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A study of immune responses during antiviral treatment for hepatitis C virus infection

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A STUDY OF IMMUNE RESPONSES DURING ANTIVIRAL TREATMENT FOR HEPATITIS C VIRUS INFECTION

by

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Dedicated

to

the memory

of

my parents and brother

Author's Declaration

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A Study of the immune responses during antiviral treatment for hepatitis C virus infection

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Abstract

Hepatitis C virus (HCV) is an RNA virus that primarily infects the liver, affecting 70 million people worldwide, according to new estimates (Polaris Observatory HCV Collaborators., 2017). Only 15-25% of persons infected with HCV will spontaneously clear the infection with the remaining developing chronic infection. Chronic hepatitis C often results in progression to liver fibrosis and ultimately cirrhosis, with risk of developing liver failure and hepatocellular carcinoma (HCC). As a consequence, HCV is the most common indication for liver transplantation in developed countries.

Chronic hepatitis C infection is characterised by a failure of HCV specific T cell responses caused by viral escape and T cell exhaustion considered to be mediated by constant antigen stimulation. Hepatitis C virus has developed strategies to escape innate immune responses leading to chronic infection in the face of ongoing innate immune activity. HCV protease NS3/4A and NS5A efficiently cleaves signalling molecules involved in pathways following activation of toll-like receptors and RIG-I by viral PAMPs to induce Interferons. HCV proteins also block signalling pathways initiated by endogenous interferon. Interferon stimulated genes induced despite these viral evasion mechanisms remain

ineffective in viral clearance. These mechanisms lead to viral persistence in chronic hepatitis C infection.

Interferon based antiviral therapies failed to recover HCV specific CD8+ T cell function suggesting that the damage to T cells may be permanent even after antigen removal.

Chronically infected patients show poor response to treatment with pegylated interferon-α and ribavirin, particularly in the presence of endogenously activated innate immune pathways which are poorly responsive to exogenous interferon-α. The standard of treatment for chronic HCV has seen an extraordinary phase of development in recent years with new direct acting antiviral drugs achieving cure rates of over 96%. However, the role of adaptive and innate immune responses in preventing relapse, sustaining viral response and the nature and extent of immune restoration that occurs with interferon free treatment regimens remains to be defined.

This thesis sets out work to analyse the impact of direct inhibition of ongoing viral replication by interferon-free therapy including direct acting antiviral agents on expression of T cell exhaustion markers and function of HCV specific T cells amongst treatment relapsers and responders. Understanding these mechanisms will determine their role in treatment outcomes and preventing viral relapse post treatment.

Expression of T cell exhaustion markers, Programmed cell death protein – 1 (PD-1) and Galectin-9, on T cells was studied using flow cytometry and demonstrated restoration of T cell phenotype following sustained viral response. Further work established a difference in PD-1 and Galectin 9 expression on CD8+ and CD4+

T cells between treatment relapsers and responders. The functional effect of these findings was further confirmed by HCV specific T cell reactivity against HCV overlapping peptides using IFN-γ ELISpot assays, demonstrating the restoration of HCV specific T cell function following successful viral clearance. There was a distinct difference in baseline ELISpot responses between treatment responders and relapsers, showing a rapid reduction in ELISpot responses at week 2 in relapsers with early augmentation in T cell reactivity in treatment responders. These findings may predict cases which are likely to relapse, thereby guiding duration of treatment.

To investigate innate immune responses, cytokine response to synthetic TLR and RIG-I ligands in a cohort of patients with sustained viral response was studied. There was an increase in the secretion of pro-inflammatory cytokines very early at treatment week 2 in the absence of synthetic TLR receptor ligands amongst treatment responders suggesting restoration of cytokine responses by use of direct acting antiviral agents, which may contribute towards viral clearance in addition to direct viral replication inhibition. This study also demonstrated at least partial restoration of innate immune responses after successful treatment of chronic HCV with interferon-free therapy shown by increase in secretion of a range of pro-inflammatory cytokines using TLR7/8 and RIG-I ligands 4 weeks after successful antiviral treatment.

Overall, studies described in this thesis demonstrate both innate and adaptive immune restoration with treatment regimens not including interferon alpha and distinct early differences in treatment relapsers and responders, which may be predictive of viral clearance.

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Abbreviations

7-AAD	7 - Aminoactinomycin
ALT	Alanine aminotransferase
AP-1	Activated protein 1
Apo-E	Apolipoprotein E
BDCA	Blood dendritic cell antigen
CLDN1	Claudin 1
CTLA-4	Cytotoxic T-lymphocyte associated antigen
DAA	Direct acting antivirals
DC	Dendritic cell
DCIR	DC immunoreceptor
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E1	Envelope protein 1
E2	Envelope protein 2
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EIA	Enzyme immunoassay

ELISpot	Enzyme linked ImmunoSpot analysis
EMCV	Encephalomyelitis virus
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FasL	Fas ligand
FCS	Foetal calf serum
FMO	Fluorescence minus one
FWB	FACS wash buffer
GAS	Gamma-activated sequence
Gp130	Glycoprotein 130
Gp80	Glycoprotein 80
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCV cc	Hepatitis C virus cell culture
HCVpp	HCV pseudoparticles
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
IDU	Injection drug use

IFN	Interferon
IL	Interleukin
IRAK	Interleukin-1 receptor associated kinase
IRES	Internal ribosomal entry site
IRF 9	Interferon response factor 9
IRF-3	Interferon regulatory factor - 3
ISG	Interferon stimulated genes
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response elements
JAK 1	Janus kinase 1
KIR	Killer immunoglobulin like receptor
LDL	Low density lipoproteins
MAM	Mitochondrial associated membrane
MAVS	Mitochondrial antiviral signalling protein
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
MoDC	Monocyte derived dendritic cell
MyD88	Myeloid differentiation primary response 88
NANBH	Non-A Non-B Hepatitis

NK cells	Natural killer cells
NK-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKT cell	Natural killer T cell
NNPI	Non-nucleoside polymerase inhibitor
NPC1L1	Niemann-Pick C1-Like1
NPI	Nucleoside polymerase inhibitor
NS2	Non-structural protein 2
NS3	Non-structural protein 3
NS4A	Non-structural protein 4A
NS4B	Non-structural protein 4B
NS5A	Non-structural protein 5A
NS5B	Non-structural protein 5B
OCLN	Occludin
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

pDC	Plasmacytoid dendritic cell
Peg IFN	Pegylated interferon
PHA	Phytohaemagglutinin
PI	Protease inhibitor
Poly (I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptors
RIBA	Recombinant immunoblot assay
RIG-I	Retinoic acid inducible gene I
RNA	Ribonucleic acid
RPMI	Royal Park Memorial Institute
RT PCR	Reverse transcription PCR
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
SRB1	Scavenger receptor B1
STAT	Signal transducer and activator of transcription
SVR	Sustained viral response
SVR	Sustained viral response
T reg	Regulatory T cells
TBK1	TANK binding kinase 1

TCR	T cell receptor
TfR1	Transferrin receptor protein 1
Th1	T helper 1
Th2	T helper 2
TIM-3	T cell immunoglobulin and mucin domain containing 3
TLR	Toll like receptors
TRAIL	TNF related apoptosis inducing ligand
TRIF	TIR – domain – containing adapter-inducing interferon-β
TYK 2	Tyrosine kinase 2
USP 18	Ubiquitin specific peptidase 18
UTR	Untranslated region
VLDL	very low density lipoproteins
WHO	World Health Organisation

Chapter 1

Introduction

1.1 Introduction

Hepatitis C virus (HCV) is an RNA virus that primarily infects the liver, affecting 70 million people worldwide, according to new estimates (Polaris Observatory HCV Collaborators., 2017). Only 15-25% of persons infected with HCV will spontaneously clear the infection with the remaining developing chronic infection. Chronic hepatitis C infection often results in progression to liver fibrosis and ultimately cirrhosis, with risk of developing liver failure and hepatocellular carcinoma (HCC). As a consequence HCV is the most common indication for liver transplantation in developed countries.

The standard of treatment for chronic HCV infection has undergone an extraordinarily rapid phase of development in recent years with the discovery of new direct acting antiviral drugs achieving cure rates of over 96%. Despite the development of highly effective direct acting antiviral (DAA) drugs, such treatment may not be easily accessible for most patients in resource limited regions. There is no available vaccine for hepatitis C, hence the main strategies of prevention of HCV focus on reduction of exposure. Thus, ongoing research in understanding immunological mechanisms predicting both natural and treatment induced clearance of hepatitis C virus remains important.

This chapter will outline virological and clinical features of hepatitis C virus infection, followed by a description of current understanding of immunological factors that determine the outcome of HCV infection and the concept of immunological changes during HCV treatment.

1.2 Discovery of hepatitis C virus

The hepatitis C virus is a hepatotropic RNA virus which was first discovered in 1989. Prior to this, the majority of transfusion related cases of hepatitis were termed as Non-A Non-B hepatitis (NANBH). Early attempts to identify and characterise this infectious particle used complementary DNA clones constructed from RNA extracted from chimpanzees infected with NANBH. Despite many initial failures using this technique, a clone was eventually isolated which reacted with specific antibodies in the serum of patients with NANBH (Choo et al., 1989). HCV specific antibodies were later identified in the serum of individuals with NANBH (Alter et al., 1989, Miyamura et al., 1990) confirming it to be the cause of a majority of the cases with transfusion related hepatitis. HCV was classified as a flavivirus due to distant sequence similarities with other viruses in the Flaviviridae family of virus. It was later classed as the only virus in the Flaviviridae family under the genus Hepacivirus (Choo et al., 1991).

1.3 Virology

1.3.1 HCV genome

The HCV genome is a single stranded RNA molecule composed of a 9kb open reading frame (ORF) which is translated into a 3011 amino acid long polyprotein. The open reading frame is flanked by 5' and 3' untranslated regions (UTR) of 341 and 230 nucleotides length respectively. Both 5' and 3' are highly conserved regions essential for polyprotein translation and genome replication. The 5' UTR contains an internal ribosomal entry site that binds the 40S ribosomal subunit and initiates polyprotein translation. The polyprotein precursor is co-translationally and post-translationally processed by both cellular and viral proteases at the level of endoplasmic reticulum membrane to yield 10 mature viral proteins. These are divided into structural and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Lindenbach and Rice, 2013). The structural proteins include core, which forms viral nucleocapsid, and the envelope viral glycoproteins E1 and E2. They are released by host cell endopeptidases. The single strand of positive sense RNA is contained within the nucleocapsid. Figure 1.1 demonstrates HCV genome, corresponding viral proteins and their function.

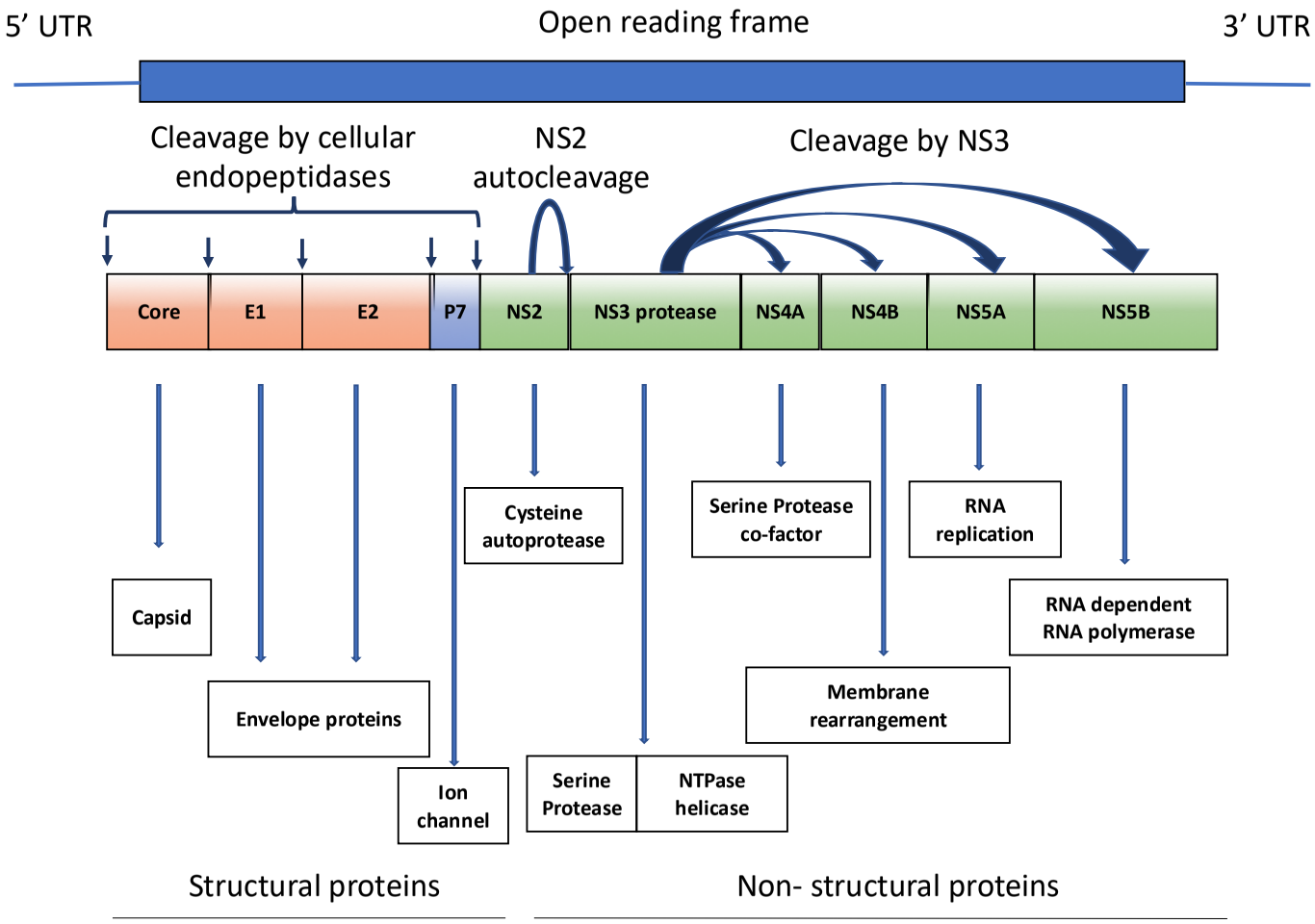

Figure 1. 1 HCV genome and corresponding viral proteins

HCV proteins encoded by open reading frame of HCV genome and their functions are shown

1.3.2 HCV cell entry and life cycle

HCV has selective cellular tropism. In humans, it principally gains entry into hepatocytes which provide an effective site for continued viral replication. Many have also reported extrahepatic sites of viral entry and replication, particularly peripheral blood mononuclear cells, however this remains controversial.

Advances in developing in-vitro HCV cell culture systems allowed greater understanding of HCV cell entry mechanisms and intracellular viral lifecycle. Initial studies used HCV like particles called HCV pseudoparticles (HCVpp). These HCVpp were composed of HCV glycoproteins (E1 or E2) attached to a lenti- or retroviral core particle. As the E1/E2 proteins were structurally intact in these HCVpp, they closely mimicked the attachment and entry of HCV into the cell, allowing to study steps of cell surface binding and viral entry (Bartosch et al., 2003b).

HCV particles are 50-80nm which can be found in various forms in the serum of infected host, including (i) free virus; (ii) virions bound to very low density lipoproteins (VLDL) or low density lipoproteins (LDL) which appear to represent the infectious fraction; and (iii) virions bound to immunoglobulins (Thomssen et al., 1993, Andre et al., 2002). This interaction of HCV with lipoproteins could contribute to the shielding of HCV glycoproteins from host immune response and explain poor detection and availability of HCV glycoproteins at the virion surface. More importantly, they play a role in HCV cell entry.

