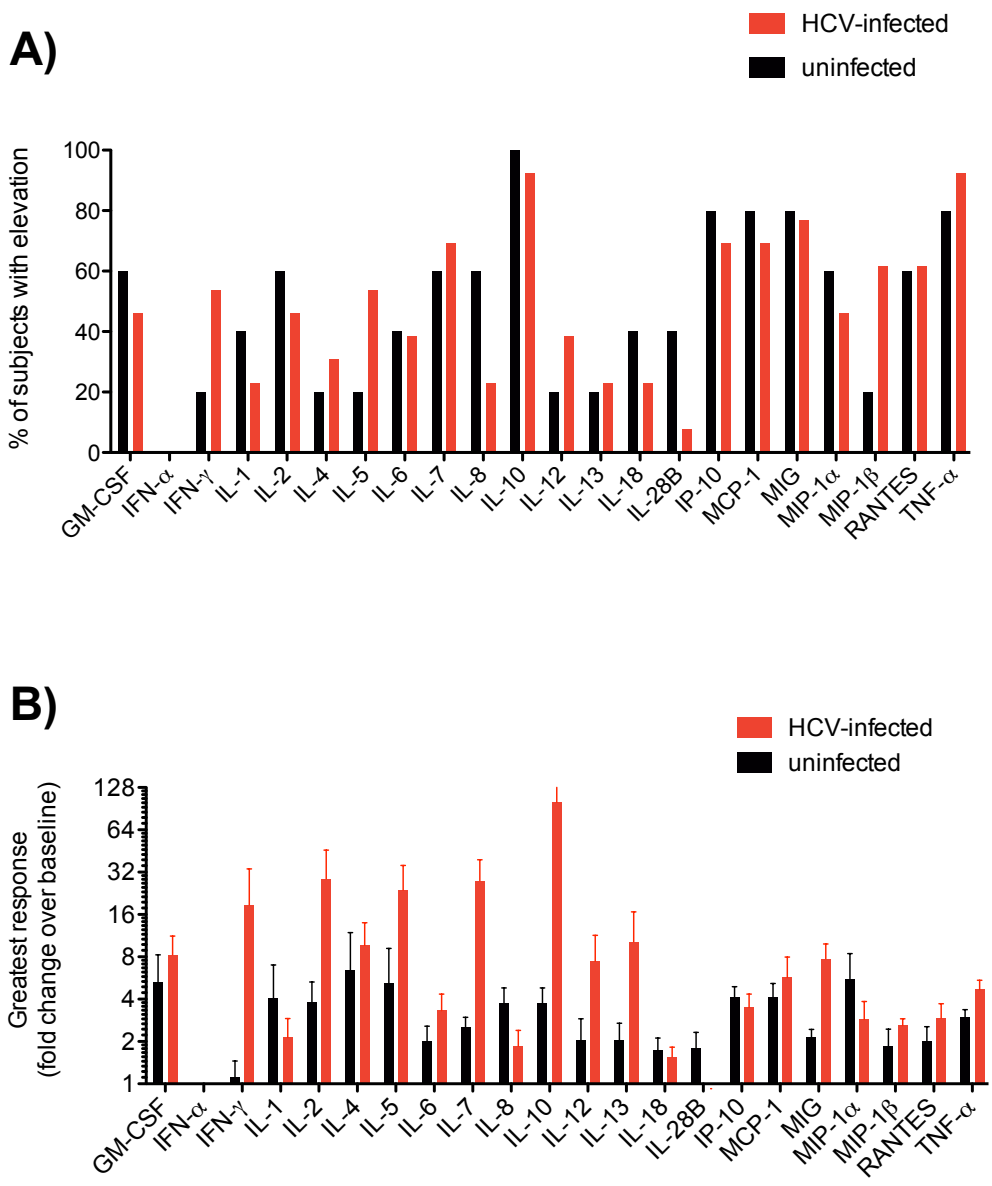

Figure 6-6 Chemokine and cytokine upregulation after liver transplantation in HCV+ and HCV- subjects

Systemic levels of 22 analytes were analysed in 13 HCV-infected and 5 uninfected patients undergoing LT. A) Percentage of subjects with ≥2-fold elevation over baseline and B) greatest magnitude of response (as fold change over baseline) during the 30 day time frame. Results are plotted as mean and the error bars indicate the SEM.

One of the things that was noted and made analysis of these data more difficult than in the primary acute HCV group was that sometimes pre-transplant levels of a given analyte in a given donor were very high, then analyte levels dropped at the first timepoint post-LT that was analysed (day 3) before beginning to rise again and did not recover in the time frame studied. The latter type of analyte increase was not revealed by analysing the response relative to baseline. Interestingly, this pattern was especially evident for type I and type III interferons in some HCV+ patients but not HCV- patients, suggesting that that there may be on-going production of these analytes in the context of CHC infection. In these patients, the levels of IFN-α and IL-28B were typically high to begin with, then dropped after LT and began to recover shortly thereafter, implying that HCV may induce systemic type I and III IFN production in some individuals in a LT setting *in vivo*. Temporal changes in circulating levels of 15 analytes are shown in one donor in Figure 6-7 to illustrate the different patterns of analyte perturbation observed.

In summary, the study with LT subjects showed that there might be some HCV-specific cytokine and chemokine responses that are induced during the burst of acute HCV replication that occurs as the new liver becomes infected but that there is also a high response induced in uninfected subjects owing to liver transplantation. Interpreting the data is hence difficult, which hampers use of the LT setting as a model for analysis of innate immune responses in primary acute HCV infection. Nevertheless, the data generated provided me with an opportunity to correlate cytokine/chemokine levels responses prior to and after LT to viral kinetics and clinical parameters such as fibrosis score.

