DENDRITIC CELL FUNCTION IN THE SETTING OF CHRONIC HEPATITIS C INFECTION AND CHANGES DURING ANTIVIRAL TREATMENT

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Faculty: MEDICINE

Field: HEPATOLOGY

A thesis submitted to the University College London for the degree of Master of Philosophy

October 2008

ABSTRACT

The role of the innate immune system and in particular of dendritic cells, in the establishment of chronic hepatitis C has been studied quite extensively over the last few years but controversy remains as to whether a potential virus-induced defect in DC function leads to sub-optimal initiation of adaptive immune responses and finally to the establishment of chronic infection. In the present thesis we examined monocyte-derived DC (MDDC), generated from patients with chronic hepatitis C (CHC), and healthy subjects in order to gain a better understanding of dendritic cell functions in the setting of hepatitis C infection. We initially observed a defect in CHC patients' DC capacity to stimulate 'naïve' CD4+ T-cells into proliferation. Investigating for factors potentially contributing to this defect we demonstrated that HCV genome (both positive strand HCV RNA and the replicative intermediate negative strand HCV RNA) and HCV protein products (HCV core protein) are present in DC without though any obvious correlation to DC functions. We further proccedeed to investigate for potential influence of other, host-related, parameters on DC and tested liver fibrosis, ethanol consumption, HCV viraemia levels and HCV protein products' concentration in the serum demonstarting significant influences of HCV viraemia / HCV core serum concentration and ethanol consumption on DC functional and phenotypic output. After successful antiviral treatment (with pegylated interferon α and ribavirin) our patients restored their DC functions (allostimulatory capacity, HCV-specific immune-reactivity and IL-12production). Performing 'cross-over' experiments we suggest that antiviral treatment exerted its effect by improving mainly DC capacities and not effector $CD4^+$ T-cell reactivity. We also tested plasmacytoid dendritic cell cytokine output after HCV infection and we observed a controversial effect of the virus on the Th1-skewing capacity of this DC subset.

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DECLARATION

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of London or any other University or Institute of learning. All the work presented is my own and any collaboration has been acknowledged.

ACKNOLEDGEMENTS

I am indebted to many people who helped me enormously throughout this project. First, Prof. Nikolai V. Naoumov, without whose help, guidance, conscientious advice and encouragement, I would never have embarked upon the wonderfull adventure of basic scientific research and would definetly never have completed the present work. Mrs Shilpa Choksi, Mrs Helen Cooksley, Dr. Gabrielle Pollara, Dr Kwok Tang, Dr Dimitrios Farmakiotis and Mrs Sandra Proto, I cannot thank enough for their time and advice in planning and carrying out this work, and of course for their kindness and patience with my continuous calls for help. I would also like to thank Mr. Nick Tatman for his invaluable help with the Hepatitis Clininic at UCLH.

To my wife Maria, I owe eternal gratitude for supporting me in every possible way and for patiently putting up with the endless hours of seclusion the completion of the present work demanded from me.

To my family,for always being there for me.

ABBREVIATIONS

APC	antigen-presenting cells
BDCA-2,4	blood dendritic cell-2,4 antigens
CD40L	CD40-ligand
CHC	chronic hepatitis C
Core	hepatitis C virus core (nucleocapsid) protein
CpG ODN	CpG oligo-deoxy-nucleotides
DC	dendritic cells
DC-SIGN	dendritic cell-specific intracellular adhesion molecule 3 grabbing non-integrin
dsRNA	double-stranded ribonucleic acid
E1,2 proteins	hepatitis C virus envelope 1,2 glycoproteins
ELISPOT	enzyme-linked immunospot assay
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
HBV	hepatitis B virus
HCV	hepatitis C virus
HCVcc	cell culture-grown HCV
HCV RNA	hepatitis C virus ribonucleic acid
HVR1 E2	hyper-variable region 1 of envelope 2 protein
IFN-α,β,γ	interferons-α,β,γ
IL-6,12	interleukins-6,12
LDL-R	low density lipoprotein receptor
LPS	lipopolysaccharide
L-SIGN	liver/lymph node-specific intracellular adhesion molecule 3 grabbing non-integrin
LSEC	liver sinusoidal endothelial cells
MDDC	monocyte-derived dendritic cells
MDC	myeloid dendritic cells
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
NF-kB	nuclear factor-kappa B
NK	natural-killer cells
NK T-cells	natural-killer T-cells
NS 2,3,4,5	hepatitis C virus non-structural proteins 2,3,4,5
PBDC	peripheral blood dendritic cells
PBMC	peripheral blood mononuclear cells
PDC	plasmacytoid dendritic cells
Poly I:C	polyinosine-polycytidylic acid
RT- PCR	reverse transcription polymerase chain reaction
RT-PCR-NAH	RT-PCR-nucleic acid hybridisation
R848	resiquimod
SR-BI	human scavenger receptor class B type I
ssRNA	single-stranded ribonucleic acid
TNF-α	tumour necrosis factor-α
TLR	toll-like receptor
Th1,2	T-cell helper 1,2 responses

CHAPTER 1

Introduction and review of the literature

1.1 Hepatitis C virus infection

1.1.1 Natural history of hepatitis C virus (HCV) infection

Hepatitis C virus (HCV) was first identified in 1989 as the major causative agent of parenterally transmitted and community-acquired non-A, non-B hepatitis. The only natural host of HCV is man. Infection with HCV occurs mainly through transfusion of blood and blood-derived products but also through in-apparent parenteral exposure (high-risk sexual activity, 'vertical' transmission from mother to child, tattoos and body piercing, occupational exposure). Rarely the infection is controlled by the host immune system with viraemia clearance. More frequently chronic infection develops, cirrhosis and hepatocellular carcinoma may ensue and overall HCV appears to represent a major health problem as 170-200 million persons worldwide are estimated to be infected [1,2].

Six major genotypes (genotype 1 to genotype 6) of HCV have been described (based upon the phylogenetic analyses of the core, E1, and NS5 regions of the HCV genome), with further divisions into subtypes (1a, 1b, 2c et.c)[3]. All HCV genotypes have a common ancestor virus. However, HCV genotypes 1, 2, and 4 emerged and diversified in Central and Western Africa, genotype 5 in South Africa, and genotypes 3 and 6 in China, South-East Asia and the Indian subcontinent. In these areas, a large number of subtypes of these genotypes are found [3]. Industrialized areas harbor a small number of HCV subtypes that could widely spread because they met an efficient route of transmission, such as blood

transfusion or the intravenous use of drugs. They include genotypes 1a, 1b, 2a, 2b, 2c, 3a, 4a and 5a.

1.1.2 Biology of HCV

1.1.2.1 Virion structure

The HCV virion, some 30 to 60 nm in diameter, is made of a single-stranded positive RNA genome, contained into an eicosahedral capsid, itself enveloped by a lipid bilayer within which two different glycoproteins are anchored[4]. The genome, approximately 9,500 nucleotides, contains three distinct regions: **(A)** a short **5′ non-coding region** that contains two domains, a stem-loop structure involved in positive-strand priming during HCV replication and the internal ribosome entry site (IRES), the RNA structure responsible for attachment of the ribosome and polyprotein translation; **(B)** a long, unique open reading frame **(ORF)** of more than 9000 nucleotides which is translated into a precursor polyprotein, secondarily cleaved to give birth to the structural proteins (the capsid protein C and the two envelope glycoproteins E1 and E2) and to the non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The functions of the non-structural proteins have been elucidated by a large number of studies and by analogy to related viruses; only NS4B and NS5A have no well-defined functions to date. **(C)** Finally, a short **3′ non-coding region,** principally involved in negative-strand priming during HCV replication, is detected in the HCV genome. The 3'-untranslated region (UTR) has been divided into three regions: a variable

sequence of approximately 40 bases, a variable length poly-UC rich tract, and a highly conserved 98 base region[4].

1.1.2.2 HCV proteins

i. Structural proteins

The first 191 amino acids of the HCV polyprotein encode the putative nucleocapsid or **core protein** (protein of 21kd molecular weight or p21) which is highly conserved and immunogenic, containing several B-cell epitopes near the N-terminus[5-7]. A hydrophobic domain at the C terminus is responsible for membrane-dependent processing of core. C-terminal truncation of p21 produces two forms of p19 terminating at a hydrophobic domain at residues 179 and 182 resulting in proteins associated but not integrated into the ER membrane. Localisation involves the cytoplasm as well as the perinuclear region and has also been identified in the nucleus (C-terminally truncated forms lacking the hydrophobic region) of tissue culture cells. The N-terminus contains several nuclear localisation signals and a DNA binding motif that may result into translocation to the nucleus[8-11]. Various functions of core protein have been described. Suppression of apoptotic cell death[12], infiltration of the liver with T lymphocytes and suppression of the systemic adaptive immune response[13], translocation to the nucleus via nuclear localisation signals[11], HBV virus suppression[14], interaction with cellular proto-oncogenes[15,16] with potential tumorigenesis, RNA (viral and host) binding activity playing a putative structural role in encapsidation of the RNA genome[9,17], are some of them.

The glycoproteins (gp) **E1 (gp31) and E2 (gp70)** represent the viral envelope (surface) proteins. Cleavage from the primary polyprotein may produce two possible precursors of E2, E2-NS2 and E2-p7[6,18-21]. A hydrophobic sequence in E2-p7 probably directs translocation into the endoplasmic reticulum (ER). The hydrophobic sequences also contribute to membrane anchoring. E1 and E2 have been shown to interact to form a complex and this interaction requires that both proteins be membrane-anchored[22]. An unusually high degree of amino acid variation has been observed in the N terminus of E2 protein. This hypervariable region, referred to as HVR1, is located between aa 384-410 of the polyprotein (aa 1-27 of E2). HVR1 varies enormously both within any one isolate, generating quasispecies, and between different genotypes. No specific HVR1 sequence has been associated with a certain genotype. Observations that specific antibody to this region changes during antiviral treatment suggests susceptibility to immunological pressure with the potential for escape mutants[23].

ii. p7

p7 is a 63-amino acid polypeptide located at the junction between the structural and non-structural region. It is unknown whether p7 is packaged into viral particles. It is composed of two trans-membrane domains and has recently been reported to form hexamers with ion channel activity[24,25] It is believed that p7 could be important for viral assembly because the corresponding protein of the related bovine viral diarrhea virus (BVDV) is essential for the production of infectious progeny virus but not for RNA replication[26].

iii. The ARFP/F protein

The synthesis of a protein encoded by an alternative reading frame within the core region was reported by several groups[27-29]. It was designated ARFP (alternative reading frame protein) or F (frameshift) protein and comprises up to 160 amino acids. The ARFP/F protein is dispensable for HCV RNA replication. Whether it is expressed during natural HCV infection has still to be clarified.

iv. Non-structural proteins

The **NS2 region** forms part of an HCV encoded protease which overlaps with the NS3 serine protease. The NS2-3 protease is believed to be responsible for auto-proteolytic cleavage at the 2/3 site[30,31] and requires zinc. The activity of NS2/3 protease, at least in vitro, appears to be dependent on the presence of microsomal membranes[32].

The **NS3 region** has been shown to have serine protease, nucleotide triphoshpatase (NTP-ase) and RNA helicase activities. Serine protease is apparently essential for HCV processing. For a flavivirus homologue the serine protease has been previously shown to be indispensable for viral growth[6,33,34] .

The activity of the NS3 region resides in the N-terminal part of the protein. First there is the cleavage of the NS2/3 site, in conjunction with NS2, for which the part of the poly-protein between amino-acids (aa) 827-1207, spanning both NS2 and NS3 regions, appears to be responsible and second the release of the remaining down-stream NS proteins, by mediating cleavage at the 3/4A, 4A/4B, 4B/5A, and 5A/5B sites[31,35]. Each of the NS3-dependent cleavage sites contain residues that are

conserved between genotypes, suggesting an importance for substrate specificity[6,34] . The NS4 protein acts as a cofactor in NS3 activity. It targets NS3 to intracellular membranes, is essential for the NS3/4A and NS4B/5A cleavage events and enhances cleavage at the NS4A/4B and NS5A/5B sites[36-38]. Surprisingly, the NS3 serine protease recently turned out to influence the innate cellular host defence by inhibition of RIG-I and TLR3 signalling [39,40]. This observation renders the NS3 protease particularly attractive as an antiviral target [41]. Serine protease inhibitors have emerged as potentially efficient antiviral components in studies on patients with chronic hepatitis C [42,43].

NTPase[44,45] and RNA helicase[45-47] activities of NS3 reside at the C-teminal 465 amino acids of the protein and appear similar to helicase activities of other positive strand RNA viruses[44,45]. Viral RNA helicases are thought to be involved in strand separation during viral replication and transcription, perhaps resolving secondary-structure prior to ribosome engagement, in order to facilitate translation. In general helicases contain NTPase activity, which hydrolyses nucleoside triphosphates to provide the energy source for unwinding nucleic acids. The HCV helicase has been shown to act 3’ to 5’ directionally unwinding DNA-RNA and DNA-DNA substrates with apparently the same efficiency as an RNA-RNA substrate[47]. This DNA helicase activity would have no function in the HCV life cycle, but would affect cellular activities. It has been suggested thus that NS3 might play a role in the pathogenesis of hepatocellular carcinoma[48].

The **NS4 region** encodes two viral proteins, NS4A and NS4B. The function of NS4B is largely unknown. So far, it has been shown that NS4B is a 27-kDa integral membrane protein that localizes to an endoplasmic reticulum (ER) derived membranous compartment while the C-terminal part of it is cytoplasmic [49,50]. Interestingly, the expression of NS4B induces a specific membrane alteration, designated as membranous web, that serves as a scaffold for the formation of the viral replication complex [51]. NS4A has been observed to act as a cofactor in NS3 protease activity. As mentioned before NS4A enhances cleavages at the NS3/4A, NS4B/5A, NS4A/4B and NS5A/5B sites which are mediated by the NS3 serine protease. Clearly the interaction of NS3 with NS4A is vital for the viral replication [51,52]. Recent studies have also shown the presence of NS4A in mitochondria, not only in the ER, of hepatocytes and also suggested induction of caspace-3 mediated apoptosis of Huh7 hepatoma cells transfected transiently with either NS4 alone or in combination with NS3 (NS3/4A) or harbouring a sub-genomic replicon [53]. Some other authors also suggest that the C terminus of hepatitis C virus NS4A encodes an electrostatic switch that regulates NS5A hyperphosphorylation and viral replication [54], and even observed that NS4B, and NS4A to a lesser extent, significantly transactivated the IL-8 promoter, which resulted in enhanced production of IL-8 protein, probably contributing to the establishment of chronicity of HCV infection by inhibiting the antiviral activity of IFNα [55,56].

The **NS5 region** is processed into NS5A and NS5B.

The primary function of **NS5A** is unknown but is suggested to be related to sensitivity to interferon (IFN). NS5A is a phosphorylated zinc metalloprotein of unknown function. Numerous potential functions have been described [reviewed in ref. [57]. NS5A has initially attracted considerable interest because of its potential role in modulating the IFN response {reviewed in ref. [58]}. These findings are still controversial, however. A striking observation was the concentration of cell culture adaptive replicon mutations within the central part of NS5A [59,60]. Taking into consideration the fact that NS5A phosphorylation also has an impact on replication efficiency, it is suggested by various studies that NS5A plays an important role in the regulation of viral replication [61-63]. The membrane association of NS5A is mediated by a unique amphipathic alpha-helix which is localized at the N-terminus [64,65]. Recent experiments, implementing partial proteolysis, allowed the definition of three protein domains within the cytosolic domain [66]. The three-dimensional structure of the N-terminal domain I was resolved by crystallography and it was shown that after dimerization, it forms a basic groove facing the cytosol at the surface of the membrane [67]. This structure is believed to provide an RNA binding site that could be involved in regulated genome targeting within the replication complex.

The key enzyme of the replicase that promotes synthesis of new RNA genomes is the **NS5B** RNA dependent RNA polymerase (RdRp). NS5B is a tail-anchored protein, characterized by a transmembrane domain at the C-terminus of the protein responsible for posttranslational membrane targeting [68-70]. The structural organization of NS5B is a typical 'right

hand' polymerase shape with finger, palm, and thumb subdomains surrounding a completely encircled active site. Replication proceeds via synthesis of a complementary minus-strand RNA using the genome as a template and the subsequent synthesis of genomic plus strand RNA from this minus-strand RNA intermediate. As central component of the HCV replicase, NS5B has emerged as a major target for antiviral intervention [41].

1.1.3 HCV lifecycle

1.1.3.1 Entry receptors

The lifecycle of the virus begins with the attachment of the virion to the surface of the host cell, via specific receptors. Several molecules have been suggested to mediate the attachment. HCV E2 binds with high affinity to the large external loop of **CD81**, a tetraspanin found on the surface of many cell types including hepatocytes and lymphocytes[71]. However, CD81 may not be sufficient to mediate cell entry and several cofactors appear to be required[72]. Experiments demonstrating inhibition of entry of HCV pseudo-particles in hepatoma cell lines by anti-CD81 monoclonal antibodies confirm the importance of CD81 as an HCV entry receptor[73]. The low density lipoprotein receptor (**LDLR**)[74] and scavenger receptor class B type I (**SR-BI**)[75,76] have been proposed as components of a putative HCV receptor complex. The concept, that lipoproteins could play an important role for cell entry is supported by recent data from studies on HCV pseudotypes that demonstrate an enhancement of infectivity by certain components of human

serum[77,78]. In particular, association to high density lipoprotein (HDL) seems to enhance SR-BI guided cell entry and could protect viral particles from neutralizing antibodies[79]. Recently, two c-type lectin receptors, **DC-SIGN** (dendritic cell-specific intracellular adhesion molecule 3 grabbing non-integrin; expressed on dendritic cells)[80] and **L-SIGN** (liver/lymph node-specific intracellular adhesion molecule 3 grabbing non-integrin; expressed on liver sinusoidal endothelial cells), have been shown to bind HCV[81]. Studies in mice have shown that DC-SIGN, initially thought to be expressed only on myeloid dendritic cells, is also expressed on plasmacytoid dendritic cells[82]. The viral ligands to DC-SIGN and L-SIGN are HCV envelope glycoprotein 1 (E1) and envelope glycoprotein 2 (E2)[83]. HCV binding to DC-SIGN, followed by internalisation, may target the virus to non-lysosomal compartments (tranferrin-positive, LAMP-1 negative, early endosomal compartments) within dendritic cells. Thus, HCV may target DC-SIGN to “hide” within dendritic cells and facilitate viral dissemination[84,85].

1.1.3.2 Intracellular lifecycle of HCV – mechanisms of viral replication

After entering the host cell, the HCV virion becomes decapsidated and the genomic HCV RNA is used both for polyprotein translation and replication in the cytoplasm. Replication and post-translational processing have been shown to happen on a complex that is often called ‘replication complex’ and consists of a membranous web [a specific membrane alteration recently identified as the site of RNA replication in Huh-7

{human hepatoma) cells harboring subgenomic HCV replicons[51]}, viral proteins, replicating RNA and additional host cell factors[51,52]. This complex appears to be located in close proximity to perinuclear membranes. Thus, intracellular membranes play a crucial role in HCV replication. Recent data underline the importance of a specific lipid environment for HCV RNA replication [86,87]. Additionally to coordinated protein-protein and protein-membrane interaction, essential *cis*-acting replication elements (CRE) of the RNA genome were recently discovered. For instance, the sequence coding for the C-terminal domain of NS5B consists of an essential stem-loop, designated SL3.2, within a larger cruciform RNA element, designated SL3[88]. Detailed characterization of the SL3.2 domain indicated a functionally important 'kissing loop' interaction with the 3' NCR(non-coding region)[89]. Several host cell factors, including hVAP-A(human vesicle-associated membrane protein, a cellular factor attachment protein receptor that interacts with NS5A for RNA replication), CyPB (cyclophilin B, a cellular peptidyl-prolyl cis-trans isomerase, which is critical for the RNA binding activity of the HCV RNA polymerase NS5B), FBL2 (a geranyl-geranylated host protein that forms a complex with NS5A and is probably essential for HCV replication) have been identified[90,91]. However, the regulation of replication, the switch to translation or assembly and the release of viral particles are still poorly understood. It appears that HCV RNA encapsidation takes place in the endoplasmic reticulum and nucleocapsids are enveloped and matured into the Golgi apparatus before viral progeny is released in the pericellular space by exocytosis

[4]. Prior to the development of HCV replicon systems, that allow for transfection of cell lines with either the full HCV genome or sub-genomic sequences, little was known about the intracellular life of HCV. In the last 6 years, a large panel of different replicon systems has been developed. These include replicons from genotype 1a[92] and 2a[93], transient systems expressing easily quantifiable marker enzymes[94], replicons with green fluorescent protein (GFP) insertions in NS5A that detect replication complexes in living cells[95], and full length replicons. In addition, the spectrum of permissive host cells has been expanded[96]. The replicon system revolutionized the research on basic replication processes. However, the step of infection and entry as well as the release of viral progeny could not be analysed to date. Recently however a genotype 2a replicon (JFH-1) was developed that was isolated from the serum of a patient with fulminant hepatitis C[93]. This system appears to replicate very efficiently in different cell types. Furthermore, the full-length JFH-1 sequence produced infectious viral particles that could be passaged in cell culture[73,97]. Chimeric constructs with the structural region of the J6 genotype 2a clone significantly improved the infectivity of this system[98]. This recombinant infectious HCV cell culture system represents the last major milestone in the field and renders the complete viral life cycle accessible to detailed analysis in vitro. It remains a fact though that, to date, infection of cell-cultures with patient isolates has not been feasible. JFH-1 is an exceptionaly infectious strain of HCV.

1.2 Role of the immune system in the control of HCV infection

After the entry of HCV in the infected host, the immune system of the latter is mobilised in an attempt to control the spread of the virus and clear viraemia. The two main parts of the human immune system, the innate and the adaptive immune systems, appear to become sequentially activated by the interaction with the virus and its products.

1.2.1 Innate immune response. The first line of defence

Natural Killer (NK) cells

Natural Killer (NK) cells, intra-epithelial TCR-γ:δ cells and NK T-cells represent parts of the innate immune system that are not restricted by MHC-complex antigen presentation for their cytolytic effect on virus-infected hepatocytes. NK cells are activated early after viral infection by type I interferons (IFN-α/β) and interleukin-12 (IL-12) and in turn secrete cytokines [i.e interferon-gamma (IFN-γ), tumour necrosis factor-α (TNF-α)] [99] and chemokines-cytokines with chemotactic functions-[macrophage inflammatory factor 1α (MIP-1α) [100,101] also known as CCL3 and interferon-gamma-inducible protein 10 (IP-10)] contributing along with other cellular populations of the innate immune system, such as the dendritic cells (DC), in a first-line immune response against the virus[102] . IFN-γ secreted by activated NK and NKT cells has been shown to inhibit the replication of HCV through a non-cytolytic mechanism [103]. The fact that during the first four to six weeks after the

entry of HCV into the infected host no significant liver inflammation can be detected along with the observation of an active and un-hindered replication of HCV, may not only underline the non-cytopathic nature of HCV but also suggest that the contribution of the NK / NK T-cells in the initial control of the infection, is rather minimal[104,105]. Some authors suggest that binding of HCV-E2 protein on CD81, on NK cell surface, leads to direct inhibition of NK cell functional activation, proliferation, cytokine production and cytotoxic granule release[106,107]. Other studies have also shown a reduction in the intra-hepatic numbers of $CD56^{+}CD3^{-}$ NK cells and $CD56^{+}CD3^{+}$ NK T-cells with histological progression of HCV infection. Once cirrhosis develops NK / NK-T cell numbers are further diminished with subsequent defect in the ability to secrete IFN-γ and in cytolytic capacity with potential implications even in hepatoma formation [108,109].

Dendritic cells (DC) that are a part of the innate immune system of paramount importance in the generation of adaptive immune responses against HCV, and are also the subject of the present study, will be discussed in detail separately.

1.2.2 Adaptive immune responses

The harmonious activation both of the humoral and the cellular part of the adaptive immune system appears to be essential for the containment of HCV infection [110]. The result of the complex interactions between the various parts of the adaptive immune system is that failure of one component may lead to generalised dysfunction. Defective $CD4^{+}$ T-cells

lead to impairment in $CD8^+$ T-cell activity and antibody production[111], while failure of the $CD8^+$ T-cells leads to high viraemia levels that cannot be contained by antibody responses alone[112].

Humoral Responses

After exposure to HCV, viraemia develops within a week. A few weeks later elevated transaminases are seen followed by a delayed antibody seroconversion within four weeks[110]. Chronic HCV infection is characterised by high titers of HCV-specific antibodies. Although studies of the humoral response have shown antibodies to both structural and non-structural regions of the virus, no antibody conferring immunity to HCV has been detected[113]. Spontaneous HCV clearance after acute infection, was shown to be temporally associated with production of anti-envelope antibodies[114] and sera with high titers of anti-HCV (specific for viral envelope) can prevent HCV infection[115]. On the other hand the fact that spontaneous viral clearance after acute HCV infection has been demonstrated in hypo-gammaglobulinaemic patients, challenges the importance of antibody responses in the control of viraemia[116]. It has also been shown that humoral anti-HCV responses are shorter-lived than ($CD4^+$ and $CD8^+$) cellular immune responses[117]. Persisting cellular immune responses, with negative anti-HCV antibodies and cleared viraemia (undetectable HCV RNA assessed with PCR assays) have been reported by some other authors in healthcare workers, spouses and healthy family members of patients infected acutely or chronically with HCV[118-120].

Cellular immune responses

Clearance of HCV can occur spontaneously during acute HCV infection, and is associated with a vigorous, long-lasting, $CD4^+$ and $CD8^+$ cellular immune response against multiple HCV epitopes and a T-helper type 1 (Th1) profile of cytokine production[121-125].

$CD4^+$ T-cell responses

$CD4^+$ T-cell responses, against multiple structural (e.g HCV core protein) and non-structural (e.g HCV NS3, NS4 and NS5 proteins) HCV antigens have been reported, both among peripheral blood mononuclear cells (PBMC's) and within liver-infiltrating lymphocytes (LIL's)[126-128]. Most studies though have focused on the analysis of peripheral blood responses. Whether conclusions on intrahepatic phenomena could be safely drawn by studying PBMC is questionable. The presence of activated $CD4^+$ T-cells (i.e after recognition of viral peptides presented by HLA class II molecules on the surface of professional antigen presenting cells, mainly dendritic cells), in the periphery, has been shown to significantly augment CTL (cytotoxic T-lymphocyte, $CD8^+$) responses[129,130]. Skewing of the immune response, by $CD4^+$ T-cells, towards Th1 (characterised by IFN-γ predominant secretion by $CD4^+$ T-cells), during acute infection, leads to the control of viraemia and resolution of the infection (via promotion of neutrophil recruitment and macrophage activation that results in inflammatory response, cytotoxic $CD8^+$ T-cell activation and possibly by control of viral replication 'non-cytolytically' as will be discussed later. On the other hand Th2 skewing

(i.e IL-4, IL-10 predominant secretion by $CD4^+$ T-cells) leads to viral persistence and reduction of liver inflammation[131]. The administration of subcutaneous IL-10 in patients chronically infected by HCV led to reduction of the intrahepatic inflammation and improvement in the fibrosis score but also to an increase in HCV viraemia[132]. As already mentioned, vigorous, multi-specific (e.g against E2, NS3 and NS4 proteins) $CD4^+$ T-cell responses, and in particular responses against non-structural HCV proteins[133], have been observed in individuals that clear HCV after acute infection[131,134-136], and also in chronically infected patients that achieve Sustained Viral Response-SVR-after antiviral treatment with interferon and ribavirin[137]. Individuals that initially presented a strong $CD4^+$/Th1 response, cleared HCV and subsequently failed to maintain the $CD4^+$ response, demonstrated a relapse of HCV infection[138]. Defective $CD4^+$ T-cell responses during acute HCV infection are related to viral persistence and chronicity of hepatitis[123,131].

$CD8^+$ T-cell reponses

$CD8^+$ T-cell (Cytotoxic Lymphocyte, CTL) responses are MHC class I-restricted, peptide-specific and represent an essential part of antiviral defence. CTL migrate into infected tissues, recognise viral antigens presented by infected cells as peptides associated with MHC class I molecules and contribute to the containment of the infection both by lysing (cytolytic control) infected cells and by secreting cytokines such as IFN-γ and TNF-α (non-cytolytic control) that inhibit viral replication. Evidence of non-cytolytic control of viraemia, that has been consistently

observed in HBV infection[139-141], is also emerging for HCV infection from studies in chimpanzees[124] and inhibition of replication of sub-genomic and genomic HCV replicon systems in Huh-7 cells ex-vivo[142]. $CD8^+$ T-cells are more difficult to study due to technical limitations [i.e requirement of extensive in-vitro expansion, HLA-A2 MHC class I-peptide tetramer complex limitations, although other recent methods such as measurement of secreted cytokines with enzyme-linked immunospot assay (ELISPOT) and intra-cytoplasmic fluorescence activated cell sorting (FACS) staining facilitated considerably the study of $CD8^+$ T-cells], thus our knowledge of the role of this particular cell population in the natural history of HCV infection is considerably more restricted than the understanding we have of the role of $CD4^+$ T-cells. In a manner similar to that described for $CD4^+$ T-cells, $CD8^+$ T-cells directed against multiple, structural and non-structural HCV antigens, are observed in individuals that resolve acute HCV infection[143]. Lack of selective pressure by CTL's on HCV is suggested by the lack of evolution of viral sequences encoding immunodominant epitopes during established HCV infection[144]. More recent studies question that concept though suggesting that impaired viral fitness may be responsible for the apparent lack of circulating mutant type HCV, in particular of variants with mutations in immunodominant sites such as the NS3 1073-1081 epitope[145]. Some authors introduced the concept of activated HCV-specific CTL that fail to eradicate viraemia, due to HCV-induced impairment in their ability to produce IFN-γ and TNF-α [146] {the so-

called ‘stunned’ phenotype[125]}, but can still act cytolytically against infected hepatocytes causing damage to the patients’ livers[147].