Initial attachment of HCV particles onto hepatocytes is mediated by the heparan sulfate proteoglycan syndecan-1 or syndecan-4 (Shi et al., 2013, Lefevre et al., 2014) or by the scavenger receptor B1 (SRB1) (Dao Thi et al., 2012). It was

initially thought that HCV glycoproteins are responsible for virion binding to heparan sulfate proteoglycans or SRB1, however more recent data suggests that apolipoprotein E (Apo-E) rather than HCV glycoproteins themselves could be involved in the initial contact (Jiang et al., 2013). After the initial attachment to cell surface, cell entry is facilitated by the co-ordinated action of cellular factors. These include SRB1, Tetraspanin, CD81, tight junction proteins Claudin-1 (CLDN1) and Occludin (OCLN), and epidermal growth factor receptor (EGFR). CD81 and OCLN determine the tropism of HCV for human cells (Pileri et al., 1998, Scarselli et al., 2002, Evans et al., 2007, Ploss et al., 2009). Sainz et al also identified cholesterol transporter Niemann-Pick C1-Like 1 (NPC1L1) as an additional entry factor (Sainz et al., 2012). In addition, Transferrin receptor 1 (TfR1) has also been reported to be involved in cell entry (Martin and Uprichard, 2013). However, precise role of these additional factors remains to be determined.

On cell binding the virus forms an endosome composed of host cell lipid membrane. This endosome is then discharged into the cytoplasm in a pH dependent manner. Following this, the internal ribosomal entry site (IRES) on the 5'UTR of the RNA virus genome enables translation of the HCV genome into a single polyprotein which is subsequently cleaved by the host cell endopeptidases and viral proteases into the non-structural and structural proteins of the virus. The later stages of replication continue in the cytoplasm in a complex termed as the "membranous web", forming a scaffolding framework of viral replication, and is made up of host cell proteins and viral components. Viral proteins are also thought to play a role in the formation of this complex as discussed in section 1.3.3. The virus and its constituent proteins are then assembled in the

membranous web, which is associated with the endoplasmic reticulum. The new virus particles are released by budding at the cell membrane. Virus turnover is very rapid and reported to be at the rate of 10^{10} to 10^{12} virions produced per day (Hoofnagle, 2002). Figure 1.2 summarises HCV entry and lifecycle.

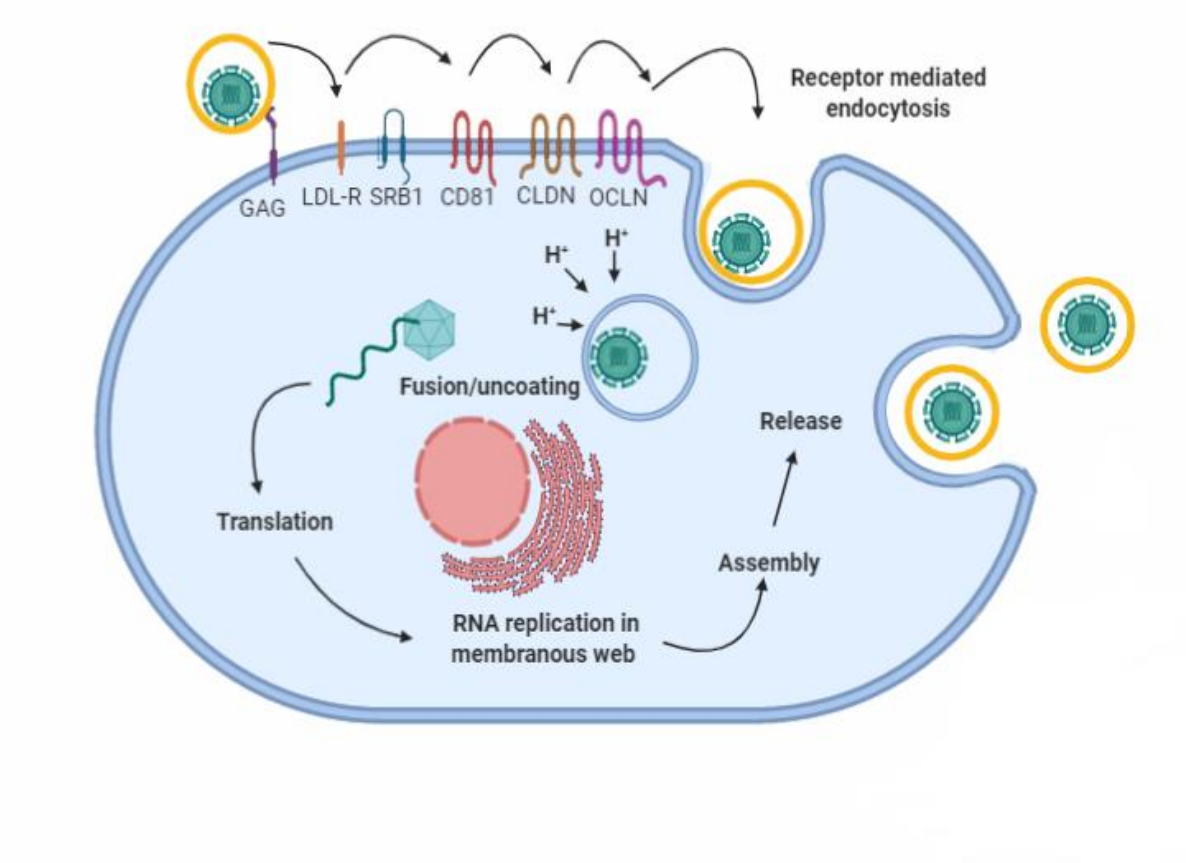

Figure 1. 2 HCV cell entry and life cycle

HCV lipoviralparticle enters the cell by engagement of attachment and entry factors followed by Clathrin mediated endocytosis. Subsequent to internalization there is fusion and pH mediated release of viral genome in cytoplasm. Engagement with Ribosomes allows translation of viral polyprotein. RNA replication and assembly continues in membranous web associated with endoplasmic reticulum. Intact infectious viral particles are then released from the cell as lipoviralparticles.

1.3.3 Role of HCV proteins

HCV non-structural proteins are involved in polyprotein processing and viral replication.

NS3 is an important non-structural viral protein. The N-terminal third is occupied by a serine protease essential for the cleavage of the non-structural region of the polyprotein. The C-terminal segment forms the NTPase/RNA helicase thought to be required for organisation of replicated RNA by unwinding of single and double stranded RNA (Brass et al., 2006). NS4A is a co-factor for the NS3 serine protease in releasing non-structural proteins. NS4B is an integral membrane protein, thought to play a role in the formation of membranous web necessary for the later stages of viral replication and virion assembly (Egger et al., 2002). NS5A is poly-phosphorylated protein also involved in viral replication.

NS5B is an RNA-dependent RNA polymerase which is important for HCV replication via synthesis of complementary plus RNA strand from complementary minus RNA strand using the genome as template.

Variability in subgenomic regions such as E1, NS4 and NS5 is responsible for HCV heterogeneity with 7 distinct major subtype genotypes with genetic variability of 30-50% and more than 100 further sub-types (Simmonds, 1995, Smith et al., 2014).

P7 has been shown to create selective ion channels in the host cell membrane, but exact role of p7 and that of NS2 in HCV viral replication remains unclear (Pavlovic et al., 2003, Steinmann and Pietschmann, 2010).

The structural proteins include the core, which forms viral nucleocapsid, the envelope glycoproteins E1 and E2. They are released following cleavage by host cell endopeptidases. The two envelope glycoproteins E1 and E2 are thought to play pivotal roles at different steps of HCV replicative cycle (Bartosch et al., 2003a). There is strong evidence that they are essential for host cell entry. Hypervariable regions have been identified in the E2 envelope glycoprotein sequence. The amino acid stretches differ by up to 80% among HCV genotypes, and even among subtypes of the same genotype (Weiner et al., 1991, Kato, 2001).

Function of HCV proteins encoded by HCV genome is also denoted in Figure 1.1.

1.4 HCV transmission

HCV is a blood borne virus and is transmitted mainly by the parenteral route with blood transfusion and injection drug use (IDU) being the highest risk modes of transmission. Since screening of blood donors for HCV became widespread between 1991 and 1992, IDU has been the main route of transmission of new infections in developed countries (Alter et al., 1990).

In healthcare settings, occupational needle stick injuries, use and re-use of non-sterile needles, syringes and haemodialysis can be a route of transmission of the virus. A striking example of this is in Egypt, where intravenous tartar was used as part of national treatment programme between 1950 and 1980 in an attempt to eradicate Schistosomiasis. As disposable needles had not come into common

use at the time, a very large number of population were inoculated with HCV (Frank et al., 2000).

Sexual transmission of HCV is rare, particularly in those in a monogamous relationship (0-0.6% per year), but the risk is increased in those with high risk sexual behaviour (0.4-1.8% per year), such as men who have sex with men or those with multiple sexual partners (Wyld et al., 1997).

Vertical transmission from mother to child in uncommon. However, co-infection with Human immunodeficiency virus (HIV) appears to increase the risk of both vertical and sexual transmission. Finally, non-sexual household contact transmission has also been reported. Overall, previous reports have also demonstrated that route of transmission cannot be identified in as many as 20% of the cases (MacDonald et al., 1996).

1.5 Epidemiology

Chronic hepatitis C virus infection is a major cause of cirrhosis and hepatocellular carcinoma (HCC) globally. Previous estimates reported 2.8% (range of estimates 2.6% - 3.1%) of the world population, more than 184 million persons as being infected with HCV (2002). Worldwide prevalence of HCV infection varies widely, and precise global estimates are limited due to undiagnosed disease and lack of appropriate data. More recent estimates HCV to be affecting 1% of the world population, corresponding to a lower number of 71 million active cases (2017). 2.3 million people worldwide are co-infected with HIV and HCV. There are major regional disparities in the prevalence of HCV. Globally, Egypt has the highest

estimated prevalence of 30%, mainly nosocomial because of treatment administration during eradication of Schistosomiasis (Thursz and Fontanet, 2014). Prevalence is also high in India, Pakistan, China and Indonesia but markedly lower in Japan, Northern and Western Europe, North America and Australia (1999).

The overall mortality attributable to viral hepatitis in 2015 is approximately 720,000 deaths from cirrhosis and 470,000 deaths from hepatocellular carcinoma (HCC), with an increase of 22% since 2000.

1.6 Natural history of HCV infection

Following HCV infection, active virus replication is evident by virus RNA being detectable in the serum as early as 1-2 weeks after infection (Hoofnagle, 2002). This is followed by a rise in liver enzymes within 1-3 weeks, predominantly Alanine aminotransferase (ALT), signalling acute hepatic inflammation and necrosis. This stage is most often asymptomatic, although about a third of cases develop an acute viral illness composed of constitutional symptoms such as nausea, malaise, anorexia and fatigue. Only a minority of these cases will have a significant enough hyperbilirubinaemia to present with jaundice (Thimme et al., 2001). Emergence of HCV antibody responses occur at or shortly after the onset of symptoms. Evolution from acute to chronic hepatitis is asymptomatic in most and occurs up to 85% of cases (Seeff, 2002). The chronic course is characterised by fluctuating levels of viral RNA and ALT with chronic infection being defined as persistence of viral RNA in serum for 6 months or more. In only a small proportion, the initial immune response to the virus results in spontaneous clearance (15-

25%), which is marked by undetectable virus in serum measured by polymerase chain reaction (PCR). The majority of those who clear the virus have persistent positive HCV antibody after clearing the infection, although antibody titres can wane with time (Takaki et al., 2000).

Once chronic infection is established the progression to liver fibrosis and ultimately cirrhosis occurs over a variable time scale. In most, it occurs over a period of decades with an estimated incidence of 5-20% after 20 years of infection (Seeff, 2002). A number of factors can accelerate the progression to cirrhosis including age at onset of greater than 40 years, male gender, daily consumption of alcohol of 30g or more per day, co-infection with HIV and/or hepatitis B virus, insulin resistance, presence of non-alcoholic steatohepatitis and Haemochromatosis. HCV cirrhosis is a major risk factor for development of hepatocellular carcinoma (HCC). It has an annual incidence of 1-4% in those with established HCV cirrhosis (Fattovich et al., 1997, Chiaramonte et al., 1999).

1.7 Testing for HCV infection

The confirmation of established infection with HCV requires demonstration of antibody to HCV and evidence of viral RNA in plasma or serum. The first serological tests to detect HCV antibody is a major advancement, drastically reducing the incidence of transfusion related transmission. The first generation enzyme immunoassay (EIA-1) utilized antibody reactivity to recombinant NS4 antigen termed as c100-3. The specificity of EIA-1 was low and high false positive rates were a major concern, being reported to be as high as 70% in low risk populations. Thus positive tests often required a supplementary confirmatory test,

the recombinant immunoblot assay (RIBA). RIBA uses an immunoblot platform based on four recombinant HCV antigens. A positive RIBA test was present when reactivity to two or more antigens was demonstrated. In a low prevalence setting, RIBA was helpful in detecting false positives. Later development of the 3rd generation EIA made the need for RIBA testing obsolete, due to its high sensitivity of 97-99% and specificity approaching 99% (Colin et al., 2001). EIA-3 detects antibody reactivity to structural and non-structural viral proteins including core, NS3, NS4 and NS5.

Molecular assays have been the mainstay in detecting circulating viral RNA, principally using reverse transcription (RT) PCR techniques. HCV RNA assays can be divided into those that detect absence or presence of viraemia (qualitative) and those that detect quantity of viral nucleic acid (quantitative) termed viral load. Quantitative assays are useful for monitoring during treatment. There has been rapid development in HCV RNA assays with both quantitative and qualitative tests becoming increasingly sensitive with similar levels of detection. Prior to 1999, viral titres were reported as number of viral copies in 1ml of serum (copies/ml). In order to standardise all available assays, the WHO collaborative group developed an international standard allowing viral load to be expressed in IU/ml providing comparison across all assays (Saldanha et al., 1999). The conversion factor for IU/ml to copies/ml varied between the assays used. There are a number of commercially available highly sensitive and reliable assays that are able to quantify HCV RNA at a level as low as 10 IU/ml (Cobas v2.0, Roche Molecular Systems).

1.8 Treatment of HCV

Interferon-α was used to treat patients with chronic HCV infection, then known as non-A non-B hepatitis, years prior to the identification of the virus itself (Hoofnagle et al., 1986). Treatment outcomes improved significantly after the nucleoside analog Ribavirin was added to prevent relapse (Reichard et al., 1998), and later after IFN-α's pharmacokinetics were optimised by pegylation (Pegylated IFN-α) (Zeuzem et al., 2000).

Until 2011, recombinant pegylated-interferon-α and Ribavirin were the standard of treatment for HCV infection. Successful treatment outcome is defined as undetectable HCV RNA 6 months following completion of treatment and is termed as sustained virological response (SVR). This combination resulted in an SVR rate of 40-45% in genotype 1 patients with 48 weeks of treatment and 75% with 24 weeks of treatment in genotypes 2 and 3, and was frequently associated with intolerable side effects.

The treatment of HCV has revolutionised in recent years with development of a number of effective oral antiviral treatment regimens directly inhibiting non-structural viral proteins involved in viral replication, targeting NS3/4A, NS5A and NS5B, thereby disrupting viral replication. These treatments are categorised as direct acting antiviral agents (DAA) and result in SVR rates of over 95% just after 8 - 12 weeks of treatment.

There are four classes of currently available DAAs, defined by their mechanism of action and therapeutic target. These include NS3/4A protease inhibitors (PIs),

NS5A inhibitors, NS5B nucleoside polymerase inhibitors (NPIs), NS5B non-nucleoside polymerase inhibitors (NNPIs).