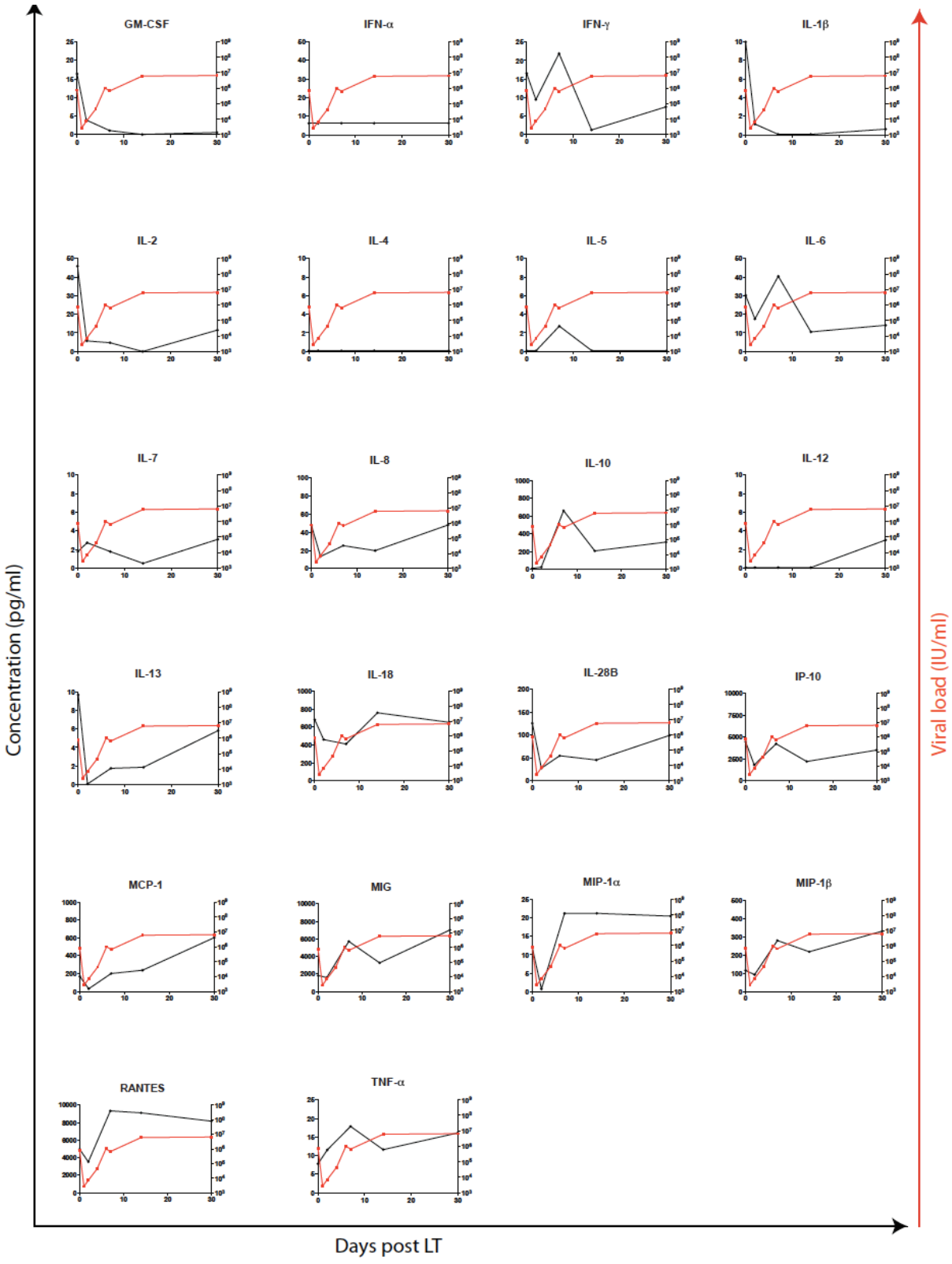

Figure 6-7 Temporal changes in systemic cytokine/chemokine levels in an individual HCV+ LT patient

Systemic levels of 15 analytes (black) and the viral load (red) in one HCV-infected patient are shown immediately prior to LT (day 0) and at the indicated timepoints post-LT.

6.2.2.2 Comparison of cytokine/chemokine profiles in HCV-infected patients with slow or fast viral replication kinetics after liver transplantation

As seen in Figure 6-5, viral loads decline after liver transplantation and subsequently rise but the kinetics of the increase in viremia in different patients vary considerably. In some patients, viral loads start to rise again as soon as 1 day after LT, whereas in others viral loads do not rise until 14 days post LT. To evaluate whether there were any relationships between cytokine/chemokine profiles and kinetics of viral replication (which could reflect either an influence of one or more analytes on virus replication or effects of viral replication on analyte production), the patients were divided into groups with fast, intermediate and slow viral replication after LT, and differences in pre-LT analyte levels. The longitudinal viral titres of the patients in these groups are shown in Figure 6-8.

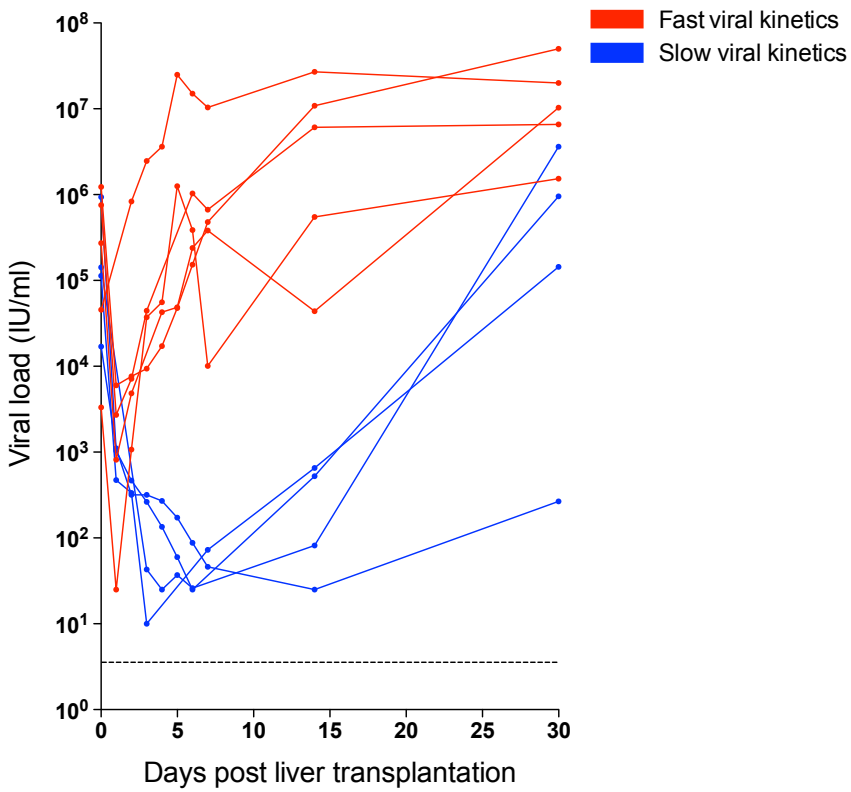

Figure 6-8 Longitudinal viral titres in HCV-infected subjects exhibiting slow and fast viral replication kinetics after LT

Viral loads from 9 patients exhibiting slow (blue) or fast (red) viral kinetics post-LT are shown. The horizontal dotted black line indicates the LLD for the viral load assay (25 IU/ml).

First, cytokine and chemokine levels at the pre-LT timepoint in the two groups were compared to determine whether pre-existing levels of any of the analytes differed in subjects exhibiting different kinetics of viral replication post-transplant (Figure 6-9). The general trend was for pre-LT levels of several analytes, particularly IL-10, to be slightly higher in the group with fast viral kinetics post-transplant, although certain chemokines (IP-10, MIP-1β and RANTES) were present at higher levels in the group that showed slow viral kinetics post-LT. Statistical analysis showed that the only analyte present at significantly different levels prior to LT in the two groups was RANTES ($p=0.0317$). There was thus no evidence of a relationship between levels higher pre-existing levels of innate and Th1 cytokines and slower viral kinetics, although high pre-LT levels of certain chemokines (especially RANTES) did seem to be associated with slow viral replication kinetics post-transplant.

Next, the magnitude of the greatest elevation (fold change over baseline) of each analyte during the time frame studied was compared in the two groups (Figure 6-10). Interestingly, although there was a trend for pre-LT levels of many analytes to be lower in the subjects exhibiting slower replication kinetics post-LT, there was a trend for these subjects to exhibit greater maximal elevations in analyte levels post-LT. There were no statistically significant differences between the two groups in the magnitude of the maximum elevation observed in any of the analytes tested, although low p values were observed for IL-28B ($p=0.0635$), IL-8 and MIP-1α, (both $p=0.1905$) and IFN-α ($p=0.2496$), all of which showed a greater maximal upregulation in the subjects with slower viral replication kinetics, and IL-12 ($p=0.1706$), which showed a greater maximum upregulation in the subjects with faster viral replication kinetics. It is thus tempting to speculate that the former analytes might have a negative effect on viral expansion, although further studies in a larger number of subjects would be required to confirm the trends in this small study and also to investigate the causality of the association.