1.3 The role of dendritic cells in the generation of adaptive immune responses against viral infections

As a result of the data pointing to a role of cellular immunity in the control of viral infections in general and HCV infection in particular, recent efforts have concentrated on understanding the process that generates these responses. It has been appreciated for some time now that the generation of adaptive cellular immune responses is dependent on the stimulation of T cells by ‘professional’ antigen presenting cells (APC). Specifically, the initial activation of antigen-specific naïve T cells in vivo is carried out predominantly by dendritic cells (DC), the most potent known ‘professional’ APC’s.

1.3.1 Dendritic cells

Originally identified on the basis of their morphology[148] [‘dendro’ is the Greek word for ‘tree’ referring to the tree-like appearance of the cells due to multiple branch-like, processes the ‘dendrites’], dendritic cells are now accepted as the most potent APC, particularly in their capacity to activate naïve T cells. Several of their cell biological properties, such as the stability of MHC-peptide complexes, explain this role[149,150]. Other important features include the cytokine cocktail secreted at the time of T cell contact, and the motile and plastic DC cytoskeleton. Recent intravital

dynamic imaging in lymph nodes has shown that rapid dendrite extensions by DC permit scanning of around 5000 T cells per hour[151]. The interaction time between DC and T cells is increased both by the presence of MHC-antigen complexes on DC surface and the maturation state of DC, such that mature DC induce formation of stable immunological synapses that last for >8 minutes at a time[152-155]. The increased length of interaction ensures that T cells receive appropriate stimulatory signals from the DC, both in the form of surface molecule interaction and via the paracrine activity of DC-secreted cytokines.

Dendritic cells (DC) represent a heterogeneous cell population, residing in most peripheral tissues and in particular at sites of interaction with the environment like the skin and the mucosae[156]. Constitutively, DC 'patrol' through the blood, peripheral tissues, lymph nodes and secondary lymphoid organs, internalise self and allogeneic antigens by receptor mediated endocytosis[80,157,158], macropinocytosis[157] or phagocytosis[159-161], and present them to the adaptive part of the immune system, that is the T cells. Antigen presentation is the processing of antigens and loading of antigenic fragments on major histocompatibility complex (MHC) class I and class II molecules for interaction with / stimulation of T-cells. Resident DC are found in an 'immature' state characterised by low expression of MHC and co-stimulatory molecules. On the occasion of local inflammation, circulating DC are recruited in response to local chemokine production[162,163]. When specific receptors on DC surface [e.g toll-like receptors (TLR), CD40, tumour necrosis factor receptors (TNF-R)] are exposed to certain (usually

combinations of) bacterial and viral products, inflammatory cytokines and other stimuli (e.g lipolysaccharide, CD40L, TNF-α), which are collectively described as 'danger signals', a process of phenotypical and functional DC changes, referred to as dendritic cell maturation, begins in the periphery and is in progress during the migration of DC to the secondary lymphoid organs[164-168]. This migration of DC takes place along a gradient of lymph node-derived chemokines[169]. Modifications in the expression of chemokine receptors and adhesion molecules as well as changes of the DC cytoskeleton, during maturation, have been shown to be responsible for the migration process. Mature DC demonstrate an increased capacity of loading antigenic peptides on MHC complexes and delivering the latter to the cell surface. The expression, on DC surface, of T-cell co-stimulatory molecules (e.g CD80 and CD86) also rises while the antigen-uptake capacity diminishes[157]. As a result of those changes DC become very efficient antigen presenting cells (APC's) and the only ones capable of activating naïve T lymphocytes (both $CD4^+$ and $CD8^+$ T-cells) and of initiating adaptive immune responses. DC antigenic presentation capacity is limited to antigens taken up and processed during the DC maturation process.

1.3.2 Dendritic cell subsets

Different DC subsets at various maturation stages express distinct surface molecules and secrete different cytokines determining the type of the induced immune-response.

It was known for many years that two types of antigen presenting cells reside in lymphoid organs, one with a pronounced ‘dendritic’ (i.e. tree-like) morphology, and another with a more round shape. By electron microscopy (EM), this second cell type was shown to have plasma cell-like features, with a smooth outline and a large amount of endoplasmic reticulum (ER) in the cytoplasm [170] and was thus termed the “plasmacytoid monocyte” or “plasmacytoid T cell”. At the same time other authors reported that a small proportion of cells in peripheral blood could produce large amounts of type I interferons (IFN-α,β) when stimulated with viruses and these cells were described as “natural interferon producing cells” (NIPC)[171]. Eventually it was established that the plasmacytoid cells are in fact the NIPC[172] and the precursors of plasmacytoid DC (PDC). PDC are critical in bridging innate and adaptive immune responses in the context of viral or bacterial infections[173,174]. This is caused in part by the production of IFN’s, as well as their ability to participate in the recruitment of NK and activated T-cells[175]. Production of IFN’s by PDC has been demonstrated to support myeloid DC in the priming of $CD8^+$ T-cells[174,176]. The extensive ER of plasmacytoid dendritic cells appears to be essential for the production of large quantities of type I IFN upon viral stimulation, while the exposure to virus may also promote their differentiation into mature DC, with dendritic morphology and capacity to activate T cells[149,170,177,178]. These cells will be referred to as PDC at all stages of their differentiation.

Human DC ontogeny is quite heterogeneous and the picture in that particular area of DC-precursor immunobiology is quite unclear. In general it is accepted that the two main subsets are the 'classical', $CD11c^{high}$, myeloid (MDC) and the, $CD11c^{low}$, plasmacytoid DC (PDC). The terminology comes from the definitive myeloid origin of 'classical' DC [e.g expression of CD13, CD33, immunoglobulin-like transcript receptor 1 (ILT1) and low levels of the IL-3R chain CD123 and the β_2-integrin CD11c][179],whereas PDC originally were believed to derive from lymphoid progenitors, as they lack myeloid markers, and expressed T and B cell molecules, such as the preTCRα and Spi-B[180] high levels of CD4, CD62 ligand (L), and CD123[170,181]. However developmental origin does not appear so rigid. Thymic MDC and PDC can arise from progenitors distinct from those committed to T cell development[182]. In murine models, easier access to lymphoid organs has allowed the definition of a large number of subsets[183]. However, the ontogeny of many of these cell types is also still unspecified. Mouse DC subsets had originally been defined on the basis of CD8α expression, and this was also believed to signify a distinction between those of myeloid and lymphoid origin. However, as both subsets could be reconstituted by either myeloid or lymphoid progenitor cells, this hypothesis is probably incorrect[184]. Furthermore, tissue-derived, migrating murine myeloid $CD11b^+$ DC may differentiate into $CD8\alpha^+$ DC in the lymph nodes (LN)[185]. As the murine equivalent to human PDC can also be reconstituted from both myeloid- and lymphoid-committed

progenitors[186], a more plastic model of DC differentiation is now accepted in both mouse and man.

Irrespective of their ontogeny, the presence of DC subsets with divergent functions is likely to represent an evolutionary advantage to the host. Different organs are populated with varying proportions of DC subsets, and therefore their function must be analysed in the appropriate anatomical context in relation to the viral route of entry/transmission, in order to understand the relative roles of these two cell types. Infectious agents can be sub-classified broadly into those that enter via mucosae or skin and those that enter directly into the blood stream. In the resting state, MDC are ubiquitous in the skin, respiratory and gut mucosa, as well as peripheral blood and lymphoid organs. PDC, on the other hand, have only been found in peripheral blood and lymphoid organs[187,188]. Few, if any, PDC are present in the skin or mucosae in the resting state [189-195]. Furthermore, their migration pattern is different to that of MDC. Whereas the latter circulate from peripheral sites via afferent lymphatics to draining lymphoid organs[196], PDC enter lymph nodes predominantly from blood through high endothelial venules (HEV) [197] and like MDC, they can also migrate into areas of inflammation in the periphery [193,198,199].

In the initial stages of a skin/mucosal infection, MDC are likely to be the predominant DC subtype to be exposed to the invading virus. PDC could come into contact with the virus at later time points, in the periphery,

following inflammation-mediated migration[189,191,200] and in secondary lymphoid organs, after cells such as MDC, are productively infected with a virus[201,202]. However, for viruses that are transmissible via the blood route, interaction with PDC may be more relevant at the earliest stages of infection. The in vitro observations that this cell type can secrete very large quantities of type I IFN's may reflect such an in vivo scenario. Secretion of large quantities of this cytokine may compensate for the dilutional effect of many litres of blood, achieving a sufficient functional concentration of type I IFN's systemically.

The importance of PDC responses, in the control of systemic viral infections in humans, has been shown recently [203,204].When PDC are exposed to viral products and inflammatory stimuli, such as Toll like receptor (TLR) ligands or CD40 ligand (CD40L) expressed on T-cells, they differentiate into potent APC capable of stimulating memory responses [205,206]. However, the relative potency of mature PDC in stimulating naïve T cells remains controversial. Murine studies demonstrate that mature PDC are inferior to MDC in this capacity [206], whereas other authors report that mature human PDC are only inferior in stimulating naïve T cells to exogenous, but not endogenous antigens[207], implying inefficiency in cross-presentation by this subset. Thus, in the early stages of infection, the main role of uninfected PDC may not be to cross-present antigen, but rather to amplify MDC activation, most probably through the secretion of type I IFN [208], which in turn may amplify cross-priming to exogenous antigens by MDC[209].

Although they do not represent a separate subpopulation of DC, liver-resident DC are of particular interest in the context of hepatitis C infection. Fetal liver hosts stem cells that have the potential to give rise to a population of native DC [210]. Adult liver DC are derived from the bone marrow [211]. The function of liver resident DC can be directly affected by their microenvironment. Specifically, IL-10 and TGF-β are constitutively expressed by Kupffer cells and Liver sinusoidal endothelial cells (LSEC) and are inducible in Ito, stellate cells in conditions of cellular stress, such as infection. Thus the liver provides a unique cytokine microenvironment that can render the resident DC tolerogenic[212-214]. Studies in mice have shown that the liver resident DC may skew responses away from Th1 [215,216]. Therefore, this indigenous population of DC may play an important role in the chronicity of viral hepatitis. In that respect, chronic HBV infection that results from inadequate T-cell response to the virus [217], may be an interesting parallel to HCV. Although direct viral infection may also inhibit these cells' function, the immunosuppressive microenvironment of the liver may contribute towards the impaired function observed in DC derived from chronic HBV patients [218].

1.3.3 Dendritic cell activating ligands

DC activation is likely to be a cumulative process, such that several weak stimuli may add up to increase the maturation / activation state of the DC or that several stimuli cancel each other's effect with a net outcome quite different to the one predicted by a 'single receptor-single ligand' interaction model. Therefore the ligand environment in which the DC

encounters antigen is important in determining the degree of DC maturation and subsequently the skewing of the immune response towards T-helper 1 (Th1), T-helper 2 (Th2) or T-helper 0 (Th0). Lipopolysaccharide (LPS)-expressing Gram negative bacteria may activate DC fully, whereas other pathogens may induce the secretion of cytokines (e.g. tumour necrosis factor α - TNFα) that promote the maturation of DC[219]. In recent years, the number of DC activating ligands has greatly increased. Initial studies examined the ability of these ligands to upregulate the expression of co-stimulatory molecules on DC surface (i.e CD80, CD86), thus inducing 'maturation'. More recently it was shown that ligands DC encounter can affect DC physiology differentially, particularly with regard to cytokines secreted by DC, and subsequently tailor the immune response appropriately for the pathogen encountered.

It has been suggested that the immune system is activated via pattern recognition receptors (PRRs), that recognise conserved pathogen associated molecular patterns (PAMPs) [220]. Evidence to back up this theory emerged with the identification of Toll-like receptors (TLRs) and C-type lectins, receptors that recognise specific components of a wide variety of pathogens and even molecular patterns that are endogenous and host derived [221,222]. There is now experimental data to support the function of both exogenous and endogenous activators, which may act to complement each other.

1.3.3.1 Pattern recognition receptors

1.3.3.1.1 TLR family

This category of receptors was identified initially on the basis of its homology to the drosophila Toll protein [223]. The currently known Toll-like receptors (TLR) in vertebrates are divided in six major groups of receptors, also called ‘families’ (the ‘TLR1’, ‘TLR3’, ‘TLR4’, ‘TLR5’, ‘TLR7’, and ‘TLR11’ ‘families’). Members of each ‘family’ recognise a general class of PAMP’s. For example the ‘TLR7 family’ (comprising of TLR7, TLR8 and TLR9) includes receptors that recognise nucleic acids and heme motifs[224]. Notably, not all the structures recognised are pathogen specific, with a growing list of endogenous ligands being described. The 11 most important TLR in humans, along with the corresponding ligands, both pathogen-associated and endogenous, are summarised in table 1.1.

TLR	Pathogen associated ligand	Endogenous ligand
1	Mycobacterial lipoproteins, triacylated lipopeptides [225]	-
2	PGN, LTA[226] MV + HCMV envelope protein[227,228]	Heat shock proteins* [229]
3	dsRNA[230] Poly I :C[230]	siRNA [231] mRNA [232]
4	LPS[233] RSV envelope protein [234]	Heat shock proteins* [235] Hyaluronan* [236]
5	Flagellin[237]	-
6	Cooperates with TLR2 in recognition of PGN and other diacylated lipopeptides [238,239]	-
7	ssRNA [240,241]	
8	ssRNA[241]	
9	CpG DNA [242]	Chromatin IgG complexes [243,243]
10	-	-
11	Uropathogenic bacteria [244]	

(*) The complete absence of microbial contaminants that could bind TLR2 and TLR4 in the preparation of these proteins could not be confirmed, and therefore, the role of these proteins as endogenous TLR ligands remains controversial.

Table 1.1 TLR ligands

Peptidoglycan (PGN), Lipoteichoic acid (LTA), Measles virus (MV), Respiratory syncytial virus (RSV), double stranded RNA (dsRNA), single stranded RNA (ssRNA), short interfering RNA (siRNA), Poly I:C (polyinosine-polycytidylic acid)

One effect of TLR ligation on DC may be the regulation of antigen uptake and processing. Two recent studies have demonstrated that TLR ligation, through p38 activation, promote uptake of bacteria in macrophages. This occurs: a) through up-regulation of expression of scavenger receptors, such as SR-1 and MARCO, and the mannose receptor, that effectively increases uptake efficiency of bacteria [245] and b) through maturation of the phagosome [246]. In DC, this TLR signalling effect may induce the delivery of degraded antigen to MHC class II loading compartments. It is interesting that MARCO (macrophage receptor with collagenous structure) is also involved in cytoskeletal rearrangement in DC [247], suggesting that TLR ligation may also prime DC for emigration from the periphery.

Despite the activation of (nuclear factor-kappa B) NF-kB after ligation of most TLR and the close association between the activation of this pathway with DC maturation [248-250], it has been demonstrated that simultaneous activation of other signalling pathways by different TLR results in diverse functional outcomes. The initial TLR ligation can condition the DC for the signals it will pass on to engaging T cells, in the forms of cytokines and surface molecule expression. An example of the latter includes the Th1 promoting ICAM-1 [251], whereas OX40 binding on T cells by OX40L on DC promotes Th2 responses [252].

TLR signalling is mediated primarily by the adaptor protein myeloid differentiation factor 88 (MyD88), which triggers the activation of transcription factors, such as NF-κB, that are essential for the expression of pro-inflammatory cytokine genes

[253]. This pathway also leads to the potent production of type I interferon (IFN) through the activation of IFN regulatory factor 7 (IRF7) upon stimulation of TLR7 or TLR9 [254]. In contrast, Toll/interleukin-1 (IL-1) receptor homology domain-containing adaptor-inducing IFN-β (TRIF/TICAM-1) mediates the production of type I IFNs primarily through the activation of IRF3 in response to TLR3 or TLR4 stimulation [253]. Type I IFN induces the maturation of DCs by increasing both the expression of co-stimulatory molecules such as CD80, CD86, and CD40 and antigen presentation via major histocompatibility complex class I in addition to classical endogenous antigen presentation; it also facilitates the cross-presentation of viral antigens. A cumulative report has shown that DC activation via TLR signaling is a prerequisite for the subsequent induction of vigorous T-cell responses [255]. Some viral proteins have been shown to inhibit the TLR-dependent signalling pathway through interactions with the downstream adaptor molecules, suggesting that the alteration of TLR-mediated signals is one of the mechanisms of virus-induced immune modulation [256].

1.3.3.1.2 c-type lectin family

This family of receptors is important for the recognition of a wide range of carbohydrate structures. These receptors play a role in facilitating phagocytosis and endocytosis, and hence also antigen processing and presentation. However, recent evidence suggests that the downstream signalling events they induce may also play a significant role in shaping the ensuing immune response. C-type lectins include the mannose receptor (MR or CD 206), DEC-205 (CD 205), DC-SIGN (CD 209), L-SIGN (CD 209L), BDCA-2 (CD 303), Dectin-1 and the

Langehans cell specific receptor Langerin (CD 207). Most of these are expressed on monocyte-derived DC (MDDC), although this does not exactly reflect a corresponding expression on circulating (peripheral blood) myeloid DC (MDC). This observation may suggest, taking into consideration the already discussed plasticity in DC ontogeny, a differential expression of c-type lectins at various stages of DC development. Furthermore, some are specific to certain DC subsets, such as Langerin on Langerhans cells, whereas others are noted by their absence, such as the lack of expression of DC-SIGN on PDC. They all possess at least one carbohydrate recognition domain and recognise sugars in a variety of secondary and tertiary structures {reviewed in[257]}.

Like for TLRs, c-type lectin ligands can be endogenous, such as ICAM-2 and ICAM-3 for DC-SIGN [258], or pathogen-derived. Recent work has focused on the field of pathogen recognition. Initial studies utilised soluble sugars, such as mannan, to compete for binding to the MR, although this approach is not selective, notably also prevented interaction with DC-SIGN [259]. As a result, only studies using neutralising antibodies to these receptors can be interpreted as showing an interaction definitively.

Many bacteria and fungi can interact with c-type lectins[257]. For virus recognition, the MR may play a role in binding to HSV-1[260] and influenza virus[261], although the interaction with either of these viruses has not been proved definitively. On the other hand, DC-SIGN binds to envelope glycoproteins of human immunodeficiency virus (HIV)[262], Ebola virus[263], HCMV [264], hepatitis C virus (HCV)[265] and DV[266]. Functionally, it has been suggested

that viral DC-SIGN binding protects viruses from degradation, ‘preserving’ them in non-lysosomal compartments in DC and thus promotes efficient trans-infection of other target cells (e.g T-cells), by infected DC, in secondary lymphoid organs[262,264].

The signalling events downstream of c-type lectin receptors have attracted interest recently. Endocytic mannose receptor (MR) may interact with TLR2/6, inside phagosomes, to specify and amplify host immune response against Staphylococcus aureus[267]. Dectin-1 and TLR2 can co-operate to increase zymosan induced signalling and cytokine secretion[268]. Cross-linking the mannose receptor, despite inducing upregulation of co-stimulatory molecules, inhibits IL-12 secretion and induces secretion of IL-10[269,270]. Cross-linking BDCA-2 also inhibits IFNα production by PDC[271], whereas Mycobacterium tuberculosis capsid sugar, mannose-capped lipoarabinomannan (ManLAM), binding to DC-SIGN can inhibit IL-12 secretion by DC stimulated with LPS[259,269]. These observations demonstrate how pathogens may have evolved mechanisms to subvert host immune responses by binding c-type lectin receptors.

1.3.4 Signalling pathways involved in dendritic cell physiology

The regulation of DC maturation and migration lies at the level of intracellular signalling. Different PAMPs, as well as viruses can activate or block intracellular signalling pathways and alter DC function.

NF-κB

The family of inducible, dimeric, NF-κB, transcription factors is made up of 5 members (belonging to the Rel family of DNA-binding proteins): p65 (RelA), p50/p105, c-Rel, p52/p100, and RelB, and represents the most important intracellular signalling pathway in DC. The classical regulation of these members is quite similar. They are retained in the cytoplasm with the nuclear localisation signals 'masked' by the inhibitory proteins IκBα and IκBβ. Phosphorylation of IκBα and IκBβ results in their ubiquitnation and rapid proteasomal degradation with subsequent translocation of NF-κB to the nucleus. Phosphorylation of the IκB proteins is in turn regulated by the multisubunit IκB kinase (IKK) family, consisting of two catalytic subunits, IKKα and IKKβ, both of which are able to correctly phosphorylate IκB. A variety of other signaling events, including phosphorylation of NF-κB, hyperphosphorylation of IKK, induction of IκB synthesis, and the processing of NF-κB precursors, provide additional mechanisms that modulate the level and duration of NF-κB activity[272].

DC maturation is preceded by an increase in DNA binding activity of RelB, p50, p52, and c-Rel[273], suggesting that NF-κB activation positively regulates DC maturation. Furthermore, transfecting DC with IκBα expressing adenoviral vectors prevented both the upregulation of molecules associated with antigen presentation / maturation (HLA class II, CD80, CD86, CD40) and the secretion of inflammatory cytokines, including IL-12, IL-6 and TNFα[274]. The role of the p40 subunit in IL-12 secretion by DC in response to LPS has been formally demonstrated using small interfering RNA (siRNA)[275]. It should be noted that

the pathways become increasingly divergent and stimulus-specific upstream of the effector molecules/transcription factors, as exemplified by the role of IKK2 in CD40L but not LPS induced maturation of DC[276]. In this respect, it is interesting that Protein Kinase Cε (PKCε) is phosphorylated rapidly (within 15 min) after LPS stimulation of DC, and promotes TNFα and IL-12 secretion by facilitating degradation of IκB and therefore NF-κB access to the nucleus. However, this enzyme does not play a role in the upregulation of costimulatory molecules by LPS and subsequent inhibition of its activity only prevented Th1 skewing of co-cultured T cells, but not their proliferation, demonstrating how NF-κB can differentially activate effector pathways [277]. Indeed, NF-κB subunits are differentially recruited upon DC maturation by various stimuli [273,278]. The role of individual NF-κB subunits has only been addressed in the murine immune system using knockout mice. Interestingly, large degree of redundancy has been observed. Mice lacking c-Rel showed no impairment in IL-12 secretion [279], whereas mice deficient in both p50 and c-Rel demonstrated reduced IL-12 secretion, despite DC from these mice expressing normal levels of MHC and costimulatory molecules [280]. It should also be noted that the role of the NF-kB subunits may vary between DC subsets, as c-Rel is important in IL-12 p35 expression in CD8α+ DC (c-Rel-dependent) [281], but not in bone marrow-derived DC (BMDC)(c-Rel independent)[279].

NF-κB is also involved in maintaining DC viability. Fas ligation is a pro-apoptotic event in most cell types. Fas induced NF-κB activation in DC prevents these cells from dying when they encounter FasL on T cells[282]. Interestingly, this is a

pathway that may be targeted by viruses, as MV infection renders DC susceptible to T cell mediated induced apoptosis by FasL [283].

1.4 Dendritic Cells in the setting of chronic HCV virus infection

One of the main characteristics of HCV is the high propensity to establish chronic infection. Clearance of the virus, as mentioned earlier, can occur spontaneously during acute HCV infection and is associated with a vigorous, long lasting cellular immune response against multiple HCV epitopes [121-125]. However in the great majority (up to 80%) of cases, the acute infection leads to chronicity. Once chronic infection is established, there is no spontaneous resolution of the viraemia. The impairment of proliferative, cytokine mediated and cytolytic T-cell functions persist long-term in patients with chronic HCV infection [123,284,285] .

Mechanisms by which HCV establishes chronic infection have not been determined yet. Several hypotheses have been proposed to explain how an inefficient cellular immune response allows HCV to establish a chronic infection:

i) High rate of HCV replication leading to primary failure or exhaustion of the immune system through the production of overwhelming quantities of HCV antigens [125,138,286]

ii) High replication rate of HCV, generating increased viral variation and emergence of escape mutations [287,288]

iii) Suppression of HCV-specific Th1 cell expansion by regulatory T-cells (T-regs) or by the tolerogenic cytokine environment of the liver [e.g liver sinusoidal endothelial cells (LSEC)] [289]. In particular HCV-

specific T-regulatory 1 (Tr1) response has been shown recently to occur as early as 1-2 months after acute infection, suppressing virus-specific CD4+ T-cell responses[290].

iv) Intrahepatic compartmentalization and induction of apoptosis of virus specific CD8+ T-cells [291]

v) Production of immunomodulatory proteins by HCV (i.e HCV core protein) [292,293]

vi) Inability of the innate immune response to promote timely and appropriate T-cell priming [110].

The role of the innate immune response for the high rate of chronic HCV persistence is not yet well defined. The initial phase of the immune response plays a critical role for effective containment of infections. DC being the most potent known antigen-presenting cell (APC), is a critical component of the innate immune system and essential to the initiation of adaptive immune responses [156]. Consequently, its functions may be targeted by viruses to disrupt the generation of cellular immune responses.

1.4.1 Viruses exploit DC to establish chronicity

Studies on the function of DC following infection by viruses have aided the understanding of the pathogenesis and establishment of several human viral infections, such as Human Immune-deficiency Virus (HIV), Measles Virus (MV), Hepres Simplex Virus (HSV), and Cytomegalovirus (CMV). Infection of, or attachment to, the migratory DC in peripheral tissues or circulating blood could

facilitate the initial viral dissemination to lymphoid organs[262], giving the virus access to its target cell of infection (e.g. CD4+ T cells for HIV). Although DC are permissive for infection by a variety of viruses of different phylogeny [294-298], the capacity to support replication is often lower than other cell types [294,299]. Therefore, it is unlikely that infection of DC contributes significantly to the production of viral progeny. Rather, its importance may be in targeting the function of the cell specifically. Analysis of DC ability to stimulate T cells following infection by many viruses demonstrates that this is disrupted, independent of the virus' capacity to replicate in DC [294,297,299-301].

To disrupt the T cell stimulating capacity of DC, viruses can target various functions of the cell (Table 1.2). Inhibiting cytokine secretion, specifically, IL-12, would prevent efficient activation of Th1 effector T cells[302]. However, the most common phenomenon observed is interference with the DC ability to respond to maturation stimuli by upregulating MHC and co-stimulatory molecules [294,297,303,304]. This renders the DC less potent APC for the stimulation of antiviral T cells and could therefore delay the generation of an effective antiviral immune response.

	HSV-1	HCMV	Vaccinia	HBV	Measles	Influenza	Dengue
Phenotype	Impaired maturation [294]	Impaired maturation [299]	Impaired maturation [297,305]	Not known	Maturation [295] Impaired CD40L maturation [304]	Maturation [306]	Maturation [307]
Replication	Low [294]	Low[299]	No/abortive replication [297,305]	Yes [217]	Increased by CD40L [301,304]	Not known	Yes/equivalent to epithelial cell[307]
Viability	Apoptosis [294]	Not known	Apoptosis [297]	Not known	Apoptosis – accelerated by T cell interaction [283]	Apoptosis [306]	Apoptosis [307]
IL-12	Not induced by infection [294] Reduced in response to LPS [294]	Not induced by infection Reduced in response to LPS + CD40L[303]	Not known	Reduced in response to T cell stimulation + cytokines [217,308,309]	Weakly induced by infection [310] Augmented with LPS [295] Reduced in response to CD40L [301]	Induced by infection [311]	Nil or low level induced by infection [307,312]
T cell stimulation	Impaired [294]	Impaired [299,303,313]	Impaired	Impaired [309,314,315]	Impaired [300,316,317]	Enhanced [306]	Not known

Table 1.2 Disruption of DC functions by viruses

1.4.2 Role of DC in Flavivirus infection

HCV is a member of the *flaviviridiae* family of viruses. In this respect, the data from the interaction of other flaviviruses with DC provides useful information. Bovine viral diarrhoea virus (BVDV) and classical swine fever virus (CSFV) do not disrupt the DC's ability to stimulate T cells, but can replicate in these cells to generate viral progeny [318,319]. Dengue virus (DV), also a member of *flaviviridiae*, infects skin DC [320] and monocyte derived DC in vitro [312], which results in production of infectious virus [312]. However, the infection is not silent; maturation of DC is initiated along with proinflammatory cytokine secretion (TNFα and IFNα) [307,312]. Therefore, the examples from BVDV, CSFV and DV suggest that flaviviruses can infect and replicate in DC. However, the different transmission, pathogenesis and anatomy of viral infection make it difficult to extrapolate the functional outcome of HCV infection from those of other flaviviruses. Nevertheless, it is noteworthy that the absence of a PDC response in humans was recently linked with more severe DV-derived disease [203], demonstrating that the interaction of circulating DC subsets in blood with blood-borne flaviviruses, like HCV, may be critical in determining the disease outcome.