NS3/4A protease inhibitors are inhibitors of the NS3/4A serine protease, an enzyme involved in post-translational processing and replication of HCV. Protease inhibitors disrupt HCV by blocking NS3 catalytic site or NS3/4A interaction.

The first generation of DAAs, NS3/4A protease inhibitors were co-administered with pegylated interferon and Ribavirin, thereby adding to the side effect burden. However, subsequent DAAs, used in combination can be administered without pegylated interferon, are more efficacious with shorter duration of treatment and limited side effect profile.

Second generation protease inhibitors currently available include Simeprevir for genotype 1 infection, pangenotypic Glecaprevir available in combination with Pibrentasvir (NS5A inhibitor), pangenotypic Grazoprevir available in combination with NS5A inhibitor Elbasvir, while pangenotypic Voxilaprevir is available in combination with NS5A inhibitor Velpatasvir and NS5B inhibitor Sofosbuvir.

NS5A inhibitors are potent drugs with low barrier to viral resistance and associated with high SVR rates when used in combination with other direct acting anti-viral agents. Currently available drugs in this class include Daclatasvir, Elbasvir, Ledipasvir, Ombitasvir, Pibrentasvir and Velpatasvir.

NS5B, as discussed previously, is an RNA dependent RNA polymerase which is highly conserved across all HCV genotypes and essential for viral replication. Thus, giving NS5B inhibitors efficacy against all HCV genotypes. The enzyme

has a catalytic site for nucleoside binding and four other sites where non-nucleoside compounds can bind and cause allosteric alteration. Hence NS5B inhibitors are divided into Nucleoside polymerase inhibitors, which includes Sofosbuvir and non-nucleoside inhibitors, which has only been studied as an adjunct to other more potent compounds and includes Dasabuvir used with combination of Ombitasvir-Paritaprevir-Ritonavir.

Currently available direct acting antiviral agents, fixed dose combination regimens available and their therapeutic target proteins are outlined in Table 1.1

Table 1. 1 Direct acting antiviral agents and their mechanism of action

Drug Class /Therapeutic target		Names
NS3 Protease Inhibitors		Glecaprevir Grazoprevir Paritaprevir Simeprevir Voxilaprevir
NS5A Inhibitors		Daclatasvir Elbasvir Ledipasvir Ombitasvir Pribentasvir Velpatasvir
NS5B Inhibitors	Nucleoside polymerase inhibitors	Sofosbuvir
	Non-nucleoside polymerase inhibitors	Dasabuvirr
Fixed dose combinations available		Elbasvir-Grazoprevir Glecaprevir-Pibrentasvir Ledipasvir-Sofosbuvir Ombitasvir-Paritaprevir-Ritonavir Sofosbuvir-Velpatasvir Sofosbuvir-Velpatasvir-Voxilaprevir

1.9 Models for the study of HCV infection

1.9.1 In-vivo models

Since its discovery, the study of HCV has always been in need of robust small animal model for the study of HCV cell entry, replication and cell virus interactions. The only established animal model for hepatitis C infection is Chimpanzee (Pan Troglodytes) (Houghton, 2009). They can be infected with isolates of the 6 epidemiologically important hepatitis C virus genotypes and have innate and adaptive immune responses similar to those observed in infected humans (Bukh, 2004). Conservation issues, high expenses and limited availability are the limitations to study HCV in these animals. Hence, National Institute of Health (NIH) has stopped all research involving Chimpanzees.

A wild smaller mammal related to primates, the tree shrew (Tupai b. chinensis) has been shown to be infected by HCV although viraemia is transient and clinical illness is difficult to establish without immunosuppression (Xie et al., 1998). Attempts have also been made to study HCV in immunodeficient mice carrying engrafted human hepatic cells. These models have numerous limitations and are difficult to translate to human infection (Bukh et al., 2001).

1.9.2 In vitro models

Early in-vitro models used primary cells from humans and chimpanzees to study the establishment of HCV infection. However, these models were limited as infection was frequently transient (Castet et al., 2002). The challenge was to develop a stable model to support replication as well as production and release

of intact virus. The development of replicon has been a great breakthrough. Lohmann et al developed subgenomic clones of HCV genotype 1b containing only non-structural portion of the genome and combined it with a heterologous encephalomyelitis virus (EMCV) internal ribosomal entry site sequence (IRES) to facilitate the translation of non-structural proteins in-vitro (Lohmann et al., 1999).

Upstream of the ECMV IRES, a coding sequence for the neomycin phosphotransferase gene was used for later selection of replicating clones. Intracellular replication of viral RNA was demonstrated when this subgenomic replicon construct was transfected into the human hepatoma cell line, Huh 7. Although these systems provide autonomous replication which could be sustained beyond a year, it became clear that effective replication of clones reduced with time. This problem was solved by adaptive mutations in the non-structural region of the genotype 1b clone which greatly increased replication levels (Lohmann et al., 2001, Blight et al., 2000).

Wakita developed a genotype 2a full length replicon (JFH-1) isolated from a Japanese patient with fulminant hepatitis. This full length HCV genome replicates efficiently producing virus particles in Huh-7 (Wakita et al., 2005). Further developments have led to Huh 7 derived cell line Huh 7.5.1 which is highly permissible to JFH-1 virus infection and releases higher viral titres of $10^4 – 10^5$ infectious units per ml of culture supernatant. This led to the development of infectious hepatitis C virus cell culture system (HCVcc). This is a cell culture infection system based on JFH-1 clone and Huh 7 derived cell lines that allows production of the virus which can be efficiently propagated in tissue culture (Zhong et al., 2005).

HCV pseudo particles (HCV pp) allowed study of early stages of viral life cycle, which were produced by transfecting three vectors encoding retroviral Gag and Pol proteins, reporter protein luciferase, and HCV glycoproteins E1 and E2 in human embryo kidney cells (293T). 293T cells then secrete viral pseudo particles which can be used to infect Huh-7 cells. Infectivity is evaluated by quantification of the amount of luciferase expressed in Huh-7 cells (Bartosch et al., 2003a).

1.10 Outcome of HCV infection – Non-immune factors

A number of non-immune factors have been recognized, mainly from epidemiological studies that may influence the natural clearance of HCV infection. This includes a number of host and viral factors described further in this section.

The most influential immunogenetic host factor linked to viral clearance is single nucleotide polymorphism (SNP) close to locus for IL28B gene. This is discussed in detail in section 1.12.3. In addition, two key factors that determine viral clearance are female gender and symptomatic acute infection (Micallef et al., 2006). Several studies have demonstrated that clearance is more likely in those with acute jaundice and other symptoms (Villano et al., 1999, Santantonio et al., 2003). Earlier reports have suggested that this could be due to stronger immune responses to the virus seen in these symptomatic individuals, however these responses can be similarly demonstrated in asymptomatic individuals. Numerous studies identify female gender as an independent predictor of viral clearance (Alric et al., 2000, Micallef et al., 2006, Grebely et al., 2006).

Additional predictor was found to be ethnicity, Caucasians are more likely to clear the virus compared to Afro-Caribbeans (Villano et al., 1999, Piasecki et al., 2004). The advantageous IL28B allele is also more frequently found in mixed European descent and Asian populations relative to the African-Americans. Hence, some of the race related differences in response to therapy with pegylated interferon and ribavirin can be explained by these population differences.

Co-infection with HIV also favours HCV chronicity, likely to be related to immunosuppression due to reduced CD4+ T cell counts (Grebely et al., 2014, Thomas et al., 2000). Conversely, co-infection with HBV increases the likelihood of spontaneous clearance of HCV particularly in acute setting (Piasecki et al., 2004).

A 5 year prospective follow up study where more than half of the individuals had symptomatic infection demonstrated that acquisition of infection at an older age was predictive of resolution of infection (Santantonio et al., 2003). This was not replicated in other studies (Thomas et al., 2000, Rolfe et al., 2011).

1.11 Outcome of HCV infection - Immune factors

Immune responses are vital in achieving viral clearance. However, chronic HCV infection usually results from the inability of the host to mount an effective immune response and due to the propensity of the hepatitis C virus to escape or subvert immune responses. The following sections will discuss innate and adaptive immunity which may influence HCV outcome spontaneously and in context of antiviral treatment.

1.12 Innate immunity

Innate immune responses are the first line of defence against viral infections. Interferons are the central cytokines responsible for the induction of antiviral state in cells and for activation and regulation of cellular components of innate immunity such as natural killer (NK) cells (Stetson and Medzhitov, 2006).

1.12.1 HCV recognition by PRR and signalling pathways

HCV is sensed as non-self by specific innate immune receptors called pathogen recognition receptors (PRRs) which bind to conserved motifs within viral particles called pathogen associated molecular patterns (PAMPs) leading to coordinated activation of innate immune response. Recognition of PAMPs by PRR activates the antiviral immune mechanisms and enables early viral clearance as well as the priming of adaptive responses. A number of PRRs sense viruses as foreign invaders leading to activation of innate immune signalling pathways. Following internalization of viral antigens by antigen presenting cells or infected hepatocytes, viral RNA is recognised by PRRs. These include Toll like receptors (TLR3 and TLR7/8 for RNA) and cytosolic receptor RNA helicase Retinoic acid inducible gene-I (RIG-I). HCV core and non-structural proteins are important PAMPs for these pattern recognition receptors. A single point mutation in RIG-I and a lack of TLR3 expression in the human hepatocellular carcinoma derived cell line Huh-7.5 and its derivatives contribute to a 50-fold greater permissiveness for HCV replication (Bartenschlager and Pietschmann, 2005, Li et al., 2005).

HCV is recognised by RIG-I within hours of infection and activates downstream signalling(Loo et al., 2006). HCV recognition by RIG-I occurs upon its binding with the exposed 3'UTR of HCV RNA rich in polyuridine motifs poly U/UC ribonucleotides (Uzri and Gehrke, 2009). HCV binding with RIG-I induces a conformational change that promotes its translocation from cytosol into intracellular membranes facilitating the interaction of RIG-I with Mitochondrial antiviral signalling protein (MAVS) (Gack et al., 2007). The RIG-I/MAVS interaction promotes the formation of a MAVS signalosome that propagates activation of downstream effector molecules including interferon regulatory factor 3 (IRF-3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Loo and Gale, 2011), which in turn induce the production of type I and type III IFNs via IRF-3, and pro-inflammatory cytokines and chemokines via NF-κB and AP-1. The endoplasmic reticulum (ER) contains a specialised domain, the mitochondrial associated membrane (MAM) which physically links ER to mitochondria and has been implicated in inflammasome signalling (Horner et al., 2011).

TLR3 is an endosomal sensor of dsRNA expressed on peripheral immune cells including myeloid dendritic cells (mDCs) and natural killer (NK) cells. TLR3 is also expressed on cells within the liver, including hepatocytes and the liver resident macrophages called Kupffer cells. TLR3 signals are transmitted through the adaptor protein Toll/interleukin-1 receptor (TIR) domain containing adaptor protein including interferon beta (TRIF), which activates IRF3 and NF-κB leading to the production of type I interferons, proinflammatory cytokines and chemokines (Takeuchi and Akira, 2009). While synthetic dsRNA ligands of TLR3 can induce

IRF3 dependent signalling in cells within 24 hours, HCV infection triggers this response 3-4 days after the infection (Wang et al., 2009).

TLR7 and 8 recognise viral ssRNA. These are expressed predominantly on pDCs and mDCs and also on NK cells and Hepatocytes. HCV RNA leads to the activation of TLR7 and TLR8 through HCV RNA polyuridine tail (Zhang et al., 2009, Zhang et al., 2005, Decalf et al., 2007, Meier et al., 2007, Judge et al., 2005, Hornung et al., 2005). TLR7 and 8 use myeloid differentiation factor 88 (MyD88) as main adaptor protein to induce activation of IRF7 and NF-κB leading to production of IFN-α (Zhang et al., 2009). There is also increased expression TLR7 and 8 on monocytes in HCV infection, although the significance of this remains unclear (Sato et al., 2007).

1.12.2 Role of interferons

Interferons are a family of cytokines grouped in three classes: type I, II and III. Type I interferons include IFN-α, IFN-β, IFN-ε, IFN-κ. Type I interferons are produced by most mammalian cells in response to viral and bacterial infections, with increased levels found in liver in both acute and chronic HCV (Bigger et al., 2001, Mihm et al., 2004, Su et al., 2002). Type II IFN, IFN-γ is produced by NK and natural killer T (NKT) cells as part of the innate immune response, and by activated antigen specific T cells (both CD4+ Th1 and CD8+ cytotoxic T cells) in response to viral infections. Type III IFN include IFN-λ1 (IL-29), IFN-λ2 (IL28A) and IFN-λ3 (IL28B), which are also produced in viral infections by cells of innate immune system, amongst which IFL-λ3 is most potent in inhibiting JFH-1

replication (Thomas et al., 2012, Marcello et al., 2006). All types of interferons induce an antiviral state by transcriptional activation of hundreds of interferon stimulated genes (ISGs). Macrophages and dendritic cells (DCs) do not have to be infected by viruses in order to produce interferons. Instead, they constantly sample material from outside, including intact viral particles as well virus containing remnants of apoptotic cells. Degradation processes in the endosomes then exposes viral nucleic acid to recognition by Toll like receptors. Type I and II interferons are essential for the defence against viruses. Knockout mice that lack the receptors for IFN-α or IFN-γ or components of IFN signalling pathways succumb to otherwise harmless viruses (Muller et al., 1994, Durbin et al., 1996).

Type I interferons bind with heterodimeric transmembrane receptors IFNA-R1/IFNA-R2 leading to activation of receptor associated protein tyrosine kinases, Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which phosphorylate transcription factors known as signal transducers and activators of transcription (STATs). IFN-γ binds to widely expressed IFN-γ receptor. IFN-λ binds to a receptor consisting of the ubiquitously expressed IL-10R2 chain shared with Interleukin 10 (IL-10) receptor and a unique IFN-λ receptor 1 chain whose expression is mainly restricted to epithelial cells (Kotenko et al., 2003, Donnelly et al., 2004). IFN-λ expression has been shown to be very low in control liver biopsy samples but significantly increased in the setting of chronic viral infections (Duong et al., 2014). All IFNs activate STAT1 to form homodimers that translocate into the nucleus and bind to the gamma-activated sequence (GAS) elements in interferon stimulated genes (ISGs). Type I and II IFNs additionally activate STAT 1 and STAT 2 triggering their stable association with interferon response factor 9 (IRF-9). The resulting IFN-stimulated gene factor 3 (ISGF3)

transcription factor complex localises to the nucleus and binds to Interferon stimulated response elements (ISRES) leading to transcription of Interferon stimulated genes (ISGs). The ISG encoded proteins inhibit viral transcription, translation and replication through a variety of mechanisms (Levy and Darnell, 2002, Schoggins et al., 2011, Stark and Darnell, 2012, MacMicking, 2012). These also promote further release of IFN-α and IFN-β in an autocrine fashion.

The subtype of Interferon driving permanent expression of ISGs in chronic hepatitis C is not entirely clear. IFN-γ can be excluded because the set of ISGs induced in chronic HCV are not typical type II interferon induced genes (Sarasin-Filipowicz et al., 2008, Dill et al., 2012, Bigger et al., 2004). IFN-α can be cautiously excluded because IFN-α signalling is subject to negative feedback inhibition by Ubiquitin specific peptidase (USP18) which would prevent long lasting activation of ISGs (Dill et al., 2012, Sarasin-Filipowicz et al., 2009). Interestingly, USP18 does not inhibit IFN-λ signalling (Makowska et al., 2011). Therefore, Type III interferons are considered to be the primary drivers of ISG induction in chronic hepatitis C patients with an activated endogenous IFN system.