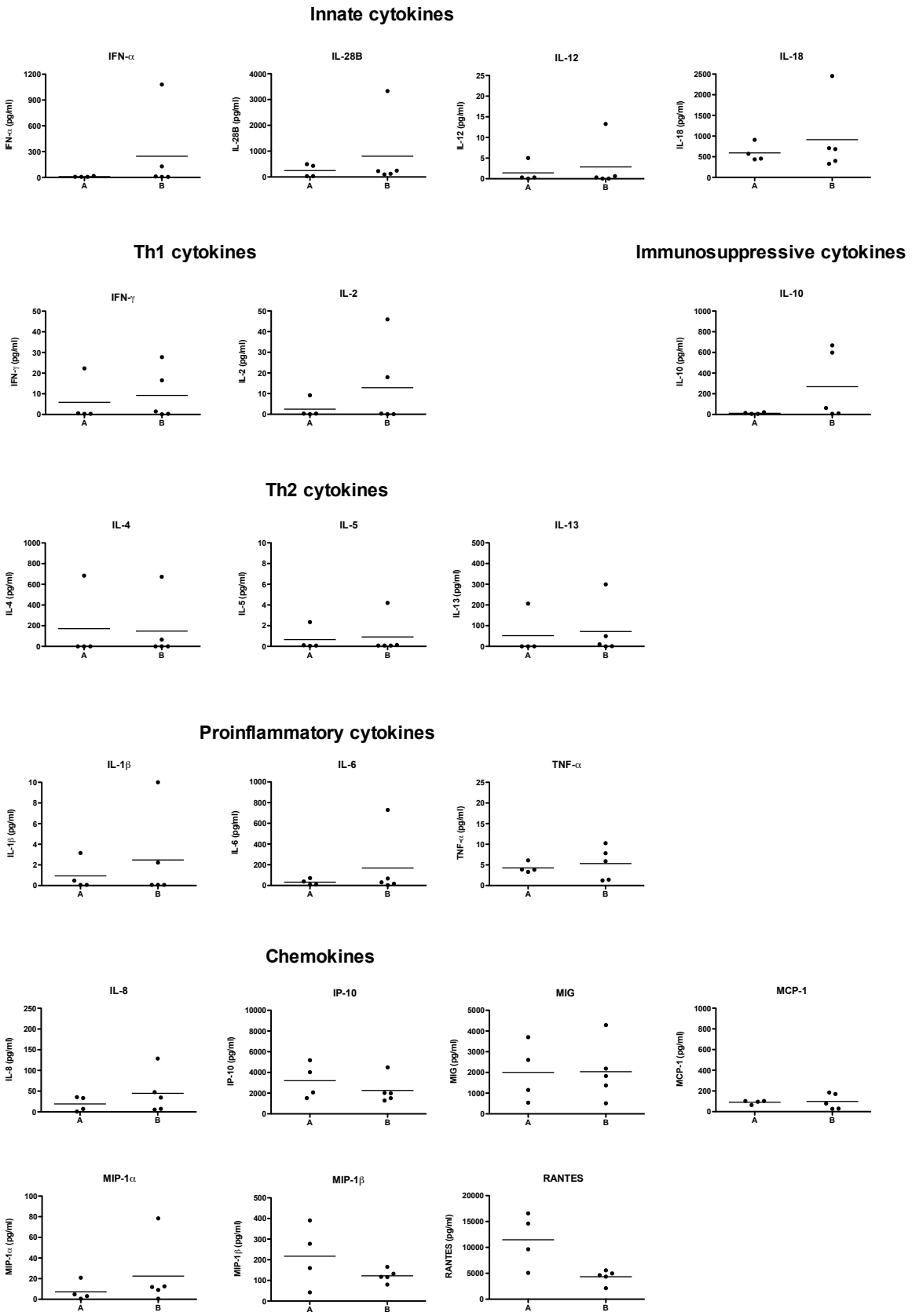

Figure 6-9 Pre-LT levels of cytokines/chemokines in HCV+ LT subjects with slow and fast viral kinetics

The pre-LT systemic levels of 20 analytes in 9 HCV-infected patients who exhibited slow (A) or fast (B) kinetics of viral replication post LT are shown. The horizontal lines represent the mean analyte levels.

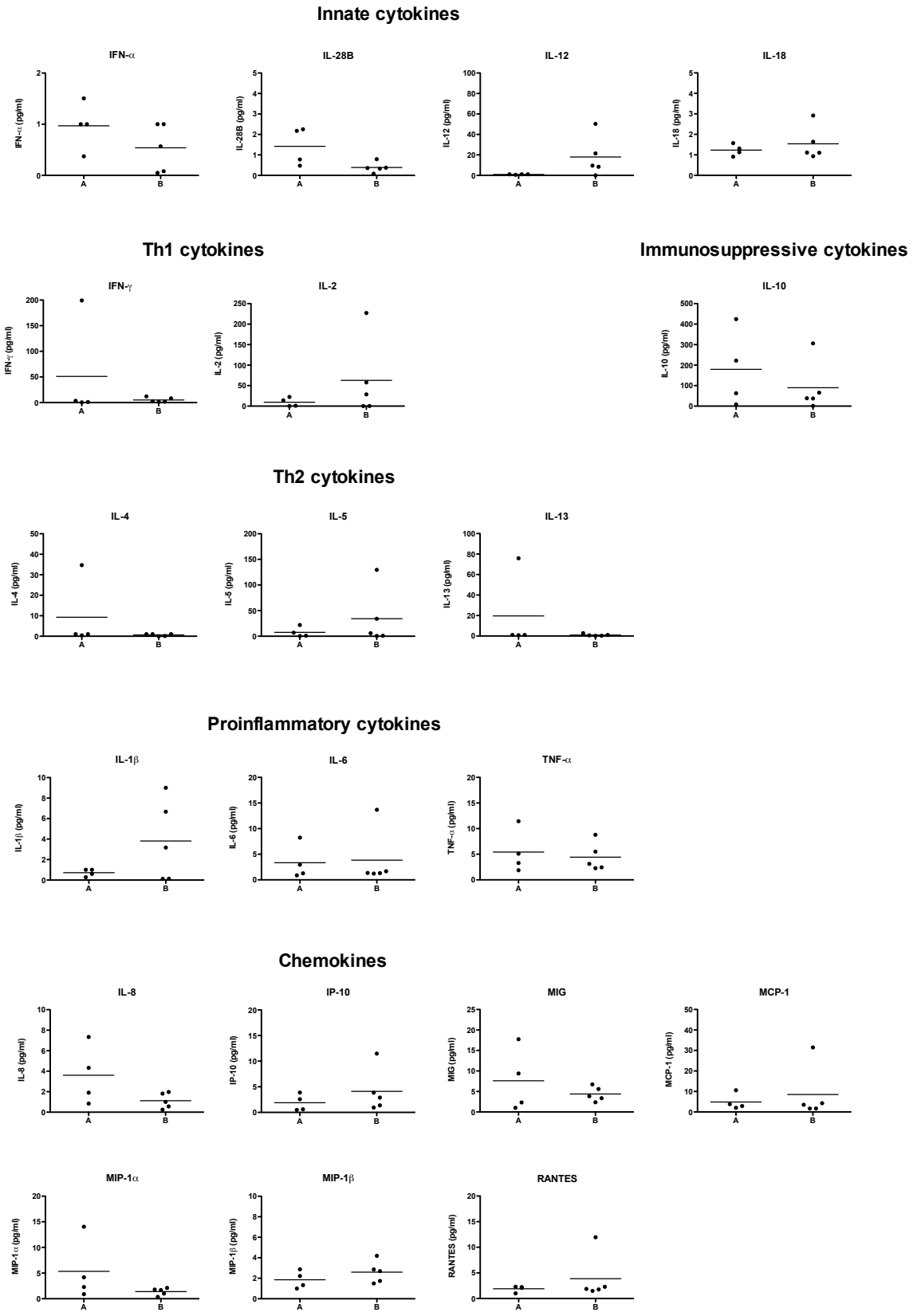

Figure 6-10 Magnitude of the cytokine/chemokine response in HCV+ LT subjects with slow and fast viral kinetics

The greatest magnitude of response (as fold change over baseline) of 20 analytes in 9 HCV-infected patients who exhibited slow (A) or fast (B) kinetics of viral replication post LT during the 30-day time frame are shown. The horizontal lines represent the mean analyte levels.

6.2.2.3 Comparison of cytokine/chemokine upregulations between HCV-infected patients with a mild or severe fibrosis score 1 year post liver transplant

HCV infection is associated with the development of liver disease and liver damage is typically accelerated in LT patients [147]. It was thus of interest to evaluate whether there were any relationships between pre-LT or post-LT cytokine and chemokine profiles and the subsequent development of liver fibrosis, which could reflect a role for particular analytes in promoting or preventing disease pathogenesis. This was addressed by studying the 13 patients in whom cytokine/chemokine levels had already been analysed (Figure 6-6) and additional 18 HCV+ patients in whom cytokine/levels at the pre-LT timepoint only.