1.4.3 The role of DC in HCV infection

1.4.3.1 How does HCV bind to DC?

The binding of HCV to a wide spectrum of cells was thought to be mediated by several receptors: a) CD81, a tetraspanin expressed both on parenchymal and non-parenchymal liver cells, which has been shown recently to be

essential in the binding of cell culture-grown HCV (HCVcc) [73,97,98], b) Low density lipoprotein receptor (LDL-R), which is expressed on hepatocytes (although there is no evidence of direct binding of HCV envelope proteins), c) human scavenger receptor class B type I (SR-BI) [which binds high density lipoproteins (HDL), native or modified LDL and very low density lipoproteins (VLDL)], expressed on hepatocytes, lympocytes, dendritic cells et.c and potentially acts as a co-factor for CD81 binding of HCV[321-323], d) highly sulphated heparin sulphate which has been shown to serve as an initial docking site ('capture molecule') for HCV attachment, while transfer of the virus to a second high affinity receptor is probably essential for triggering entry[324] and finally e) 'tight-junction' proteins claudin-1/-6 and -9 have also been shown to act as entry co-factors / 'capture molecules' for CD81 binding of HCV[325,326].

Recently two trans-membrane, mannose-binding, C-type lectin receptors, DC-SIGN [Dendritic Cell Specific Intracellular adhesion molecule-3 (ICAM-3) Grabbing Non-integrin], expressed on DC[262], and L-SIGN [Liver/Lymph node Specific Intracellular adhesion molecule-3 (ICAM-3) Grabbing Non-integrin, formerly known as DC-SIGN-related molecule expressed on Liver Sinusoidal Endothelial Cells (LSEC) and lymph nodes, have also been shown to bind HCV [81],[327]. DC-SIGN is an adhesion receptor that was primarily shown to serve the interactions between DC and T-cells and between DC and epithelial cells as well as the internalisation of viral products in late-endosomal compartments for processing and presentation to T-cells[327]. DC-SIGN is also shown to act as a rolling receptor mediating ICAM-2-dependent DC

migration[328]. Similar to DC-SIGN, L-SIGN has been shown to mediate interactions between liver sinusoidal epithelium and trafficking T-cells as well as with viruses such as HIV[329].

HCV envelope glycoproteins 1 and 2 (E1 and E2) have been shown to be ligands of DC-SIGN and L-SIGN[83,84,265]. It has also been suggested recently that HCV binding to DC-SIGN and internalisation targets the virus to non-lysosomal compartments (early endosomal compartments) within DC. Thus HCV may use DC-SIGN to 'hide' within DC, a process that facilitates viral dissemination[84,85]. Other authors though suggest that the SIGN receptors simply bind and transfer HCV to succeptible cells (e.g hepatocytes' 'trans'-infection) that eventually become infected, without mediating entry of the virus into the cells expressing SIGN (i.e DC)[84,265].

On the other hand some studies, on HIV, question even the expression and importance of DC-SIGN in in vivo conditions, implying lack of expression of the lectin on circulating myeloid DC[330].

Recently a study using an HCV replicon cell culture system demonstrated that scavenger receptor class B type I(SR-BI), expressed in high levels on DC, is essential for uptake of HCV by DC and for cross-presentation of viral antigenic products[322]. Despite that data other recent studies, using cell culture produced HCV, question receptor mediated entry of HCV in DC[331].

Unlike HIV, HCV probably enters DC with the potential of replication / production of viral progeny within the latter and also possibly affects the functions of dendritic cells.

1.4.3.2 Does HCV replicate in DC?

Most of the studies looking into DC function in HCV-infected patients have focused on DC derived in vitro from monocytes. Several studies have suggested that PBMC and monocytes in particular, can be infected by HCV[332-334]. In addition, HCV genome has been detected in DC derived from monocytes [335,336]. As monocyte differentiation to DC occurs in vivo [337], data acquired from monocyte-derived dendritic cells may be relevant.

One of the approaches used to detect HCV RNA and to investigate whether there is active replication of HCV genome within DC, is the detection of virus specific sequences in total RNA extracts from DC. The presence of both 'positive strand' HCV RNA and the replicative intermediate 'negative strand' HCV RNA was detected in peripheral blood DC (PBDC, isolated directly from PBL without generation from monocytes) [335,338]. Moreover a study looking into predilection of specific 'quasispecies' [as these are defined by sequence variation within the hyper-variable region 1 (HVR1) of E2] of HCV in PBDC suggested viral tropism and implied active viral replication, as opposed to acquisition of viral genome through phago-, pino-cytosis, within DC by demonstrating that quasispecies of the virus infecting DC (2 out of 7 sequenced quasispecies) were not detected in the corresponding patients' sera and DC-depleted PBMC[335]. The presence of HCV RNA in DC was only observed though in a very small proportion of the patients investigated [335]. However other authors showed that positive strand HCV RNA was consistently found in DC of patients presenting with the most profound defect in T cell stimulation [336].

It has to be taken into consideration though that the low viral loads detected in DC after total RNA extraction from quite considerable numbers of cells (100-350 HCV genomic molecules from 10^4-10^6 DC or 0.1-35 genomic molecules for every 1000DC) challenge the sensitivity of the detection methodology used[335,336]. The latter is supported by other studies that have not found HCV RNA in DC in both humans [339] and chimpanzees [340].

More recently the implementation of ultra-sensitive methodologies such as reverse transcription (RT) PCR-nucleic acid hybridisation (NAH)[341,342] or very sensitive real-time, nested RT-PCR[343] in some studies has shown presence of HCV RNA sequences in total peripheral blood mononuclear cells (PBMC) and their subsets (including $CD4^+$/$CD8^+$ T-cells, B-cells, monocytes and monocyte-derived DC) and confirmed the replication of the virus in PBMC by demonstrating the presence of negative strand sequences [341,342].

As no viraemia differences are noted between subjects with defective DC function and subjects with normal DC function it seems reasonable to hypothesise that DC dysfunction - defective cytokine production as observed by some authors - is probably due to the presence of HCV genome within DC and not due to chronic hepatitis C in general[343].

As a result of what has been mentioned so far about the low grade of HCV replication in DC, the possibility of the virus infecting DC for the production of progeny is highly unlikely. It is probably the function of DC that is targeted by HCV.

1.4.3.3 Effects of chronic HCV infection on DC function

1.4.3.3.a Capacity to stimulate T cells

A widely used approach to assess the T-cell stimulating capacity of DC is the study of their ability to induce proliferation of allogeneic naive T cells in the context of Mixed Lymphocyte Reaction (MLR). In this assay - formerly used for the detection of alloreactive donor T cells in allogeneic bone marrow transplantation - DC from chronically infected patients, usually irradiated or treated with the cytostatic mytomycin C to prevent them from proliferating, are incubated with naïve T-cells from healthy individuals. A proportion (1%-10%) of the effector T-cells used in the assay is presumed to cross-react to non-self MHC molecules on stimulator DC and will thus expand and proliferate. The effector cell proliferation is usually assessed with radioactive [methyl-^{3}H]-thymidine incorporation in proliferating T-cells, as measured by a beta-counter. It has to be mentioned here that a serious limitation of the MLR is that it does not reflect a virus-specific response, as DC are not pulsed with HCV-specific antigens, but rather gives an overall estimation of the functional efficiency of the studied DC population.

Most of the studies carried out so far have demonstrated that this function, also known as the ‘allostimulatory capacity’ of DC, is impaired in monocyte-derived DC from patients with chronic HCV infection [336,340,344,345]. However, while the alloreactive capacity of DC from HCV infected patients (HCV-DC) is reduced when compared to DC from healthy donors (N-DC), autologous responses to HCV antigens (stimulation of T-cells from infected patients after incubation with autologous DC and HCV-specific antigens) can still be elicited, and recall responses to heterologous antigens, such as

influenza A, cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are not impaired [344,346]. Interestingly, the allostimulatory capacity of HCV-DC returns to normal after treatment-induced HCV clearance[336], an observation indicating that HCV may be responsible for the initial defect. These findings are also supported by a study in HCV-infected chimpanzees [340], whereby although the impairment of allostimulatory function is minimal for most of the investigated animals, the subjects presenting with the highest viral loads present with significantly decreased allostimulatory capacity, implying an etiologic correlation of DC functional impairment to HCV infection.

On the other hand several authors have demonstrated that chronic HCV patients' T-cell response to recall antigen, is comparable to those of antiviral treatment-cleared subjects[339,344,347-349].

Furthermore, recent studies were not able to reproduce the impairment in allostimulatory capacity, nor in any other DC functional output, in patients with chronic hepatitis C[331,349,350]. In order to explain the differences to previously published data, inadequate maturation of DC studied in the latter, as evidenced mainly by low expression of CD83 on DC surface, was suggested. When strong maturation stimuli were used and high CD83 expression on mature monocyte-derived DC surface was achieved, the allostimulatory capacity was restored.

In another recent study it is also suggested that DC defects, observed previously in the setting of chronic HCV infection, were probably attributable to the immune-modulatory effect of on-going or recent (even if antiviral treatment was discontinued more than six months prior to patients' inclusion to the study) use of Ribavirin or even immune-suppression caused by

advanced hepatic fibrosis and not to chronic hepatitis C. It is also pointed out by the same authors that potential host variation in DC function may account for the differences seen in previous studies. This observation is supported in the same paper by the study of a small cohort of 'high risk' intravenous drug users that acquired HCV while they were being followed-up. In those patients no significant changes in DC functional capacities were seen before and after the establishment of chronic infection[350].

The controversy on the effect of chronic HCV infection on DC functional output remains to the day though and the necessity of more studies is more than obvious.

1.4.3.3.b Surface marker expression on infected DC

Although inseparable from the functional output of DC - due to the role of surface molecules expressed on DC on the interaction of the latter with other components of the immune system, such as T cells - DC surface molecule expression has been extensively studied and is thus reviewed separately here.

A lower level of co-stimulatory molecule expression (B7.1 / CD80 and B7.2 / CD86) - molecules that are essential for T cell activation - on DC surface could explain potential impairment in the function of the latter as T-cell stimulator. While some studies have shown that HCV infected DC express lower levels of CD86 and CD80[344,351,352] and fail to mature upon TNF-α stimulation[344,351], as evidenced by reduced CD83 expression, these observations are not born out in all the studies nor do they appear to correlate to the degree of functional alteration observed [336,339,350]. A recent study

even suggests induction of DC maturation upon binding to HCV E2 protein[353].

Despite these data another study suggests decreased expression of HLA-DR on plasmacytoid DC, directly isolated from circulating PBMC, that affects their capacity to stimulate $CD4^+$ T-cell proliferation and IFN-γ production, adding some evidence of an altered DC phenotype contributing to a potential functional defect, a hypothesis that has been discouraged by most previous so far[354].

1.4.3.3.c Cytokine secretion

IL-12 is one of the main cytokines secreted by DC and plays a critical role in priming Th1 immune responses [355]. Thus, an HCV-induced defect in DC secretion of IL-12 might be detrimental for the induction of anti-HCV responses. Several studies so far have shown that defective IL-12 production from DC in patients with chronic HCV infection was responsible for the defective T-cell allostimulatory capacity and IFN-γ production[344,356]. However, another study failed to reproduce those findings[336]. This discrepancy of data between various studies may be partly attributed to the complex signal regulation that governs secretion of this cytokine by DC. Other authors demonstrated that disrupted type I IFN production by plasmacytoid DC, in the setting of viral infections such as HSV, acting in a paracrine manner, may cause reduced IL-12 production by myeloid and plasmacytoid DC[294,357], those observations though remain to be shown in the setting of HCV infection.

Recently data from a study implementing direct ex vivo investigation of DC isolated from peripheral blood, after TLR-3 and TLR-4 stimulation with polyinosinic-polycytidilic acid (Poly I:C, a known TLR-3 ligand) and lipopolysacharide (LPS, a known TLR-4 ligand) respectively, suggested defective IL-6, IL-12 and TNF-α production, probably attributable to the attenuation of 'innate sensing' by TLR as a result of HCV infection[343].

1.4.3.3.d NK cell stimulation

Recent evidence has demonstrated a close association between resolution of HCV infection and NK cell activity [358]. One mechanism of activating NK cells in vivo is through the interaction with DC. Therefore, it is noteworthy that DC generated from HCV carriers are defective in upregulating the NK activating ligands MHC class-I related chains A and B (MICA and MICB) in response to IFNα. Lack of these molecules results in reduced NK cell activation and consequently may contribute to HCV persistence and insufficient response to IFNα treatment[359]. More recent studies confirm the functional defect of NK cells in the setting of CHC and attribute discrepancies in various previous studies that fail to uniformly recognise the defect, to altered NK subset distributions between chronically infected patients, healthy controls and treatment-cleared individuals (i.e lower total numbers of NK cells but higher frequencies of IFN-γ producing CD56bright NK in CHC patients)[360]. Defective NK / NKT / NT-cell function has been associated with HCV persistence[108]. The existence and properties of (Natural T)NT cells, a predominantly intrahepatic T-cell population that expresses NK cell surface markers (CD56) and thus posses both innate and adaptive properties,

appears in some studies to represent an important parameter in the setting of HCV infection with some authors suggesting the correlation of a defective response in part of this T-cell population in acute HCV infection to the establishment of persistent infection[361].

More recently studies observing normal DC function in the setting of chronic HCV infection have suggested mechanisms to explain defects in DC shown by other authors. In particular it is claimed that antiviral treatment and in particular Ribavirin with its significant immunomodulatory properties[362] may persist in DC for more than 6 months[363] after the completion of treatment and may thus affect DC function of patients included in various studies[336,351]. Also host parameter variability such as the extent of liver damage (inflammation grade and fibrosis stage on liver biopsy) are being suggested as potential factors influencing DC function with unpredictable influence in the results of studies assessing DC in the setting of chronic HCV infection. Other host differences, such as inter-individual variation in the stimulatory capacity of DC (e.g allogeneic MLR assay), and not primary DC defect due to CHC, may also be responsible for the controversy[350].

1.4.4 Potential mechanisms of HCV-induced disruption of DC function

Several studies have raised the possibility that HCV proteins could modulate some DC functions. The background for this hypothesis is that infection and HCV replication in DC may not be a prerequisite for the impaired DC allostimulatory capacity. Considering that a 1,000- to 10,000-fold excess of viral proteins relative to positive-strand RNA is observed with full-length HCV replicon cell clones[364], it is tempting to speculate that even the presence of

a few viral genomes per DC may be sufficient to produce concentrations of viral proteins required to effectively interfere with innate defence function at the single-cell level without the need for productive infection (replication).

Individual HCV antigens, namely Core and NS3, have been shown to inhibit the allostimulatory function while at the same time trigger the production of TNF-α and IL-10 from DC. A quantitative relationship between the amount of HCV core and NS3 protein that DC are pulsed with, and the corresponding production of IL-10 and TNF-α has been suggested[356]. This observation bears significant consequences in the amount of liver injury and the skewing of the immune response towards tolerance (IL-10). In particular IL-10 inhibits both the production of IL-12 by DC and T cell proliferation [365], possibly leading to T cell anergy through direct down-regulation of the CD28 co-stimulatory pathway[366]. Furthermore it was shown that treatment of immature DC with IL-10 leads to a 'tolerogenic' DC population [216]. Interestingly, the immunomodulatory role of core and NS3 proteins may also lie at the level of DC differentiation, as addition of these proteins at the monocyte stage can render monocyte-derived DC (MDDC) poor T cell stimulators. This seems to occur through a process analogous to the one leading to the 'exhausted' DC phenotype[367]. The ability of HCV proteins to down-regulate the NF-κB pathway may explain the low IL-12 secretion by DC generated in this way. It should be noted that maturation of DC from healthy, non-infected individuals, pulsed with HCV core and NS3, recovered the defect in T cell stimulation. On the other hand, DC from chronic HCV carriers do not regain function as efficiently following maturation. Therefore, core and NS3

are unlikely to be the only proteins involved in modulating DC function in vivo [356].

Alternative approaches have included expression of HCV Core and E1 genes in healthy donor DC. Adenoviral vectors encoding HCV core and E1 were used to transfect myeloid DC. The transfected DC presented poor $CD4^+$ and $CD8^+$ stimulating capacity, with regard to both alloreactive and autologous T-cell proliferation as well as IL-2 production, and failed to mature upon stimulation by TNF-α and CD40L [368,369].

Exposure to soluble HCV core antigen as well as intracellular expression of HCV core in myeloid DC was also shown to cause a significant impairment in the expression of MHC class-I/II, co-stimulatory molecules (CD80/86) and (programmed death ligand) PD-L1 expression on myeloid DC surface along with a defective intracellular expression and secretion of IFN-γ, IL-6, IL-12. $CD4^+$ and $CD8^+$ T-cells primed by those DC were sub-optimally activated (reduced expression of CD69 and CD25), were defective in Th1 cytokine production (IFN-γ) while at the same time expressing increased intracellular levels of IL-10 and overall presented a regulatory T-cell (Treg) phenotype[352].

Another recent study explores the inhibitory effect of HCV NS5A protein on TLR-MyD88-dependent signalling, through the IFN-sensitivity-determining region (ISDR), causing impaired cytokine (IL-6) production in macrophage cell-lines expressing HCV proteins[370], suggesting a potential novel mechanism of interference of HCV with DC function.

In support of the suggested mechanisms of interference of HCV with DC function, suppression of plasmacytoid DC(PDC) TLR9L(CpG ODN 2216 / HSV)-induced (MyD88-dependent) IFN-α production after interaction with ultra-violet light inactivated (i.e unable to replicate) HCV cell-culture produced (HCVcc) virions, was reported recently and was not abrogated by human sera containing HCVcc-neutralising antibodies, suggesting again that PDC infection was not required [331]. The above data were generated in the acute setting, with HCVcc impairing PDC response within as little as 42hours.

Even more recently the chemotactic behaviour of MDC and PDC was reported defective in the setting of chronic HCV infection, with immature DC migrating towards inflammatory sites. Treatment with IFN-α resulted in maturation of DC and re-directed their migration from inflamed portal tracts towards secondary lymphoid organs, in order to initiate adaptive immune responses against HCV[371].

Blockade of the inhibitory receptor 'programmed death - 1' (PD-1), expressed on $CD8^+$ T-cells preventing the interaction with its ligand B7-H1, expressed on liver resident DC, resulted in functional improvement of $CD8^+$ T-cell reactivity in HCV core protein transgenic mice, suggesting one more potential mechanism of HCV proteins inteference with DC function, contributing to the known $CD8^+$ T-cell reactivity defect[372]. A synergistic effect of IFN-α treatment with PD-1/PD-1ligand interaction blockade, in the setting of acute HCV infection as shown recently[373], may prove important in the development of potential therapeutic strategies.

1.5 Aims of the study

The aim of this study is to test monocyte-derived dendritic cells (MDDC) generated from patients with chronic hepatitis C (CHC) and from healthy subjects, in order to contribute to a better understanding as to:

1. whether there is a functional defect in DC from CHC patients, in particular in their capacity to stimulate 'naïve' CD4+ T-cells into proliferation as compared to healthy individuals
2. whether the virus is actually present and replicating within DC
3. whether there is a potential correlation of host factors to DC function. To that effect patients' characteristics such as fibrosis on liver biopsy, ethanol consumption and viraemia levels were tested against DC function
4. whether infection of DC by HCV or HCV viraemia levels bear an impact on DC function
5. whether the presence of viral products such as HCV Core protein either inside DC, or circulating in patients' plasma affect DC function
6. whether potential restoration of DC function after antiviral treatment contributed to the containment of the infection (viraemia clearance by week 12 of antiviral treatment)
7. whether the chronic infection affects primarily the antigen-presenting DC or the effector T-cells

Finally the effect of HCV on the function of the second subset of human DC, the plasmacytoid dendritic cells (PDC) is also explored aiming at contributing to our understanding of the effect of HCV on dendritic cell function.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Clinical material - patients

Thirty five consecutive, treatment-naïve patients with chronic hepatitis C, monitored at the Hepatitis Clinic, University College Hospital, London, were enrolled in the study (patients' characteristics described in table 2.1). All subjects included were positive for anti-HCV (tested with a 3rd generation ELISA assay) and HCV RNA (Amplicor HCV v2.0; Roche Molecular Systems, Pleasanton, CA). The HCV genotype was determined by a restriction-fragment-length-polymorphism (RFLP) methodology {as in[374]}. All patients tested negative for HBsAg and antibodies against HIV1,2, by commercially available enzyme assay (Abbott Diagnostics, Maidenhead, UK). All patients included in the study also underwent a liver biopsy, prior to the initiation of antiviral treatment, as part of the baseline routine diagnostic assessment. The study was approved by the University College London Ethics Committee, and all patients gave informed consent prior to enrolment.

2.1.2 Materials

Unless otherwise stated, all chemicals and reagents were obtained from Sigma Ltd. (Poole, Dorset, UK). Disposable pipettes, pipette tips and 50ml tissue culture tubes were from Sarstedt AG, Numbrecht, Germany. 60-ml sterile plastic syringes were TERUMO Leuven, Belgium. 6-well culture plates were FALCON (Beckton Dickinson labware, NJ, USA), 96-well U-bottom culture plates and 75cm^3 cell-culture flasks were from Nunc (Nunclon, Gibco BRL, Glascow, Scotland), 30ml centrifuge tubes (Sterilin) were from Bibby LTD, UK.

2.2 Methods

2.2.1 Cell Cultures

All work involving human cells was performed in a Microflow (Bioquel, Hampshire, UK) Laminal Flow cabinet Class II. All short and long term cell cultures were carried out at a temperature of 37°C in a humidified incubator atmosphere comprising 95% air and 5% CO_2.

Cell cultures were conducted in Roswell Park Memorial Institute medium-1640 (RPMI-1640) (Gibco BRL, Paisley, UK) that was supplemented with 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine and HEPES (free acid) buffer (Fisher Bioreagents, Fisher Scientific, Loughborough,UK) (hereof described as sRPMI-1640 medium) with either 10% v/v heat inactivated foetal bovine serum (FBS) (Gibco BRL, Paisley, UK), or 10% Human AB serum (Nabi Biopharmaceuticals, Florida, USA), depending on the conducted experiment.

All cell samples were counted using a Neubauer haemocytometer (Fisher Scientific, Loughborough, UK). 10μl of cell suspension were mixed thoroughly with 190μl of 0.1% Trypan Blue solution. This technique allowed for a rapid assessment of cell viability, since Trypan Blue is excluded from live cells.

2.2.2 Cell separation and storage

Peripheral blood mononuclear cells (PBMC) were isolated from patients with chronic HCV infection and healthy volunteers by density gradient centrifugation. Peripheral blood, 60-80ml at each venesection session, was obtained using a 21g 'butterfly' needle into heparinised (approx. 0.1ml or 100 Units of sterile, pyrogen- and preservative-free sodium heparin per tube), 'brown-topped' glass vacutainers (BD labware,NJ,USA). This was diluted into an equal volume of Normal Saline (0.9% NaCl). 30 ml of this dilute blood solution were layered carefully, using 60ml syringes, over 17.5 ml of Lymphoprep 1077 (Nycomed Pharma, Oslo, Norway) in a 50 ml tissue culture tube and were centrifuged for 30 minutes at 600g (brake off) at room temperature in a Heraeus Mistral 3000i centrifuge (SANYO Inc, Japan). The interface over the Lymphoprep, containing PBMC, was aspirated with a sterile pipette, washed with sRPMI 1640 and centrifuged at 1000g/10minutes in a 30ml tissue culture tube initially. The supernatant was discarded and the cell pellet was resuspended with vigorous pipetting in a small volume of sRPMI 1640. The tube was filled with media (sRPMI 1640) and centrifuged again at 750g/10minutes. The supernatant was discarded and the cell pellet resuspended in 3-4ml sRPMI 1640 / 10% v/v heat inactivated foetal bovine/calf serum (FBS or FCS) (Gibco BRL, Paisley, UK). 25µl of the PBMC suspension were added to 475µl acetic acid 'counting' solution (5ml Acetic Acid + 245ml distilled water + 100µl Trypan Blue) and counted in a Neubauer haemocytometer.

Whenever PBMC were not used in experiments immediately, they were 'cryo-preserved' according to the following protocol.

A solution of two volumes of Dimethyl-sulfoxide (DMSO) (Sigma, Ayshire, UK) and three volumes sRPMI 1640 was prepared and left for at least 20min at room temperature before use. The solution is described as 'freezing mix'. PBMC, after centrifugation at 1000g/10min, were resuspended in FCS ($5x10^6$ PBMC per 750µl FCS). 'Freezing mix' was added, 250µl for every 750µl PBMC/FCS suspension. Samples were subsequently transferred to 1ml cryovials (Simpor Plastics, Canada) and frozen overnight in a -80°C REVCO (Thermo Fisher Scientific Inc, USA) freezer in an isopropanol container (5100 Cryo 1°C Freezing Container, "Mr. Frosty'', Nalgene Labware Ltd. Hereford,UK). Frozen cryovials were transferred to a 'liquid nitrogen' (vapour phase nitrogen, BOC Industrial, UK) container (lindy) for long-term storage.

When frozen cells were to be used in subsequent experiments, cryovials were transferred from the liquid nitrogen lindy into a polystyrene box containing 'dry ice' (solid CO_2, at ~ -78°C, from BOC Industrial, UK) and were thawed gradually with immersion into a pre-warmed (37°C) water-bath. The thawed content of the cryovials was quickly transferred with sterile pipettes into 30ml centrifuge tubes (Sterilin) containing sRPMI 1640 / 20% FCS medium ('thawing medium'), pre-warmed to 37°C. The tubes were subsequently centrifuged at 750g and the resulting cell-pellets were resuspended in sRPMI 1640 / 10% human AB serum or sRPMI 1640 / 10% FCS ('culture media', depending on the assay the cells were to be used into). Small PBMC aliquots were used for Trypan Blue staining (0.1% Trypan Blue in sRPMI solution) and assessment of the dead-cell content of the thawed PBMC suspension, in a Neubauer haemocytometer. Cells with intact cell membranes do not take up

Trypan Blue (a diazo-dye derived from toluidine). Disrupted cell-membranes of dead cells allow trypan blue infiltration thus staining dead cells blue.

2.2.3 Generation of monocyte-derived dendritic cells (MDDC)

PBMC isolated from 60ml-80ml of whole blood, as described previously, were resuspended in Dulbecco's Phosphate Buffered Saline (D-PBS) without calcium or magnesium (-$CaCl_2$, -$MgCl_2$) (GIBCO, Pasley, UK) supplemented with 2% human AB serum and 50mM ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate (Fisher Scientific, Leicester, UK), a medium that is used at 4^0C, and is hereof described as 'MACS buffer'. $CD14^+$ monocytes were subsequently isolated from total PBMC implementing immuno-magnetic bead separation methodology ('positive selection' protocol) according to the manufacturer's instructions ($CD14^+$ Microbeads and MS MACS immunomagnetic separation columns both from Miltenyi Biotec, Gladbach, Germany, under the influence of a strong magnetic field). Briefly, anti-$CD14^+$ monoclonal antibodies, ligated to microscopic metal beads ($CD14^+$ Microbeads), were mixed, at ratios described by the manufacturer, with the PBMC suspension and passed through MS columns allowing for the specific retention of the $CD14^+$ monocytes in the columns. Subsequently the columns were moved out of the magnetic field and washed with ice-cold MACS buffer. Eluted cells were peleted with centrifugation and resuspended in sRPMI 1640 10%FCS. Viability and purity of the resulting $CD14^+$ monocytes were assessed with trypan blue staining and fluorescence-activated cell sorting (FACS) flow cytometry, respectively. Viability was ≥90% and purity of the isolated $CD14^+$ cells was between 90% and 97%. Isolated $CD14^+$ monocytes

were subsequently cultured in sRPMI 1640 / 10%FBS culture medium supplemented with 100 ng/ml human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and 100 ng/ml human recombinant interleukin-4 (IL-4) (both from R&D), in 6-well tissue culture plates (Falcon, Becton-Dickinson, Mountain View, CA, USA). Each well contained 3ml of $CD14^+$ monocyte cell-suspension, approximately 3 million $CD14^+$ cells. The plates were incubated for 6 days at 37^0C, 5% CO_2 and the morphological differentiation of monocytes to dendritic cells[219] was monitored with an inverse- idol optical microscope. At the end of the 6-day incubation period, non-adherent and loosely adherent cells were removed with a pipette from the plates and the wells were washed twice with ice-cold D-PBS (-$CaCl_2$, -$MgCl_2$) to remove any remaining DC. The non-adherent and loosely adherent cells were subsequently centrifuged in sterile 30ml tissue culture tubes (Sterilin), at 750g, room temperature and the cell pellets were resuspended in sRPMI 1640 10% human AB serum or in sRPMI 1640 10% FCS ('complete' culture media) in order to be used for further applications. At the end of this process MDDC viability was >95%, while purity of the MDDC was 90% - 97%.