1.12.3 Role of Interferon-λ

IFN-λs display type I interferon-like antiviral activity, although are structurally closer to members of Interleukin-10 (IL-10) cytokine family. Receptor signalling leading to production of ISGs is discussed in Section 1.12.2. Following both in-vitro and in-vivo HCV infection, type III are upregulated at the mRNA and protein

levels to an even greater extent than type I IFNs (Thomas et al., 2012, Park et al., 2012). Type III interferons in addition to inducing well known ISGs activate a distinct set of genes in primary human hepatocytes from the type I interferons, including those involved in chemotaxis and antigen presentation, suggesting possible divergent signalling pathways following receptor engagement (Thomas et al., 2012). The induction of type III interferons as the predominant antiviral pathway and driver of ISG induction may render hepatocytes refractory to further type I interferon action, which is supported by the observation that blocking type III interferon enhances the antiviral activity of exogenous IFN-α (Thomas et al., 2012).

One of the major discoveries in the area of HCV immune response has been the finding that SNPs within or near IFNL3 gene locus encoding IFN-λ3 or IL28B are highly predictive of both spontaneous resolution of infection and antiviral treatment response. A genome wide association study demonstrated that patients homozygous for the C allele at the rs12979860 SNP of IFNL3 had a 2 fold greater chance of cure with previous standard of care pegylated IFN and Ribavirin (Ge et al., 2009). This favourable IFNL3 variant is associated with lower ISG expression in pre-treatment liver biopsies (Urban et al., 2010) and exogenous IFN-α is seen to induce a rapid antiviral state (Sarasin-Filipowicz et al., 2008).

1.12.4 HCV evasion of innate immune signalling

Hepatitis C virus has evolved important interactions with host cell that benefits the viral lifecycle by disarming and evading innate immune responses. Once

sufficient viral proteins have accumulated, HCV uses its NS3/4A protease to target MAM anchored synapse, thereby cleaving MAVS from MAM (but not from the mitochondria) and ablating RIG-I mediated innate immune signalling (Horner et al., 2011). NS3/4A also interferes with TLR3 signalling mediated by binding of activated TLR3 to the adaptor TRIF (Toll/interleukin-1 receptor domain-containing adapter-inducing IFN-β), which is then cleaved by NS3/4A (Horner and Gale, 2009). In addition, NS3/4A also interacts directly with TANK-binding kinase 1 (TBK1) to reduce TBK1-IRF3 interaction and therefore inhibits IRF3 activation (Otsuka et al., 2005).

HCV also interferes with the TLR-MyD88 pathway through NS5A interaction with MyD88 to prevent IRAK (Interleukin-1 receptor associated kinase) recruitment and cytokine production (Abe et al., 2007).

In addition, HCV also escapes immunomodulatory effects of type I interferons via variety of mechanisms. The induction of endogenous IFN system in the liver has little antiviral efficacy in chronic HCV infection and HCV persists for decades despite the expression of hundreds of ISGs (Sarasin-Filipowicz et al., 2008, Chen et al., 2005, Asselah et al., 2008).

HCV core protein induces synthesis of suppressor of cytokine signalling 1 and 3 (SOCS1 and 3), which inhibits STAT 1 phosphorylation thereby interfering with JAK-STAT signalling and contributes to diminished binding of ISGF3 to nuclear IFN stimulated response elements (ISREs) (Horner and Gale, 2009, Bode et al., 2003).

Furthermore, NS5A and E2 proteins have been shown to impair the function of ISGs, protein kinase R (PKR) and 2'-5'-oligoadenylate synthetase (Gale et al.,

1997, Taylor et al., 1999). In cell culture experiments, HCV triggers phosphorylation and activation of the RNA-dependent protein kinase PKR which phosphorylates eukaryotic translation initiation factor, eIF2a (Garaigorta and Chisari, 2009). Because eIF2a inhibits cap-dependent translation, no proteins are produced from ISG mRNAs. HCV protein production is however not impaired because HCV RNA translation occurs through IRES dependent mechanism, which is not impaired by eIF2a (Garaigorta and Chisari, 2009).

These mechanisms suggest that HCV directly affects signal transduction cascades following virus sensing thereby attenuating innate immune responses leading to chronicity and failure to clear the virus during acute infection.

Recent extraordinary development of drugs including NS3 serine protease inhibitors, NS5A and NS5B inhibitors used as part of direct acting antiviral therapy inhibit viral replication by directly acting against these viral proteins. Due to the role of these proteins in immune evasion by HCV, DAA treatment may also be expected to restore innate immune responses. Such immune restoration may also play a role in relapse prevention. However, the nature and extent of such immune restoration remains to be defined.

Innate immune signalling pathways and the mechanisms of immune evasion by hepatitis C virus are demonstrated in Figure 1.3 and Figure 1.4.

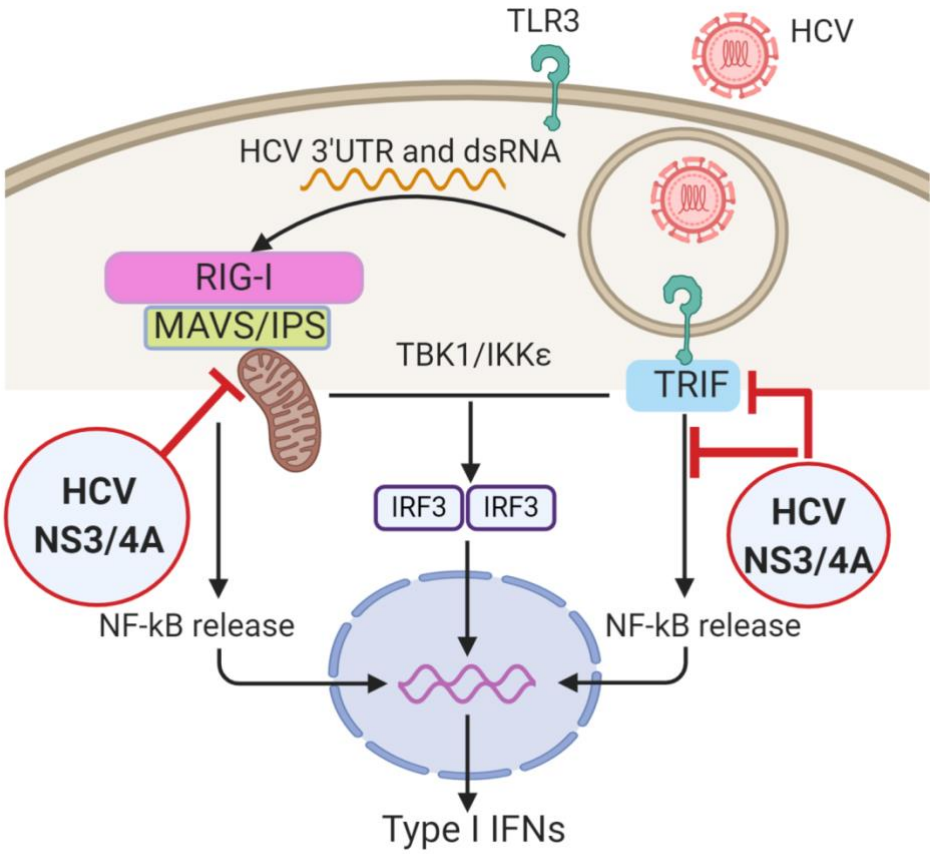

Figure 1. 3 HCV recognition by TLR3 and RIG-I signalling pathways

Intracellular HCV recognition occurs through dsRNA sensors including RIG-I and TLR. Endosomal toll like receptor 3 (TLR3) via adaptor molecule TRIF (Toll/IL-1 receptor domain containing adaptor inducing IFN-β) activates a cascade of signalling proteins, leading to phosphorylation of interferon regulatory factor 3 (IRF3) which homodimerizes and translocates to the nucleus, where it stimulates the expression of type I interferons.

Retinoic acid inducible gene-I (RIG-I) receptor, situated in the cytoplasm, senses viral RNA resulting in transduction via mitochondrial associated adaptor proteins (MAVS/IPS-1), with downstream signalling pathways resulting in expression of type 1 interferons.

HCV NS3/4A protease cleaves both TRIF and MAVS, and interferes with signal transduction and interferon production.

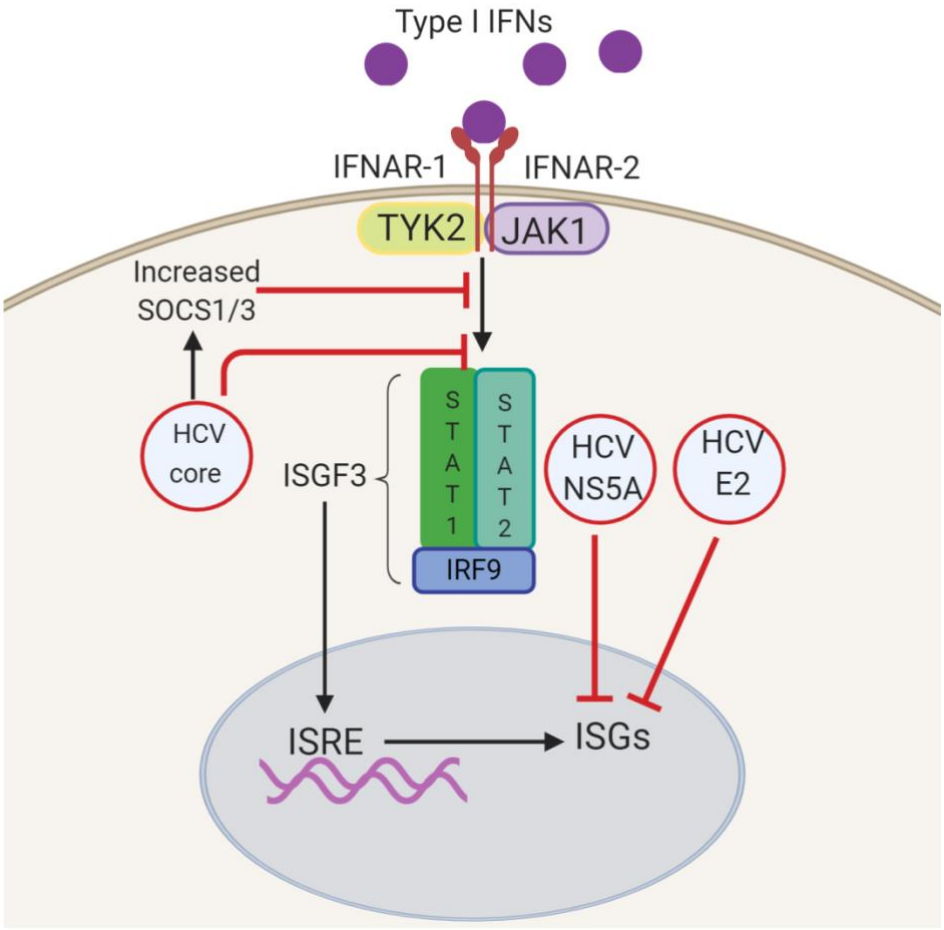

Figure 1. 4 Type I interferon mediated signalling and HCV evasion by HCV

Binding of type I interferons to IFN-α/β receptors results in the activation of JAK/STAT pathway, conferring stable association with IRF-9. The resultant IFN-stimulated gene factor 3 (ISGF3) localises to the nucleus, where it binds to the ISREs within the promoter /enhancer region of hundreds of ISGs.

HCV core protein subverts immunity by the induction of suppressors of cytokine signalling (SOCS1/SOCS3) and HCV NS5A impairs the production of ISGs by inhibiting binding of ISGF3 to ISRE.

1.12.5 Role of dendritic cells

Dendritic cells (DCs) play crucial roles in innate pathogen sensing as well as in the initiation of adaptive immunity (Lande and Gilliet, 2010). They circulate in the peripheral blood as either mature or immature forms. In humans, DCs are differentiated into two subsets, plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) (Kadowaki, 2009), which make up a small proportion of the total peripheral blood mononuclear cells (PBMCs) in the blood (0.5-1% and 0.2-0.5% respectively) (Woltman et al., 2010). As immature cells they express low levels of MHC class I and II antigens but following maturation after detection of pathogen, MHC expression is increased with increased ability to present antigens to T cells, activate NK cells, and produce IFNs. Both subsets differ in their phenotype, antigen presenting characteristics and effector functions. mDCs express high levels of MHC class II, interact with T cells to induce strong Th1 cellular response. pDCs express high levels of MHC class I molecules and are potent secretors of Type I interferons, which then exert a direct antiviral effect and stimulate other immune cells, such as NK cells.

pDCs are the main producers of type I interferons which is mediated by TLR7 activation in pDCs, and is independent of HCV viral replication within pDCs (Takahashi et al., 2010). HCV RNA containing exosomes produced by infected hepatocytes have been shown to transfer their RNA to pDCs which subsequently respond by producing IFN-α (Dreux et al., 2012). In addition, HCV subgenomic replicon cells that replicate viral RNA without producing infectious viral particles can trigger type I interferons by pDCs. A previous study has demonstrated that intracellular sensing of HCV PAMPs by pDCs leads to robust type I and III

interferon production and is mediated by signalling through cytosolic RIG-I (Stone et al., 2013, Takeuchi and Akira, 2008).

A host of studies have been performed to determine mechanism and role of dendritic cell dysfunction in preventing viral clearance leading to chronic HCV infection. However, the results remain conflicting. It has been shown that the ligation of C-type lectin immunoreceptors, Blood Dendritic Cell antigen 2 (BDCA2), and Dendritic Cell Immunoreceptor (DCIR) on pDCs by HCV E2 glycoprotein antagonizes production of interferons (Florentin et al., 2012), and the relative expression of these receptors likely affects the ability of pDCs to respond to HCV.

Further studies have also looked at the role of dysfunctional dendritic cells in the lack of a strong adaptive immune response in HCV. The data remains controversial. Monocyte derived dendritic cells (MoDCs) from patients with HCV have shown to be impaired with reduced capacity to activate and induce proliferation of autologous T cells (Bain et al., 2001, Kanto et al., 1999). However, multiple later studies failed to confirm these findings (Larsson et al., 2004, Barnes et al., 2008, Echeverria et al., 2008). The main criticism being that MoDCs are in-vitro generated from culture of monocytes with IL-4 and GM-CSF and do not represent in-vivo circulating DCs. Later, Kanto et al and others reported reduced circulating levels and function of both mDCs and pDCs in chronic HCV (Kanto et al., 2004, Murakami et al., 2004). Several mechanisms proposed include direct HCV infection of DCs and interference with viral signalling through TLR pathways (Albert et al., 2008). In contrast to this, Longman et al and others have reported reduced frequency of circulating DCs, but preserved function(Longman et al.,

2004, Decalf et al., 2007). Such differences more likely represent differences in study groups and experimental models and require further evaluation.

1.12.6 Role of NK cells

NK cells are large granular lymphocytes, and an important cellular component of the innate immune system providing early host defence against viral pathogens. NK cells account for 5-20% of peripheral blood mononuclear cells and represent 30-50% of lymphocytes in the liver (Corado et al., 1997). They are potently cytolytic with the ability to lyse virally infected or tumour cells and unlike T cells, they do not require prior sensitisation. They exert their function by degranulation of cytotoxic granules such as perforin and granzyme B as well as inducing apoptosis by expression of death receptors, Fas ligand (FasL) and TNF related apoptosis inducing ligand (TRAIL). NK cell function is amplified by IFN-α released by dendritic cells and virally infected cells. NK cells are also rapidly activated by other monocyte and dendritic cell derived cytokines such as IL-1, IL10, IL-12, IL-15, IFN-α and IL-18. In addition, NK cells exert antiviral effect indirectly by secreting immunostimulatory cytokines, chiefly IFN-γ and TNF-α, which can exert an effect on innate as well as adaptive arms of the immune system (Biron and Brossay, 2001, Cooper et al., 2001).