The stage of fibrosis was determined at one year post-transplant by the hospital clinic Barcelona using the Metavir scoring system based on a scale of 0–4 (0 = no fibrosis and 4 = liver cirrhosis) as previously described [415]. At one year post-transplant, all patients had a fibrosis score between 0 and 3, where a score of 0-1 was classified as mild fibrosis and a score of 2-3 was classified as severe fibrosis.

Cytokine/chemokine levels at the pre-LT timepoint in patients who subsequently developed mild fibrosis and severe fibrosis are shown in Figure 6-11. At the pre-LT timepoint there were no major differences in the levels of Th1 cytokines, chemokines, proinflammatory cytokines, innate cytokines or IL-10, but there was a trend for Th2 cytokines to be present at higher levels in the group with mild fibrosis. None of the differences reached statistical significance, although the p value for IL-4 was low ($p=0.0705$). These results would be consistent with the hypothesis that a strong pre-existing Th2 response might reduce the development of liver disease, although further work would be needed to confirm this. As the fibrosis score was measured one year after transplantation, it was also of interest to analyse the cytokine/chemokine levels after LT, so analyte levels in patients who subsequently developed mild fibrosis and severe fibrosis were also compared at one month post LT (Figure 6-12).

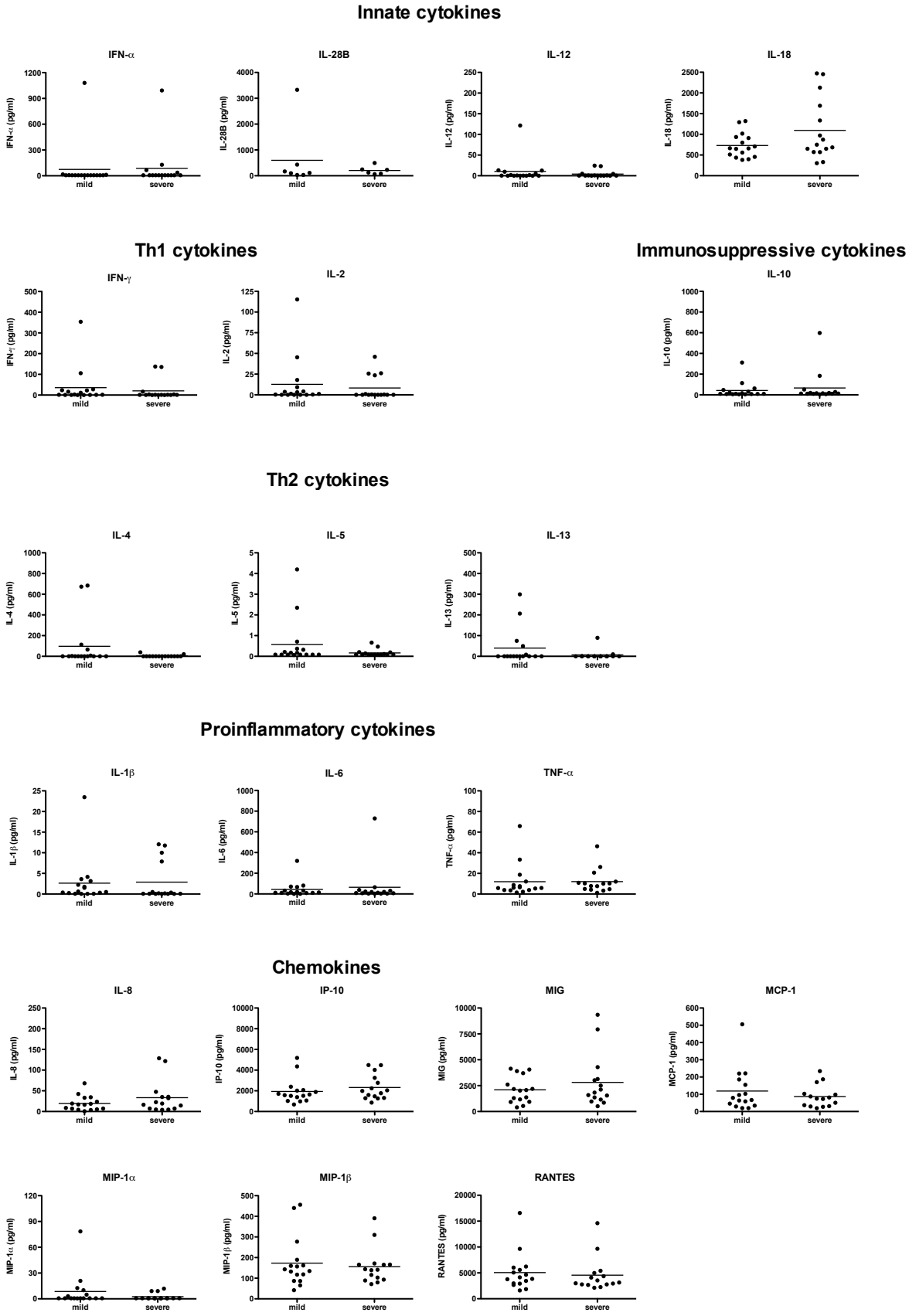

Figure 6-11 Pre-LT levels of cytokines/chemokines in HCV+ LT subjects with mild or severe fibrosis

The pre-LT systemic levels of 20 analytes in 31 HCV-infected patients who exhibited mild or severe fibrosis (as measured at one year post-LT) are shown. The horizontal lines represent the mean analyte levels.