2.2.4 Isolation of $CD4^+$ T cells

PBMC not selected during the previously described 'positive selection' immunomagnetic $CD14^+$ cell-isolation protocol, were utilised in subsequent experiments for the isolation of autologous $CD4^+$ T cells. These 'effluent' cells were frozen and stored, as described previously (section 2.2.2), in the vapour phase of liquid nitrogen. When required, frozen vials were thawed, as described previously, and $CD4^+$ T cells were isolated with a 'negative

selection' immunomagnetic separation protocol, implementing a methodology quite similar to the one described in section 2.2.3 for $CD14^+$ cell isolation. Briefly non-$CD4^+$ T cells, i.e., $CD8^+$ T cells, γ/δ T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes, and erythroid cells, were magnetically labeled by using a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, and CD235a (glycophorin A). These cells were subsequently magnetically labeled with Anti-Biotin MicroBeads for depletion. The isolated $CD4^+$ T cells at the end of the separation process were >95% viable and between 90% and 98% pure.

2.2.5 CD40-ligand (L) - expressing human lymphoid cells

J558 lymphoid hybridoma cells which are stably transfected with CD40-ligand(L) (a gift from Dr.Peter Lane) were defrosted from the vapour phase of liquid nitrogen, as previously described and were subsequently cultured upright in $75cm^3$ tissue culture flasks as they are suspension cells. The medium used for each cell culture was 20mL IDDM / 10%FCS supplemented with L-Histidinol at 10umMol, 2mM glutamine (2mM), 100U/ml penicillin and 100μg/ml streptomycin in 37^0C, 5% CO_2, humidified incubator. The CD40L-expressing cell cultures were split 1 in 5 every 3-4 days.

2.2.6 Plasmacytoid dendritic cell (PDC) separation

In order to isolate and study the rare population of plasmacytoid dendritic cells (PDC) (0.03%-0.5% of total PBMC) from peripheral blood, large quantities of whole blood are essential. To that effect blood from patients suffering either from haemochromatosis or essential polycythaemia and were undergoing

regular venesections at the Department of Haematology at University College Hospital, was utilised with their written permission. In particular approximately 500ml of whole blood, removed on a single venesection session, were used for PBMC isolation and subsequently for PDC isolation, with the patients' written permission. Subjects donating blood tested negative for anti-HCV antibodies, HBsAg and anti-HIV antibodies before being included in the study. Total PBMC were subsequently cryo-preserved. PDC were isolated at a later stage from total PBMC, using a 'positive selection' immunomagnetic bead separation protocol according to the manufacturer's instructions. In particular the CD304 (BDCA-4/Neuropilin-1) MicroBead Kit (Miltenyi Biotec, Gladbach, Germany) was used. The kit includes FcR Blocking Reagent and CD304 (BDCA-4/Neuropilin-1) MicroBeads. CD304 (BDCA-4/Neuropilin-1) is exclusively expressed by PDC in human blood, bone marrow, and cord blood. Briefly, PBMC from the aforementioned subjects were suspended in ice-cold (4^0C) MACS buffer and were passed through MACS (LS) columns, under the influence of a strong magnetic field, after the addition of BDCA-4 Microbeads / FcR Blocking Reagent. Positive cells (PDC) retained in the columns were vigorously washed out of the columns with ice-cold MACS buffer, after removing the columns from the influence of the magnetic field. The cell population isolated at the end of the process contained significant numbers of dead cells, as assessed by trypan blue staining, presumably due to the large numbers (~10^8) of PBMC used and the fact that the implemented 'positive selection' protocol usually results in the retention in the columns of most of the dead / apoptotic PBMC. Thus the eluted, at the end of the PDC isolation protocol, cell fraction underwent dead-cell depletion with a similar

immunomagnetic bead separation protocol using a commercially available kit (MACS 'dead-cell removal kit, Miltenyi Biotech). The final effluent contained PDC that were at least 95% viable, and ~90% pure by FACS flow-cytometry as discussed in section 2.2.14 [anti-CD303(BDCA-2)- phycoerythrin(PE) mouse IgG1 antibody, and anti-CD123-fluorescein isothiocyanate (FITC) dual staining, both from Miltenyi Biotech].

2.2.7 Cytokine assays

2.2.7.1 Enzyme-linked immunospot (ELISpot) assay

In order to assess the autologous $CD4^+$ T cell responses to HCV specific antigens, dendritic cells and $CD4^+$ T cells, that were generated / isolated as described previously (sections 2.2.3, 2.2.4), were used in ELISpot assays. The assay is measuring the frequency of cytokine-producing $CD4^+$ T-cells after antigenic stimulation, in the form of cytokine specific 'spots' on the surface of membrane bound $CD4^+$T cells.

Monocyte-derived dendritic cells (MDDC) (10^4/well) were incubated overnight (for 16-20hours) with autologous $CD4^+$ T cells (10^5/well), in 96-well round-bottom tissue culture plates, in triplicates, in the presence of recombinant HCV antigens, core (amino acid 1-115) and NS3 (aa 1007- 1534) (Mikrogen, Munich, Germany), at a concentration of 1 μg/ml each, and also in the presence of tetanus toxoid (TT) (0.5μg/ml) (Connaught International Laboratories, Ontario, Canada) and phytohaemoagglutinin (PHA) (1 μg/ml) (Sigma) as positive control antigens for memory and non-specific $CD4^+$ T-cell stimulation responses respectively. The cultures were incubated at 37°C with 5% CO_2. Lipopolysaccharide (LPS) (Salmonella Abortus Equi, 0.1μg/ml,

Sigma-Aldrich, Gillingham, UK) was added in each well as MDDC maturation factor (successful maturation was confirmed by significant increases in CD80, 83 and 86 expression on DC on FACS flow-cytometry, table 2.2, as described in section 2.2.14). In parallel, 96-well Immobilon-P membrane microtitre plates (Millipore, Bedford, UK) were coated with 100μl of 5μg/ml anti-interferon-γ (IFN-γ) monoclonal antibody [Beckton Dickinson (BD) Biosciences, Oxford, UK]. The plates were incubated for 16 hours at 4 °C, and were subsequently washed and blocked with sRPMI 10% human AB serum for 2 hours. The medium was then discarded from the plates and the pre-incubated MDDC-$CD4^+$ T-cell cultures were transferred to the antibody coated plates and incubated for another 20 hours at 37°C, 5% CO_2.

Following this incubation, the plate was washed with PBS/0.5% Tween, 100μL of 2μg/ml biotinylated anti-IFNγ antibody (BD Biosciences) was added and a 2 hours incubation at room temperature ensued. The plates were then washed again and 100μL of 1:100 dilution of streptavidin horseradish peroxidase (HRP) conjugate (BD Biosciences) was added to each well with a subsequent incubation for 1h at room temperature. Unbound conjugate was removed by further thorough washings and finally 100μl of AEC substrate [3-Amino-9-ethylcarbazole (AEC) is a substrate for peroxidase and is widely-used chromogen for ELISpot, from BD Biosciences] was added to each well. The plate was incubated for 15 minutes, and subsequently the reaction was terminated with extensive distilled water washing. Plates were then dried overnight at room temperature and the spots counted with an AID ELISpot reader system (AID Diagnostika GmbH, Strassberg, Germany). The number of specific spot-forming cells (SFC) was determined as the mean number of

spots in the control wells with medium subtracted from the mean number of spots in the control wells, and expressed per 10^6 $CD4^+$ T cells. An IFN-γ response was judged to be positive if the average of SFC in triplicate wells after antigen stimulation was greater than the average of control wells' SFC plus 2 standard deviations, determined from ELISpot assays performed previously with PBMC from 10 anti-HCV negative healthy controls.

It has to be noted that other maturation stimuli were tried along with Salmonella abortus equi lipopolysacharide that was finally selected. Recombinant human TNF-α (R&D Systems) at 50ng/ml and 100ng/ml was used in several initial experiments. The resulting maturation status of MDDC on FACS (CD80,83,86,HLADR) with 50ng/ml was not comparable to that achieved by the implementation of Salmonella LPS. When 100ng/ml recombinant human TNF-α were used the maturation status of DC was comparable to that of the much cheaper Salmonella LPS at 0.1μg/ml.

2.2.7.2 Enzyme-linked immunosorbent Assay (ELISA)

Measurement of cytokines in the supernatants of cell cultures was performed according to the following protocol.

After 16-24 hours (depending on the experiment) of antigenic stimulation, supernatants of cell cultures were harvested and used to test for various cytokines. Secreted interleukin-12 (IL-12) and interleukin-6 (IL-6) were measured using commercially available ELISA kits according to the manufacturer's instructions: [human IL-12 (detection range 5pg/ml - 500 pg/ml) and human IL-6 (detection range 0.7pg/ml - 300pg/ml) Quantikine

ELISA kits, implementing quantitative sandwich enzyme immunoassay technique, both from R&D Systems Europe, Abingdon, UK].

For IL-6 ELISA a mouse monoclonal antibody specific for IL-6 has been pre-coated onto a 96-well polystyrene microplate. Standards and samples were pipetted into the wells and any IL-6 present was bound by the immobilized antibody. After washing away any unbound substances, a polyclonal antibody specific for IL-6, conjugated to horseradish peroxidase (HRP), was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution containing stabilized chromogen tetramethylbenzidine (TMB), was added to the wells and colour developed in proportion to the amount of IL-6 bound in the initial step. The colour development was stopped and the intensity of the colour was measured on a TECAN ELISA reader, using Magellan™ software (both from TECAN UK Ltd, Reading, UK).

IL-12 (p70) is a 75 kDa glycoprotein heterodimer composed of two genetically unrelated subunits linked by a disulfide bond. The smaller subunit (p35) has homology to IL-6 and G-CSF while the larger subunit (p40) demonstrates similarity to the soluble receptor for IL-6. The secretion of IL-12 (p70) from MDDC cultures was measured, as described, under two different stimulation conditions. When the capacity of MDDC from chronic hepatitis C patients to produce IL-12, and thus skew the immune response towards Th1, was assessed, necessitating maximal stimulation conditions, the stimulus used was lipopolysaccharide (LPS) (Salmonella Abortus Equi, 1µg/ml, Sigma-Aldrich, Gillingham, UK), at 10-fold concentrations than the ones used for induction of MDDC maturation in other cell cultures (0.1µg/ml) and

preconditioning of the MDDC with 2ng/ml IFN-γ was also implemented for better stimulation. When the capacity of isolated circulating plasmacytoid DC to produce IL-12p70 was assessed, PDC were stimulated in co-culture with CD40L-expressing cells and under the influence of various stimuli such as sera from patients with high levels of HBV / HCV viraemia, TLR -9 / -7,8 ligands, et.c.

Secreted interferon-α (IFN-α), produced by directly isolated from circulating PBMC and ex-vivo stimulated plasmacytoid dendritic cells (pDC), was measured by another commercially available ELISA kit according to the manufacturer's instructions (PBL, Biomedical Laboratories, NJ, USA) (detection range 10pg/ml - 500 pg/ml). The kit quantitates human interferon alpha (IFN-α) in media using a sandwich immunoassay, practically identical to the one described previously for IL-12 and IL-6 detection [based on an ELISA with anti-secondary antibody conjugated to horseradish peroxidase (HRP) and tetramethylbenzidine (TMB) as substrate]. The human-IFN-a multi-subtype ELISA kit is specifically formulated to detect 14 out of 15 identified human IFN-a subtypes, namely IFN-aA, IFN-a2, IFN-aD, IFN-aB2, IFN-aC, IFN-aG, IFN-aH, IFN-aI, IFN-aJ1, IFN-aK, IFN-a1, IFN-a4a, IFN-a4b, and IFN-aWA.

2.2.8 Cell proliferation assays

All proliferation assays [for the purposes of the present study mixed lymphocyte reactions (MLR)] were conducted using 10^5 purified $CD4^+$ T cells incubated with two-fold dilutions of MDDC (10,000DC/well, 5,000DC/well and 2,500DC/well), in 96-well U-bottom plates (Nunc) for six days. Quantification

of cell proliferation was performed by measuring incorporation of radio-labelled [methyl-^{3}H] thymidine (Amersham Pharmacia, UK) into proliferating $CD4^{+}$ T-cells. Cells were pulsed with 1μCi/well (10μl of 100μCi/ml per well) [methyl-^{3}H] thymidine in the evening of the fifth day of the culture, for the final 16 hours of incubation. All proliferation assays were performed in triplicate wells. MDDC used for the purposes of MLR in the present study are described as either 'immature' or 'mature' depending on whether they were used in the experiments without of with, respectively, the addition of 0.1μg/ml LPS as maturation stimulus. The maturation status of DC was confirmed by FACS flow-cytometry [increases in mean fluorescence intensity (MFI), and in the % of gated events for DC surface markers CD40, 80, 83, 86 and HLA DR]. After the addition of the maturation stimulus (LPS), dendritic cells up-regulated the expression of: CD40, CD80, CD83, CD86 and HLA DR on their surface (table 2.2).

Cells were harvested, and transferred from the 96-well cell-culture plate on to glass fibre filters (Filtermats) using a TomTec cell harvester (Wallac, Turkum Finland). "Melt-on" scintillant ('MeltiLex', Wallac) was added to the filters, at 90^{o}C on a hot plate, and incorporation of radiolabelled [methyl-^{3}H] thymidine into cells was subsequently quantitated using a 1450 Microbeta liquid scintillant counter (Wallac). Results are expressed as counts per minute (cpm).

It has to be mentioned that although in the literature in general, inactivation of DC with irradiation or mitomycin-C incubation is suggested in MLR in order to eliminate DC proliferation, our observations suggested no difference in proliferation detected whether we used irradiated (at 5,500 rad) or non-

irradiated DC. Taking this observation into consideration we did not irradiate the MDDC we used in MLR assays.

2.2.9 Total RNA extraction from PBMC and MDDC

2.2.9.1 Using the Qiagen RNeasy® Minikit

The RNeasy Minikit (Qiagen, Crawley, UK) was used according to the manufacturer's instructions. Briefly, cells were first lysed and homogenized in the presence of a highly denaturing guanidine thiocyanate–containing buffer, (RLT buffer), supplemented with 0.1% β-mercaptoethanol (β-ME) (to increase the efficiency of RNase elimination from the lysate) after repeated passages (between 5 and 10) through a 25G needle with the help of a 1ml syringe. The RLT buffer immediately inactivates RNases to ensure purification of intact RNA. Ethanol 70% was added to the homogenised lysate to provide appropriate binding conditions, and the sample was then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants were efficiently washed away during centrifugation in a bench-top microcentrifuge (with rotor for 2 ml tubes), at 10,000rpm. Subsequent steps of elution/centrifugation, as suggested by the manufacturer, followed using RPE (diluted with 96%-100% ethanol), RW1 (diluted with 96%-100% ethanol and containing a small amount of guanidine thiocyanate) and RFW buffers. At the end of the procedure high-quality RNA was eluted in 30–100 µl RNase-free water. The final elute was frozen in a 1ml RNase-free Eppendorf tube at -80^{0}C. Care was taken throughout the RNA isolation procedures, and the RNA quantitation assays that will be described in this section, that RNase contamination was avoided with the use of certified RNase-free consumables

(pipette-tips, syringes, centrifugation tubes) and the frequent change of gloves.

2.2.9.2 Using a TRIzol® chloroform-isopropanol precipitation protocol

DC lysates, cryopreserved at -80^{0}C in QIAGEN RLT / 0.1% β-mercaptoethanol buffer in our lab, were sent on dry ice to a collaborating group in Canada and total RNA was extracted according to a TRIzol® (guanidinium thiocyanate-phenol-chloroform) (Invitrogen, Carlsbad, CA, USA) protocol for working with lysates of less than 10^6 cells, aiming to use the extracted RNA in highly sensitive nested PCR-nuclear acid hybridisation assay. TRIzol works by maintaining RNA integrity during tissue homogenization, while at the same time disrupting and breaking down cells and cell components. It reacts strongly with chloroform and is thus used in this form of RNA extraction. Cell lysate (0.5 ml) was added to 0.75 ml Trizol LS and chloroform-isopropanol precipitation followed overnight at -20^{o}C with rehydration for at least 2 hours at -80 C. Frozen samples were subsequently thawed and the extracted RNA was used in PCR assays. It has to be noted that some differences in the amount of RNA recovered from the same number of cells may be due to a mismatch between the two different products, i.e., Qiagen RLT buffer with TRIzol.

2.2.10 Total RNA extraction from plasma

Total RNA was extracted from 1ml of reconstituted lyophilised HCV positive plasma from the WHO Second International Standard for hepatitis C virus RNA, using the QIAamp Ultrasens® Virus Kit (Qiagen, Crawley, UK),

according to the manufacturer's instructions in a centrifugation-elution method similar to the one described previously for RNA extraction from PBMC/MDDC. The sample was first lysed under the highly denaturing conditions provided by Buffer AC. Carrier RNA, added to buffer AC, improved the binding of viral RNA to the kit's mini-spin column membrane, limited possible degradation of the viral RNA due to any residual RNase activity and is suitable for a wide range of RNA viruses. Buffers AR+ proteinase κ, AB, AW1 and AW2 were also used. Subsequent steps of elution/centrifugation of the total RNA on the mini-spin columns resulted in the elution of RNA in 30μl of buffer AVE that can be stored at -20^{o}C to -70^{o}C for up to a year.

With a methodology quite similar to the one described for the QIAamp Ultrasens® Virus Kit, total RNA was also extracted from smaller volumes of patients' plasma (140μl) with the QIAamp Viral RNA Minikit (Qiagen, Crawley, UK) in order to be used as a positive control for real-time RT-PCR implemented for the detection of HCV-specific genomic sequences in MDDC lysates.

2.2.11 Real-time RT-PCR for the detection of HCV RNA in PBMC and MDDC

2.2.11.1 Real-time RT-PCR assay

We first attempted to detect and quantitate HCV RNA, both in PBMC and MDDC, with a real-time, quantitative, reverse-transcription polymerase chain reaction (RT-PCR), methodologically similar to previously described assays[375], with a lower limit of detection of 20 IU/ml.

Briefly, purified HCV RNA was extracted with the RNeasy® mini-kit (Qiagen) as described previously and all samples were eluted in 30μl RNase-free water. Subequently HCV RNA was reverse-transcribed and PCR amplified using QuantiTect Probe™ RT-PCR Kit (Qiagen, Crawley, UK), with dual-labelled probes. The RT-PCR Master Mix and RT mix allows both reverse transcription and PCR to take place in a single-tube one-step process, thus avoiding carry-over contamination, and allowing for highly efficient amplification of the total cDNA from reverse-transcribed HCV RNA 5'UTR template. HCV RNA quantitation was performed in an ABI Prism® 7700 sequence detection system (Applied Biosystems, Warrington, UK). This assay amplifies an 89-base target sequence defined by forward primer (nucleotides 60-76) 5'–GTCTAGCCATGGCGTTA-3' and reverse primer (nucleotides 149-133) 5'-GTACTCACCGGTTCCGC-3'; and the TaqMan probe (nucleotides 107-128) 5'-FAM-CCCTCCCGGGAGAGCCATAGTG-TAMRA-3'. The fluorescent reporter dye 6-carboxyfluorescein (FAM) is attached covalently to the 5' end of the probe. The quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) is attached to the 3' end of the probe. During PCR, the probe hybridises to the template and is digested as *Taq* DNA polymerase extends the PCR primer. Digestion of the probe releases the reporter from the activity of the quencher, and successive PCR cycles result in exponential amplification of the PCR product and fluorescence intensity. Data is collected by continuous fluorescence monitoring and processed with sequence detection system software according to the manufacturer's instructions (Applied Biosystems). Sample quantitation values are determined from an internal quantitation standard curve, run in parallel on the same 96-well plate,

which has previously been validated against the WHO International Standard for HCV [96/798] (National Institute for Biological Standards and Control, Potters Bar, UK) All samples were tested by RT-PCR in duplicate wells with negative controls.

The 25μl / well RT-PCR reaction mixture comprised QuantiTect Probe™ RT-PCR Master Mix (HotStarTaq DNA polymerase, RT-PCR buffer {Tris-Cl, KCl, $[NH_4]_2SO_4$, 8mM $MgCl_2$, pH 8.7 (20°C)}, dNTP mix, ROX passive reference dye), QuantiTect RT Mix (Omniscript and Sensiscript reverse transcriptases), forward and reverse primers, TaqMan probe, RNase-free water, and HCV RNA template from either standardized plasmid HCV 5'UTR RNA transcript (standard curve) or HCV RNA extracted from MDDC or PBMC as described previously. These components were mixed in the concentrations shown in table 2.3.

Five microlitres of extracted plasma HCV RNA or diluted standard HCV 5'UTR transcript were added to an ABI PRISM 96-well reaction plate containing 20μl reaction mix, in duplicate wells, and sealed with ABI PRISM optical adhesive cover. The samples were amplified and detected with ABI 7700 SDS using the following thermal cycling parameters: 50°C for 30 minutes (reverse-transcription), 95°C for 15 minutes (Taq DNA polymerase and reverse transcriptase inactivation). PCR amplification consisted of 45 cycles of 94°C for 15 seconds (denaturation) and 60°C for 60 seconds (annealing/extension). The ABI 7700 SDS measured fluorescent emissions, which increase in direct proportion to the increase of amplified product, continuously during PCR amplification.

2.2.11.2 Standardisation / normalisation of the real-time RT-PCR assay

a. Sensitivity of real-time RT-PCR and calibration against International Standard

The analytical sensitivity of the real-time RT-PCR we implemented was determined using a dilutional series of HCV 5'UTR RNA transcripts - prepared previously by Dr.KH Tang in our lab from cloned plasmids containing cDNA of genotype 1 HCV 5'UTR that were linearized by restriction enzyme *Eco* R1 digestion (Amersham-Pharmacia, Buckinghamshire, England) - from 10 to 10^7 molecules and tested five times, each in duplicate (table 2.4). The detection limit was consistently found at 100 copies per reaction mixture with 100% detection ability (10 out of 10 replicates). The standard curve constructed using the mean threshold cycle values (C_T) against the starting quantity of HCV 5'UTR RNA template demonstrated excellent correlation coefficient (r^2) of 0.9945.

The detection limit of our assay in International Units (IU) was determined by quantitating the number of HCV RNA copies-molecules in a known volume of WHO International Standard for HCV RNA by real-time RT-PCR, against the prepared dilutional standard of transcripts from cloned plasmids. The WHO Second International Standard for HCV RNA, 96/798, (NIBSC, Potters Bar, UK) consists of lyophilized HCV positive (genotype 1) human plasma diluted in HCV-negative human cryo-supernatant and assigned a titre of $5x10^4$ International Units (IU) per vial. This standard was reconstituted in 0.5ml of distilled water, and 250μl of the reconstituted solution was removed and added to 750μl HCV-negative plasma to make 1ml total. HCV RNA was

extracted from this sample using the QIAGEN Ultrasens® virus kit as previously described, and eluted in 60μl of RNase-free buffered water.

Four aliquots of 5μl each from this WHO HCV RNA standard were subsequently used as unknown template for real-time RT-PCR assay, and C_T values determined on a standard curve constructed from a 10-fold dilution series of HCV 5'UTR RNA plasmid transcripts, as previously mentioned.

The four International Standard replicates of 5μl each, showed a mean of 124490 copies per reaction (5μl template/reaction), equivalent to 1493880 copies per 60μl RNA template, or 59.8 copies/IU HCV RNA (bearing in mind that the eluted 60μl RNA template resulted from the extraction of total RNA from half of the reconstituted WHO standard, containing effectively 25,000IU HCV RNA). The detection limit of our real-time RT-PCR assay hence equates to 20 IU/ml [124490x12=1493880; /25000=59.8copies/IU. Lower limit of detection is 100 copies/reaction = 100x12 (out of 60μl of eluted RNA, 5μl were used as template/reaction) =1200copies/60μl or 1200copies/ml plasma or DC lysate. Therefore 1200/59.8~20IU/ml lower limit of detection]. (table 2.5).

b. Normalisation against cellular GAPDH gene expression

In order to demonstrate the presence of cellular genome in real-time RT-PCR assays that failed to detect any HCV-specific genomic sequences in MDDC lysates, cDNA template produced by reverse transcription of three out of the ten tested (n=3), randomly selected, patients' total RNA samples extracted from MDDC, was tested for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression by real-time RT-PCR. This is a widely used control housekeeping gene present in all cells.

Reverse transcription was carried out by mixing 300ng MDDC RNA, measured by spectrophotometer, with 1.5μl 100mM random hexamers (Promega, Southampton, UK), made up to 12.75μl with RNase-free water. This mixture was then added to 12.25μl reverse-transcriptase mix consisting of 5μl 2.5mM dNTPs, 1μl reverse-transcriptase (Promega, Southampton, UK), 5μl 5xRTbuffer, 0.25μl RNase-inhibitor and 1μl water, and incubated at 37°C for 1 hour to produce 25μl cDNA. The RT-PCR reaction mix amplifies GAPDH mRNA by single probe RT-PCR, using Pre-Developed Assay Reagents (PDAR) and an ABI Prism® 7700 sequence detection system (both from Applied Biosystems, Warrington, UK). The components of the reaction mix are added in the proportions described in table 2.6.

2.2.12 HCV RNA quantitation in patients' plasma and HCV genotype determination

Quantitation of HCV RNA in patients' sera, both prior to the initiation of antiviral treatment and at treatment week 12, was performed at the Department of Medical Virology, University College London Hospitals, implementing a similar real-time RT-PCR assay (Amplicor HCV v2.0; Roche Molecular Systems, Pleasanton, CA) as the one described previously for the quantitation of HCV RNA in cell lysates

HCV genotyping was determined by a restriction fragment length polymorphism (RFLP) method at the Department of Medical Microbiology, University of Edinburgh, UK[374].

For the purposes of this thesis the HCV RNA quantitation and genotype data that appear in table 2.1, come from this source.

2.2.13 Nested RT-PCR-nucleic acid hybridization (RT-PCR-NAH) for the detection of HCV-specific sequences in PBMC and MDDC

Nested RT-PCR for the detection of HCV specific sequences in DC / PBMC was performed by a collaborating group in Canada (Dr.T.Michalak's group) as previously described by them [342]. The same group performed the initial total RNA extraction from DC / PBMC lysates, sent to them frozen in QIAGEN RLT buffer from our lab, according to the modified TRIzol LS® protocol from cell-lysates described previously. Two different methods of nested PCR were implemented:

a) nested PCR with 5'UTR primers. The product was detected by EB-agarose gel electrophoresis (n/gel) and detection was always finally confirmed by Southern blot hybridization (sensitivity 10-100 viral genomes/mL).

b) nested PCR followed by Southern blot hybridization (n/s) for detection and validation of PCR products (sensitivity $\leq$10 viral genomes/mL).

PCR assays were performed using 2 μg RNA for cDNA synthesis; all used for direct PCR. 20 μL of direct PCR product was used as template for nested PCR. For negative strand detection, 4 μg was used as a standard, however, when there was not enough, all residual RNA was used after the positive strand was detected.

2.2.14 Surface marker expression on MDDC and PBMC

Within individual experiments, the number of cells stained in all samples was the same to ensure a constant cell / antibody ratio. The usual number of cells stained was $5x10^4$ – 10^5 cells per well, depending on the experiment conducted. These cells were harvested and resuspended in 50μl of blocking buffer (PBS 1% foetal calf serum) per well at 4°C. 50μl of primary monoclonal antibody solution directly conjugated to fluorochromes, (5μl of antibody in 45μl PBS 1%FCS per well) were added to the cell suspension in a 96-well round-bottom plate (Nunc), and then incubated for 30 min at 4°C. At the end of the incubation period, 100μl of wash buffer (PBS 1%FCS) were added to each well and the plates were subsequently centrifuged at 1400 rpm, 4^0C, for 7 min. Supernatants were flicked off and pelleted, stained cells were resuspended in another 100μl of wash buffer (PBS 1%FCS) and centrifuged again. Finally, cells were fixed by adding 40μl of PBS containing 3.7% (v/v) formaldehyde (BDH, Poole, UK) per well. Samples were stored at 4°C in the dark and analysed within 24 hours. Primary mouse, anti-human, monoclonal antibodies conjugated with fluorochromes used were : anti-CD1α fluorescein isothiocyanate (FITC), anti-CD14 phycoerythrin (PE), anti-CD40 FITC, anti-CD80 FITC, anti-CD83 FITC, anti-CD86 PE, anti-HLA DR FITC along with the corresponding isotype controls (mouse IgG_1, IgG_{2a}, IgM) (all from BD, Biosciences Pharmingen), and anti-BDCA 2 PE, anti-CD123 FITC (with the corresponding isotype controls (mouse IgG_1 and IgG_{2a}) all from Miltenyi Biotech, Gladbach, Germany).