NK cell function is regulated by a combination of regulatory receptors with either inhibitory or stimulatory effect which interact with MHC-I alleles and other ligands on a variety of cells with viral infections or tumours. This receptor/ligand interaction provides a mechanism of self-tolerance and inhibition of NK cell autoreactivity.

Table 1.2 outlines key NK cells receptors, their function and ligands.

Table 1. 2 Key NK cell activating and inhibitory receptors and their ligands

NK cell receptor family	Receptor	Receptor ligand
	Activating receptors	
Killer immunoglobulin-like receptors (KIR)	sDS1	HLA-Bw4
C-type lectin receptors	NKG2D	MICA A/B
	CD94:NKG2C/E	HLA -E
Natural cytotoxicity receptors	NKp30, NKp44, NKp46	Viral haemagglutinin
		BAT-3, B7-H6, CMV pp65
Others	CD16	IgG (Fc)
	Toll like receptors	PAMPs
	Inhibitory receptors	
Killer immunoglobulin-like receptors (KIR)	2DL1/2/3	HLA – C
C-type lectin receptors	CD94:NKG2A	HLA-E

Current data indicate that NK cells play central role in every stage of HCV infection, from protection against infection in injection drug users to prediction of antiviral success with previously used IFN-based therapies. The original implication of NK cells in the outcome of HCV infection was associated with killer Ig-like receptor (KIR) genes and their human leucocyte antigen – C (HLA-C) ligands, which are highly polymorphic. Therefore, certain combinations are protective from HCV infection. Khakoo et al showed that genes encoding the inhibitory NK cell receptor KIR2DL3 and its human leukocyte antigen C group 1 (HLA – C1) ligand directly influence resolution of hepatitis C virus infection in patients with low volume inoculum (Khakoo et al., 2004). Several studies have shown increased expression of NKG2A on NK cells in chronic HCV infection causing reduction in NK cell mediated cytotoxicity and dendritic cell activation (Jinushi et al., 2004).

CD56+NK cells have been assigned to functional categories on the basis of cell surface density of CD56. These subsets include $CD56^{dim}$ NK cells and $CD56^{bright}$ NK cells. $CD56^{dim}$ NK cells are predominant in peripheral blood representing 90% of circulating NK cells and display potent cytolytic activity whereas the $CD56^{bright}$ NK cells are enriched in tissues, are poorly cytolytic and are responsible for cytokine production. Compared to peripheral blood and spleen, NK cells present in liver exist in a hyporesponsive state, are less cytotoxic and produce lower level of IFN-γ and higher levels of IL-10. This feature may contribute to the development of chronic hepatotropic viral infections.

Injecting drug users repeatedly exposed to HCV infection but who remain uninfected have proportionally higher circulating levels of $CD56^{dim}$ mature NK cells (Golden-Mason et al., 2010). This study also found that activating receptor

NKp30, which is induced by IL-2 is most highly expressed on the NK cells and NKT cells of exposed uninfected subjects (Golden-Mason et al., 2010). The NKp46 receptor is considered the major human natural cytotoxicity receptor involved in NK cell mediated killing (Sivori et al., 1999, Mandelboim et al., 2001) and is more highly expressed on the NK cells of people with mixed European descent and women i.e. populations who are known to demonstrate high rate of spontaneous resolution. NKp46 ligand expression is induced on hepatocytes following HCV infection (Golden-Mason et al., 2012). TLR stimulation of purified $NKp46^{hi}$ cells is associated with increased transcription of cytotoxicity related genes as compared with $NKp46^{lo}$ counterparts. Two further studies (Golden-Mason et al., 2012, Kramer et al., 2012) have highlighted that $NKp46^{hi}$ NK cells have increased anti- HCV activity in vitro, a process mediated by IFN-γ. Intrahepatic accumulation of $NKp46^{hi}$ NK cells is also found to be inversely correlated with HCV RNA levels (Kramer et al., 2012).

HCV has a number of strategies through which it can evade NK cell activity. HCV core can upregulate HLA-E leading to CD94:NKG2A mediated inhibition of NK cells (Nattermann et al., 2005). The result is an increase in IL-10 production which promotes Th2 as opposed to Th1 differentiation, an effect that can be restored following inhibition of NKG2A (Jinushi et al., 2004, De Maria et al., 2007). Earlier studies had also demonstrated the ability of HCV E2 to reduce IFN-γ production by linking to CD81 on NK cells. These experiments used plate bound E2 and subsequent studies using infectious particles refuted this theory (Yoon et al., 2009, Crotta et al., 2002). In chronic HCV, NK cells are polarised towards cytotoxicity with deficient IFN-γ secretion as a consequence of chronic exposure to endogenous IFN-α (Oliviero et al., 2009, Ahlenstiel et al., 2010). This

phenomenon is caused by type I interferon induced phosphorylation of STAT-1, which displaces STAT4 at the interferon α/β receptor resulting in decreased phosphorylated STAT4 (pSTAT4) dependent IFN-γ production, and increased pSTAT1 dependent cytotoxicity (Miyagi et al., 2010).

Our understanding of NK cell function in HCV infection is still emerging and there remains a difference in NK cell number, activation and cytokine profile between acute and chronic HCV.

1.12.7 Natural Killer T cells

Natural killer T cells (NKT) are a subset of lymphocytes that possess both NK markers and CD1d-resticted T cell receptor (TCR). NKT cells recognise lipids derived from pathogens, tumours or allergens that are presented to them by CD1d, a non-classical MHC class I molecule. Ligation of NKT cell TCR leads to rapid and copious secretion of Th1 and Th2 cytokines. In addition, they express cytotoxic granules containing granzyme and perforin which are able to kill target cells.

1.13 Adaptive immunity

Adaptive immune response to HCV consists of CD4+ and CD8+ HCV specific T cell responses as well as humoral responses. In contrast to the innate responses which are induced within hours or days after infection, there is a delay of 6-8 weeks before adaptive immune responses become detectable (Thimme et al.,

2002, Shin et al., 2011). T cell responses coincide with a reduction in viral load indicating the role of adaptive responses in viral clearance. HCV elimination in individuals who spontaneously resolve infection is associated with strong and high amplitude CD4+ and CD8+ T cell responses targeting multiple epitopes in HCV proteins (Thimme et al., 2001, Cooper et al., 1999, Diepolder et al., 1997, Lechner et al., 2000, Missale et al., 1996, Takaki et al., 2000), which are sustained over time and detectable long after resolution of infection (Takaki et al., 2000). In contrast, individuals who develop chronic infection may have strong responses initially, but these give way to low amplitude T cell responses with a restriction in the HCV epitopes targeted (Diepolder et al., 1996, Lechmann et al., 1996, Missale et al., 1996, Thimme et al., 2002).

1.13.1 T cell responses in HCV clearance

CD4+ and CD8+ T cells recognise antigens presented to T cell receptors (TCR) by MHC class II and I molecules on the surface of antigen presenting cells (APC) respectively.

Naïve CD4+ T cells further differentiate following activation and depending on the cytokine milieu of the microenvironment. T helper 1 cells (Th1) produce IFN-γ which leads to macrophage activation, promote development of cytotoxic T lymphocytes and NK cells in addition to activation of CD4+ and CD8+ T cells. T-helper 2 (Th2) cells secrete a variety of cytokines which promote B cell activation, proliferation and antibody production. CD8+ T cells are main effector cells.

Both T cell subsets, CD4+ and CD8+ T cells are essential for successful cell mediated response to HCV infection. This is supported by a clear temporal association between the onset of peripheral and intrahepatic virus specific T cell responses and HCV clearance (Thimme et al., 2001, Thimme et al., 2002, Cooper et al., 1999, Lechner et al., 2000). Antibody mediated CD8+ T cell depletion in Chimpanzees prior to experimental infection led to HCV persistence until CD8+ T cell response recovered (Shoukry et al., 2003).

However, out of the two, CD4+ T cells are central regulators while virus specific CD8+ T cells primarily function as the key effector cells (Gerlach et al., 1999). This was demonstrated by chimpanzee studies where depletion of CD4+ cells in previously protected chimpanzees led to the persistence of HCV following re-infection despite the presence of strong intrahepatic memory CD8+ T cell responses (Grakoui et al., 2003). The important role of CD4+ T cell responses is also supported by a study showing an association between expansion of IL-17 and IL-21 producing CD4+ T cell responses and viral clearance (Kared et al., 2013).

Resolution of HCV infection is seen in individuals who mount a strong CD4+ T cell response (Diepolder et al., 1995, Missale et al., 1996, Urbani et al., 2006). Studies have shown that this response needs to be sustained over time and loss of initial strong responses can lead to rebound viraemia (Gerlach et al., 1999) and viral escape mutations in MHC class I epitopes emerge leading to CD8+ T cell escape (Grakoui et al., 2003). Breadth and specificity of HCV epitopes targeted is equally important. In order to be effective, these responses should be targeted towards multiple immunodominant epitopes in non-structural HCV

proteins such as NS3, NS4 and NS5 (Diepolder et al., 1995, Lechner et al., 2000, Day et al., 2002).

CD8+ T cell responses are also vital to effective clearance of acute HCV infection. Their functions include direct cytotoxicity and production of antiviral cytokines, including TNF-α and IFN-γ. Their activation requires prior sensitization to HCV antigens presented by antigen presenting cells on MHC Class I molecules.

Further studies established that as with CD4+ T cells, mounting vigorous and multispecific CD8+ T cell responses in periphery and liver is an important determinant of viral clearance (Gruner et al., 2000, Lechner et al., 2000). HCV specific CD8+ T cells have also been shown to inhibit the replication of HCV in replicon model by Liu et al (Liu et al., 2003). Jo et al used subgenomic replicon containing cell line transduced with common MHC class I allele HLA-A2 gene to demonstrate that HCV specific CD8+ T cells exert strong antiviral effects primarily by IFN-γ secretion, and to a lower extent by cytolytic effector functions (Jo et al., 2012).

Virus specific CD8+ T cells are seen to emerge in acute stages irrespective of the outcome of viral infection. However, these initial CD8+ T cells may show impaired function including cytotoxic function and inability to produce IFN-γ. These are often referred to as ‘stunned phenotype’. Active CD8+ T cell phenotype is only seen in individuals who clear infection which coincides with peak CD4+ T cell responses and a decline in viral load (Gruner et al., 2000, Lechner et al., 2000, Wedemeyer et al., 2002, Urbani et al., 2006). Persistence of this stunned phenotype is only seen in those who progress to chronic infection.

1.13.2 Failure of T cell responses in chronic HCV infection

Multiple mechanisms have been suggested to explain T cell failure leading to viral persistence and chronic HCV infection although the relative contribution of each of these are not entirely clear.

As discussed in previous section, several studies have demonstrated that CD4+ and CD8+ T cell responses are of low amplitude or completely absent in cases progressing to chronic infection. If present, the response is oligo-specific and directed towards a contracted number of viral epitopes (Cramp et al., 1999, Day et al., 2002, Ulsenheimer et al., 2006). It is often difficult to differentiate between primary T cell failure and early T cell exhaustion following initial priming (Thimme et al., 2001).

Studies on early phase of acute HCV infection in Chimpanzees (Thimme et al., 2002) and in health care workers infected via needlestick injury (Thimme et al., 2001) support the concept that in some individuals virus specific T cells are weakly primed during acute infection. This may be due to functional impairment of antigen presenting cells such as dendritic cells or macrophages (Rosen, 2013, Sarobe et al., 2002, Longman et al., 2004) or interference of HCV with interferon signalling pathways impacting on downstream T cell responses as discussed in section 1.12.4.

Emergence of viral escape mutations has also been associated with development of chronic infection, while absence of these mutations is related to viral clearance (Cox et al., 2005a). As previously discussed HCV RNA replicates by its RNA dependent polymerase which lacks proof reading and natural selection leads to evolution of variants resistant to cellular and humoral immune responses. Earlier

studies in chimpanzees and humans had shown that substitution of amino acids that inhibit CD4+ and CD8+ T cell recognition is linked to chronic HCV infection (Weiner et al., 1995a, Tsai et al., 1998, Chang et al., 1997). However, viral escape is usually focussed on a single epitope which is not seen in T cell responses in acute HCV infection, and hence loss of a single epitope would probably not be sufficient for the survival of viral escape mutants (Neumann-Haefelin et al., 2005). In addition, viral escape is also limited by viral fitness cost e.g. inability of the virus to tolerate certain mutations and have a high cost to viral replicative fitness (Neumann-Haefelin and Thimme, 2013).

1.13.3 T cell exhaustion

T cell exposure to persistent antigen or inflammatory signals in chronic infections has been associated with deterioration of T cell function leading to a state of T cell 'exhaustion'. Higher and sustained expression of inhibitory receptors and negative immune check points is a hallmark of exhausted T cells. Programmed cell death protein 1 (PD-1) is an inhibitory receptor, expression of which is substantially higher on effector or memory CD8+ T cells in chronic infection (Blackburn et al., 2008). Sustained upregulation of PD-1 is usually dependent on continued epitope recognition (Blattman et al., 2009). The mechanisms by which PD-1 mediates T cell exhaustion are incompletely understood. Studies have implicated a role of PD-1 signalling in modulating phosphoinositide 3-kinase (P13K), AKT and RAS pathways (Parry et al., 2005, Patsoukis et al., 2012); as well as cell cycle control (Patsoukis et al., 2012).

In addition to PD-1, exhausted T cells express a range of other cell surface inhibitory molecules. Exhausted T cells can co-express PD-1 with lymphocyte activation gene 3 protein (LAG3), 2B4, CD160, T cell immunoglobulin domain and mucin domain containing protein 3 (TIM3) and Cytotoxic T lymphocyte associated protein 4 (CTLA-4). Such co-expression is a cardinal feature of T cell exhaustion. The higher the number of inhibitory receptors co-expressed by exhausted T cells, the more severe the exhaustion.

The intracellular domain of PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). Inhibitory receptor B and T lymphocyte attenuator (BTLA) also contains both ITIM and ITSM, whilst 2B4 contains ITSM and T cell immunoreceptor with Ig (TIGIT) contains ITIM in their intracellular domains. Other receptors have specific motifs, such as YVKM for CTLA4 and KIEELE for LAG3. The molecular mechanism of inhibitory receptor signalling includes binding with their cognate ligands expressed on APCs during infection and modulation of intracellular mediators resulting in attenuation of positive signals from activating receptors such as T cell receptors and co-stimulatory receptors; and induction of inhibitory genes. Molecular pathways of inhibitory receptors associated with T cell exhaustion are illustrated in figure 1.5.

