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# **Interferon lambda, Dendritic Cells and Hepatitis C Virus Infection**



**A thesis submitted to the University of Dublin for the  
Degree of Doctor of Philosophy**

**2013**

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*Thesis 10295*

## Abstract

It is estimated that ~3% of the world's population is infected with Hepatitis C virus (HCV), a hepatotropic virus which can lead to liver fibrosis, cirrhosis and hepatocellular carcinoma. Of those infected, ~20% clear the virus naturally whereas the majority develop chronic infection. Response to the current therapy, the important antiviral cytokine interferon alpha (IFN- $\alpha$ ), also varies with response rates of less than 50% in some cohorts. The inter-individual variation in clinical outcome to HCV infection highlights the contribution of genetic variation to the host antiviral response to infection.

The antiviral effect of IFN- $\alpha$  is mediated by the induction of antiviral genes. We investigated if variation in a key antiviral gene, *2'-5' oligoadenylate synthetase (OAS)*, could account for some of the variation in HCV infection outcome. A single nucleotide polymorphism (SNP) in the *OAS1* gene, rs10774671, is known to give rise to splice variants with different antiviral activity. In a homogenous cohort of Irish women, infected with HCV from a single source, no association was found between *OAS* rs10774671 genotype and natural clearance of infection or markers of disease severity.

In the meantime, in late 2009, genome-wide association studies (GWAS) identified a number of SNPs in the interferon lambda (IFN- $\lambda$ ) gene region strongly associated with HCV outcome. The IFN- $\lambda$ s functionally resemble IFN- $\alpha$ , despite using different receptor systems. However much about IFN- $\lambda$  biology remains unclear and it is not known if they possess any additional properties differing from IFN- $\alpha$ . Within immune cells, Type I IFNs are known to utilise different patterns of cell-specific STAT activation to activate different genes and subsequent biological responses. However, the response of individual immune cells to IFN- $\lambda$  is unknown. While IFN- $\lambda$  receptor expression could be demonstrated on peripheral blood mononuclear cells (PBMCs), a lack of STAT phosphorylation was evident in CD45<sup>+</sup> PBMCs. Additional analysis of STAT phosphorylation in the immune cell subsets revealed that only the rare plasmacytoid dendritic cell (pDC) population was

responsive to IFN- $\lambda$ , with phosphorylation of STAT1, STAT3 and STAT5 observed. While STAT1 activation is key for an antiviral response, phosphorylation of STAT3 and STAT5, which have anti-apoptotic properties, suggest that IFN- $\lambda$  could have a function in promoting pDC survival in viral infection.

The novel CD141<sup>+</sup> subset of myeloid DCs (mDCs) has recently been shown to specialise in producing IFN- $\lambda$  in response to the TLR3/RIG-I agonist poly(I:C). CD141<sup>+</sup> DCs express high levels of TLR3 and are superior in cross-presentation and activation of a CD8<sup>+</sup> cytotoxic T cell response, thus, suggesting an important role in antiviral and anti-tumour immunity. Although CD141<sup>+</sup> DCs have been characterised in human blood, spleen and skin, little is known about this subset in the liver. We characterised DC subsets in healthy and diseased liver perfusates. CD141<sup>+</sup> DCs were present at an increased frequency in liver compared to blood. This subset was capable of activating pro-inflammatory Th1 and Th17 T cell responses in a mixed-lymphocyte reaction. However CD141<sup>+</sup> DCs were significantly depleted in liver disease whereas other DC subsets were increased. Despite their depletion, CD141<sup>+</sup> DCs from explant livers produced markedly increased poly(I:C)-induced IFN- $\lambda$  compared with donor liver DCs, indicating these cells are primed to produce IFN- $\lambda$  in disease.

In conclusion, we have found that IFN- $\lambda$  has little effect on peripheral immune cells, restricted only to pDCs, but induces robust antiviral responses in hepatocytes suggesting a tissue-specific effect. CD141<sup>+</sup> DCs, shown to be major producers of IFN- $\lambda$ , were characterised in liver and found to be decreased in disease suggesting a role for both this DC subset and IFN- $\lambda$  production in tissue homeostasis. This work places CD141<sup>+</sup> DCs and IFN- $\lambda$  production at the forefront of the innate immune response and holds exciting potential for the development of future vaccination strategies and immunotherapy.



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## Abbreviations

AIM2	Absent in Melanoma 2
ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
AP1	Activator Protein-1
APC	Antigen-presenting cell
AST	Aspartate aminotransferase
BCR	B cell receptor
BDCA-	Blood dendritic cell antigen
BLAST	Basic Local Alignment Search Tool
BMI	Body mass index
BSA	Bovine serum albumin
BST2	Bone marrow stromal antigen 2
CLDN-1	Claudin-1
CLEC9A	C-type lectin domain family 9A
CLR	C-type lectins
ConA	Concanavalin-A
CTL	Cytotoxic lymphocyte
CX3CR1	CX3C chemokine receptor
DAAs	Direct acting antiviral agents
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid

ds	Double-stranded
EMCV	Encephalomyocarditis virus
FCS	Foetal calf serum
FLT3	FMS-related tyrosine kinase 3
GAG	Glucosaminoglycan
GAS	Gamma-activated sequence
GWAS	Genome wide association study
HAI	Histological activity index
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HFLC	Human foetal liver cell
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMNCs	Hepatic mononuclear cells
hrs	Hours
IFI16	IFN-inducible protein 16
IFNL4	Interferon lambda 4
IFN- $\alpha$	Interferon alpha
IFN- $\beta$	Interferon beta
IFN- $\gamma$	Interferon gamma
IFN- $\lambda$	Interferon lambda
IKK $\epsilon$	IKB kinase $\epsilon$
IL-	Interleukin



ILT	Immunoglobulin-like transcript
IRES	Internal ribosomal entry site
IRF	Interferon regulatory factor
ISGF3	IFN-stimulated gene factor 3
ISGs	Interferon stimulated genes
JAK	Janus kinase
KIR	Killer cell immunoglobulin-like receptor
LD	Linkage disequilibrium
LDLR	Low density lipoprotein receptor
LGP-2	Laboratory of Genetics and Physiology 2
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cell
MAPK	Mitogen activated protein kinase
MAVS	Mitochondrial antiviral signalling
M-CSFR	Macrophage colony-stimulating factor receptor
MDA5	Melanoma differentiation-associated gene
mDC	Myeloid DC
MDDCs	Monocyte-derived dendritic cells
MHC	Major Histocompatibility Complex
min	Minutes
MLR	Mixed lymphocyte reaction
MxA	Myxovirus resistance gene
MyD88	Myeloid differentiation factor 88
NASH	Non-alcoholic steatohepatitis
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells

NK cell	Natural killer cell
NKT cell	Natural killer T cell
nr	Non-redundant
OAS	Oligoadenylate synthetase
OASL	Oligoadenylate synthetase-like
OCLN	Occludin
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death 1
pDC	Plasmacytoid dendritic cell
PFA	Paraformaldehyde
PKC- $\beta$	Protein kinase C beta
PKR	Protein kinase R
Poly(I:C)	Polyinosinic:polycytidylic acid
PRRs	Pattern recognition receptors
RFLP	Restriction fragment length polymorphism
RIG-I	Retinoic acid inducible gene-1
RLRs	Retinoic acid-inducible gene-I-like receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SARS	Severe acute respiratory syndrome
sec	Seconds

SNP	Single nucleotide polymorphism
SOCS	Suppressor of Cytokine Signalling
SR-B1	Scavenger receptor class B type 1
ss	Single-stranded
STAT	Signal Transducer and Activator of Transcription
SVR	Sustained virological response
SVUH	St. Vincent's University Hospital
TBK1	TANK-binding kinase 1
TCR	T cell receptor
TGF- $\beta$	Transforming growth factor- $\beta$
Th	T helper
Tim-3	Mucin domain-containing molecule 3
TLRs	Toll-like receptors
TNF- $\alpha$	Tumour necrosis factor alpha
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TRIF	TIR domain-containing adaptor inducing IFN- $\beta$
UTR	Untranslated region
WNV	West Nile Virus



# **Chapter 1: Introduction**

## **1.1 Hepatitis C virus infection: a global health burden**

It is estimated that ~160 million people worldwide are infected with Hepatitis C virus (HCV), a hepatotropic virus causing a broad range of disease <sup>1</sup>. While up to 20% of individuals exposed to HCV are capable of clearing infection, the virus persists in the majority of individuals <sup>2,3</sup>. Chronic infection leads to liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC) in a proportion of patients as well as extra-hepatic manifestations such as lymphoma and mixed cryoglobulinaemia <sup>4</sup> (**Figure 1.1**). Nevertheless, many chronically-infected patients experience mild health effects for many years. The initial acute infection is often characterised by non-specific flu-like symptoms but can also be asymptomatic, making diagnosis difficult. Indeed, it is estimated that millions worldwide are infected but undiagnosed. HCV is a blood-borne infection, transmitted by infected blood products and the use of infected needles and, less frequently, via sexual and perinatal transmission. Since the introduction of routine screening of blood products for HCV in the early 1990's, transmission through contaminated blood in the developed world has been greatly reduced.

Six major genotypes of HCV have been defined which can be divided into more than 50 subtypes, which vary in terms of geographical distribution and response to treatment. Genotypes 1-3 are distributed worldwide, genotypes 4 and 5 are mostly found in Africa and genotype 6 is more prevalent in Asia <sup>1</sup>. Genotype 1 is the most common genotype in Europe and the United States while genotype 3a is common in European intravenous drug users <sup>5</sup>. HCV is endemic in some countries such as Egypt where there is a very high burden of infection with genotype 4, with one in ten individuals estimated to be chronically infected <sup>6</sup>.

In patients chronically infected with HCV, progression of liver disease occurs over several decades, and is influenced by co-factors such as obesity, alcohol consumption and co-infection with human immunodeficiency virus (HIV) or Hepatitis B virus (HBV). Between 10% and 20% of patients with chronic HCV infection will develop cirrhosis, characterised by replacement of healthy tissue with fibrotic, scar tissue <sup>3</sup>. The liver is capable of regeneration and can compensate for the liver damage. However, once cirrhosis occurs, there is a risk of the development of hepatic decompensation where the liver is damaged and ceases to function. Development of hepatocellular carcinoma (1% to 3% per year) is also a potential consequence of chronic HCV infection <sup>7</sup>. In the case of either decompensated liver cirrhosis or hepatocellular carcinoma, a liver transplant is necessary. In countries such as the US, the demand for liver transplants outweighs the organs available and HCV almost always re-infects the liver upon transplantation of a donor organ <sup>8</sup>.

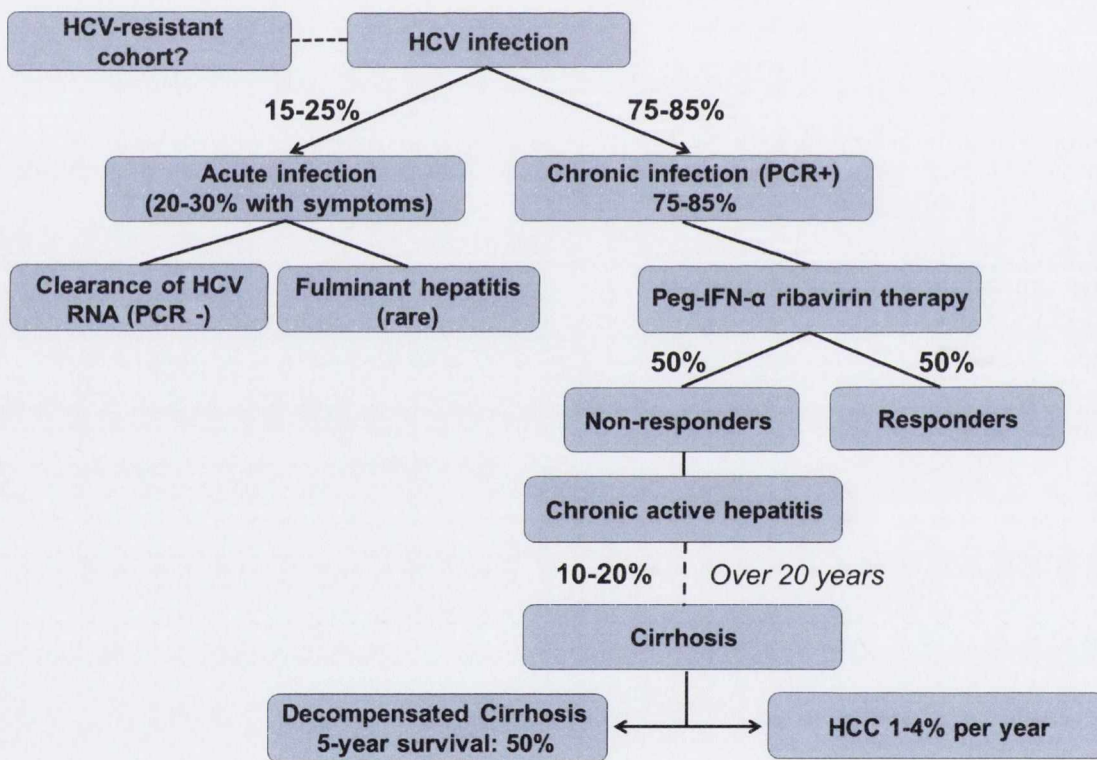
The current standard treatment for HCV infection is pegylated interferon alpha (IFN- $\alpha$ ) and ribavirin. IFN- $\alpha$  is a critical component of the innate immune antiviral response, activating interferon-stimulated genes (ISGs), which establishes an antiviral state within cells. Ribavirin is a nucleoside analogue which is incorporated into the RNA of replicating virions, thereby increasing the mutation frequency and reducing the specific infectivity of new virions <sup>9</sup>. Various alternative mechanisms have been described regarding the mechanism of action of ribavirin, including immunomodulation promoting Th1 over Th2 phenotype and direct inhibition of HCV RNA polymerase. HCV genotype has an important impact on the patient response to treatment, with genotype 1 generally being considered



the most difficult to treat. Therapeutic response is variable in infected individuals, with, on average less than 50% of genotype 1-infected individuals responding to treatment. In contrast, an almost 80% response rate is observed in those infected with HCV genotype 3<sup>10</sup>. The IFN- $\alpha$  treatment regime involves a 24 or 48-week course of pegylated (peg)-IFN- $\alpha$  and ribavirin, depending on virus genotype, and is associated with significant side-effects such as flu-like symptoms, fever, headaches, cytopaenias, fatigue, anorexia, depression and anxiety, which can result in discontinuation of therapy<sup>11</sup>. Response to IFN- $\alpha$  therapy is known as sustained virological response (SVR), which is defined as being HCV RNA negative 6 months post-treatment<sup>12</sup>. SVR is associated with a 99% chance of being HCV RNA negative during long-term follow-up<sup>13</sup>. As yet, there is no available vaccine for HCV but recent progress has been made in the development of more effective therapies such as protease inhibitors<sup>14</sup>. Nevertheless, HCV is still a substantial global health burden, and remains the primary indication for liver transplantation in the US and Europe<sup>8</sup>.

The high rate of progression to chronic HCV infection is partially due to the genetic diversity of the virus and its tendency towards rapid mutation, allowing HCV to escape immune recognition. HCV replicates rapidly during infection with up to  $10^{12}$  viruses estimated to be produced daily in an infected individual<sup>15</sup>. This rapid replication, along with the high error rate of virus transcription generates quasispecies, facilitating viral immune escape. In addition to differences in viral genotype, it has been documented that individuals exposed to the same viral genotype from a single source have greatly divergent outcomes to infection<sup>16</sup>. The variation observed in outcome to HCV infection begs the question: why are some

people able to clear the infection naturally without treatment while others develop chronic infection? And of those who undergo treatment, what determines whether or not there will be a successful outcome? This would point to host variation playing a major role in outcome. Understanding the genetic basis of immune variation may offer predictors of viral clearance and we could learn more about antiviral immune response which could have implications for many viral infections.



**Figure 1.1: Differential clinical outcome to HCV infection**

Approximately 20% of individuals infected with hepatitis C virus (HCV) are capable of clearing the infection whereas the majority develop chronic infection. Fulminant liver injury, resulting in acute liver failure, is rare and occurs in less than 1% of patients. Of those chronically infected, only ~50% respond to interferon alpha-ribavirin therapy (genotype 1). Those who remain chronically infected potentially develop cirrhosis over a period ranging up to a couple of decades, with chronic active hepatitis capable of leading to hepatocellular carcinoma or decompensated liver cirrhosis, thus requiring liver transplantation.

Adapted from <sup>3</sup>



## 1.2 Hepatitis C virus

HCV was first identified as the causative agent of non-A, non-B hepatitis following cloning of the novel virus in 1989 <sup>17</sup>. HCV is an enveloped positive sense, single-stranded (ss)RNA virus of ~9.6 kb in length and is part of the genus *Hepacivirus* in the *Flaviviridae* family. Following the discovery of HCV, study of the virus was initially hampered by the inability to culture the virus *in vitro* and the absence of a small animal model. Complete replication in culture was not achieved until 2005, when a strain of HCV derived from a case of fulminant hepatitis was shown to replicate in a hepatoma cell line <sup>18</sup>. Availability of the replicon system, and the more recently described humanised mouse model <sup>19</sup>, has furthered progress in understanding virus life cycle which ultimately has led to elucidating novel therapeutic targets.

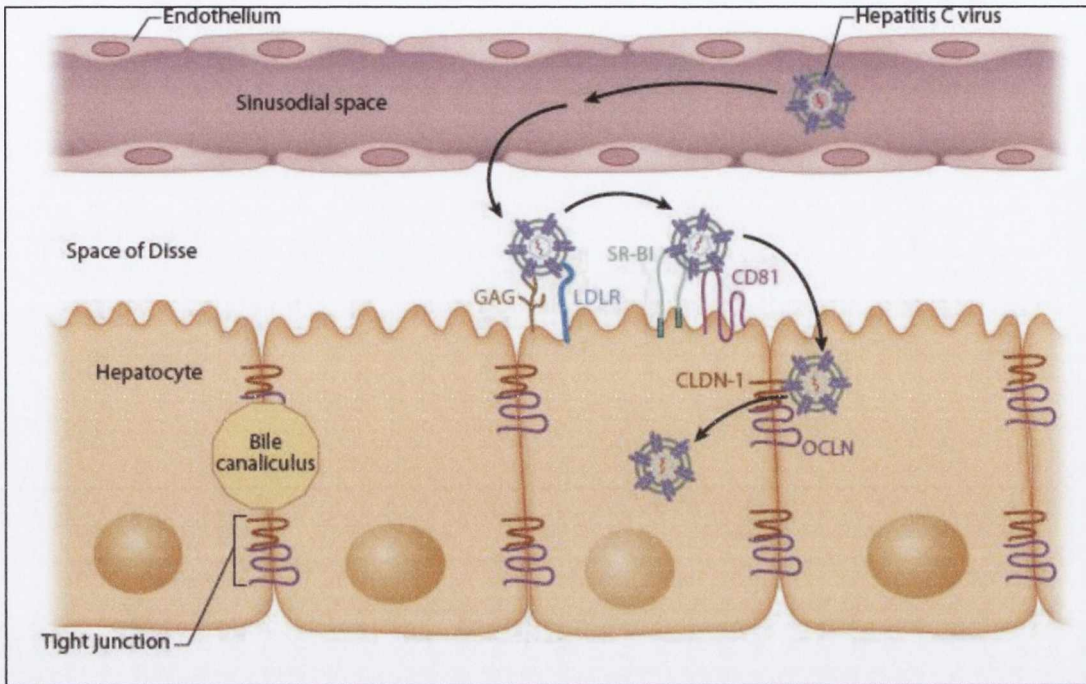
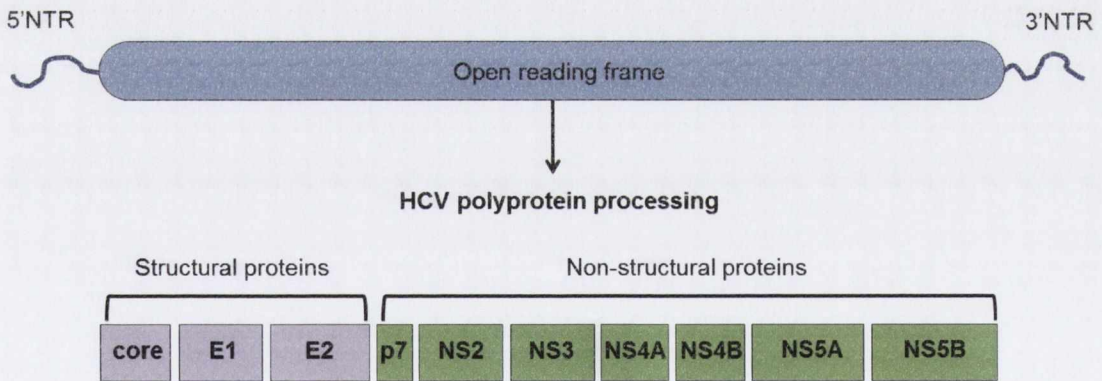
### 1.2.1 HCV entry and life cycle

Although evidence is continuing to emerge that HCV can replicate in extra-hepatic areas such as in peripheral blood mononuclear cells (PBMCs) <sup>20</sup> and the nervous system <sup>21</sup>, the virus is hepatotropic, primarily targeting the liver. HCV is an enveloped virus that displays two glycoproteins on its surface, the E1 and E2 proteins, which are responsible for receptor binding. The proteins are embedded in a lipid bilayer surrounding a nucleocapsid composed of core protein and the genomic RNA. The virus is often associated with serum lipoproteins which play a role in entry, virus assembly and possibly in immune evasion <sup>22</sup>. Glucosaminoglycans (GAGs) and low density lipoprotein receptors (LDLRs) on the hepatocyte surface are thought to mediate initial attachment. The virus enters the



bloodstream and then migrates to the liver, passing through the endothelium and the space of Disse before entering hepatocytes. The virus particle enters the hepatocyte via interaction with GAGs, LDLRs <sup>23</sup>, the scavenger receptor class B type 1 (SR-B1) and the tetraspanin CD81 on the basolateral surface of the cell <sup>24</sup>, followed by movement to the tight junctions formed by claudin (CLDN-1) <sup>25</sup> and occludin (OCLN) <sup>26</sup> (**Figure 1.2**). DC-SIGN (CD209) and DC-SIGN-R have also been shown to have a role as a receptor for HCV by binding to HCV E2 glycoprotein <sup>27-29</sup>. DC-SIGN is expressed on some dendritic cells, while DC-SIGNR is localized to certain endothelial cell populations, including hepatic sinusoidal endothelial cells.

Following viral entry into hepatocytes by endocytosis, HCV translation is initiated through an internal ribosomal entry site (IRES) and generates a single polyprotein of ~3,000 amino acids. This polyprotein is cleaved by cellular (signal peptidase and signal peptide peptidase) and viral proteases (NS2/3 and NS3/4A) to give ten proteins; three structural (core protein and envelope proteins E1 and E2), the small hydrophobic protein, p7, which is thought to function as an ion channel and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B), which coordinate the intracellular process of the HCV life cycle <sup>30</sup>. Following synthesis and maturation, non-structural proteins and viral RNA form membrane-associated replication complexes, producing a negative-stranded RNA copy of the genome, which is used to form positive-strand RNA as the virus replicates. Capsid proteins and genomic RNA assemble to form a nucleocapsid which buds through intracellular membranes into cytoplasmic vesicles. Enveloped mature virions then leave the cell via the secretory pathway <sup>31</sup>.

**A****B****Figure 1.2: HCV entry and genome**

(A) The HCV virus particle enters the hepatocyte via interaction with glucosaminoglycans (GAGs), low density lipoprotein receptors (LDLR), the scavenger receptor class B type 1 (SR-B1) and the tetraspanin CD81 on the basolateral surface, followed by movement to the tight junctions formed by claudin (CLDN-1) and occludin (OCLN). (B) Following entry into the cell and uncoating, HCV RNA is translated into a polyprotein of ~3,000 amino acids and is cleaved to 10 mature proteins.

Figure A taken, and figure B, adapted from <sup>22</sup>



Understanding the HCV replication and life cycle has led to a recent revolution in HCV therapy with the approval of boceprevir and telaprevir, two direct-acting antiviral agents (DAAs) against the NS3/4A serine protease for use in treating genotype 1 HCV. DAAs with different viral targets, including NS3 protease inhibitors, nucleoside/nucleotide analogues and non-nucleoside inhibitors of the RNA-dependent RNA polymerase, and NS5A inhibitors are in development <sup>22</sup>. While these drugs are showing promising results, there are concerns about drug resistance, requiring that a cocktail of drugs be administered, and significant side-effects are still observed. At the moment the use of DAAs is restricted to genotype 1 infection therefore necessitating the need to develop a drug regime suitable for all viral genotypes. Peg-IFN- $\alpha$ -ribavirin still remains the backbone of all HCV therapy, continuing to be administered alongside the newer DAAs.

### **1.3 Antiviral immune response**

Viruses are particularly adept at gaining entry to the body and possess many mechanisms of evading host detection and persisting to cause chronic infection. However, the host immune response is also equipped with a myriad of immune processes capable of mounting a successful immune response, limiting host damage and eradicating viral infection. The host immune response has evolved exquisite mechanisms of distinguishing self from non-self and commensal microbial agents from pathogenic invaders, thus protecting the body from infection with viruses and other pathogens. This detection is mediated by microbial sensing pathways, capable of maintaining host-microbe homeostasis required to tolerate commensal and non-pathogenic antigens, yet with the potential to induce anti-microbial defence mechanisms. The immune system is



generally divided into two interlinked systems – the innate immune response and the adaptive immune response. The very first level of immune defence is the epithelial barriers on skin and mucosal membranes such as gastrointestinal tract etc., protected by the secretion of mucus and various anti-microbial peptides. However if these barriers are breached, the innate immune response is poised to recognise and respond to the invariant features of the invading pathogen. Cells of the innate immune system include dendritic cells (DCs), macrophages, natural killer (NK) cells, the granulocytes neutrophils, eosinophils, basophils and mast cells as well as a number of innate lymphoid cells. This response is instantaneous and powerful but essentially non-specific. If the microorganism succeeds in overcoming the innate immune response and persists, the adaptive immune response is employed, a process which takes days to weeks compared to the rapid innate response. The adaptive immune response comprises B and T lymphocyte responses which are highly specific to target the invading microbe.

### **1.3.1 Innate immune response**

#### *Macrophages*

Macrophages are resident phagocytic cells in lymphoid and non-lymphoid tissues and are believed to be involved in steady state tissue homeostasis, via the clearance of apoptotic cells. For example, a large population of macrophages called Kupffer cells are resident in the liver. Macrophages are equipped with a broad range of pathogen recognition receptors (PRRs) that make them efficient at phagocytosis and inflammatory cytokine production. Upon PRR activation,

macrophages up-regulate major histocompatibility complex (MHC) II on their surface, thus allowing them to present antigen to activate T cell responses.

### *Dendritic cells*

DCs are important phagocytic cells found in small numbers in the circulation and throughout tissues regularly exposed to infectious agents such as the skin, lungs, gastrointestinal tract and the liver. DCs are pivotal in pathogen recognition, expressing high levels of PRRs and transcription factors poised to initiate an immune response upon detection of invading microorganisms. DCs are critical to successful antiviral immunity, being potent producers of the antiviral cytokine interferon and stimulators of the adaptive immune response. In addition to activating immunity, another important function of DCs is promoting tolerance to self-antigens. Although rare, DCs, in particular myeloid DCs, are considered the most highly specialised or 'professional' antigen-presenting cell (APC), capable of providing T cells with all three signals required for their activation <sup>32</sup>. DC activation by pathogen up-regulates the co-stimulatory molecules CD80 and CD86 on their surface, providing the signals necessary for lymphocyte activation in addition to those provided through the antigen receptor. DCs are also motile and, when activated following antigen capture, can migrate to the lymph node where they interact with T cells to mount an adaptive immune response.

### *NK cells*

NK cells are important innate immune cells, capable of killing virally-infected and malignant cells. NK cells can kill their targets by releasing their cytotoxic granules onto the surface of a bound target cell, as determined by altered MHC I expression,



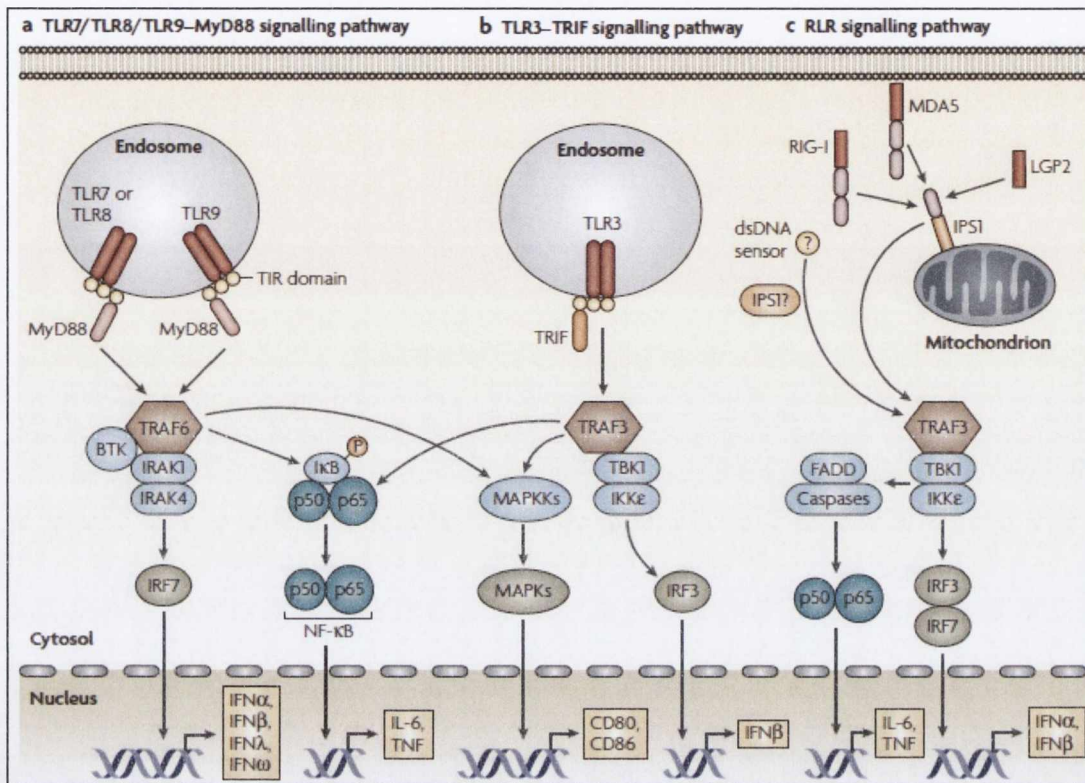
or by another mechanism known as antibody-dependent cellular cytotoxicity through binding of their Fc receptors to antibody-coated pathogens. NK cells can be activated by Type I IFNs and IL-12, both of which have been shown to increase NK cell killing. In humans, NK cells can be divided into CD56<sup>dim</sup> and CD56<sup>bright</sup> populations; around 90% of peripheral blood and spleen NK cells are CD56<sup>dim</sup> CD16<sup>+</sup> and express perforin. These CD56<sup>dim</sup> NK cells are cytotoxic and produce IFN- $\gamma$  upon interaction with tumour cells *in vitro*. In contrast, most NK cells in lymph nodes and tonsils are CD56<sup>bright</sup>CD16<sup>-</sup> and lack perforin<sup>33</sup>. These cells readily produce cytokines such as IFN- $\gamma$  in response to stimulation with interleukin IL-12, IL-15 and IL-18.

#### **1.3.1.1 Pattern Recognition Receptors**

The immune system recognises invading viruses via PRRs expressed on various immune cells which sense pathogen-associated molecular patterns (PAMPs) such as viral nucleic acids. PRRs also detect damage associated molecular patterns (DAMPs) from cell components released during cell damage and death. The major families of PRRs capable of detecting viruses are Toll-like receptors (TLRs) and Retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). C-type lectins (CLRs) are an additional family of PRRs, capable of sensing DAMPs, as well as PAMPs, in cytopathic viral infection. For example, C-type lectin domain family 9A (CLEC9A) is capable of recognising necrotic cells and presenting dead cell-associated antigens to activate T cells<sup>34</sup>. The TLRs are a family of transmembrane proteins, among which, several are capable of detecting viral components; TLR3, TLR7, TLR8 and TLR9 are situated on the endosomal membranes and detect viral nucleic acid. TLR3 recognises double stranded (ds) RNA, TLR7/8 recognises ssRNA and



TLR9 recognises viral DNA containing CpG motifs. TLR2 and TLR4 are present on the cell surface and can recognise viral envelope proteins <sup>35</sup>. The RLRs are cytosolic receptors consisting of three family members; Retinoic acid inducible gene-I (RIG-I), melanoma differentiation-associated gene (MDA) 5 and, the most recently described, Laboratory of Genetics and Physiology 2 (LGP-2). RIG-I is activated by the 5'-triphosphate (5'-PPP) of viral dsRNA <sup>36</sup> whereas MDA5 detects long dsRNA <sup>37</sup>. An important distinction between TLRs and RLRs is that TLRs are expressed in a cell-type specific manner, mainly on DCs, thereby allocating recognition capabilities to various cell types <sup>38</sup>. In contrast, the RLRs are ubiquitously expressed. While PRR recognition of RNA viruses is well established, sensors of DNA viruses have only recently been discovered. These DNA PRRs include DNA-dependent activator of IFN-regulatory factors (DAI), Absent in Melanoma 2 (AIM2) and IFN-inducible protein 16 (IFI16), among others <sup>39</sup>. Engagement of the PRRs leads to the recruitment of adaptor molecules; all TLRs activate a common signalling pathway via myeloid differentiation factor 88 (MyD88). TLR3, however, recruits TIR domain-containing adaptor inducing IFN- $\beta$  (TRIF). Activation of RLRs leads to signalling through the adaptor protein mitochondrial antiviral signalling (MAVS). Recruitment of adaptors to their cognate receptors triggers the downstream activation of the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), Interferon Regulatory Factors (IRF) -3 and -7 and Activator Protein-1 (AP-1) leading to the transcription of pro-inflammatory cytokines and Type I IFNs such as IFN- $\alpha$  and IFN- $\beta$  (**Figure 1.3**).



**Figure 1.3: Pathogen recognition receptor sensing of viral infection**

Viral RNA is detected by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and RIG-I like receptors (RLRs), while viral DNA is detected by the DNA sensors of IFN-regulatory factors (DAI), Absent in Melanoma 2 (AIM2) and IFN-inducible protein 16 (IFI16) (not shown). Endosomal TLR7, TLR8 and TLR9 signal through the adaptor molecule myeloid differentiation factor 88 (MyD88), which leads to the activation of interferon regulatory factor (IRF)-7, nuclear factor kappa B (NF- $\kappa$ B) and mitogen-activated protein kinases (MAPKs), resulting in the production of IFNs and pro-inflammatory cytokines. TLR3 senses double stranded RNA and signals through the adaptor TIR domain-containing adaptor inducing IFN- $\beta$  (TRIF), activating IRF-3, leading to IFN- $\beta$  production. This pathway also activates NF- $\kappa$ B, resulting in the production of pro-inflammatory cytokines. Retinoic acid inducible gene-1 (RIG-I), melanoma differentiation-associated gene (MDA) 5 and Laboratory of Genetics and Physiology 2 (LGP-2) signal through the common adaptor mitochondrial antiviral signalling (MAVS) to activate IRF-3 and NF- $\kappa$ B, resulting in the production of Type I IFNs and other cytokines.

Figure taken from <sup>40</sup>



### 1.3.1.2 Interferons

The reliance on IFN- $\alpha$  for treatment of HCV emphasises the important role this cytokine plays in antiviral immunity. IFNs play a major role in antiviral immunity by activating antiviral mechanisms including induction of antiviral proteins and activation of NK cells which kill virally-infected cells. IFNs thereby act to inhibit viral replication and protect neighbouring cells during the early stages of infection. The innate immune response is therefore often capable of clearing viral infection without requiring the generation of an adaptive immune response. The IFN family consists of the Type I, Type II and Type III IFNs. Type I IFNs were first described in 1957 as an agent that 'interfered' with viral infection of cells that had already been infected with a different virus. This soluble factor could then be transferred to render uninfected cells resistant to viral infection <sup>41</sup>. Type I IFNs are comprised of 12 subtypes of IFN- $\alpha$  and one subtype each of IFN- $\beta$ , IFN- $\epsilon$ , IFN- $\kappa$ , and IFN- $\omega$  <sup>42</sup>. Type I IFNs also combat viral infection indirectly by stimulating the adaptive immune system via increase of MHC class I molecule on all cells, thus aiding cytotoxic lymphocyte (CTL) killing of cells expressing MHC I complexed with viral peptide <sup>43</sup>. While Type I IFNs can be produced by any virally-infected cell, it is known that plasmacytoid DCs (pDCs) are the major IFN-producing cells in the body, capable of producing up to one thousand times more IFN- $\alpha$  than any other cell type <sup>44</sup>. Type II IFNs consist of the sole member, IFN gamma (IFN- $\gamma$ ), which promotes NK cell activity and activates macrophages. The more recently described Type III IFNs or the IFN-lambdas (IFN- $\lambda$ s) consists of the cytokines IL-28A, IL-28B and IL-29, which are also produced in response to viral infection and are thought to act in a similar way to Type I IFNs <sup>45, 46</sup>.



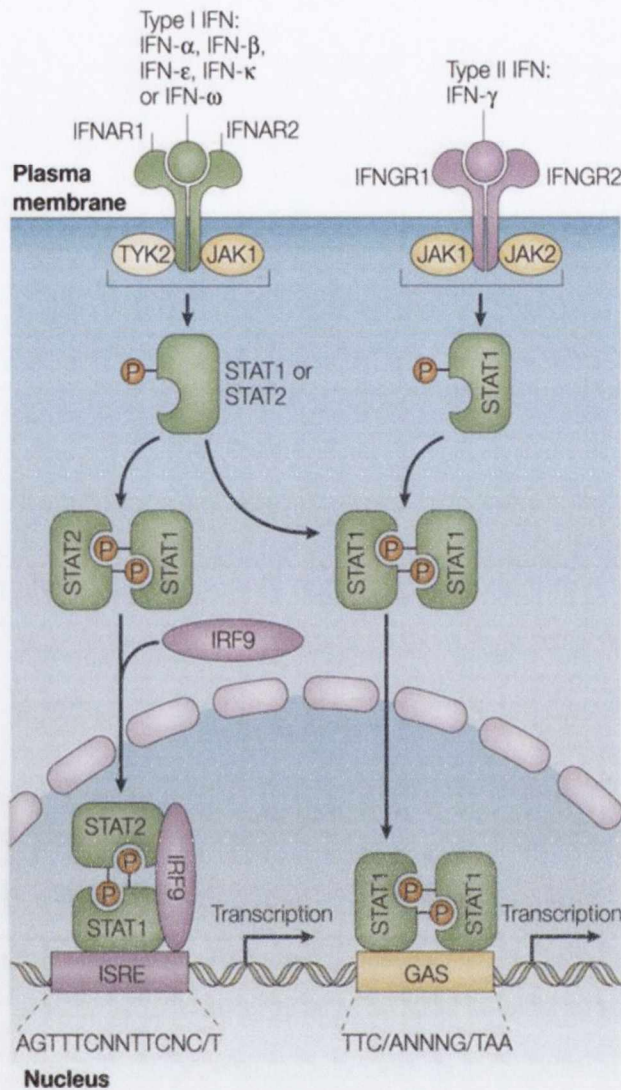
### 1.3.1.3 Signalling pathways activated by IFNs

Following production from an infected cell, Type I IFNs signal through the Janus Kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) pathway to up-regulate antiviral genes and protect uninfected cells. Type I IFN signalling is initiated by binding of cytokine to their cell surface receptor composed of IFNAR1 and IFNAR2c, a specific splice variant. The IFNAR consists of an extracellular ligand binding domain and an intracellular kinase domain, activated after ligand-induced dimerization. Each receptor subunit binds constitutively to JAKs, IFNAR1 to tyrosine kinase 2 (TYK2) and IFNAR2 to JAK1. Ligand binding induces the phosphorylation of JAK1, TYK2 and STATs which direct the phosphorylation and assembly of STAT homodimers or heterodimers. There are seven STAT proteins, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT 6. STATs are proteins present latent in the cytoplasm which become activated by phosphorylation and subsequently function as transcription factors, translocating to the nucleus where they bind to specific DNA sequences to activate gene expression. IFN- $\alpha$  is unique in that it can activate all seven depending on conditions and cell type, forming many combinations of hetero- or homo- dimer pairs <sup>47</sup>. This explains how Type I IFNs can stimulate anti-proliferative and immunoregulatory responses as well as antiviral responses. Activated STATs dimerise, dissociate from the receptor and translocate to the nucleus to induce the expression of ISGs. Type I IFNs activate a major transcription factor, IFN-stimulated gene factor 3 (ISGF3), a complex of phosphorylated STAT1, STAT2 and unphosphorylated IRF-9. The ISGF3 complex translocates to the cell nucleus, where it binds to the IFN-sensitive response element (ISRE) (**Figure 1.4**). ISREs are highly conserved sequences of 12-15bp which are present in the promoters of many target genes of IFN to direct the

expression of ISGs, the genetic effectors of the host response <sup>47, 48</sup>. In all, over five hundred genes are up-regulated by IFN- $\alpha$ , a proportion of which are antiviral <sup>43</sup>. The IFN-signalling proteins such as IRF-7, RIG-I, MDA-5 and STAT1 are also ISGs themselves, thus amplifying JAK-STAT signalling.

#### **1.3.1.4 Interferon-stimulated genes: antiviral effectors**

Of the hundreds of genes upregulated by IFN, the exact function of only a fraction of these genes is known. Some of the best characterised antiviral genes include *2'-5' oligoadenylate synthetase (OAS)*, *Myxovirus resistance (MxA) protein* and *Protein kinase R (PKR)*. For example, OAS enzymes activate the cytoplasmic ribonuclease RNase L which cleaves viral RNA <sup>49</sup>. MxA is a GTPase involved in blocking viral gene transcription by trapping essential viral components and degrading them <sup>50</sup>. PKR inhibits viral replication by phosphorylating and thereby inhibiting factors required for translation.



**Figure 1.4: Interferon signalling through the JAK-STAT pathway**

Type I and Type II interferons (IFNs) bind to their receptors. The Type I IFN receptor is composed of the IFNAR1 and IFNAR2 chains whereas Type II IFNs bind to IFNGR1 and IFNGR2. Ligand binding induces the phosphorylation of receptor-associated JAK proteins (JAK1 and TYK2 on IFNAR; JAK1 and JAK2 on IFNGR) leading to the phosphorylation and assembly of STAT homodimers or heterodimers. The Type I IFNs activate a major transcription factor, IFN-stimulated gene factor 3 (ISGF3), a complex of phosphorylated STAT1, STAT2 and unphosphorylated IRF-9. The ISGF3 complex translocates to the cell nucleus, where it binds to the IFN-sensitive response element (ISRE) in the promoter regions of IFN stimulated genes (ISGs). IFN- $\gamma$  signalling activates STAT1 homodimers which bind to gamma activated sequences (GAS) to activate gene transcription.

Figure taken from <sup>51</sup>



### **1.3.2 Adaptive immune response**

Persistence of viral infection requires the employment of the adaptive immune response. T and B lymphocytes are the major effector cells of this arm of the immune response. The adaptive immune response is highly specific and tailored towards clearance of the particular pathogen causing infection. B cells are important for the generation of antibodies against viruses. B cells can recognise pathogens directly via their B cell receptor (BCR) and can act as APCs. B cells, with the help of signals from T cells, differentiate into plasma cells capable of making virus-neutralising antibodies, which forms the basis for many current viral vaccinations. T cells also have a very important role in antiviral immunity. The role of CD8<sup>+</sup> cytotoxic T cells is well characterised. They recognise viral peptides complexed to MHC I on infected cells and can kill cells via release of granzyme and perforin. CD4<sup>+</sup> T cells also play a role in antiviral adaptive immunity, providing help for B cells and CD8<sup>+</sup> T cells but many T cell subsets are also involved, with virus-specific Th1 and Th17 cells promoting an inflammatory response <sup>52</sup>. Additional more recently described T cell subsets include Th9 cells (secrete IL-9), Th22 cells (secrete IL-22) and the specialised B cell helpers, T follicular cells. To mobilise the adaptive immune response into action, signals from the innate immune response are required, showing how these two fractions of immunity are intertwined.