Stained cells were then acquired on a FACS Calibur (Becton-Dickinson) flow-cytometer, using CellQuest software version 3.3 (Becton-Dickinson). For each

sample at least 5000 events were acquired. Analysis of the data was carried out using WinMDI software (Joseph Trotter, Scripps Research Institute, La Jolla, CA). The amount of fluorescence emitted by the cells in the appropriate channels was determined by the median fluorescence intensity (MFI). Where appropriate, a marker was set such that <2% of negative control cells gave a fluorescence signal beyond this level. The percentage fluorescence (% of gated events staining positive for the tested fluorochrome) refers to the percentage of cells with fluorescence above this marker.

2.2.15 Intracellular and plasma HCV Core antigen quantitation

In order to quantify the presence of HCV core protein in DC and PBMC, aliquots of cells were lysed according to the following protocol. Pelleted cells were resuspended in 1ml of ice-cold lysis buffer consisting of 50mM Tris(hydroxymethyl)aminomethane TRISMA^{TM} Base (Sigma, Ltd. Poole, Dorset, UK), 150mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 1% NP40 (Nonidet P40) in D-PBS (PBS without $CaCl_2$ and $MgCl_2$) at 4^{o}C. The lysis of the cells was additionally helped with repeated passages through a 25G needle, adding the effect of mechanical membrane disruption to that of chemical lysis. Subsequently the lysate was incubated at 4^{0}C for 30min and then it was centrifuged, in a bench-top micro-centrifuge (with rotor for 2 ml tubes), at 10,000rpm for 5min. The supernatant was removed with a pipette and immediately frozen at -80^{0}C.

The quantitation of HCV core protein in cell lysates (from aliquots of DC and PBMC) and from 500μl of plama from the corresponding patients and timepoints, was performed by a collaborating group in Germany (Drs

C.Sarrazin's and S.Zeuzem's group, Medizinische Klinik und Poliklinik, Innere Medizin II, Universitatskliniken des Saarlandes, Homburg/Saar, Germany), with the commercially available Ortho trak C assay (Ortho Clinical Diagnostics, Raritan, NJ) (range of detection 1.5 to 300 pg/ml), as previously described [376-378].

2.2.16 Antiviral treatment

Twenty two of the 35 studied patients received standard antiviral treatment. Genotype 3 patients were treated for 24 weeks and genotype 1 patients for 48 weeks[2,379-381]. Treatment consisted of Pegylated Interferon-α 2b at a dose of 1.5μg/kgr of body weight and Ribavirin at a dose of 1,000mg (body weight<70kgr) or 1,200mg (body weight>70kgr) (Peg-Intron® and Rebetol® respectively, both from Schering-Plough, Welwyn Garden City, UK). Dosage adjustments were undertaken according to clinical and laboratory parameters assessed during follow-up visits to our out-patients' clinic. Pegylated Interferon-α 2b dosage was reduced when neutropenia (<500 neutrophils/ml) or very low platelet counts (<5X10^4/ml) were seen, initially to 1.35μg/kgr and if the blood count abnormalities persisted to 1.0μg/kgr. Ribavirin dosage had to be adjusted when Hb was <10g/dl or even discontinued when Hb was <8g/dl. Neither neutrophil growth factors (granulocyte colony stimulating factor, G-CSF) nor erythropoietin were used to correct blood count abnormalities.

For the purposes of the study antiviral treatment effectiveness was considered the achievement of undetectable HCV RNA levels, using real-time TaqMan® RT-PCR (as described previously), by treatment week 12 (TW12). This particular time-point has been shown to be critical for the establishment of a

sustained response after the end of the antiviral treatment (SVR)[382-386]. 'Good responders' to antiviral treatment hereof will be described patients with undetectable levels of HCV RNA by TW12, while 'poor responders' are patients with still detectable viraemia at TW12. HCV RNA quantitation, at baseline of antiviral treatment (BL) and at TW12 was performed, as described previously, at the Department of Virology, University College London Hospitals NHS Trust.

2.2.17 *Liver biopsy*

For the purposes of the study percutaneous liver biopsy was performed to all included patients, not more than 6 months before the initiation of antiviral treatment, in order to quantify hepatic inflammation and fibrosis. Histology specimens were acquired using the Menghini percutaneous aspiration technique[387], with a 1.6mm bore liver biopsy needle (Hepafix®,Braun Melsungen AG) under aseptic conditions. The acquired specimens were reviewed by experienced histopathologists at the Department of Histopathology, University College London Medical School and were scored according to the modified Ishak scale [388].

2.2.18 Statistical analyses

Non-parametric tests, in particular Mann-Whitney (for comparisons between two different groups) and Wilcoxon signed ranks (for comparisons before and after treatment) tests, were used as appropriate. In the 'cross-over' experiments of our study, a 2-way mixed model-based ANOVA was used, with 'treatment' ('treated' vs 'untreated') and 'cell type' (DC or $CD4^+$ T-cells) as the

within-group factors. P values equal to or less than 0.05 were considered statistically significant. P values above 0.05 but less than 0.1 were considered indicative of a trend. Results were analysed using SPSS for Windows v.13.0 (SPSS Inc, Chicago, IL).

	N	=	35
Gender (males / females)	23	/	12
Age (years)	45.5	±	10.2 (range: 24-69)
Race (Caucasian / non-Caucasian*)	34	/	1
Genotype (1/3)	16	/	20
Pre-treatment plasma HCV RNA (IU/mL)	$1.39x10^6$	±	1.14×10^6
Pre-treatment ALT levels (IU/L)	83	±	52.7 (range:30-280)
Inflammation (grade)**	3.4	±	1.4
Fibrosis (stage)**	2.03	±	1.07
	Stage 0-3:n=18,Stage 4-6: n=17		

* Asian, ** Modified Ishak scale

Table 2.1 Patients' characteristics. Scale data are presented as mean ± standard deviation.

	iDC		***mDC***	
	MFI	**%gated events**	**MFI**	***%gated events***
CD40 FITC	17.65	86.04	35.77	*95.49*
CD80 FITC	16.56	51.08	57.32	*96.21*
CD83 FITC	14.85	17	28.36	*69.95*
CD86 PE	86.81	79.68	546.93	*99.18*
HLADR FITC	*20.45*	*59.92*	*27.11*	*79.46*

iDC: immature MDDC, **mDC**: MDDC matured with 0.1µg/ml LPS, **MFI**: mean fluorescence intensity, **%gated events**: percentage of the gated 'positive' events for the tested fluorochrome/surface marker

Table 2.2 Maturation of MDDC with 0.1µg/ml LPS. Representative FACS data from a single patient (GS).

REAGENT	Neat concentration	Final concentration	25μl
QuantiTect Probe RT-PCR Master Mix	2x	1x	12.50
QuantiTect Probe RT Mix	5x	0.5ul/reaction	0.25
HCV 5'UTR forward primer	10μM	400nM	0.50
HCV 5'UTR reverse primer	10μM	400nM	0.50
HCV 5'UTR TaqMan probe	10μM	200nM	0.50
RNase-free water			5.75
HCV RNA template	-	-	5.0

Table 2.3 Real-time TaqMan RT-PCR reaction mixture

Run	Threshold cycle (C_T)						
	10^1	**10^2**	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**
1	45	35.82	29.53	26.02	22.36	19.20	15.90
2	45	35.25	30.05	26.18	22.40	18.91	15.94
3	45	35.02	30.08	26.84	22.81	19.95	16.20
4	45	33.96	29.83	25.70	22.26	19.10	15.98
5	45	33.62	28.84	27.44	23.35	19.57	16.30
Mean	45	**34.73**	**29.67**	**26.44**	**22.64**	**19.35**	**16.06**
SD		*0.92*	*0.51*	*0.70*	*0.45*	*0.41*	*0.18*
CV		*2.64*	*1.72*	*2.64*	*1.99*	*2.14*	*1.09*

The mean *coefficient of variation (CV)* of the 10 replicates is 2.04

Table 2.4 Determination of the analytical sensitivity of real-time RT-PCR using a dilutional series of the HCV 5'UTR RNA transcripts from 10 to 10^7 molecules and tested five times, each in duplicate

Standards	C_T	Copies	Log_{10}
1.00E+08	14.2	100000000	8
1.00E+08	14.21	100000000	8
1.00E+07	17.12	10000000	7
1.00E+07	17.25	10000000	7
1.00E+06	20.55	1000000	6
1.00E+06	20.76	1000000	6
1.00E+05	24.17	100000	5
1.00E+05	22.72	100000	5
1.00E+04	31.34	10000	4
1.00E+04	30.72	10000	4
1.00E+03	33.35	1000	3
1.00E+03	35.6	1000	3
1.00E+02	35.9	100	2
1.00E+02	38.55	100	2
1.00E+01	45	0	-
1.00E+01	45	0	-
No-template control	45	0	-
No-template control	45	0	-
96/798 (1)	**25.27**	**111583**	**5.0476**
96/798 (2)	**24.99**	**130188**	**5.1146**
96/798 (3)	**25.15**	**119598**	**5.0777**
96/798 (4)	**24.19**	**136590**	**5.1354**

Table 2.5 Determination of the detection limit of the real-time RT-PCR assay International Standard replicates [**(1) to (4)**]: mean of 124490 copies per reaction, i.e per 5µl template→ 124490x12=1493880copies/60µltemplate.

/225000IU=59.8copies/IU. Lower limit of detection is 100 copies/reaction (5µl RNA template) →100x12=1200copies/60µl or 1200copies/ml plasma. Therefore 1200/59.8~20IU/ml lower limit of detection

Component	**Volume (μl)**
20x GAPDH probe	0.75
PBMC / MDDC cDNA	3.00
RNase-free water	3.75
2x TaqMan Universal Master Mix	7.50
Total	**15**

Table 2.6 Reaction mixture used for real-time RT-PCR for the detection of 'house-keeping' GAPDH genomic sequences in MDDC

CHAPTER 3

DENDRITIC CELL FUNCTION IN THE SETTING OF CHRONIC HCV INFECTION

3.1 Introduction

As discussed in Chapter 1, literature on the functional status of DC in chronic HCV is still quite controversial. While some authors report defective DC function in chronic hepatitis C (CHC), others observe comparable functional capacity to DC generated from healthy, non-infected individuals.

Defects in DC function, under the influence of HCV, may lead to sub-optimal virus-specific immune responses and eventually to viral persistence and development of chronic infection. Disruption of many aspects of DC physiology could impinge on the outcome of this interaction between DC and T cells. Interference with DC maturation process[344,351,389], including secretion of relevant cytokines[344,356,390], may prevent the generation of a potent, mature APC. Other potential, HCV-related, mechanisms mediating interference with DC function include active viral replication in DC[335,342,391-393] or even the presence inside DC of virus-specific genomic sequences and / or structural (e.g HCV core protein) and non-structural (e.g HCV NS3 protein) HCV proteins[347,369]. Protein products of HCV have been suggested to interfere with DC function through induction of Interleukin (IL)-10 and tumour necrosis factor (TNF)-α[366,394] secretion. IL-10 may skew DC function towards tolerance leading to T-cell anergy[365,366]. Down-regulation of the CD28 co-stimulatory pathway[366] or NF-κB inhibition[394] by HCV have also been implicated by some authors in DC functional defects.

One of the most important functions of DC, practically forming the basis of adaptive immune responses, is their ability to stimulate naïve T cell proliferation. Investigating that critical aspect of DC function we tested the

allostimulatory capacity of monocyte-derived DC (MDDC) from patients chronically infected by HCV (n=30), with Mixed Lymphocyte Reaction (MLR) assays, and compared it to the corresponding function of MDDC generated from a group of healthy, non-infected, individuals (n=12).

We subsequently assessed viral and host-related factors that may potentially interfere with efficient DC function. In particular we tested: a) the presence of HCV genomic sequences and replicative forms of the viral genome (negative-strand HCV RNA) inside dendritic cells, b) HCV core protein concentration in DC lysates and in patients' plasma, c) HCV viraemia levels, d) the degree of liver damage, assessed by liver biopsy (modified Ishak scoring system) and e) alcohol intake. Our intention was to explore the relation between those parameters and various outputs of DC activity namely a) allostimulatory capacity, b) autologous $CD4^+$ T-cell stimulation, c) IL-12 secretion and d) expression of activation / maturation and co-stimulatory markers (CD40, CD80, CD83, CD86, HLA DR) on MDDC surface.

Taking a step further the findings of previous studies that showed improvement of host adaptive immune responses after treatment-induced viraemia clearance [137], we proceeded to test the effect of antiviral treatment on DC functions, in particular their ability to stimulate autologous $CD4^+$ T-helper cells to produce IFN-γ, by monitoring a group of 22 (n=22) of our patients prospectively, prior to and twelve weeks after the initiation of standard antiviral treatment with pegylated-Interferon α2b and Ribavirin. Our working hypothesis was that on-treatment viraemia clearance improves DC functions and as a result of that restores HCV-specific T-cell response.

We subsequently studied the differential contribution of DC and $CD4^+$ T-cells in the establishment of a defective virus-specific immune response that favours HCV persistence. In most of the studies so far, including our own, experiments testing the role of DC in CHC are performed with both DC and T-cells originating from infected patients. Under these circumstances it is difficult to dissect whether it is mainly DC functional failure or primary T-cell dysfunction, resulting from chronic HCV infection that affects the outcome of the assays testing the interaction of the two cell populations and effectively the integrity of the virus-specific adaptive response. As patient groups with acute HCV infection or, ideally, groups prospectively followed-up prior to and after the transmission of HCV infection, are quite rare, in order to define the influence of the infection on 'naïve', non-infected DC and effector T-cells individually, we used PBMC generated after early, treatment-induced [at week 12 of antiviral treatment, (TW12)] viraemia resolution, considering them to be 'treated', and effectively 'cleared', from the virus and its influence. To that effect and in order to address the issue of DC / T-cell differential contribution to the establishment of CHC we implemented a novel methodology we called 'cross-over' experiments.The intention was to explore the interaction of 'treated' and 'un-treated' MDDC both with 'treated' and 'un-treated' autologous $CD4^+$ T-cells, in all possible combinations, in IFN-γ Elispot assays, in order to dissect the role of DC and $CD4^+$ T-cells in CHC.

As myeloid DC (MDC) are ubiquitously expressed on self-environment interfaces such as the skin and mucosal surfaces and plasmacytoid DC (PDC) are mainly found circulating in blood or in secondary lymphoid organs, it would reasonable to assume that MDC represent the 'first line of defence'

against invading pathogens, with PDC being involved in the shaping of immune responses at a later stage. However, for viruses, such as HCV, that are transmissible via the blood route, interaction with PDC may be more relevant at the earliest stages of infection.

In vitro observations that PDC [originally described as 'natural interferon producing cells' (NIPC)] can secrete very large quantities of type I IFN's (cytokines essential for initial antiviral control and the subsequent shaping of a Th1 adaptive immune response[395]), suggest that PDC may be important in the control of HCV infection. Defective PDC function may be potentially critical for the establishment of chronic hepatitis C (CHC).

In the literature so far there is a controversy as to whether the function and numbers of PDC are affected in the setting of chronic HCV infection, contributing thus to the observed virus-specific immune defect. Some studies support defective function and reduced numbers of circulating host PDC in chronic hepatitis C (CHC) [345,351,396-401] while other authors observe comparable levels of PDC numbers and function in the setting of CHC to those of non-infected individuals [348,349].

As an extension of our study of MDDC we also made a first attempt to test the relevance of PDC in chronic hepatitis C, in particular in terms of cytokine production under the influence of HCV-infected plasma (plasma from a single, chronically infected patient with high viraemia titre), as compared to the corresponding production stimulated by other known PDC stimuli, i.e TLR 7/8 and TLR9 ligands.

In summary the aim of this chapter is to determine whether HCV influences MDDC ability to stimulate T cells and to define potential parameters, either

virus- or host-related, that could account for the altered MDDC function. The dissection of the differential contribution of MDDC and $CD4^+$ T-cells in the establishment of chronic infection along with an initial approach in the understanding of the role of PDC in the setting of chronic HCV infection, are also attempted.

3.2 Results

3.2.1 MDDC allostimulatoty capacity

The capacity of monocyte-derived DC (MDDC), generated from chronic hepatitis C (CHC) patients, to stimulate proliferation of naïve allogeneic $CD4^+$ T-cells was assessed with a Mixed Lymphocyte Reaction as described in section 2.2.8. In this assay MDDC generated from 30 CHC (n=30) (CHC-DC) patients and 12 healthy non-infected individuals (n=12) (N-DC) were cultured with $CD4^+$ T-cells from the same healthy non-infected subject. Both CHC-DC and N-DC were tested as immature DC - without the addition of a maturation stimulus - and as mature DC - following stimulation with LPS (0.1μg/ml). The proliferation rate of naïve, allogeneic $CD4^+$ T-cells was assessed by the incorporation of [methyl-^{3}H] thymidine, measured using a beta radiation detector and was expressed as Counts Per Minute (CPM).

The mean proliferation rate of 10^4 $CD4^+$ T-cells incubated with all two-fold dilutions of CHC-DC (as described in section 2.2.8) was compared, both for mature and immature DC, to the corresponding proliferation triggered by N-DC.

Immature DC from healthy controls (N-DC) showed a trend (not reaching statistical significance though) for higher allostimulatory capacity than CHC-

DC (P=0.063).The difference between the two groups was greater, and statistically significant, when testing mature (after stimulation with LPS) DC from the same subjects (P=0.015) (Mann-Whitney U test) (fig. 3.1).

We effectively observed a reduced allostimulatory capacity of CHC-DC as compared to the corresponding function of N-DC, when both the ratio of stimulator - effector cells and the 'microenvironment' (i.e maturation stimuli, LPS) were kept constant.

3.2.2 Assessment of factors potentially interfering with DC function in the setting of chronic HCV infection

3.2.2.1 Presence of HCV-specific genomic sequences in MDDC from chronically infected patients (CHC-DC)

As mentioned previously, data on the presence of HCV-specific sequences in DC and the potential effect of HCV genome and active viral replication in DC on the functional integrity of the latter, has been quite controversial, with some authors not being able to detect any HCV RNA in DC while others report both presence of viral genome and active HCV replication in DC, resulting in defective DC function.

In our study we investigated the relation between the presence of HCV-specific genomic sequences within DC and DC functional and phenotypic parameters.

Total RNA was extracted with the QIAGEN RNeasy total RNA extraction kit, as described in section 2.2.9.1, from different numbers of MDDC ($2.5x10^5$ to 10^6) generated from 10 (n=10) of the 35 CHC patients that were included in our study. Extracted total RNA was subsequently used as template in an one-

step, ‘real-time’, reverse-transcription, TaqMan® polymerase chain reaction (RT-PCR) with a lower limit of detection of 20IU/ml, as described in section 2.2.11 [briefly: lower limit of detection was 100copies per reaction when 5μl (from 60μl eluted RNA) of template RNA were used; x12=1200copies/60μl i.e. 1200copies/ml of MDDC lysate initially used for total RNA extraction ($5x10^5$ MDDC) or $2.4x10^3$copies per 10^6 MDDC. Standardisation of HCV 5’UTR RNA plasmid transcripts we used for preparing the standard curve of our assay against the WHO Standard showed 59.8copies/IU, 1200/59.8~20IU/ml. In the same real-time RT-PCR assays total RNA extracted from 10^6 PBMC (as in 2.2.9.1) as well as total RNA extracted from 140ml of plasma (as in 2.2.10), were also tested as positive controls, while at the same time for three, randomly selected, of the tested patients (n=3) real-time RT-PCR mRNA expression for the house-keeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (as in 2.2.11) was performed in order to prove the presence of cellular (MDDC) genome.

The sensitivity of our in-house real-time Taq-Man RT-PCR assay was $2.4x10^3$ viral copies per 10^6MDDC [1200 viral copies per ml of MDDC lysate (per $5x10^5$MDDC)] or 40IU per 10^6MDDC (20IU per ml of DC lysate i.e per $5x10^5$ MDDC).

None of the ten tested patients presented any detectable HCV RNA in MDDC lysates while all tested PBMC lysates and plasma samples had detectable levels of HCV genomic sequences (table 3.1).

MDDC lysates of all three tested patients had detectable levels of GAPDH, and thus host, genome.

Previous studies[342], implementing more sensitive RT-PCR-nucleic acid hybridisation (NAH) methodology, showed presence of small quantities of HCV genome in DC from chronic HCV patients. Taking those observations into consideration we asked for the input of a collaborating group in Canada with considerable experience in the detection of minute quantities of HCV genomic sequences in DC (Dr T.Michalak's laboratory in Molecular Virology and Hepatology Research, Division of Basic Medical Science, Faculty of Medicine, Memorial University, St. John's, Newfoundland, Canada). MDDC lysates in RLT+β-mercaptoethanol(ME) lysis buffer (used with the QIAGEN RNeasy total RNA extraction kit), generated from 30 (n=30) out of the 35 chronically infected patients of our study group, were sent frozen on 'dry-ice' to Canada. Subsequently a modified TRIzol chloroform-isopropanol extraction protocol was undertaken by the Canadian group as described in 2.2.9.2 and the extracted total RNA was tested for the presence both of positive-strand HCV RNA and the replicative intermediate of the virus negative-strand HCV RNA. Direct and nested RT-PCR were performed with subsequent agarose-gel electrophoresis and Southern blot hybridisation with HCV 5'-untranslated region- and HCV envelope(E)2 region-specific probes for the detection of PCR products (as in[342]). Detection levels of the assay were as low as $\leq$10 viral genomes/mL of MDDC lysate (i.e $\leq$10 viral copies per $5x10^5$MDDC).

Positive strand HCV RNA was detected in MDDC lysates from 13 (n=13) and was undetectable in the remaining 17 tested patients. Presence of the replicative intermediate negative strand HCV RNA was also detected in DC lysates in 5 (n=5) patients tested (table 3.2).

Having shown the presence of HCV genomic sequences and low-levels of replication of the virus in MDDC we further investigated potential relation to phenotypic parameters and functional outputs of DC in the 30 (n=30) tested patients.

In particular the relation between the presence of HCV-specific sequences (positive and negative strand HCV RNA sequences individually) and a) MDDC allostimulatory capacity, assessed by MLR, b) autologous responses to HCV and recall antigens assessed by IFN-γ ELIspot assays following recombinant HCV core and NS3 proteins or tetanus toxoid pulsing respectively (described in 2.2.7.1), c) Interleukin(IL)-12p70 production, assessed by ELISA assays on cell culture supernatants (described in 2.2.7.2) and finally d) the expression of, CD40, CD80, CD83, CD86 and HLA DR antigens, by FACS flow-cytometry, on the surface of the studied MDDC, were statistically explored. All functional and phenotypic parameters testing was performed on MDDC generated from patients prior to the initiation of antiviral treatment, with or without LPS stimulation as described in the corresponding sections of Chapter 2.

Patients were stratified as: with ‘presence’ or ‘absence’ of detectable positive strand HCV RNA and with ‘presence’ or ‘absence’ of detectable negative strand HCV RNA; statistical correlation with functional and phenotypic DC parameters followed.

Both ‘immature’ and ‘mature’ (after stimulation with LPS 0.1μg/ml that triggered a corresponding increase in CD80, CD83 and to a lesser extend in CD86 and HLA DR expression on tested MDDC surface) MDDC were assessed, without any significant differences between the two groups.

Testing MDDC allostimulatory capacity with MLR assays, proliferation of $CD4^+$ T-cells (10^4/well) was assessed in co-culture with two-fold dilutions of MDDC (10,000/well, 5,000/well and 2,500/well). $CD4^+$ T-cell proliferation for each of the two-fold MDDC dilutions was statistically tested individually against the presence of etheir positive or negative strand HCV RNA in DC lysates and so was the mean (corresponding to the mean of the co-cultered two-fold MDDC dilutions) proliferation rate of $CD4^+$ T-cells.

Statistical analysis demonstrated that the presence of positive strand and negative strand HCV RNA in DC lysates had no significant effect on MDDC allostimulatory capacity.

Autologous $CD4^+$ T-cell responses - both virus-specific (after HCV core and NS3 pulsing) and recall (after tetanous toxoid pulsing of the cultures) - assessed in IFN-γ Elispot assays with LPS-matured DC were also not shown to be affected by the presence of HCV sequences.

The same was observed for DC capacity to produce IL-12p70 as tested in ELISA assays performed on stimulated DC culture supernatants and for the expression of activation, maturation and co-stimulatory markers on DC surface (CD40, CD83, CD80, CD86) as well as DC antigen presenting capacity assessed by the expression of HLA DR on DC surface.

Data from the correlations of the presence of ‘positive’ and ‘negative’ strand HCV RNA sequences to the various functional parameters and surface marker expression on the studied population of MDDC are summarised in table 3.4.

3.2.2.2 HCV RNA levels in patients' plasma

Viraemia (HCV RNA) levels were tested in plasma samples from 30 (n=30) patients included in our study group, prior to the initiation of antiviral treatment, with a TaqMan® real-time RT-PCR assay performed at the Department of Virology, University College London Hospitals (implementing a methodology quite similar to the one we used in our lab for quantitation of viral genome in MDDC lysates), and with a lower limit of detection of 20IU/ml.

Patient stratification according to viraemia levels was made on the basis of a cut-off level of HCV RNA of $6x10^5$ IU/ml (generally accepted as an independent predictive factor of response to antiviral treatment in the relevant literature so far[402]). 'Low viraemia' was considered to be an HCV RNA level $<6x10^5$ IU/ml (n=16), while 'high viraemia' was $\geq 6x10^5$ IU/ml (n=14). Viraemia levels were tested against the same MDDC functional and phenotypic parameters that were discussed in 3.2.2.1.

HCV viraemia was observed to influence significantly surface marker expression on MDDC. In particular, CD40 and CD80 expression (tested with FACS flow-cytometry, as described in section 2.2.14) on immature DC was significantly higher at 'high viraemia' levels compared to the corresponding immunological surface marker expression [as assessed by mean fluorescence intensity (MFI)] from patients with 'low viraemia' (P=0.04 and 0.007, respectively) (figs 3.2.a and 3.2.b). Scatterplots of raw data, including correlation coefficient and P value [Kendall's τ (tau) correlation], corresponding to data presented in figures 3.2.a and 3.2.b are shown in figure 3.2.c.

With regard to the rest of the tested parameters of DC function including

allostimulatory capacity, stimulation of autologous virus-specific and recall antigen (tetanous toxoid) $CD4^+$ T-cell responses and IL-12p70 production, the influence of HCV viraemia on them was not statistically significant.

3.2.2.3 Ethanol consumption

Alcohol is considered in general to enhance the severity of damage caused to the liver by chronic HCV infection also influencing negatively antiviral immune responses against HCV. Multiple mechanisms for those actions have been proposed including interference with innate and adaptive antiviral immune responses and in particular the IFN-α triggered anti-HCV response as well as increased rates of viral replication[403-405]. Exploring for the purposes of our study host-related factors that may interfere with DC function in CHC, we stratified our patients into 'heavy alcohol consumers' when their intake was ≥20units/week (n=24) and to 'light alcohol consumers' when their intake was <20units/week (n=11) [one unit of alcohol is the equivalent of a small glass of wine (125ml) or half a pint of beer (~284ml)].

Alcohol consumption was statistically correlated to the same functional parameters and cell-surface markers of MDDC described in 3.2.2.1.

Analysis revealed a significant inverse correlation between ethanol consumption and IL-12p70 secretion (P=0.001)(fig.3.3), in particular heavy alcohol consumption was observed to be related to reduced IL-12p70 secretion from MDDC, as that was measured in cell culture supernatants with an ELISA assay described in 2.2.7.2. Our observations were effectively in agreement with previous studies[406-408];.

No more significant correlations between alcohol intake and any other

functional outputs (autologous $CD4^+$ T-cell responses and allostimulatory capacity both for mature and immature DC) of the studied MDDC population were established and the same was true for the expression of activation, maturation, co-stimulatory marker and HLA DR expression on the surface of mature and immature DC as well.

3.2.2.4 HCV core protein levels in MDDC lysates

Another parameter potentially affecting DC function has been demonstrated by some authors to be the presence of HCV protein products inside DC. Studies so far have shown that DC generated from healthy subjects and subsequently transfected with adenoviral vectors encoding HCV structural proteins such as Core and envelope (E)1 are poor stimulators of allogeneic reactions and of autologous primary and secondary proliferative responses[369].

In our study we did not implement adenoviral transfection, instead we assessed the actual presence of HCV protein products (HCV core protein in particular) in chronically infected subjects' MDDC lysates, attempting to approximate the in vivo conditions.

Accordingly we lysed MDDC generated from eleven (n=11) chronically infected patients, implementing a protocol described in 2.2.15, and shipped the frozen lysates on 'dry-ice' to a collaborating group in Germany (Drs C.Sarrazin's and S.Zeuzem's group, Medizinische Klinik und Poliklinik, Innere Medizin II, Universitatskliniken des Saarlandes, Homburg/Saar, Germany) for HCV core protein quantitation with the commercially available Ortho trak-C™ assay as previously described[376-378].