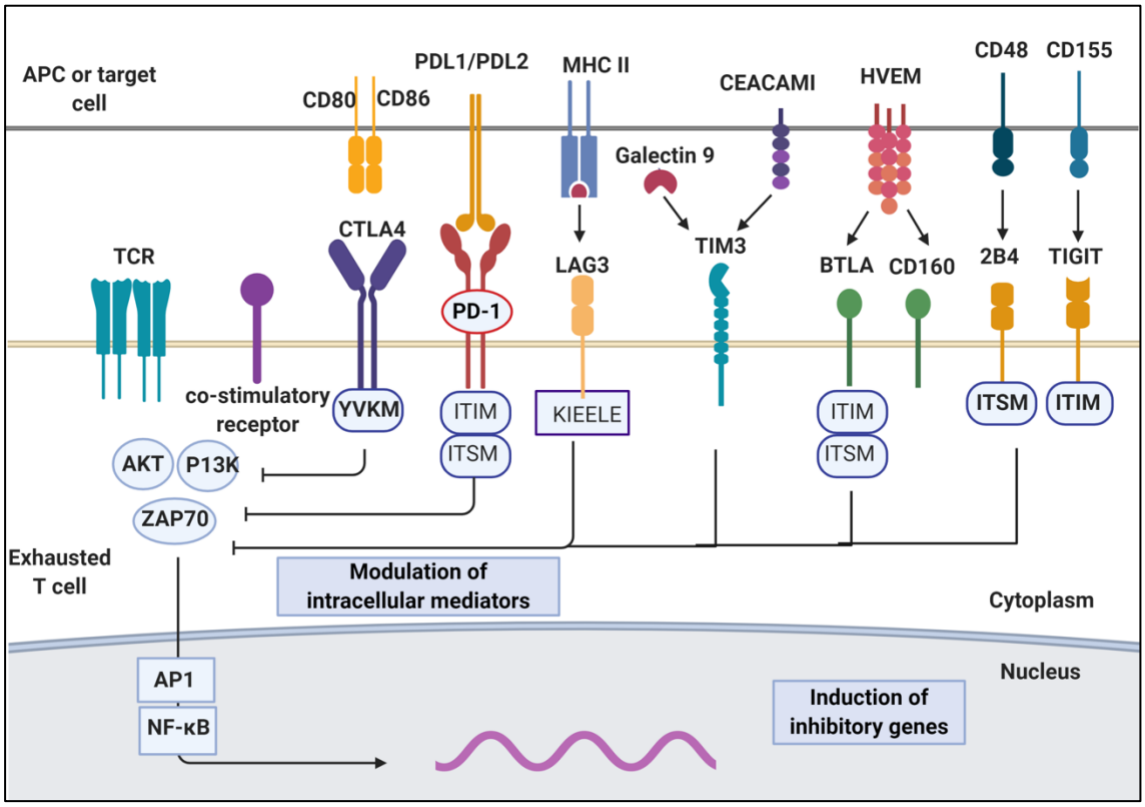

Figure 1. 5 Molecular pathways of inhibitory receptors associated with T cell exhaustion

Ligand and receptor pairs for inhibitory pathways and intracellular domains of receptors that contribute to T cell exhaustion are shown. The molecular mechanisms of inhibitory receptor signalling are also depicted including; inhibitory receptor sequestration of target receptors or ligands, modulation of intracellular mediators causing attenuation of signals from T cell receptors and co-stimulatory receptors, and induction of inhibitory genes.

AP-1, activator protein 1; BTLA, B and T lymphocyte attenuator; CEACAM1, carcinoembryonic antigen related cell adhesion molecule 1; HVEM, herpes virus entry mediator; NF-κB, nuclear factor – kappa B; PD-1, programmed cell death protein-1; PDL1, PL1 ligand 1; P13K, phosphoinositide 3-kinase; TIGIT, T cell immunoreceptor with ITIM domains; TIM3, T cell immunoglobulin and mucin domain containing protein 3.

ITSM, Immunoreceptor tyrosine based switch motifs; ITIM, immunoreceptor tyrosine based inhibitor motifs.

In addition to inhibitory receptors, immunosuppressive cytokines such IL-10 and transforming growth factor-β (TGFβ) also regulate T cell exhaustion (Tinoco et al., 2009, Brooks et al., 2008), suggested by the evidence that blockade of IL-10 restores T cell function and improves control of chronic viral infections (Brooks et al., 2006, Ejrnaes et al., 2006, Brooks et al., 2008). However, the molecular events downstream of IL-10 signalling which shape T cell exhaustion remain to be clearly defined.

As discussed earlier, a prominent feature observed in chronic HCV infection is presence of functionally impaired or 'stunned' CD8+ T cells which are characterised by their inability to proliferate, lack of cytolytic activity and inability to secrete cytokines such as IFN-γ (Klenerman and Thimme, 2012, Rehermann, 2009). These 'stunned' or 'exhausted' T cells are characterised by upregulation of inhibitory receptor Programmed death-1 (PD-1) (Radziewicz et al., 2007, Golden-Mason et al., 2007) and a low expression of CD127 (Golden-Mason et al., 2007, Radziewicz et al., 2007). These HCV specific CD8+ T cells with high expression of PD-1 are prone to apoptosis (Radziewicz et al., 2008). Blockade of PD-1 by antibodies targeting PD-1 increases the response of these CD127-PD1+HCV specific CD8+ T cells to antigenic stimuli (Penna et al., 2007, Nakamoto et al., 2008, Golden-Mason et al., 2007). In addition to PD-1, T cell exhaustion is mediated by co-expression of several different inhibitory receptors. Targeting of these additional receptors including cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and T-cell immunoglobulin and mucin containing-3 (TIM-3) is required in addition to PD-1 blockade in order to restore T cell function (Nakamoto et al., 2009, McMahan et al., 2010). A previous study has also associated expression of 2B4 with HCV specific CD8+ T cell exhaustion

(Schlaphoff et al., 2011). Another study showed co-expression of inhibitory receptors 2B4, KLRG1, CD160 and PD-1 on CD127-HCV specific CD8+ T cells in chronic HCV infection (Bengsch et al., 2010).

Regulatory T cells (T reg) expressing CD4+CD25+ are responsible for maintaining a balance between the inflammatory response and viral control. They can induce immune tolerance in viral epitopes in case of excessive host damage. They inhibit antigen presenting cell maturation and T cell activation. A higher frequency of suppressive CD4+CD25+ T cells has been found in chronic HCV (Boettler et al., 2005, Cabrera et al., 2004, Rushbrook et al., 2005). In vitro studies have demonstrated that depletion of CD25+ leads to enhanced CD8+ HCV specific T cells responses (Rushbrook et al., 2005, Boettler et al., 2005), suggesting their role in development of T cell exhaustion.

Another type of regulatory T cells detected in the liver of HCV infected individuals are virus specific regulatory CD8+T cells expressing high levels of IL10 and neutralizing IL10 antibodies can block their suppression of virus-specific CD8+ effector T cells (Accapezzato et al., 2004, Abel et al., 2006).

1.14 Humoral immunity

Antibodies to HCV appear late in the course of infection between 8 – 12 weeks (Chen et al., 1999). These are of low titre, restricted to IgG1 isotype and do not affect the outcome of infection (Cooper et al., 1999, Cramp et al., 1999). All cases develop an antibody response, however this does not correlate with viral clearance. Previous studies suggested that serum antibodies from patients with

acute HCV could neutralize and protect chimpanzees from HCV infection (Farci et al., 1994). However, chimpanzees who had cleared HCV and were re-challenged with the same or different HCV strain, antibody did not provide any protection from subsequent infections (Farci et al., 1992). Further evidence that a humoral response was not required to clear the virus was evident from resolution of infection in agammaglobulinaemic patients (Semmo et al., 2006).

Newer evidence suggests early production of broad neutralising antibodies targeting epitopes within envelope glycoproteins E1 and E2 (Osburn et al., 2014, Giang et al., 2012). One study suggested that spontaneous clearance of HCV infection is associated with the appearance of neutralising antibodies and reversal of T cell exhaustion (Raghuraman et al., 2012). There is also data suggesting that individuals with apparent resistance to HCV infection can produce neutralising anti-envelope antibodies, but whether these antibodies contribute to host immunity is yet to be determined (Swann et al., 2016).

Most of the neutralising antibodies target epitopes in hypervariable region of E2. Even though neutralisation of HCV by antibodies targeting envelope glycoproteins is demonstrable in vitro (Pestka et al., 2007); in vivo this antibody response lags behind as virus continuously escapes due to emergence of glycoprotein variability termed as quasispecies (von Hahn et al., 2007).

The virus also has other ways of evading humoral immune response. These include glycosylation shielding and lipid shielding of epitopes targeted by neutralising antibodies (Helle et al., 2007, Nielsen et al., 2006).

Overall, antibodies directed against HCV do not appear to provide protective immunity from subsequent infection, nor do they influence the outcome of acute HCV.

1.15 Cytokines and chemokines in HCV infection

Cytokines are small soluble regulatory proteins, secreted mainly by immune cells which act locally in an autocrine and paracrine fashion facilitating intercellular communication to orchestrate an immune response to infections. In HCV infection, cytokines play an important role in viral clearance, and can also induce tissue damage.

More than 100 cytokines have been identified and can be roughly classified according to their main function and source, although strict classification is challenging due to frequently overlapping actions and cells of origin.

1.15.1 Helper T cell cytokine profiles in HCV infection

Activated CD4+ helper T cells can be subdivided into different subsets dependent on cytokines they produce. A T-helper 1 (Th1) cytokine profile is recognised by release of IL-2, IL-12 and IFN-γ, which are important in generating cytotoxic CD8+ T cell responses, as well as activating NK cells. It is this phenotype which leads to strong and polyclonal CD4+ and CD8+ T cell responses associated with HCV clearance. A T helper 2 (Th2) response results in release of IL-4, IL-5, IL-10 and

IL-13 which principally mediates humoral immune responses by promoting B cell activation, proliferation and production of HCV specific antibodies.

Many groups have investigated whether a skewed Th1/Th2 contributes towards outcome of infection, including HCV. In acute HCV infection, polarization of CD4+ T cell response towards Th1 phenotype with release of IL-2 and IFN-γ is crucial to activation of CD8+ T cell responses and subsequent viral clearance (Urbani et al., 2006). However, these Th1 responses were also seen in intrahepatic lymphocytes in chronic infection (Bertoletti et al., 1997, Penna et al., 2002). In the later situation, Th1 response results in liver injury, inflammation and progression to liver fibrosis. This also illustrates that the timing of Th1 responses is crucial in enabling self-limiting infection as well as reducing tissue damage. However, findings from other studies do not support this theory and have shown lower levels of IFN-γ in chronic HCV (Osna et al., 1997, Sofian et al., 2012). The discrepancies between these studies may be due to composition of study populations.

In contrast, findings regarding a skewed Th2 response are more consistent and associated with viral persistence (Cramp et al., 1999) and reduced liver inflammation. Diminished liver inflammation is a consequence of suppression of inflammatory Th1 responses.

The mechanism through which polarization of Th2 cells in chronic HCV infection occurs is unclear, but dendritic cell function has been shown to have the profound ability to prime IL-10 producing T cells in the context of viral infection (Kanto et al., 2004).

Overall, as mentioned previously, cytokine biology and pathways are complex. Th1/Th2 categories are not clear cut and often the function of both can show overlap and heterogeneity.

1.15.2 Innate cytokines in HCV infection

Type I interferons are the main innate immune cytokines released early in HCV infection. Their role, signalling pathways and ability of HCV to evade their action is described in sections 1.12.2 and 1.12.4.

More recently described Type III interferons, also called IFN-λ have been described as a further group of interferons with potent anti-HCV effects. IFN-λ mediated signalling and responses in HCV infection as well as role spontaneous and treatment induced HCV clearance is discussed in section 1.12.3.

Interleukin 6 (IL-6) was previously named Interferon β2 due to its initially described interferon type effects. This proinflammatory cytokine, is overexpressed and exhibits pleiotropic effects in patients with HCV infection. Similar to interferons, it is released by both immune and non-immune cells, such as hepatocytes and sinusoidal epithelial cells. The receptor complex mediating biological activities of IL-6 consists of the transmembrane glycoprotein 80 (gp80) and glycoprotein 130 (gp130). Receptors for IL-6 have also been found in many immune and non-immune cells, including hepatocytes. Intracellular signal transduction is similar to type 1 interferons involving activation of JAK/STAT pathway except STAT 3, which is the main coupling protein with JAK-TYK as opposed to STAT1/STAT2. This results in the production of IL-6 stimulated

genes, the expression of which consequently impacts apoptosis, cell differentiation, cell proliferation, cell recruitment and the acute phase response.

IL-6 has shown to directly reduce viral replication in vitro (Hosel et al., 2009). There is good evidence to suggest that IL-6 plays an important role in transition from innate to adaptive immunity. In the initial phase of immune response, IL-6 attracts neutrophils, but subsequently switches from neutrophil to monocyte recruitment and skews their differentiation towards macrophages (Chomarat et al., 2000). IL-6 directly stimulates NK cells and dendritic cells that are necessary for T cell recruitment. Additionally, IL-6 plays a role in B cell proliferation, differentiation into antibody secreting plasma cells and antibody production (McLoughlin et al., 2005).

IL-6 is an acute phase reactant found to be raised in inflammatory responses to multiple stimuli. The dysregulation of IL-6 production has been described in numerous inflammatory conditions including inflammatory bowel disease and rheumatoid arthritis. In relation to the liver, IL-6 induces growth and proliferation of hepatocytes and has a protective role in liver injury by enhancing hepatocyte survival during stress (Xia et al., 2015).

IL-6 has complicated pleiotropic effects in the context of hepatitis C infection, associated with HCV clearance, poor response to antiviral therapy and development of progressive fibrosis and hepatocellular carcinoma (Nakagawa et al., 2009, Ueyama et al., 2011, Giannitrapani et al., 2013).

1.15.3 Chemokines in HCV infection

An effective and vigorous intrahepatic T cell response is required for clearance of HCV, as discussed in section 1.12.1. As the liver a has propensity to immune tolerance, the activation of T cells is likely to occur in secondary lymphoid tissues under the influence of activated dendritic cells (Bowen and Walker, 2005). Hence the mechanisms to co-ordinate migration of T cells to lymphoid tissue and subsequently to liver are crucial to facilitate intra-hepatic immune responses.

Chemokines are a family of chemoattractant cytokines which play an important role in trafficking and retention of these effector immune cells to the site of inflammation. In addition chemokines also play a role in immune cell activation, differentiation and proliferation, angiogenesis and tissue regeneration.

Structurally chemokines can be divided into four subgroups; C-X-C motif (CXC), C-C motif (CC), (X)-C motif ((X)C) and C-X3-C motif (CX3C) depending on the position of the two N-terminal cysteine residues. Functionally, chemokines can be divided into inflammatory or homeostatic. Inflammatory chemokines facilitate recruitment of leucocytes to inflamed tissues, whereas homeostatic chemokines are constantly expressed in lymphoid organs mediating migration of various cells. However, there remains a degree of overlap in function amongst some chemokines.

Chemokines exert their effect by binding to seven transmembrane spanning receptors. Homeostatic chemokine receptors bind only one or two chemokines, while inflammatory chemokine receptors bind several ligands. Chemokine receptor expression is variable depending on stage of infection. A list of human

chemokines that act on immune cells infiltrating the liver tissue is summarised in Table 1.3.

Table 1. 3 Chemokines, their receptors, and predominant effector cells

Chemokine	Alternative name	Chemokine receptors	Lymphocyte subsets
CCL2	MCP-1 monocyte chemoattractant protein-1	CCR2	Monocytes
CCL3	MIP-1α Macrophage inflammatory protein-1α	CCR1, CCR5	T, NK, NKT, DC, monocytes & macrophages
CCL4	MIP-1β Macrophage inflammatory protein-1β	CCR1, CCR5	T, NK, NKT, DC, monocytes & macrophages
CCL5	RANTES – Regulated upon activation, normal T cell expressed, and secreted	CCR1, CCR5	T, NK, NKT, monocytes
CXCL8	Interleukin-8	CXCR1, CXCR2	Neutrophils, lymphocytes
CXCL9	Mig, Monokine induced by interferon-γ	CXCR3	T (activated and memory), NK, NKT
CXCL10	Interferon-γ inducible protein-10	CXCR3	T (activated and memory), NK, NKT
CXCL11	ITAC, interferon-γ inducible T-cell α chemoattractant	CXCR3	T (activated and memory), NK, NKT

Note: NKT, natural killer T cells; DCs, dendritic cells; NK, natural killer cells

Inflammatory chemokines are released early in HCV infection resulting in recruitment of immune cells including neutrophils, dendritic cells, NK cells and NKT cells to the liver (Decalf et al., 2007). CXCL8, CXCL16, CXCL2 and CXCL3 are the main cytokines responsible for this effect. Recruitment of dendritic cells to the liver enables sampling of HCV antigens by them, allowing sensitization of T cells when these DCs subsequently migrate to lymph nodes. These activated T cells then migrate to the liver under influence of an array of chemokines. Chemokines that recruit activated T cells include the ligands for CXCR3, namely CXCL9, CXCL10 and CXCL11, and those for CCR5 including CCL3, CCL4 and CCL5. Chemokines with CXCR3 as their ligand have been linked to favourable Th1 T cell phenotype. Th1 cytokine IFN-γ is also a strong inducer of chemokine CXCL9, CXCL10 and CXCL11. No clear links between HCV clearance and these chemokines has been established so far.