#### **1.3.2.1 Dendritic cells: link from innate to adaptive immunity**

DCs play an essential role in initiating antigen-specific immunity and tolerance. DCs are unique in that they are the only cell type capable of activating naïve T cells.

While macrophages and B cells also function as APCs, they are only capable of expanding T cells that have already been activated<sup>53</sup>. Thus, DCs are considered 'professional' APCs and are essentially the initiators and modulators of the immune response. DCs are capable of sensing pathogen via PRRs which activates the DC, causing maturation and subsequent migration to T cell rich areas to initiate an adaptive immune response. Pathogens detected by PRRs on DCs are phagocytosed and processed and antigen presented in the context of MHC molecules. The antigen is processed intracellularly into short peptides by means of proteolytic cleavage before it is presented by MHC molecules on the surface of DCs. MHC class I is present on all nucleated cells whereas MHC class II is present only on APCs. DCs process and present pathogens in the context of MHC II but are also capable of processing antigens and presenting to CD8<sup>+</sup> cytotoxic T cells via MHC class I, a process termed cross-presentation<sup>54</sup>. The MHC class II molecules present the peptides to the T cell receptor (TCR) on the surface of helper T cells, thus linking the innate and adaptive immune systems. This presentation occurs in the context of several signals that are induced by the TCR and that are required for naïve T cell activation, including co-stimulatory signals and cytokines.

### **1.3.2.2 Dendritic cell activation of T cell responses**

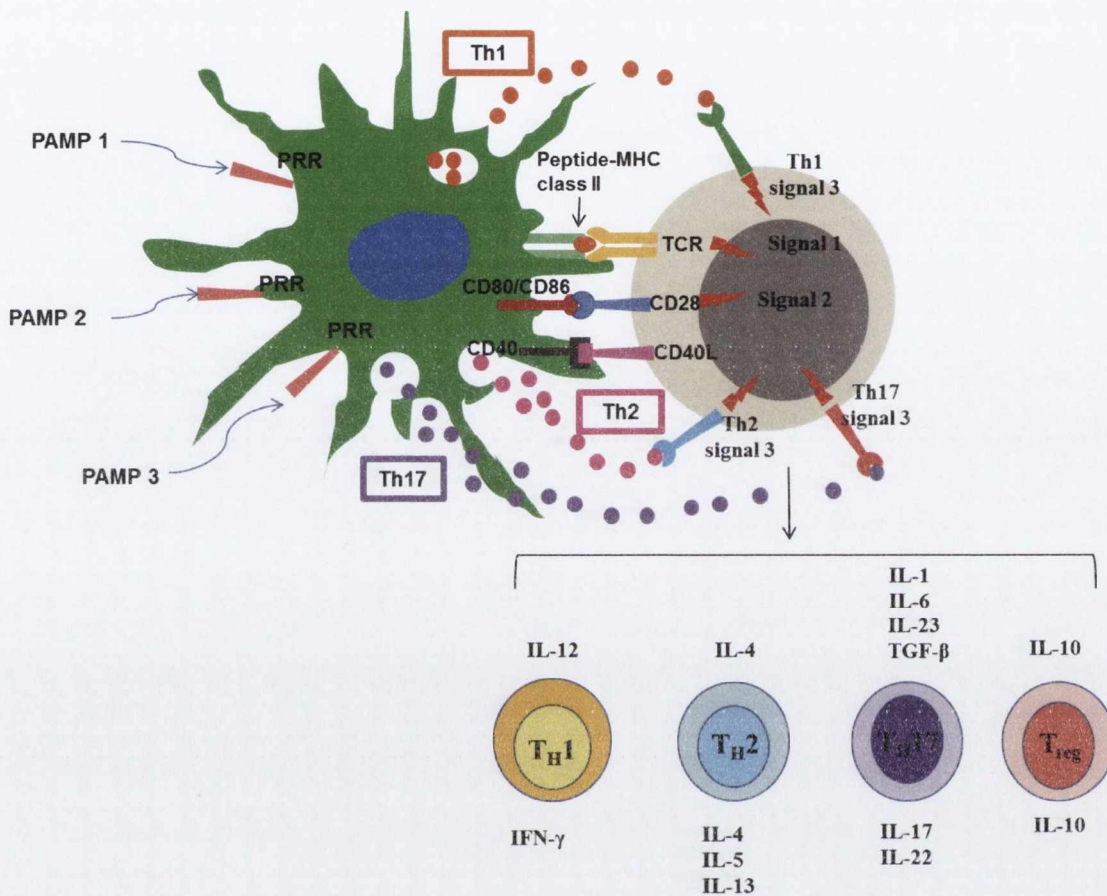
DCs are capable of delivering the three signals required to activate naïve T cells. The TCR on T cells recognises peptide fragments (antigen) bound to the MHC molecules expressed on the DC. Signal 1 is generated when the TCR engages an appropriate peptide-MHC complex. Binding of MHC I-peptide complexes activates CD8<sup>+</sup> cytotoxic lymphocytes whereas binding of MHC II-peptide complexes initiates a CD4<sup>+</sup> T helper response. CD4<sup>+</sup> T helper cells are the major effector cell



of the adaptive immune response, capable of providing help to B cells to produce antibodies and promote the development of CD8<sup>+</sup> cytotoxic lymphocytes which kill infected or transformed cells. Signal 2 is referred to as 'co-stimulation' and involves interaction of CD28 on T cells with CD80/CD86 on APCs. Together, signal 1 and signal 2 favour immunity, with the absence of co-stimulatory signal leading to T cell anergy and tolerance <sup>32</sup>.

Signal 3 refers to signals delivered from the APC to the T cell that determine its differentiation into an effector cell. DC activation of T cells leads to T cell clonal expansion and differentiation into effector cells. CD4<sup>+</sup> T helper cells can differentiate into a number of different effector subtypes, all with functional specialities: T helper 1 (Th1) cells, Th2 cells and Th17 cells (**Figure 1.5**). IL-12 is an example of a mediator that delivers a signal 3 that can promote Th1-cell development which is responsible for cell-mediated immunity. Although not known to be produced by DCs, it is established that IL-4 promotes the development of Th2 cells which produce the Th2 cytokines IL-4, IL-5 and IL-13 and play a role in defence against helminths. One of the most recently described Th subsets is Th17 T cells, which are driven by cytokines such as TGF- $\beta$ , IL-1, IL-6 and IL-23. Th17 cells produce IL-17 and play a role in antimicrobial defence against extracellular bacteria. T regulatory (T reg) cells are another type of CD4 cell which are driven by and promote anti-inflammatory cytokines such as IL-10 to dampen down immune responses, promote tolerance and prevent autoimmunity.





**Figure 1.5: Dendritic cell activation of T cell responses**

T cell responses are governed by the type of pathogen associated molecular patterns on invading pathogens recognised by pattern recognition receptors on DCs. This signal leads to upregulation of MHC class II and co-stimulatory molecules CD80/CD86 and CD40 which interact with the T cell receptor, CD28 and CD40L respectively on T cells. DCs can take up and process pathogens and present peptides complexed with MHC II on the cell surface. DCs also secrete cytokines which promote differentiation of T cells into the required T cell subset. Interaction with the TCR and costimulatory molecules as well as secretion of T cell polarising cytokines leads to the activation of antigen-specific T cells. IL-12 promotes Th1 cells, IL-4 Th2 cells, IL-6/IL-23 Th17 cells and IL-10 T regulatory cells. The signature cytokines secreted by each of the T cell subsets is indicated.

Figure adapted from <sup>55</sup>

## **1.4 Immune response to HCV infection**

### **1.4.1 Hepatic immune system**

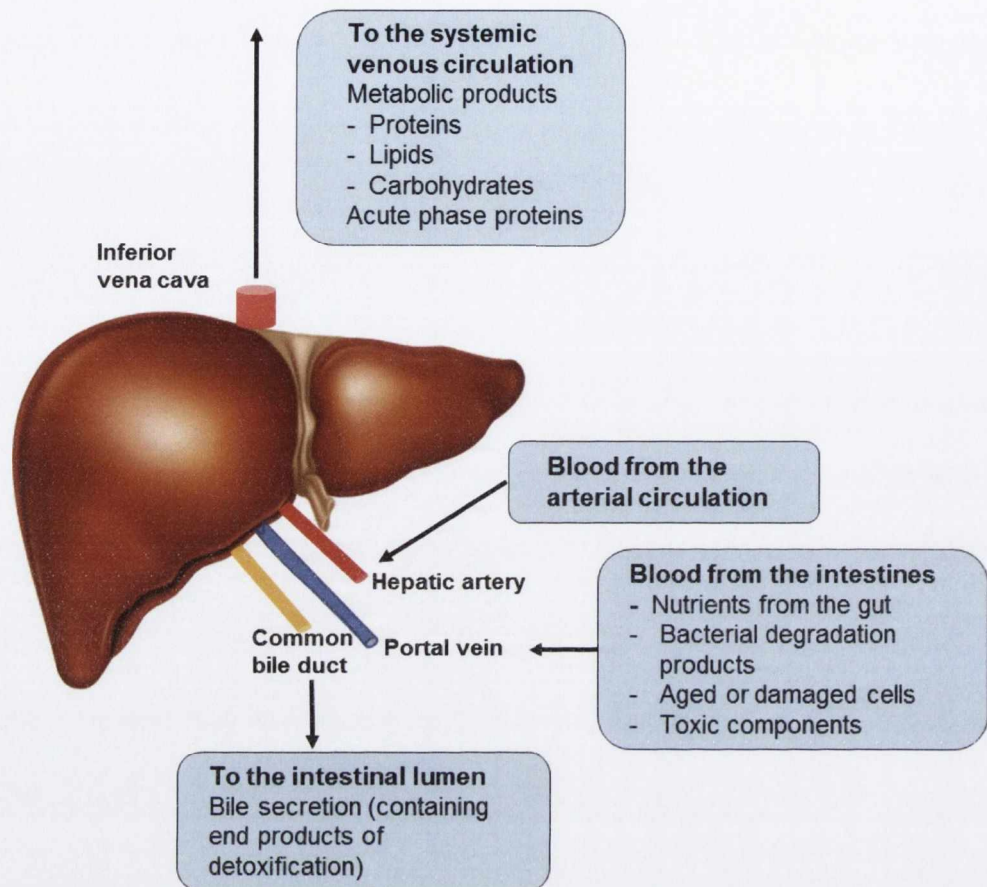
The HCV virus is hepatotropic and the liver is the main site of viral replication and HCV-related disease development. The liver has two major blood supplies; the majority of the blood arrives in the liver (80%) via the portal vein and originates from the intestines. The additional 20% of blood is supplied to the liver via the hepatic artery from the systemic circulation, therefore meaning that a mixture of arterial and venous blood occurs in the liver. This leads to low oxygen tension, low perfusion pressure and slow and irregular blood flow. Following entry of blood into the liver, it passes through hepatic sinusoids, finally exiting via the inferior vein<sup>56,57</sup>. The anatomy and functions of the liver are described in **Figure 1.6**.

The liver is now recognised to have a major immunological function including its role as a tolerogenic organ<sup>58</sup>, being able to tolerate many antigens from the products of digestion, along with antigens and microbial products from bacteria in the intestines, including lipopolysaccharide (LPS) in the cell wall. In support of its tolerogenic potential, liver transplantation requires less immunosuppressive drugs and results in less rejection episodes than with other organs<sup>59</sup>. Furthermore, transplantation of another organ at the same time as a liver transplant makes it more likely that the other organ will be accepted, suggesting that the liver confers tolerance and may have a role in central tolerance in the immune system<sup>60</sup>.

These factors therefore make the liver an attractive site for persistent infection due to its rich source of nutrition and oxygenated blood supply. Although most

pathogens that reach the liver via the blood are eliminated or controlled by the local liver innate and adaptive immune responses, some pathogens escape immune control and persist in the liver, such as HCV, HBV and malaria parasites, which target the liver to establish chronic infection. Therefore, the liver immune system is required to switch between tolerating innocuous antigen from the gut and recognising harmful hepatotropic pathogens. We do know that the liver immune response is capable of clearing infection based on the self-limiting nature of Hepatitis A virus (HAV) infection and the clearance, in a proportion of people, of HBV and HCV <sup>61</sup>. It is therefore vital to study HCV infection in terms of the local hepatic immune response.





**Figure 1.6: Anatomy and functions of the liver**

The major function of the liver is in the metabolism of carbohydrate, lipid and protein and the breakdown of toxic waste products. The majority of the liver's blood supply is derived from the intestines, entering via the hepatic vein while blood from the arterial circulation is delivered to the liver through the hepatic artery. Blood perfuses through the liver sinusoids and exits via the inferior vein.

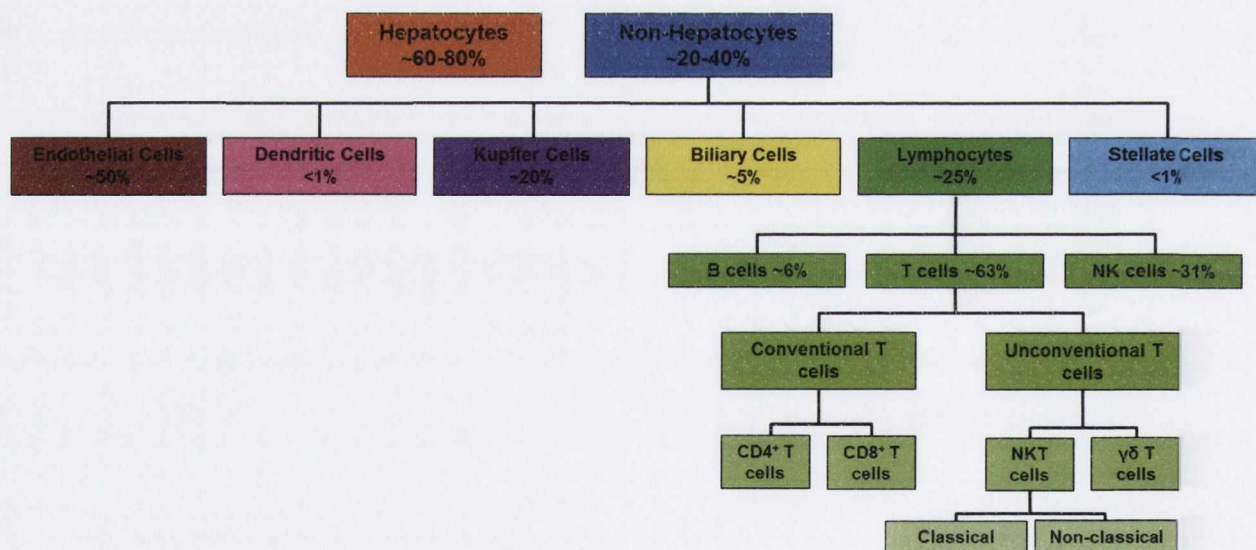
Figure adapted from <sup>56</sup>

### *Cell populations in the liver*

Extensive populations of immune cells in the liver are critical for its role in detecting, initiating and regulating innate and adaptive immunity to hepatotropic pathogens. Key components of the immune system present in the liver include liver-specific macrophages, known as Kupffer cells, DCs, as well as NK cells, and Natural Killer T (NKT) cells <sup>62</sup>. Kupffer cells are resident and immobile hepatic macrophages that are located in the sinusoidal lumen and are the most abundant APC present in the liver. Kupffer cells are activated by IFN- $\gamma$  and produce inflammatory mediators such as IL-12 and IL-18. Additional liver APCs are liver sinusoidal endothelial cells (LSECs), stellate cells and DCs, found preferentially in the periportal and pericentral area <sup>63</sup>. It has been established that liver DCs have an immature phenotype and are therefore poor inducers of adaptive immunity. Continuous exposure to LPS and other gut-derived antigens is thought to induce a state of endotoxin tolerance, owing in part to decreased expression of TLR4 <sup>64, 65</sup>. These tolerogenic DCs produce increased IL-10, lower IL-12 and have an immature phenotype, expressing low co-stimulatory molecules and are poor stimulators of naive allogeneic T cells <sup>56</sup>. The cytokine microenvironment also contributes to the tolerogenic phenotype of hepatic DCs, with IL-10 and TGF- $\beta$  being constitutively expressed by Kupffer cells and LSECs <sup>66</sup>.

The liver contains many populations of conventional and unconventional lymphocytes, being selectively enriched with NK and NKT cells, which play critical roles in the innate immune defence against invading pathogens. NK cells are important innate immune cells in the liver, capable of killing virally-infected and malignant cells. NK cell frequency is higher in the liver than anywhere else in the

body, accounting for 30-50% of liver lymphocytes compared to 10% in the blood<sup>67</sup>. Additional cells involved in the innate immune response in the liver include a group of cells termed innate lymphocytes such as CD1d-restricted NKT cells, NK receptor-positive T cells and gamma delta ( $\gamma\delta$ ) T cells. NKT cells express an invariant TCR alpha chain paired with one of three beta chains and are able to recognise glycolipid antigen. These cells rapidly secrete cytokines such as IL-4, IL-10 and IFN- $\gamma$ . NKT cells are enriched in the liver, constituting up to 30% of intrahepatic lymphocytes<sup>68</sup>.  $\gamma\delta$  T cells express invariant TCRs which recognise a limited range of antigen. Interestingly there is also an increased frequency of CD8<sup>+</sup> T cells in the liver which outnumber CD4<sup>+</sup> T cells. The liver CD4:CD8 T cell ratio is 1:3.5 compared to 2:1 for blood T cells<sup>67, 69</sup>. An overview of the cellular composition of the liver is presented in **Figure 1.7**.



**Figure 1.7: Cell populations in the liver**

The liver consists of hepatocytes and immune cells, including a large proportion of resident macrophages, known as Kupffer cells, as well as conventional and unconventional lymphocyte populations. Figure adapted from<sup>62</sup>



#### **1.4.2 Innate immune response to HCV**

Failure to eradicate HCV infection can be attributed to a combination of host and viral factors. Viruses have evolved to co-exist with their host through the development of immune evasion strategies. HCV is a very successful pathogen, due in part to its rapid mutation rate and the ability of HCV proteins to circumvent both IFN production and IFN-mediated antiviral response by targeting host signalling proteins limiting the efficacy of the IFN response and accounting for some of the treatment failure observed <sup>70</sup>.

HCV is a single stranded virus, detected via TLR7 but also produces double stranded intermediates, thereby also activating RIG-I and TLR3 in hepatocytes. Recognition of HCV RNA by RIG-I and TLR3, results in the phosphorylation and activation of IRF-3 by the protein kinases TANK-binding kinase 1 (TBK1) or I $\kappa$ B kinase  $\epsilon$  (IKK $\epsilon$ ). IRF-3 dimers then translocate to the cell nucleus and upregulate IFN expression and secretion from the infected cell. The importance of these viral sensors is highlighted by the fact that the HCV enzyme NS3/4A is capable of cleaving the adaptor proteins TRIF and MAVS downstream from TLR3 and RIG-I respectively. This leads to greatly attenuated downstream signalling, resulting in an inhibition of IFN production and consequently failure to signal through the JAK-STAT pathway to up-regulate antiviral genes <sup>71, 72</sup>.

The HCV core protein is further capable of targeting JAK-STAT signalling by inducing suppressor of cytokine signaling-3 (SOCS3), a negative regulator of IFN signalling, and by directly binding to STAT1 to prevent its phosphorylation and subsequent activation of downstream ISGs <sup>73</sup>. Our research group has shown that

HCV further targets STAT proteins by encoding a viral E3 ligase capable of targeting STAT3 for proteasomal degradation (Stevenson *et al*, unpublished). Additional HCV proteins with well-characterised immune evasion strategies are NS5A and E2. The HCV NS5A protein directly interacts with the proteins PKR and OAS, thus preventing their antiviral function <sup>74</sup>. The HCV glycoprotein E2 acts as competitive substrate with eIF2 $\alpha$ , the substrate of activated PKR, therefore inhibiting PKR kinase activity <sup>75</sup>. An overview of how HCV evades the immune response is presented in **Figure 1.8**.

This HCV-mediated inhibition of innate viral sensing, IFN production and JAK-STAT signalling in hepatocytes brings viral detection and IFN production by immune cells to the forefront. For instance, viral recognition by APCs and subsequent IFN production by DCs inhibits viral replication in hepatocytes via induction of antiviral ISGs. However, it has been suggested that the virus may also target the DCs disabling their key function of linking innate and adaptive immunity through pattern recognition, T cell activation and IFN secretion. This may explain the sub-optimal T cell response and defective IFN signalling that are evident in chronic HCV infection. Studies on DC phenotype and function in HCV have been carried out on peripheral blood from HCV-infected patients, often with conflicting results <sup>76</sup>. Decreased T-cell stimulatory capacity, increased IL-10 secretion, deficiency in co-stimulatory molecules and failure of patient monocyte-derived DCs (MDDCs) to secrete IFN- $\alpha$  in response to poly(I:C) or IFN- $\beta$  stimulation in chronic HCV-infected individuals have all been described <sup>77-83</sup>. However, not all investigators are in agreement with these findings, with some concluding that DCs

from HCV individuals are phenotypically and functionally comparable to healthy DCs <sup>84-86</sup>.

In HCV infection, IFN activates NK and NKTs which are among the first line of antiviral defence, capable of inhibiting spread of HCV via secretion of IFN- $\gamma$  and cytotoxic killing of infected hepatocytes. NK killing is further increased via the IFN-induced tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on NK cells, which causes apoptosis and killing of HCV-infected hepatocytes <sup>87</sup>. The HCV E2 protein also binds to CD81 on human NK cells, inhibiting NK-mediated cytotoxicity and IFN- $\gamma$  production by NK cells <sup>88</sup>.



### 1.4.3 Adaptive immune response to HCV

The major factors leading to HCV clearance are effective detection of the virus, an adequate innate immune response and the generation of an effective adaptive response, in particular a robust CD4<sup>+</sup> and polyclonal CD8<sup>+</sup> T cell response and IFN- $\gamma$  production <sup>89</sup>. The initiation of an effective adaptive T cell response is dictated largely by the DCs via recognition of HCV RNA, causing activation and maturation of the DCs and secretion of cytokines such as IL-12p70, a key cytokine required for a Th1 response. Multi-specific and sustained CD4<sup>+</sup> Th1 cells are required to aid CD8<sup>+</sup> CTLs and are required for spontaneous viral control. HCV-specific CTLs recognise viral peptides in the context of Human Leukocyte Antigen (HLA) class I molecules and are critically important for successful control of acute HCV infection through direct cytotoxicity and secretion of antiviral cytokines.

An 'exhausted' T cell response is characteristic of chronic viral infection, whereby T cells initially display normal effector mechanisms but progressively lose function over time due to sustained exposure of T cells to viral antigen <sup>90</sup>. CTLs that express high levels of the inhibitory receptor molecules, programmed death 1 (PD-1), a member of the CD28 family, and mucin domain-containing molecule 3 (Tim-3) are functionally impaired and accumulate in the liver of patients with chronic HCV infection <sup>91</sup>. To escape immune surveillance, HCV alters its epitopes so that it escapes detection by T cells and neutralising antibodies. Mutations within HLA-restricted epitopes represent one of the most potent immune evasion strategies utilised by HCV <sup>92</sup>. The role of CTL responses, restricted by HLA class I alleles, is recognised as highly significant in the successful clearance of HCV. IL-10-secreting T reg cells, as well as Th1 cells, have been reported to be induced against the core

protein in HCV infection <sup>93</sup>. Furthermore, IL-10 producing T cells were detected in a higher proportion of patients with chronic infection than in those who had cleared the virus <sup>93</sup>. It has also been shown that virally induced TGF- $\beta$  and IL-10 suppress Th1 and Th17 responses in HCV-infected patients, thereby representing an immune subversion mechanism by the virus to evade host protective immune responses <sup>94</sup>. Additional targeting of T cell activation has been demonstrated by HCV E2 inhibition of IL-2 secretion by targeting the translocation of protein kinase C  $\beta$  (PKC $\beta$ ) which is essential for IL-2 secretion <sup>95</sup>.

Despite the success of the HCV virus in evading both the innate and adaptive immune response, the antiviral response is still capable of clearing the virus in a proportion of individuals, making it critical to identify the host immune factors involved in achieving successful outcome to viral infection.







## 1.5 Host factors associated with HCV clearance

While viral factors, such as infecting viral genotype and viral load, contribute to the varying outcome to HCV infection, people inadvertently inoculated with the same dose and genotype of the virus have greatly differing responses indicating that host factors are largely at play in determining outcome to this infection <sup>16</sup>. Racial differences are important as Asian patients appear to have considerably better response to therapy than Caucasians treated with the same drugs and infected with the same viral genotype. Furthermore, an inferior response to antiviral therapy is well-documented in Africans compared to Caucasians, further highlighting that ethnic differences play a role in treatment response <sup>96</sup>.

Host factors that predict failure to respond to treatment include male gender, advanced age, increased Body Mass Index (BMI), the presence of co-morbidities and African ethnicity. The activation state of the immune system has also been associated with HCV clearance and treatment response with those who have high basal peripheral and hepatic ISG activation and high serum CXCL10 predicting chronicity and non-response to treatment <sup>11</sup>. This underlines the important role that immune system variation plays in determining outcome to HCV infection. In addition to these factors, genetic factors have been implicated with many studies associating genetic variation in key immune system molecules with HCV clearance/persistence. While much of the human genome is conserved, significant variation does exist, with the most common variation being single nucleotide polymorphisms (SNPs) whereby there is a base difference at a nucleotide locus, which differs within a population. A common SNP is defined as having a minor allele frequency of at least 5%, of which, approximately ten million are thought to

exist<sup>97</sup>. Since the discovery and characterisation of the HCV virus and disease process, many studies have examined frequency of variation in genes involved in the immune response to viral infection and genetic associations have been established between HLA molecules, cytokines and antiviral genes (summarised in **Table 1.1**). However, while variation has been associated with outcome in the populations studied, no single factor had been identified in these studies and it seems that a combination of different variables might be responsible or, possibly, that there is an as yet undiscovered association. Despite the progress made in determining genetic association with HCV outcome, the factors identified cannot account for all the variability observed and further research is required to identify key components of the immune response to HCV infection which may in the future be harnessed for therapeutic use.

**Table 1.1: Genetic variants associated with outcome to HCV infection**

<b>HLA Class I</b>		
A*0301	Viral clearance	98
A*1101	Viral clearance	
B*07	Viral clearance	98
B*27	Viral clearance	98
B*57 (B*5702, B*5703)	Viral clearance	99
Cw*01	Viral clearance	98, 99
A*2301	Chronic infection	100
<b>HLA Class II</b>		
DQB1*0301	Viral clearance	101, 102
DRB1*1101	Viral clearance	102
DR4	Treatment non-response	103
DRB1*0404 (DR4)	Treatment response	104
DRB1*1104	Normal ALT	105
DRB1*11	Normal ALT/less severe liver disease	105
DRB1*1101	Normal ALT/less severe liver disease	106
<b>Cytokines</b>		
TNF $\alpha$ -238A, -863A	Viral clearance	107, 108
IFN $\gamma$ -764G	Spontaneous resolution/treatment response	109
IL-12 1188C	Viral clearance	110, 111
IL-18 -607A, -137C	Viral clearance	112
IL-18 binding protein	Viral clearance	113
IL-10 592A	Viral clearance	114
IL-10 -1082T	Chronic infection	115, 116
IL-10 promoter (-1082A, -819T, 592A)	Treatment response	117
IL-28B rs12979860 C allele	Viral clearance/Treatment response	118, 119
<b>Antiviral genes</b>		
OAS rs2660 G allele	Viral clearance	120
PKR -168 CT	Viral clearance	120
MxA -88T	Viral clearance /Treatment response	120
<b>Other</b>		
KIR 2DS3	Chronic infection	121
DC-SIGN (CD209) -336G	Increased ALT/more severe liver disease	122

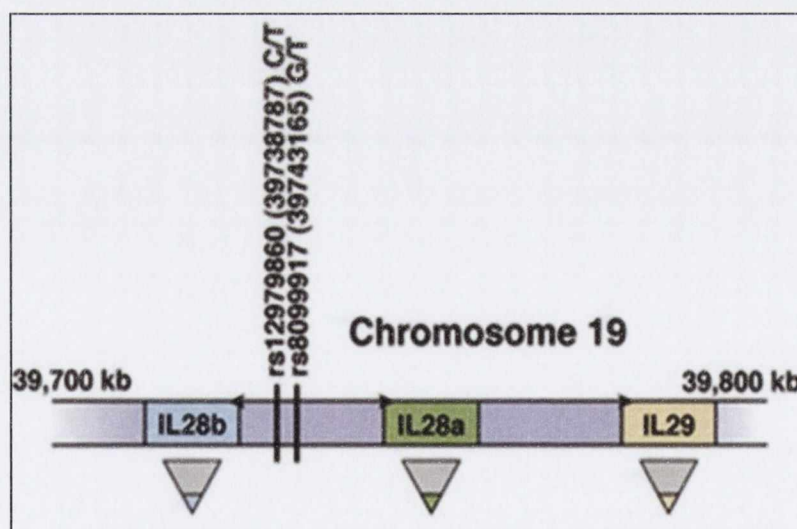
Table adapted from <sup>123</sup>



## 1.6 *IL28B* polymorphism

Instead of the 'candidate gene' approach that had been used previously, advances in genotyping technology and large databases of genetic variants make it possible to search for new genetic influences across the entire human genome<sup>123</sup>. Genome-wide association studies (GWAS) examine the association of large numbers of genetic variants, 500,000 to 2 million<sup>124</sup>, with a specific disease phenotype in large populations without any prior hypothesis<sup>123</sup>. A series of GWAS studies exploring variation in outcome to HCV infection were published in late 2009. What was remarkable about these studies was that, from the number of SNPs which showed some association with natural and treatment-induced clearance of HCV, seven of the top hits were in the region of the novel IFN- $\lambda$  family, with rs12979860 and rs8099917 being the most significant in several reports<sup>118, 119, 125, 126</sup>. Since the *IL28B* studies were first published simultaneously by a number of groups in 2009, the association between the *IL28B* SNP and outcome to HCV infection has been replicated by other researchers in various different cohorts, with over one hundred papers being published since the initial link was made. *IL28B* genotyping is now routinely carried out prior to HCV treatment as a predictor of treatment outcome. The effect of both SNPs in the *IL28B* region appear to be independent of ethnicity, gender, baseline viral load, degree of liver fibrosis and viral genotype which are accepted determinants of treatment response. Despite the proliferation of data concerning *IL28B* polymorphism and HCV infection, critical questions remain regarding clinical implications and the underlying biological mechanisms [83].

Interestingly, these SNPs are not even located in a gene; rs12979860 is located 3kb upstream from the *IL28B* gene but it is hypothesised that the association is due to the high linkage disequilibrium (LD) within this gene area. LD is when markers that are physically close tend to remain associated with an ancestral mutation and the rate of recombination decreases in that region over time <sup>127</sup>. It was speculated, therefore, that the *IL28B* SNP may be linked to or affect other polymorphisms within the IFN- $\lambda$  genes and promoter region. Both SNPs are strongly associated with a non-synonymous SNP in the *IL28B* gene, where the favourable haplotype corresponds to a lysine changing to an arginine at position 70 (rs8103142) <sup>124</sup>. However, very recently it has been discovered that the SNPs are in fact located in the previously undiscovered *IFNL4* gene region, leading to a loss of function in this molecule <sup>128</sup>. This recent breakthrough demonstrates how much we still have yet to uncover about the important biological functions of this new IFN family.



**Figure 1.9: Genetic location of IL28B polymorphisms rs12979860 and rs8099917**

The IFN- $\lambda$  genes *IL28A*, *IL28B* and *IL29* are located on human chromosome 19. As depicted in the figure, the two SNPs most highly associated with HCV outcome and treatment response, rs12979860 and rs8099917, are located upstream of the *IL28B* gene, in between *IL28A* and *IL28B*. Figure adapted from <sup>124</sup>

## 1.7 The IFN family and the novel IFN- $\lambda$ cytokines

The IFN- $\lambda$  family was discovered simultaneously by two groups in 2003, in the quest to identify additional members of the IL-10 family of cytokines <sup>45, 46</sup>. The IFN- $\lambda$ s consist of three cytokines designated IL-28A (IFN- $\lambda$ 2), IL-28B (IFN- $\lambda$ 3) and IL-29 (IFN- $\lambda$ 1), which form a gene cluster on chromosome 19. IL-28A and IL-28B are closely related to each other, having 96% amino acid identity, and both of these cytokines are more distantly related to IL-29, sharing ~81% homology <sup>46</sup>. Type III IFNs were first described as having similar antiviral properties to the Type I IFNs, due to their up-regulation of MHC class I expression, induction of ISGs and antiviral protection <sup>45</sup>. As mentioned above, a new family member IFNL4 was recently discovered. While this protein is more related to the IFN- $\lambda$ s than any other protein, it is still quite different, showing only 29% amino acid identity, being most related to IL-28B <sup>128</sup>.

A major difference between the Type I and Type III IFNs is at the level of receptor expression. The IFN  $\alpha/\beta$  receptor is comprised of the IFNAR1 and IFNAR2 subunits while the IFN- $\lambda$  receptor is formed of a distinct receptor complex consisting of the specific IL-28R $\alpha$  chain and the IL-10R $\beta$  chain, the latter being common to some members of the IL-10 superfamily. The IFN  $\alpha/\beta$  receptor is ubiquitously expressed. However, the specific IL-28R $\alpha$  receptor chain is mainly restricted to cells of epithelial origin and pDCs, signifying a role in localised tissue response rather than the peripheral effects of the Type I IFNs <sup>129</sup>. A comparison of the three IFN families is presented in **Table 1.2**.



**Table 1.2: Summary of Type I, II and III Interferons**

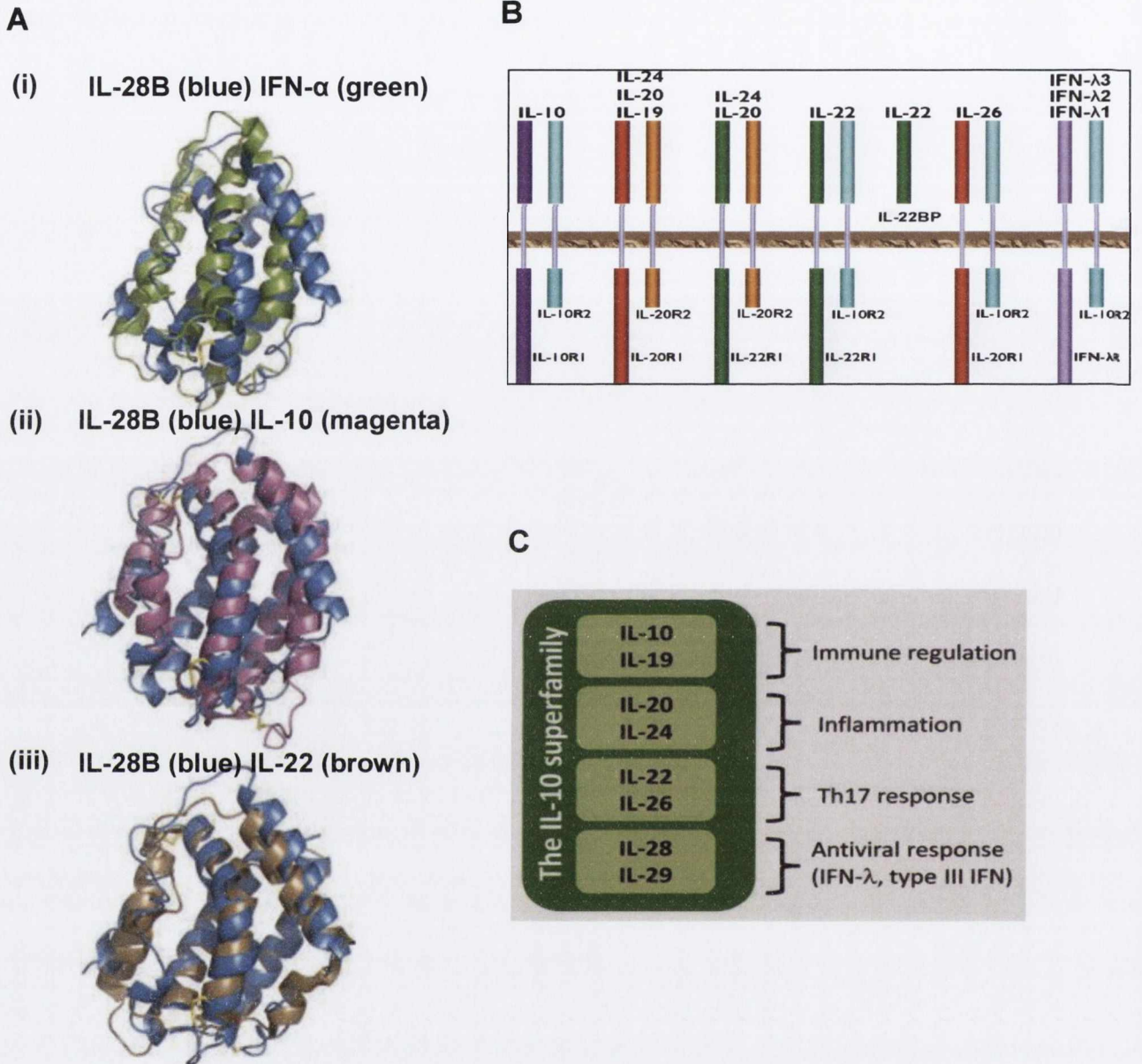
	Type I IFNs	Type II IFNs	Type III IFNs
<b>Family members</b>	12 IFN- $\alpha$ , IFN- $\beta$ , IFN- $\kappa$ , IFN- $\omega$ , IFN- $\epsilon$	IFN- $\gamma$	IFN- $\lambda$ (IL-28A, IL-28B, IL-29, IFNL4*)
<b>Gene location</b>	Chromosome 9	Chromosome 12	Chromosome 19
<b>Gene structure</b>	No introns	4 exons	5-6 exons, similar to IL- 10 superfamily
<b>Receptor</b>	IFNAR1 and IFNAR2	IFNGR1 and IFNGR2	IL-28R $\alpha$ and IL-10R $\beta$
<b>Signalling pathways</b>	Mainly JAK-STAT pathway	JAK-STAT pathway	JAK-STAT and MAPK pathways
<b>Cells responsible for production:</b>	Most cells can produce IFN- $\alpha$ but pDCs x1000	NK, NKT and T cells	DCs
<b>Target cells</b>	Ubiquitous receptor expression - effects almost every cell in the body	NK, activated T cells macrophages	Epithelial cells and DCs
<b>Biological function</b>	<ul style="list-style-type: none"> <li>- Antiviral</li> <li>- Increase in lytic potential of NK cells</li> <li>- Increase in MHC I expression, promoting Ag presentation, T cell survival and stimulates DC maturation</li> <li>- Anti-proliferative</li> </ul>	<ul style="list-style-type: none"> <li>- Important role in activation of NK cells and macrophages</li> <li>- Promotes Th1 differentiation</li> </ul>	<ul style="list-style-type: none"> <li>- Antiviral</li> <li>- Modulates Th1/Th2 response</li> <li>- Anti-proliferative</li> </ul>

\* Biological function of IFNL4 yet to be established

The Type III IFNs are related to both Type I IFNs (15-19% amino acid identity) and the IL-10 superfamily (11-13% identity), and are thereby said to represent an evolutionary intermediate between the two cytokine families <sup>46</sup>. The low percentage homology to each family explains the delay in their identification, which depended on structural studies rather than identification based on sequence alone <sup>130</sup>. While officially classified by Nomenclature Committee of the International Society for Interferon and Cytokine Research as an IFN <sup>130</sup>, and having slightly more sequence homology to the Type I IFNs (31-33%) than the IL-10 family (23%) , the multi-exon gene structure of IFN- $\lambda$  is more similar to that of the IL-10 superfamily compared to the lack of exons seen with Type I IFNs. The IL-10 superfamily consists of IL-10, IL-19, IL-22, IL-24 and IL-26. Indeed, crystallisation of the molecular structure of IL-28B revealed that it is structurally more similar to members of the IL-10 family than to Type I IFN, being particularly related to IL-22 <sup>131</sup>. The IFN- $\lambda$ s share the property of binding to and signalling via the class II cytokine receptor IL-10R $\beta$  with IL-10, IL-22 and IL-26 (**Figure 1.10**). This family is a pleiotropic group of cytokines with conserved signalling cascades that are classified based on sequence alignment, structural homology and binding to shared class II cytokine receptor chains that activate the JAK-STAT pathway <sup>132</sup>. Despite shared receptor usage, these cytokines have quite a broad range of biological functions such as antiviral activity, secretion of antibacterial proteins, cell-growth stimulation, acute phase response, wound healing, anti-tumour activity and apoptosis induction, all of which are quite distinct from the anti-inflammatory properties of IL-10 <sup>133</sup>. The members of the IL-10 superfamily are compared in **Figure 1.10**.

The restriction of the IFN- $\lambda$  response largely to epithelial cells suggests that the IFN- $\lambda$  system may have evolved to specifically protect epithelia. Evidence for an antiviral role at epithelial surfaces comes from work using IL-28R $\alpha$  knock-out mice which demonstrated that IFN- $\lambda$  is effective against influenza virus, if the virus is administered through the intranasal route, but not if it is administered systemically <sup>134</sup>. Thus IFN- $\lambda$ s might represent key contributors to the prevention of viral infection of skin and mucosal surfaces. Interestingly, IL-22, shown to be highly structurally similar to IFN- $\lambda$ , is known to regulate protection and reorganisation of tissue at outer body barriers and is critical in establishing an efficient antibacterial defence in lung and gut epithelia <sup>135 136</sup>. Thus, it seems likely that IL-22 and IFN- $\lambda$  play critical parallel roles against bacterial and viral infections, respectively, in the innate immune defence of epithelial cells, tissues regularly exposed to pathogens.





**Figure 1.10: The IL-10 superfamily: members, function and receptors**

(A) The molecular structure of IL-28B reveals that it is structurally more similar to members of the IL-10 family than to Type I IFNs, being particularly related to IL-22. (B) The IL-10 superfamily receptors; IFN- $\lambda$  signals through IL-10R $\beta$ , as does IL-10, IL-22 and IL-26. (C) The IL-10 superfamily members have diverse functions in immune regulation, inflammation, Th17 response and in the antiviral response.

Figures adapted from <sup>131, 133, 137</sup>

## 1.8 Biological effects of IFN- $\lambda$

IFN- $\lambda$  induces phosphorylation of STAT1 and STAT2, inducing the ISGF3 complex, elevating expression of antiviral genes and displaying antiviral activity *in vitro*. These findings, together with the observation that Type III IFNs are induced by a range of viral infections have initiated studies focusing on the antiviral properties of these cytokines. However, the IFN- $\lambda$  usage of the IL10R $\beta$  receptor chain and their ability to phosphorylate additional STAT proteins such as STAT3, STAT4 and STAT5 suggest more complex properties of the IFN- $\lambda$  family, in particular that they may also exhibit immunomodulatory activity. IFN- $\lambda$  also holds promise as a therapeutic agent in cancer due to their cell specific anti-proliferative properties, similar to IFN- $\alpha$  which is currently used in cancer therapy<sup>138</sup>.