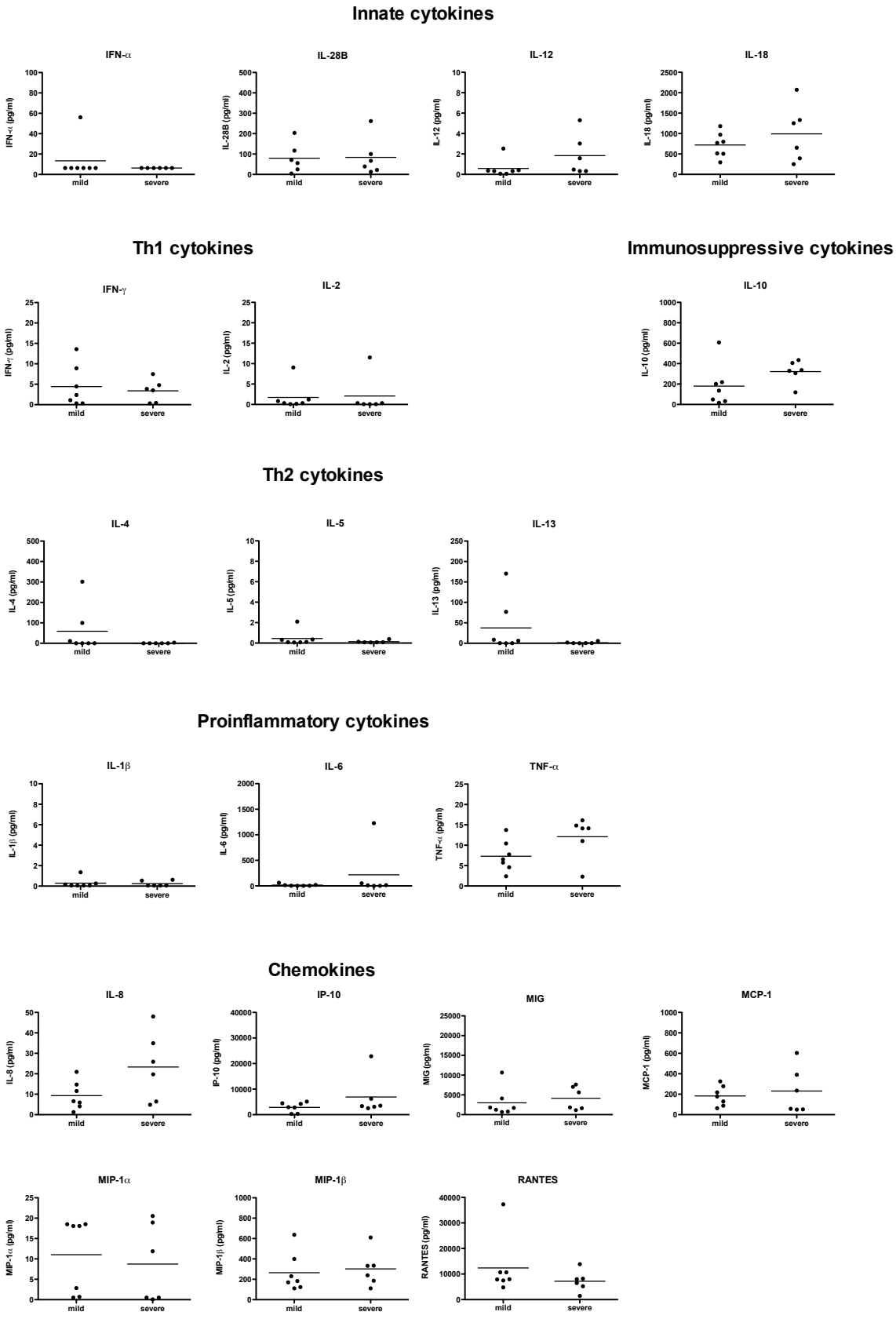

Figure 6-12 Cytokine/chemokine levels one month post-LT in HCV+ LT subjects with mild or severe fibrosis

The systemic levels of 20 analytes in 31 HCV-infected patients who exhibited mild or severe fibrosis (as measured at one year post-LT) at one month post-LT are shown. The horizontal lines represent the mean analyte levels.

There was a trend for higher levels of IL-8 (p=0.1375), IL-10 (p=0.1014), IL-12 (p=0.1277) and TNF-α (p=0.0734) to be present at this timepoint in the group with severe fibrosis, which might indicate a role for these analytes in liver pathogenesis, although none of the analytes studied were present at significantly different levels in the two groups.

In summary, these studies were hampered by the small number of patients from whom samples were available, which resulted in group sizes being too low for conclusive statistical analysis. Nonetheless, the results from these analyses performed here raised some interesting hypotheses, suggesting putative roles for type I and type III IFNs in restricting viral replication post-LT and for a Th2 bias prior to LT in protecting against and pro-inflammatory cytokine production post-LT in helping to drive the development of liver fibrosis. However more extensive studies in larger numbers of subjects would be required to confirm the associations observed here and determine whether relationships between cytokine/chemokine levels and *in vivo* phenotypes are causal or not.

6.3 Discussion

In this chapter the systemic cytokine/chemokine responses activated during the acute burst of HCV replication in both plasma donors who acquired HCV infection and in HCV-infected patients undergoing LT were studied over time. In primary acute HCV infection modest responses were observed but the five analytes that were upregulated at greatest magnitude and in the greatest number of subjects (compared to non-infected controls) were IL-7, IL-10, IL-18, IP-10 and TNF-α. Other analytes, including IL-1, IL-2, IL-12 and MIP-1β were also upregulated, although in fewer subjects and to a lesser extent. It has been reported by a recent study looking at a broad range of cytokines/chemokines in CHC infection that many analytes, including some of those above, are also upregulated in chronic HCV infection compared to healthy controls [416]. The difference between acute HCV infection and HIV infection (where a large cytokine/chemokine response is induced [204]) might be due in part to differences in the

principal sites of viral replication (HIV replicates in lymphoid tissues throughout the body, whereas HCV replication is localised primarily to the liver) and in part to differences in virus-mediated activation of innate immune subsets (e.g. HIV stimulates IFN-α production in pDCs better than HCV).

In the liver transplant patients, many analytes were upregulated in both the HCV- and the HCV+ subjects, although the magnitude of the response was higher in the HCV+ group for several analytes, including MIG, IFN-γ, IL-7, IL-8, IL-10 and MIP-1β. Many of these differences in analyte levels between groups failed to reach statistical significance but this was likely a consequence of the relatively small numbers of subjects in each group. Notably, there was no strong concordance between those analytes most strongly upregulated in primary infection and those upregulated during the post-LT burst of viral replication. Generally, it was harder to evaluate which cytokine/chemokine responses were actually induced by HCV in the LT setting than it was in primary acute infection. Firstly, LT induced a strong cytokine/chemokine response on its own and furthermore high pre-LT levels of many analytes could be seen in the HCV+ patients (presumably induced by chronic infection), complicating the evaluation of responses. It was also noted that despite the significant immunosuppression after LT there was increased expression of almost all cytokines/chemokines in the HCV+ LT patients as compared to the subjects who acquired primary HCV infection, indicating an environment of a heightened immune response in the presence of increased viral replication and the newly transplanted graft. Since the pathogenicity of HCV infection is immune-mediated, this increased immune response could explain the faster course of liver damage seen after liver transplantation as compared to primary infection, leading to accelerated cirrhosis and need for a new liver transplant [147]. This is the first study examining temporal fluctuations in a large number of cytokines and chemokines in a post-LT setting. My main objective for carrying out this work was to determine whether the LT setting could be used as a model to study the innate immune

response activated in the context of acute HCV replication. Unfortunately it proved not to be ideal for this purpose, although it might still be a valuable model to study other aspects of HCV-host interactions.

When comparing the cytokine/chemokine responses in the groups of HCV+ LT patients with different viral kinetics post-transplant, it was found that the group with slower viral kinetics post-transplant exhibited higher maximal increases, including IFN-α, IL-28B, IL-8 and MIP-1α. Conversely, there was a trend for faster viral kinetics to be associated with a larger maximal increase in IL-12. Finally, after comparing fibrosis outcomes in two groups (mild and severe fibrosis), it was noted that those subjects who progressed to mild fibrosis after a year had higher levels of the Th2 cytokine IL-4 at the pre-LT timepoint. Elevations in levels of IL-8, IL-10, IL-12 and TNF-α were seen in the group with severe fibrosis at one month post LT, although again differences between groups were not statistically significant and would require confirmation.

In neither primary infection nor post-transplant reinfection could a systemic type I or type III IFN response above baseline be detected. Type I and type III IFNs are usually one of the first cytokines to be induced in viral infections and both are very important in HCV infection. It is known that high ISG expression is induced in the liver of chimpanzees as quickly as 2 days post infection [185-187]. One study showed that although IFN-α and ISG expression could be observed intrahepatically, no systemic IFN-α could be detected in chimpanzees [188], indicating that the response might remain local. Type I and III IFNs can be produced by most cells in the body when they become infected, and in addition there are immune cells such as pDCs that are specialised for production of very high levels of IFNs following pathogen recognition. The latter are thought to produce the majority of IFN-α found in the circulation during virus infections. The *in vitro* studies carried out in chapter 5 (and reports in the literature) suggest that HCV is a weak inducer of IFN-α in pDCs, which may explain why IFN-α was not found systemically during acute HCV replication. HCV has also been shown to inhibit both type I

IFN production in the cells it infects and downstream IFN-triggered responses by several mechanisms, including cleavage of adaptor molecules of the TLR3 and RIG-I pathways [278-280,282], inhibition of the JAK-STAT signalling pathway [291-293] and inhibition the action of several ISGs [295,296,297]. The fact that there is some production of IFNs and upregulation of ISG expression in the infected liver suggests that these mechanisms for inhibition of type I IFN production are not entirely effective and/or that type I IFN production is triggered by other means (e.g. it has been shown that pDCs mount a robust IFN-a response following contact with HCV-infected cells [189]). Nonetheless, although ISGs are induced, they might be induced at much lower levels than in the absence of IFN-subverting strategies, and the action of these ISGs might be subsequently inhibited, blunting the overall response. Interestingly I found a larger (although not statistically significant) IFN-α and especially IL-28B response in those LT subjects who had slower viral kinetics post-transplant. Two SNPs upstream of the *IL-28B* gene have been shown to be predictors of both spontaneous clearance and SVR after treatment [169,170]. Interestingly, a recent study showed that one of the SNPs that predicts clearance and SVR also results in higher systemic levels of IL-28A/B [417], suggesting that this is the mechanism by which this SNP mediates its beneficial effects in HCV infection.