Despite this role of chemokines as potent chemoattractants, certain chemokines have been associated with HCV evasion from immune responses. Although CXCL8 (or IL-8) is important for the initial recruitment of neutrophils and lymphocytes to the liver (Heydtmann and Adams, 2009), it facilitates HCV evasion by inhibiting IFN-α activity and suppressing ISGs (Polyak et al., 2001).

In addition, HCV induced secretion of CCL5 has been shown to attract immature DC to the liver unresponsive to CCR7 ligand as a result of HCV-E2 and CD81 interaction and delays activation of T cells (Nattermann et al., 2006). HCV-E2 also attracts CD8+ T cells which co-express CCR5 and inhibitory NKG2A receptor, where the latter leads to their inactivation (Nattermann et al., 2008).

As with perpetual Th1 responses due to failed immune mechanisms, continued secretion of chemokines perpetuates ongoing liver inflammation and disease progression. Many studies have implicated elevated chemokine levels in continued hepatic necro-inflammation, progression to liver fibrosis and risk of developing hepatocellular carcinoma. Such studies have associated levels of CXCL10 (also called Interferon-inducible protein-10 or IP-10), CXCL11 and CXCL8 with fibrosis progression and hepatocellular carcinoma (Li et al., 2007, Ren et al., 2003, Apolinario et al., 2002).

1.15.4 Role of cytokine gene polymorphisms in HCV infection

Cytokine and chemokine gene polymorphisms can influence their production and thereby alter disease outcome. Several such gene polymorphisms have been studied in HCV and linked to HCV clearance, progression and response to treatment.

IL-10 forms part of Th2 response, and is produced by macrophages and monocytes in addition to T cells. As discussed previously it promotes humoral responses and limits Th1 response. The gene responsible for IL -10 production is located on chromosome 1. Few studies have linked the variations in the promoter region with HCV outcome. The G/G genotype is known to be related to increased IL-10 production and has been associated with high risk of HCV persistence as well as resistance to interferon based therapy (Oleksyk et al., 2005, Paladino et al., 2006). However, this effect has not been confirmed in other studies and may be gender and ethnicity related (Constantini et al., 2002, Paladino et al., 2006).

IL-12 forms part of Th1 responses which favour HCV clearance. The single nucleotide polymorphism (SNP) at position 1188 (A/C) of the 3'-UTR of the IL12B gene has been found to be associated with differential production of IL-12 (Morahan et al., 2002, Seegers et al., 2002). A/A genotype corresponds to low IL-12 production and is more frequent in those with chronic HCV infection (Yin et al., 2004, Houldsworth et al., 2005). Yin and co-workers also found that A/C genotype, with C allele is associated with increased levels of IL-12 associated with spontaneous resolution of HCV infection.

In relation to other Th1 cytokines, Huang et al reported a SNP (-766G/C) in the non-coding promoter region of the IFN-γ gene to be associated with spontaneous clearance (Huang et al., 2007). INF-α gene polymorphisms have also been studied, but failed to demonstrate any association with HCV infection outcome (Constantini et al., 2002).

Il-6 gene is located on chromosome 7 and more than 150 different gene polymorphisms have been identified. The presence of SNP in the promoter region at position -174 has been extensively studied for its effect on HCV outcome with mixed results. Two phenotypes G/G and G/C are associated with high circulating IL-6 while C/C is associated with low circulating IL-6. Barret et al found that low circulating IL-6 phenotype was associated with SVR, whereas presence of G/G and G/C SNPs were associated with persistent infection which became more apparent when combined (Barrett et al., 2003). Counter-intuitively in another study Nattermann et al showed that genotype associated with high circulating IL-6 CC is associated with higher SVR rates following treatment (Nattermann et al., 2007). Yee et al subsequently examined IL-6 promoter region haplotypes in

relation to treatment response and spontaneous viral clearance. They found that specific IL-6 haplotype combinations are associated with decreased chance of spontaneous clearance, however did not comment on whether there is correlation with the levels of IL-6 produced (Yee et al., 2009).

As cytokine gene expression is often a complex process involving multiple transcriptional and post translational events, it is difficult to associate a multi-step process with a single nucleotide polymorphism. This highlights the need for evaluating such SNPs in combination rather than individually.

1.15.5 Role of chemokine gene polymorphisms in HCV infection

Chemokine receptor polymorphisms have also been studied. In HCV, no studies have identified gene polymorphisms associated with HCV outcome. Gene wide association studies have reported that CCR5 and CXCL5 polymorphisms are associated with chronic HCV but the data are controversial (Woitas et al., 2002). One study in an Irish cohort of women acquiring HCV via blood transfusion has shown that CCR5d32 variant (with 32bp depletion) is associated with SVR (Goulding et al., 2005).

1.15.6 Role of Major histocompatibility complex genes in the outcome of HCV infection

Host genetic factors may influence the immune responses to various pathogens. The major histocompatibility complex (MHC) genes regulate both innate and adaptive immune responses in HCV infection. They are located on chromosome

6 and are among the most polymorphic in human genome. These genes encode human leucocyte antigen (HLA) class I and II molecules which are responsible for effective presentation of antigen to T cells for recognition via T cell receptors. HLA class II presents HCV antigens to CD4+ T cells while HLA class I presents HCV antigens to CD8+ T cells.

Several HLA polymorphisms have been demonstrated to influence the outcome of HCV infection. This includes many class II alleles and haplotypes associated with spontaneous resolution. The HLA alleles most commonly associated with spontaneous HCV clearance are DQB1*0301 and DRB1*1101, which positively influence the presentation of immune-dominant HCV epitopes to T cells (Cramp et al., 1998, Minton et al., 1998, Yee, 2004, Mangia et al., 1999, Alric et al., 2000). This observation was found to be consistent across all HCV genotypes and other ethnically diverse study populations (Yoon et al., 2005, Yenigun and Durupinar, 2002, Vejbaesya et al., 2000).

There is less data reporting polymorphisms of HLA class I, as opposed to class II, alleles in the outcome of HCV infection. HLA class I molecules both present antigen to CD8+ T cells and are cognate ligands for NK cell receptor. Genetic variations in HLA class I locus can influence responses of both innate and adaptive immune responses to HCV. Thio et al performed molecular genotyping of HLA class I loci in 231 individuals with well documented spontaneously resolving HCV infection and 444 matched chronically infected patients. They found HLA class I alleles HLA-A*1101, HLA-B*57 and HLA-Cw*0102 to be associated with viral clearance and HLA-Cw*04 with viral persistence (Thio et al., 2002). HLA-B*27 has been shown to be associated with viral clearance in a cohort of Irish women who acquired genotype 2b from contaminated Rhesus-D

immunoglobulin (McKiernan et al., 2004). At a functional level, they later found greater IFN-γ secretion from HLA-B27 restricted CD8+ T cells amongst those with spontaneous clearance of infection (Neumann-Haefelin et al., 2006).

1.15.7 NK cell receptor polymorphisms in HCV infection

The role of NK cells in the outcome of HCV infection is described in section-. NK cell function is regulated by a combination of regulatory receptors which interact with MHC molecules and other ligands on a variety of cells with infections or tumours, and the net balance of signals will determine the threshold at which NK cells will be activated.

KIR genes, amongst NK receptor genes are the most polymorphic. There are 14 KIR genes located on chromosome 19, encoding for both inhibitory and activating receptors (Kulkarni et al., 2008). These genes are highly polymorphic at the allele and haplotype level which generates a substantial level of population diversity in expression of KIR. In addition, the MHC class I molecules, which are ligands of KIR receptors, include HLA-A, B and C are encoded on chromosome 6. These molecules are additionally highly polymorphic. It is possible to have a KIR in an individual which lacks its cognate class – I ligand. This multiple layer diversity of KIR/HLA receptor system provides NK cells with a broader capacity to response to multiple different organisms in a specific manner. Many association studies have been carried out in role of KIR genetics in infectious diseases. Khakoo et al genotyped KKIR genes and their corresponding HLA in cases with chronic HCV and spontaneous resolvers. They determined that homozygous KIR receptor KIR2DL3, and its corresponding class 1 HLA ligand HLA-C1 was found to be

associated with spontaneous resolution of HCV infection. KIR2DL2 is another ligand for HLA-C1, however there was no association found between infection resolution and the compound KIR2DL2/HLA-C1 genotype (Khakoo et al., 2004). However, this effect was only shown in individuals that acquired HCV infection through small inoculums by injection drug use or needle stick injuries as opposed to through blood transfusion thereby suggesting a threshold of HCV inoculum beyond which this immune defence mechanism could be overcome.

Knapp et al validated the role of KIR and HLA-C in individuals achieving SVR following treatment for HCV and individuals spontaneously clearing infection. They demonstrated that KIR2DL3/HLA-Cw*03 was associated with SVR and spontaneous resolution of HCV infection (Knapp et al., 2010).

In conclusion, there is a great polymorphic diversity and a number of genetic variations and haplotypes may be associated with different HCV outcomes. How this affects outcomes at a functional level, however still needs to answered.

1.16 Immune responses and antiviral treatment

Most previous studies evaluating immune responses on antiviral treatment and immune predictors of treatment outcome are based on interferon alpha based treatment.

1.16.1 Innate responses and antiviral treatment

The co-existence of high viral loads and high ISG expression has been interpreted as a proof that innate interferon response in ineffective against HCV. A higher hepatic ISG expression is predictive of a non-response to exogenous IFN-α treatment (Sarasin-Filipowicz et al., 2009, Afdhal et al., 2011) suggesting refractoriness of hepatocytes to any further type I interferon action. A study of paired liver biopsies obtained before treatment and 4 hours after the first injection of pegylated interferon alpha (Peg IFN-α) revealed that patients with an activated endogenous IFN system had hundreds of ISGs expressed at high levels already, which was not increased by exogenous pegylated interferon alpha (Sarasin-Filipowicz et al., 2008). In addition, staining for phosphorylated STAT1 revealed faint staining in pre-treatment hepatocyte nuclei which did not increase after administration of Pegylated IFN-α. In contrast, no phospho-STAT1 signals were detected in pre-treatment biopsies of 'treatment responder' patients, but Peg IFN-α injections induced a very prominent and strong activation and nuclear translocation within 4 hours (Sarasin-Filipowicz et al., 2008).

Similar to the predictive value of high pre-treatment ISG levels, NK cell responses have also been studied to evaluate predictors of treatment response. Higher pre-treatment levels of inhibitory receptors, such as NKG2A predict treatment failure with IFN-alpha based therapy (Golden-Mason et al., 2011). In addition, dynamic changes in NK cells are observed during such treatment demonstrating an association between higher NK perforin content, higher natural and antibody dependent NK cell cytotoxicity with a virological response (Oliviero et al., 2013). Also, treatment responders show greater levels of NK cell degranulation than

non-responders in first 12 weeks of therapy (Edlich et al., 2012, Ahlenstiel et al., 2011).

As described in section 1.12.6, there is a net “functional dichotomy” of NK cells in chronic HCV infection due to chronic exposure to endogenous IFN-α characterised by failure to produce IFN-γ and TNF-α while demonstrating enhanced cytolytic activity leading to persistence of HCV infection. IFN-α based therapies further accentuate NK cell dichotomy, resulting in decrease in IFN-γ production early in treatment which does not recover for at least several weeks after treatment (Ahlenstiel et al., 2011).

Recently, subsequent to the development of new interferon free direct acting antiviral therapy, Serti at al demonstrated that the expression of several NK cell activating receptors decreased to normal levels within hours of commencement of treatment with direct acting antiviral agents including NS3 protease inhibitor and NS5A inhibitor. IFN-γ production was also shown to normalise by week 2 of therapy, whereas markers of cytotoxicity lagged behind to normalise by treatment week 8 (Serti et al., 2015).

Further studies are required to establish the effect of new interferon free direct acting antiviral treatments on innate immune signalling and NK cell profile in treatment responders and those who fail treatment.

1.16.2 T cell immune responses and antiviral treatment

As discussed in section 1.8, pegylated interferon-α based therapies were standard of care for treatment of chronic HCV until 2014 when second generation direct acting antiviral agents became available.

Previous studies on HCV specific CD8+ T cell function have demonstrated lack of restoration of T cell function following treatment with pegylated type Interferon-α (Badr et al., 2008, Seigel et al., 2013, Abdel-Hakeem et al., 2010). Barnes et al demonstrated that there was a profound decline in IFN-γ secreting HCV specific T-cells during high dose IFN-α treatment, although noted that these responses recovered to baseline after cessation of treatment (Barnes et al., 2009). This indicates that restoration of T cell responses is unlikely to be linked to an early response or sustained virological response to IFN-α based therapy. Further studies showed lack of complete restoration of antiviral T cell function following antiviral therapy with pegylated interferon-α in chronic hepatitis C (Missale et al., 2012, Abdel-Hakeem et al., 2010), suggesting irreversible damage to the HCV specific memory T cell response associated with chronic HCV infection.

In recent years, there has been a significant development of potent new direct acting interferon free antiviral therapy regimens which lead to sustained viral response rates reaching almost 100%. These therapies have been discussed in section 1.8. It has been observed that detection of low levels of HCV RNA at the end of treatment does not preclude achievement of sustained virological response with these treatment options suggesting role of restored innate and adaptive immune responses contributing towards ongoing treatment success and prevention of relapse. There have been some initial recent studies suggesting

positive effect of DAA therapy on T cell mediated immune responses. Fard et al demonstrated an increase in overall circulating T helper and T cytotoxic cells producing IFN-γ, IL-17 and IL-22 (Najafi Fard et al., 2018). Burchill et al studied T cell phenotype in patients undergoing DAA treatment when they showed re-differentiation of memory T-cells towards a more effector phenotype and a reduction in expression of co-inhibitory molecule TGIT on bulk lymphocytes (Burchill et al., 2015). Meissner et al also demonstrated a significant increase of CD4+ and CD8+ T lymphocytes in the peripheral blood early after initiation of the DAA treatment, however percentage of both activated cell types with activated phenotype decreased (Meissner et al., 2017). These studies focussed on the bulk T-cell populations without specifically focussing on HCV specific T cells. While there is currently no published data on DAA induced alterations in HCV-specific CD4 T cells, Martin et al demonstrated changes in CD8+ T cell compartment. They demonstrated that while the amount of circulating HCV specific CD8 T cells does not significantly change following DAA therapy, their functional capacity, in particular proliferation, is restored (Martin et al., 2014).

Taken together, although this initial data suggest at least partial restoration of different T cell populations following DAA therapy, the nature and extent of immune restoration with these treatment regimens remain to be fully defined. In addition, the role of immune responses in preventing relapse and sustaining viral response with treatment not containing interferon alpha also remains unclear.