### 1.8.1 IFN- $\lambda$ , the liver and HCV

The association of *IL28B* with HCV clearance and treatment response has initiated numerous detailed studies on the role of IFN- $\lambda$  in the immune response to this hepatotropic virus. Liver hepatocytes are one of the cell types shown to highly express IL-28R $\alpha$ <sup>139</sup>. IFN- $\lambda$  has been shown to inhibit HCV replication in a dose- and time-dependent manner<sup>140, 141</sup>. Some reports suggest that IFN- $\lambda$  can synergistically enhance IFN- $\alpha$  antiviral activity<sup>140, 141</sup> while others find more of an additive effect, instead showing that IFN- $\lambda$  in combination with IFN- $\gamma$  shows the greatest decrease in HCV viral RNA<sup>142</sup>. However, the signalling kinetics and gene induction of IFN- $\lambda$  were distinct from that of IFN- $\alpha$ , with IFN- $\lambda$  inducing steady state increases in levels of ISGs whereas IFN- $\alpha$ -induced ISGs peaked early and declined rapidly, outlining differences in the antiviral states induced by IFN- $\lambda$  and IFN- $\alpha$ <sup>141</sup>. There are limited data comparing signalling and antiviral properties of



IL-28A/B and IL-29 but a report from Diegelmann *et al* found that while IL-29 activated antiviral genes in the Huh7 hepatoma cell line, IL-28A was in fact a potent gene repressor <sup>143</sup>. Recent studies using primary human foetal liver cell (HFLC) cultures show that the cell culture produced HCVcc induced expression of Type III IFNs and ISGs but in contrast, very little Type I IFN production was detected <sup>144</sup>. This gene induction was dependent on actively replicating virus and the level of IFN- $\lambda$  produced was sufficient to inhibit HCVcc infection of uninfected liver cell cultures.

## **1.9 IFN- $\lambda$ production**

IFN production is triggered via PRR sensing of viral nucleic acid. Key PRRs are RIG-I and MDA-5 in the cytoplasm and TLR3, 7, 8 and 9 on endosomal membranes, as well as the more recently described DNA sensors which can also induce IFN expression <sup>39</sup>. Key transcription factors involved in the induction of IFN- $\alpha$  and IFN- $\lambda$  are the IRFs and NF- $\kappa$ B. While IRF-7 is considered the master regulator of IFN- $\alpha$  production <sup>145</sup>, IFN- $\lambda$  utilises both IRF-3 and NF- $\kappa$ B <sup>146</sup>, being strongly reliant on NF- $\kappa$ B compared to IFN- $\alpha$  <sup>147, 148</sup>.

IFN- $\lambda$ s have been reported to be produced following viral infection and in response to synthetic TLR agonists . The Type III IFNs are expressed by PBMCs after infection with Encephalomyocarditis virus (EMCV), Sendai virus or influenza A or stimulation with the synthetic TLR3 agonist, poly(I:C), but not TLR2 agonist Pam3Cys or TLR7/8 agonist R848 <sup>149</sup>. It is apparent that DCs are major producers of IFN- $\lambda$  but epithelial cells are primarily responsive to IFN- $\lambda$ , in addition to pDCs. Pre-treatment with IFN- $\alpha$  has been reported to increase IFN- $\lambda$  production <sup>150, 151</sup>.



Many experiments involved the use of MDDCs, CD14<sup>+</sup> monocytes expanded *in vitro* for 6 days using IL-4 and GM-CSF, and pDCs and it was shown that TLR9 stimulation induced the expression of IFN- $\alpha$ , IFN- $\beta$  and IFN- $\lambda$  in pDCs whereas TLR4 stimulation by LPS or TLR3 stimulation by poly(I:C) induced only IFN- $\beta$  and IFN- $\lambda$  expression in MDDCs due to differential TLR expression on the different types of DCs. Additional work by Makela *et al*, 2010 reported that stimulation of MDDCs with R848 and with TLR3 or TLR4 ligands leads to a synergistic expression of IFN- $\beta$  and IFN- $\lambda$  mRNAs <sup>152</sup>. An interesting experiment by Medjugorac *et al* showed that IL-4 treatment during stimulation of PBMCs significantly increased IL-29 transcription <sup>153</sup>. Further analysis showed that monocytes were responsible for responding to IL-4 by producing IL-1Ra which acts on pDCs to increase IL-29 production. Interestingly, *in vivo* mouse experiments where CD11c<sup>+</sup> DCs were depleted identified that mDCs are an important source of Type I and Type III IFN in the vaginal epithelium during HSV infection and following TLR9 stimulation <sup>154</sup>. A report by Lauterbach *et al* in 2010 identified that a minor myeloid DC subpopulation, CD141<sup>+</sup> (BDCA-3) DCs, rather than the pDCs, as being the major producers of IFN- $\lambda$  in response to poly(I:C) <sup>155</sup>. This little known DC subset is known to express high TLR3 in contrast to the high constitutive expression of TLR7 and TLR9 by pDCs, and also to be capable of cross-presentation <sup>156</sup>.

### **1.10 IFN- $\lambda$ and DCs**

DCs are known to be responsive to and produce IFN- $\lambda$  thereby underlining the important role DCs seem to play in mediating the biological effect of IFN- $\lambda$  <sup>157</sup>. IL-28R $\alpha$  is reported to be up-regulated when monocytes are differentiated into DCs

by GM-CSF and IL-4, meaning that immature DCs are responsive to IFN- $\lambda$  <sup>158</sup>. IFN- $\lambda$ -treated MDDCs promote the generation of tolerogenic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> naturally occurring T reg cells, capable of inhibiting T cell proliferation, suggesting that IFN- $\lambda$  may suppress inflammatory responses. In addition, IFN- $\lambda$ -treated MDDCs express high levels of MHC I and MHC II, low co-stimulatory molecules and express CCR7, indicating that they are capable of migration to the lymph nodes <sup>158</sup>. pDCs are one of the few immune cell types to have high expression of IL-28 $\alpha$ . IFN- $\lambda$  was reported to affect pDCs by altering expression of the co-stimulatory molecules, upregulating CD80 and inducing expression of ICOS-L and, in synergy with IFN- $\alpha$ , upregulating CD83. Human pDCs secrete high levels of IL-29 in response to TLR7 and TLR9 agonists but not TLR3 or TLR4. Many initial studies identify pDCs as the source of IFN- $\lambda$  rather than mDCs but MDDCs generated *in vitro* are used in these studies, rather than primary mDCs, which we are now aware, comprise functionally distinct subsets <sup>149</sup>.

### **1.11 Human dendritic cell subsets**

DCs are traditionally divided into mDCs and pDCs. The DC originally discovered by Steinman and Cohn in 1973 corresponds to the mDC subset, also referred to as 'classical' or 'conventional' DCs <sup>159</sup>. These cells were initially identified as being different in morphology to macrophages by appearance of their dendrites and proved to be the missing 'adapter cell' responsible for initiating T cell responses <sup>160</sup>. pDCs are quite different. As their name suggests, they resemble plasma cells, displaying lots of golgi apparatus and secretory ability and being much smaller than mDCs. pDCs particularly resemble B cells with which they share some characteristics and a similar gene expression profile <sup>161, 162</sup>. pDCs were only



unequivocally identified in 1999, with their signature function being potent IFN production <sup>44</sup>. While the origin and development of DCs is still being elucidated, the current understanding is that DCs develop from haematopoietic stem cells in the bone marrow. Myeloid-derived progenitors produce committed DC progenitors, giving rise to pre-DCs which leave the bone marrow and migrate to the blood. These pre-DCs then differentiate into the various DC subsets, a process controlled by specific transcription factors and cytokines <sup>163-165</sup>.

DC subsets differ in their expression of pattern recognition receptors, cytokine production and T cell-polarising abilities, which points to specialised roles in host defence. Human DCs have been defined traditionally as HLA-DR<sup>+</sup> cells that lack the hematopoietic cell lineage (lin) markers CD3, CD14, CD16, CD19 and CD56. DCs can be subdivided into mDCs (CD11c<sup>+</sup> CD123<sup>low</sup>) and pDCs (CD11c<sup>-</sup> CD123<sup>high</sup>) <sup>166</sup>, with pDCs being traditionally defined as specialist IFN-producers whereas mDCs are potent APCs. Four additional surface antigens specific to human DCs are used to further distinguish DC subsets, Blood Dendritic Cell Antigen (BDCA) -1, -2, -3 and -4 <sup>167</sup>. Myeloid DCs can be divided into two subsets based on expression of either CD1c (BDCA-1) or CD141 (BDCA-3) whereas CD303 (BDCA-2) and CD304 (BDCA-4) are expressed by all pDCs. CD11c<sup>+</sup> mDCs are important antigen-presenting cells whereas CD123<sup>+</sup> pDCs are potent producers of IFN- $\alpha$  and are considered 'professional' interferon-producing cells due to their high expression of the viral sensor TLR7 and TLR9 and their constitutive expression of the necessary transcription factor IRF-7 resulting in their ability to potently produce Type I IFNs in response to TLR ligation <sup>168</sup>. CD1c expression defines the most abundant mDC subset in peripheral blood. An additional mDC subset, CD141<sup>+</sup> DCs (BDCA-3 or



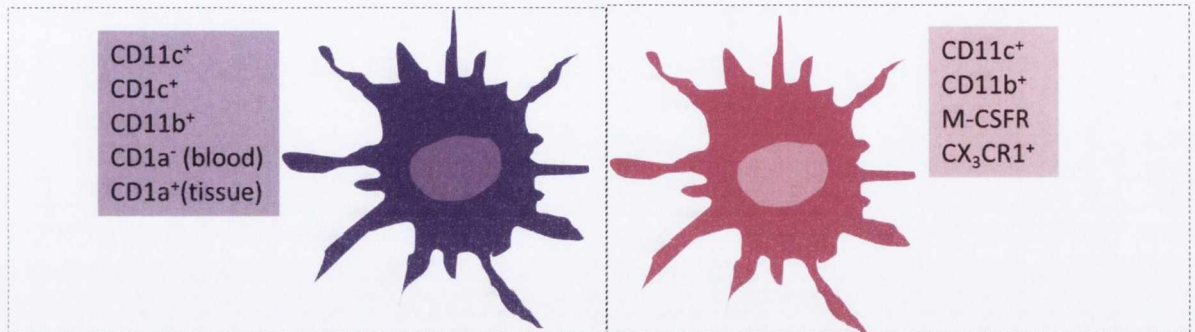
thrombomodulin), has been identified. Human CD141<sup>+</sup> DCs are homologous to mouse CD8 $\alpha$ <sup>+</sup> DCs, which highly express TLR3, and possess superior cross-presenting ability to activate CD8 cytotoxic T cell responses<sup>156, 169, 170</sup>, thus making them important in antiviral and anti-tumour immunity.

While blood DCs are well characterised, less is known about human tissue DCs, with most DC characterisation studies carried out on mice, due to the difficulty in obtaining sufficient human tissue to study human DCs. Frustratingly, while murine DC subsets have been revealed to comprise complex subsets, they do not match the human system, raising a question mark over the relevance of such studies in terms of human disease. Mouse DCs can be broadly divided into B220<sup>+</sup> plasmacytoid DCs and CD11b<sup>+</sup> CD8 $\alpha$ <sup>-</sup> myeloid DCs and CD8 $\alpha$ <sup>+</sup>, CD11b<sup>-</sup> lymphoid DCs. Further subtypes of CD8 $\alpha$ <sup>-</sup> DCs are based on expression of CD4. Other markers used for segregating mouse DC subsets are CD11c and CD205 (DEC205). The spleen contains three of these subsets; CD4<sup>-</sup>CD8 $\alpha$ <sup>+</sup> CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>-</sup> whereas the lymph nodes contain two extra DC subsets; CD4<sup>-</sup>CD8<sup>-</sup>CD11b<sup>+</sup> and in skin-adjacent lymph nodes a further DC subset exists expressing high levels of langerin<sup>168</sup>. CD103<sup>+</sup> DCs are another DC subset found in non-lymphoid organs related to CD8 $\alpha$ <sup>+</sup> found in lymphoid organs. A breakthrough in reconciling the mouse DC system to human was accomplished in 2010 with a series of papers describing the human equivalent of the mouse CD8 $\alpha$ <sup>+</sup> subset<sup>155, 156, 170, 171</sup>. This mouse subset, it is now agreed, is the equivalent of a tiny population of human blood DCs, CD141<sup>+</sup> or BDCA-3<sup>+</sup>. The characterisation of human and mouse DC subsets are shown in **Figure 1.11** and details of the surface markers used for human DC characterisation in **Table 1.3**.

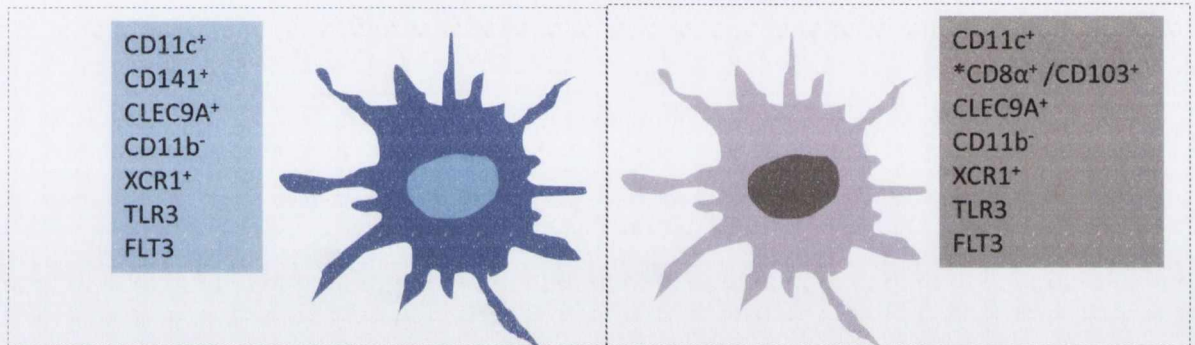
## Human DCs

## Mouse DCs

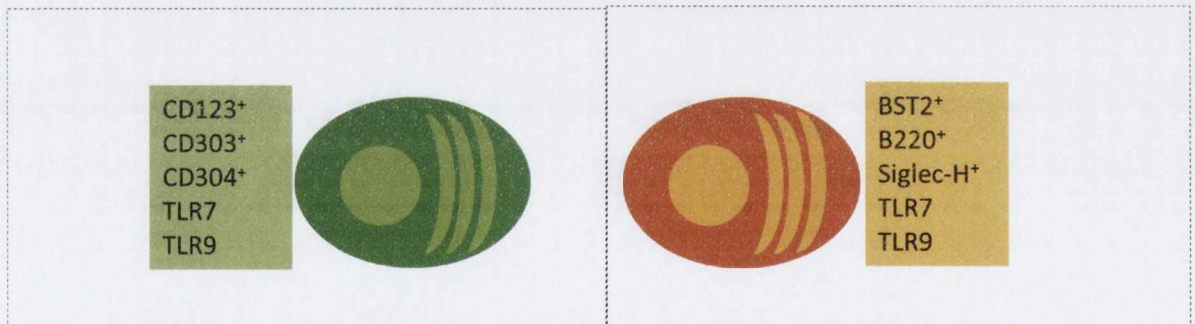
### Myeloid dendritic cell subset



### Myeloid dendritic cell subset



### Plasmacytoid dendritic cells



**Figure 1.11: Summary of human and murine dendritic cell subsets**

Dendritic cells (DCs) can be broadly divided into myeloid and plasmactoid DCs (mDCs and pDCs). Human DCs consist of two mDC subsets, CD1c<sup>+</sup> and CD141<sup>+</sup>. CD1c share CD11b expression and functional properties with their CD8α<sup>-</sup> murine counterpart, specialising in antigen-presentation via MHCII and activation of CD4<sup>+</sup> T cell responses. CD141<sup>+</sup> DCs share CLEC9A, XCR1, FLT3 and high TLR3 expression with the CD8α<sup>+</sup> murine DC subset, which is also related to CD103<sup>+</sup> DCs, found in non-lymphoid tissues. CD141<sup>+</sup>/CD8α<sup>+</sup> DCs specialise in cross-presentation of antigen via MHC Class I to activate CD8<sup>+</sup> T cell responses. Human and mouse pDCs have conserved expression of TLR7 and TLR9. CLEC9A, C-type lectin domain family member 9; BST2, bone marrow stromal antigen 2; CX3CR1, CX3C-chemokine receptor 1; FLT3, FMS-related tyrosine kinase 3; M-CSFR, macrophage colony-stimulating factor receptor

Adapted from <sup>172</sup>

**Table 1.3: Human blood DC subset nomenclature**

DC subset	DC marker(s)	Marker function
<i>Plasmacytoid DCs (pDCs)</i>	BDCA-2 (CD303/CLEC4C)	Strictly expressed on human pDCs. CD303 (BDCA-2) is a type II transmembrane C type lectin. pDCs can uptake ligand via CD303 (BDCA-2) and process and present ligand to T cells <sup>173</sup>
<i>Myeloid DCs (mDCs)</i>		
	BDCA-1 (CD1c)	Expressed on a major subpopulation of human mDCs (~0.3% of white cells) <sup>167</sup> . CD1c is a member of the CD1 family of proteins that are structurally related to MHC Class I proteins and mediate the presentation of non-peptide antigens to T cells <sup>174</sup>
	BDCA-3 (CD141/ Thrombomodulin)	Expressed at high levels on a minor subpopulation of human mDCs (~0.02% of white cells) <sup>167</sup> . CD141 (thrombomodulin) was described to mediate co-agglutination by interaction with thrombin and protein C <sup>175</sup> .



### 1.11.1 CD141 (BDCA-3) DCs

A series of papers published in late 2010 describing the human equivalent of a well-characterised mouse DC subset, CD8 $\alpha$ <sup>+</sup>, has begun to reconcile the mouse and human DC systems <sup>156, 169-171</sup>. This mouse subset, it is now strongly suggested, is the equivalent of a tiny population of human blood DCs, CD141<sup>+</sup> or BDCA-3<sup>+</sup>. This has led to renewed interest in DC subsets and their role in the immune response to infection and their therapeutic potential. This CD141<sup>+</sup> DC subset has been described to have quite a unique set of functions compared to other DC populations. Human CD141<sup>+</sup> DCs are specialised in cross-presentation; they express CLEC9A, which recognises F-actin exposed on dead cells <sup>176, 177</sup>, and can take up antigen and present via MHC I to activate a CD8<sup>+</sup> T cell response making them an important cell type in the context of antiviral and anti-tumour immunity <sup>178</sup>. While pDCs are specialised IFN- $\alpha$  producers and are considered at the forefront of DC antiviral immunity, mouse CD8 $\alpha$ <sup>+</sup> and human CD141<sup>+</sup> DCs appear to be the major producers of IFN- $\lambda$ , which we now know is also an important antiviral cytokine <sup>155</sup>. Further study of CD141<sup>+</sup> DCs is necessary to establish the functional role of IFN- $\lambda$  production by this DC subset and the role this plays in antiviral immunity.

#### **Rationale for thesis:**

HCV infection results in differential outcomes in infected individuals. It is not known why a proportion of those infected are capable of viral clearance whereas the majority become chronically infected and, furthermore, why a significant percentage of chronically infected fail to respond to IFN- $\alpha$  therapy. Genetic

variation in the IFN pathway and key innate immune genes has been associated with differential HCV outcome. In addition, polymorphisms in the gene region encoding the antiviral IFN- $\lambda$  cytokines have been shown to be closely associated with outcome to HCV infection but, as yet, much remains to be determined about the biology of IFN- $\lambda$  and what exact role it has in the innate immune response. IFN- $\lambda$  may hold great potential for therapeutic application but its biological function first needs to be better understood, in particular the effect of these cytokines on immune cell function and the factors involved in driving IFN- $\lambda$  production. The parallel description of a minor DC subset, specialist in IFN- $\lambda$  production is an interesting development and the study of this little-characterised subset in humans, their localisation and function, may shed light on the role of IFN- $\lambda$  association in the immune response to HCV. Linking these two novel immune system components may provide a much sought immunological therapy in targeting a specific DC subset with desirable cytokine production which will have benefits in treating viral infection, cancer and autoimmune disease.

**Specific aims:**

- To evaluate the association between genetic variation in the antiviral gene *OAS* and differential outcome to HCV infection
- To investigate the responsiveness of immune cells and hepatocytes to IFN- $\lambda$  in terms of IFN- $\lambda$  receptor expression, activation of signalling pathways and gene expression
- To characterise DC populations in the liver and evaluate CD141<sup>+</sup> DCs as a source of IFN- $\lambda$  in the local hepatic antiviral response

## **Chapter 2: Materials and Methods**



## **2.1 *OAS1* SNP genotyping of Anti-D cohort**

### **2.1.1 Patient Information**

*OAS1* SNP genotyping was performed on a homogenous group of women infected with the same dose and genotype 1b of HCV virus via contaminated anti-D immunoglobulin. All were females of child-bearing age with similar genetic background, and lacking confounding factors making them an ideal cohort for genetic studies. Blood samples from HCV-infected patients (n=178) were collected at the Liver Unit of St. Vincent's University Hospital. All patients tested positive for antibodies to HCV using a third-generation enzyme immunoassay (Abbott Diagnostics, Wiesbaden, Germany), confirmed with an immunoblot assay (RIBA-3) (Chiron Corp., Emeryville, CA, USA). Some patients remained chronically infected with the virus (PCR+; n=98), as confirmed by testing consistently positive for HCV RNA by a qualitative reverse transcriptase polymerase chain reaction (RT-PCR) (Amplicor; Roche Diagnostic Systems, Nutley, NJ, USA), whereas others cleared the virus either naturally or following treatment (PCR-; n=80). Ethical approval was obtained from the Research and Ethics Committee at St. Vincent's University Hospital in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

### **2.1.2 Genomic DNA extraction from whole blood**

Genomic DNA was extracted from whole blood using either the QIAamp DNA Blood Mini Kit (Qiagen, Crawley, UK) as per manufacturer instructions, or a modified salting out method. Briefly, 10 ml red cell lysis buffer (RCLB) (0.32 M sucrose, 10 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 0.75% Triton-X-100, dH<sub>2</sub>O, pH adjusted to

7.6) was added to 3 ml blood and incubated at room temperature (RT) for 10 min, mixing gently. The sample was centrifuged at 3,166 x *g* for 10 min at 4°C and supernatant discarded, repeating this process twice. To the cell pellet, 500 µl of RCLB was added and incubated at RT for 5 min, mixing gently. The sample was centrifuged for 5 min at 15,700 x *g*, supernatant removed and 500 µl of proteinase K buffer (20 mM Tris-HCl, 4 mM Na<sub>2</sub>EDTA, 100 mM NaCl, pH adjusted to 7.4) added. All reagents for red cell and proteinase K buffer solutions were purchased from Sigma, Dublin, Ireland. To the sample, 10µl of 20 mg/ml proteinase K was added, followed by 35 µl 10% SDS (both Sigma). Following 2 hr incubation at 55°C, 170 µl of 5.3 M NaCl was added and samples were shaken vigorously for 15 sec. Samples were centrifuged for 10 min at 15,700 x *g* and 700 µl of isopropanol was added, mixing gently by inversion until DNA had precipitated out of solution. The DNA was pelleted by centrifuging for 10 min at 15,700 x *g*. Following removal of supernatant, 1 ml of 70% ethanol was added and centrifuged for 5 min at 15,700 x *g*. The ethanol was then removed and the DNA pellet was allowed to air dry (~15 min). Finally, the DNA pellet was re-suspended in 100 µl sterile Tris-EDTA (TE) buffer and allowed to dissolve O/N at RT. The extracted DNA concentration and purity was measured using the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, MA, USA) and samples were stored at -20°C until use.

### **2.1.3 Restriction fragment length polymorphism (RFLP)**

*OAS* genotyping was carried out using RFLP. A region of the *OAS1* gene incorporating the rs10774671 SNP was first amplified using the primers (5'-3') F: GGGATCCAGATGGCATGTCA, R: CTGGATCAAGAGTCCCACCTG in a PCR reaction



consisting of 2.2  $\mu$ l dNTPs, 2.2  $\mu$ l primer mix, 2.2  $\mu$ l PCR Buffer (10X), 2.2  $\mu$ l  $MgCl_2$  (50 mM), Taq polymerase 0.11 $\mu$ l (Invitrogen; 5 units/ $\mu$ l), 1  $\mu$ l DNA template, made up to a final reaction volume of 20  $\mu$ l. PCR reaction conditions were as follows: 94°C 3 min, 94°C 45 sec, \*62°C 30 sec for 35 cycles, 72°C for 90 sec and 72°C for 10 min. This amplified product contains an *AluI* restriction site resulting in fragments of 52bp and 295bp. In the presence of the *OAS* rs10774671 A allele, a second restriction site is created, which results in the 295 bp fragment being digested into fragments of 51 bp and 244 bp. The restriction digest reaction was set up as follows: 10  $\mu$ l PCR product, 1  $\mu$ l restriction enzyme *AluI* (10,000 units/ml), 1.25  $\mu$ l NEBuffer 4 (10X) (New England Biolabs) made up to a final reaction volume of 12.5  $\mu$ l with DNase-free water. The reaction was incubated at 37°C for ~4 hours (<8 hours) followed by heat inactivation at 65°C for 20 min. An uncut DNA sample was included as a control with each batch of samples.

#### **2.1.4 Applied Biosystems SNP genotyping allelic discrimination assay**

Genotyping of the *OAS* rs10774671 SNP was carried out using Applied Biosystems SNP genotyping primer/probe assay x 40 C\_2567433 (Applied Biosystems, Foster City, CA). Prior to use, the supplied AB primer/probe mix (40X) was diluted to 20X in TE buffer. The PCR reaction consisted of: 5.0  $\mu$ l TaqMan Universal PCR Mastermix (P/N 4304437), 0.5  $\mu$ l 20X AB primer/probe mix, 2.5  $\mu$ l PCR-grade water, 2.0  $\mu$ l DNA (diluted 1-70 ng/ $\mu$ l) for a total reaction volume of 10  $\mu$ l. Samples were analysed on AB 7500 Fast Real-Time PCR System using the appropriate AB Fast optical plates (Applied Biosystems).



### **2.1.5 Statistical analysis**

P-values for comparison of healthy and HCV- and HCV+ genotype and allele frequency were generated using the  $\chi^2$  test (P-value <0.05) using GraphPad Prism software, version 5.01 (GraphPad Software, La Jolla, CA, USA). Clinical disease parameters were compared between each genotype using one way ANOVA Kruskal Wallis test (P-value <0.05).

## **2.2 Cellular responsiveness to IFN- $\lambda$**

### **2.2.1 Culture of Huh7 hepatocyte cell line**

The Huh7 hepatocyte cell line expressing T7 polymerase was kindly gifted by Prof. Ralf Bartenschlager, University of Heidelberg. Huh-7s are a hepatocyte cellular carcinoma cell line derived from a 57 year old Japanese male in 1982 <sup>179</sup>. Huh7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, Life Technologies, Paisley, UK) supplemented with 10% foetal calf serum, 250U/ml penicillin, 250 $\mu$ g/ml streptomycin and 6 $\mu$ g/ml zeocin (all Gibco, Life Technologies) at 37°C in 5% CO<sub>2</sub>. Cells were passaged every 3-4 days when ~80% confluent. To remove from flask, cells were incubated with trypsin-EDTA (Gibco, Life Technologies) for 10 min at 37°C and cell pellet re-suspended in DMEM. Cell counts were carried out on a light microscope using trypan blue exclusion assay.

### **2.2.2 Preparation of peripheral blood mononuclear cells (PBMCs)**

PBMCs were from buffy coats from the Irish Blood Transfusion Service or were isolated from healthy donors recruited locally. The PBMC fraction was isolated from whole blood using density centrifugation. Briefly, 15 ml of Ficoll-Paque Plus™ separation medium (GE Healthcare, Uppsala, Sweden) was added to a Leucosep® tube (Greiner Bio-One) and centrifuged at 290 x *g* for 2 min. Blood, collected in lithium heparin blood tubes, was added directly onto the Leucosep® tubes and centrifuged at 1,580 x *g* for 5 min at RT with brake at 2. The interface 'buffy coat' layer (containing T and B lymphocytes, monocytes, NK cells and DCs) was removed and added to a 50 ml tube using a sterile Pasteur pipette. The cells were re-suspended gently in 30 ml Roswell Park Memorial Institute medium (RPMI) (Gibco, Life Technologies). The cells were centrifuged for 5 min at 300 x *g* at RT, supernatant discarded and cells re-washed in 30 ml RPMI at 300 x *g* for 5 min at RT. The final cell pellet was re-suspended in 1-10 ml RPMI supplemented with 10% foetal calf serum, 250U/ml penicillin, 250µg/ml streptomycin and cell number and viability determined using the trypan blue exclusion assay.

### **2.2.3 Isolation of immune cell populations by magnetic bead separation**

T cells, B cells and monocytes were isolated from freshly prepared PBMCs using the CD3, CD19 and CD14 Microbead kits (Miltenyi Biotec, Bergisch Gladbach, Germany) respectively, as per manufacturer's instructions. pDCs were isolated using the Diamond Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec). Isolation of pDCs involved a number of steps; non-pDCs were indirectly magnetically labelled with a cocktail of biotin-conjugated antibodies against

lineage-specific antigens and anti-biotin Microbeads. Additionally, non-pDCs were directly magnetically labelled with a cocktail of Microbead-conjugated antibodies against antigens that are not expressed on pDCs. The labelled cells were subsequently depleted. In the second step, the pre-enriched pDCs were labelled with the pDC-specific surface marker CD304 (BDCA-4/Neuropilin-1). PDCs were isolated based on BDCA-4 expression because antibody binding to BDCA-2 significantly alters cell function, with a reduction in IFN- $\alpha$  observed<sup>173, 180</sup>.

MDDCs were generated by culturing isolated CD14<sup>+</sup> monocytes by culturing at a concentration of 1 million/ml in 3 ml in 6-well plates in 10% RPMI supplemented with 20 ng/ml IL-4 and 50 ng/ml GM-CSF (Peprotech, Rocky Hill, NJ) for 6 days, with fresh media and cytokines added on day 3.

#### **2.2.4 Stimulation of PBMCs and Huh7 cells with IFN- $\lambda$ and IFN- $\alpha$**

Healthy PBMCs were cultured in a 12-well plate at a density of  $2 \times 10^6$ /ml. Isolated immune populations were cultured at  $1 \times 10^6$  in a 12-well plate for T cells and monocytes, 24-well plate for B cells and 96-well plate for pDCs. Total and isolated peripheral immune populations were cultured in RPMI with 10% foetal calf serum, 250U/ml penicillin and 250 $\mu$ g/ml streptomycin at 37°C in 5% CO<sub>2</sub>. Isolated pDCs were supplemented with recombinant human IL-3 (1 ng/mL; Immunotools, Friesoythe, Germany) to maintain pDC viability<sup>180</sup>. The Huh7 hepatocyte cell line was cultured at a density of 125,000/well in 12-well plate O/N before treatment. Stimulations consisted of either 100 ng/ml, 500 ng/ml IL-29, IL-28A (Peprotech) or IL-28B (R&D Systems) and 1000U/ml IFN- $\alpha$  (Roferon-A, IFN- $\alpha$ -2a, Roche) for 4



hours after which time TRIzol<sup>®</sup> reagent (Invitrogen) was added to cell pellets for RNA extraction.

### **2.2.5 Characterisation of IFN- $\lambda$ receptor by flow cytometry**

Expression of the IFN- $\lambda$  receptor chains IL-28R $\alpha$  and IL-10R $\beta$  was characterised on Huh7 cells and peripheral immune cell populations by flow cytometry. For Huh7 cells, 300,000 cells were removed by scraping from bottom of well and were re-suspended in cold PBS. Following centrifugation at 300 x g for 5 min, cells were re-suspended in staining buffer (1% FCS, 0.1% sodium azide in PBS), washed once and incubated with IL-28R $\alpha$ -PE (Biolegend, San Diego, CA) and IL-10R $\beta$ -PE (R&D Systems, Abingdon, UK) antibodies, or isotype controls IgG1-PE and IgG2a-PE (BD Biosciences, Erembodegem, Belgium) for 20 min at 4°C. Cells were washed in staining buffer and acquired on the Canto II flow cytometer. MDDCs were stained following cell harvest using the same staining protocol.

A whole blood staining protocol was utilised to characterise IFN- $\lambda$  receptor expression on immune cell populations. Briefly, fresh 1X BD FACS Lysing solution (BD Biosciences) was prepared by diluting the 10X solution in the ratio of 1:10 with sterile, endotoxin-free water before use, taking care to keep 1X FACS Lysing solution and 1X PBS on ice at all times. Two hundred microlitres of whole blood was pipetted into each tube and antibodies against the receptor chains and cell surface markers were added to the blood samples as follows: IL-28R $\alpha$ -PE (Biolegend), IL-10R $\beta$ -PE (R&D Systems), CD3-pacific blue, CD14-FITC, CD19-APC-Cy7 (BD Bioscience), CD56-PE-Cy7 (eBioscience, Hatfield, UK), HLA-DR-PerCP-Cy5.5, Lin1-FITC (BD Bioscience), CD11c-eFluor450, CD123-PE-Cy7 (eBioscience).

Controls used included: isotype controls, IgG1-PE and IgG2a-PE (both BD Bioscience), as well as fluorescence minus one (FMO) controls. The mixture was incubated in the dark for 20 min at 15-20°C. Red blood cells were lysed by adding 2 ml of 1X FACS Lysing solution per 100 µl of blood sample used and incubated for 10 min at 15-20°C with inversion of tubes every 2-3 min, ensuring that cells were well re-suspended for proper lysis. After incubation, the solution was centrifuged at 350 x *g* for 5 min at 15-20°C. The supernatant was discarded, and the cell pellet re-suspended in 1ml of cold, endotoxin-free 1X PBS for each 100 µl of whole blood sample used and mixed well. The cells were centrifuged at 350 x *g* for 7 min at 4°C, supernatant discarded and the cell pellet re-suspended in 300 µl of 1% paraformaldehyde (PFA) (Sigma). The fixed cells were then stored at 4°C for up to 24 hr before flow cytometric analysis using the Canto II instrument (BD Biosciences) and Flowjo software (Flowjo, Treestar Inc, Ashland, OR, USA).

#### **2.2.6 Intracellular staining for IFN-λ-induced STAT phosphorylation: Huh7**

Huh7 cells (300,000 cells/well in 6-well plate) were stimulated with IFN-λ (IL-29) over a time-course from 15-90 min. IFN-α treatments were carried out in parallel as it is known that IFN-α strongly phosphorylates STAT1 and STAT3 15 min following treatment (Stevenson *et al*, unpublished data). Following stimulation, cells were placed on ice and 4 ml of cold staining buffer was added. Media was removed and cold PBS was added to the cells following stimulation and cells scraped from the plate. The cells were centrifuged for 8 min at 285 x *g* at 4°C. The supernatant was removed and cells washed with 2 ml staining buffer. Cells were fixed by incubating in an equal volume (250 µl) of Cytofix (BD Biosciences) at 37°C for 10-15 min. Cells were washed in 2 ml staining buffer and centrifuged at 285 x



*g* for 5 min at 4°C. The supernatant was removed and cells permeabilised by adding 500 µl cold Phosflow Perm Buffer III (BD Biosciences) and incubating on ice for 30 min. The cells were centrifuged for 8 min at 285 x *g* at 4°C and washed once with 2 ml staining buffer. Antibodies against STAT1 (pY701) and STAT3 (pY705) (both BD Bioscience) were added to 100 µl of cells per test and incubated at RT for 30 min. Following staining, cells were washed once with staining buffer and re-suspended in 500 µl staining buffer for analysis using the Cyan flow cytometer (Beckman Coulter).

### **2.2.7 Whole blood staining to detect STAT phosphorylation**

Whole blood staining was carried out to detect STAT phosphorylation in immune cell populations, according to the manufacturer's protocol. Briefly, whole blood (200 µl) was untreated or treated with 1000U/ml IFN- $\alpha$  or 100 ng/ml IL-28A, IL-28B or IL-29 and incubated at 37°C for 30 min. After the stimulation period, the cells were fixed immediately by adding 10 volumes of pre-warmed Lyse/Fix Buffer (BD Bioscience) to the samples. The blood was lysed by inverting 5 to 10 times or by vortexing, followed by an incubation at 37°C for 10 to 12 min. The tubes were centrifuged at 600 x *g* min, supernatant was removed and cell pellet vortexed. The cells were washed twice, first by adding a volume of PBS equivalent to the volume of Lyse/Fix Buffer used. Samples were centrifuged at 600 x *g* for 6 min, supernatant removed and pellet vortexed, followed by an additional wash using stain buffer. The cells were re-suspended in a residual volume of stain buffer, to which surface marker antibodies were added: CD45-AmCyan, CD3 Pacific blue (BD Bioscience), CD303 (BDCA-2)-PE (Miltenyi Biotec), CD14-FITC/Lin-1-FITC/CD19-FITC (BD Bioscience). Samples were incubated at RT for 60 min protected from



light, after which time, cells were washed with 3 ml of Stain Buffer, centrifuged at  $600 \times g$  for 6 min and the supernatant removed. The cell pellet was vortexed and cells permeabilised by adding 1 ml of pre-chilled Perm Buffer III (BD Bioscience), vortexing and incubating for 30 min on ice. The cells were washed by adding 3 ml of Stain Buffer for every 1 ml of Perm Buffer used. The samples were centrifuged at  $600 \times g$  for 6 min, supernatant removed and pellet vortexed. The cells were washed once more with 3 ml stain buffer, followed by centrifugation at  $600 \times g$  for 6 min. The supernatant was removed and cells vortexed and re-suspended in the residual volume of stain buffer. The following BD Bioscience Phosflow antibodies (10  $\mu$ l) were added: STAT1-Alexa fluor 647 (pY701), STAT3-PE (pY705), STAT4-PE (pY693), STAT5-Alexa fluor 647 (pY694), STAT6 (pY641). Samples were incubated at RT for 60 min protected from light. Cells were washed with 3 ml of Stain Buffer, centrifuged at  $600 \times g$  for 6 min. Supernatant was removed, cells vortexed and re-suspended in approximately 500  $\mu$ l of Stain Buffer. Samples were acquired immediately using a Canto II flow cytometer and data analysed using FlowJo software.

### **2.2.8 RNA extraction**

RNA was extracted from pDCs using the RNeasy micro kit (Qiagen) and from B cells using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. RNA was extracted from Huh7s and PBMCs using the TRIzol® method. Cell pellets from which RNA was to be extracted were re-suspended in 1 ml of Trizol and left at RT for 5 min. Two hundred microlitres of Chloroform (Sigma) was added to cells and shaken vigorously for 15 sec, left at RT for 3 min and then centrifuged at  $13,400 \times g$  for 15 min at  $4^{\circ}\text{C}$ . The upper aqueous layer was

carefully transferred to a second eppendorf tube, mixed with 500  $\mu$ l isopropanol (Fisher Scientific) and shaken. Following 10 min incubation at RT, the solution was centrifuged at 13,400  $\times g$  for 10 min at 4°C. The supernatant was removed and discarded and the RNA pellet re-suspended in 75% ethanol (Sigma) and vortexed. The suspension was once again centrifuged at 5,100  $\times g$  for 5 min at 4°C. The supernatant was removed and the pellet air-dried for approximately 4 min. Finally, the RNA pellet was dissolved in 30  $\mu$ l RNase-free water and vortexed. The pellet was dissolved by incubating at 60°C for 5-10 min then placed on ice immediately. RNA quality and yield was assessed using the Agilent 2100 Bioanalyser (Agilent Technologies Ireland Ltd., Cork, Ireland) and ND-1000 NanoDrop® spectrophotometer, respectively as well as visualising the ribosomal RNA bands on 1.5% agarose gel. RNA was stored at -80°C.

### **2.2.9 Complementary DNA (cDNA) synthesis**

For downstream applications such as gene expression studies, cDNA was synthesised from the extracted RNA using Qiagen Omniscript® reverse transcription kit (Qiagen). RNA samples were diluted to give 1  $\mu$ g RNA in 12  $\mu$ l water and the standard protocol for cDNA synthesis from 50 ng-2  $\mu$ g RNA was followed. Briefly, all reagents were thawed on ice and then 2  $\mu$ l 10X buffer, 2  $\mu$ l oligo dT primer, 2  $\mu$ l dNTP and 1  $\mu$ l Omniscript was added per reaction. Samples were incubated for 1 hr at 37°C following which the enzyme was inactivated by incubating for 5 min at 95°C. Samples were stored at -20°C until further use.

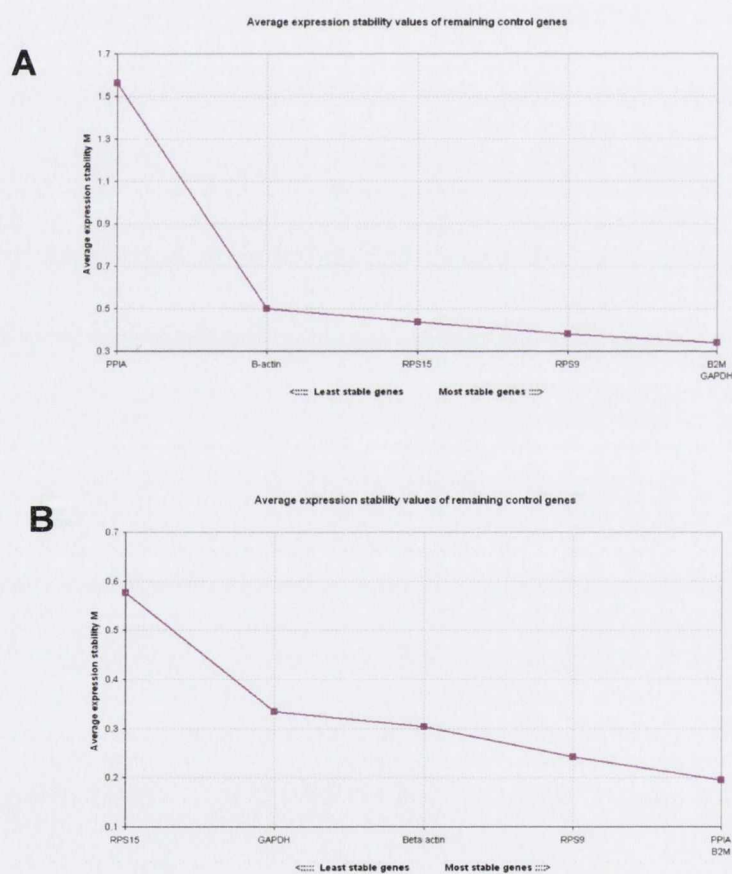
### 2.2.10 Real-time quantitative PCR (RT-PCR)

Following cDNA synthesis, samples were measured by NanoDrop® ND-1000 spectrophotometer and diluted in RNase-free water to give a concentration of 40 ng/μl. RT-PCR was carried out using SYBR Green (Roche, Switzerland) chemistry and the MX3000P® instrument (Stratagene, CA, USA). Each reaction was carried out in duplicate in a total volume of 25 μl with 2 μl of cDNA (40 ng/μl), 12.5 μl SYBR Green and 10.5 μl primer. Primers were designed to be intron-spanning using Primer3 or Primer Express® v3.0 software (Applied Biosystems) and purchased from IDT. To determine optimal primer concentration, concentrations of 100nM, 300nM, 600nM and 900nM were titrated using qRT-PCR. Amplification of a single PCR product was confirmed by analysing dissociation curves and visualisation on agarose gels. A full list of primers is given in **Table 2.1**. A non-template control (NTC) was included for each primer set to confirm absence of DNA contamination. The following cycling parameters were used: 95°C for 30 sec, 60°C for 1 min and 72°C for 30 sec, followed by amplicon dissociation. For calculation of gene induction of a stimulated sample versus an unstimulated sample, data was analysed using the  $2^{-\Delta\Delta C_t}$  method by normalising gene expression of test gene to a housekeeping gene<sup>181</sup>, selected using GeNorm analysis. For calculation of basal gene expression, a ratio of gene of interest relative to housekeeping gene was generating by normalising the gene of interest to a stable internal housekeeping control gene using the formula  $2^{-(C_t \text{ gene of interest})} / 2^{-(C_t \text{ housekeeper})}$ .



### 2.2.11 GeNorm analysis

All gene amplifications for human samples were normalised to  $\beta 2$  microglobulin ( $B2M$ ), which was selected as the most stably expressed gene across samples from a panel of potential normalisers:  $\beta$ -Actin, ribosomal protein  $S15$  ( $RPS15$ ), glyceraldehyde 3-phosphate dehydrogenase ( $GAPDH$ ), peptidylprolyl isomerase A protein ( $PPIA$ )  $\beta 2$  microglobulin ( $B2M$ ) and ribosomal protein  $S9$  ( $RPS9$ ) using GeNorm version 3.4 (**Figure 2.1**). Gene stability is measured by the M value, with a stable gene measured as having an M value  $< 1.5$  and the lowest M value signifying the most stable gene.