Other innate cytokines include IL-12 and IL-18. IL-12 is primarily produced by mDCs and drives Th1 development and IL-18 has also been shown to synergise with IL-12 to drive Th1 development [418]. Both IL-12 and IL-18 have been implicated in clearance of HCV infection. A SNP in the *IL-12B* gene that results in lower IL-12 production had a negative effect on the outcome of HCV infection [419]. Furthermore, two regulatory SNPs in the IL-18 promoter were also found to be associated with clearance of infection [413]. IL-12 and IL-18 also seem to have a beneficial role in treatment outcome, with subjects expressing more IL-12 [414,420] and more IL-18 [414] being more likely to develop a SVR. Although I saw an IL-12 increase in both the HCV-infected plasma donors and the LT patients, higher IL-12 expression was not observed in the LT subjects with slower viral replication kinetics than those with faster kinetics.

Conversely, there was a trend for a high IL-12 response to be correlated with faster viral replication and subsequent development of more severe fibrosis, suggesting that higher IL-12 production was not protective in HCV-infected LT patients. IL-18 was upregulated in primary acute infection but not in the HCV+ LT subjects (compared to the uninfected subjects) and was not correlated with either liver damage or viral replication kinetics.

Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α can mediate inflammatory effects in the liver [421,263]. TNF-α was found to be upregulated in primary acute infection but not in the HCV+ LT subjects (as compared to the HCV-LT subjects). I saw a trend for higher levels of TNF-α to be present at one month post LT in the group of HCV+ LT subjects that developed more severe fibrosis, an observation consistent with the reported role of TNF-α mediating liver inflammation [421,263].

Th1 responses are very important and predominate in HCV infection [422]. Furthermore, a recent study published examining a broad range of cytokines and chemokines before and after antiviral treatment using longitudinal cohorts found that IFN-γ and TNF-α were higher and IL-4 lower in the group that achieved SVR compared to the group that did not achieve SVR [416]. An induction of systemic IFN-γ and IL-2 was observed in both cohorts, although IFN-γ was also induced in one of the uninfected plasma donors and IFN-γ and IL-2 were also induced in the HCV- LT patients to a lesser extent. As only a very early timeframe was studied in most patients, later Th1 responses might have been missed, although also the two plasma donors studied during a longer period after infection only showed minimal Th1 cytokine upregulation. There was also little evidence of induction of a Th2 response in primary acute infection or in the HCV+ LT subjects (as compared to the HCV-LT subjects). Interestingly, higher levels of the Th2 cytokine IL-4 were present at the pre-LT timepoint in patients who subsequently developed more mild fibrosis, indicating that Th2 responses may be counteracting immunopathogenic Th1 responses in chronic infection.

Chemokines are small proteins that are best known for their chemotactic potential in directing leukocytes to sites of infection. Th1 cells, which are very important and predominate in HCV infection [422], preferentially express CXCR3 and CCR5, which bind to Th1-associated chemokines MIG, IP-10 and I-TAC and MIP-1α, MIP-1β and RANTES, respectively. These chemokines seem to be upregulated in HCV infection *in vivo* [204,422-428], and also *in vitro* induction of IP-10, I-TAC, MCP-1, MIG and RANTES by HCV proteins or full-length virus has been observed [427,429-431]. IL-8 has also been shown to be induced *in vivo* and *in vitro* [239, 270, 432]. My results showed that IP-10 and MIP-1β were induced in primary acute infection in the time frame studied, although MIG and I-TAC weren't analysed in this cohort. In the LT patients, induction of all the chemokines studied was seen in both the uninfected and the infected subjects, with MIG being upregulated to a significantly greater extent in the HCV+ than in the HCV- LT patients. Chemokine levels have also been correlated with the outcome of HCV treatment. Several studies have shown that high expression of IP-10 was correlated with a non-response [424,433-436]. In other studies higher levels of MIP-1α [437], MIP-1β [437,438] and RANTES [437] were correlated with SVR. Similarly, I found a trend for high pre-LT levels of RANTES to be observed in patients with slower viral kinetics post-LT ($p=0.0317$) and trends towards higher maximal upregulation of IL-8 and MIP-1α post-LT in patients with slower viral replication. While being necessary in the acute phase of infection to attract lymphocytes that eliminate virus-infected cells, once chronic infection has been established, prolonged secretion of chemokines can play a role in liver inflammation and fibrosis. Most prominently, IP-10 has been correlated with both inflammation and fibrosis in several studies [422,423,426,439], but also I-TAC, MIG and RANTES have been correlated with these parameters [422,425,426,439]. In this study no such clear correlation was found at any timepoint, but this might have been due to the small number of subjects studied at all timepoints post-LT.

IL-10 suppresses immune functions and is thought to be important to prevent inflammation. In HCV infection it has several roles. Firstly it benefits viral replication by suppressing

immune responses. At the same time, it can have a very beneficial role in preventing liver inflammation and resulting damage [359], as it can decrease inflammatory and Th1 cytokine production [440] and has anti-fibrotic activity [441]. The influence of both IL-10R and IL-10 polymorphisms on the outcome of HCV infection have been demonstrated [442,443]. Higher baseline IL-10 expression has also been shown to correlate with non-SVR after treatment [414]. Similarly, I observed a trend towards higher levels of IL-10 at the pre-LT timepoint of those LT subjects who showed faster viral replication kinetics post-LT. Other studies found that increased histologic recurrence was also associated with increased systemic IL-10 levels post-LT [444] or increased intrahepatic IL-10 RNA [445]. I also found a trend towards heightened IL-10 expression at one month post LT in the group of HCV+ LT patients who developed more severe fibrosis. This might be explained by the fact that high IL-10 levels are induced in response to the inflammation in the liver to counteract inflammation and fibrosis.

It is evident from these studies that cytokines and chemokines play complex roles in protection and pathogenesis of HCV infection. Whilst this and other studies have given some insight into the nature and dynamics of cytokine/chemokine responses during acute HCV replication, further *in vivo* studies in larger cohorts coupled with analyses of the cellular sources and causality of associations between *in vivo* analyte levels and differing disease courses are needed to understand the cytokine/chemokines in HCV infection more completely.

7 GENERAL DISCUSSION

HCV establishes a chronic infection in about 70% of infected individuals that is associated with the development of liver disease and a number of extrahepatic complications, including lymphoproliferative disorders, skin diseases and autoimmune disorders, the most common of which is mixed cryoglubulinemia [148]. The predominant manifestation of HCV infection is chronic liver damage leading to liver cirrhosis and hepatocellular carcinoma, and HCV infection is the most common indicator for liver transplantation [143]. Furthermore, HCV recurrence in HCV+ LT patients is almost universal and liver disease is accelerated in HCV+ LT patients, leading to accelerated cirrhosis and the need for a new liver transplant [147]. Currently available therapies for HCV infection are inadequate, and there is no prophylactic vaccine to block the spread of infection. The development of an effective prophylactic vaccine and improved therapeutic strategies to combat HCV infection and the associated pathological manifestations would be facilitated by a better understanding of interactions between this virus and the host immune system. The fact that the immune response is not effective at eradicating HCV infection in about 70% of cases suggests that the virus possesses strategies for impairing and/or evading the host immune response. However, the contribution of different arms of the immune response to control virus replication has not been fully defined, and viral immune evasion strategies are also not fully understood. In addition, as HCV is a non-cytopathic virus, liver damage and the other pathologies associated with HCV infection are largely immune-mediated. The mechanisms by which they are driven also need to be better understood, so that appropriate intervention strategies can be developed.