Patients were stratified according to the concentration of HCV core antigen in DC lysates as with: 'low HCV core concentration' (<1.4pg/ml, n=8) or 'high HCV core concentration' (≥1.4pg/ml, n=3). The 1.4pg/ml cut-off limit was based on the median of the measured titres of HCV core in DC lysates.

Subsequently we investigated potential relations between HCV core protein concentration in MDDC and MDDC functional outputs (autologous $CD4^+$ T-cell responses and allostimulatory capacity both for mature and immature DC, IL-12 secretion) as well as activation / co-stimulatory phenotypic marker expression on mature and immature MDDC surface, finding no statistically significant correlations.

3.2.2.5 HCV core protein levels in patients' plasma

During HCV infection, HCV proteins, particularly core, enter the circulation at detectable levels[409].

Some studies have suggested that circulating HCV core and NS3 proteins inhibit DC differentiation in the absence of the intact virus and mediate some of the inhibitory effects of HCV on DC (i.e reduced allostimulatory capacity) via interleukin(IL)-10 induction[394].

Taking these observations into consideration we had HCV core protein concentration in patients' plasma measured in fourteen (n=14) chronically infected patients from our study group by the same collaborating group in Germany. Patients' stratification according to core plasma levels was into: 'low plasma levels'<184pg/ml (n=8) and 'high plasma levels'≥184pg/ml (n=6), with the cut-off of 184pg/ml based on the median of the measured titres.

This time statistical analysis revealed a significant correlation between plasma

levels of HCV core protein and the DC allostimulatory capacity (P=0.014) (fig. 3.4.a). In particular high levels of HCV core in patients' plasma were related to a more potent DC allostimulatory capacity.

With regard to other functional and phenotypic parameters of MDDC tested, HCV core plasma concentrations were observed to be directly related to CD83 expression on mature MDDC (total n=7, 'low' HCV core levels n=3, 'high' HCV core levels n=4, P=0.05)(fig.3.4.b) and CD86 expression on immature MDDC surface (total n=7, 'low' HCV core levels n=3, 'high' HCV core levels n=4, P=0.05)(fig.3.4.c). Scatterplots of raw data, including correlation coefficient and P value [Kendall's τ (tau) correlation], corresponding to data presented in figures 3.4.a and 3.4.b&c are shown in figures 3.4.d and 3.4.e respectively.

3.2.3 Effect of antiviral treatment on DC functions

Investigating DC function in the setting of CHC and practically extending findings of previous studies[137], we explored the hypothesis that early (by week 12 of antiviral treatment) viraemia clearance may improve DC function and thus restore adaptive immune responses against HCV. In particular in the present study we investigated the impact of MDDC phenotypic and functional changes in relation to early on-treatment viraemia clearance on $CD4^+$ T-cell reactivity.

MDDC from 22 (n=22) of the 35 chronic HCV patients of our study group were tested both before the initiation of antiviral treatment and at treatment week 12 (TW12). Patients were divided into: 'good responders' to treatment (n=17) [with undetectable HCV RNA in plasma (by TaqMan®, real-time RT-PCR as

described in 2.2.12) at TW12] and 'poor responders' to treatment (n=5) (with still detectable HCV RNA at TW12).

Early viraemia clearance was tested in relation to autologous $CD4^+$ T-cell responses. The frequency of IFN-γ-producing $CD4^+$T-cells at TW12, as compared to corresponding baseline (pre-treatment) levels was assessed with ELIspot assays, after recombinant HCV core, HCV NS3 and tetanous toxoid pulsing of MDDC. In that manner both virus-specific and recall responses were assessed. A significant increase in the frequency of IFN-γ-producing $CD4^+$ T-cells at TW12 was observed in 'good responders', when MDDC were pulsed with recombinant HCV core, compared to pre-treatment (P=0.036), while there were no significant changes in the 'poor-responder' group (fig. 3.5.a). The corresponding function for HCV NS3 pulsing presented a non-significant increase for the 'good responder' group (fig. 3.5.b).

Furthermore a statistically significant difference was observed between 'good responders' and 'poor responders' in Δ(Delta) (TW12 - pre-treatment) values for the number of IFN-γ-producing $CD4^+$ T-cells after recombinant HCV core pulsing (P=0.038) (fig.3.5.a), demonstrating in a more graphic manner the restorative effect of viraemia clearance on DC function. Non-significant differences were observed when MDDC were pulsed with recombinant HCV NS3 or tetanous toxoid.

It has to be mentioned that interleukin(IL)-10 ELIspot assays were also undertaken initially, for about 10 of the subjects included in the study, to test the hypothesis that this cytokine, present in increased quantities in the setting of chronic HCV infection, may skew the immune response towards tolerance promoting the establishment of chronic HCV infection[356]. Methodology

implemented was identical to the one described for IFN-γ ELIspots in 2.2.7.1. Data from the IL-10 ELIspots were not interpretable as very low numbers of spot-forming cells (SFC) were detected with quite significant 'backgroud bruit' (increased numbers of spot-forming cells in the negative control wells) on numerous occasions.

Early viraemia clearance was also observed to be associated with improved allostimulatory capacity of DC. Allogeneic $CD4^+$ T cell proliferation, tested with MLR assays as described in section 2.2.8, at TW12 was increased in 'good responders', while 'poor responders' showed a non-significant decrease, as compared to corresponding pre-treatment values. The difference between 'good' and 'poor-responders' in Δ(Delta) (TW12 - pre-treatment) values for 'naïve' $CD4^+$ T cell proliferation (MLR) was statistically significant (P=0.048) (fig.3.6).

Finally both 'good responders' and 'poor responders', demonstrated a decrease in IL-12p70 secretion at TW12 compared to baseline (fig.3.7.a). Pooling the two groups, the decrease in IL-12p70 secretion after treatment reached statistical significance (P=0.041). This defect possibly represents a consequence of standard antiviral treatment with pegylated-Interferon-α and Ribavirin on DC function (fig.3.7.b).

3.2.4 'Cross- over' experiments

Having observed defective $CD4^+$ T-cell reactivity in the setting of chronic HCV infection we proceeded further to test whether it is the restoration of DC or of $CD4^+$ T-cell functions, after treatment-induced viraemia containment, that is

responsible for the improvement of the tested parameters. To that effect we implemented a novel approach we named ‘cross-over’ methodology.

The basis of the hypothesis leading to the ‘cross-over’ experiments was our observations in section 3.2.3, confirming previous studies[137] showing that early viraemia clearance (by week 12 of antiviral treatment) is associated with improved autologous and allogeneic $CD4^+$ T-cell responses. Assuming that individuals with resolved HCV viraemia after antiviral treatment effectively had DC and $CD4^+$ T-cells with restored functions, we proceeded to test MDDC and $CD4^+$ T-cells, isolated both before the initiation of antiviral treatment and at TW12, from patients presenting with undetectable levels of HCV RNA at TW12 (‘good responders’), in all possible combinations. The aim was to observe the interaction between ‘treated’ and ‘untreated’ MDDC and $CD4^+$ T-cells trying to understand their differential contribution to the evolution of the immune responses observed in the setting of CHC.

Having cryopreserved PBMC from all the patients we treated in our study, both at the baseline of antiviral treatment and at TW12, we used the ones isolated from eight (n=8) patients - whose characteristics are summarised in table 3.3 - that presented resolution of the viraemia by week 12 of antiviral treatment (TW12) and we generated/isolated MDDC and $CD4^+$ T-cells, as described in sections 2.2.3 and 2.2.4.

The interaction of generated MDDC and $CD4^+$ T-cells was subsequently tested with IFN-γ Elispot assays in the following combinations for each patient: i) MDDC (BL) with $CD4^+$ T-cells (BL); ii) MDDC (BL) with $CD4^+$ T-cells (TW12); iii) MDDC (TW12) with $CD4^+$ T-cells (BL) and iv) MDDC (TW12) with $CD4^+$ T-cells (TW12), as graphically demonstrated in fig. 3.8. For the

purposes of the Elispot, MDDC were pulsed with recombinant HCV core and NS3 proteins as well as tetanous toxoid, matured with LPS, and the frequency of IFN-γ-producing, HCV-specific and recall, $CD4^+$T-cells was determined as described in section 2.2.7.1.

No significant increase was observed with regard to the frequency of IFNγ-producing $CD4^+$ T-cells, between 'treated' and 'untreated' $CD4^+$ T-cells. In contrast, when MDDC were generated from PBMC isolated from patients that cleared HCV viraemia early on treatment ('treated' DC) and were tested against the same two groups of autologous - isolated at baseline visit ('untreated') and at TW12 ('treated) - $CD4^+$ T-cells, after pulsing with recombinant HCV core antigen, a trend (P=0.084) towards an increased frequency of IFNγ-producing $CD4^+$ T-cells was observed (fig.3.9.a) while for recombinant HCV NS3 pulsing the increase was non-significant (fig. 3.9.b). Pooling the data for HCV core and HCV NS3 pulsing, we observed that the frequency of IFNγ-producing $CD4^+$ T-cells (both 'untreated' and 'treated') associated with 'treated' DC was increased significantly (P=0.045; fig. 3.9.c). As far as recall responses, tested in the same Elispot assays with tetanous toxoid pulsing, are concerned, no significant differences between 'treated' and 'untreated' DC or T-cells were observed.

3.2.5 Plasmacytoid dendritic cells

Attempting an initial approach to the role of the second sub-population of dendritic cells, the plasmacytoid dendritic cells (PDC), in the setting of HCV infection, we isolated this cell population directly from PBMC separated from large amounts of blood that was removed therapeutically from six patients

(n=6) suffering from haemochromatosis or essential polycythaemia. The patients did not have a previous history of exposure to chronic viral hepatitis viruses (negative testing for HBsAg, anti-HBc and anti-HCV) or any other potentially immune-modulatory pathology. Subsequently we 'infected' the isolated PDC ex vivo by incubating them with plasma from a patient with high HCV viraemia levels (-7log_{10}), at variable MOI's (4, 2 and 0.4) and studied the cytokine secretion capacity of the HCV-infected PDC comparing it to the corresponding function of PDC that were pulsed with various stimuli known to stimulate this specific DC sub-population.

Viral interactions with PDC are known to be mediated by toll-like receptors (TLR), as discussed in section 1.3.3.1.1. In particular some authors have shown that TLR7/8- ligands such as ribonucleoside analogs[410] and single-strand RNA (ssRNA) viral genome such as those of influenza and vesicular stomatitis virus[240,411] interact with PDC via theTLR7/8 subfamily. TLR 7/8 have been reported located in endosomal compartments of PDC. In the same studies, simple synthetic, non-viral RNAs [polyuridylic acid, poly(U)][240], were also shown to stimulate TLR7.

Those data, considered in combination to the fact that HCV is a single-strand RNA virus, led us hypothesize that the potential effect of HCV infection on PDC may be mediated by TLR 7/8.

We incubated the PDC isolated from our patients with an appropriate activation stimulus [CD40-ligand(L)-expressing cells, 10^4 CD40L-transfected cells/well (a gift from Dr.Peter Lane)], and HCV plasma, as described above, for 24hours at 37^oC / 5% CO_2. Subsequently we harvested and tested culture supernatants for IL-12p70, IFNα and IL-6. The cytokines tested were chosen

on the basis: a) of their importance in the skewing of the host immune response either towards Th1 {IL-12p70 [412-414] and IFN-α [413,415,416]} - a process vital for subsequent control and clearance of HCV - or towards Th2 {IL-6[417-420]}, an adaptive immune response that facilitates viral persistence, quenching at the same time hepatic inflammation and b) of the recently observed secretion of the mentioned cytokines from PDC after TLR7/8 ligation[410]. A property unique to PDC is their ability to produce considerable amounts of type I interferons (IFN-α) in response to TLR7/8 and TLR9 ligation, while MDC, after stimulation of the same TLR, mainly produce IL-12[421].

As 'positive' control of cytokine production from PDC we used a known synthetic TLR7/8-ligand(L), R848 - resiquimod (Bioquote Limited, York, UK). R848 is an imidazoquinoline compound with known antiviral activity that has been shown to activate human immune cells via the TLR7/8 - MyD88-dependent signalling pathway[422,423]. In our cell culture R848 was used in ten-fold dilutions (10μl, 1μl and 0.1μl of a 500μg/ml solution).

As an additional 'positive' control we tested PDC cytokine secretion after stimulation with synthetic oligonucleotides that contain unmethylated cytosine-phosphodiester-guanine (CpG) dinucleotides and in particular specific sequence motifs [CpG motifs, CpG oligo-deoxy-nucleotide(ODN) type A: ODN 2216, Bioquote Limited, York, UK, (20μl, 5 μl, 2.5 μl from a solution of 100μg/ml) for 24hours]. CpG, a known TLR9-ligand(L), has been shown to represent a potent PDC stimulus. Recent studies have demonstrated the effect of type A CpG ODN on the induction of IFNα secretion from PDC[424-

426]. Other authors have also shown mediation of CpG effects via TLR9 ligation[427].

TLR9 is closely related to TLR7/8 as they are all located in endosomal compartments and share a common dependence of their function on acidification and maturation of endosomes[240,428]. Stimulating PDC with CpG effectively provides evidence of integrity of the common pathway shared by TLR7/8 and TLR9.

Another virus, also causing chronic hepatitis, the DNA virus HBV, was comparatively tested for its interaction with PDC. We chose to use HBV as it has been shown by other groups to affect PDC function[315,429] and also that the immune system uses similar strategies for detecting ssRNA and DNA viruses[428]. The latter is strongly suggested by the similarity of recognition of influenza virus (ssRNA) [240] and human immunodeficiency virus-1 (HIV-1) derived guanosine (G)- and uridine (U)-rich ssRNA oligonucleotides[410] by TLR7/8 in PDC endosomal compartments, to the recognition of unmethylated DNA (CpG) genome by TLR9, also found in PDC endosomes, as proposed for herpes simplex virus (HSV-1,2, DNA viruses)[427,430].

To that effect serum from a single donor with known high HBV viraemia levels ($\sim 9 \log_{10}$ IU/ml) was used at variable MOI's (400, 200 and 40), similarly to what was undertaken for HCV plasma.

Finally, as Ribavirin constitutes a part of the currently used standard antiviral treatment (pegylated-IFN-α2a/2b with Ribavirin) and has been shown to have significant immune-modulatory potential[123,431,432], we also tested PDC

cytokine production after pulsing PDC cultures with clinically relevant Ribavirin concentrations (20μM, 5μM and 2μM)[431].

The small number of subjects tested would not allow for any statistical analyses. Graphic representation of the data, as shown in fig. 3.10, permitted a rough assessment as follows:

A. mean **IL-6** secreted levels were comparable when PDC cultures were pulsed with CpG ODN 2216, HBV serum and R848. These levels were 3-4 times higher than the ones elicited by all the implemented HCV plasma volumes. The effect of Ribavirin on IL-6 production from PDC was comparable to levels triggered by HCV plasma.

B. mean **IL-12p70** secretion under R-848 stimulation was twice as high as the corresponding levels observed after incubation with HCV serum and comparable to the levels elicited by HBV serum. Also very high levels of IL-12p70 were shown after stimulation with Ribavirin; in fact levels of IL-12p70 secretion triggered by 5μg/ml of Ribavirin, a concentration with clinical relevance, were 2-4 times higher than the ones elicited by all other stimuli used (CpG ODN 2216, R848, HBV serum, HCV plasma).

C. mean **IFN-α** secretion stimulated by CpG ODN and R-848 was comparable to the one observed with HBV and HCV serum pulsing, respectively. Ribavirin on the contrary was not observed to trigger secretion of any significant quantities of IFN-α from circulating PDC.

Acknowleging the fact that our data on PDC so far would not allow for any safe conclusions on the role of the latter in the establishment of chronicity of HCV infection we decided to present them nevertheless as we feel that discussion of those preliminary data would be quite usefull in terms of DC

subpopulation analysis in the setting of chronic HCV infection while on the other hand we believe that it will help us generate hypotheses that will potentially guide further studies.

3.3 Discussion

The mechanisms HCV uses to down-regulate antiviral immunity, thus promoting viral persistence, are still not well defined although recent work from several groups has contributed significantly to the definition of some of the potential immune-evading tactics of the virus such as: a) the development of escape mutations[433-435], b) the antagonism of effector T-cell stimulation by viral proteins[436,437], c) intra-hepatic recruitment and deletion of activated virus-specific $CD8^+$ T-cells[291], d) immune-suppressive effect of certain viral proteins[292] and possible subsequent late induction of virus-specific responses[110], et.c.

The role of a potential defect caused by HCV in the innate immune system and in particular the dendritic cells, the most potent known antigen-presenting cells, in the establishment of chronic infection, is emerging over the last few years. To gain a better understanding of the still contradictory role of dendritic cells in the establishment of chronic HCV infection, we compared the allostimulatory capacity of monocyte-derived dendritic cells (probably the most important function of DC as it reflects their potential to stimulate naïve $CD4^+$ T-cells) generated from chronically infected patients (CHC-DC) to the corresponding function of MDDC generated from healthy non-infected individuals (N-DC). Our data confirm previous studies showing down-regulation of DC allostimulatory capacity in the setting of chronic hepatitis

C[336,344,345]. It is widely accepted though that patients suffering from chronic hepatitis C do not present a global immune defect; the suppression of the immune response is rather virus-specific. One possible explanation of this observed discrepancy in the immune resposes may be that probably even a minor defect in the host's DC allostimulatory potential may prove critical for the induction of the complex immune response required for the containment of the sophisticated HCV while responses against other pathogens, possibly less complicated and thus less fragile, retain a status adequate for infection control. On the other hand immunological mechanisms restoring the defect caused by HCV on DC allostimulatory capacity may preserve responses to other pathogens.

Having found a significantly reduced allostimulatory function in CHC-DC (fig.3. 1), we further proceeded to investigate host and virus-related parameters that may contribute to the observed defect.

The assessment in our study of the influence of HCV proteins and in particular of the highly immunogenic, structural Core protein constitutes one of the novelties of our approach. Direct quantitation of HCV core antigen levels in patients' sera and in MDDC lysates, and exploration of potential correlations to DC function and phenotype in our study reflects an attempt to approach the in-vivo condition avoiding methodologies adopted previously such as the transfection of naïve DC with either adenovirus vectors expressing HCV proteins[347,369] or liposomal transfection agents[438]. Our observations indicate that increased concentration of HCV core (≥1.4pg/ml) present inside DC, does not influence DC function and phenotype as compared to low (<1.4pg/ml) concentration, while high HCV core plasma levels (≥184pg/ml)

were shown to be related to increased maturation (CD83, fig.3.4.b; P=0.05 and fig. 3.4.e), and co-stimulatory (CD86, fig.3.4.c; P=0.05 and fig 3.4.e) marker expression on DC surface as well as to enhanced DC allostimulatory capacity (fig.3.4.a; P=0.001 and fig 3.4.d). Thus, contrary to previous reports [347,369,394] and in line with other studies[438,439] our experiments show a 'stimulatory' effect of circulating HCV viral products (plasma HCV core protein) on DC phenotype and function.

Expectedly, in a similar manner, HCV viraemia appears directly related to increased expression on MDDC surface of activation and co-stimulatory markers (CD40 and CD80 on immature DC with P values 0.04 and 0.007 respectively, figs. 3.2.a & 3.2.b and fig 3.2.c) but not to any other functional output of DC.

Investigating further we implemented an ultra-sensitive PCR methodology[342] that detected positive strand HCV RNA in MDDC lysates in 13 of 33 patients. Our observations also demonstrated the presence of low level replication of HCV (detection of the replicative intermediate, negative strand HCV RNA) in monocyte-derived dendritic cells, supporting previous studies claiming both presence and replication of the virus in DC [336,392]. The detection of the replicative intermediate, negative strand HCV RNA, in MDDC lysates argues against the assumption that the presence of HCV-specific genomic sequences and antigens in DC is nothing but the result of non-specific scavenging of virions and circulating viral proteins by the 'professional' antigen-presenting dendritic cells. We have though to be careful when interpreting those data bearing in mind that potential contamination of the tested MDDC population may be responsible for the detection both of the

presence of virus-specific genomic sequences and the low-grade HCV replication in MDDC observed in our experiments. MDDC generated from $CD14^+$ monocytes after the 6-days incubation protocol we implemented were 90%-97% pure, effectively allowing for 3%-10% of other contaminating PBMC subsets. On the other hand no influence of the presence of HCV-specific genomic sequences, either positive or negative strand HCV RNA, in MDDC lysates, on DC functions or surface marker expression, was demonstrated. The presence and replication of HCV in DC, as suggested by our data, apparently does not affect either the functional output (at least the functional parameters that we tested) or the expression of DC surface markers. Those observations may imply that HCV entry and low grade replication within DC could be a mechanism the virus implements to evade immunological control. It was shown previously[85] that HCV is internalized in non-lysosomal compartments inside DC via binding to DC-SIGN receptors and thus may escape degradation 'hiding' from the immune system facilitating viral dissemination to the effector cells (T-cells) in secondary lymphoid organs. As an 'immunologically privileged' reservoir of the virus, DC may also contribute to viral persistence even after successful control of viraemia, through antiviral treatment, or spontaneous clearance[342].

With regard to host characteristics affecting DC function in CHC, we observed a suppressive effect of alcohol on DC IL-12p70 secretion (P=0.005) (fig.3.3), potentially leading to impaired induction of Th1 response against HCV.

Overall among the various parameters tested, only high HCV viraemia levels and increased HCV core protein concentration in patients' serum appear to affect, rather stimulate vital MDDC functions such as their allostimulatory

capacity and the expression on MDDC surface of activation / maturation / antigen-presenting and co-stimulatory molecules (CD40, CD83, HLA DR, CD80, CD86). In conclusion our data suggest a stimulatory effect of circulating HCV genome and HCV protein products on the tested MDDC functions, and surface marker expression, contrary to the initially observed in our study defect of allostimulatory capacity. Apparently parameters related to HCV infection, other than the ones we tested, contribute the observed defect in DC function. Another observation we were also not able to explain was the discrepancy between the effect of intracellular and the effect of soluble HCV core protein and plasma HCV genomic sequences on DC function and surface marker expression. We may hypothesize that methodological shortcomings in the cell lysis protocols and in HCV core and HCV RNA extraction from DC as well as the small number of patients studied could be responsible. The corresponding detection methodologies for serum and plasma samples are better established and involve less elaborate, and thus less error-prone, protocols.

In order to further investigate the effect of HCV on DC function we explored potential restoration of antiviral immune responses after viraemia clearance-mediated improvement of CHC-DC function. Practically extending previous findings that rapid viraemia clearance is associated with enhanced HCV-specific T-cell responses[137,440], we assessed the impact of MDDC phenotypic and functional changes in relation to early on-treatment (pegylated-Interferon α2b + Ribavirin) viraemia clearance on $CD4^+$ T-cell reactivity. Implementing ELIspot assays with HCV core, HCV NS3 and tetanous toxoid (tt) pulsing we effectively tested the status of both virus-

specific (core, NS3) and other recall responses (tt). Mixed Lymphocyte Reactions (MLR) at baseline of antiviral treatment and at TW12 provided insight in the non-specific $CD4^+$ T-cell reactivity in relation to treatment. Our data demonstrate that in patients who cleared the virus by week twelve of antiviral treatment (TW12) ('good responders'), the enhanced adaptive immunity observed is related to restoration of DC functions [both non-specific allostimulatory capacity (MLR) (fig.3.6) and virus-specific autologous $CD4^+$ T-cell stimulation against HCV antigens (figs. 3.5.a & 3.5.b)], an observation that was not born out in patients who remained viraemic ('poor responders'). Recall responses, reflected by tetanous toxoid-induced frequency of IFNγ-producing $CD4^+$ T-cells, remained potent and unaffected after treatment.

The observed improvement in $CD4^+$ T-cell reactivity could either be attributed to improved DC or improved $CD4^+$ T-cell function due to treatment-induced viraemia clearance. This controversy effectively reflects the argument on whether HCV primarily affects DC function or $CD4^+$ T-cell reactivity leading to defective virus-specific adaptive immune response and viral persistence. In our study we attempted to approach this issue implementing a novel methodology we called 'cross-over' experiments. In those experiments MDDC and $CD4^+$ T-cells isolated both before the initiation of antiviral treatment and after treatment-induced viraemia resolution at TW12, were tested in IFN-γ Elispot assays, in all possible combinations. Analysis of the resulting data pointed primarily towards the significance of 'treated' MDDC in the improvement of autologous $CD4^+$ T-cell responses (figs.3.9.a&b&c). Taking this observation into consideration we may argue in favour of a defect in DC function caused by HCV infection being responsible for the defective $CD4^+$ T-

cell responses and in particular for a defect in the production of the Th1-skewing IFNγ, instead of a potential virus-induced primary $CD4^+$ T-cell dysfunction.

An interesting observation regarding IL-12p70 production from DC, both in 'good responders' and 'poor responders' to antiviral treatment, is that it appears decreased at TW12 compared to baseline levels (figs.3.7.a&b), probably as an effect of the antiviral treatment itself, an observation confirming previous studies [362].

With regard to our preliminary data on PDC functional capacities in the setting of HCV infection we observed low levels of IL-12p70 secretion in pDC-HCV culture supernatants as compared to levels of the same cytokine triggered by R848 (3-4 times higher). We also documented very high levels of secreted IL-12p70 after pDC stimulation with clinically relevant concentrations of Ribavirin (twice the levels triggered by R848). On the other hand CpG ODN 2216 and R848 stimulation caused IFNα secretion from pDC at levels comparable to those triggered by HCV (fig.3.10). Our observations suggest that although IFNα secretion by pDC is probably not affected by HCV, a significant defect to the Th1 skewing capacity of pDC, as manifested by defective IL-12 production, is probably related to the interaction with HCV, as shown in previous studies[345,401]. We also suggest that Ribavirin may exercise its immune-modulatory effect, as part of standard antiviral treatment, via PDC.

Patient initials	HCV RNA in $5x10^5$ MDDC lysate(IU/ml)	HCV RNA in $2.5x10^5$ MDDC lysate (IU/ml)	HCV RNA in 10^6 PBMC lysate (IU/ml)	Plasma HCV RNA (IU/ml)
MF	0	0	**$5.7x10^4$**	$5.4x10^5$
PL	0	0	**$4.9x10^3$**	$15x10^6$
TJ	0	0	**$8.3x10^3$**	460
GS	0	0	**$7..2x10^2$**	$1.8x10^5$
MP	0	0	**$7.6x10^3$**	**-***
JM	0	0	**$4.6x10^2$**	**-***
SA	0	0	**$3.6x10^3$**	$1.1x10^5$
GA	0	0	**$4.1x10^3$**	$2.4x10^5$
SW	0	0	**$7.4x10^2$**	$3.8x10^5$
MM	0	0	**$4.8x10^3$**	**-***

(* : sample not available)

Table 3.1 HCV RNA detection by TaqMan real-time RT-PCR in cell lysates (MDDC and PBMC) and in patients' plasma. Lower limit of detection of the assay: 20IU/ml.