**Figure 2.1: GeNorm analysis of reference genes**

GeNorm analysis showing stability of reference genes across (A) Huh7s and (B) PBMCs, treated with IFN- $\lambda$ . M values  $< 1.5$  are considered stably expressed genes.

### 2.2.12 Antiviral gene array

The RT<sup>2</sup> Profiler PCR Array Human Antiviral Response array (SA Biosciences, Frederick, MD, USA) was used to identify gene expression induced by IFN- $\lambda$  (IL-29) in the Huh7 hepatocyte cell line. These arrays are low-density arrays of qPCR primers that amplify a defined set of 96 genes (**see Appendix**). Huh7 cells (125,000) were either untreated or treated with 500 ng/ml IL-29. RNA was extracted as per **section 2.2.8** and purified using the RNeasy mini kit clean-up protocol as per manufacturer's instructions. The RNA (375 ng) was converted to cDNA using the RT<sup>2</sup> First Strand cDNA synthesis kit (SA Biosciences). Real time PCR reactions were prepared using RT<sup>2</sup> SYBR Green ROX qPCR Mastermix (SA Bioscience) and performed on the Mx3000P instrument, as per the manufacturer's instructions. Analysis of the PCR array gene expression data was carried out using the data analysis web portal provided by SA Biosciences (<http://www.sabiosciences.com/pcrarraydataanalysis.php>). The array was run using one untreated Huh7 sample, and one treated with IL-29 500 ng/ml. Gene expression induced was validated by real-time PCR in n=3 Huh7 samples treated with 1000 U/ml IFN- $\alpha$ , and 100 ng/ml or 500 ng/ml IL-28A, IL-28B and IL-29.

## **2.3 IFN- $\lambda$ -producing DC subsets in the liver**

### **2.3.1 Liver perfusate collection**

Samples were collected from donor livers (n=22) during orthotopic liver transplantation at St. Vincent's University Hospital. During retrieval, the donor aorta and superior mesenteric vein were initially flushed with University of Wisconsin (UW) solution (Bristol-Myers Squibb, Uxbridge, UK) at the time of exsanguination. The liver was flushed again with UW solution after excision of the organ until all blood was removed and the perfusate appeared clear, at which time the liver was placed in a container with UW solution and packed on ice for transportation. Donor livers were transplanted within 12 hours. At implantation, after completion of the upper inferior cava anastomosis, livers were flushed with normal saline through the portal vein to wash out the UW before reperfusion. This wash-out fluid was collected from the inferior vena cava; the UW transportation solution was also collected. Diseased livers (n=12) were also perfused with normal saline and cells collected. Upon explantation, the diseased liver was perfused with saline through the portal vein, similar to the donor organ, and comparable volumes of perfusate collected (600-1,200 mls). Clinical details from donors and recipients are presented in Table 1. All protocols were approved by St. Vincent's University Hospital Ethics Committee in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.



### **2.3.2 Isolation of hepatic mononuclear cells (HMNCs) from liver perfusate**

HMNCs were isolated by filtration of liver perfusate through 70  $\mu$ M filters (BD Biosciences) followed by centrifugation at 300 x *g* for 10 min to remove debris. HMNCs were separated from the resulting solution by density gradient centrifugation using Ficoll-Paque™ PLUS and any red blood cells were removed by red cell lysis solution (Sigma).

### **2.3.3 Characterisation of liver DC subsets by flow cytometry**

HMNCs isolated from liver perfusates collected from donor livers (n=22) and explant livers (n=12) were characterised. Following incubation of cells with 5% mouse serum (eBioscience) for 10 min, 500,000 HMNCs were stained for 20 min at 4°C using fluorescently labelled antibodies: Lin1-FITC, HLA-DR-PerCP-Cy5.5, (BD Biosciences), CD11c-PE-Cy7, CD123-eFluor®450, (eBioscience), CD1c (BDCA-1)-PE, CD141 (BDCA-3)-APC, (Miltenyi Biotec) and CLEC9A-PE (R&D Systems). ILT3 and ILT4 (both R&D systems) expression was also measured. At least 100,000 events were acquired for each sample on a Canto II flow cytometer and analysis was performed using Flowjo software.

### **2.3.4 Whole blood staining for DC subsets**

Healthy blood donors (n=10) were recruited locally. DC subsets were characterised by flow cytometric analysis of 200  $\mu$ l fresh whole blood, stained within 4 hours of collection, as described previously<sup>182</sup> and in **Section 2.25**. Antibodies used to stain DC subsets included: Lin1-FITC, HLA-DR-PerCP-Cy5.5, CD11c-PE-Cy7, CD123-eFluor450, BDCA1-PE, -APC, BDCA2-PE and BDCA3-APC.

### **2.3.5 Magnetic bead separation of CD141<sup>+</sup> DCs**

CD141<sup>+</sup> DCs were isolated by positive selection using the CD141 (BDCA-3) Microbead kit (Miltenyi Biotec) as per manufacturer's instructions. Cells were cultured in RPMI 1640 medium (Invitrogen) with 10% FCS and 250U/ml penicillin and 250µg/ml streptomycin (both Invitrogen) at 37°C in 5% CO<sub>2</sub>.

### **2.3.6 TLR activation of HMNCs, PBMCs and CD141<sup>+</sup> DCs**

Isolated HMNCs (n=3) were cultured at a density of 1x10<sup>6</sup>/ml in 24-well plates and activated with TLR 3, 4 and 7/8 ligands: 50 µg/ml poly(I:C), 1 µg/ml LPS and 1 µg/ml CLO97 (Invivogen) for 18 hrs, after which time supernatants were removed and stored at -20°C until analysis. Total PBMCs and isolated CD141<sup>+</sup> DCs were treated with 50 µg/ml poly(I:C) in 250 µl 10% RPMI in a 96-well plate for 18 hrs. After treatment, cell supernatants were collected and stored at -20°C until further analysis.

### **2.3.7 Cytokine measurement**

IL-1β, IL-23 and IFN-λ (IL-29) levels were measured in supernatants from poly(I:C)-treated CD141<sup>+</sup> DC cultures and T cell secretion of IFN-γ, IL-17A and IL-17F measured in MLRs using Ready-SET-Go!<sup>®</sup> ELISAs (eBioscience) according to the manufacturer's instructions. DC production of IL-12p70 and secretion of IL-10 from both T cell and DC supernatants were measured using DuoSet<sup>®</sup> ELISAs (R&D Systems). IFN-α, IL-1β, IL-10 and IL-12p70 were additionally quantified in the supernatants of poly(I:C)-treated cells using FlowCytomix bead arrays

(eBioscience). Samples were acquired on a Canto II flow cytometer and results analysed using FlowCytomix Pro 3.0 software (eBioscience).

### **2.3.8 Confocal microscopic analysis of hepatic DCs**

HMNCs isolated from liver perfusate were coated on a BD BioCoat™ poly-D-lysine-coated chamber slide (BD Biosciences) overnight at a density of 600,000 cells/well. The cells were fixed with 4% PFA and then permeabilised and blocked in 1% BSA, 0.1% Triton X-100 (Sigma) and 1% goat serum for 30 min at room temperature. The slides were stained overnight with anti-human CD141 (Miltenyi Biotec). Following five washes in PBS, cells were stained with goat anti-mouse Alexa Fluor 488 and Alexa Fluor 594-labelled wheat germ agglutinin membrane stain (both Invitrogen) at room temperature for 60 min. Slides were washed and mounted using Prolong gold anti-fade reagent containing DAPI (Invitrogen). Microscopy was performed on an Olympus FV1000 laser scanning confocal microscope, using an UPlanSApo 20X/0.75 NA and a UPlanSApo 60X/1.35 NA oil objective, and images were analysed using Imaris software version 7.4.2 (Bitplane AG, Zurich, Switzerland).

### **2.3.9 Mixed lymphocyte reaction: CD141<sup>+</sup> DCs: CD3<sup>+</sup> T cells**

CD141<sup>+</sup> DCs were isolated from liver perfusate as described above. Allogeneic CD3<sup>+</sup> T cells were isolated from healthy donor blood using CD3 Microbeads (Miltenyi Biotec) as per manufacturer's instructions and labelled with 0.5µM CFSE (eBioscience) as described previously<sup>94</sup>. T cells and CD141<sup>+</sup> DCs were co-cultured at a ratio of 20:1 for 7 days; cells were then re-stimulated with 10 ng/ml PMA and



500 ng/ml ionomycin (both Sigma) with 1 $\mu$ l/ml Golgi plug (BD Biosciences). Cells were harvested and stained for intracellular cytokines using IL-17A-PE (eBioscience), IFN- $\gamma$ -PE-Cy7, IL-10-APC (both BD Bioscience), CD8a-APC-eFluor®780 and CD3-PerCP-Cy5.5 (eBioscience) antibodies. Samples were acquired using the Canto II flow cytometer and analysed using Flowjo software.

### **2.3.10 Statistical analysis**

Statistical differences between DC subset frequencies in donor and explant perfusates were evaluated using one way ANOVA (Kruskal-Wallis test with Dunns post-test) and between blood and liver by Mann-Whitney *U* test using GraphPad Prism software, version 5.01. A p-value of  $\leq 0.05$  was considered significant. Principal component analysis was performed in MATLAB R2011b (The Mathworks, Inc., Natick, MA, USA) with the PLS-Toolbox version 6.7.1 (Eigenvector Research Inc., Wenatchee, WA, USA).

**Table 2.1: List of primer sequences**

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<b>Primer</b>	<b>Primer sequence (5'-3')</b>
B-actin F	AGATGACCCAGATCATGTTTGAGA
B-actin R	CGTCACCGGAGTCCATCAC
GAPDH F	CCACCCATGGCAAATTCC
GAPDH R	CAGCATCGCCCCACTTG
PPIA F	GCAAATGCTGGACCCAACAC
PPIA R	GCCATTCTGGACCCAAAG
RPS9 F	TGACCGGGTGGTTTGCTTA
RPS9 R	ATACTCGCCGATCAGCTTCAG
RPS15 F	CGGACCAAAGCGATCTCTTC
RPS15 R	CGCACTGTACAGCTGCATCA
B2M F	GTGCTCGCGCTACTCTCTCTTT
B2M R	GTCAACTTCAATGTCGGATGGA
IL-28R $\alpha$ F	CCAGCCAGTCCAGATCACTCT
IL-28R $\alpha$ R	ACAGCAGTATCAGAAGCGATGG
IL-10R $\beta$ F	AGGTCCTCAGAAACCTGGAGCCA
IL-10R $\beta$ R	TCCAGGAGGGGACCGTTTCGTC
MxA F	GGTGGTGGTCCCCAGTAATG
MxA R	ACCACGTCCACAACCTTGTCT
OAS2 F	GAAGCCCTACGAAGAATGTCAGA
OAS2 R	TCCGAGTTGCCTCTTAAGACTGT
MDA5 F	CAGTGTGCTAGCCTGTTCTGG
MDA5 R	GTTCCCCAAGCCTGGCCACAT

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**Chapter 3: Association of Genetic  
Variation in the Antiviral Gene *OAS1*  
with Outcome to HCV Infection**



### 3.1 Introduction

Of individuals infected with HCV, ~20% are capable of viral clearance whereas ~80% develop chronic infection. Combined IFN- $\alpha$ -ribavirin therapy resolves disease in only ~50% of treated genotype 1-infected individuals, suggesting involvement of host genetic factors. Variation in innate immune genes upon initial detection and response to viral infection may account for differential outcome in HCV infection. Type I IFNs, induced upon HCV infection, activate ISGs including the *OAS* genes, which are a critical component of the innate antiviral response. *OAS* enzymes catalyse the synthesis of 2'-5'-linked oligoadenylates from ATP which then bind and activate latent RNase L resulting in the degradation of viral RNAs (**Figure 3.1**). There are four *OAS* genes in humans; *OAS1*, *OAS2* and *OAS3*, which are clustered together on chromosome 12q24.1, and *OAS*-like (*OASL*) gene, located at 12q24.2<sup>183</sup>. There is one copy of *OAS1* in the human genome but alternative splicing gives rise to several isoforms, thereby potentially signifying differences in the innate immune response to viral infection. Splice variants include exons 1-5 but vary with regard to downstream exons and thus produce proteins of various sizes: p42, p44, p46, p48 and p52<sup>184</sup>.

SNPs give rise to variation in the *OAS1* gene. The *OAS1* SNP, rs10774671 is a prime candidate for being a functional SNP as the A allele is at an intron-exon boundary, resulting in alternative splicing, generating isoforms with varying *OAS* activity. The G allele allows splicing to occur leading to generation of the p46 isoform, reported to have higher *OAS* enzymatic activity, whereas the A allele prevents splicing at this site. Instead, splicing occurs further downstream, giving rise to the p48 and p52 isoforms, associated with lower *OAS* enzymatic activity<sup>184</sup>. Genetic variation

in *OAS1* has previously been associated with disease outcome in HCV <sup>185, 186</sup> and other viral infections such as West Nile Virus (WNV) <sup>187</sup> and severe acute respiratory syndrome (SARS) <sup>188</sup> as well as in the immune response to rubella vaccination <sup>189</sup> and autoimmune diseases such as Type I Diabetes <sup>190</sup> and Multiple Sclerosis <sup>191, 192</sup>, both of which have been linked with viral aetiology (summarised in **Table 3.1**). While rs10774671 has been studied intensely and found to be functional in a number of diseases, limited study has been carried out in HCV. One study found association with the rs2660 SNP in the 3' untranslated region (UTR) of *OAS1*, which is known to be in LD with rs10774671. An additional study, focused specifically on rs10774671, found an association with HCV treatment outcome and disease severity <sup>193</sup>. However, the disadvantage of such studies is that the cohorts often vary in ethnicity, sex, infecting viral genotype and presence of co-morbidities.

In this study, we aimed to examine the influence of rs10774671 in determining HCV outcome by genotyping a homogenous cohort of HCV-infected Irish women. This cohort was infected with HCV genotype 1b from a single source in 1977-78, after administration of HCV-contaminated anti-D immunoglobulin at childbirth. Of the thousands potentially infected and screened, 704 women had evidence of exposure to HCV and 55% of these were chronically infected as measured by presence of HCV RNA (PCR+) <sup>16</sup>. Individuals in this cohort generally had mild disease but some went on to develop fibrosis, cirrhosis and HCC <sup>16</sup>. All of these women were of child-bearing age without co-morbidities such as alcohol-related liver disease and co-infection with additional viruses. Therefore, access to this well-characterised cohort of patients exposed to a single inoculum of HCV allows

us to examine the role of genetic variation in immune response to the outcome of HCV.

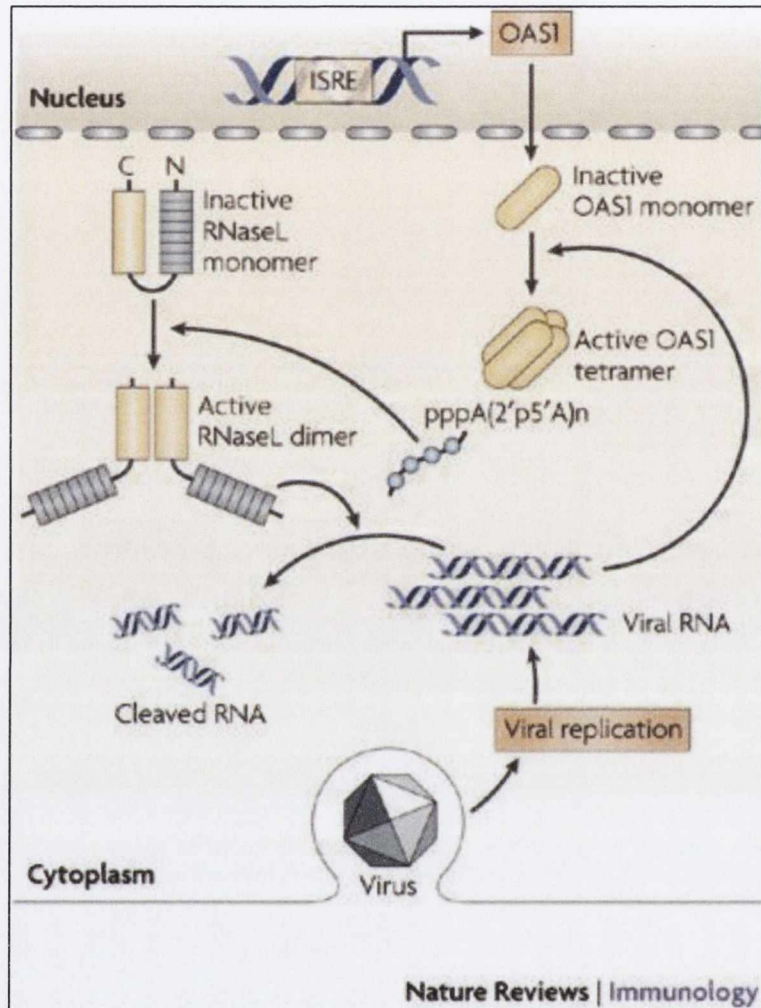
**Aims:**

- To genotype HCV patients for *OAS1* rs10774671 SNP using RFLP and real time PCR
- Observe if a correlation exists between rs10774671 genotype and natural clearance of HCV
- Correlate rs10774671 genotype with markers of disease severity

**Hypothesis:**

The *OAS1* rs10774671 GG genotype, leading to high antiviral activity, is more common in patients who spontaneously clear HCV infection





**Figure 3.1: Overview of the OAS/RNase L pathway**

Binding of 2'-5' oligoadenylate synthetase (OAS) proteins to viral double-stranded (ds)RNA triggers the synthesis of 2'-5' oligoadenylates which bind and activate RNase L. RNase L dimerises and degrades viral RNA present in the cell leading to the inhibition of protein synthesis.

Figure taken from <sup>50</sup>

**Table 3.1: Summary of OAS SNP associations with disease**

<i>OAS</i> SNP	Disease	Clinical Association	Reference
rs10774671	Type 1 Diabetes	G allele increased in diabetics	190
	Multiple Sclerosis	(a) G allele associated with increased risk of MS in haplotype with rs3741981	191
		(b) The AA genotype may confer susceptibility to and the GG genotype may protect against increased disease activity	192
	WNV	A allele increased in symptomatic and asymptomatic WNV seroconverters - AA associated with initial infection and not severity of clinical outcome	187
	HCV	AA associated with treatment non-response and progressive HCV disease in Egyptian cohort	193
Rubella vaccine Response	A allele was associated with lower rubella virus-specific IL-2 secretion and higher IL-10 levels	189	
rs2660	HCV	GG genotype found in 9% of people with self-limiting infection compared to 18.8% with chronic hepatitis Caucasian patients, n=437 Mixed population with regard to patient gender, virus genotype and source of infection	120
	SARS	G allele confers protection against SARS	188
rs3741981	HCV	Patients with GG genotype were at increased risk of suffering from higher ALT, AST levels, higher degree of liver fibrosis and presence of liver cirrhosis	186

## 3.2 Results

### 3.2.1 *OAS1* rs10774671 is not associated with natural clearance of HCV infection

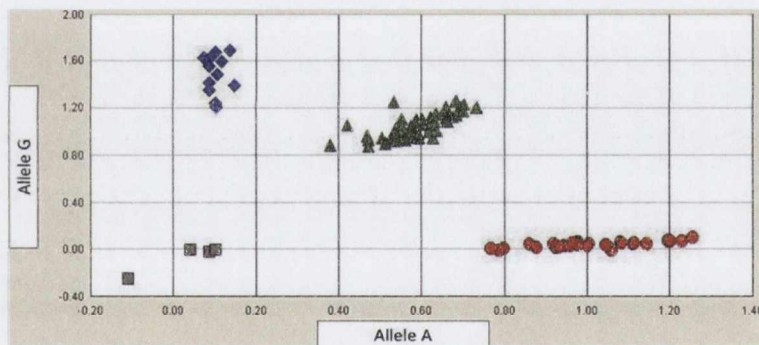
DNA samples from the Irish anti-D cohort were genotyped for the *OAS1* SNP rs10774671 using the Applied Biosystems Allelic Discrimination SNP genotyping assay, whereby samples clustered into AA, AG or GG genotypes (**Figure 3.2A**). SNP determinations were based on quality values of >98%, indicating adequate DNA quality for analysis. The genotype of a proportion of samples were also determined using RFLP (n=20) and were found to correlate 100% with the Applied Biosystems assay (**Figure 3.3**). When the genotyping results were correlated with clearance of the virus (PCR-) or development of chronic infection (PCR+) using the  $\chi^2$  test for statistical analysis, there was no association between rs10774671 genotype and clearance of HCV infection indicating that this SNP does not have any bearing on HCV outcome in this cohort (**Table 3.2** and **Figure 3.2**).



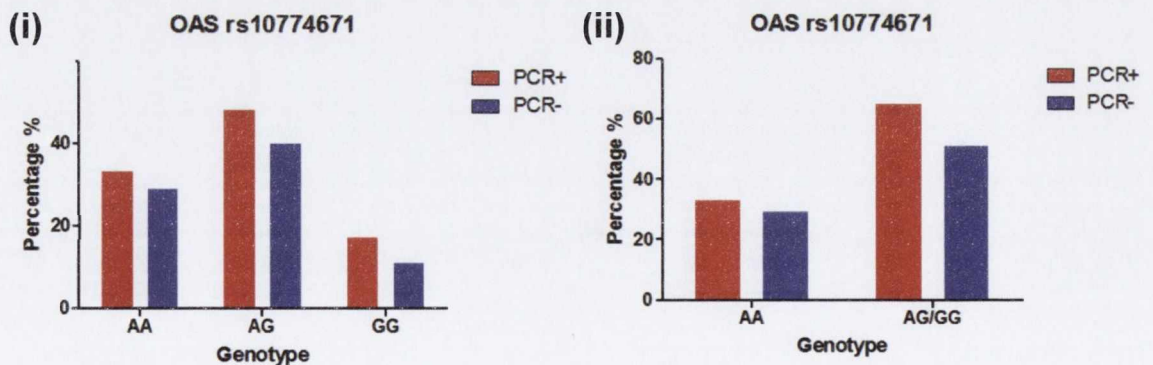
**Table 3.2: Allelic and genotype frequencies of *OAS* rs10774671 in a homogenous cohort of Irish patients who were exposed to HCV contaminated Anti-D**

	Total (%) n=178	PCR+ (%) n=98	PCR- (%) n=80
AA	62 (34.8)	33 (33.7)	29 (36.3)
AG	88 (49.5)	48 (49.0)	40 (50.0)
GG	28 (15.7)	17 (17.3)	11 (13.7)
Total	178 (100)	98 (100)	80 (100)
AA	62 (34.8)	33 (33.7)	29 (36.2)
AG/GG	116 (65.2)	65 (66.3)	51 (63.8)
Total	178 (100)	98 (100)	80 (100)

**A**



**B**



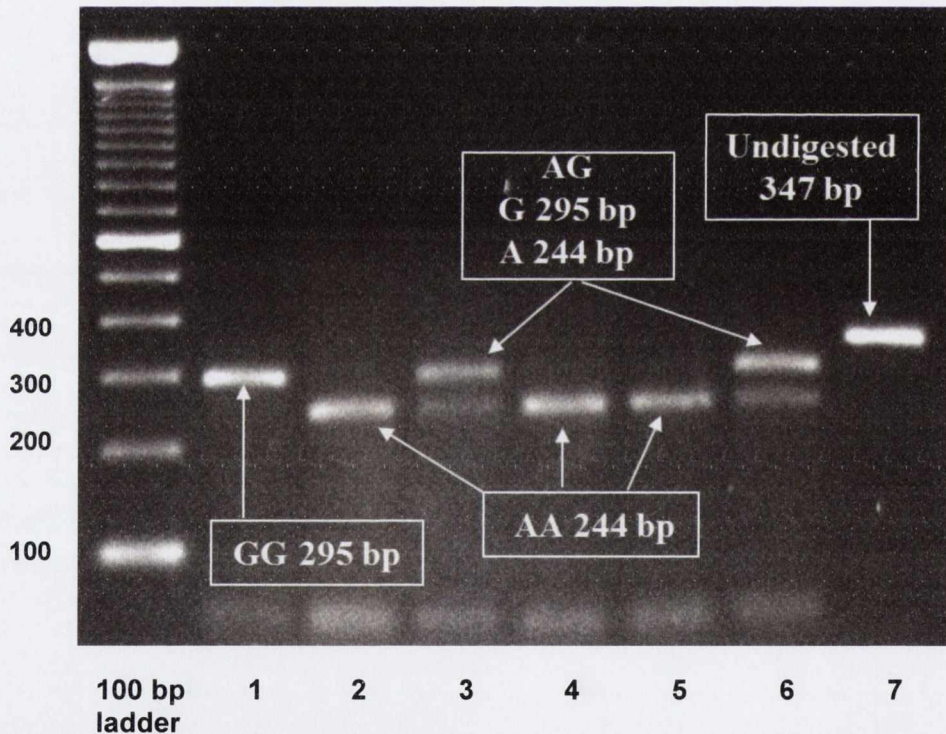
**Figure 3.2: *OAS1* rs10774671 SNP genotyping frequencies**

(A) HCV-infected individuals were genotyped for rs10774671 using Applied Biosystems Allelic Discrimination assay. (B) No significant differences in (i) genotype or (ii) allele frequency were observed between the groups as measured by  $\chi^2$  test (genotype,  $p=0.7963$ ; allele,  $p=0.7534$ ).

**A**



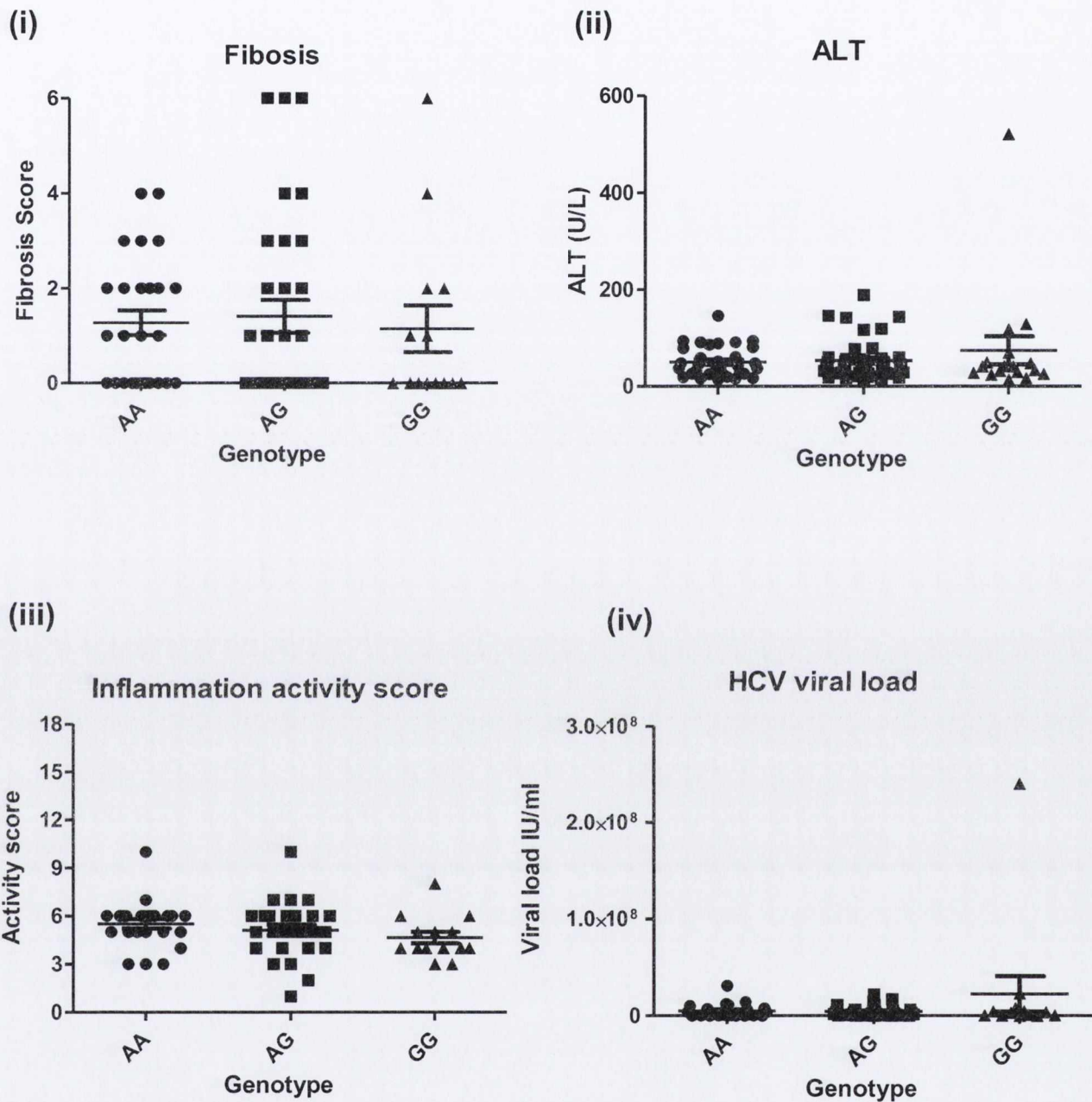
**B**



**Figure 3.3: *OAS1* rs10774671 SNP genotyping by RFLP**

(A) A region of the *OAS1* gene, containing rs10774671, was amplified and the PCR product digested using *AluI* restriction enzyme which cuts the DNA product in the presence of the sequence 'AGCT'. (B) Digested products were visualised on a 1.5% agarose gel. A constant restriction site resulted in bands of 52bp and 295bp. The presence of the A allele created a second restriction site with the 295 bp fragment being further digested into bands of 244 bp and 51 bp in size.





**Figure 3.4: *OAS* rs10774671 and clinical outcome of HCV infection**

Clinical parameters of liver disease were correlated with the rs10774671 AA, AG or GG genotype in HCV PCR+ patients. Data was available for n=25 patients with the AA genotype, n= 33 AG patients and n=14 patients with the GG genotype. Biopsy specimens were scored according to Ishak's grading and staging method and the modified histological activity index (HAI) system. The most recent ALT results were obtained from medical records. Each parameter was compared between the three groups using one way ANOVA Kruskal Wallis test (P-value <0.05).



### 3.3 Discussion

This aim of this study was to determine if there was any association between variation in the host antiviral gene *OAS1* and the clinical outcome to HCV infection. Genetic variation in the immune response to HCV has been shown to be multifactorial, with no one factor responsible for viral clearance. However, determining key markers of disease outcome is likely to be useful as a predictor of therapeutic response. Currently, HCV is treated with peg-IFN- $\alpha$  and ribavirin, which is hugely expensive, associated with significant side-effects and is ineffective in up to 50% of individuals infected with genotype 1 of the virus. Predicting the patients more or less likely to respond to treatment could help guide administration of therapy.

We selected a candidate gene, *OAS1*, known to have a functional SNP, rs10774671, previously associated with host outcome to a number of viral infections (**Table 3.1**). The aim of our study was to determine if there was any association between *OAS1* rs10774671 genotype and clearance of HCV infection or disease severity. We genotyped a homogenous cohort of 178 HCV-infected Irish women for the *OAS1* rs10774671 SNP in order to investigate if the G allele, which results in high enzymatic activity, was more frequent in individuals who spontaneously cleared the virus. No significant association was correlated between the rs10774671 genotype and initial HCV infection. Furthermore, when rs10774671 genotype was correlated with markers of disease severity and progression such as ALT level, viral load, fibrosis score and inflammation score, no association was evident with any of these factors.

While we found that *OAS1* rs10774671 does not influence natural clearance of HCV or disease severity, it is possible that this SNP is associated with HCV treatment outcome, as *OAS1* is a key antiviral gene upregulated by IFN- $\alpha$  treatment. Some studies have found treatment association in HCV cohorts of different genotypes and treatment regimes. In a study of an Egyptian cohort infected with HCV genotype 4a (n=70), the AA genotype was associated with non-response to treatment and progressive liver disease <sup>193</sup>. It is noteworthy that the GG genotype is the most frequent in this population and AA is rare, which is the complete opposite to Caucasian populations. However, due to the general mild disease of the Anti-D cohort, not enough individuals have been treated in order to make this assessment in our study.

Continued analysis may reveal a relationship between this *OAS1* SNP and response to treatment. However, during the course of this study, a number of GWAS studies were published, identifying SNPs in the IFN- $\lambda$  gene region as having a highly significant association with natural and therapeutic outcome to HCV <sup>118</sup>. This type of large scale study has now mostly superseded candidate gene studies and has proven a revolution in the search for genetic markers for disease outcome. The successful identification of a genetic marker of disease outcome has resulted in *IL28B* genotyping being adopted into the treatment algorithm in the clinic. Continued study of the *IL28B* SNP its biological function may reveal a novel candidate therapeutic target for HCV.

## **Chapter 4: Cellular responsiveness to IFN- $\lambda$**



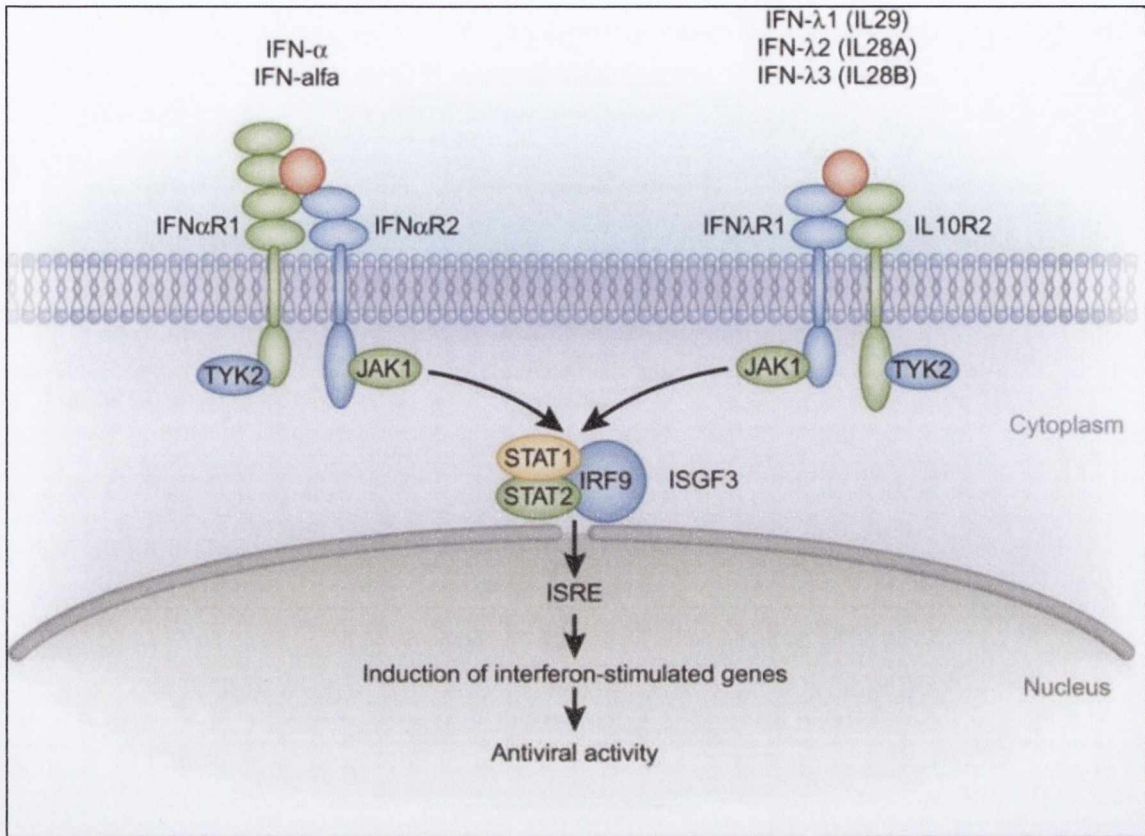
## 4.1 Introduction

IFNs are important pleiotropic cytokines with a wide range of biological functions. Their best known function is in antiviral immunity, however, they can also have immunomodulatory function, and can block or promote cell proliferation<sup>194</sup>. As a result, IFNs are used in the treatment of a range of diseases. For example, IFN- $\alpha$  is used to treat viral infections, such as HBV and HCV, and some cancers including hairy cell leukaemia<sup>195</sup>, chronic lymphocytic leukaemia<sup>196</sup> and melanoma<sup>197</sup>. IFN- $\beta$  is used as therapy for the autoimmune disease, Multiple Sclerosis<sup>198</sup>. The role of the Type I and Type II IFNs in the immune response is well-characterised, but the major functions of the most recently discovered IFN, the Type III IFNs or IFN- $\lambda$ , are less well known.

The Type III IFNs are related to both the Type I IFNs and the IL-10 superfamily, but, show less than 20% amino acid identity with either family<sup>46</sup>. Similar to Type I IFNs, IFN- $\lambda$  signals through the JAK-STAT pathway to induce ISGs, upregulate MHC expression and inhibit viral replication<sup>45, 46</sup>. However, it uses a receptor chain shared with IL-10, IL-22 and IL-26 and has a multi-exon gene structure that is more similar to the IL-10 superfamily<sup>131</sup>. Following recent genetic association of polymorphisms in the IFN- $\lambda$  family gene region with clinical outcome to HCV infection, it is apparent that these cytokines play an important role in the antiviral immune response to HCV but its relationship to the IL-10 family implies that it may have additional immunomodulatory properties. Therefore, a major question is which cell types does IFN- $\lambda$  exert its effect on and how does this differ from IFN- $\alpha$ , the best-characterised Type I IFN, with which IFN- $\lambda$  has demonstrated functional similarity.

#### 4.1.1 IFN- $\lambda$ receptor expression

Despite many shared characteristics, a major difference exists between IFN- $\alpha$  and IFN- $\lambda$  at the level of receptor expression. In order for a cytokine to exert its effect on a particular cell type, the cell must express the necessary receptor. The Type I IFN receptor consists of two chains, IFNAR1 and IFNAR2, and is expressed on almost every cell in the body. The IFN- $\lambda$  receptor comprises the IL-10R $\beta$  chain, shared with members of the IL-10 superfamily and ubiquitously expressed, and the specific IL-28R $\alpha$  chain, expressed on only a subset of cells (**Figure 4.1**). In studies aimed at identifying target cells of IFN- $\lambda$  on epithelial barriers and the immune system, the liver, specifically hepatocytes<sup>139, 199</sup>, the skin, intestine and lungs<sup>129, 139</sup> were among the tissues with the highest IL-28R $\alpha$  expression, whereas expression was low in tissues such as the brain. The main organs of the immune system (spleen, thymus, PBMCs), as well as the skin, were also among those organs expressing above average IL-28R $\alpha$  levels<sup>139</sup>. Reports showing IFN- $\lambda$  receptor expression on immune cells have been conflicting; data is largely based on gene expression studies<sup>139, 200</sup> with little known about expression of the IL-28R $\alpha$  protein on individual immune populations.



**Figure 4.1: IFN- $\lambda$  and IFN- $\alpha$  receptors**

IFN- $\alpha$  binds to its receptor, composed of IFNAR1 and IFNAR2 chains, activating downstream antiviral activity. Binding of the Type III IFNs, IL-28A, IL-28B and IL-29, to their receptor comprising the IL-10R $\beta$  chain and the specific IL-28R $\alpha$  chain, appears to activate similar biological activity to IFN- $\alpha$ .

Figure taken from <sup>201</sup>



#### 4.1.2 IFN activation of signalling pathways

Type I, type II and type III IFNs primarily signal through the JAK-STAT pathway to up-regulate hundreds of ISGs, a proportion of which are antiviral, contributing to IFN-mediated inhibition of viral replication in infected cells <sup>43</sup>. Binding of IFNs to their receptor induces the phosphorylation of receptor-associated JAK proteins and STAT proteins. The classical activation of antiviral immunity in response to Type I IFNs occurs via activation of a STAT1-STAT2 heterodimer in complex with IRF-9, forming the major transcription factor ISGF3, which binds to ISRE elements in the promoters of antiviral genes. The Type II IFN, IFN- $\gamma$ , activates STAT1 homodimers which bind to GAS elements in the gene promoter region. STAT1 and STAT2 have a well-characterised role in antiviral defence, with STAT1<sup>-/-</sup> and STAT2<sup>-/-</sup> mice showing increased susceptibility to viral infection <sup>202-204</sup>. However, not all genes activated by Type I IFNs are antiviral. Differential use of STAT proteins occurs, with Type I IFNs being capable of activating all STATs depending on conditions of activation, which accounts for their additional immunomodulatory and anti-proliferative properties <sup>205</sup>. In particular, unique STAT activation patterns are evident in immune cells, which contribute to their different biological functions. Regulation of recruitment of different signalling pathway components may account for the pleiotropic biological effect of IFN <sup>205</sup>. STAT activation by Type I IFNs results in the formation of different combinations of homo- and hetero-dimer pairs. These STAT complexes can then translocate to the nucleus and bind to specific response elements, present in ISG promoter regions.

Type I IFNs, in addition to inducing antiviral genes, are involved in directing T cell polarisation, cell survival and, paradoxically, induction of apoptosis. The relative concentrations of STATs in immune cells can account for their differential responses to Type I IFNs. For example, Type I IFN activates STAT4 in CD8<sup>+</sup> T cells to promote cell survival, proliferation and IFN- $\gamma$  production, important in antiviral immunity. However, this activation only occurs in the presence of low STAT1 <sup>206</sup>. Similarly, high STAT4 is a characteristic of NK cells, which is important for IFN- $\gamma$  induction. STAT1 increase in viral infection leads to decreased STAT4 access, thus, representing a key role for STAT1 in the regulation of IFN- $\gamma$  production <sup>207</sup>. Furthermore, it has been shown that Type I IFNs have an anti-proliferative effect on T cells, but have an anti-apoptotic effect in the absence of STAT1, mediated by the activation of STAT3 and STAT5 <sup>208</sup>.

Additional signalling pathways other than JAK-STAT also contribute to the biological functions of IFNs. Type I IFNs can activate the mitogen-activated protein kinases (MAPK), p38 and ERK (**Figure 4.2**), and the phosphoinositide-3 kinase (PI3K) pathway, which participate in mediating the growth-inhibitory and antiviral effects of Type I IFNs <sup>209</sup>.

While Type I and Type II IFN effects have been characterized in immune cells, to date <sup>47, 210</sup>, minimal information is known about the specific responsiveness of immune cells to IFN- $\lambda$ . Activation of signalling pathways in individual cell populations and downstream gene induction has not been demonstrated. It has been reported that IFN- $\lambda$ , although expressing a distinct receptor to IFN- $\alpha$ , has an

almost identical signalling profile, reported to activate STAT 1, 2, 3, 4 and 5<sup>45, 199</sup> and to up-regulate antiviral genes<sup>45, 46</sup>. Multiple activation of STATs by IFN- $\lambda$ , similar to Type I IFN, suggests diverse biological effects, similar to Type I IFNs. Prolonged activation of the JAK-STAT pathway has been reported in IFN- $\lambda$ -treated cells compared to IFN- $\alpha$ <sup>211</sup>. Similar to IFN- $\alpha$ , the IFN- $\lambda$ s also trigger different MAPK pathways: ERK, JNK and p38 kinases<sup>212</sup>. These various signalling studies have been carried out on a number of cell types including hepatocytes<sup>141, 213</sup>, intestinal epithelial cells<sup>214</sup> as well as various cell lines<sup>211, 215</sup>. However, almost nothing is known about the IFN- $\lambda$  response in immune cells, and as yet it is unclear how IFN- $\lambda$  may differ to IFN- $\alpha$  in terms of signalling pathway usage.