In this project, I focused predominantly on interactions between HCV and components of the innate immune system, using *in vitro* approaches to address the capacity of HCV to infect, activate and modulate the functions of innate cell subsets. HCV particles could potentially interact with immune cells in several different ways: they could bind to surface

attachment/entry receptors and infect the target cells, or they could be recognised by PRRs and trigger cellular activation, or they could engage receptors that modulate the functions of the cells, co-stimulating or inhibiting their responses to infection-associated or other stimuli. All of these possibilities were addressed. Chapter 3 explored the potential for HCV to infect PBMC subsets, whereas chapters 4 and 5 explored HCV-mediated immunomodulation of NK cell and DC responses. Finally, certain aspects of the immune response activated during acute HCV replication *in vivo* were addressed in chapter 6.

Many viruses that persist *in vivo* infect cells of the immune system [275]. They may exploit these cells to facilitate their spread and/or modulate their functions to impair virus clearance. In chapter 3, I explored the potential for HCV to infect PBMC subsets. The potential for MLV-based HCVpp expressing glycoproteins from hepatotropic HCV clones to enter PBMCs and the potential for HCVcc produced from a hepatotropic HCV clone to infect PBMCs was examined *in vitro*. My results showed that pseudoparticles were generally very inefficient at entering PBMCs (as compared to cell lines) and that HCVcc (J6/JFH-1) did not infect PBMCs. These observations are in line with findings from other studies where it has also been shown that PBMCs and hematopoietic cell lines could not be infected by retroviral [9,117] and lentiviral HCVpp [136,138] or by HCVcc [138,139]. The inability of JFH-1 HCVcc to infect PBMCs is likely due in part to the fact that none of the PBMC subsets I tested expressed all four of the principal receptors required for HCV entry into hepatocytes, although HCV replication in PBMC subsets is likely also restricted at post-entry steps, as one study showed that transfection of HCV RNA into PBMCs also did not result in productive infection [138].

However, there is evidence to suggest that PBMC subsets may be infected by HCV *in vivo*. The primary site of infection is the liver, where HCV replicates in hepatocytes [114], but HCV has also been detected in extrahepatic compartments including PBMCs [118]. Several studies (although not all [122]) have shown that PBMC subsets are associated with HCV RNA or proteins *in vivo* [123-126], and *de novo* infection of PBMCs with plasma-derived HCV has also

been reported [128-131]. The most likely explanation for these two seemingly inconsistent sets of observations is that there are lymphotropic variants of HCV that, unlike the hepatotropic viruses predominantly utilised in *in vitro* studies of HCV tropism, are capable of infecting and replicating in PBMC subsets. This is supported by the establishment of B cell lines from HCV-infected patients that continuously release HCV [132,133], and the derivation of a virus from these cells (SB) that is reported to infect Raji cells and PBMCs [132]. I obtained a recently cloned chimeric virus (SB/JFH-1), which like SB-HCV, should be able to infect PBMCs, and tried to investigate the susceptibility of different PBMC subsets to SB/JFH-1 infection, but the very low titres of this virus precluded the planned work.

Study of lymphotropic HCV variants represents an interesting and important area for future research. The existence of lymphotropic HCV variants needs to be confirmed and the proportion of infected individuals who harbour such viruses, their relative abundance in these subjects and the stage of infection at which they arise (or whether they are present from the start of infection) explored. Whether they infect some or all PBMC subsets and the attachment/entry receptors that they use to infect these cells also need to be defined. One way in which these questions could be addressed would be by developing methods for isolation and *in vitro* growth of lymphotropic HCV variants. Lymphotropic HCV clones or chimeras could then be constructed, and these viruses and their glycoproteins could be studied *in vitro*. If HCV isolates could be generated, which efficiently infected PBMC subsets, these could also be used to explore the effects of HCV infection on the functional properties of infected cells. Together, studies of this type could lead to the development of novel entry inhibitors to block HCV infection of PBMC subsets, and may identify targets for therapeutic strategies aimed at counteracting detrimental effects of infection on the functions of immune system cells.

Furthermore, other ways of HCV uptake, such as antibody-mediated and DC-SIGN-mediated uptake could be explored in more depth.

In chapters 4 and 5, I carried out *in vitro* studies to address how HCV may modulate the activation and functions of two important innate cell types, DCs and NK cells.

DCs play a central role in the innate immune response [190], being adapted to sense the presence of infection and mediate rapid antiviral effector activity and stimulate the induction of other innate and adaptive responses. I found that pDCs were very poorly activated and hardly produced any IFN-α following exposure to HCVcc *in vitro*, and that HCV particles were also very weak inducers of maturation and cytokine responses in mDCs, observations also supported by results from other studies [202,206]. It is also very likely that HCV particles fail to stimulate high-level activation of pDCs and mDCs *in vivo*, as in chapter 6 I showed that the increase in viral titres during acute HCV replication *in vivo* was not associated with any significant increase in circulating levels of IFN-α or IL-28B, and although there were increases in certain other cytokines/chemokines, these were very modest in primary infection and occurred with relatively delayed kinetics. This is in marked contrast to the rapid and dramatic upregulation in plasma levels of type 1 IFNs and multiple innate cytokines and chemokines accompanying the acute burst of virus replication during primary infection with HIV-1 but similar to what is seen in HBV infection [204].

The failure of HCV particles to trigger mDC and pDC activation may be one of the factors that contributes to the relatively delayed activation of adaptive responses in acute HCV infection [405]. Although there is some local activation of innate responses in the liver (perhaps due to triggering of DCs or macrophages in the liver by HCV-infected cells [189,206]) this alone may be inadequate to trigger robust activation of adaptive responses in lymphoid tissues. The delay in the mobilisation of the host adaptive response could give the virus a crucial advantage in its fight to establish a persistent infection *in vivo*, enabling amplification of infection to levels that are difficult for the host immune response to eliminate before adaptive effector mechanisms come into play, and promoting "exhaustion" of T cell functions [446]. It is

thus of great importance to understand the mechanisms responsible for the relative lack of pDC and mDC activation following exposure to HCV.

One possibility is that HCV particles may impair pDC and mDC activation/functions. I found that pre-treatment with HCVcc did not result in inhibition of pDC or mDC maturation or cytokine production in response to TLR ligation. Results of similar studies by other groups have yielded confusing results, with inhibition of pDC (but not mDC) responses to certain TLR ligands being observed by some but not other investigators [202,203,333]. This controversy may be due to differences in the HCV preparations and experimental conditions employed in different studies and requires resolution. There are also conflicting results in the literature about whether DC functions are impaired in chronic HCV infection. Some reports do show impaired DC function in chronic HCV infection [309-322], whereas other reports do not [323-327], and the findings about which DC functions are impaired also vary between the studies. If HCV does impair DC functions *in vivo*, the deficiency cannot be very profound, as CHC patients do not exhibit generalised deficiencies in immune responses to other pathogens. Nonetheless, if HCV does impair DC functions it will be of importance to understand the mechanisms by which this is achieved, so that strategies can be developed to prevent this impairment and boost immune responses *in vivo*.

Although it cannot be excluded that HCV may cause some impairment of DC functions *in vivo*, it seems more likely that the virus fails to stimulate rather than actively blocks pDC and mDC activation. It is not clear why DCs fail to recognise HCV particles. My results (and those of others [184,189]) have shown that HCV RNA can stimulate pDCs to produce IFN-α when it is transfected into these cells. If HCV genomic RNA does act as a TLR7/8 ligand, it is possible that HCV particles are just not taken up efficiently enough by DCs for the viral RNA to trigger their activation. However, I found that HCV RNA did enter MDDCs when these cells were magnetofected with HCVcc particles and there is also a report in the literature showing that HCVcc can be taken up by both mDCs and pDCs [384]. Furthermore, the fact that

HCV does not replicate within DCs would preclude their activation via autophagy-mediated transfer of viral RNA into TLR-containing endosomes. If, in future, DC-tropic HCV isolates were identified, it would be of great interest to perform experiments with these to determine whether HCV variants that can enter or replicate within DCs do trigger their activation.