Initials	Total RNA (µg)	HCV RNA + strand	HCV RNA - strand
GA	2.6	- n/s	**NT**
KW	4.0	- n/s	**NT**
TJ	24.8	+ n/s	**-**n/s
SA	13.3	- n/s	**NT**
MH	4.6	+ n/s	**-**n/s
GFo	5.9	+ n/gel	+ n/gel (2 µg)
UM	7.1	+ n/gel	-n/s
DS	28	- n/s	**NT**
KD	5.3	- n/s	**NT**
PJ	3.0	+ n/s	**-**n/s
GS	8.3	- n/s	**NT**
MA	5.4	- n/s	**NT**
KA	15.3	- n/s	**NT**
MSS	2.4	+ n/gel	-n/s
RC	2.8	+ n/gel	-n/s
CM	6.0	+ n/gel	+ n/gel (2µg)
JM	4.6	- n/s	**NT**
SL	6.2	- n/s	**NT**
RA	7.2	+ n/gel	- n/s (3 µg)
BS	6.1	+ n/s	+ n/gel (4µg)
PL	9.0	- n/s	**NT**
TM	10.4	- n/s	**NT**
MSM	4.3	- n/s	**NT**
MHO	10.7	- n/s	**NT**
JC	3.8	- n/s	**NT**
WB	9.8	+ n/gel	+ n/gel (2µg)
MPort	11.4	+ n/gel	smear (4µg)
JCM	1.5	- n/s	**NT**
RT	6.6	+ n/s	- n/s (4µg)
DL	12.2	- n/s	**NT**

Table 3.2 Total RNA recovery from MDDC lysates ($4x10^5$ – $5x10^5$ MDDC generated before treatment) and HCV RNA positive strand and negative strand detections

NT: not tested (HCV RNA positive strand not detected)

n/gel: nested PCR with 5'UTR primers, product detected by EB-agarose gel electrophoresis always finally confirmed by Southern blot hybridization

n/s: nested PCR followed by Southern blot hybridization for detection and validation of PCR products (as in [342])

Lower limits of sensitivity: nested PCR/EB agarose gel (**n/gel**): 10-100 viral genomes(vge)/mL, nested PCR/ Southern blot hybridization (**n/s**) : ≤10 vge/mL

Initials	Gender (male, female)	Age (years)	Race*	Geno-type	Plasma HCV RNA (IU/mL)	ALT levels (IU/L)	Inflam-mation (grade**)	Fibrosis (stage**)
RB	M	47	C	1a	$5x10^5$	68	3	3
PA	M	42	C	3a	520	80	2	0
JM	F	42	C	3a	$1.2x10^5$	51	3	0
RK	M	50	C	3a	10^6	80	6	3
GF	M	41	C	3a	$1.4x10^6$	198	5	4
MT	M	72	C	3a	$9x10^4$	135	6	3
KW	F	40	C	3a	$8.9x10^4$	66	2	2
AH	M	32	C	3a	$1.7x10^4$	61	4	2

* Caucasian (C), non-caucasian (NC)
** Liver biopsy, modified Ishak scale

Table 3.3 Patients' characteristics for the 'cross-over' experiments (prior to the initiation of antiviral treatment)

a. Grouping Variable: HCV RNA positive strand presence in MDDC lysates						
	CD1a im.DC*	CD40 im.DC	CD80 im.DC	CD83 im.DC	CD86 im.DC	HLA DR im.DC
P value	**0,1**	**0,69**	**0,51**	**0,36**	**0,66**	**0,84**
	CD1a mat.DC**	CD40 mat.DC	CD80 mat.DC	CD83 mat.DC	CD86 mat.DC	HLA DR mat.DC
P value	**0,43**	**0,19**	**0,59**	**0,36**	**0,11**	**0,6**

b. Grouping Variable: HCV RNA positive strand presence in MDDC lysates			
	MLR 10e4DC†	MLR 0.5e4DC	MLR 0.25e4DC
P value	**0,98**	**0,75**	**0,66**
IL12 secreted in DC culture supernatant			
P value	**0,29**		
	ELIspot HCV core‡	ELIspot HCV NS3	ELIspot tetanous toxoid
P value	**0,69**	**0,36**	**0,45**

c. Grouping Variable: HCV RNA negative strand presence in MDDC lysates						
	CD1a im.DC*	CD40 im.DC	CD80 im.DC	CD83 im.DC	CD86 im.DC	HLA DR im.DC
P value	**0.34**	**1**	**0.85**	**0.07**	**0.41**	**0.21**
	CD1a mat.DC**	CD40 mat.DC	CD80 mat.DC	CD83 mat.DC	CD86 mat.DC	HLA DR mat.DC
P value	**0.51**	**0.61**	**0.61**	**0.63**	**0.62**	**0.61**

d. Grouping Variable:HCV RNA negative strand presence in MDDC lysates			
	MLR 10e4DC†	MLR 0.5e4DC	MLR 0.25e4DC
P value	**0.51**	**0.51**	**0.71**
IL12 secreted in DC culture supernatant			
P value	**1**		
	ELIspot HCV core‡	ELIspot HCV NS3	ELIspot tetanous toxoid
P value	**1**	**0.61**	**1**

(*)CD1a expression on immature MDDC surface, **(**)**CD1a expression on LPS-matured MDDC surface, **(†)**MLR 10e4DC: mixed lymphocyte reaction with 10,000MDDC co-cultured with 100,000 allogeneic $CD4^+$ T-cells from the same, non-infected, donor, **(‡)**ELIspot HCV core: 100,000 autologous CD4+ T-cells co-cultured with 10,000 mature MDDC stimulated with HCV core antigen

Table 3.4 Correlation of the presence of positive-strand HCV RNA and negative-strand HCV RNA in MDDC lysates to: cell-surface marker expression (**a**. and **c.** respectively) and functional parameters of MDDC (**b**. and **d.** respectively) (P values significant if ≤0.05, **Mann-Whitney U test**)

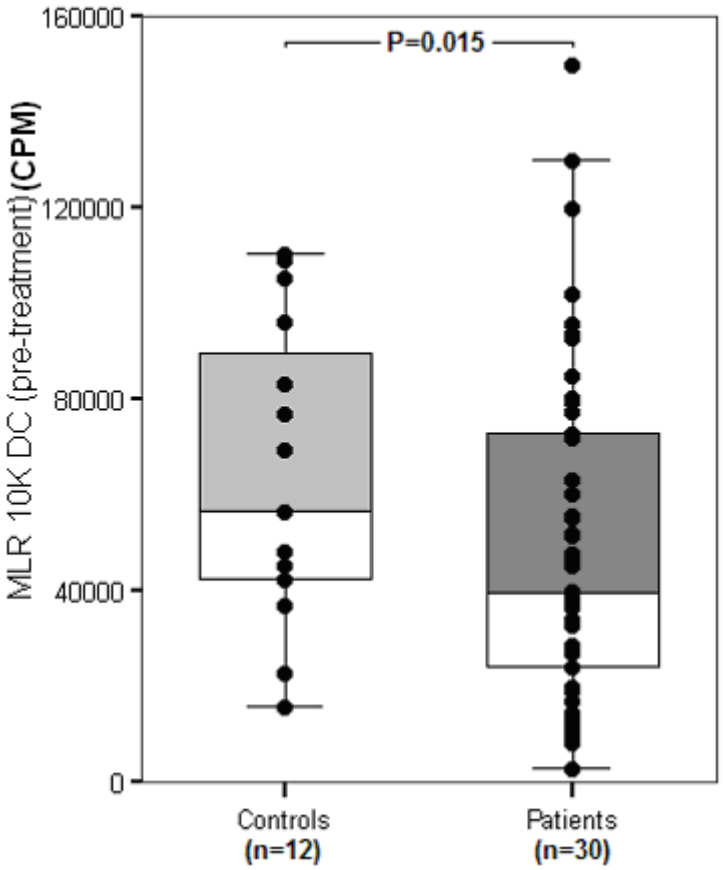


fig.3.1 Allostimulatory capacity of LPS-matured DC from 30 patients with chronic HCV infection (Patients) and LPS-matured DC from 12 non-infected healthy individuals (Controls).

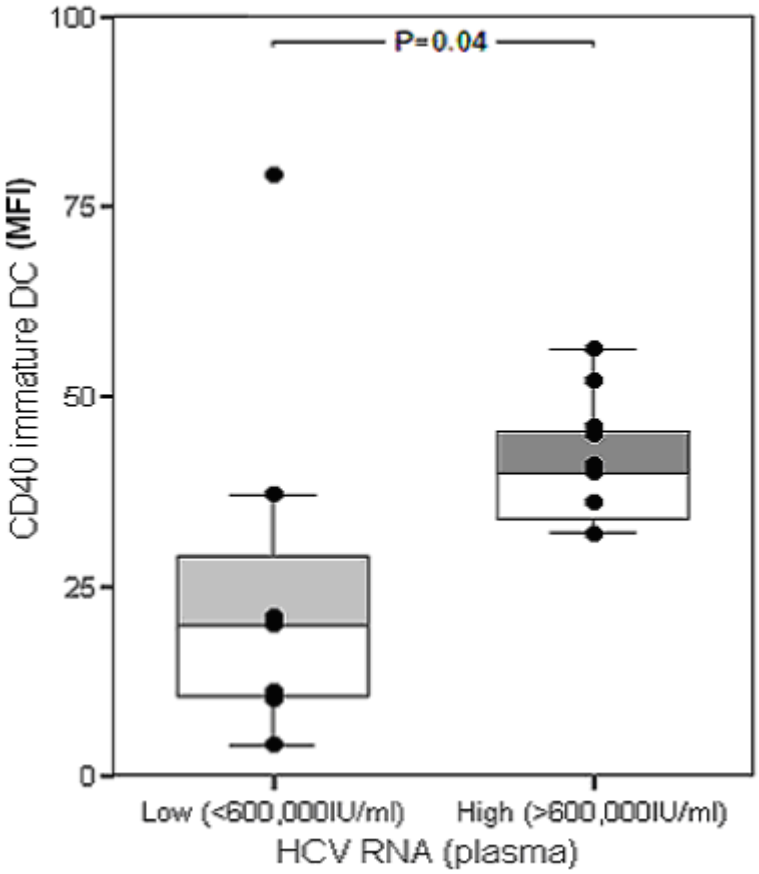


fig.3.2.a HCV RNA plasma levels and effect on CD40 expression on immature DC

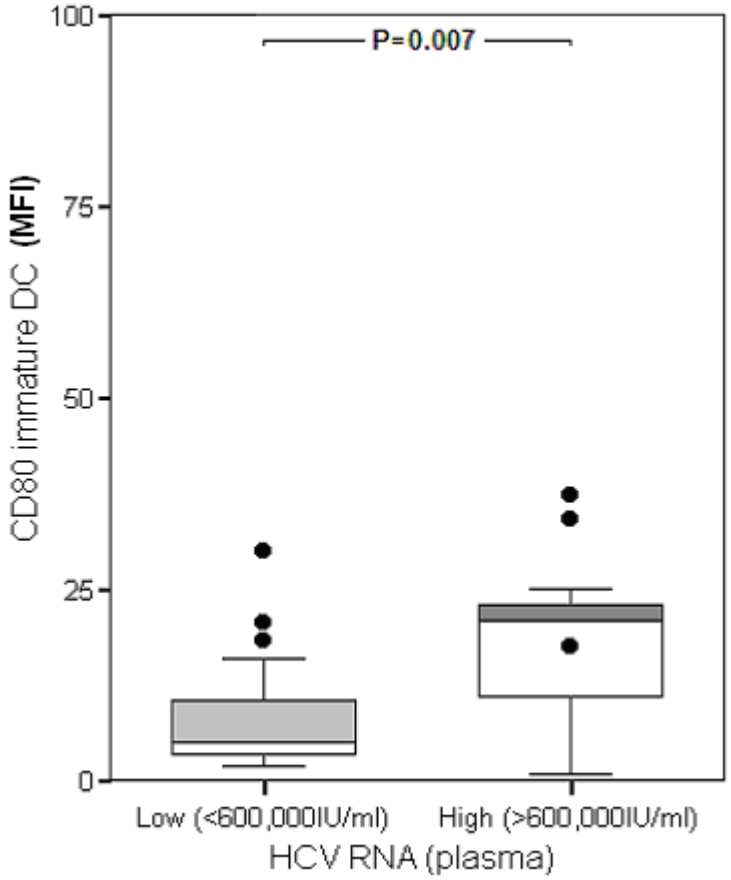


fig.3.2.b HCV RNA plasma levels and effect on CD80 expression on immature DC.

For both figures (3.2.a & b): scatterplot and boxplot extending from the 25th to the 75th percentile and whiskers to the largest and smallest observed values within 1.5 box lengths; the solid line is the median.

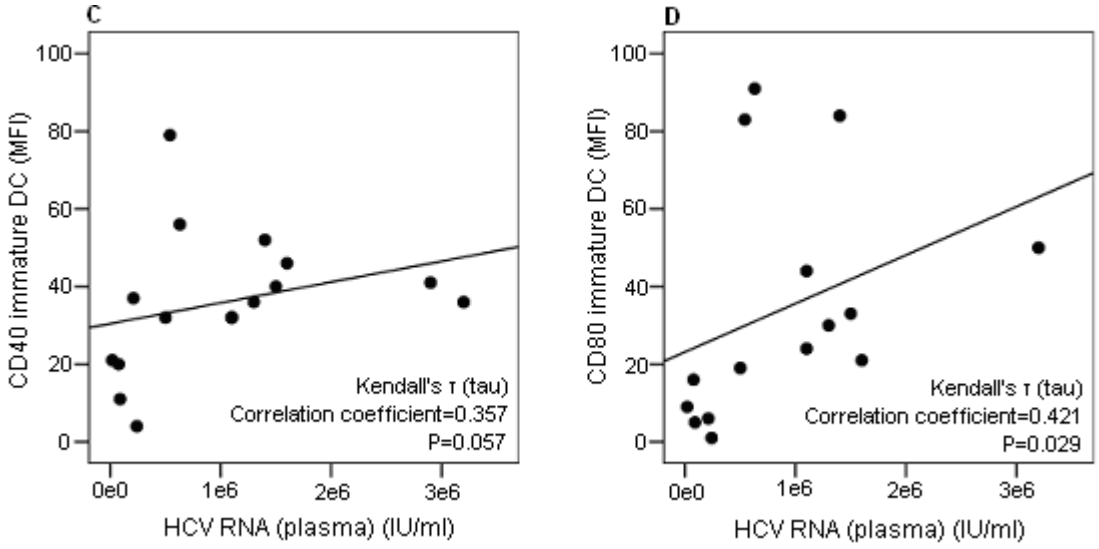


fig.3.2.c HCV RNA plasma levels and effect on CD40 and CD80 expression on immature DC. Scatterplot of raw data, including correlation coefficient and P value [Kendall's τ (tau) correlation], corresponding to data presented in figures 3.2.a and 3.2.b.

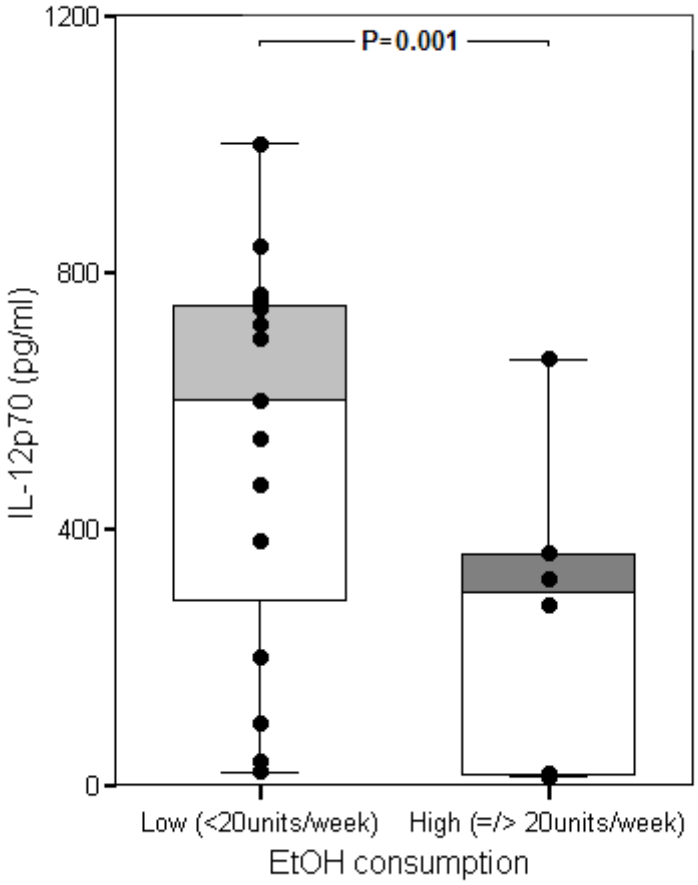


fig.3.3 Effect of weekly ethanol (EtOH) consumption on IL-12p70 production. Scatterplot and boxplot extending from the 25th to the 75th percentile and whiskers to the largest and smallest observed values within 1.5 box lengths; the solid line is the median

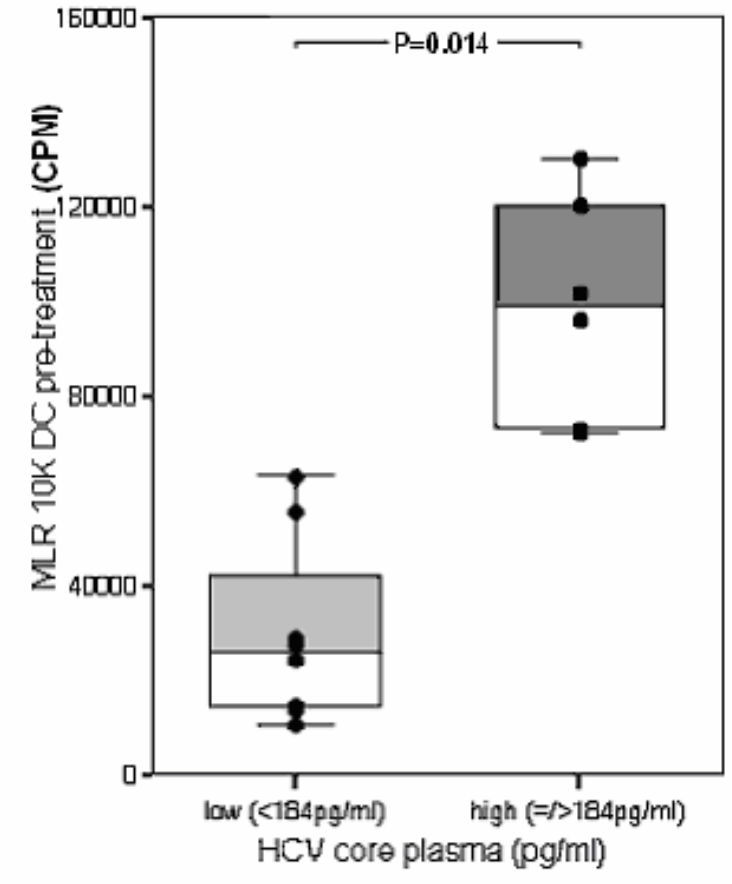


fig.3.4.a Effect of HCV core plasma levels on DC allostimulatoty capacity (MLR with LPS-matured DC)

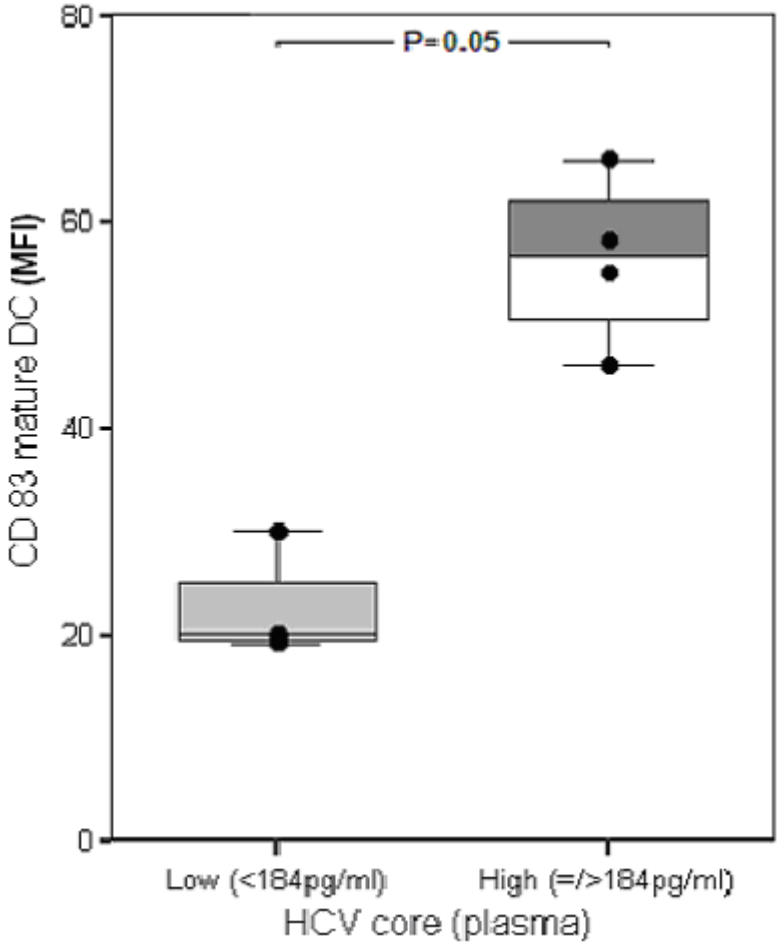


fig.3.4.b HCV core plasma levels and effect on CD83 expression on LPS-matured DC

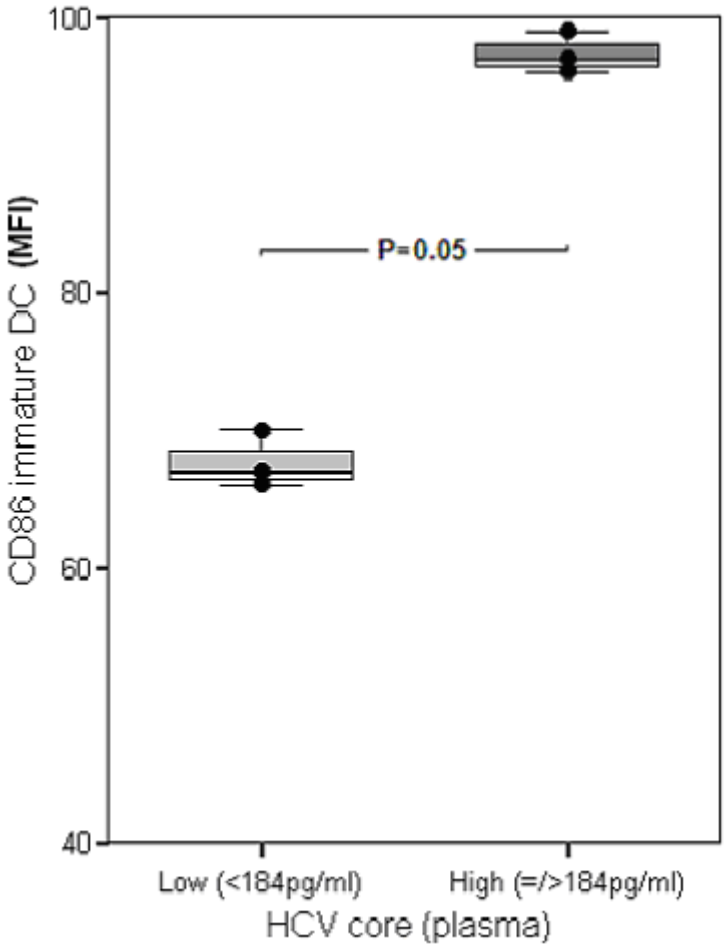


fig.3.4.c HCV core plasma levels and effect on CD86 expression on immature DC

For all three figures (3.4.a&b&c): scatterplot and boxplot extending from the 25th to the 75th percentile and whiskers to the largest and smallest observed values within 1.5 box lengths; the solid line is the median

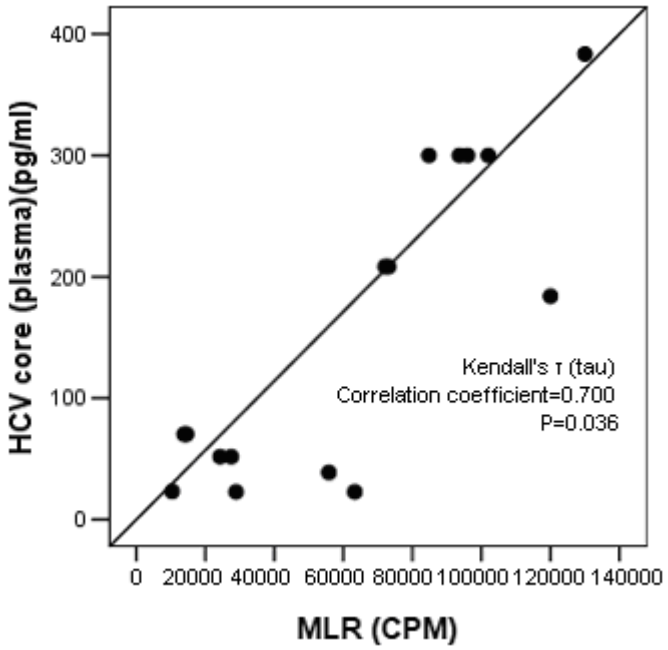


fig.3.4.d Effect of HCV core plasma levels on DC allostimulatoty capacity (MLR with LPS-matured DC). Scatterplot of raw data, including correlation coefficient and P value [Kendall's τ (tau) correlation], corresponding to data presented in figure 3.4.a.

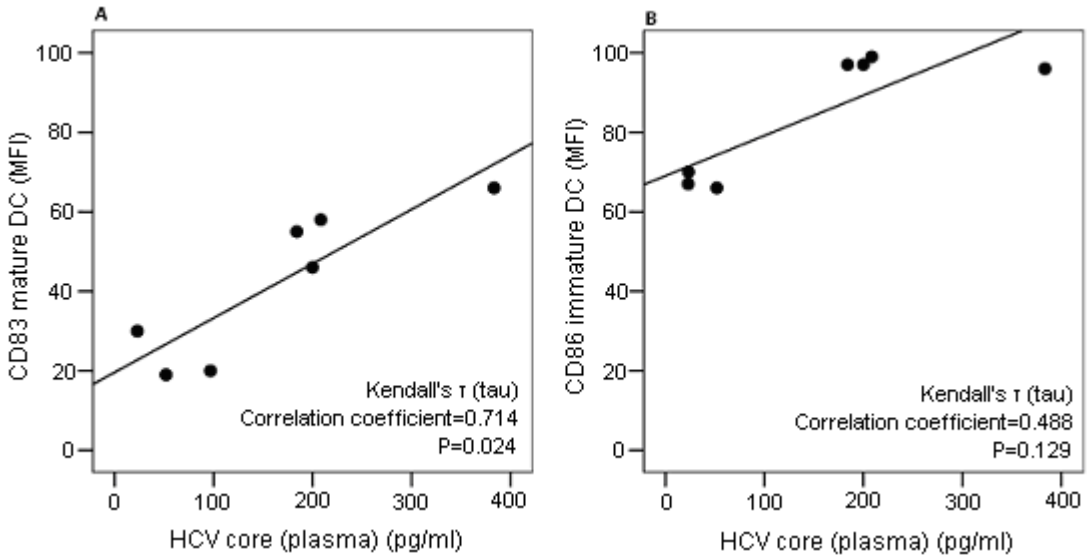


fig.3.4.e HCV core plasma levels and effect on CD83 expression on mature DC and CD86 expression on immature DC. Scatterplot of raw data, including correlation coefficient and P value [Kendall's τ (tau) correlation], corresponding to data presented in figures 3.4.b and 3.4.c.

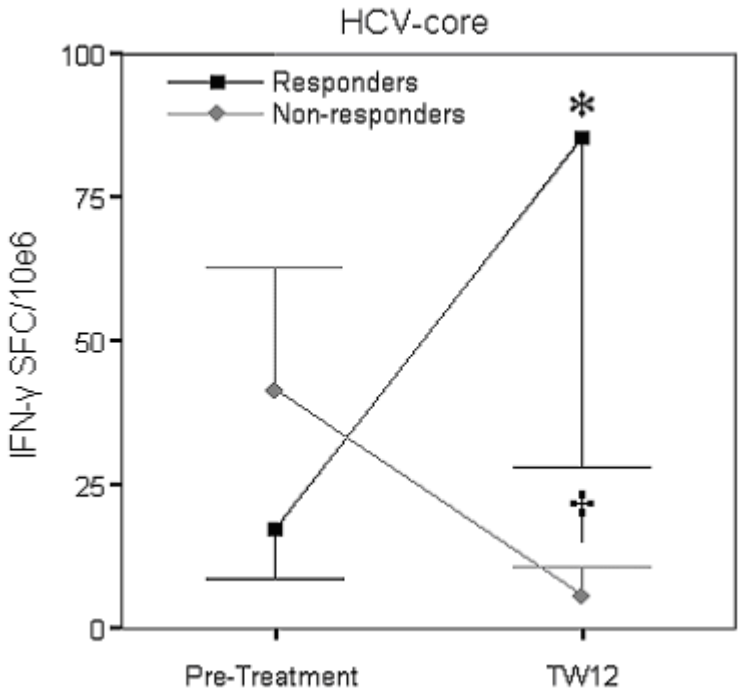


fig.3.5.a Autologous $CD4^+$ T-cell response, tested with IFN-γ Elispot assay, for 'good' and 'poor' responders to antiviral treatment, with DC pulsed with HCV core protein. *Significant difference (P=0.036) at TW12, compared with pre-Rx values; †Significant difference (p=0.038) in Δ(Delta) (TW12-preRx) values between responders and non-responders

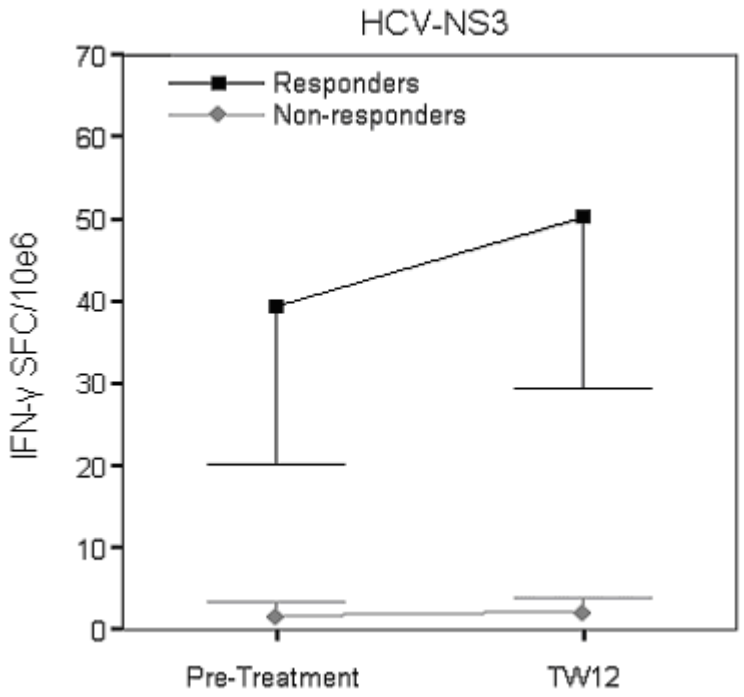


fig.3.5.b Autologous CD4+ T-cell response, tested with IFN-γ Elispot assay, for 'good' and 'poor' responders to antiviral treatment, with autologous DC pulsed with HCV NS3.