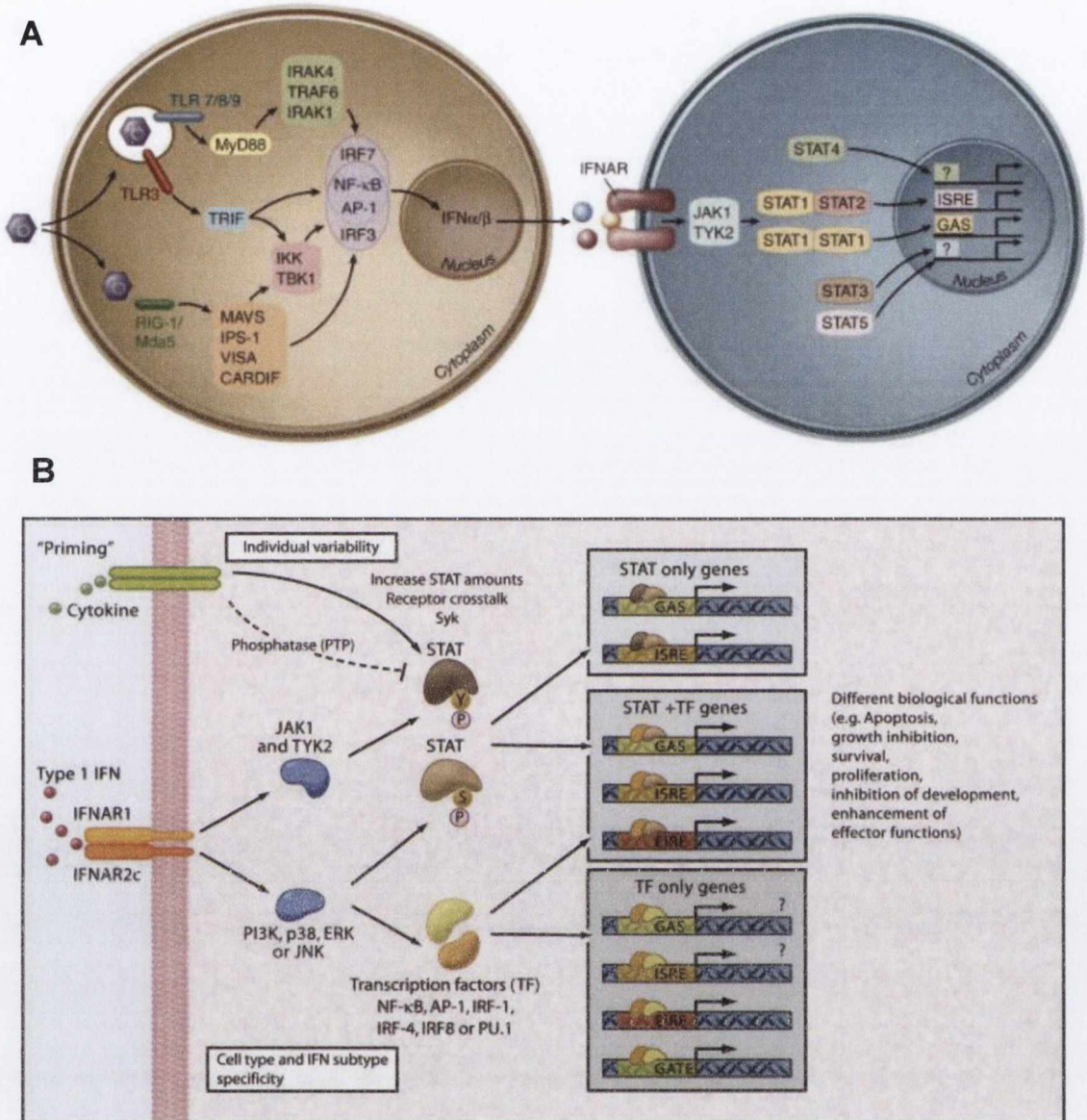
#### **Aims:**

- To characterise IFN- $\lambda$  receptor expression on hepatocytes and peripheral immune cell populations
- Measure phosphorylation of STAT proteins in response to IFN- $\lambda$  in hepatocytes and immune cells to ascertain cellular responsiveness
- Determine gene expression profile induced in response to IFN- $\lambda$  in responsive immune cells

#### **Hypothesis:**

IFN- $\lambda$  effects on immune cells may be restricted by receptor and transcription factor expression, and may unveil key differences to IFN- $\alpha$  and thus attribute a unique function to IFN- $\lambda$  in the immune response.





**Figure 4.2: Activation of JAK-STAT and MAPK pathways by IFNs**

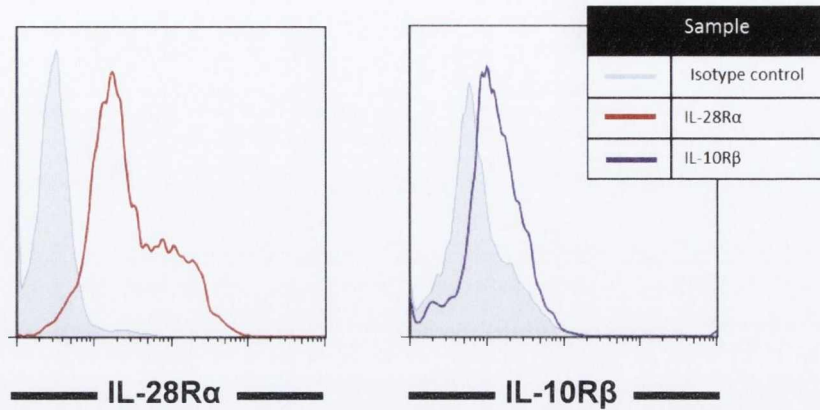
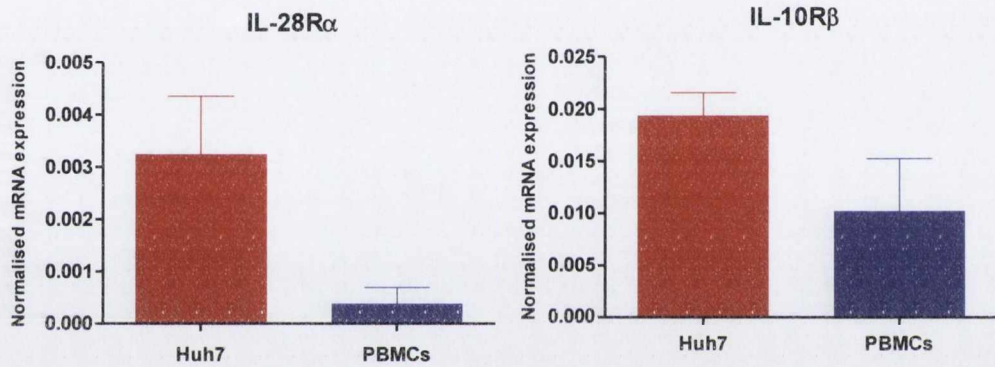
(A) Type I IFN (IFN- $\alpha$ ) is produced in response to viral infection. It then binds to its receptor on the cell surface, activating various components of the JAK-STAT pathway, depending on cell type and conditions, resulting in antiviral gene induction. (B) Type I IFNs also signal through the PI3K and MAPK pathways in addition to the JAK-STAT pathway resulting in different gene expression and therefore biological functions.

Figures taken from <sup>47,205</sup>

## 4.2 Results

### 4.2.1 IFN- $\lambda$ receptor is expressed on the Huh7 hepatocyte cell line

It is known that IFN- $\lambda$  acts on hepatocytes <sup>213</sup> but not as much is known about its effect on individual immune cell populations. First, using the Huh7 hepatocyte cell line we confirmed IFN- $\lambda$  receptor expression on hepatocytes. Huh7 hepatocyte cells were stained with antibodies specific for IL-28R $\alpha$  and IL-10R $\beta$ , followed by flow cytometric analysis. The data obtained confirms Huh7 expression of both receptor chains, with expression of the specific IFN- $\lambda$  receptor, IL-28R $\alpha$ , showing much higher expression than IL-10R $\beta$  (**Figure 4.3A**). Expression of the *IL28RA* and *IL10RB* genes by Huh7 cells was also confirmed by real-time PCR. Hepatocyte expression of each receptor gene was higher than PBMCs, suggesting greater potential for responsiveness by hepatocytes to IFN- $\lambda$  than immune cells (**Figure 4.3B**).

**A****B**

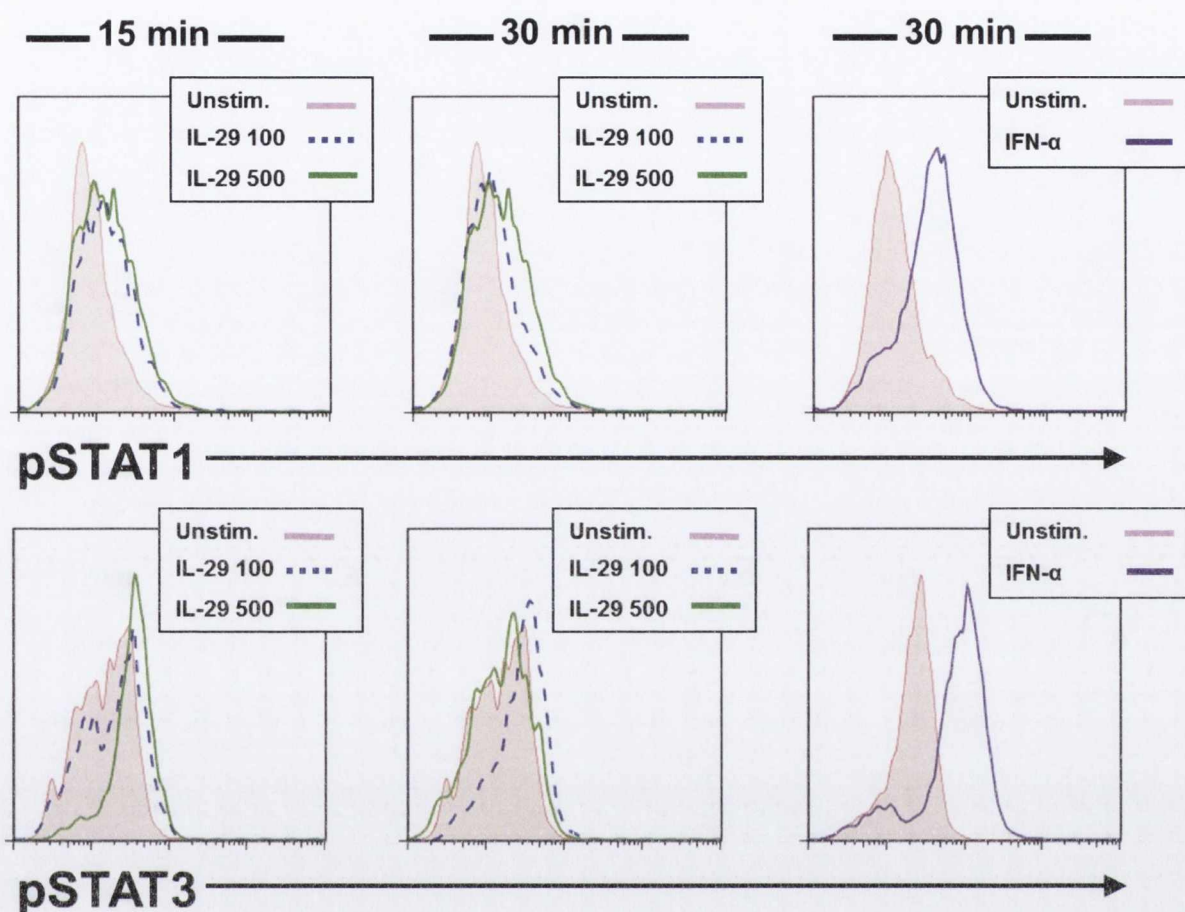
**Figure 4.3: Expression of IFN- $\lambda$  receptor chains IL-28R $\alpha$  and IL-10R $\beta$  on the Huh7 hepatocyte cell line**

(A) IFN- $\lambda$  receptor expression was examined on Huh7 cells by flow cytometry. Cells were stained with antibodies against the IL-28R $\alpha$  and IL-10R $\beta$  receptor chains. (B) Expression of the *IL28RA* and *IL10RB* genes on Huh7 cells was confirmed by real-time PCR and compared to IFN- $\lambda$  receptor expression on peripheral blood mononuclear cells (PBMCs). Gene expression was normalised to the housekeeping gene *beta-2-microglobulin* (*B2M*). Huh7 results shown are from four independent experiments and PBMC expression from n=3 donors.



#### **4.2.2 IFN- $\lambda$ induces phosphorylation of STAT1 and STAT3 in the Huh7 cell line**

To measure hepatocyte responsiveness to IFN- $\lambda$ , Huh7 cells were treated with either 100 ng/ml or 500 ng/ml of IFN- $\lambda$  (IL-29) for 15 and 30 min and then STAT1 and STAT3 phosphorylation was measured by intracellular staining with fluorescently-labelled specific antibodies. Cells were analysed by flow cytometry. IFN- $\alpha$  treatment (1000 U/ml) for 30 min was included as a positive control. IFN- $\lambda$  treatment of Huh7 cells resulted in increased levels of both pSTAT1 and pSTAT3 compared to the untreated sample, indicating these cells respond to IFN- $\lambda$ . At both concentrations and time-points tested, IFN- $\lambda$  treatment yielded similar results, with phosphorylation much weaker than observed for IFN- $\alpha$  (**Figure 4.4**). Continued treatment until 90 min did not yield increased phosphorylation (data not shown).



**Figure 4.4: STAT1 and STAT3 phosphorylation following treatment of Huh7 cells with IL-29 or IFN- $\alpha$**

Huh7 cells were treated with 100 ng/ml or 500 ng/ml IL-29 for 15 and 30 min and 1000 U/ml IFN- $\alpha$  for 30 min. Cells were harvested, fixed and permeabilised and intracellular staining carried out using antibodies against phospho-STAT1 (p-STAT1) and p-STAT3. Cells were analysed immediately by flow cytometry to detect phosphorylation. Flow plots show overlay of unstimulated pSTAT1 or pSTAT3 levels (pink), phosphorylation induced by 100 ng/ml IL-29 (dashed blue line) and 500 ng/ml IL-29 (green line). IFN- $\alpha$ -induced STAT phosphorylation is indicated by the purple line.

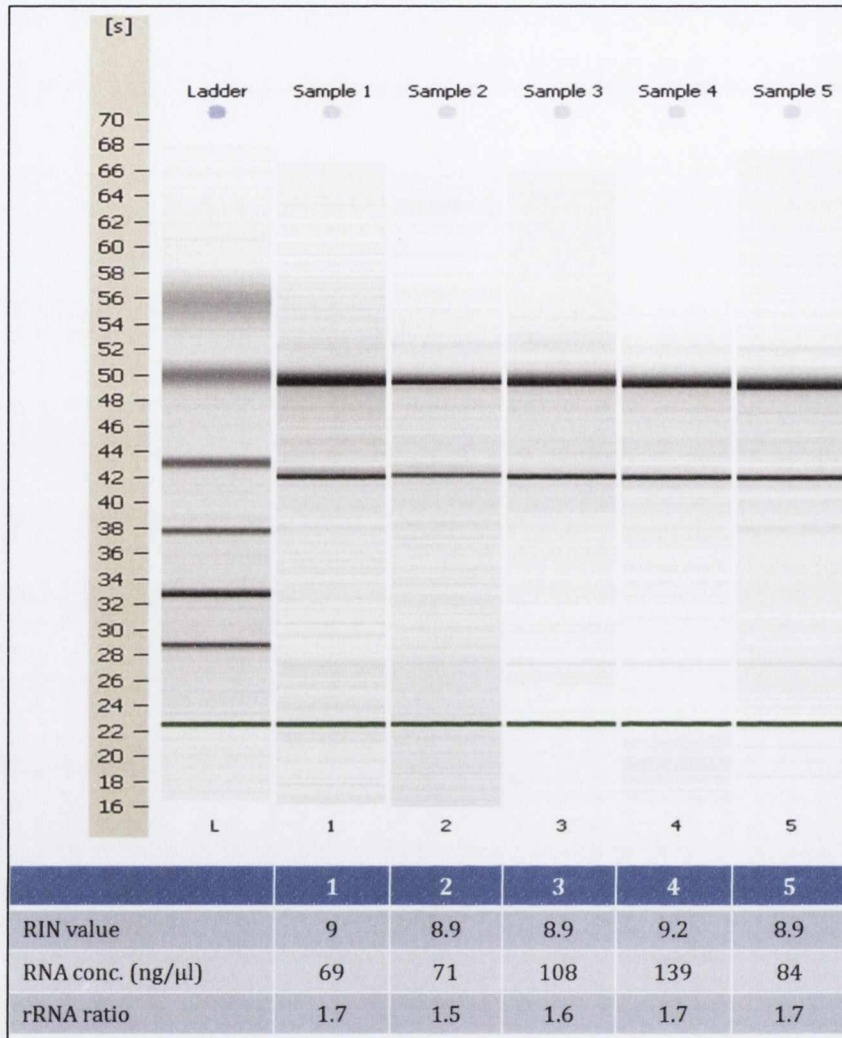
### 4.2.3 Induction of antiviral genes in IFN- $\lambda$ -treated Huh7s

To assess the downstream consequences of Huh7 responsiveness to IFN- $\lambda$ , we assessed gene expression following IL-29 treatment of Huh7s using a real time PCR based gene array. This array measures a selection of genes including receptors, chaperones, downstream signalling and responsive genes involved in TLR, NLR and RIG-I signalling, as well as components of Type I IFN signalling and response, including a number of ISGs (**see appendix**). Huh7 cells were treated with 500 ng/ml of IL-29 for 4 hrs after which time RNA was extracted, purified and cDNA synthesised. RNA quality was assessed using the Agilent Bioanalyser (**Figure 4.5**). All samples yielded an RNA integrity number (RIN) value of >8.9, meaning that the samples were suitable for use with the PCR array (a RIN value of 7 or higher is recommended). Upregulation of the genes *MDA-5* (*IFIH1*), *MxA* (*Mx1*) and *OAS2* was observed (16.6-, 16.5- and 4.5-fold respectively) (**Figure 4.6**). A number of genes were down-regulated (see **Table 4.1** for full list of genes).

Up-regulation of *MxA*, *OAS2* and *MDA-5* was validated by real time PCR of triplicate Huh7 samples treated with IFN- $\alpha$  (1000U/ml) and IL-28A, IL-28B and IL-29 (100 ng/ml and 500 ng/ml). Induction of *OAS2* was much higher in response to IFN- $\alpha$  than the IFN- $\lambda$ s, with a mean fold change of 68 (range 21-130.2) over gene expression in untreated cells, compared to a mean fold change of 32.8 (range 18.3-50.9) induced by IL-29 100 ng/ml (**Figure 4.7A**). *MxA* was the gene most induced by the IFN- $\lambda$ s, with maximal induction occurring in response to 500 ng/ml of IL-29 (mean 129.2; range 35.8-289), which tended to have greater gene induction than that induced by 1000 U/ml IFN- $\alpha$  (mean 82.5; range 39.3-124.9) (**Figure 4.7B**). A dose-dependent response is apparent for *MxA* and *MDA-5*, with increased gene



induction observed for 500 ng/ml compared to 100 ng/ml for each of the IFN- $\lambda$ s. *MDA-5* induction was higher upon IFN- $\alpha$  treatment (mean 138; range 68.8-216.8) compared to the IFN- $\lambda$ s (**Figure 4.7C**). The highest *MDA-5* induction by any of the IFN- $\lambda$ s was again seen with IL-29 500ng/ml (mean 105.7; range 27.9-160.9). Overall, IL-29 tended to induce higher gene expression than the other IFN- $\lambda$  family members, IL-28A and IL-28B, which both showed similar levels of antiviral gene induction.



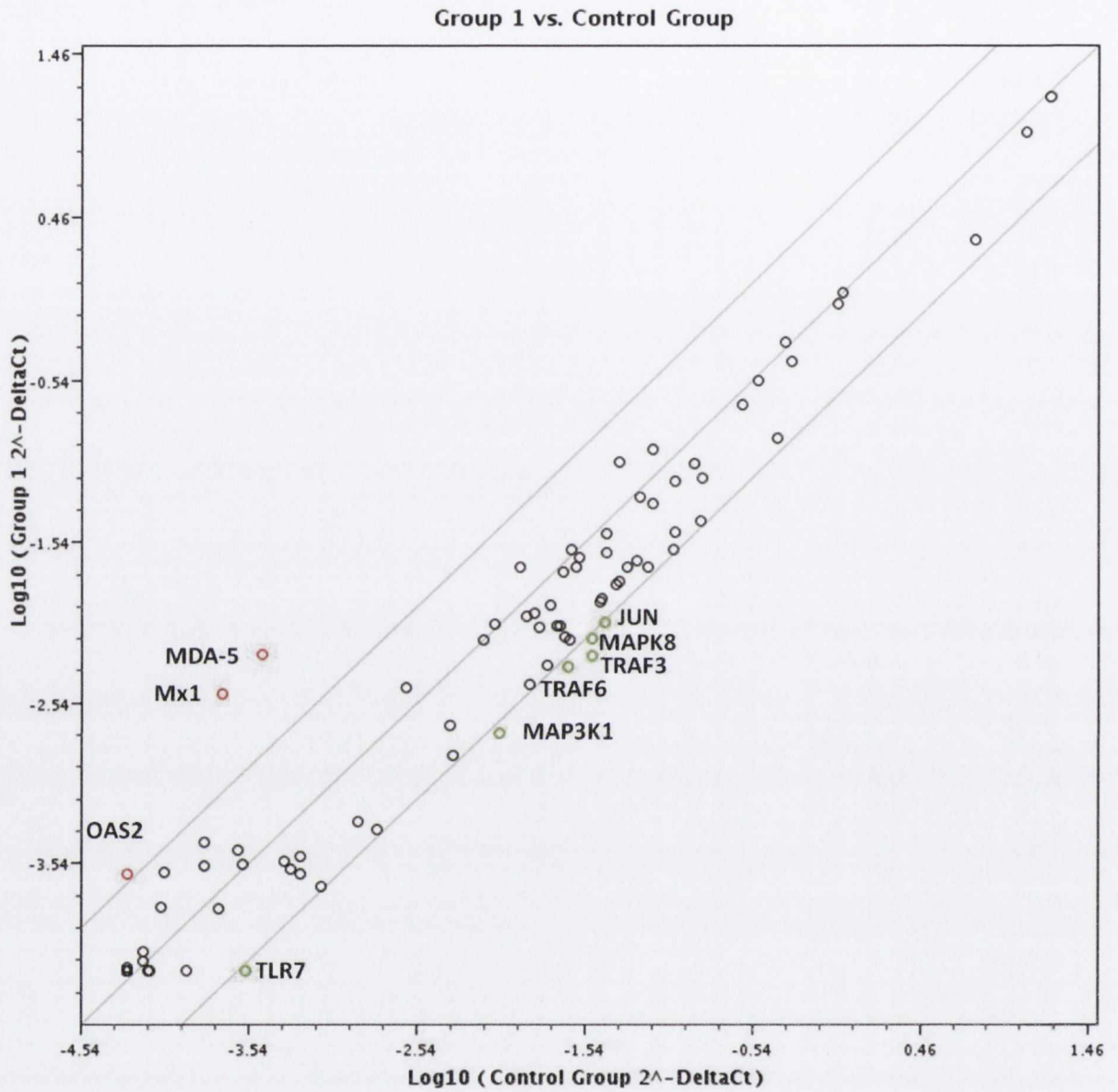
**Figure 4.5: Assessment of RNA quality using Agilent Bioanalyser**

RNA was extracted from Huh7 cells which were either untreated (lanes 1-3) or treated with 500 ng/ml IL-29 (lanes 4,5) using Trizol and purified using the RNeasy mini kit. The quality of the RNA extracted was assessed by the Agilent Bioanalyser, whereby high quality RNA is evident by clearly visible 28S and 18S ribosomal RNA bands. The level of RNA degradation is reported as RNA integrity number (RIN value), ranging from 1 (low-quality degraded RNA) to 10 (high-quality intact RNA).

**Table 4.1: Changes in antiviral gene response in Huh7 hepatocyte cell line after treatment with 500 ng/ml IL-29 for 4 hours**

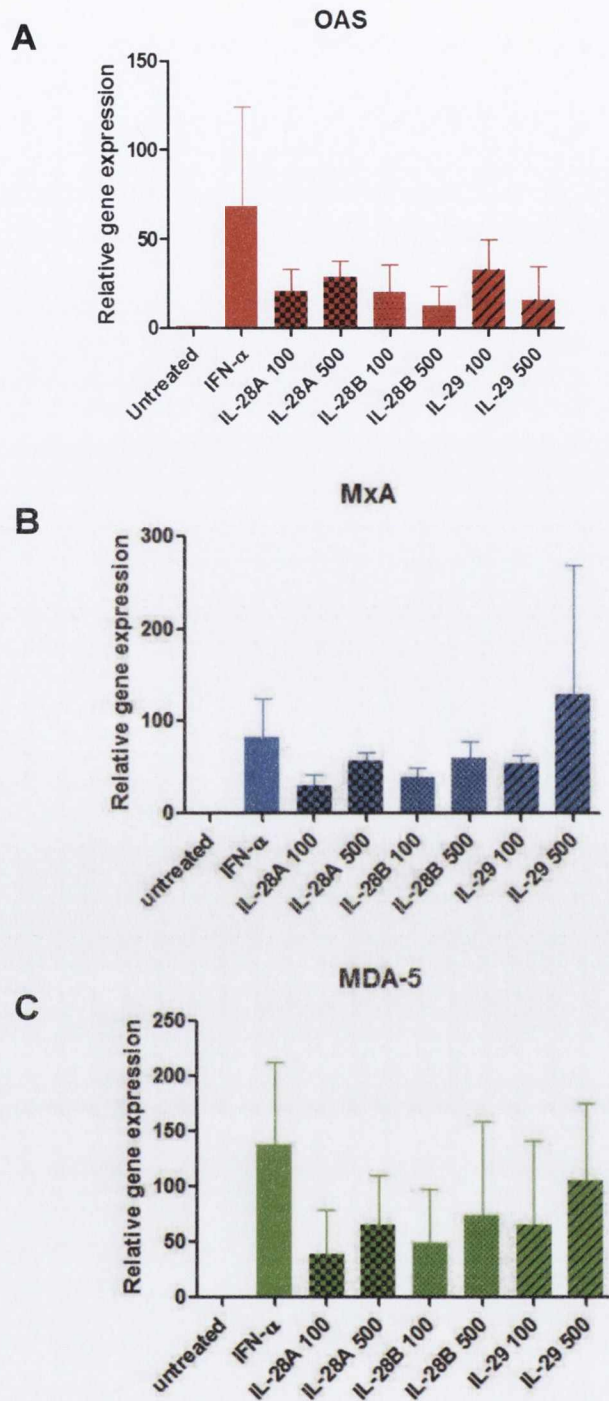
<b>Gene symbol</b>	<b>RefSeq</b>	<b>Fold change</b>	<b>Gene description</b>
IFIH1	NM_022168	16.6795	Hlcd, IDDM19, MDA-5, MDA5, MGC133047
MX1	NM_002462	16.5642	IFI-78K, IFI78, MX, MxA
OAS2	NM_002535	4.5948	MGC78578
NOD2	NM_022162	2.8679	ACUG, BLAU, CARD15, CD, CLR16.3, IBD1, NLR2, NOD2B, PSORAS1
TLR9	NM_017442	2.4453	CD289
STAT1	NM_007315	-2.0139	DKFZp686B04100, ISGF-3, STAT91
IKKBK	NM_001556	-2.1735	FLJ33771, FLJ36218, FLJ38368, FLJ40509, IKK-beta, IKK2, IKKB, MGC131801, NFKBIKB
IL15	NM_000585	-2.2038	IL-15, MGC9721
IRF5	NM_001098629	-2.3295	SLEB10
MYD88	NM_002468	-2.3784	MYD88D
TRADD	NM_003789	-2.4623	Hs.89862, MGC11078
MAPK14	NM_001315	-2.5491	CSBP, CSBP1, CSBP2, CSPB1, EXIP, Mxi2, PRKM14, PRKM15, RK, SAPK2A, p38, p38ALPHA
FADD	NM_003824	-2.639	MGC8528, MORT1
CHUK	NM_001278	-2.7511	IKBKA, IKK-alpha, IKK1, IKKA, NFKBIKA, TCF16
RELA	NM_021975	-2.7702	MGC131774, NFKB3, p65
IFNAR1	NM_000629	-2.7895	AVP, IFN-alpha-REC, IFNAR, IFNBR, IFRC
SPP1	NM_000582	-2.8481	BNSP, BSPI, ETA-1, MGC110940, OPN
TICAM1	NM_182919	-2.8879	MGC35334, PRVTIRB, TICAM-1, TRIF
GAPDH	NM_002046	-2.8879	G3PD, GAPD, MGC88685
IL18	NM_001562	-3.0105	IGIF, IL-18, IL-1g, IL1F4, MGC12320
MAPK1	NM_002745	-3.2043	ERK, ERK2, ERT1, MAPK2, P42MAPK, PRKM1, PRKM2, p38, p40, p41, p41mapk
IRAK1	NM_001569	-3.249	IRAK, pelle
DAK	NM_015533	-3.4105	DKFZp586B1621, MGC5621, NET45
MAP2K3	NM_002756	-3.4105	MAPKK3, MEK3, MKK3, PRKMK3
FOS	NM_005252	-3.4343	AP-1, C-FOS
RIPK1	NM_003804	-3.5801	FLJ39204, RIP, RIP1
PYCARD	NM_013258	-3.605	ASC, CARD5, MGC10332, TMS, TMS-1, TMS1
IRF3	NM_001571	-3.6301	-
MAP2K1	NM_002755	-3.7321	MAPKK1, MEK1, MKK1, PRKMK1
IRF7	NM_001572	-3.8106	IRF-7H, IRF7A
JUN	NM_002228	-4.1699	AP-1, AP1, c-Jun
MAPK8	NM_002750	-4.3772	JNK, JNK1, JNK1A2, JNK21B1, 2, PRKM8, SAPK1
TLR7	NM_016562	-4.5002	-
MAP3K1	NM_005921	-4.724	MAPKKK1, MEKK, MEKK1
TRAF6	NM_004620	-4.7568	MGC:3310, RNF85
TRAF3	NM_003300	-5.6569	CAP-1, CD40bp, CRAF1, LAP1





**Figure 4.6: *Mx1*, *MDA-5* and *OAS2* expression was upregulated in the Huh7 cell line in response to treatment with IL-29**

Graph shows genes upregulated (red) and genes downregulated (green) in response to 500 ng/ml IL-29 using antiviral response PCR array (n=1 control versus n=1 treated sample).

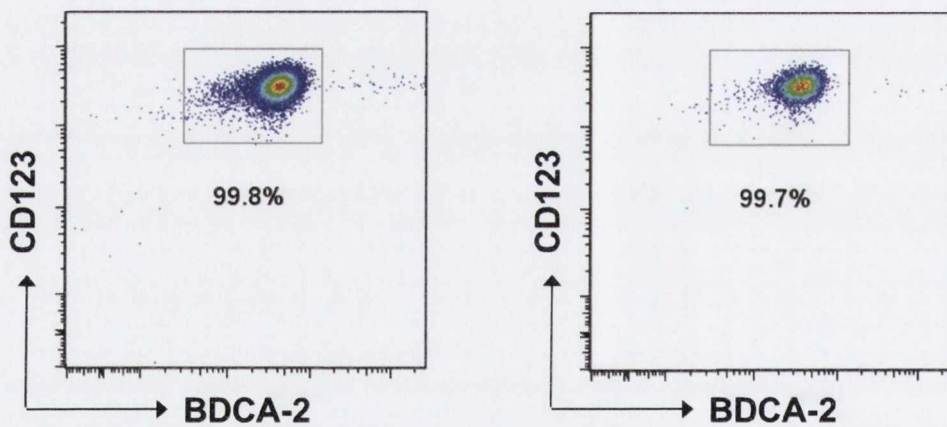


**Figure 4.7: IFN- $\lambda$  and IFN- $\alpha$  induction of the interferon-stimulated genes *OAS2*, *MxA* and *MDA-5* in the Huh7 cell line**

Huh7 cells were treated with 1000 U/ml IFN- $\alpha$  and 100 ng/ml or 500 ng/ml IL-28A, IL28B and IL-29 for 4 hours. RNA was extracted, cDNA synthesised and expression of the antiviral genes *OAS2*, *MDA-5* and *MxA* was measured by real time PCR. Relative gene expression was calculated using the delta delta Ct method, with samples normalised to the *beta-2-microglobulin (B2M)* housekeeping gene. Results shown represent three independent experiments.

#### 4.2.4 Isolation of cell subpopulations from PBMCs to measure IFN- $\lambda$ responsiveness

Having demonstrated pSTAT1 and pSTAT3 induction, and the resulting effect on gene expression in hepatocytes in response to IFN- $\lambda$  treatment, we next aimed to establish the effects of these cytokines on peripheral immune cells. In order to measure IFN- $\lambda$  receptor expression and induction of antiviral genes in immune cell subpopulations, cells were isolated and RNA extracted for gene expression studies. Purity of CD3<sup>+</sup> T cells, CD19<sup>+</sup> B cells and CD14<sup>+</sup> monocytes was >97% (data not shown) while pDC purity was >99% (**Figure 4.8**).



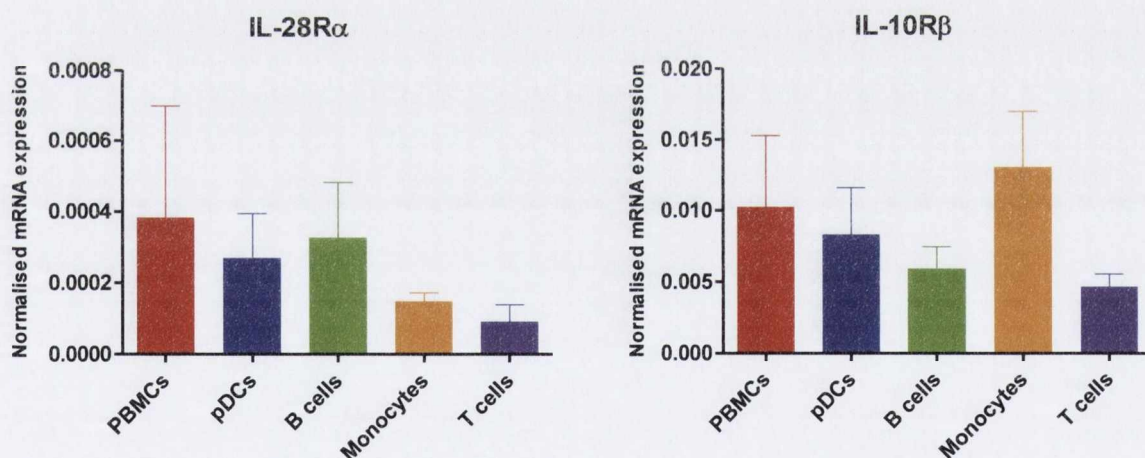
**Figure 4.8: Isolation of plasmacytoid dendritic cells**

Plasmacytoid dendritic cells (pDCs) were isolated from 120ml fresh blood by magnetic bead separation using the Diamond Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec). Cell purity was assessed by staining cells with antibodies against CD123 and the specific pDC surface marker BDCA-2 and analysing expression by flow cytometry, gating out dead cells and doublets. Flow plots shown represent the pDCs of two donors used in experiments.



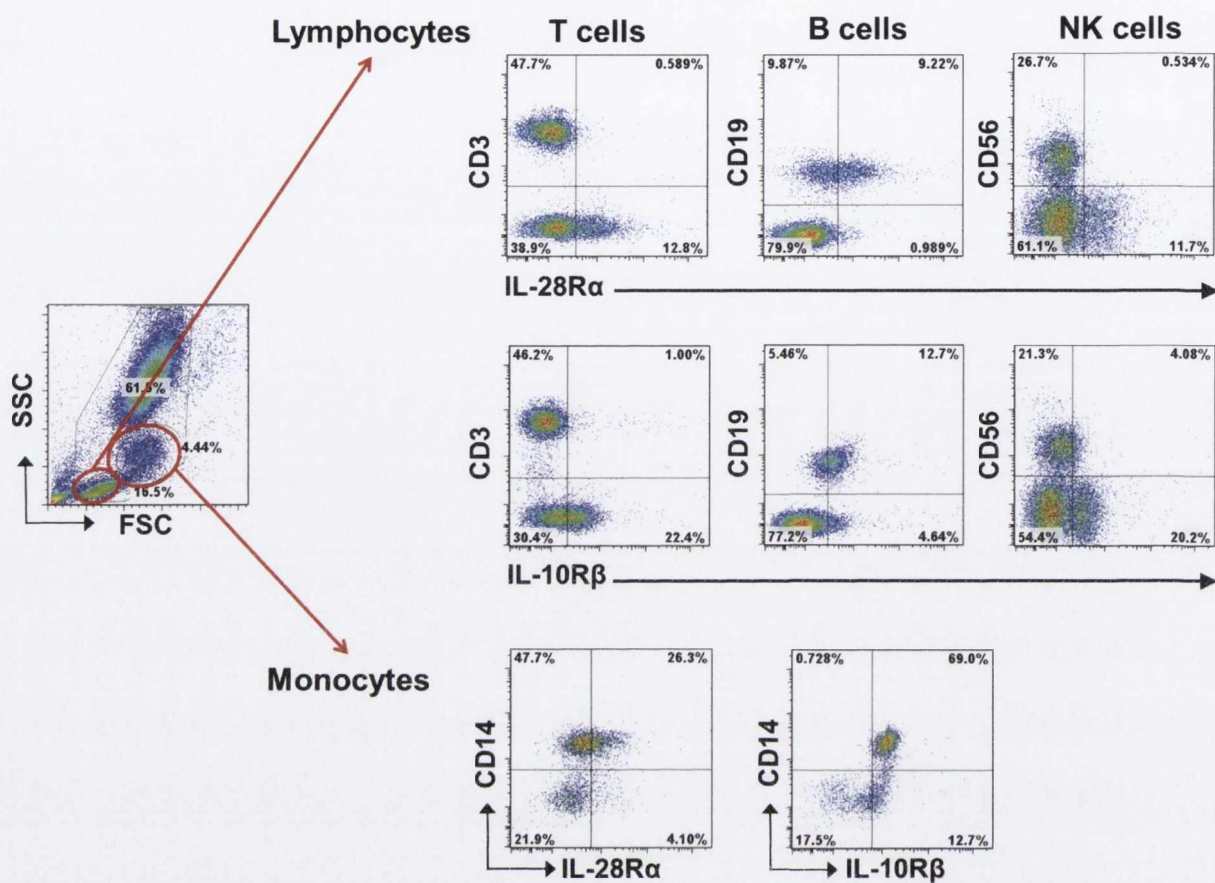
#### 4.2.5 The IFN- $\lambda$ receptor is expressed on PBMCs

First, we measured expression of both IL-28R $\alpha$  and IL-10R $\beta$  in PBMCs by real time PCR. The *IL28RA* gene is expressed on total PBMCs and is also evident on pDCs, B cells, monocytes and T cells, with the highest expression on pDCs and B cells and very low expression on T cells (**Figure 4.9**). The second chain of the IFN- $\lambda$  receptor, *IL10RB* is also expressed on peripheral immune cell populations, with the highest levels seen in monocytes. However, measurement of IL-28R $\alpha$  protein expression by flow cytometry revealed that expression was restricted to B cells within the lymphocyte population (6.5%; range 3.9-9.2%), while being clearly absent from T cells and NK cells. Expression was also seen on monocytes (23.3%; 16-27.7% range) (**Figure 4.10**), pDCs (>30%) (**Figure 4.11A**) and on MDDCs (**Figure 4.11B**).



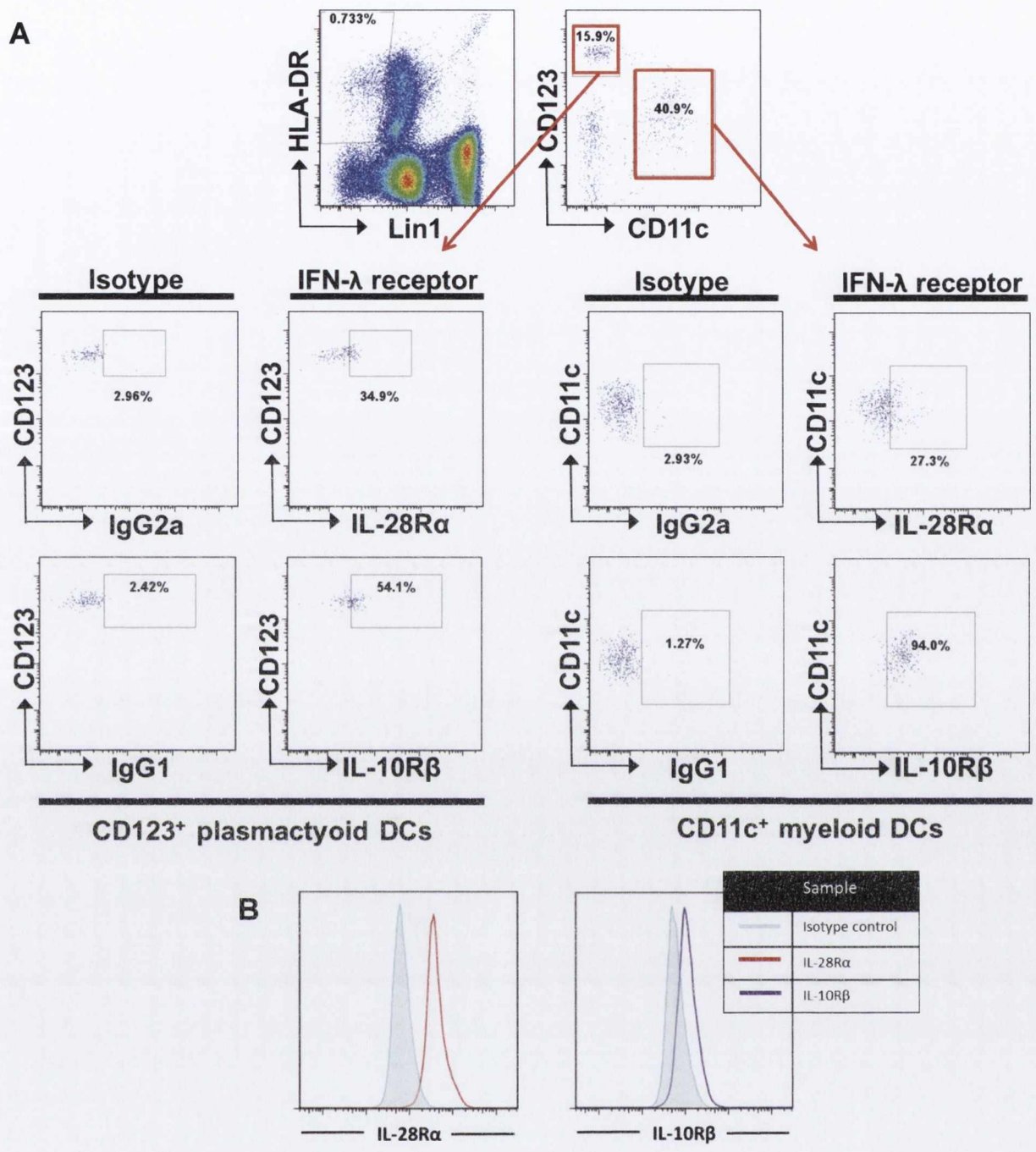
**Figure 4.9: Expression of *IL28RA* and *IL10RB* on PBMCs**

(A) CD3<sup>+</sup> T cells, CD19<sup>+</sup> B cells and CD14<sup>+</sup> monocytes were isolated from peripheral blood mononuclear cells (PBMCs), derived from healthy buffy coats (n=3), by magnetic bead separation. BDCA-4<sup>+</sup> plasmacytoid dendritic cells (pDCs) were isolated using the Diamond Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec). RNA was extracted and cDNA synthesised for gene expression studies. Expression of the *IL28RA* and *IL10RB* receptor genes was measured on each cell population by real time PCR, normalised to the house keeping gene *beta-2-microglobulin* (*B2M*).



**Figure 4.10: IFN- $\lambda$  receptor is expressed on B cells and monocytes**

Expression of the IFN- $\lambda$  receptor was measured by flow cytometry on CD3<sup>+</sup>, CD19<sup>+</sup> and CD56<sup>+</sup> cells within the lymphocyte gate and CD14<sup>+</sup> monocytes within the monocyte/dendritic cell gate using whole blood staining on fresh healthy blood (n=4).