Elucidation of the mechanisms responsible for the failure of HCV to activate DC subsets could suggest some novel approaches for therapeutic eradication of HCV infection. It may be difficult to re-invigorate adaptive responses in chronic infection but if innate responses could be boosted, either by releasing HCV-mediated inhibition of DC functions if this does occur, or by providing an alternative stimulus to activate them, this could help to eradicate infection. IFN-α therapy is partially effective in controlling HCV replication, but by effectively triggering DC activation *in vivo*, it may be possible to induce *in vivo* production of a mix of IFN subtypes that are capable of suppressing virus replication more effectively than exogenously administered IFN-α.

NK cells play an important role in many viral infections [387], and a beneficial effect of NK cells on the outcome of HCV infection has been suggested by several studies [211-214,219-222,224,234]. How NK cells recognise and eliminate HCV-infected cells is not well understood. This was something I would have liked to address, but I was unable to find a good system for studying NK cell interaction with HCV-infected cells *in vitro*. An ideal system would be one that utilised NK cells and autologous HCV-infected hepatocytes – but given the difficulties associated with obtaining the latter, I had hoped that if I was able to infect PBMC subsets with the SB/JFH-1 virus I could potentially study the interaction of NK cells with autologous HCV-infected B cells, and perhaps also HCV-infected DCs. These are experiments that may become feasible if lymphotropic HCV isolates that show robust replication in cell culture are developed in future.

If NK cells do play an important role in control of HCV replication *in vivo*, it is likely that HCV will have evolved strategies for evading this arm of the immune response. Many viruses

employ mechanisms for reducing NK triggering by the cells in which they are replicating, e.g. by maintaining surface expression of MHC class I molecules that are ligands for inhibitory receptors or impairing expression of stress proteins such as MICA/B that are ligands for activating NK cell receptors [387]. The expression of ligands for activating and inhibitory NK cell receptors on HCV infected cells has not been well-characterised, and is something that would be of great interest for future studies to address. Viruses can also induce alterations in NK cell numbers and/or functions, e.g. in chronic HIV infection, NK cells have been shown to express enhanced levels of inhibitory receptors and reduced levels of activating receptors, which impairs their functions [447].

One mechanism proposed to contribute to NK impairment in CHC was NK inhibition via E2-mediated crosslinking of CD81. Antibody-mediated cross-linking of CD81 on NK cells was shown to inhibit their response to activating stimuli in vitro [351-353] (in contrast to the function of CD81 on T cells, where CD81 crosslinking costimulates T cell activation [351,388,394]). Arrays of immobilised sE2 were also shown to inhibit NK responses via CD81 cross-linking *in vitro* [353,388], and on this basis it was suggested that contact of virions with NK cells might inhibit their functions by cross-linking of CD81 *in vivo*. To test the validity of this hypothesis, I addressed whether the inhibitory effects of sE2 on NK responses were also mediated by whole virus particles. I found that HCVpp, HCVcc and plasma-derived HCV all had no specific effect on T cell activation through CD3 or NK cell activation through CD16, NCRs or cytokines, suggesting that HCV particles probably do not modulate NK cell or T cell activation in response to these stimuli *in vivo*. Notably, hyper-stimulation of T cell activity is not observed in CHC, and although there are some studies that report impairments in NK cell functions in CHC, there are also others that do not, suggesting that major impairment of NK cell functions is not a common feature of this infection. Nonetheless, it cannot be excluded that localised effects on immune functions are mediated via this mechanism. A recent study showed that if virions are immobilised, they can inhibit NK responses via CD81 cross-linking

[214], raising the possibility that if high concentrations of virions are trapped on surfaces in the liver they may cause a localised inhibition of NK cell-mediated control of virus replication.

The controversies in the literature as to whether DC and NK cell functions are impaired in HCV-infected individuals emphasise the need for more studies to understand the extent to which these cell types are activated and/or their functions dysregulated *in vivo*. It is of particular interest to understand the nature of the innate response activated during the critical acute phase of HCV infection, and whether/how this may differ in subjects who subsequently clear virus replication and those who go on to become chronically infected. There have been relatively few studies of the innate response activated during acute HCV infection in humans, largely due to the difficulty in identifying infected individuals during this phase of infection. However as alluded to above, what is known from my work and prior data presented in chapter 6 is that there is hardly any systemic increase in innate cytokines such as type I/III IFN and IL-12 during acute HCV infection, and although levels of some analytes do increase, these elevations are modest and occur with delayed kinetics relative to the increase in plasma viremia. I compared the changes in circulating cytokine/chemokine levels during the acute burst of HCV replication following LT in HCV+ patients to those observed in primary HCV infection to determine the utility of the LT setting as a model for analysis of the innate responses activated in primary infection. Although there were similarities between the two, e.g. systemic type I and III IFN responses were not detected in either setting, the perturbations in systemic analyte levels observed in both HCV- and the HCV+ LT patients complicated the use of the LT setting as a model to study the innate immune responses activated in the context of acute HCV replication. In future, more work could be carried out in NHP models to study the innate immune responses activated in acute HCV infection; and efforts could be made to obtain more samples from subjects acquiring HCV infection so that critical early events can also be studied in humans.

In conclusion, it seems that, rather than directly impairing the activation of a systemic innate response, HCV largely avoids detection by DCs in blood and lymphoid tissues, hence delaying the generation of systemic antiviral immune responses, which favours virus persistence. HCV does possess strategies for actively impairing type I IFN and ISG induction in the cells it infects to reduce innate control in the liver [278-282,291-296297,300,301]; nonetheless there is evidence from NHP models that at least some IFN synthesis does occur in the liver in the early stages of infection [185-187]. This may be produced by innate cell subsets within the liver, as several reports indicate that DCs can recognise and respond to HCV-infected cells [189,206]. Study of intrahepatic DC and NK cell responses in acute HCV infection would be of great interest, as the nature of the intrahepatic innate response may be a critical determinant of whether HCV infection is resolved or persists after the acute phase. Conversely, prolonged activation of innate responses in the liver during CHC may be one of the factors that contributes to liver damage over time. Understanding how this is stimulated could enable the development of therapeutic strategies aimed at blocking on-going immune activation to improve patient prognosis.

There are several approaches that can be pursued in future to combat HCV infection and its associated pathological consequences. First, it should be possible to develop a prophylactic vaccine to block the spread of HCV infection. If adaptive responses have been induced prior to infection, the pre-existing antibodies should at least limit the acquisition of infection, and if there is an effector-memory T cell response, this may be able to eradicate the virus from the liver before infection becomes very widespread. Although it may be challenging to induce adaptive responses that are capable of recognising the diversity of circulating HCV genotypes and variants, and HCV does have strategies for evading control by neutralising antibodies and T cells, the fact that HCV infection is spontaneously cleared in ~30% of cases suggests that development of an effective prophylactic vaccine for this infection should be achievable. Second, improved therapeutic strategies can be developed to eliminate infection in chronically

infected individuals. A new drug, Boceprevir, has just been approved for the therapy of HCV [160,161], giving way to triple therapy. Other novel antiviral drugs are also likely to be developed in future; and a better understanding of the potential for innate effector mechanisms to contain HCV replication and the strategies used by the virus to evade innate control *in vivo* may also pave the way for design of therapeutic strategies to harness endogenous innate defence mechanisms. Finally, it should be possible to develop strategies that prevent or ameliorate the diverse pathologies associated with chronic HCV persistence. As more is understood about how chronic inflammation is driven in the HCV-infected liver and the mechanisms by which these pathologies are triggered, this will reveal opportunities for development of novel interventions to enable infected individuals to avoid the pathological consequences of chronic infection.

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