For both figures (3.5.a&b) values are expressed as mean ± standard error.

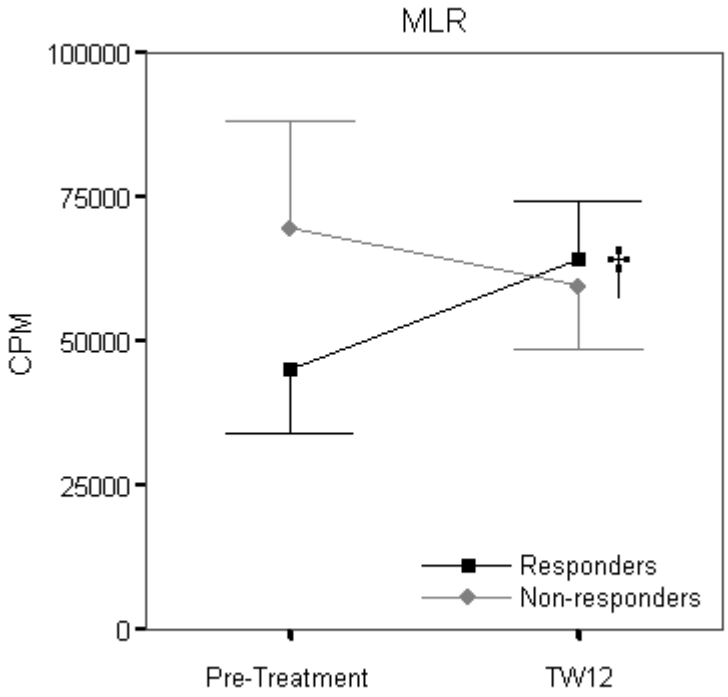


fig.3.6 Allostimulatory capacity in 'good' and in 'poor' responders to antiviral treatment. † Significant difference (P=0.048) in Δ(Delta) (TW12-preRx) values between responders and non-responders. Values are expressed as mean ± standard error

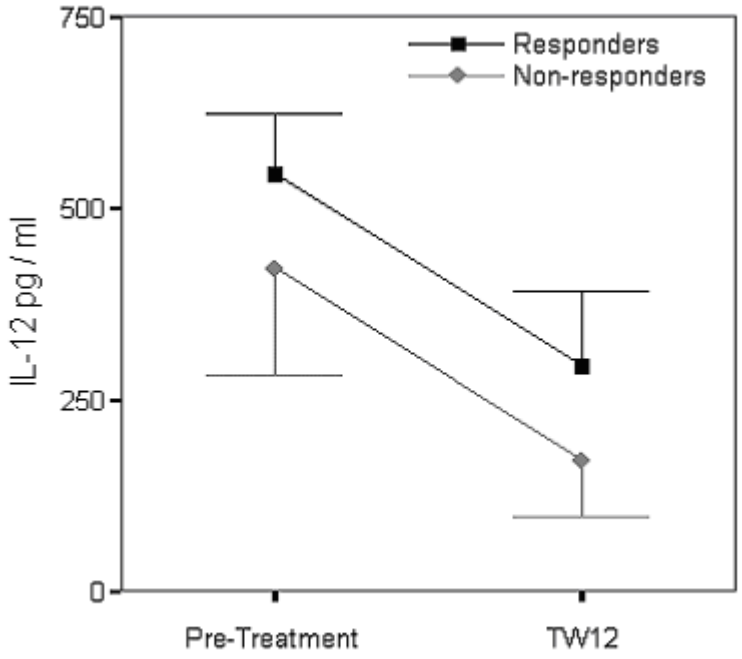


fig. 3.7.a IL-12p70 production under antiviral treatment, in 'good' and in 'poor' responders

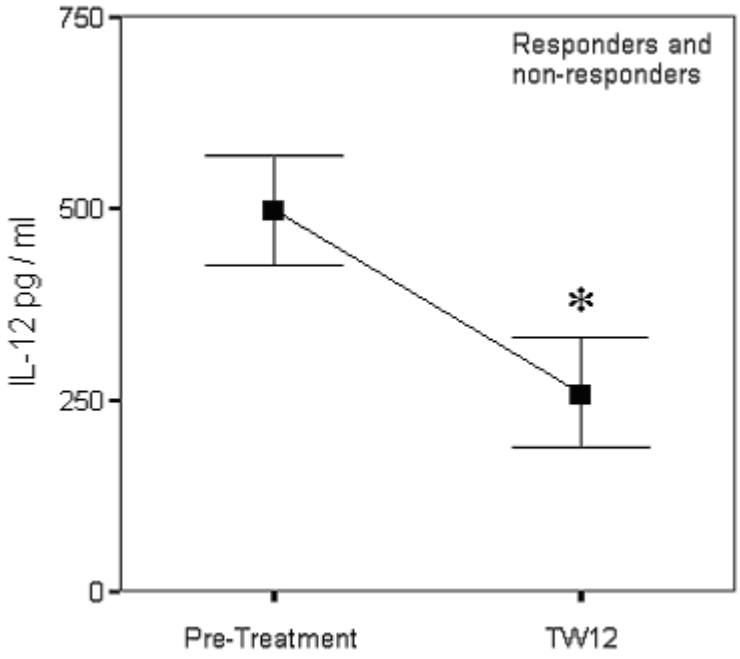


fig.3.7.b IL-12p70 production under antiviral treatment, in 'good' and in 'poor' responders collectively (pooled data).*Significant decrease (P=0.041) of IL-12p70 production at TW12, compared to pre-Rx values, both in 'good' and 'poor' responders. Values are expressed as mean ± standard error.

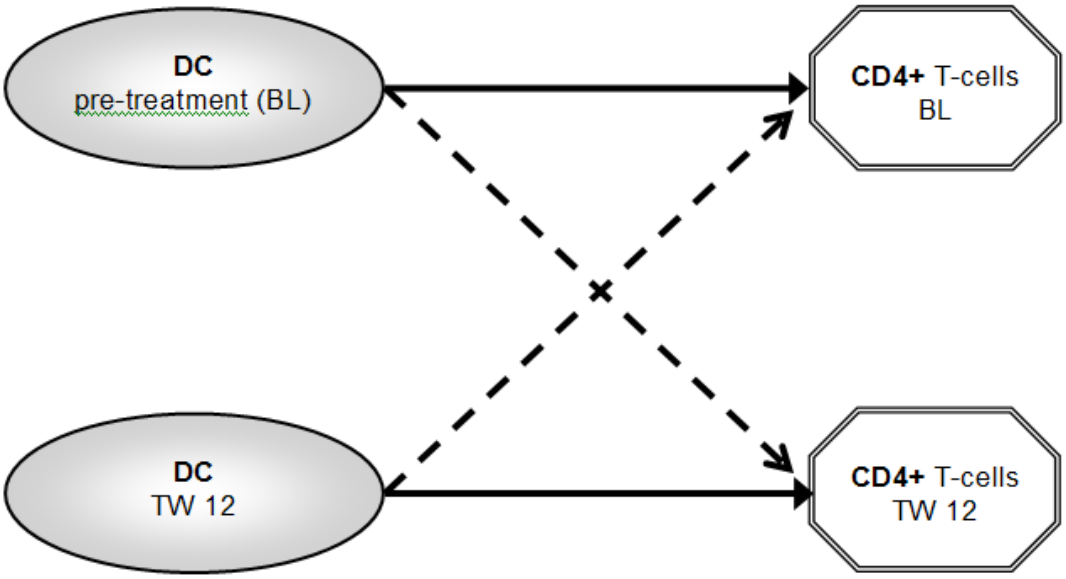


fig.3.8 Design of the 'Cross-over' experiments

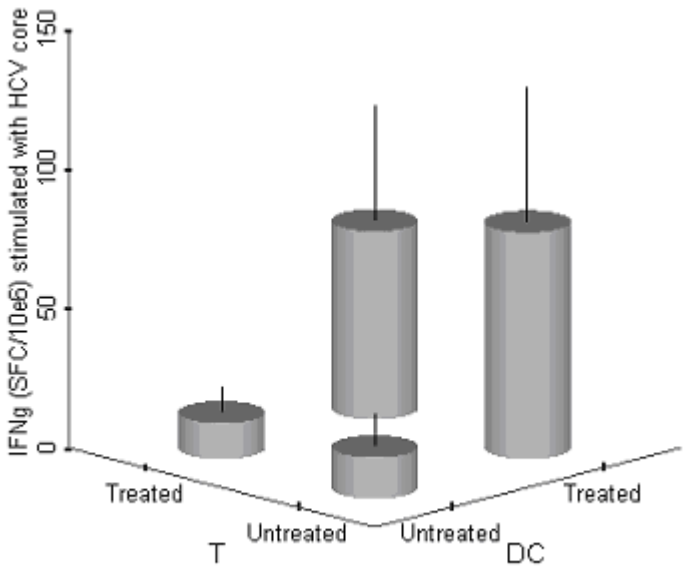


fig.3.9.a Cross-over' experiment with rHCV core pulsing (DC and CD4+ T-cells both from BL - pre-treatment - and from TW12 - week 12 of antiviral treatment).

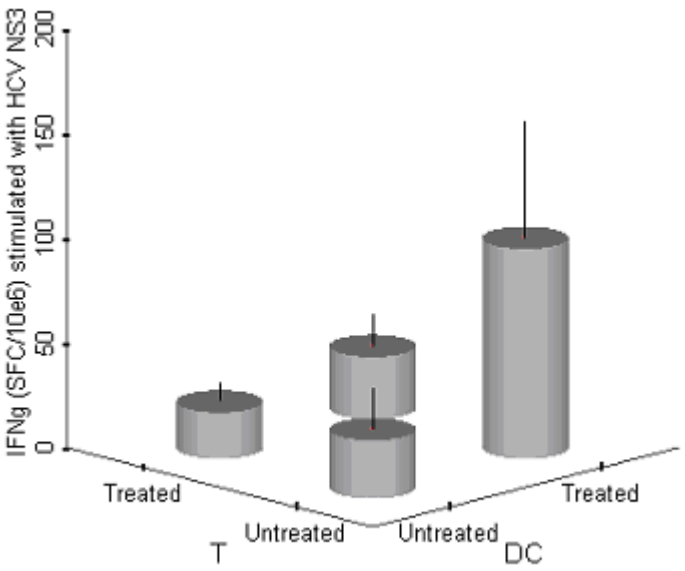
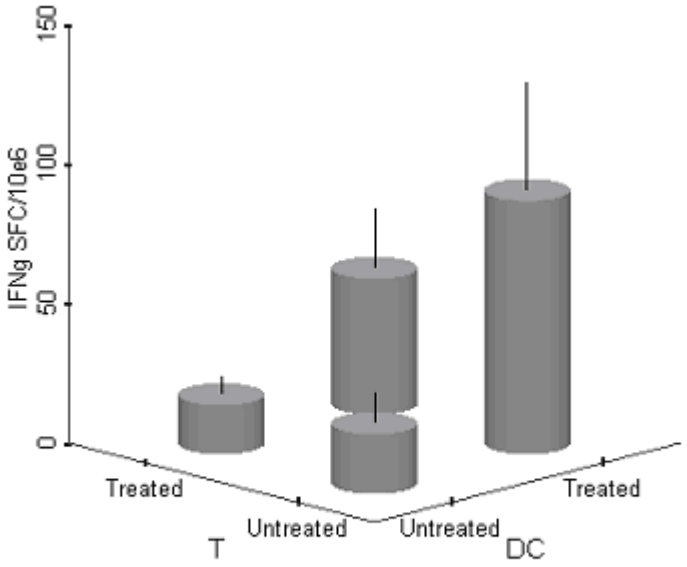


fig.3.9.b 'Cross-over' experiment with rHCV NS3 pulsing (DC and CD4+ T-cells both from BL - pre-treatment - and from TW12 - week 12 of antiviral treatment).



Pooled data for HCV core and NS3

fig.3.9.c Pooled data from the two previous 'cross-over' experiments, assessing the overall individual contribution of DC and CD4+ T-cells in the development of an efficient Th1 (IFN-γ producing CD4+ T-cells) adaptive immune response.

For all three figures (3.9.a&b&c) values are expressed as mean + standard error.

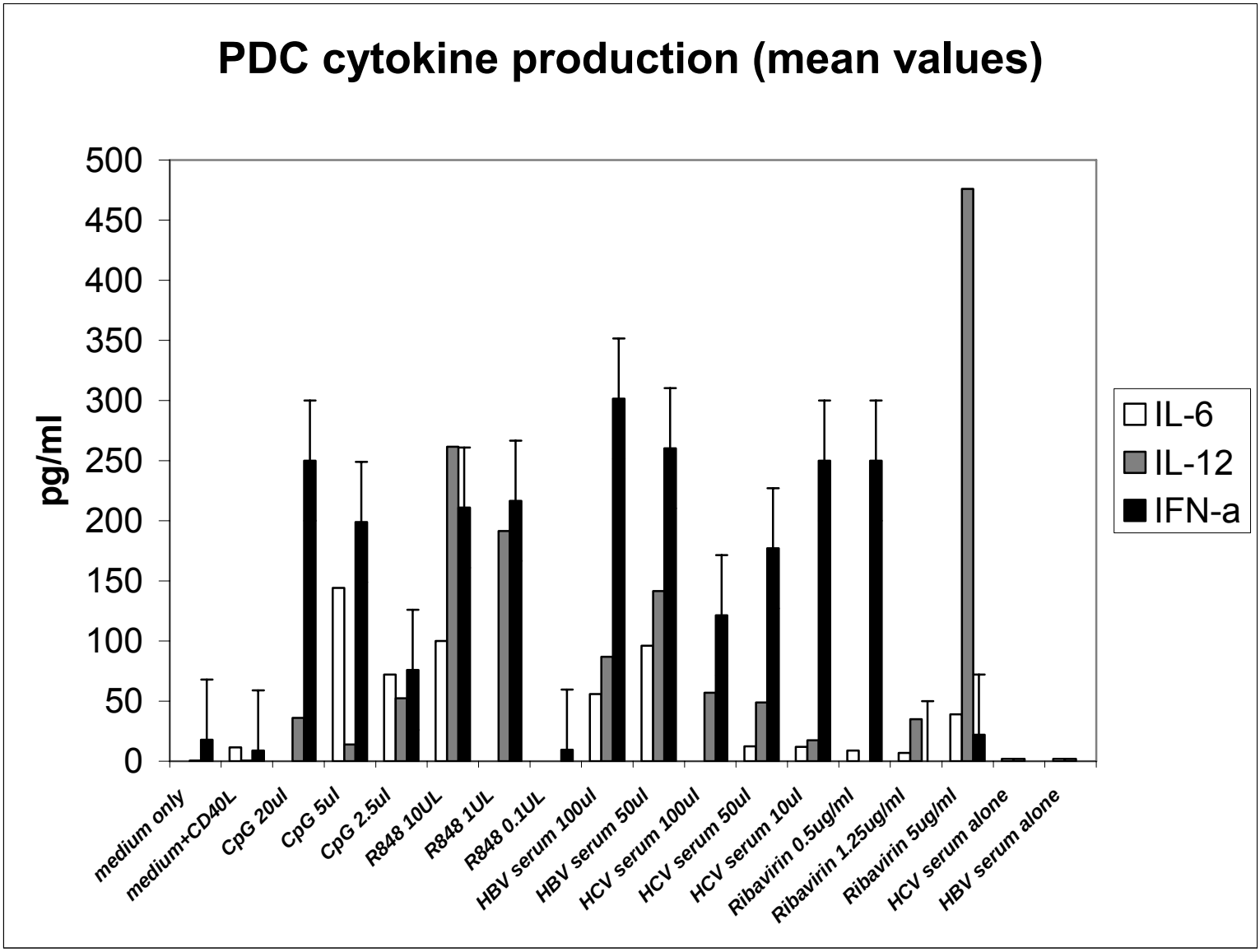


fig.3.10 Cytokine production from PDC under the influence of various stimuli. Values displayed in bars are mean (+/- standard error) values of cytokine (IL-6, IL-12p70, IFNα) production, in pg/ml, under the influence of various stimuli: a. TLR9-ligand CpG oligodeoxynucletides, b. TLR7/8-ligand resiquimod (R848), c. dilutions of HBV serum, d. dilutions of HCV plasma, d. Ribavirin

CHAPTER 4

SUMMARY AND CONCLUSIONS

4.1 Summary

The mechanisms that HCV uses to down-regulate antiviral immunity, thus promoting HCV persistence, are still not well defined. To gain a better understanding, along the line of recent studies pointing out the emerging importance of the innate immune system in the setting of chronic hepatitis C, we initially tested the capacity of monocyte-derived dendritic cells (MDDC) from chronically infected patients to stimulate naïve $CD4^+$ T-cells to proliferation in MLR assays and found it defective compared to the corresponding function of MDDC from healthy, non-infected individuals. As the capacity of DC to stimulate $CD4^+$ T-cell proliferation, and in particular 'naïve' to HCV allogeneic $CD4^+$ T-cells, is probably the most important function of dendritic cells, the obvious defect we observed in those initial experiments led us investigate further a potential role of DC in the establishment of chronic HCV infection.

The litterature on this issue so far is quite controversial. While most studies, using either monocyte-derived dendritic cells (MDDC) or myeloid dendritic cells isolated directly from peripheral blood mononuclear cells, demonstrate defective DC function[344,345,347,369,391], other studies report comparable capacity to healthy controls[339,348-350].

Our data confirm defective DC allostimulatory capacity in the setting of chronic HCV that is not virus-specific though (allogeneic $CD4^+$ T-cell MLR assay). As overwhelming evidence precludes global immune-suppression in chronically infected by HCV patients, while at the same time the HCV virus-

specific responses appear in most relevant studies attenuated allowing for HCV viraemia persistence[121-125], we may hypothesize that other immunological mechanisms, not yet defined, restore or rather preserve immune responses to other recall antigens.

We subsequently proceeded to investigate the impact of MDDC functional changes in relation to early on-treatment viraemia clearance on $CD4^+$ T-cell reactivity, implementing IFNγ Elispot and MLR assays, effectively hypothesizing that control of viraemia improves DC functions allowing for better $CD4^+$ T-cell reactivity. This hypothesis was based on previous studies showing that rapid viraemia clearance is associated with enhanced HCV-specific, T-cell responses[137,440]. Our data suggest that HCV viraemia clearance by TW12 is related to restoration of autologous and allostimulatory $CD4^+$ T-cell responses. We did not observe the same in patients who remained viraemic during treatment with peg-interferon alpha/ribavirin.

Taking things a step further we analysed virus-related and host-related factors that may interfere with DC function in the setting of chronic HCV infection. For this purpose we quantitated, in collaboration with a group in Germany, HCV core antigen levels directly in patients' plasma and in MDDC lysates, and searched for potential corelations to DC function and phenotype. Most previous studies used transfection of non-infected DC with either adenovirus vectors expressing HCV proteins[368,369] or plasmid transfection[370,438] or even pulsed cultures of non-infected DC or PBMC with recombinant HCV core protein[344]. In that aspect our experiments were probably closer to the in-vivo situation and that was one of the novelties of our study. Our data indicate that HCV core protein can be found inside DC, probably as a result of

either non-specific scavenging from the DC environment or of active viral replication however it does not influence DC function and phenotype. On the other hand high HCV core plasma levels were shown to be related to increased maturation (CD83), and co-stimulatory (CD86) marker expression on DC surface as well as to enhanced DC allostimulatory capacity, tested in MLR assays. Contrary to previous reports[347,356,369] and in line with other studies[438,439], our experiments suggest that circulating HCV viral products (plasma HCV core protein) may have a 'stimulatory' effect on DC phenotype and function.

Hypothesizing that HCV genome presense, replication and transcription inside DC is responsible for at least a part of the detected intra-DC HCV core protein we decided to also test the presence of viral genome and potential active viral replication within DC[338,341,342,391] in order to establish possible influences on DC functional capacities. Initially, we used real-time, TaqMan RT-PCR (lower limit of detection 20IU/ml or 1200 viral copies/ml of MDDC lysate). That methodology failed to detect any HCV-specific sequences in MDDC lysates. As a next step, we implemented, in collaboration with a group in Canada, an ultra-sensitive RT-PCR-nucleic acid hybridisation methodology (lower limit of detection ≤10viral genomes/ml)[342]. Positive strand HCV RNA was detected with this methodology in MDDC lysates in 13 of 33 tested patients. We also observed the presence of a low level replication of HCV (presence of negative-strand HCV RNA) in MDDC, supporting findings of previous studies[392]. The detection of the replicative intermediate, negative strand HCV RNA, in MDDC lysates argues against the presence of HCV sequences and antigens in DC being only the product of non-specific

scavenging of virions by dendritic cells. On the other hand though the minute quantity of HCV-specific genomic sequences detected with ultra-sensitive PCR inside DC should be interpreted with caution as contamination levels of the studied MDDC populations ranged from 3% to 10%.

Similar to HCV core protein plasma levels, the HCV viraemia levels (determined with the same sensitive real-time TaqMan RT-PCR methodology with lower limit of detection of 20IU/ml) and not the presence of HCV genomic sequences inside MDDC, were found to correlate with increased expression of activation and co-stimulatory markers on MDDC surface (CD40 and CD80 on immature DC) but not to any other functional output of DC.

Although we showed presence of HCV RNA and HCV Core protein inside MDDC we were not able to correlate those parameters to a significant influence on DC functions, at least the functions that we tested. Our observations probably suggest that HCV enters DC and exploits their immunologically privileged milieu in order to evade detection and control by the host immune system. It was shown previously that HCV is internalized via binding to DC-SIGN receptors[83] in non-lysosomal compartments in DC, escapes degradation and probably ‘hides’ from the immune system facilitating viral dissemination[85]. As an ‘immunologically privileged’ reservoir of the virus, DC may contribute to viral persistence, even after successful control of viraemia through antiviral treatment (sustained viral response, SVR), or spontaneous clearance[342]. On the other hand we recognize that our data, based on small numbers of tested patients, are probably not statistically powered enough to draw safe conclusions on whether in fact the presence,

active replication and transcription of HCV inside dendritic cells does or does not affect the function of the latter.

One possible explanation of the discrepancy we report between the observed effect of HCV genome and HCV core protein presence inside DC on the function of the latter and the effect of HCV viraemia and HCV serum levels on the corresponding function, could be that the action of HCV on DC is mediated via transmembrane receptor ligation on DC surface without the need for the actual presence and replication-transcription of HCV inside dendritic cells.

With regard to other host characteristics affecting DC function in CHC, we confirmed a previously reported[408] suppressive effect of alcohol on DC IL-12p70 secretion, potentially leading to impaired induction of Th1 responses, making the argument against the use of alcohol in HCV infected patients even stronger.

At the same time we also made an interesting observation regarding IL-12p70 production from DC. The latter appears decreased at week 12 of antiviral treatment (TW12) compared to baseline levels both in 'good responders' and 'poor responders' to antiviral treatment, probably as an effect of the antiviral treatment itself, as suggested by other authors previously[362].

One of the novelties of the present study is the approach we adopted to dissect whether the impaired adaptive immune response to HCV is primarily due to defect of DC or of the effector $CD4^+$ T-cells. We conducted ''cross-over'' experiments with DC and $CD4^+$ T-cells isolated from patients with chronic HCV infection both prior to the initiation of antiviral treatment and after twelve weeks of antiviral treatment that resulted in undetectable levels of HCV

RNA at that timepoint. Isolated DC and $CD4^+$ T-cells were co-incubated in every possible combination hypothesizing restoration of DC and/or $CD4^+$ T-cells function after viraemia clearance with the aim to explore whether it is mainly the 'treated DC' or the 'treated $CD4^+$ T-cell' that was responsible for the observed improvement of T-cell reactivity in terms of IFNγ production from autologous $CD4^+$ T-cells. An increased frequency of IFNγ-producing $CD4^+$ T-cells was associated with 'treated' DC, but not 'treated' $CD4^+$ T-cells. Therefore data from the "cross-over" experiments, for the first time, demonstrate the importance of dendritic cells in the development of an effective adaptive response against HCV, rendering them potential primary targets for immune-modulatory therapeutic approaches to chronic hepatitis C.

The second DC subset in humans is the plasmacytoid DC (PDC). Improvement of cell separation techniques over the last few years allowed for isolation of PDC directly from peripheral blood in numbers sufficient for ex-vivo experiments. As a result an increasing number of studies on the potential role of PDC in the chronicity of HCV infection are emerging recently.

In order to gain an initial insight into the role of PDC in the setting of chronic hepatitis C we measured the cytokine secretion (IL-12p70, IL-6, IFNα) capacity of PDC isolated from non-infected individuals that were incubated with plasma from a single, higly viraemic, chronic HCV patient at various MOI's. PDC cytokine secretion after HCV plasma pulsing was compared to the corresponding outputs after TLR 7/8 and TLR9 ligand stimulation, HBV serum pulsing at various MOI's and Ribavirin co-culture at clinically relevant dilutions. For the purposes of our experiments we used TLR 7/8 and TLR 9 ligands and HBV serum as 'positive' controls taking into consideration that

those Toll-like receptors have been suggested by numerous studies to mediate viral interactions with PDC[240,315,410,411,427]. Our data suggest that: a. the Th-1 skewing capacity of PDC is diversely affected by HCV (suppressed IL-12p70 secretion, preserved IFNα production capacity), and that b. Ribavirin probably exerts an immunomodulatory role by triggering IL-12p70 (Th1-skewing cytokine) production from PDC. Although we realize that our data is too incomplete to raise any firm hypothesis we observe a discrepancy as far as PDC Th1 capacity in chronic HCV is concerned, as also shown previously [331,345,396,441], pointing out the necessity for further studies. Also, for the first time in the literature so far, we imply an immune-modulatory function of Ribavirin on PDC.

4.2 Conclusions

In conclusion, the present thesis suggests that dendritic cell functions are impaired in patients with chronic HCV infection despite the fact that HCV viraemia and HCV plasma-circulating proteins (HCV core protein) appear to exert a ‘stimulatory’ effect on DC function and cell-surface immune-phenotype. The observation of a DC functional defect, in the setting of CHC, is supported by the finding that viraemia clearance, early on-treatment, leads to improvement of DC functions an observation that was not reproduced in patients who remained viraemic while on-therapy.

The study of PDC indicates a diverse effect of the HCV on PDC and the potential mediation of that effect by TLR7/8 and TLR9 as well as an immune-modulatory effect of Ribavirin on PDC.

The data generated for the present thesis also suggest that in patients with chronic HCV infection an improvement of antiviral immunity (during successful antiviral therapy) is due to improved DC function, as a result of viraemia clearance, and not of the restoration of a primary, virus-induced, $CD4^+$ T-cell defect.

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PUBLICATIONS – PRESENTATIONS

1. IS HEPATITIS C VIRUS INFECTION OF DENDRITIC CELLS A MECHANISM FACILITATING VIRAL PERSISTENCE?
   Ioannis Pachiadakis, Gabriele Pollara, Benjamin Chain, Nikolai V Naoumov
   Lancet Infect Dis 2005;5: 296–304

2. HEPATITIS C VIRAEMIA INHIBITS DENDRITIC CELL FUNCTIONS AND THEIR ABILITY TO INDUCE ADAPTIVE IMMUNE RESPONSE TO HCV
   I. Pachiadakis, S. Chokshi, H. Cooksley, C. Sarrazzin, S. Zeuzem, NV Naoumov
   European Association of the Study of Liver Disease (EASL) meeting, 2006

3. HCV INFECTION OF DENDRITIC CELLS AFFECTS THEIR FUNCTION LEADING TO AN IMPAIRED ADAPTIVE IMMUNE RESPONSE
   I. Pachiadakis, S. Chokshi, H. Cooksley, C. Sarrazzin, S. Zeuzem,T. Michalak , NV Naoumov
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4. EFFECT OF CHRONIC HCV INFECTION ON Th SKEWING CAPACITY OF CIRCULATING PLASMACYTOID DENDRITIC CELLS (PDC). THE INFLUENCE OF ANTIVIRAL TREATMENT (RIBAVIRIV)
   I. Pachiadakis, S. Chokshi, H. Cooksley, D. Farmakiotis, NV Naoumov
   European Association of the Study of Liver Disease (EASL) meeting, 2008

5. EARLY VIRAEMIA CLEARANCE DURING ANTIVIRAL THERAPY OF CHRONIC HEPATITIS C IMPROVES DENDRITIC CELL FUNCTIONS
   Ioannis Pachiadakis, Shilpa Chokshi, Helen Cooksley, Dimitrios Farmakiotis, Kwok H. Tang, Christoph Sarrazin, Stefan Zeuzem, Tomasz I. Michalak, Nikolai V. Naoumov
   Clinical Immunology (2009) 131, 415–425