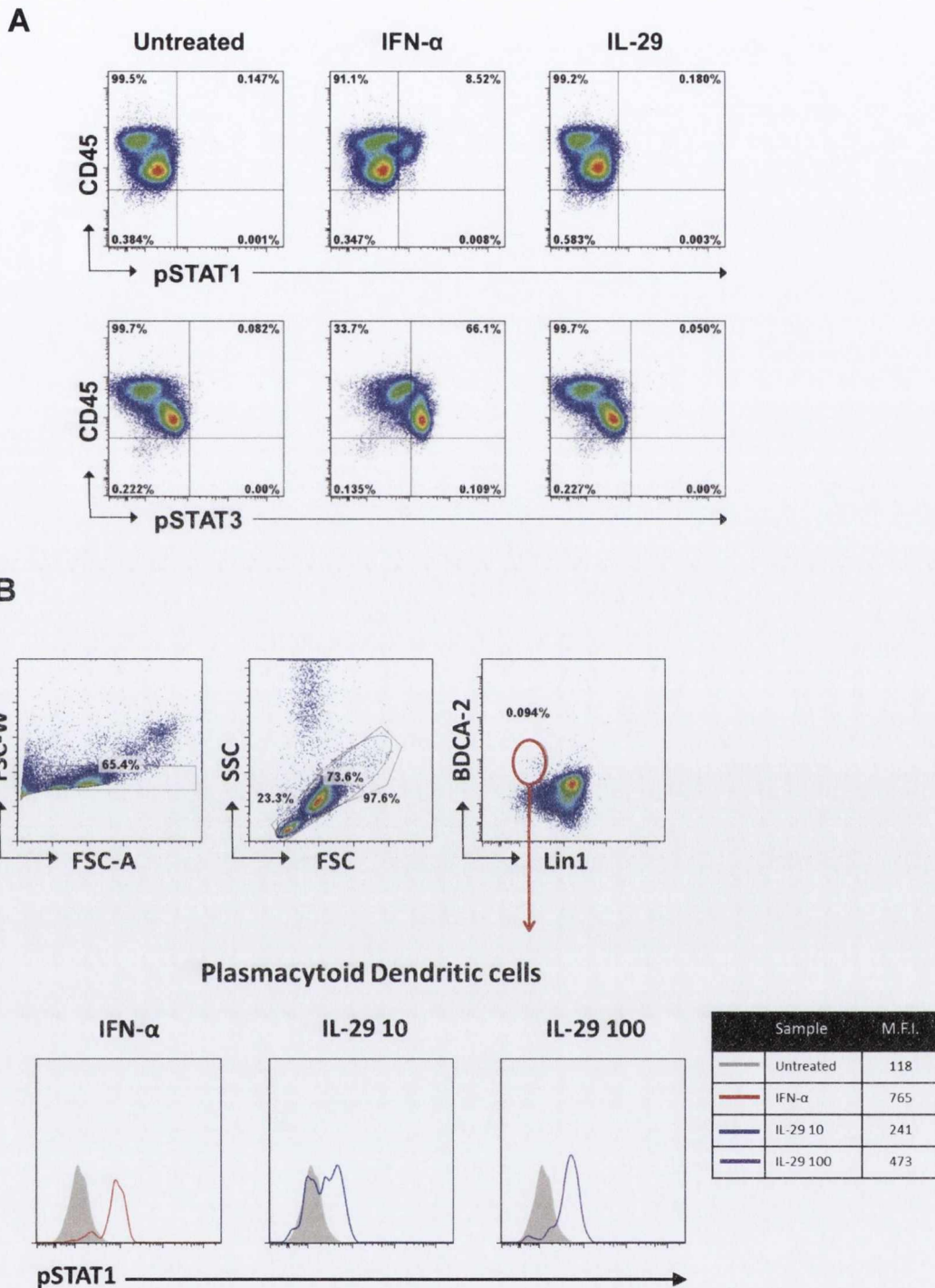
**Figure 4.11: IFN-λ receptor is expressed on dendritic cells**

(A) IL-28Rα and IL-10Rβ expression was measured on CD123<sup>+</sup> pDCs and CD11c<sup>+</sup> mDCs by first gating on Lin1<sup>-</sup> HLA-DR<sup>+</sup> population. Gates were set based on fluorescence minus one (FMO) controls and isotype controls (shown). Data is representative of four donors. (B) CD14<sup>+</sup> monocytes were isolated from healthy buffy coats (n=3) and cultured in 10% RPMI, supplemented with IL-4 and GM-CSF for 6 days. Monocyte-derived dendritic cells (MDDCs) were then analysed for IL-28Rα and IL-10Rβ expression by flow cytometry.



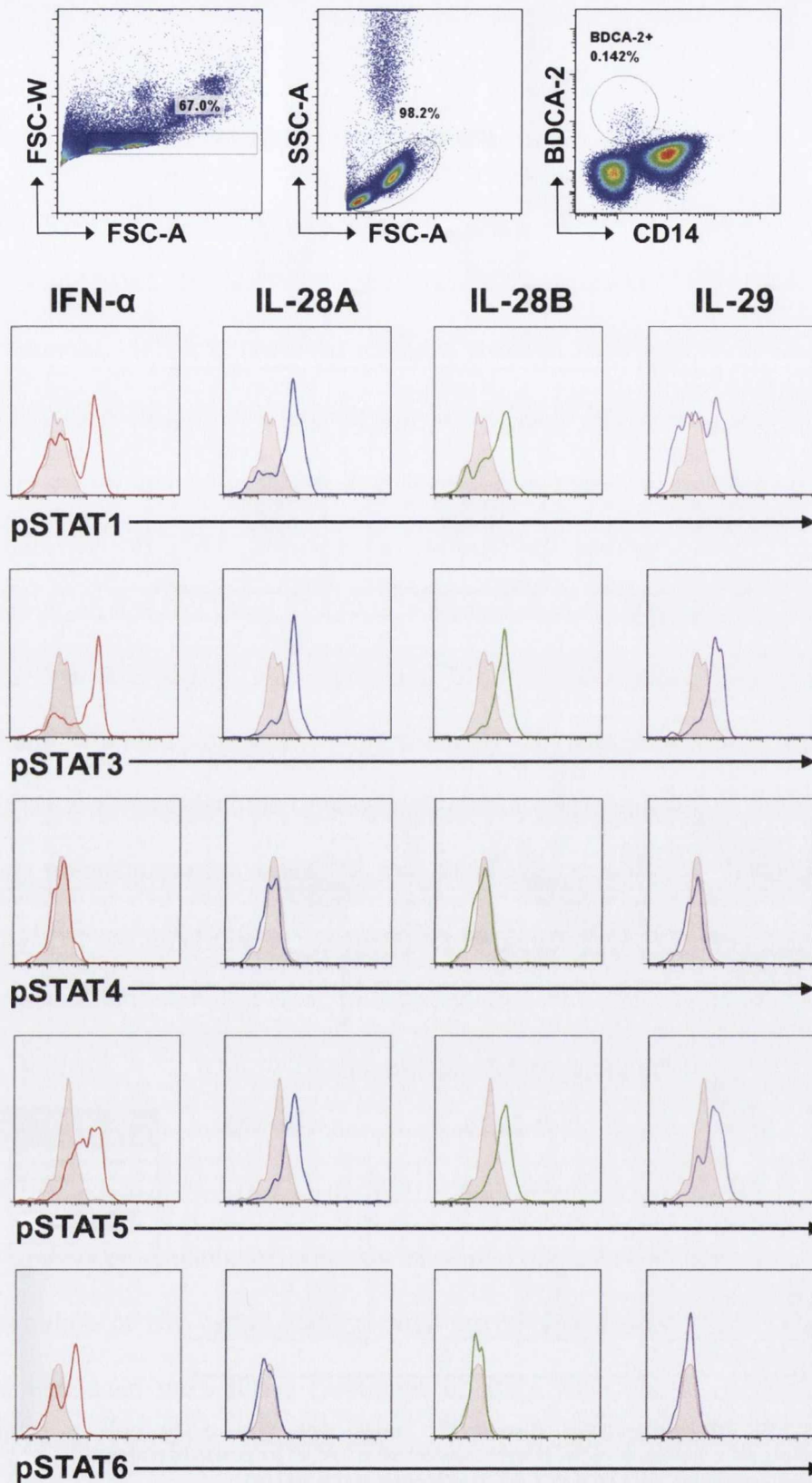
#### 4.2.6 Responsiveness of PBMC subpopulations to IFN- $\lambda$

Following confirmation of IFN- $\lambda$  receptor expression on PBMCs, specifically B cells, monocytes and pDCs, the next step was to measure immune cell responsiveness to IFN- $\lambda$  treatment. IFN- $\lambda$  is reported to signal through the JAK-STAT pathway to activate downstream antiviral activity similar to IFN- $\alpha$  but little is known about the activation of this pathway in immune cells in response to IFN- $\lambda$  and the differential use of STAT proteins in various cell populations. Therefore, phosphorylation of STAT1 and STAT3 was measured in CD45<sup>+</sup> leukocytes, CD3<sup>+</sup> T cells, CD19<sup>+</sup> B cells, CD14<sup>+</sup> monocytes and BDCA-2<sup>+</sup> pDCs using a whole blood intracellular staining protocol. Surprisingly a complete lack of STAT phosphorylation was evident in total cells (CD45<sup>+</sup>) treated with IFN- $\lambda$  compared to the strong phosphorylation of STAT1 and STAT3 in response to IFN- $\alpha$  (**Figure 4.12A**). However pDCs were shown to be responsive to IFN- $\lambda$  via JAK-STAT pathway activation, showing clear phosphorylation of STAT1 in response to as little as 10ng/ml IL-29 with increased phosphorylation evident at a dose of 100 ng/ml (**Figure 4.12B**). Additional characterisation of pDC responsiveness to IFN- $\lambda$  revealed that each of the IFN- $\lambda$ s, IL-28A, IL-28B and IL-29, acted in a similar manner and were capable of inducing phosphorylation of STAT1, STAT3 and STAT5 at a dose of 100 ng/ml when treated for 30 min (**Figure 4.13**). A similar response was seen with IFN- $\alpha$  treatment of pDCs with one exception; IFN- $\alpha$  induced the phosphorylation of STAT6 whereas the IFN- $\lambda$ s did not. Investigation of the IFN- $\lambda$  responsiveness of other immune populations revealed no phosphorylation of STATs 1, 3, 4, 5 or 6 on monocytes (**Figure 4.14**) or B cells (**Figure 4.15**) which clearly express the IFN- $\lambda$  receptor. CD3<sup>+</sup> T cells, which do not express the IFN- $\lambda$  receptor, were also non-responsive (**Figure 4.16**).



**Figure 4.12: Plasmacytoid dendritic cells are the only cell population responsive to IFN- $\lambda$  via JAK-STAT pathway activation**

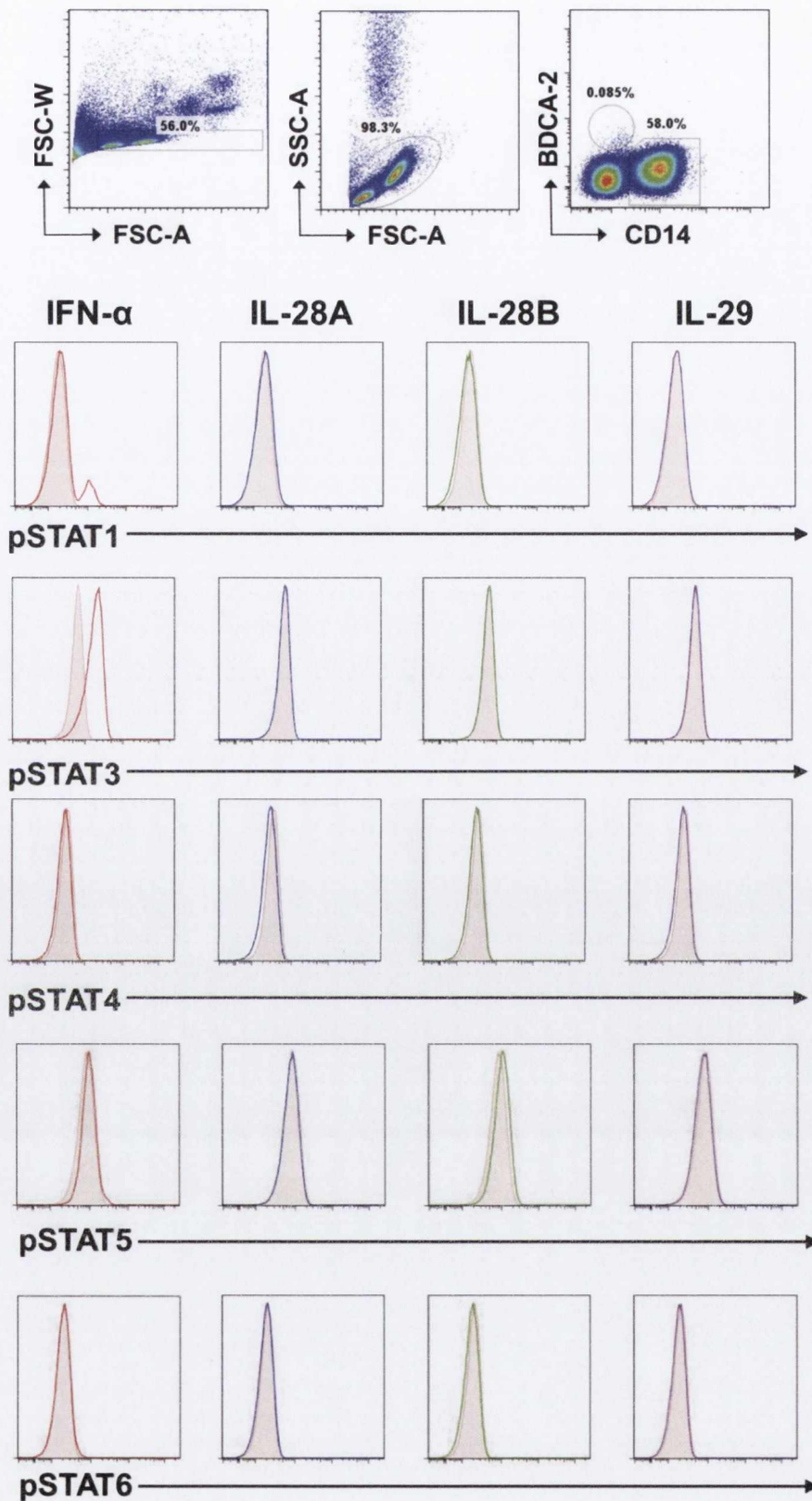
Whole blood (200  $\mu$ l) was treated with 1000U/ml IFN- $\alpha$  and 10 ng/ml and 100 ng/ml IL-29 for 30 min. Following red cell lysis, cells were then fixed and stained with the cell surface markers (A) CD45 (pan-leukocyte marker) and (B) Lineage 1 and BDCA-2 to identify plasmacytoid dendritic cells. Following permeabilisation with Phosflow perm buffer III, intracellular staining was performed using antibodies against pSTAT1 and pSTAT3. Cells were acquired immediately on a flow cytometer.



**Figure 4.13: IFN- $\lambda$  induces phosphorylation of STAT1, STAT3 and STAT5 in plasmacytoid dendritic cells**

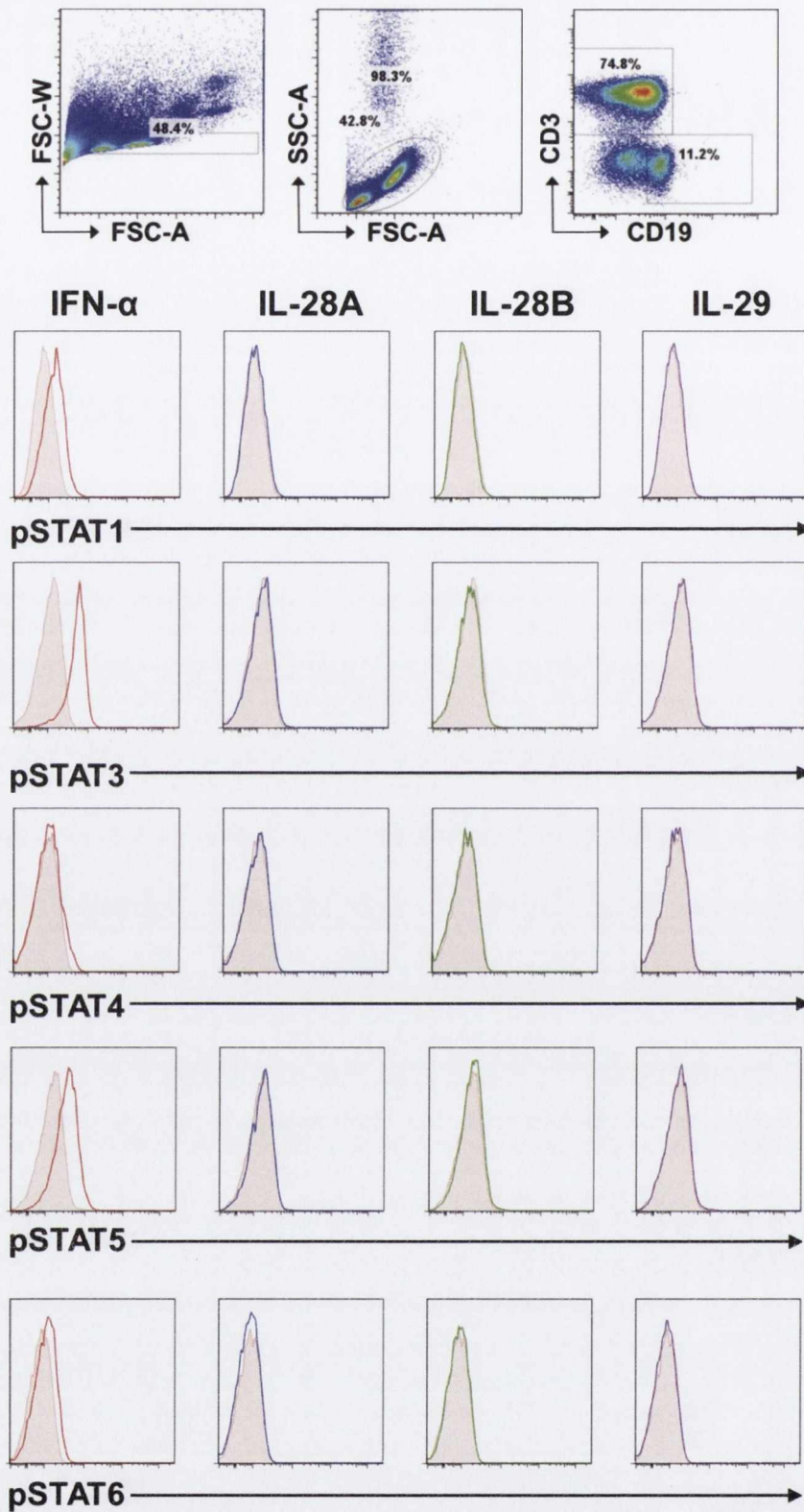
Whole blood (200  $\mu$ l) was treated with 1000U/ml IFN- $\alpha$  and 100 ng/ml IL-28A, IL-28B and IL-29 for 30 min. Following red cell lysis, cells were fixed and stained with the pDC specific surface marker BDCA-2. Following cell permeabilisation, intracellular staining was carried out using antibodies against phospho-STAT1, STAT3, STAT4, STAT5 and STAT6.





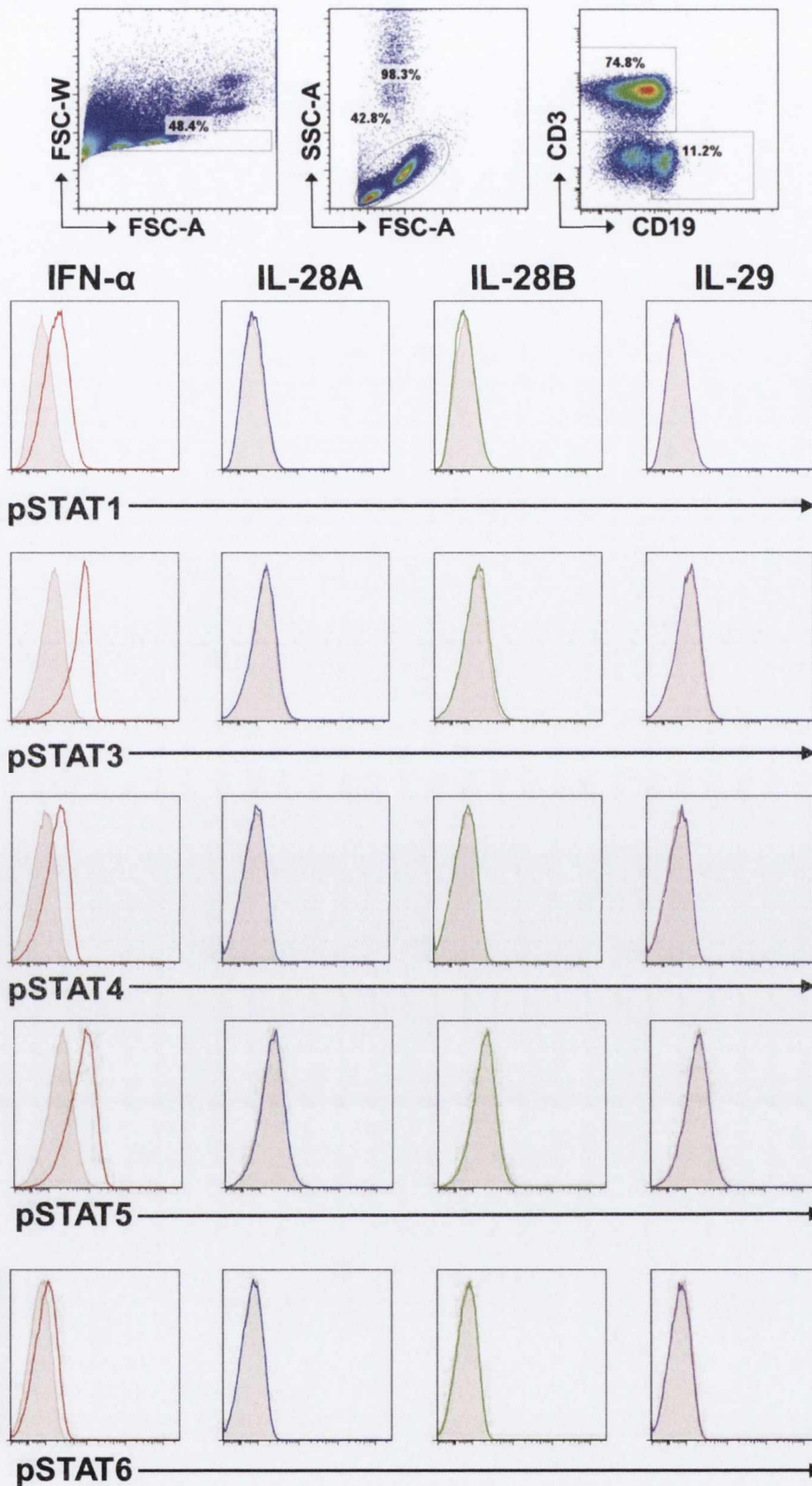
**Figure 4.14: Lack of phosphorylation of STAT 1-6 in CD14<sup>+</sup> monocytes in response to IFN- $\lambda$**

Whole blood (200  $\mu$ l) was treated with 1000U/ml IFN- $\alpha$  and 100 ng/ml IL-28A, IL-28B and IL-29 for 30 min. Following red cell lysis, cells were, fixed and stained with the monocyte surface marker CD14. Following cell permeabilisation, intracellular staining was carried out using antibodies against phospho-STAT1, STAT3, STAT4, STAT5 and STAT6.



**Figure 4.15: IFN- $\lambda$  does not induce phosphorylation of STAT1-6 in CD19<sup>+</sup> B cells**

Whole blood (200  $\mu$ l) was treated with 1000U/ml IFN- $\alpha$  and 100 ng/ml IL-28A, IL-28B and IL-29 for 30 min. Following red cell lysis, cells were fixed and stained with the B cell surface marker CD19. Following cell permeabilisation, intracellular staining was carried out using antibodies against phospho-STAT1, STAT3, STAT4, STAT5 and STAT6.



**Figure 4.16: IFN- $\lambda$  does not induce phosphorylation of STAT1-6 in CD3<sup>+</sup> T cells**

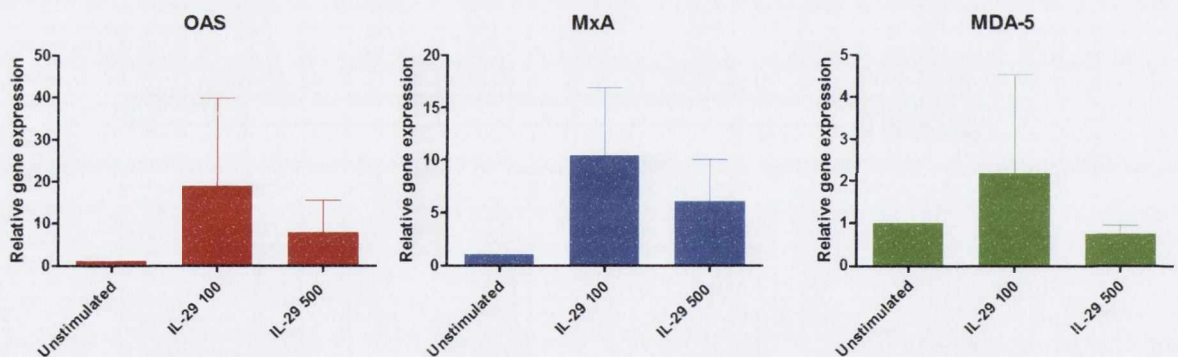
Whole blood (200  $\mu$ l) was treated with 1000U/ml IFN- $\alpha$  and 100 ng/ml IL-28A, IL-28B and IL-29 for 30 min. Following red cell lysis, cells were fixed and stained with the T cell surface marker CD3. Following cell permeabilisation, intracellular staining was carried out using antibodies against phospho-STAT1, STAT3, STAT4, STAT5 and STAT6.



#### 4.2.7 Gene expression induced by IFN- $\lambda$ treatment

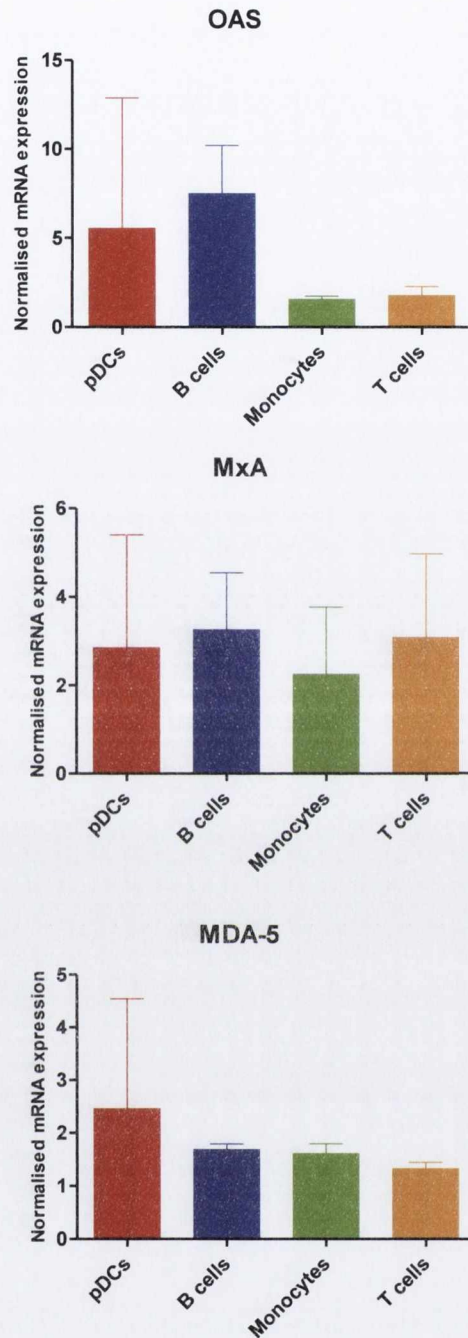
We found that treatment of Huh7 hepatocytes with IFN- $\lambda$  resulted in the induction of *OAS2*, *MxA* and *MDA-5* expression. Therefore, we examined the expression of these genes in healthy PBMCs treated with IL-29 (100 and 500 ng/ml). Minimal ISG expression was observed; expression levels were much lower than in Huh7 hepatocytes. Fold change was less than 20, with the highest induction of *OAS2*, followed by *MxA* and no induction of *MDA-5* (Figure 4.17).

ISG induction was also measured in purified immune cell populations: pDCs, B cells, monocytes and T cells. Overall, minimal induction of these genes was observed. *OAS2* expression was upregulated specifically in pDCs and B cells with low-level induction seen in monocytes and T cells. A 2-fold induction of *MxA* was observed in all cell subsets. Induction of *MDA-5* was >2-fold in pDCs and lower in other cell types (Figure 4.18).



**Figure 4.17: Antiviral gene induction in PBMCs in response to IFN- $\lambda$  treatment**

PBMCs were isolated and treated with 100ng/ml and 500 ng/ml IL-29 for 4 hours after which time cells were harvested and RNA extracted. Expression of the antiviral genes *OAS2*, *MxA* and *MDA-5* were determined by real time PCR and data analysed using the delta delta Ct method whereby gene expression was calculated relative to the housekeeping gene *beta-2-microglobulin* (*B2M*).



**Figure 4.18: Induction of the interferon-stimulated genes *OAS2*, *MxA* and *MDA-5* in pDCs, B cells, monocytes and T cells in response to IL-29**

BDCA-4<sup>+</sup> pDCs, CD19<sup>+</sup> B cells, CD14<sup>+</sup> monocytes and CD3<sup>+</sup> T cells were isolated from peripheral blood mononuclear cells (PBMCs) by magnetic bead separation and cultured either untreated or treated with IL-29 (100ng/ml) for 4 hours. RNA was extracted, cDNA synthesised and expression of the genes *OAS2*, *MxA* and *MDA-5* measured using real time PCR. Relative gene expression was normalised to the housekeeping gene *beta-2-microglobulin* (*B2M*) using the delta delta Ct method. Each bar represents the mean of n=3 blood donors and error bars show standard deviation.

### 4.3 Discussion

In this study, we show that while IFN- $\lambda$  receptor expression could be demonstrated on both PBMCs and hepatocytes, downstream signalling and gene induction were strikingly different. Huh7 hepatocytes expressed both chains of the IFN- $\lambda$  receptor, upregulated pSTAT1 and pSTAT3 in response to IFN- $\lambda$  treatment, with an associated increase observed in the expression of the ISGs, *MxA*, *OAS2* and *MDA-5*. In contrast, while PBMCs express the IFN- $\lambda$  receptor, IFN- $\lambda$  fails to induce STAT phosphorylation. This is in contrast to the clear STAT1 and STAT3 phosphorylation induced by IFN- $\alpha$  in both Huh7 cells and PBMCs. Lack of STAT phosphorylation correlated with minimal ISG induction in PBMCs treated with IFN- $\lambda$ . However, a more comprehensive analysis of STAT phosphorylation in PBMC subpopulations in response to IFN- $\lambda$  treatment revealed that pDCs were the only cell type responsive to IFN- $\lambda$  via the JAK-STAT pathway.

#### *Huh7 hepatocyte responsiveness to IFN- $\lambda$*

A number of studies have demonstrated that epithelial cells, including hepatocytes, are the main cell type responsive to IFN- $\lambda$  <sup>129, 139</sup>. Therefore, we first confirmed responsiveness of the Huh7 hepatocyte cell line to IFN- $\lambda$ . IFN- $\lambda$  receptor was expressed on the Huh7 hepatocyte cell line and treatment with IL-29 induced phosphorylation of STAT1 and STAT3, and subsequent upregulation of antiviral genes such as *MxA* and *OAS*. Similar to a recent study by Dickensheets *et al* comparing IFN- $\alpha$  and IFN- $\lambda$ -induced response in primary human hepatocytes <sup>216</sup>, we found that STAT phosphorylation and ISG expression is induced by IFN- $\lambda$  in the Huh7 cell line at a lower magnitude than IFN- $\alpha$ . The antiviral gene *MxA* was highly upregulated by IL-29, surpassing the levels induced by IFN- $\alpha$ . The fact that



comparable antiviral gene induction to IFN- $\alpha$  is observed in Huh7 liver cells despite less STAT phosphorylation indicates either different signalling kinetics or use of alternative signalling pathways to induce antiviral genes. While the JAK-STAT pathway was initially accepted as being the pathway responsible for IFN- $\lambda$ -induced signalling, it is possible that alternative pathways may be more utilised. For example, IFN- $\lambda$ s have been reported to strongly rely on both p38 and JNK MAP kinases, but not ERK, for gene induction in a B cell line <sup>212</sup>. In corresponding inhibitor experiments, IFN- $\lambda$  induction of the antiviral gene ISG56 was almost completely abolished following p38 inhibition and strongly reduced by JNK inhibition <sup>212</sup>. Brand *et al*, showed that IFN- $\lambda$  activates JNK and also ERK1/2 in intestinal epithelial cells which differs from the lack of ERK signalling observed in B cells <sup>212, 217</sup>. However, in our antiviral gene array which examined Huh7 cells either untreated or treated with IFN- $\lambda$ , we observed down-regulation of many genes after 4 hours of IFN- $\lambda$  treatment, including JAK-STAT and MAPK signalling components and target genes (see supplementary table 1 for complete list). A 4 hr timepoint was used for this array because this has previously been shown in our laboratory to be an optimal timepoint for measuring expression of the ISGs. However, it is possible that many of the signalling components activated in response to IFN- $\lambda$  treatment were upregulated at an earlier timepoint and subsequently downregulated. This could represent a feedback mechanism to down-regulate components of the signalling pathway, thus limiting signal activation and gene expression. It would be interesting to expand this type of gene expression study to include a timecourse of gene activation and compare IFN- $\lambda$  and IFN- $\alpha$ -induced gene expression. The contribution of alternative signalling

pathways, including the MAPKs, to ISG induction in response to IFN- $\lambda$  in the liver requires further investigation.

#### *PBMC responsiveness to IFN- $\lambda$*

Having confirmed hepatocyte responsiveness to IFN- $\lambda$ , we next sought to examine the effect of IFN- $\lambda$  on PBMCs. We showed that the IFN- $\lambda$  receptor was expressed by CD45<sup>+</sup> PBMCs and expression was detected by real time PCR on all cell subsets, mainly pDCs and B cells, with less on monocytes and T cells. Detection of IFN- $\lambda$  receptor expression by flow cytometry shows that IL-28R $\alpha$  expression within the lymphoid population is restricted to B cells. IL-28R $\alpha$  is also expressed on monocytes and pDCs, therefore representing potential target cells of IFN- $\lambda$ . While there are indications that the receptor is also expressed on CD11c<sup>+</sup> myeloid dendritic cells, data was difficult to interpret and unclear due to the small population of cells being gated. The IFN- $\lambda$  receptor was, however, clearly expressed on MDDCs.

Following characterisation of receptor expression on immune populations, we next sought to characterise IFN- $\lambda$  activation of the JAK-STAT pathway in various immune subsets. Historically, western blotting has been used to visualise STAT and phospho-STAT proteins by lysing the cells and probing for protein expression using specific antibodies. However, the disadvantage of this technique is that signalling activation cannot be characterised in a mixed cell population. Isolation of purified immune cells for protein extraction is not practical for many rare populations, e.g. DCs, as large volumes of blood would be required. A further disadvantage of western blotting is that it is qualitative rather than quantitative,



making it difficult to detect subtle changes in phosphorylation patterns, thus being open to subjectivity. In recent years, flow cytometry protocols have been developed which allow simultaneous detection of multiple parameters within a mixed population of cells <sup>218</sup>. While intracellular cytokines can be detected in multiple cell types, defined by their surface markers, phosphorylated STAT proteins translocate to the nucleus, thereby requiring harsher cell permeabilisation protocols to access nuclear proteins. This method can destroy some surface antigens being detected and also disrupt the fluorescent signal of the fluorochrome. We have adopted the phospho-STAT staining protocol and developed a staining strategy using antibody combinations allowing us to detect pSTAT1, 3, 4, 5 and 6 in CD45<sup>+</sup> total PBMCs, CD3<sup>+</sup> T cells, CD19<sup>+</sup> B cells, CD14<sup>+</sup> monocytes and CD303<sup>+</sup> (BDCA-2) pDCs. In addition, we have utilised a whole blood staining protocol, performed using fresh blood. This protocol preserves cell populations and minimises cell manipulation to minimise basal signal activation.

Given that the IFN- $\lambda$  receptor was detected on a number of peripheral immune populations, it was surprising that IFN- $\lambda$  did not activate STAT phosphorylation in CD45<sup>+</sup> PBMCs. This observed non-responsiveness was in stark contrast to the strong phosphorylation of STAT1 and STAT3 seen with IFN- $\alpha$ . However, further evaluation of IFN- $\lambda$ -induced STAT phosphorylation in different cell populations showed that, among the potential populations shown to express the IL-28R $\alpha$  (B cells, monocytes, pDCs), only pDCs showed a clear phosphorylation of STAT1 in a dose-dependent manner in response to IFN- $\lambda$ . The observed lack of PBMC responsiveness to IFN- $\lambda$  is explained by the fact that the sole responsive



population, pDCs, represent only 0.5-0.8% of PBMCs and therefore changes in STAT phosphorylation are masked within bulk PBMCs.

Additional characterisation of JAK-STAT activation in pDCs showed that all three IFN- $\lambda$ s (IL-28A, IL-28B and IL-29) behaved similarly in their ability to phosphorylate STAT1, and additionally induced phosphorylation of STAT3 and STAT5. IFN- $\alpha$  also phosphorylates STAT1, 3 and 5 in pDCs. A key difference observed between IFN- $\alpha$  and IFN- $\lambda$  is that IFN- $\alpha$  also induces phosphorylation of STAT6 in pDCs. STAT3 is known to be activated in response to Type I IFNs in most cell lines, whereas activation of STAT4 and STAT5 by Type I IFNs is found mostly in NK <sup>219</sup> and T cells <sup>220</sup>. The activation of STAT6 induced by Type I IFNs has been described thus far only in B cell lines <sup>221</sup>. Thus our data showing STAT6 phosphorylation in pDCs in response to IFN- $\alpha$  is a novel observation. In contrast to previous findings, that IFN- $\lambda$  activates the JAK-STAT pathway in T cells <sup>200</sup>, we find that the receptor is not expressed on T cells as measured by flow cytometry and a lack of STAT phosphorylation is observed upon IFN- $\lambda$  treatment. The different methods used to measure STAT phosphorylation may account for these conflicting results, with flow cytometry being a more sensitive and quantitative method than western blot.

The lack of IFN- $\lambda$ -induced STAT phosphorylation in total PBMCs observed in this study correlates with studies by Witte *et al* and Dickensheets *et al* where lack of responsiveness of the major PBMC populations (lymphocytes and monocytes) to IFN- $\lambda$  is reported using Western blotting <sup>139, 216</sup>. While it has been widely reported that IFN- $\lambda$  induces phosphorylation of the STAT proteins, many of these studies

have been carried out on cell lines rather than primary cells. For example, IFN- $\lambda$ s activate STAT1, STAT2, STAT3, STAT4 and STAT5 in a T cell line transfected with the IFN- $\lambda$  receptor <sup>215</sup>. Our data, to the best of our knowledge, represents the first comprehensive evaluation of the responsiveness of peripheral immune populations to IFN- $\lambda$  treatment and finds that responses are restricted to pDCs. Our findings further highlight the cell-specific response to IFNs. No phosphorylation of STATs is induced in B cells or monocytes, despite the fact that they clearly express the IFN- $\lambda$  receptor. Studies have identified that individual cell types are capable of activating different STATs in response to the same IFN. For example, it has been shown that IFN- $\beta$  has a very different effect on immune cells populations. Upon IFN- $\beta$  treatment of PBMCs, few B cells activated STAT1 in comparison to T cells and monocytes but activated STAT5 in addition to STAT3. STAT1-dependent pro-apoptotic mRNAs were induced in monocytes but not in B cells. This differential STAT activation is suggested as the reason why IFN- $\beta$  increases the survival of primary human B cells and CD4 T cells but enhances the apoptosis of monocytes <sup>222</sup>. It must be noted that B cells and monocytes are heterogenous populations composed of various subsets. Therefore it remains a possibility that a minor subset within these cell types could be responsive.

Another reason for the observed lack of phosphorylation in B cells in this study in response to IFN- $\lambda$ , is the time-point used to measure STAT phosphorylation. While most phosphorylation signals are thought to occur within 30 min of cell treatment with cytokine, it has been reported that, particularly in B cells and monocytes, these phosphorylation signals can occur later, with maximal phosphorylation of



STAT1, STAT3 and STAT5 occurring at 45 min in response to IFN- $\beta$  treatment, and levels being much lower at 25 min <sup>222</sup>. Later induction of STAT phosphorylation in response to IFN- $\lambda$  could account for our negative results, requiring a more detailed time course to be performed in future experiments.

Having studied IFN- $\lambda$ -induced cell signalling, downstream gene expression was measured in individual immune populations (pDCs, monocytes, B, T cells) treated with IL-29 100 ng/ml for 4 hours. Very low induction of the antiviral genes *OAS*, *MxA* and *MDA-5* were observed and gene expression, rather being restricted to pDCs, was detected in all cell subsets. The fact that some gene expression was observed in the absence of STAT phosphorylation raises the possibility that these genes may be activated in a STAT-independent manner in some cells, or that the response to IFN- $\lambda$  may be via an indirect rather than direct switching on of antiviral responses. For example, IFN- $\lambda$  has been known to activate ISGs independently of STATs <sup>212</sup>. Therefore, it would be necessary to look at activation of transcription factors other than STAT proteins, such as MAPK transcription factors, and examine protein expression of the antiviral genes studied. Stimulation for 4 hours was examined in this study, however, different timepoints could be used to explore the possibility that IFN- $\lambda$  upregulates some genes at an earlier or later time-point. The kinetics of ISG regulation by IFN- $\lambda$  compared to IFN- $\alpha$  remains to be explored.

Our research is in agreement with previous indications that IFN- $\lambda$  can induce functional responses in pDCs but is the first to show that they are the sole immune



population capable of activating JAK-STAT signalling in response to IFN- $\lambda$ . Yin *et al* also found receptor expression on pDCs and also show that it is functional by demonstrating pSTAT1 activation <sup>223</sup>. This study also strengthens the evidence for IFN- $\lambda$  having an effect on pDC function by showing that IFN- $\lambda$  induces upregulation of HLA-ABC (also on B cells) and protects from apoptosis. DC activation must be regulated in order to prevent over-activation of the immune system which would lead to autoimmunity. Recent studies have demonstrated STAT transcription factors as having a key role in DC subset development <sup>224, 225</sup>. Therefore the effect of IFN- $\lambda$  on immune cells may not be directly antiviral, rather, these cytokines may play a more immunomodulatory role. An interesting example of this immunomodulation was demonstrated by Liu *et al* who showed that IFN- $\lambda$  promoted TLR-induced IL-12p40 expression by monocyte-derived macrophages via upregulation of the IFN- $\gamma$  receptor while IFN- $\alpha$  had the opposite effect, instead suppressing IFN- $\gamma$ -induced pro-inflammatory cytokine production <sup>226</sup>. Both Type I and type III IFNs have also been shown to promote sensitivity of antigen-presenting cells to IL-10 via upregulation of the IL-10 receptor <sup>227</sup>. Additional study is required to determine the specific effect of IFN- $\lambda$  on pDC function and how this could be important in antiviral immunity. Megjugorac *et al* demonstrated that IL-29 was able to modulate pDC function by upregulating the maturation markers CD80 and ICOS-L on pDCs, also CCR7 and CD62L, but stimulated decreased levels of pDC signature cytokines in a mixed lymphocyte reaction; pDCs inhibited T cell production of IFN- $\gamma$ , IL-13 and IL-10 <sup>157</sup>. However, not much is known about the antigen-presenting capability of pDCs. IL-4 and IL-13 activates STAT6 resulting in Th2 polarisation. In our study, we have shown that IFN- $\alpha$  activates STAT6 in pDCs whereas IFN- $\lambda$  does not, thereby suggesting that IFN- $\alpha$  activates STAT6-dependent

genes, and thereby has a biological function, in pDCs which IFN- $\lambda$  does not. It has been shown recently that STAT6 is required for innate immune response to viral infection. STAT6<sup>-/-</sup> mice have higher susceptibility to viral infection <sup>228</sup>. Viral infection triggers STAT6 to induce a set of chemokines capable of attracting various immune cells. Future studies require a more detailed evaluation of the implications of pDC responsiveness to IFN- $\lambda$  and how this could be important in HCV infection.

## **Chapter 5: IFN- $\lambda$ -producing Dendritic Cells**



## 5.1 Introduction

### 5.1.1 Interferon production

IFNs are key antiviral cytokines, capable of being produced by almost any cell type in response to viral infection. IFN- $\alpha$  is used therapeutically for HCV infection and is capable of clearing infection in a proportion of individuals, thereby emphasising its importance as a critical component in the innate immune response to viral infection. Several recent studies have demonstrated robust production of the Type III IFN, IFN- $\lambda$ , in primary human hepatocyte cultures, which is in contrast to the minimal induction of IFN- $\alpha$  observed <sup>229-231</sup>. Production of IFN- $\lambda$  correlated with ISG induction and decrease in viraemia in the chimpanzee model <sup>229</sup>, with the level of IFN- $\lambda$  produced sufficient to inhibit the HCVcc infection of naïve uninfected hepatocytes <sup>229, 231</sup>, thereby indicating an important role for IFN- $\lambda$  in the induction of antiviral genes in the liver.

While many cell types can produce IFN in viral infection, DCs are the major producers, being fully equipped to sense viral infection and rapidly produce IFN. This potent IFN-producing capability makes DCs a key cell type in mediating the innate antiviral response to HCV. Using an *in vitro* model of HCV infection, it has been demonstrated that pDCs are capable of sensing HCV infection in infected hepatocytes and produce IFN- $\alpha$  via TLR7 <sup>232</sup>. During HCV infection, pDCs have been shown to accumulate in HCV-infected liver <sup>233, 234</sup>. However less is known about production of IFN- $\lambda$  by immune cells in the liver. It has recently been shown that, while IFN- $\alpha$  can be produced by many cells in viral infection, the production

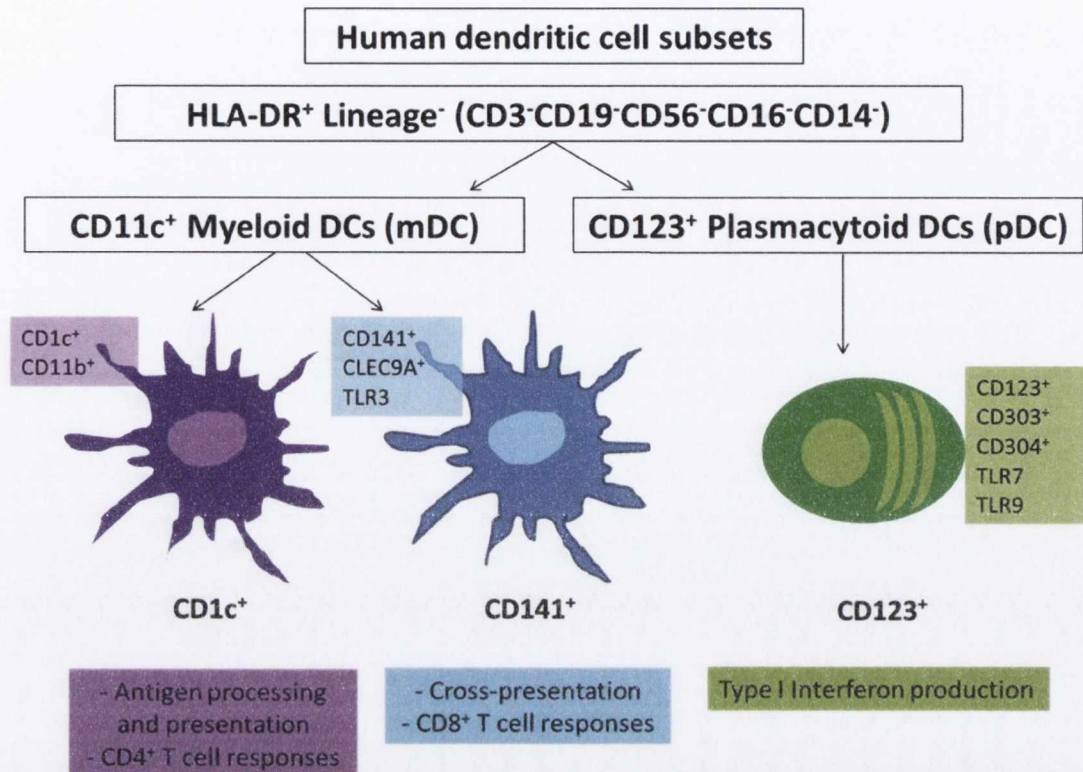
of IFN- $\lambda$  is more restricted and seems to be dependent on haematopoietic cells, in particular DCs <sup>155</sup>.

DC subsets differ in their expression of pattern recognition receptors, cytokine production and T cell-polarising abilities. CD11c<sup>+</sup> mDCs are important antigen-presenting cells whereas CD123<sup>+</sup> pDCs express high levels of the viral sensors TLR7 and TLR9 and are potent producers of IFN- $\alpha$  <sup>168</sup>. pDCs have also been shown to produce IFN- $\lambda$  in response to TLR9 ligands and in certain viral infections <sup>149, 153, 223</sup>. In recent years, a further subset of mDCs has been identified, characterised by high expression of CD141 (BDCA-3 or thrombomodulin) (**Figure 5.1**). Human CD141<sup>+</sup> DCs are homologous to mouse CD8 $\alpha$ <sup>+</sup> DCs, which highly express TLR3, and possess superior cross-presenting ability to activate CD8 cytotoxic T cell responses <sup>156, 169, 170</sup>, thus making them important in antiviral and anti-tumour immunity.

In addition to their ability to activate CD8 T cell responses, CD141<sup>+</sup> myeloid DCs are the major producer of IFN- $\lambda$  in response to the TLR3/RIG-I agonist poly(I:C) <sup>155</sup>. CD141<sup>+</sup> DCs can also express IFN- $\beta$ , but, importantly, fail to produce IFN- $\alpha$  in response to poly(I:C) <sup>156</sup>. This finding is particularly interesting because, although IFN- $\lambda$  is also produced via TLR9 in pDCs, the TLR3/RIG-I pathways represent the major mechanism by which HCV is sensed <sup>235</sup>. Thus, a DC subset specifically expressing high levels of TLR3 is particularly relevant in HCV infection. Most studies examining DC production of IFN- $\lambda$  used MDDCs, an *in vitro* laboratory model of DCs, as a representative of mDCs to compare to pDCs <sup>149, 236, 237</sup>. A recent appreciation of the existence of mDC subsets such as CD141<sup>+</sup> DCs necessitates the need to use primary isolated DCs rather than MDDCs.

Following the association of polymorphisms in the IFN- $\lambda$  gene region with clinical outcome to HCV infection, there has been much interest in the role of this antiviral cytokine in the immune response to HCV. Defining the production of IFN- $\lambda$  is important in understanding its role in innate immunity. HCV primarily infects the liver, yet many of the studies examining DC function and IFN production in HCV stem from peripheral blood, where decreased T-cell stimulatory capacity, increased IL-10 secretion and a deficiency in co-stimulatory molecules have been reported on DCs from chronic HCV-infected individuals <sup>76</sup>. In fact, the liver is a very unique immune system compared to the periphery, where cells differ in terms of frequency and functionality <sup>67</sup>.





**Figure 5.1: Human dendritic cell subsets**

Human dendritic cells (DCs) express CD45 and HLA-DR and lack the lineage markers (CD3, CD14, CD16, CD19 and CD56). Expression of the integrin CD11c and the IL-3 receptor  $\alpha$  chain, CD123, divides DC populations into myeloid DCs (mDCs) (CD11c<sup>+</sup> CD123<sup>-</sup>), which are important antigen-presenting cells and plasmacytoid DCs (pDCs) (CD11c<sup>-</sup> CD123<sup>+</sup>) which express high levels of the viral sensors Toll like receptor (TLR)7 and TLR9 and are potent producers of interferon alpha (IFN- $\alpha$ ). CD11c<sup>+</sup> myeloid DCs can be divided into CD1c<sup>+</sup> and CD141<sup>+</sup> subsets, specialising in the induction of CD4 and CD8 T cell responses respectively. Human CD141<sup>+</sup> DCs are homologous to mouse CD8 $\alpha$ <sup>+</sup> DCs, which highly express TLR3 and produce IFN lambda (IFN- $\lambda$ ) in response to poly(I:C)

Adapted from <sup>172</sup>

### 5.1.2 Dendritic cells in the liver

The immune system in the liver is required to tolerate many dietary and commensal antigens translocated from the gut; it is therefore considered to be a mainly tolerogenic organ <sup>56</sup>. This tolerogenic environment makes it an ideal hideout for many hepatotropic pathogens including HCV <sup>238</sup>. However we do know that the liver is capable of generating successful immune responses, as some individuals are capable of clearing HCV naturally. Among the prime candidates for the induction of hepatic tolerance are myeloid DCs, which govern the activation of an appropriate T cell response. To date, we have extremely limited knowledge about the frequency, phenotype and function of DCs in the livers of either healthy or HCV-infected individuals.

#### *Murine liver DCs*

Isolation of liver DCs has proven difficult. Murine studies have been aided by the ability to inject mice with the cytokine Flt3L which promotes increased DC numbers in the liver, allowing adequate numbers for isolation and characterisation <sup>239</sup>. Murine hepatic DCs have been shown by immunohistochemistry and flow cytometry to be largely immature, expressing low/undetectable levels of maturation markers such as CD40 and CD86 <sup>239, 240</sup>, secreting low IL-12, and being poor stimulators of naive allogeneic T cells <sup>241, 242</sup>. The liver environment is known to be rich in anti-inflammatory cytokines such as IL-10 and TGF- $\beta$ , contributing to the tolerogenic phenotype of hepatic DCs <sup>56</sup>. Liver DCs also express lower TLR4 than spleen DCs and require higher concentrations of LPS to be activated, thus representing an additional layer of tolerance induction in the hepatic immune environment <sup>242</sup>.

### *Human liver DCs*

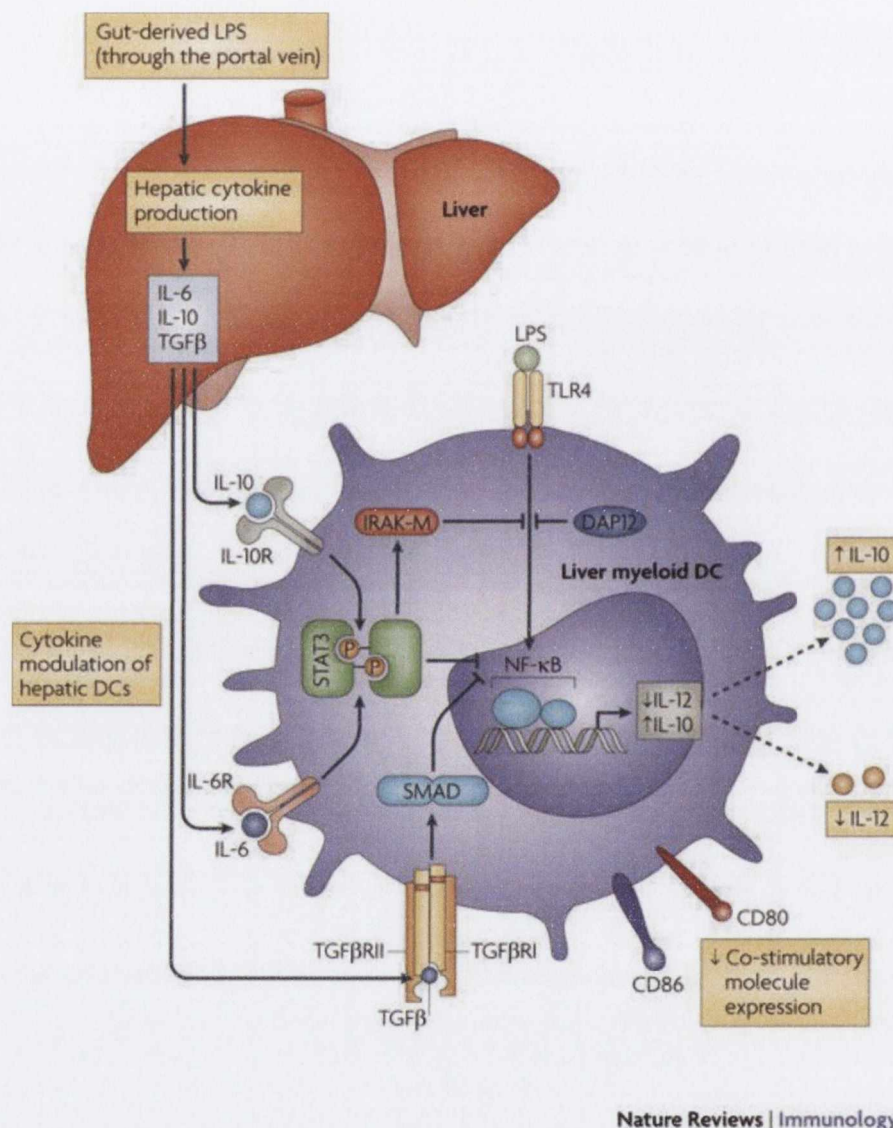
While the study of human liver DCs has proven extremely difficult, recent technological advances in DC isolation, increased availability of reagents for DC characterisation, along with the expansion of knowledge about human DC subsets<sup>243</sup> has given rise to increased interest in this area. Much of the difficulty in studying liver DCs is the lack of sufficient liver sample available from humans in order to isolate cells, especially given that DCs are present at such a low frequency. In particular, obtaining healthy human liver for research use is a difficult task as even the process of taking a liver biopsy is not without risks and complications<sup>244</sup>.

Human studies to date, similar to murine, have demonstrated that hepatic DCs are immature, poor stimulators of T cell responses and produce increased IL-10 in response to TLR4 ligands compared to the spleen and skin<sup>245, 246</sup>. As a result, liver DCs induce less proliferation of allogeneic T cells in a MLR, instead promoting the induction of T regs<sup>246</sup>. Liver DCs were also observed to be immature and inefficient at antigen capture and processing compared to blood DCs<sup>246</sup>. These data suggest key differences between DC function at different sites in the body, further underlining the more regulatory or tolerogenic nature of hepatic DCs.

Interestingly, one study overcame isolation difficulties by utilising *ex vivo* vascular perfusion of liver grafts pre-transplantation to characterise liver DCs<sup>247</sup>, a method previously exploited for the study of lymphocyte populations<sup>248, 249</sup>. The observation that the CD4/CD8 T cell ratio and the high percentage of NK cells in liver perfusate was the same as liver tissue strongly suggests that perfusate mononuclear cells are liver-derived, making it an ideal source to study liver cell



populations including DCs. Immunohistochemistry revealed the presence of BDCA-1<sup>+</sup> (CD1c<sup>+</sup>) mDCs in the liver portal fields with few found in the parenchyma and functionally, these cells produced IL-10 and were of immature phenotype <sup>247</sup>.



### Figure 5.2: Tolerogenic dendritic cells in the liver

Dendritic cells (DCs) in the liver are reported to be tolerogenic, being hypo-responsive to LPS, producing increased IL-10, decreased IL-12 and being poor stimulators of T cell responses.

Figure taken from <sup>56</sup>

### *Liver DCs in disease*

While there is growing evidence that DC function is affected in liver disease, most work to date has been performed on peripheral blood <sup>76</sup>. DCs derived from blood precursors are unlikely to reflect DCs in the liver at the site of viral persistence isolated directly from the liver of HCV-infected individuals. Immune defects in HCV may be restricted to the liver and not reflected by changes in the peripheral department, thereby justifying the efforts to specifically evaluate liver DCs.

In murine studies, both B220<sup>+</sup> pDCs and CD11c<sup>+</sup> DCs were expanded early in viral infection <sup>240</sup>. In human studies, DCs have been observed by immunohistochemistry in the immune infiltrate in the portal areas of chronic HCV-infected liver <sup>250</sup>. These DCs are present in contact with lymphocytes and hepatocytes and express T cell co-stimulatory and maturation molecules including CD83. Fewer CD83<sup>+</sup> cells were present in mild hepatitis compared to moderate hepatitis, suggesting that an increase in DC recruitment or accumulation correlates with severity of disease <sup>251</sup>.

Functional studies have been described using an overnight cell migration DC isolation technique, allowing study of un-manipulated tissue-resident DCs *ex vivo*. DCs from the livers of HCV patients were compared to non-infected, inflamed liver; mDC numbers in HCV liver were increased compared to non-HCV liver and demonstrated higher expression of MHC II, CD86 and CD123, were more efficient stimulators of allogeneic T cells and induced less secretion of IL-10 <sup>252</sup>. Interestingly, blocking IL-10 resulted in enhancement of the ability of mDCs from uninfected liver to stimulate T cells, thereby suggesting that reduced IL-10

secretion in HCV liver may be a factor in the enhanced functional properties of mDCs from HCV liver. For functional studies, *in vitro* expanded MDDCs instead of freshly-isolated blood DCs were used to compare to liver-derived mDCs which neglects to take into account the presence of more than one subset of mDC and is therefore a drawback of these experiments.

Nattermann *et al* also demonstrated enrichment of DCs in the liver, with an elevation in the number of intrahepatic BDCA-1<sup>+</sup> (CD1c<sup>+</sup>) cells, as shown using immunohistochemical techniques, in patients with chronic HBV or HCV when compared with normal subjects <sup>234</sup>. Here, it was also found that the E2 protein of HCV induced RANTES to attract CCR5 immature DCs. However, these DCs are unresponsive to the CCL21 chemokine which prevents DC migration to the lymphoid tissue for interaction with T cells and generation of an adaptive immune response. These data suggest that accumulation of DCs in the liver in HCV infection may be due to impaired migration induced by viral proteins.

While human liver DC studies to date have proven useful in providing clues to hepatic DC phenotype and function, most reports are from peri-tumoral or diseased explanted livers <sup>234, 246, 252-255</sup>, with minimal information available on the distribution of DC subsets and their functionality in healthy liver. CD141<sup>+</sup> DCs have also been described in human liver <sup>246, 253</sup> although only peritumoral liver tissue was studied and functional analyses were not performed. It has also been suggested that CD141<sup>+</sup> DCs accumulate in HCV-infected liver <sup>254</sup> but levels were compared only with other diseased livers.



Although CD141<sup>+</sup> DCs in human blood, spleen and skin have been well investigated<sup>253, 256</sup>, CD141<sup>+</sup> DCs in human liver remain difficult to study, with studies to date limited to peritumoral and diseased tissue<sup>246, 253, 254</sup>. In particular, functional analyses have been restricted by the difficulty in obtaining healthy human liver tissue. Little is known about specific DC subsets, particularly CD141<sup>+</sup> DCs, in healthy human liver and how this profile is perturbed by disease.

**Rationale:**

- IFN- $\lambda$  is the major IFN produced in HCV-infected liver
- IFN- $\lambda$  has an important role in activating antiviral gene expression in hepatocytes
- CD141<sup>+</sup> DCs are known to be potent producers of IFN- $\lambda$
- CD141<sup>+</sup> DCs express high TLR3 and cross present viral antigen to activate CD8<sup>+</sup> T cells
- pDCs are known to be an important sensor of HCV infection and producer of IFN- $\alpha$  in response to HCV-infected hepatocytes
- CD141<sup>+</sup> DCs and their production of IFN- $\lambda$  has not been characterised in healthy liver

**Hypothesis:** CD141<sup>+</sup> DCs are present in healthy liver and are important in the hepatic antiviral immune response

**Aims:**

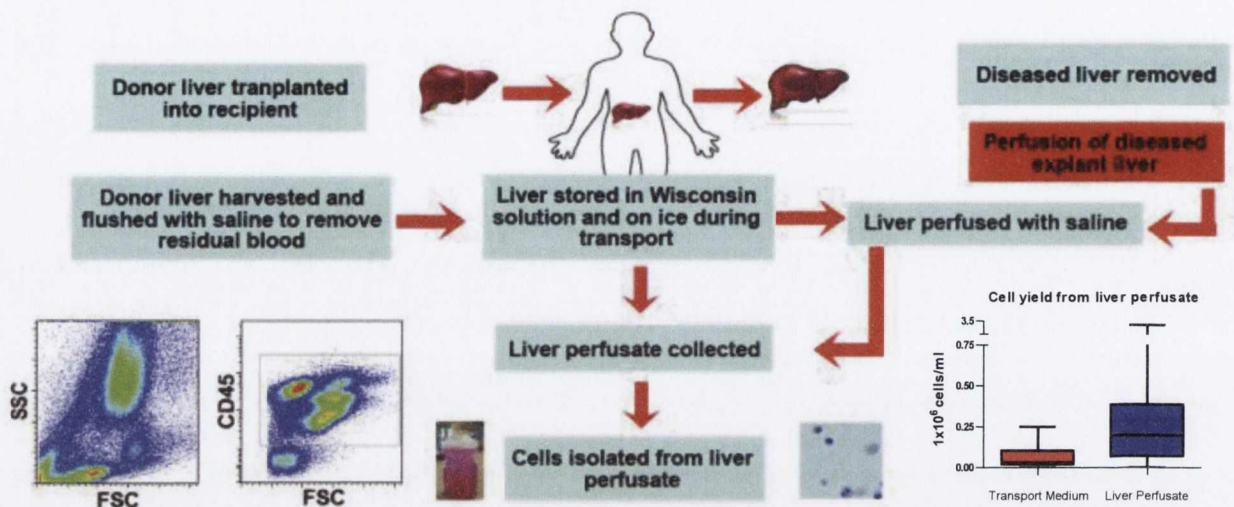
- To compare CD141<sup>+</sup> DC frequency in liver and blood
- Examine the profile of T cell responses activated by hepatic CD141<sup>+</sup> DCs
- Characterise DC subsets, including CD141<sup>+</sup> mDCs, in the perfusate of both healthy and diseased liver
- Determine IFN- $\lambda$  production in healthy and diseased liver

## 5.2 Results

### 5.2.1 Dendritic cell populations in perfusates from healthy donor livers

Perfusates from 22 donor livers were studied. On average, 850 ml (+/- 235 ml) of liver perfusate yielded up to  $1 \times 10^8$  viable HMNCs (**Figure 5.3**). We found reversed CD4:CD8 ratios as well as high proportions of NK cells amongst HMNCs, confirming their hepatic origin (Fahey *et al*, unpublished data; <sup>67, 257</sup>) as well as significant populations of myeloid cells. DCs (Lin1·HLA-DR<sup>+</sup> cells) accounted for approximately 1% of total HMNCs and were consistently detected in all donor liver perfusates (**Figure 5.4A**). Over 15% of hepatic DCs expressed the pDC marker CD123 (+/- 11.9%) (**Figure 5.4 A & C**), while almost half expressed CD11c, identifying them as mDCs. Of particular interest was the increased frequency of the CD141<sup>+</sup> mDC population in donor liver compared to peripheral blood. Almost a third of liver mDCs co-express CD141 while less expressed CD1c<sup>+</sup>. In contrast, the mDC population in the blood was predominantly CD1c<sup>+</sup> (76.3% +/- 16%), while only 4% (+/- 1.8%) expressed CD141 (**Figure 5.4 B & C**).

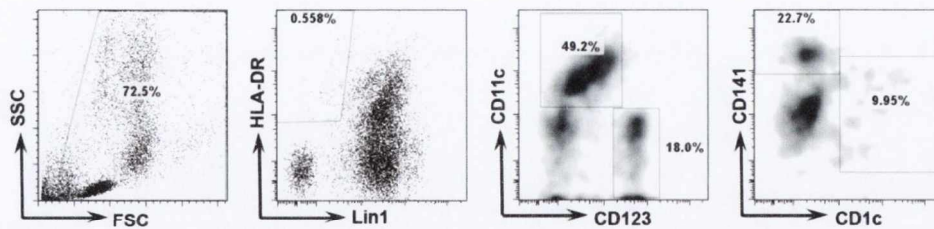




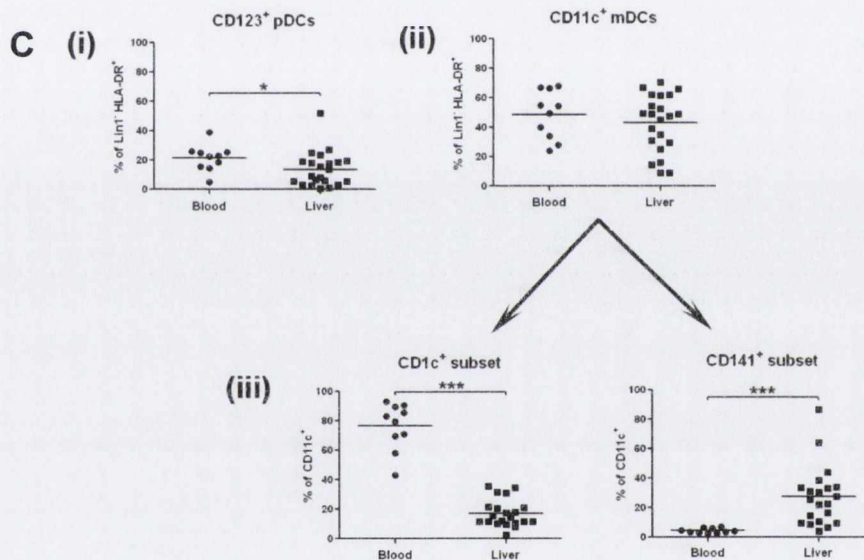
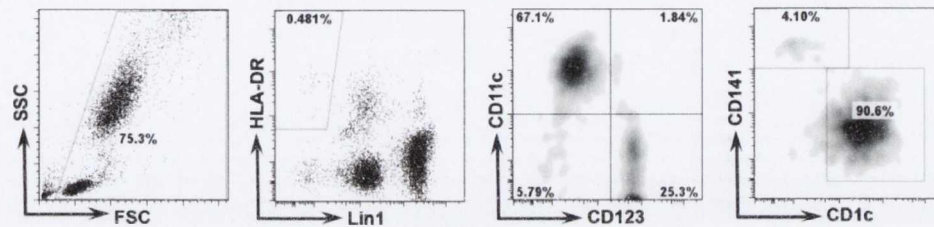
**Figure 5.3: Isolation of hepatic mononuclear cells (HMNCs) from liver perfusate**

Liver perfusate was collected during transplantation. Briefly, donor liver was harvested and perfused to remove any residual blood. The organ was then stored in preservation medium (Wisconsin's solution) on ice during transportation. Prior to transplantation, the liver was perfused with saline to remove the Wisconsin's solution and the liver perfusate was collected for research. Explant liver removed from the recipient was also perfused in a similar manner. HMNCs were isolated and cell yield and viability was calculated.

## A Liver



## B Blood



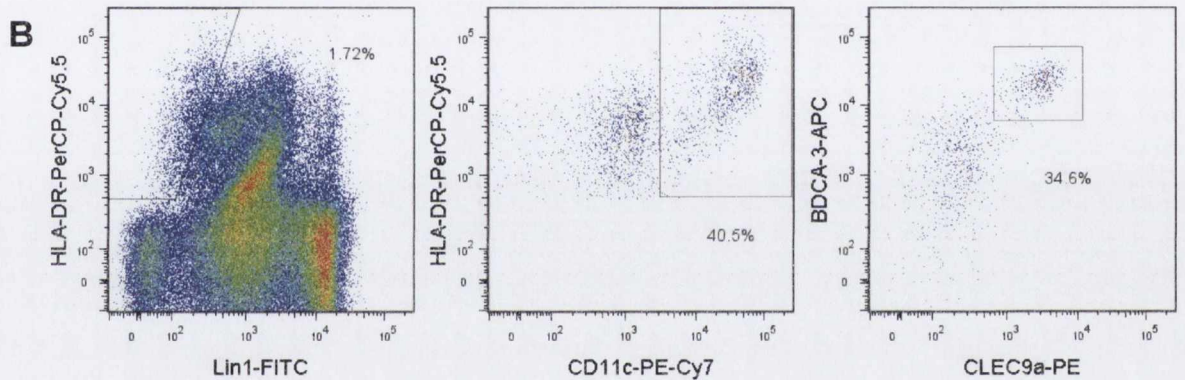
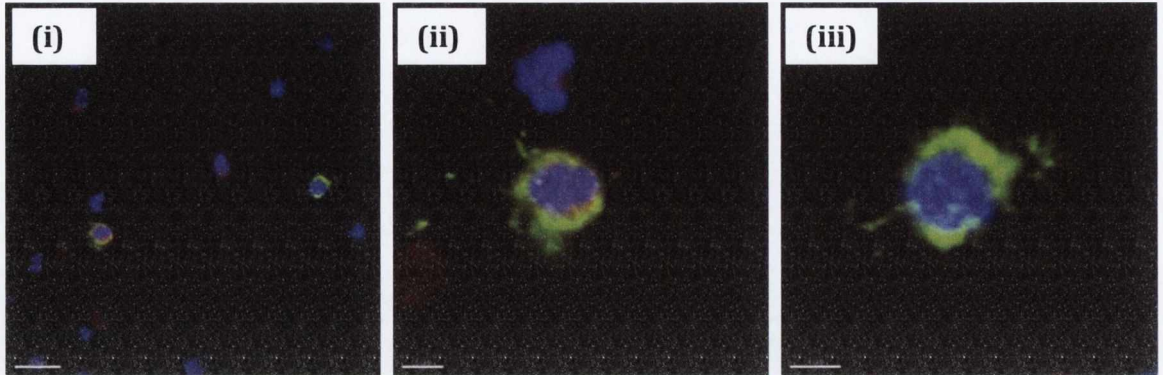
**Figure 5.4: Characterisation of dendritic cell subsets in healthy donor liver perfusate**

(A) Staining for dendritic cell (DC) subsets was carried out on hepatic mononuclear cells (HMNCs) isolated from healthy liver perfusate and compared with DC subsets in (B) blood from healthy controls. For each sample, at least 100,000 events were acquired. Following exclusion of dead cells based on light scattering and gating of Lin1-HLA-DR<sup>+</sup> cells, plasmacytoid DCs (pDCs) were identified by expression of CD123. Myeloid DCs (mDCs) were identified as Lin1-HLA-DR<sup>+</sup>CD11c<sup>+</sup>, within which CD1c<sup>+</sup> and CD141<sup>+</sup> subsets were identified. Representative flow cytometry plots are shown of CD123<sup>+</sup> pDCs and CD1c<sup>+</sup> and CD141<sup>+</sup> mDC subsets in donor liver perfusate and healthy control blood. (C) Frequency of (i) CD123<sup>+</sup> pDCs, (ii) CD11c<sup>+</sup> mDCs ( $n=22$ ) expressed as a percentage of Lin1-HLA-DR<sup>+</sup> cells and (iii) CD1c<sup>+</sup> and CD141<sup>+</sup> mDC subsets in healthy blood ( $n=10$ ) and liver ( $n=21$ ; one sample lost to analysis) expressed as a percentage of CD11c<sup>+</sup> mDCs. The horizontal bars represent the mean of each of the groups. Results were analysed using Mann-Whitney  $U$  test, \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ .

### 5.2.2 CD141<sup>+</sup> liver DCs express CLEC9A, ILT3 and ILT4

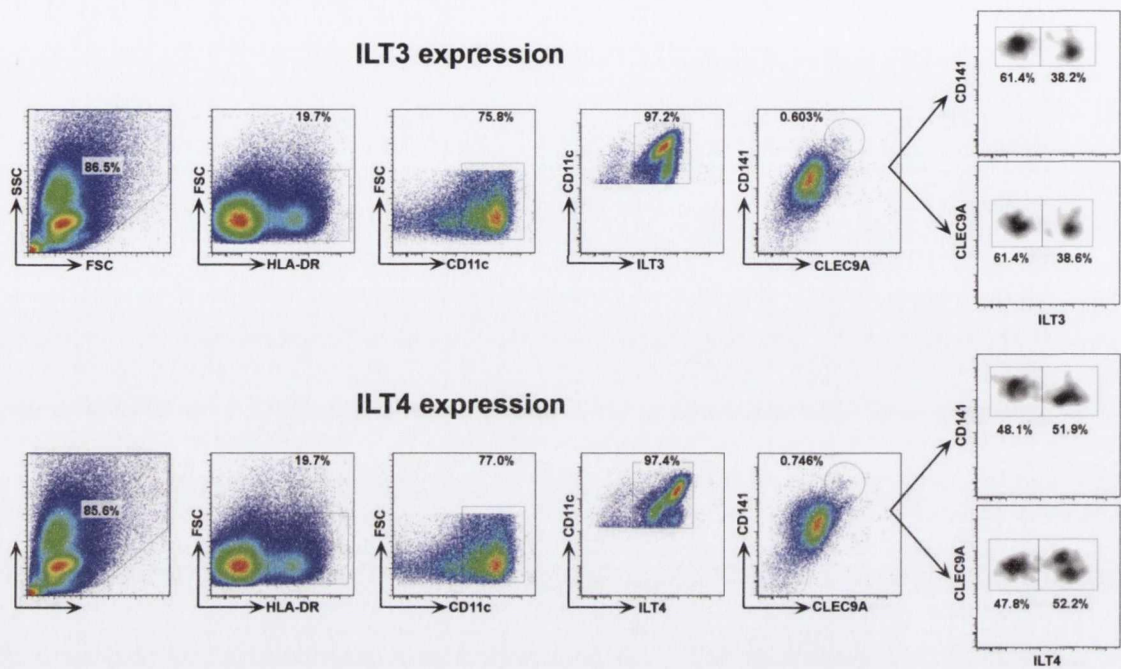
Having identified a significant population of CD141<sup>+</sup> DCs in healthy donor liver, we further characterised their phenotype. The dendritic morphology of liver CD141<sup>+</sup> DCs was confirmed by confocal microscopy (**Figure 5.5A**) and this population co-expressed the dead cell receptor CLEC9A (**Figure 5.5B**). The majority (>90%) of liver HLA-DR<sup>+</sup>CD11c<sup>+</sup> liver cells expressed immunoglobulin-like transcript (ILT)3 and ILT4, molecules which mediate immune tolerance <sup>258, 259</sup>, suggesting they contribute to the tolerogenic function of healthy liver. A small subset of DCs were negative for both markers and on closer examination, the ILT3- and ILT4-negative population was CD141<sup>+</sup>CLEC9A<sup>+</sup> (**Figure 5.6**), although a proportion of CD141<sup>+</sup> DCs express ILT3 and ILT4 (38% ILT3<sup>+</sup> and 52% ILT4<sup>+</sup>).



**A**

**Figure 5.5: Hepatic CD141<sup>+</sup> DCs display dendritic morphology and express CLE9A**

(A) Hepatic mononuclear cells (HMNCs) obtained from donor liver perfusate were incubated in a poly-D-lysine coated chamber slide overnight at 37°C, fixed and stained with anti-CD141, Alexa Fluor 488 goat anti-mouse secondary antibody (green), DAPI nuclear stain (blue) and wheat germ agglutinin (red) and analysed by microscopy at (i) x20 and (ii and iii) x60. (B) HMNCs obtained from the perfusate of donor livers (n=7) were stained for CLE9A and analysed by flow cytometry. Representative flow cytometric plots are shown of the CD141<sup>+</sup>CLE9A<sup>+</sup> double-positive DC population amongst HMNCs from liver perfusate.



**Figure 5.6: A proportion of hepatic CD141+ DCs express ILT3 and ILT4**

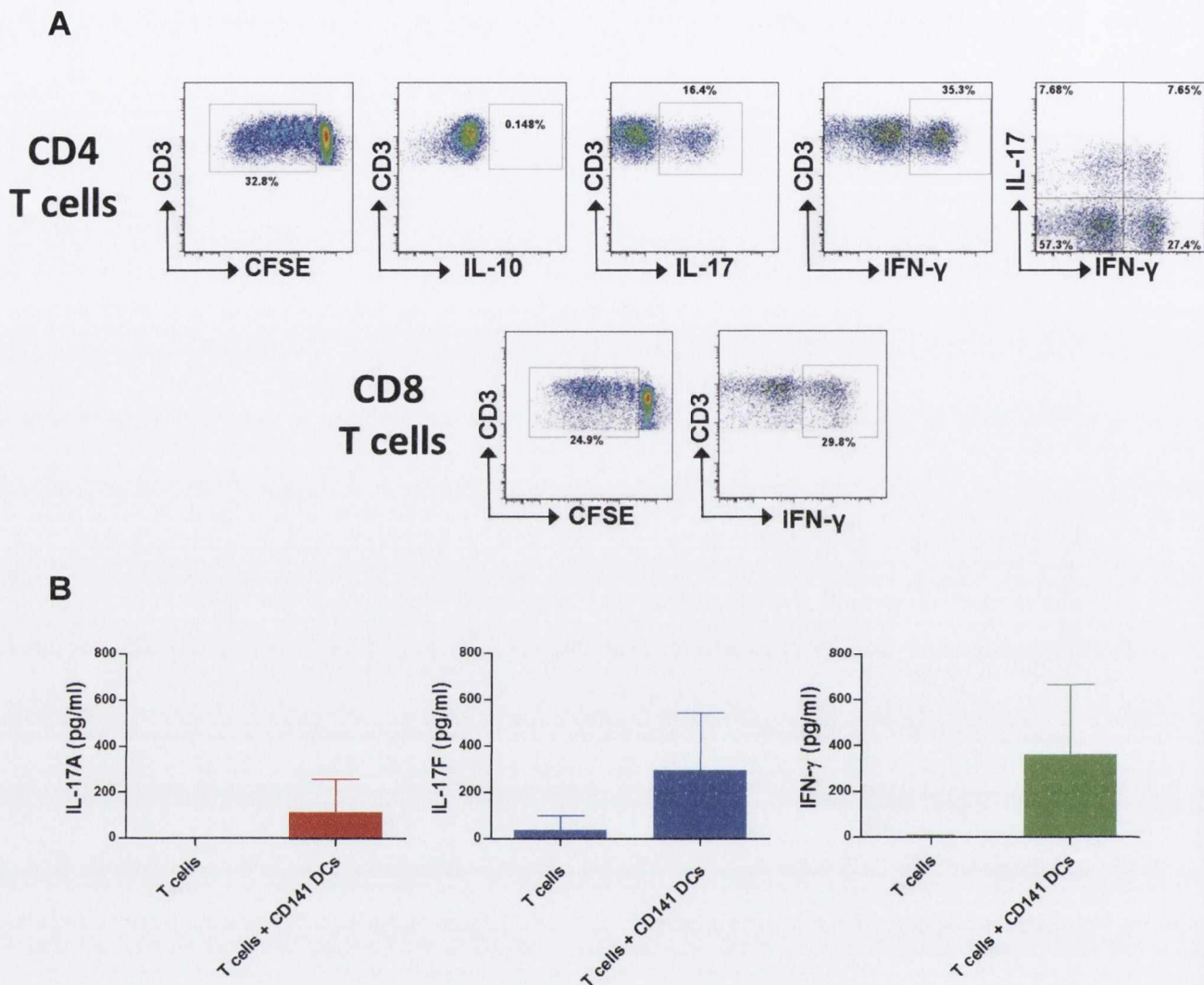
Hepatic mononuclear cells (HMNCs) were isolated from donor liver perfusate and expression of the tolerogenic markers immunoglobulin-like transcript (ILT)3 and ILT4 by HLA-DR<sup>+</sup>CD11c<sup>+</sup> cells was examined by flow cytometry. CD141<sup>+</sup> DCs were further distinguished by staining of the double positive population expressing CD141 and CLEC9A. The flow plots shown are representative of three healthy donor liver samples.

### 5.2.3 Liver CD141<sup>+</sup> DCs respond to TLR agonists and stimulate IFN- $\gamma$ and IL-17 production by T cells

DC-derived cytokines play a key role in T cell polarisation. We found that CD141<sup>+</sup> DCs obtained from healthy human liver produced low/undetectable levels of IFN- $\alpha$ , IL-10, IL-23, IL-1 $\beta$  or IL-12p70, constitutively or in response to poly(I:C) stimulation but did produce CXCL10 which is in line with other studies which have also shown production of TNF- $\alpha$ , IL-6 and IL-8<sup>156, 169, 253</sup>.

To evaluate the capacity of hepatic CD141<sup>+</sup> DCs to stimulate T cell responses, we performed allogeneic MLRs. CD141<sup>+</sup> DCs obtained from donor livers (n=3) were co-cultured with CFSE-labelled allogeneic T cells for 7 days; cells were then re-stimulated with PMA/ionomycin and T cell cytokine production was assessed by intracellular staining. Co-culture with hepatic CD141<sup>+</sup> DCs induced robust proliferation of CD3<sup>+</sup>CD8<sup>-</sup> (CD4 T cells) which produced significant levels of IL-17 and IFN- $\gamma$  but no IL-10. CD141<sup>+</sup> DCs also promoted CD8<sup>+</sup> T cell proliferation and IFN- $\gamma$  secretion (**Figure 5.7A**). T cell secretion of IFN- $\gamma$ , IL-17A and IL-17F, as well as lack of IL-10, was confirmed by measuring cytokine levels in 7 day supernatants from MLRs (n=3; **Figure 5.7B**).



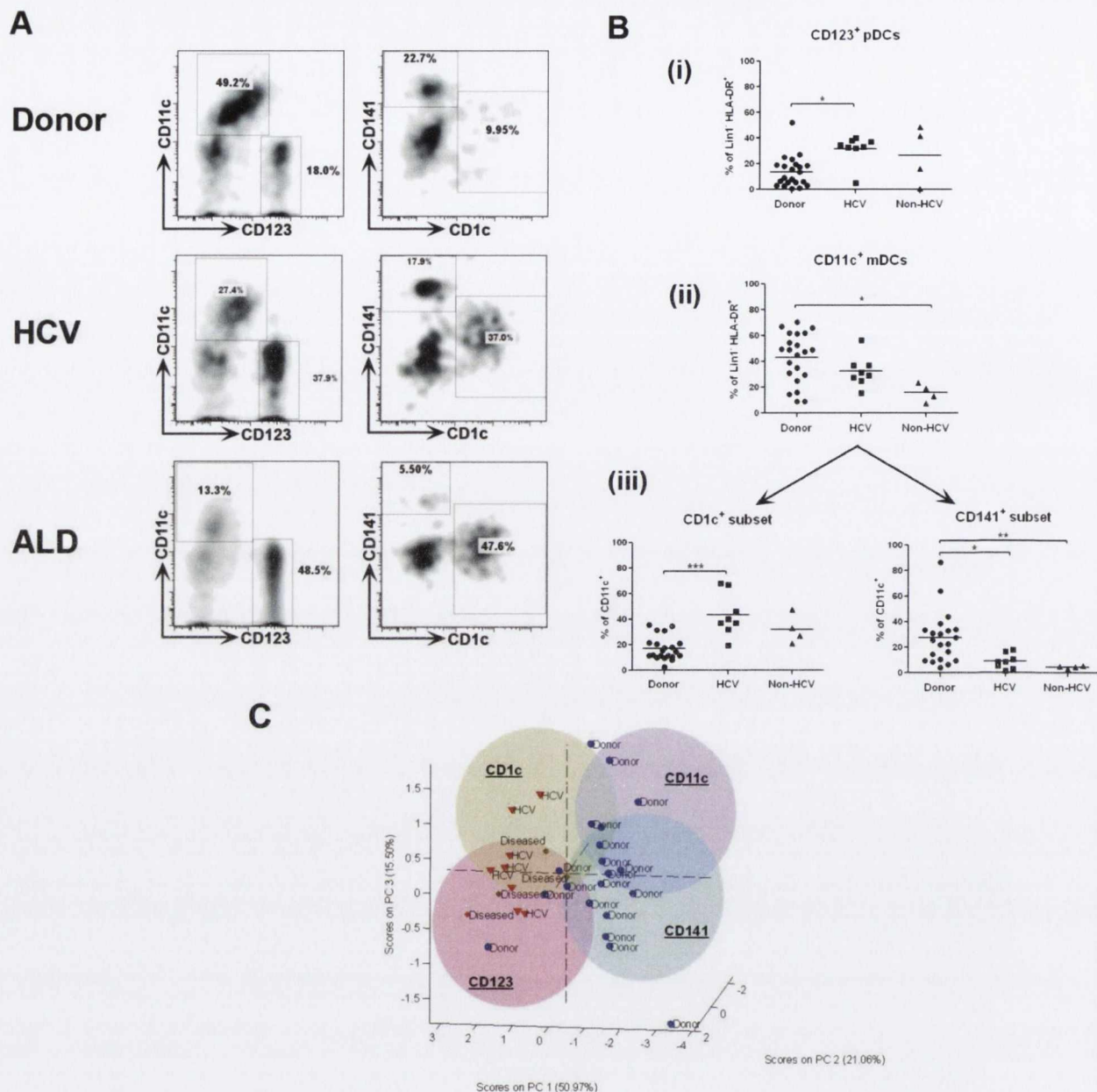


**Figure 5.7: CD141<sup>+</sup> DCs drive IL-17 and IFN- $\gamma$ -producing T cells in a mixed lymphocyte reaction**

CD141<sup>+</sup> DCs were isolated from donor liver hepatic mononuclear cells (HMNCs) by magnetic bead separation. DCs (10,000) were then co-cultured with 200,000 allogeneic CFSE-labelled CD3<sup>+</sup> T cells for 7 days. (A) Following re-stimulation with PMA/ionomycin for 5 hrs, production of the intracellular cytokines IL-10, IL-17 and IFN- $\gamma$  by CD4<sup>+</sup> and CD8<sup>+</sup> T cells was analysed by flow cytometry. The dot plots show proliferation (CFSE loss) and cytokine production by CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD8<sup>-</sup> T cells, and the data is representative of three separate experiments. (B) T cell secretion of IFN- $\gamma$ , IL-17A and IL-17F were measured by ELISA in cell supernatants from CD141<sup>+</sup> DCs and allogeneic CD3<sup>+</sup> T cells co-cultured for 7 days. Graphs show data from three independent experiments.

#### 5.2.4 Characterisation of CD141<sup>+</sup> DCs from diseased liver

Next, we wished to determine the effect of liver disease on the profile of hepatic DC subsets. Twelve explant diseased livers were perfused with saline and cells were collected. The pathologies of the explanted organs are listed in **Table 5.1**. We found that the CD123<sup>+</sup> pDC population was significantly increased in perfusates from the HCV-infected explant livers compared to donor ( $p=0.0188$ ). This population was also expanded, though not significantly, in non-HCV diseased livers (**Figure 5.8A** and **Figure 5.8B i**). In contrast, the frequency of the CD11c<sup>+</sup> mDCs was decreased in diseased livers (**Figure 5.8A** and **Figure 5.8B ii**). Even more striking was the change in mDC subset distribution in diseased liver. CD141<sup>+</sup> mDCs were significantly depleted compared with healthy liver whereas the CD1c<sup>+</sup> population was expanded; these changes were not confined to HCV infection, suggesting that depletion of CD141<sup>+</sup> DCs is a feature of end-stage liver disease rather than exclusively HCV-mediated pathology (**Figure 5.8A** and **Figure 5.8B iii**). Principal component analysis was used to classify healthy and diseased liver DC populations, based on their variances. The overlaid scores and loadings plots demonstrate differing patterns of DC subset frequency in healthy and diseased liver samples. Healthy liver positively correlates with frequency of CD11c<sup>+</sup> and CD141<sup>+</sup> subpopulations whereas liver pathology is positively correlated with frequency of CD123<sup>+</sup> and CD1c<sup>+</sup> subpopulations (**Figure 5.8C**).



**Figure 5. 8: Increased frequency of pDCs and CD1c<sup>+</sup> mDCs with a concomitant decrease in the CD141<sup>+</sup> mDC subset in hepatic mononuclear cells obtained from perfusates of diseased liver compared to donor liver perfusates**

(A) Liver perfusate from HCV-infected (n=8) and non-HCV diseased explant (n=4) liver was analysed for presence of DC subsets by flow cytometry and compared to donor liver perfusates (n=22). For each sample, at least 100,000 events were acquired. Following the exclusion of dead cells based on light scattering, Lin1<sup>+</sup>HLA-DR<sup>+</sup> cells were gated. Plasmacytoid dendritic cells (pDCs) were identified by expression of CD123 within the Lin1<sup>+</sup>HLA-DR<sup>+</sup> gate. Myeloid DCs (mDCs) were identified as Lin1<sup>+</sup>HLA-DR<sup>+</sup> CD11c<sup>+</sup>, which were then further classified as CD1c<sup>+</sup> and CD141<sup>+</sup> subsets. Representative staining of a healthy donor liver perfusate and hepatitis C virus (HCV)-infected and alcoholic liver disease (ALD) explant liver perfusates are shown. (B) (i) CD123<sup>+</sup> pDC and (ii) CD11c<sup>+</sup> mDC frequencies (n=22) are presented as a percentage of the parent Lin1<sup>+</sup>HLA-DR<sup>+</sup> population while (iii) CD1c<sup>+</sup> and CD141<sup>+</sup> are expressed as a percentage of the Lin1<sup>+</sup>HLA-DR<sup>+</sup>CD11c<sup>+</sup> myeloid DC population (n=21; one lost to analysis). Statistical analysis was performed using one-way ANOVA (Kruskal-Wallis) followed by Dunn's multiple comparison post-test. The horizontal bars represent the mean of each of the groups. \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001. (C) Principal Component Analysis was used to classify different sample sets, healthy and diseased liver DC populations, based on their variances.



**Table 5. 1: Characteristics of donor and recipient liver transplant patients**

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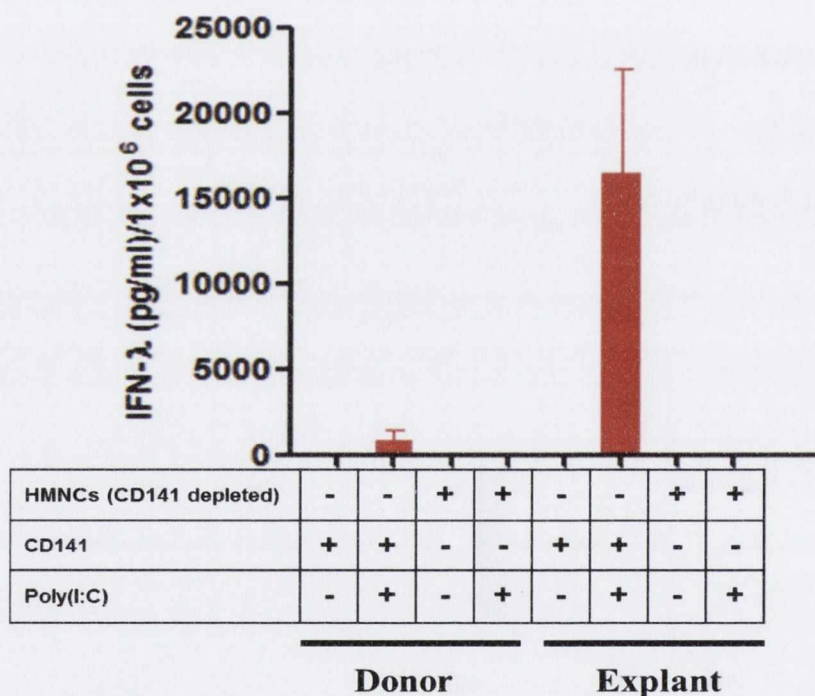
<b><u>Donors</u></b>	
<b>Number of patients</b>	22
<b>Mean donor age</b>	44 years (range 14-68)
<b>Male/female</b>	17 male, 5 female
<b>Cause of death</b>	RTA (1), SAH (13), Fall (2), CVA (2), Meningitis (1), Cardiac arrest (1), Suicide (2)
<b>Use of drugs</b>	None (16), Phenytoin (1), Amiodarone (1), Inhalers (1), Aspirin (1), Thyroxine (1), Antihypertensives (1)
<b><u>Recipients</u></b>	
<b>Number of patients</b>	12
<b>Mean recipient age</b>	51 years (range 33-65)
<b>Male/female</b>	7 male, 5 female
<b>Reason for transplant</b>	HCV HCC (3), HCV ALD (4), NASH (1), Polycystic liver disease (1), Seronegative hepatitis (1), HCV (1), ALD (1)

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RTA, road traffic accident; SAH, subarachnoid haemorrhage; CVA, cerebrovascular accident; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ALD, alcoholic liver disease; NASH, non-alcoholic steatohepatitis

### 5.2.5 IL-29 production from PBMCs and HMNCs

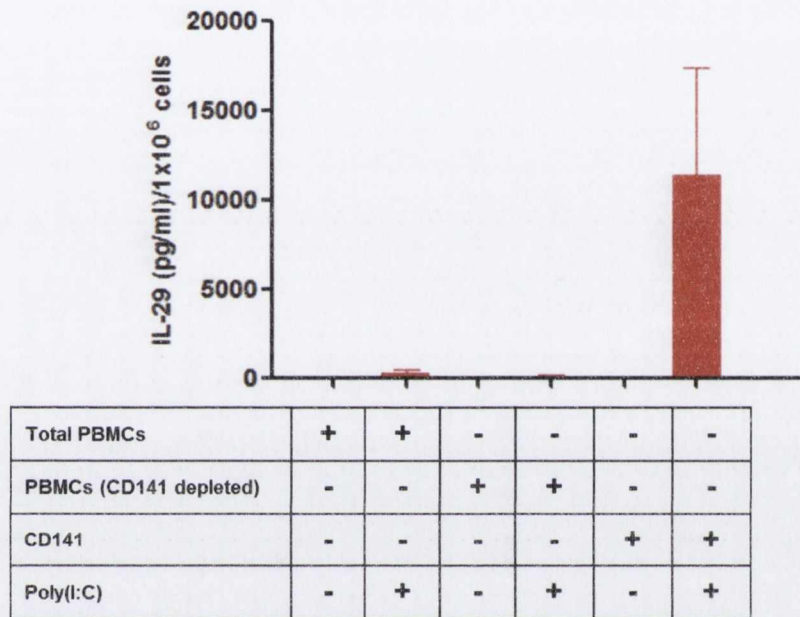
To explore ability of hepatic DCs to produce IFN- $\lambda$ , HMNCs were treated with the TLR3 agonist poly(I:C) (50  $\mu$ g/ml) for 18 hours and IFN- $\lambda$  production measured by ELISA. We found IFN- $\lambda$  was produced by hepatic DCs in response to poly(I:C) and was restricted to the CD141<sup>+</sup> subset of liver DCs (Figure 5.9). As shown by Lauterbach *et al*, 2010 neither LPS, nor, CLO97, induced IFN- $\lambda$  production by HMNCs<sup>155</sup>. However, poly(I:C)-treated CD141<sup>+</sup> DCs from diseased liver produced ten times more IFN- $\lambda$  in comparison to donor CD141<sup>+</sup> DCs (Figure 5.9).



**Figure 5.9: CD141<sup>+</sup> DCs from diseased liver produce increased IFN- $\lambda$  in response to poly(I:C) compared with donor liver DCs**

CD141<sup>+</sup> DCs were isolated from diseased explant liver hepatic mononuclear cells (HMNCs) (n=2) and treated with 50  $\mu$ g/ml poly(I:C) for 18 hrs and IFN- $\lambda$  (IL-29) production was measured in cell supernatants by ELISA and compared with IFN- $\lambda$  production from donor liver (n=3).

Similar observations were made in PBMCs, with IFN- $\lambda$  production once again restricted to the CD141<sup>+</sup> DC subset. Interestingly, blood CD141<sup>+</sup> DCs were capable of producing higher levels of IFN- $\lambda$  compared to liver CD141s (**Figure 5.10**).



**Figure 5.10: IFN- $\lambda$  production from blood CD141<sup>+</sup> DCs stimulated with poly(I:C)**

PBMCs from healthy donors (n=3) were isolated, treated with 50  $\mu$ g/ml poly(I:C) for 18 hrs, as indicated, and IFN- $\lambda$  (IL-29) measured by ELISA. Minimal IFN- $\lambda$  production was observed following poly(I:C) treatment. When CD141<sup>+</sup> DCs were isolated and treated with poly(I:C) up to 15,000 pg/ml IFN- $\lambda$  was observed compared to the lack of IFN- $\lambda$  produced from the total cells and fraction of cells lacking CD141<sup>+</sup> DCs.



### 5.3 Discussion

In this study, we found that almost one third of mDCs from healthy liver highly expressed CD141 and could stimulate Th1 and Th17 pro-inflammatory T cell responses. In diseased liver, CD141<sup>+</sup> DCs were depleted but were primed to produce IFN- $\lambda$ . To our knowledge, this is the first description of functional CD141<sup>+</sup> DCs in healthy human liver, which are depleted in end-stage liver disease.

Previous studies on human liver DCs used cells isolated from liver tissue obtained during resection for cancer metastases <sup>234, 246, 253</sup>. While these liver samples appear free from gross malignancies, tumour growth clearly influences the surrounding environment with significant perturbation of local immune cell populations and cytokine levels <sup>260-262</sup>. Isolation of HMNCs from donor liver perfusates obtained prior to organ implantation is a unique opportunity to study healthy liver-derived immune cells from disease-free organs. Cells from liver perfusates are particularly useful for DC analysis as these cells are normally difficult to release from tissue specimens, requiring the use of enzymes which often interfere with surface marker quantitation <sup>263, 264</sup> and result in high levels of autofluorescence which can complicate flow cytometric analysis.

Less than 16% of hepatic DCs co-expressed the pDC marker CD123 while almost half expressed CD11c identifying them as mDCs. Of particular interest was the expansion of the CD141<sup>+</sup> mDC population in donor liver. These cells are present in small numbers in the circulation and are also present in skin, where they are reported to constitutively produce IL-10 <sup>256</sup>. Liver CD141<sup>+</sup> DCs could initiate Th1 and Th17 expansion in MLR experiments. Therefore hepatic CD141<sup>+</sup> DCs are

clearly capable of responding to pathogenic challenge and directing adaptive immunity in the liver.

The observation that a subset of liver DCs is capable of inducing a pro-inflammatory response is interesting given that many reports find liver DCs to be poor stimulators of T cell proliferation. It has been shown in mouse liver that, while overall liver DCs are weak stimulators of T cells, the classical CD8 $\alpha^+$  and CD8 $\alpha^-$  DCs, equivalent to CD141 $^+$  and CD1c $^+$  human DCs respectively, are in the minority, representing only 19% of total liver DCs compared to over 80% in the spleen <sup>241</sup>. When these subsets are isolated, they are capable of stimulating T cell proliferation similar to their splenic counterparts. While pDCs comprise an additional 20% of liver DCs, the remainder consists of two populations of CD11b<sup>low</sup> CD8 $\alpha^{\text{low}}$  and CD11b $^-$  CD8 $\alpha^-$  DCs. The presence of these additional DCs in the liver is really striking when flow plots of liver DCs are compared to spleen, which largely consists of CD8 $\alpha^+$  and CD8 $\alpha^-$ . The difference in liver DC subset composition compared to spleen may account for the overall tolerogenic DC phenotype observed. Even within the CD8 $\alpha^+$  and CD8 $\alpha^-$  populations, a major proportion are immature, expressing low levels of maturation markers and lower levels of CD11c, while mature populations with high CD11c and co-stimulatory molecule expression are in the minority <sup>239,240</sup>.

In our study of human liver DCs, we also observe extra populations in addition to the known subsets. A significant portion of Lin $^-$  HLA-DR $^+$  CD11c $^+$  cells are present that do not segregate into CD1c $^+$  and CD141 $^+$  subsets (**Figure 5.4A**). This population is absent in flow plots of blood DCs (**Figure 5.4B**), thus raising the

possibility that a significant proportion of liver DCs are either immature precursors or additional subtypes yet to be identified. Furthermore, variable expression of the tolerogenic markers ILT3 and ILT4 was observed on CD141<sup>+</sup> CLEC9A<sup>+</sup> DCs, which suggests different cell developmental stages or additional unidentified subsets. Further investigation is required to characterise these cells which may reveal unique characteristics of human liver DCs.

CD141<sup>+</sup> DCs express high levels of TLR3 and are therefore the population of DCs most likely to respond to certain conserved viral motifs such as dsRNA. IFN- $\lambda$  production has been demonstrated by blood CD141<sup>+</sup> DCs <sup>155</sup>. Here, we found that IFN- $\lambda$  production in response to poly(I:C) was restricted to the CD141<sup>+</sup> subset of liver DCs. In MLRs, hepatic CD141<sup>+</sup> DCs induced production of IL-17 and IFN- $\gamma$  from co-cultured T cells but little IL-10. A proportion of CD141<sup>+</sup> mDCs is negative for the tolerogenic DC molecules ILT3 and ILT4, thereby indicating pro-inflammatory rather than tolerogenic properties. Our results indicate that normal liver has a significant population of CD141<sup>+</sup> DCs which have a potent pro-inflammatory phenotype likely to play an important role in hepatic antiviral immunity.

In explant livers with end-stage liver disease, pDCs were increased compared to healthy donor liver. The CD1c<sup>+</sup> subset of mDCs was also expanded significantly. In contrast, the CD141<sup>+</sup> mDC subset, which was enriched in healthy human liver, was depleted from diseased liver, whether HCV-infected or not. CD1c<sup>+</sup> mDCs and CD123<sup>+</sup> pDCs have previously been shown to be expanded in diseased livers <sup>234</sup>. Reasons for the selective depletion of the CD141<sup>+</sup> DC subset from explant livers,



while other populations are increased, are unknown. One possibility is that activated CD141<sup>+</sup> DCs have migrated to the hepatic lymph nodes or alternatively have undergone activation-induced cell death <sup>265</sup>.

Interestingly, poly(I:C)-treated CD141<sup>+</sup> DCs from diseased liver produced ten times more IFN- $\lambda$  in comparison to donor CD141<sup>+</sup> DCs which suggests that pathogenic factors ensure this cell population is primed to respond to TLR ligation. Thus it appears that even though the CD141<sup>+</sup> mDC population is depleted from diseased liver, the cells that remain are responsive to TLR ligands. CD141<sup>+</sup> DCs can also secrete IFN- $\lambda$  in response to HCV infection *in vitro* <sup>266, 267</sup>, therefore the CD141<sup>+</sup> DCs present in diseased liver may contribute to production of this important cytokine in patients exposed to HCV. IFN- $\lambda$  production in response to poly(I:C) from donor liver CD141<sup>+</sup> DCs is much lower than the same population derived from blood, whereas increased levels are observed from diseased liver CD141<sup>+</sup> DCs. The tolerogenic environment of the liver ensures that there is a high threshold for T cell activation within the liver. Increased concentrations of LPS are required to induce liver DC responsiveness compared to spleen DCs <sup>242</sup>. Therefore, it appears that pathogenic challenge or inflammation can override the steady state tolerogenic mechanisms at play in the liver, when necessary. Thus liver DCs, while overall being tolerogenic, are capable of responding to pathogenic challenge. DCs can also be activated by dead cell antigens, particularly the CD8 $\alpha$ <sup>+</sup>/CD141<sup>+</sup> subset. Therefore it is possible that continuous exposure to DAMPs in the fibrotic, diseased liver primes CD141<sup>+</sup> DCs to produce IFN- $\lambda$ . While IFN- $\lambda$  can activate antiviral genes in response to viral infection, it is unknown what the function of CD141<sup>+</sup> IFN- $\lambda$  production is in the absence of viral infection, but it is possible that

they play a role in tissue homeostasis and modulating the immune response. CD8 $\alpha$ <sup>+</sup> DCs have been shown to induce peripheral tolerance to tissue associated antigens <sup>268</sup>, thus suggesting a dual role for this subset in homeostasis and antiviral or anti-tumour immunity .

In conclusion, healthy human liver is a rich source of CD141<sup>+</sup> DCs. Co-expression of the dead cell receptor CLEC9A by hepatic CD141<sup>+</sup> DCs suggests these cells could have a role in the maintenance of liver homeostasis. A significant proportion of hepatic CD141<sup>+</sup> mDCs lack the tolerogenic markers ILT3 and ILT4 and induce T cell production of pro-inflammatory cytokines. These cells are depleted from diseased liver, while other DC subsets are increased. Despite depletion, the remaining CD141<sup>+</sup> DCs are primed to produce significantly more IFN- $\lambda$  in response to poly(I:C). Our data highlight the possibility of several different functional roles for liver DC populations which could provide important targets for the development of successful therapeutic and preventative vaccines.

## **Chapter 6: Final Discussion**



HCV is a chronic viral infection of the liver, estimated to affect over 160 million people worldwide. Variation in the host immune response has been demonstrated to have a major impact in the outcome to HCV infection. The majority of individuals develop chronic infection, but a subset is capable of naturally clearing the infection, as evidenced by detection of HCV-specific antibodies in the absence of viral RNA. It has been hypothesised that a resistant cohort may exist, whereby individuals are exposed to HCV but do not become infected. The innate immune system is capable of clearing the virus, without the need for activation of the adaptive immune response and antibody generation. Evidence for this hypothesis exists from the anti-D cohort in Ireland, where it is known that some people were exposed to infected batches of anti-D but did not develop infection. This highlights the importance of the innate immune response in clearing viral infection.

Variation in immune genes, in particular, has been shown to be associated with the clinical outcome of HCV infection (summarised in **Table 1.1**). In the first part of this study we hypothesised that variation in the antiviral gene *OAS1* would be associated with HCV outcome. A homogenous cohort of Irish women infected with HCV from a single source, were genotyped for a functional SNP in *OAS1*. This SNP, rs10774671, is known to give rise to transcript variants with variable antiviral activity<sup>184</sup>. In a cohort of 178 (98 PCR+; 80 PCR-) patients infected with HCV, we found no association with rs10774671 genotype and natural clearance of HCV infection. Some studies have shown association of this SNP with HCV treatment response<sup>193</sup>, but this was not possible in our cohort due to low numbers of patients undergoing treatment.

Large scale GWAS studies have now largely superseded single candidate gene studies. Reports published in late 2009 revealed that a number of polymorphisms near the *IL28B* gene were strongly associated with HCV viral clearance and therapeutic response<sup>118, 119</sup>. Association of the *IL28B* SNP with HCV pointed towards an important role for IFN- $\lambda$  in the immune response to HCV infection. The IFN- $\lambda$  family (IL-28A, IL-28B and IL-29) are a relatively new addition to the IFN family. Despite being classified as an IFN, the multi-exon gene structure and sharing of a chain of the IL-10 receptor resembles more the IL-10 superfamily<sup>131</sup>. Functionally IFN- $\lambda$  resembles IFN- $\alpha$ , in its ability to signal through the JAK-STAT pathway, activate ISGs, up-regulate MHC I and inhibit viral replication<sup>45, 46</sup>. To date, the major difference between IFN- $\alpha$  and IFN- $\lambda$  seems to be at the level of receptor expression. The IFN- $\lambda$  receptor is restricted to epithelial cells<sup>129</sup> and DCs<sup>7</sup>, whereas the Type I IFN receptor is widely expressed. IFN- $\lambda$  has been shown to activate an overlapping profile of ISGs as IFN- $\alpha$ <sup>199</sup>. As yet, the exact biological role of IFN- $\lambda$  in the innate immune response and whether it has a distinct non-redundant function differing from IFN- $\alpha$  is unknown.

We therefore sought to evaluate the effect of IFN- $\lambda$  on different cell types in terms of receptor expression, activation of signalling pathways and downstream gene induction, particularly focusing on peripheral blood immune cells. Because of the association with HCV, we included the Huh7 hepatocyte cell line in our studies. While the IFN- $\lambda$  receptor was present on both Huh7s and PBMCs, the activation of signalling was quite different. Huh7 cells were clearly responsive to IFN- $\lambda$ , inducing phosphorylation of STAT1 and STAT3 and activating expression of the antiviral genes *MDA-5*, *MxA* and *OAS2*. In comparison to the response of Huh7s to



IFN- $\alpha$ , much weaker phosphorylation of STAT proteins was observed upon IFN- $\lambda$  treatment, but yet, similar levels of antiviral genes were induced. This observation would suggest alternative signalling pathways are at play.

However, a major difference between IFN- $\alpha$  and IFN- $\lambda$  was observed in their differing effect on immune cells. Despite reports that IFN- $\lambda$  activates the JAK-STAT pathway <sup>45, 215</sup>, STAT phosphorylation was, surprisingly, not observed in CD45<sup>+</sup> peripheral immune cells treated with IFN- $\lambda$ , compared to the very clear phosphorylation of STATs in response to IFN- $\alpha$ . Further investigation within the immune cell subsets examined, revealed that IFN- $\lambda$  only activated the JAK-STAT pathway in pDCs. All of the IFN- $\lambda$  cytokines, IL-28A, IL-28B and IL-29, acted similarly in their ability to phosphorylate STAT1, STAT3 and STAT5. This pattern of STAT phosphorylation mirrored the response to IFN- $\alpha$ , except that IFN- $\alpha$  additionally phosphorylated STAT6, a response which up to now has only been reported in B cells <sup>47, 221</sup>. These data highlight pDCs as an important target cell of IFN- $\lambda$ .

pDCs are shown to express the IFN- $\lambda$  receptor and also to be responsive to IFN- $\lambda$  treatment via up-regulation of maturation markers and modulation of T cell cytokines in an MLR <sup>157, 223</sup>. Our work shows, for the first time, that within immune cell populations IFN- $\lambda$  activation of JAK-STAT signalling is restricted to pDCs and is not observed in T cells, B cells and monocytes. Our data shows that IFN- $\lambda$  has a very distinct, restricted effect on immune cells compared to IFN- $\alpha$ . IFN- $\lambda$  can activate STATs and up-regulate genes in epithelial cells but also has a biological effect on pDCs. A previous report showing that IFN- $\lambda$  protects from pDC



apoptosis <sup>223</sup>, signifies a possible role for IFN- $\lambda$  JAK-STAT signalling and downstream gene activation, in promoting anti-apoptotic mechanisms to sustain pDCs in viral infection. This result is interesting in light of the different effects reported on immune cells in response to IFN- $\beta$ , where the varied effect of IFN- $\beta$  on cell subsets was related to differential usage of STAT proteins, allowing IFNs to have a wide range of, sometimes paradoxical, biological functions <sup>222</sup>.

Further questions remain as to what is the function of IFN- $\lambda$  receptor on B cells and monocytes. An absence of STAT phosphorylation in B cells and monocytes was observed in response to IFN- $\lambda$ , despite our data showing clear expression of the receptor. It is therefore possible that either STAT-independent signalling mechanisms are at play or a later time-point is required to capture the p-STAT signal in these cells. Minimal STAT phosphorylation is also observed in B cells and monocytes in response to IFN- $\beta$  <sup>222</sup>. Therefore it would be interesting to directly compare STAT activation induced by IFN- $\alpha$ , IFN- $\beta$  and IFN- $\lambda$  in future experiments. Additional experiments required include looking at IFN- $\lambda$ -induced STAT phosphorylation in other populations such as CD11c<sup>+</sup> DCs and potentially different types of monocytes and B cells, where subtle differences may exist in IFN- $\lambda$  responsiveness. Although we report that IFN- $\lambda$  clearly did not affect T cells, we need to address the specific responsiveness of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and discrete subsets within these populations e.g. Tregs. There have been conflicting findings with regards to IFN- $\lambda$  receptor expression on immune cells. While some groups have shown an effect of IFN- $\lambda$  on T cells, we did not demonstrate receptor expression or STAT phosphorylation in T cells. In addition, while we clearly show receptor expression on monocytes and MDDCs, Liu *et al* did not see expression on

these cell types, instead showing expression on monocyte-derived macrophages<sup>226</sup>. The reason for this discrepancy is unclear but could be related to different culture conditions, cell maturation stage or staining protocol. Further investigation is required to clarify immune cell expression of the IFN- $\lambda$  receptor and to consider whether subsets of monocytes, B cells or T cells may specifically show expression.

Despite the *IL28B* SNP being identified in 2009 as a major factor in HCV response in 2009, it was not until early 2013 that the causal variant was identified. A novel gene has been described, designated *IFNL4*, within which the rs12979860 SNP is present. The *IFNL4* transcript is created by a common deletion frame-shift allele of ss469415590, which is a dinucleotide variant strongly linked with rs12979860<sup>128</sup>. During the course of this work we predicted the existence of a novel gene around the rs12979860 using GenScan, based on homology with proteins in the non-redundant database. Based on this prediction, we designed primers against the predicted exons and proceeded to look for expression of the novel gene in various samples such as PBMCs, healthy/HCV MDDCs, either untreated or treated with poly(I:C) and healthy and HCV-infected liver samples. Based on the findings reported by Prokunina-Olsson *et al*, poly(I:C) treatment of primary hepatocytes was required to induce expression of the novel *IFNL4* which was undetectable in healthy or infected liver samples<sup>128</sup>. The novel gene still poses a lot of unanswered questions; it is less than 30% homologous to the IFN- $\lambda$ s at the amino acid level, yet it similarly induces antiviral genes and inhibits HCV replication. Its receptor system is also as yet unidentified and target cells unknown. It is not yet



known if IFNL4 is even secreted. Further work is required to identify the function of IFNL4 and its responsive cells and production within the immune system.

In addition to the effect of IFN- $\lambda$  on immune cells, we were also interested in where and how IFN- $\lambda$  is produced. In recent years, IFN- $\lambda$  was shown to be produced by a rare subset of CD141<sup>+</sup> DCs in the periphery. Little is known about these DCs, as characterisation and functional studies have been limited by their paucity in blood. A recent study has demonstrated the presence of these cells in a number of human tissues <sup>253</sup>. However, little is known about DCs in the liver, where CD141<sup>+</sup> DCs are likely to be a key cell type in the innate immune response to HCV infection. We were interested to see was this DC subset present in the liver and how might this population be perturbed in the diseased state. Interestingly we found that CD141<sup>+</sup> DCs were enriched in the liver compared to blood. We confirmed that these cells displayed DC phenotype and also co-expressed a signature marker, CLEC9A.

We observed that while the majority of liver CD11c<sup>+</sup> cells express the tolerogenic markers, ILT3 and ILT4, a proportion of CD141<sup>+</sup>CLEC9A<sup>+</sup> DCs are negative for these markers. This would indicate either differential activation states of these cells or the presence of additional subpopulations. When we isolated CD141<sup>+</sup> DCs from healthy liver, we found that they were capable of inducing IFN- $\gamma$  and IL-17-producing T cells in MLRs, which would indicate a pro-inflammatory phenotype. This is in contrast to the many reports about liver DCs being tolerogenic, and indicates that the CD141<sup>+</sup> population could be very important in mediating hepatic pro-inflammatory responses, thus justifying the need to define DC subset-specific



responses in the liver. For example, murine studies have demonstrated that the proposed CD141<sup>+</sup> DC murine equivalent, CD8 $\alpha$ <sup>+</sup> DCs, can also activate potent T cells responses upon isolation from the liver<sup>239</sup>. CD141<sup>+</sup> DCs are superior in cross-presentation, with the dead cell receptor CLEC9A capable of binding to F-actin exposed in necrotic cells, taking up antigen and presenting via MHC I to directly activate a CD8<sup>+</sup> T cell response<sup>34, 176, 177</sup>. It is known that there is a greater proportion of CD8<sup>+</sup> T cells in the liver than in the periphery, particularly with an apoptotic phenotype<sup>269</sup>. This raises the question of what is the function of CD141<sup>+</sup> DCs in this liver and their interaction with T cells. CD8 $\alpha$ <sup>+</sup> DCs have been shown to play a role in peripheral tolerance<sup>268</sup>. Therefore human CD141<sup>+</sup> DCs could represent a key factor in the maintenance of homeostasis, via uptake of dead cell antigens, to induce tolerance in the steady state. Our demonstration that CD141<sup>+</sup> DCs can induce pro-inflammatory T cell responses would indicate that this population is also capable of switching to the induction of immunity upon pathogenic challenge. During the course of our analysis of liver perfusate, an extra population of CD11c<sup>+</sup> cells were present that did not segregate into CD1c<sup>+</sup> and CD141<sup>+</sup> subsets. Evidence from murine liver would suggest that the liver has a distinct repertoire of DCs, the majority of which are immature and poor stimulators of T cells<sup>241</sup>. Therefore it is likely that the human liver harbours additional cell types that have not yet been described. Thus the liver perfusate offers the opportunity to further explore liver immune cells, and phenotypically and functionally characterise many cell populations, with minimal manipulation.

A recent report has indicated that CD141<sup>+</sup> DCs have a very important role in HCV infection. In an *in vitro* HCV infection model, CD141<sup>+</sup> DCs co-cultured with HCV-

infected hepatocytes were capable of producing high levels of IFN- $\lambda$  and subsequently activating ISGs in the hepatocytes <sup>266</sup>. Interestingly we found that CD141<sup>+</sup> DCs were depleted in liver disease, both HCV-infected and uninfected, compared to the expansion of other DC subsets, CD1c<sup>+</sup> mDCs and pDCs. This would indicate that the localised liver DC subsets have distinct functions in liver immunity. It is possible that the CD141<sup>+</sup> DCs could have migrated to nearby lymph nodes, or they could be dying in response to chronic activation. We noted that CD141<sup>+</sup> DCs were capable of producing increased IFN- $\lambda$  in response to poly(I:C) when isolated from diseased liver, indicating that these DCs are already primed to respond to infection. This would suggest that, in the absence of infection, exposure to DAMPs present in the inflamed, fibrotic diseased liver environment drive production of IFN- $\lambda$  by CD141<sup>+</sup> DCs. Further work needs to evaluate the biological consequence of increased IFN- $\lambda$  production in disease and clarify if this is a property of CD141<sup>+</sup> DCs that is involved in promoting tolerance under specific conditions.

DCs govern the entire immune response and are known to have distinct functions. A lot of work has been carried out in mice, and it is only recently that the distinct features of human DC subsets have become apparent. Study of the different DC subsets at various locations in the body gives an insight into their distinct functional roles. It is important to study dendritic cells in terms of their microenvironment which influences the function of the cell <sup>253</sup>. The study of CD141<sup>+</sup> DCs in recent years has raised exciting potential for immunotherapy, particularly the potential use of this DC subset in vaccination. DCs have been



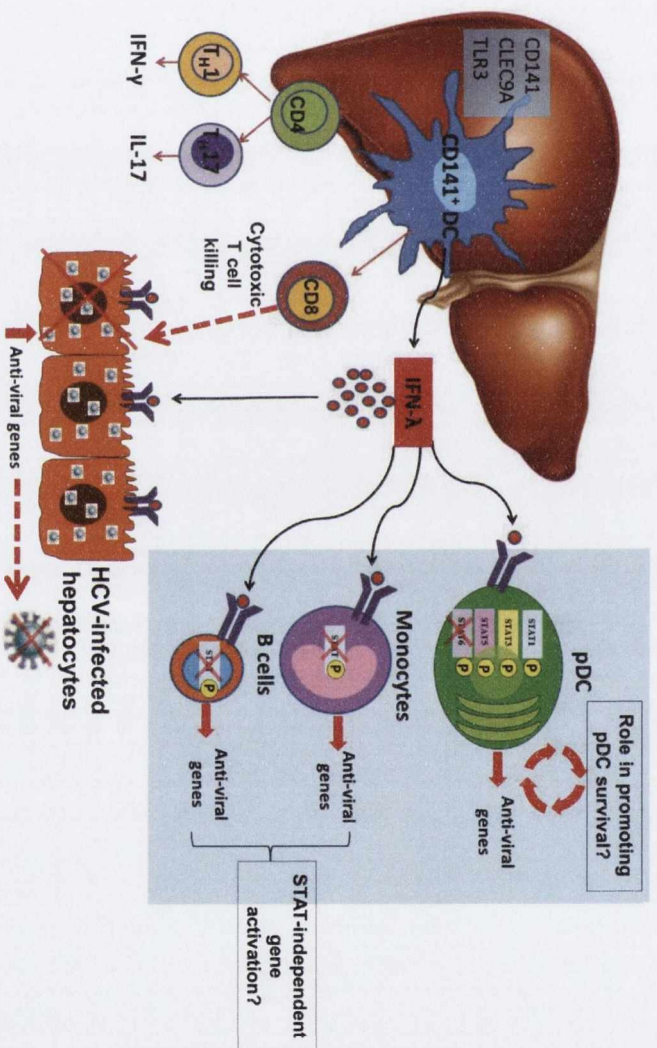
described as 'nature's adjuvants' and the use of DCs for vaccination strategies have been the subject of research for over a decade.

Current DC vaccine strategies are based on isolation of patient monocytes and culture of *in vitro* MDDCs which are exposed to tumour associated antigen and then re-infused into the patient where they can elicit antigen-specific T cell effector and memory responses. The first DC vaccine to be licensed, Sipuleucel-T (Provenge), is a prostate cancer vaccine composed of white cells partially enriched with blood DCs pulsed with a prostate cancer-associated antigen. Patients show a small but statistically significant improvement <sup>270</sup>. The marginal effects observed could be related to the fact that MDDCs are a laboratory model of DCs and while there is increasing evidence for a related population in inflammatory conditions <sup>271</sup>, their function and existence *in vivo* is still under debate. The increasing knowledge about human DCs subsets demonstrates that they have distinct functions in terms of PRR expression and cytokine secretion. Therefore, it is conceivable that targeting of specific subsets *in vivo* may be more advantageous. CD141<sup>+</sup> DCs are a prime target for vaccination strategies for both cancer and chronic viral infection <sup>272</sup>. Studies have been carried out showing antibody-mediated targeting of antigens to endocytic receptors expressed by DCs *in vivo* <sup>273</sup>, <sup>274</sup>.

To improve the therapeutic use of DC vaccination strategies it is important to understand the biology of DCs and how they regulate the innate and the adaptive immune systems, particularly in the context of the local immune microenvironment. This work has identified an important target cell for IFN- $\lambda$ ,



showing IFN- $\lambda$ -responsiveness via the JAK-STAT pathway is restricted to pDCs in the immune cell repertoire. In addition, another DC subset, CD141<sup>+</sup> DCs, has been identified in the liver which is capable of producing IFN- $\lambda$ . Therefore, we propose a model in which IFN- $\lambda$ , mostly produced by CD141<sup>+</sup> DCs, acts on other DCs, namely pDCs as well as on local epithelial tissues, including hepatocytes, to activate immunomodulatory and antiviral function respectively (**Figure 6.1**). Our finding, that a significant population of CD141<sup>+</sup> DCs in the liver is capable of inducing a pro-inflammatory response, contributes to the expanding knowledge of this subset, and widens their potential to target the hepatic microenvironment to combat hepatotropic pathogens.



**Figure 6.1: Role of CD141+ DCs and IFN-λ in the innate immune response**

Our work has shown that CD141+ DCs are enriched in the liver and are capable of producing IFN-λ in response to the synthetic dsRNA poly(I:C), as well as driving pro-inflammatory T cell responses. CD141+ DCs play an important role in cross-presenting antigen to activate CD8+ T cells. All of these factors contribute to the antiviral response to HCV in infected hepatocytes. The IFN-λ produced binds to its receptor on immune cells and activates STAT1, STAT3 and STAT5 phosphorylation, but does not phosphorylate STAT6, indicating a key difference to IFN-α. The exact functional effect of IFN-λ on pDCs requires further study but it has been suggested that it may have an anti-apoptotic effect, thus promoting pDC survival in viral infection. The IFN-λ receptor is present also on monocytes and B cells but interferon-stimulated gene (ISG) expression is evident in the absence of STAT phosphorylation upon IFN-λ treatment, thus indicating the possibility of STAT-independent mechanisms of gene induction occurring in these cells.

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# Appendix

## List of genes included in Antiviral response PCR array

Symbol	Description
AIM2	Absent in melanoma 2
APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
ATG5	ATG5 autophagy related 5 homolog ( <i>S. cerevisiae</i> )
AZI2	5-azacytidine induced 2
CARD9	Caspase recruitment domain family, member 9
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
CASP10	Caspase 10, apoptosis-related cysteine peptidase
CASP8	Caspase 8, apoptosis-related cysteine peptidase
CCL3	Chemokine (C-C motif) ligand 3
CCL5	Chemokine (C-C motif) ligand 5
CD40	CD40 molecule, TNF receptor superfamily member 5
CD80	CD80 molecule
CD86	CD86 molecule
CHUK	Conserved helix-loop-helix ubiquitous kinase
CTSB	Cathepsin B
CTSL1	Cathepsin L1
CTSS	Cathepsin S
CXCL10	Chemokine (C-X-C motif) ligand 10
CXCL11	Chemokine (C-X-C motif) ligand 11
CXCL9	Chemokine (C-X-C motif) ligand 9
CYLD	Cylindromatosis (turban tumor syndrome)
DAK	Dihydroxyacetone kinase 2 homolog ( <i>S. cerevisiae</i> )
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58
FADD	Fas (TNFRSF6)-associated via death domain
FOS	FBJ murine osteosarcoma viral oncogene homolog
HSP90AA1	Heat shock protein 90kDa alpha (cytosolic), class A member 1
IFIH1	Interferon induced with helicase C domain 1
IFNA1	Interferon, alpha 1
IFNA2	Interferon, alpha 2
IFNAR1	Interferon (alpha, beta and omega) receptor 1
IFNB1	Interferon, beta 1, fibroblast
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IL12A	Interleukin 12A
IL12B	Interleukin 12B
IL15	Interleukin 15
IL18	Interleukin 18 (interferon-gamma-inducing factor)
IL1B	Interleukin 1, beta
IL6	Interleukin 6 (interferon, beta 2)
IL8	Interleukin 8
IRAK1	Interleukin-1 receptor-associated kinase 1
IRF3	Interferon regulatory factor 3
IRF5	Interferon regulatory factor 5
IRF7	Interferon regulatory factor 7
ISG15	ISG15 ubiquitin-like modifier
JUN	Jun proto-oncogene
MAP2K1	Mitogen-activated protein kinase kinase 1

MAP2K3	Mitogen-activated protein kinase kinase 3
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
MAP3K7	Mitogen-activated protein kinase kinase kinase 7
MAPK1	Mitogen-activated protein kinase 1
MAPK14	Mitogen-activated protein kinase 14
MAPK3	Mitogen-activated protein kinase 3
MAPK8	Mitogen-activated protein kinase 8
MAVS	Mitochondrial antiviral signaling protein
MEFV	Mediterranean fever
	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78
MX1	(mouse)
MYD88	Myeloid differentiation primary response gene (88)
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor,
NFKBIA	alpha
NLRP3	NLR family, pyrin domain containing 3
NOD2	Nucleotide-binding oligomerization domain containing 2
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa
PIN1	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1
PSTPIP1	Proline-serine-threonine phosphatase interacting protein 1
PYCARD	PYD and CARD domain containing
PYDC1	PYD (pyrin domain) containing 1
RELA	V-rel reticuloendotheliosis viral oncogene homolog A (avian)
RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1
SPP1	Secreted phosphoprotein 1
STAT1	Signal transducer and activator of transcription 1, 91kDa
SUGT1	SGT1, suppressor of G2 allele of SKP1 ( <i>S. cerevisiae</i> )
TBK1	TANK-binding kinase 1
TICAM1	Toll-like receptor adaptor molecule 1
TLR3	Toll-like receptor 3
TLR7	Toll-like receptor 7
TLR8	Toll-like receptor 8
TLR9	Toll-like receptor 9
TNF	Tumor necrosis factor
TRADD	TNFRSF1A-associated via death domain
TRAF3	TNF receptor-associated factor 3
TRAF6	TNF receptor-associated factor 6
TRIM25	Tripartite motif containing 25
ACTB	Actin, beta
B2M	Beta-2-microglobulin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPRT1	Hypoxanthine phosphoribosyltransferase 1
RPLP0	Ribosomal protein, large, P0

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