1.17 Aims and Objectives

The aim of this thesis was to analyse the evolution of innate and adaptive immune responses in patients undergoing interferon free anti-viral treatment and to determine if these immune responses play a role in treatment outcomes and preventing viral relapse post-treatment.

Factors determining the course of HCV infection are still poorly understood. Improved understanding of the role that innate and adaptive immune responses play in determining treatment outcomes has the potential to translate into individually tailored treatment, including drugs used and duration of treatment.

A cohort of patients with chronic HCV undergoing interferon free antiviral treatment were recruited, allowing study of longitudinal changes in immune responses whilst on interferon free antiviral treatment.

Innate immune responses were studied in response to single TLR and RIG-I ligands as a model for virus PAMPs to establish if loss of antigenic stimuli results in restoration of these important innate immune signalling pathways. Longitudinal study of such response during treatment will allow to establish if restoration of innate responses contributes towards viral clearance or has a predictive role in determining treatment outcome.

In addition, T cells were evaluated for phenotypic changes characteristic of exhaustion and functional HCV specific T cell responses to evaluate for changes in keeping with recovery of adaptive immune mechanisms following loss of antigenic stimuli with successful interferon free treatment and whether recovery of this immune component contributes towards viral clearance. Furthermore,

longitudinal study of these characteristics throughout the treatment allowed comparison of such responses between treatment responders and treatment relapsers in order to identify patterns predictive of successful treatment outcome.

This study was carried out between 2014 and 2016, prior to the widespread availability of second generation of highly effective DAA agents.

The aims of this thesis were investigated as follows:

1. Frequency and expression of exhaustion markers on CD4+ and CD8+ T cells was studied using flow cytometry to establish if there is restoration of immune cell phenotype following interferon free treatment.
2. HCV specific T cell responses in form of cytokine production was measured using overlapping HCV peptides at baseline, and changes during and after treatment were studied to elicit differences.
3. Comparisons were made between treatment responders and relapsers for changes in phenotypic markers of T cell exhaustion and HCV specific T cell function in form of cytokine production to seek patterns predictive of differing treatment outcomes
4. Cytokine responses were measured in response to HCV related innate immune stimulation by using specific single ligands for RIG-I and toll-like receptors 3, 7 and 8 at baseline and changes whilst on interferon free DAA treatment were studied.

Chapter 2

Material and Methods

2.1 Setting of research

Patients were recruited from the Southwest Liver Unit, Derriford Hospital, Plymouth University Hospital NHS Trust attending as outpatient (Hepatology outpatient clinics or Lind Research Centre) between February 2013 and June 2015 in accordance with appropriate ethical approval.

Healthy volunteer samples matched for age and gender were obtained from colleagues at John Bull Building, University of Plymouth.

All laboratory experiments were conducted either in John Bull Building, School of Medicine and Dentistry or Davy building, School of Biomedical Sciences, at University of Plymouth.

2.2 Ethical approval

Ethical approval was obtained from local Research Ethics Committee (REC). Full written informed consent was obtained from all study participants including

patients (Appendix 1) and healthy volunteers (Appendix 2) which was maintained in the study file stored in a secure location in John Bull Building, School of Medicine and Dentistry.

2.3 Study subject recruitment

2.3.1 Patients

Adult patients with chronic hepatitis C undergoing antiviral treatment under two different protocols were recruited.

In the first protocol, patients recruited were also participants of a randomised multicentre clinical trial using Sofosbuvir (NS5B inhibitor) and Ribavirin with or without pegylated interferon alpha for treatment naïve or experienced patients with chronic hepatitis C genotype 2 or 3 (BOSON trial).

The second protocol included a cohort of patients with advanced liver disease who received interferon free combination direct acting antiviral therapy as part of Early Access Programme for treatment of chronic hepatitis C in England. Treatment regimens included NS5A inhibitor + NS5B inhibitor ± Ribavirin for 12 weeks.

Each study subject was assigned a unique study number to ensure confidentiality. Baseline characteristics of patients were recorded. Heparinised blood samples were used for isolating peripheral blood mononuclear cells (PBMCs) at baseline prior to commencing treatment, and at different time points during treatment and post treatment alongside samples obtained as part of clinical trial protocols.

Study subjects were followed prospectively during treatment on each protocol and further divided into two groups based on treatment response; treatment responders are individuals who achieved sustained virologic response (defined as undetected hepatitis C virus by polymerase chain reaction at 24 weeks after treatment) while treatment relapsers either relapsed after completing treatment or developed viral breakthrough during treatment defined by detectable viral titres/rise in viral titres in serum.

Of the total of 35 patients recruited, 6 were treatment relapsers. One of these 6 patients with relapse was on treatment regimen containing pegylated interferon alpha and was excluded from further study. The remaining 29 of 35 patients achieved sustained virologic response. 12 out of 29 cases matching in baseline and clinical characteristics with treatment relapsers had sufficient PBMCs available at all study time points and were included in this study. Baseline characteristics of all patients included are described in Table 2.1.

Table 2. 1 Demographics and baseline characteristics of cases and healthy volunteers

		SVR cases (n=12)	Relapsers (n=5)	HC (n=10)
Gender	Male: Female	8:4	3:2	6:4
Age	Mean ±SD	45.9 ± 5.0	41.6 ± 8.2	37 ± 9
HCV genotype	1	4	1	
	3	8	4	
Fibrosis	F4	9	4	
	F0-F3	3	1	
Treatment regimen	Sofosbuvir +Ribavirin	3	2	
	Sofosbuvir + Ledipasvir + Ribavirin	2	2	
	Sofosbuvir + Daclatasvir	2	1	
	Sofosbuvir + Ledipasvir	4		
	Sofosbuvir + Daclatasvir + Ribavirin	1		
Relapse time point	Week 24 post treatment		1	
	Week 4 post treatment		4	

2.3.2 Healthy controls

Healthy control heparinised blood samples for isolating PBMCs were obtained from medical school laboratory staff. Healthy volunteers did not have a history of exposure to hepatitis C virus, while majority had negative hepatitis C virus antibody screening within one year of recruitment.

2.4 Materials

2.4.1 Cell culture media

Royal Park Memorial Institute (RPMI) 1640 culture medium was purchased from Lonza Biowhittaker (Lonza, Walkersville, USA).

Supplemented RPMI 1640 (sRPMI) was made by the addition of the following to RPMI 1640: (1) 12.5 ml of 1M Hepes buffer (Lonza Biowhittaker, Walkersville, USA), (2) 3ml of 1M NaOH, (3) 1% Benzyl Penicillin/Streptomycin, (Invitrogen, Paisley, Scotland) (4) 1% L-Glutamine (Sigma, Poole, Dorset, UK).

sRPMI was stored at 4^{o}C and prepared fresh every month.

2.4.2 Serum

Human AB serum

Human AB serum (AB) was purchased from sera labs (Sera laboratories International limited, West Sussex, UK). It was frozen at -20^{o}C in aliquots of 10ml and thawed prior to use.

Foetal calf serum

Foetal calf serum (FCS) were purchased from PAA labs (Pasching, Austria), stored in aliquots of 50 ml at -20^{o}C and thawed prior to use.

2.4.3 Salt solutions

Phosphate-buffered saline (PBS)

Sterile PBS without calcium or magnesium was purchased from Lonza (Lonza Biowhittaker, Walkersville, USA).

Flow cytometry wash buffer (FWB)

FWB was made using PBS supplemented with 2%FCS, 0.5M Ethylenediaminetetraacetic acid (EDTA; Life technologies).

2.4.4 Reagents

Histopaque

Histopaque – 1077 used for PBMC isolation was purchased from Sigma (Sigma, Dorset, UK).

Dimethylsulfoxide

Dimethylsulfoxide (DMSO) was used as cryoprotectant to reduce ice formation and preventing cell death during slow freezing process of PBMCs. It was purchased from Sigma (Sigma, Dorset, UK).

2.4.5 Cell viability reagents

These fluorescent reagents were used to exclude dead cells from flow cytometry analyses by labelling non-viable cells either when fresh or frozen.

Live/dead reagent

Live/dead near infra-red (Life technologies) was reconstituted in dimethylsulfoxide (DMSO) according to the manufacturer's instructions and stored in aliquots at -20$_{o}$C. All work used a 1:1000 dilution in PBS, which was prepared prior to use.

7-Aminoactinomycin (7AAD)

7AAD (Life technologies) is used for exclusion of non-viable cells in flow cytometric analysis. Fluorescence is detected in the far red range of spectrum (650nm). It was reconstituted in 1ml ethanol and stored at -20$_{o}$C in aliquots. Stock was diluted to working concentration on 1:300 in FWB.

2.4.6 Cell counting reagents

Cell counting solution for freshly isolated PBMCs contained 1:50 1M acetic acid and 1:500 0.4% Trypan blue solution (Sigma-Aldrich, Dorset, UK) made up to a volume of 250ml with distilled water.

Cell counting solution for frozen PBMCs contained 1ml ml 0.4% Trypan Blue and 4ml sRPMI.

Trypan blue can only cross the cell membrane of dead cells staining them blue, allowing the distinction of dead from viable cells under a light microscope.

2.4.7 Drugs for PBMC stimulation experiments

Polyinosinic-polycytidylic acid (Poly (1:C))

Polyinosinic-polycytidylic acid (Poly (I:C)) was purchased from Invivogen (InvivoGen, San Diego, USA). It is a synthetic analogue of viral replication intermediate double stranded RNA (dsRNA) which is recognised by Toll like receptor 3 (TLR 3) (Alexopoulou et al., 2001, Matsumoto et al., 2002).

It is provided lyophilised, and was resuspended in sterile endotoxin free physiological water to prepare stock solution of 1mg/ml and stored in aliquots at – 20^{o}C.

Resiquimod (R848)

R848 was purchased from Invivogen (InvivoGen, San Diego, USA). It is an imidazoquinoline compound which activates immune cells via Toll like receptors 7 and 8 (TLR 7/TLR 8) (Hemmi et al., 2002, Jurk et al., 2002).

It is provided lyophilised, and was resuspended in sterile endotoxin free water. Upon resuspension, aliquots were prepared and stored at -20^{o}C.

Poly(I:C)-LMW/Lyovec complex

Polyinosinic-polycytidylic (Poly(I:C)) and Lyovec were purchased separately from Invivogen (InvivoGen, San Diego, USA). Lyovec is an efficient transfection reagent. Poly (I:C) complexed with Lyovec allows transfection of Poly (I:C) into the cells to allow stimulation of cytoplasmic RIG-I pathway (Gitlin et al., 2006, Kato et al., 2005).

Lyophilised Lyovec was resuspended in sterile de-ionised endotoxin free water and placed at 4^{o}C in aliquots. Lyovec was brought to room temperature prior to use and gently vortexed to homogenise. Poly(I:C) was added to Lyovec at concentration of 3μg poly(I:C)/100μl Lyovec and incubated at room temperature

for at least 15 minutes to allow formation of the complex prior to use in stimulation experiments.

2.5 Sample preparation

2.5.1 Sample collection

All peripheral blood samples were obtained by a trained phlebotomist at Derriford Hospital, Plymouth using standard venepuncture techniques. Blood was obtained in 2 x 9 ml sodium heparin tubes (BD, Becton Dickinson and company), which are coated on the inside wall with sodium heparin acting as anticoagulant to block clotting cascade allowing separation of peripheral blood mononuclear cells (PBMCs).

2.5.2 Peripheral blood mononuclear cell (PBMC) isolation

PBMCs were isolated from whole blood using density gradient centrifugation. 15 ml of blood obtained in sodium-heparin tubes (BD, Becton Dickinson and company, USA) was diluted 1:1 with sterile Phosphate buffered saline (Lonza Biowhittaker, Lonza, USA) in a 50 ml polypropylene falcon tube (Greiner Bio One, UK). The diluted blood was then gently layered on top of 15ml Histopaque -1077 (Sigma Dorset, UK), using a Pasteur pipette (Fisher scientific, USA). Care was taken to avoid disrupting the surface interface with Histopaque. The Falcon tube was then centrifuged for 30 mins at 750g with no brake applied (Heraeus labofuge centrifuge 400R, Newport Pagnel, UK). The centrifugation resulted in the

separation of sample into 3 layers. The uppermost layer, composed of serum was carefully removed and discarded into 5% Virkon. The second layer of opaque interface above the histopaque -1077, containing mononuclear cells was gently aspirated using Pasteur pipette into a 30ml universal container (Greiner Bio-one, Gluocestershire, UK). The supernatant was then washed with 10ml of supplemented RPMI (sRPMI) medium and centrifuged at 750g for 10 minutes with the brake activated. The supernatant was discarded into 5% Virkon. Remaining cell pellets was further re-suspended in 10ml sRPMI. This process was repeated once more. The pellet following third wash was re-suspended in 1.5 ml of supplemented foetal calf serum (FCS) and counted.

2.5.3 PBMC counting and viability testing

Freshly isolated PBMCs were counted using a counting solution containing 1M Acetate and 0.4% trypan blue (as described in section 2.4.6). The PBMC suspension was diluted to 1:20 with counting solution (25µl PBMC cell suspension in 475µL of counting solution). An Improved Neubauer haemocytometer with haemocytometer cover slip were used for cell counting and viability testing. The filling chamber was gently filled with PBMC and counting solution suspension prepared as above. Using a light microscope (AE 2000, Motic) at x100 magnification all cells within the 4 quadrants containing 16 grid squares were counted with a hand tally counter. This corresponded to number of cells in 10^{-4} ml. The number of cells in 1ml was thus calculated based on a dilution factor of 1:20. Trypan Blue is not absorbed by viable cells but traverses the membrane in dead cells, thereby staining dead cells blue giving them distinctive

blue colour under the microscope. Since live cells are excluded from staining, this is called dye exclusion method. The percentage of viable cells was calculated as the number of viable cells divided by the total of the number of non-viable and viable cells multiplied by 100.

2.5.4 Freezing and storage of freshly isolated PBMCs

The freezing solution, consisting of 3 parts of supplemented RPMI and 2 parts DMSO was prepared at least 15 minutes prior to use to allow cooling to ambient temperature. 250µL of freezing solution was added to each 1ml cryotube (Sarstedt, Germany). Freshly isolated PBMCs were re-suspended in FCS at a concentration of 5-15 x 10^6 per 750µL. 750µl of PBMC suspension was added to each cryotube already containing freezing solution. The cryotubes were then placed immediately in a Nalgene cryofreezing container (Thermofisher Scientific, Denmark) and stored at -80^{o}C for 24 hours. This allowed freezing at a rate of 1^{o}C per minute. After 24 hours the cryotubes were placed in liquid nitrogen for long term storage (BOC cryospeed/CRY/00809/APUK/0205/7.5 M). All cryotubes were labelled with the South West (SW) number of the case, date of freezing, number of PBMCs in the tube and treatment time point. All cryotubes were indexed in laboratory liquid nitrogen database with details included on cryotube labels to allow quick retrieval when required.

2.5.5 Thawing of PBMCs

Supplemented RPMI with 10% FCS (sRPMI/10% FCS) solution was removed from the fridge and warmed in a water bath at 37$_o$C. For each sample 10 ml of sRPMI/10%FCS was added to 15ml Falcon tube. The cryotubes with PBMCs to be thawed were removed from liquid nitrogen and placed on ice. Cryotubes were thawed individually by placing in 37$_o$C water bath until majority of the contents had thawed and only a small piece of ice was left in the tube. The outside of the cryotube was sprayed with 70% ethanol and dried. This partially thawed suspension was immediately transferred to a 15ml falcon tube. 1 ml of warm sRPMI/10%FCS was added dropwise with slow shaking of universal tube. A further 2ml of warm medium was added dropwise using the same technique in order to allow slow thawing of PBMC suspension. This was repeated to a final volume of 10mls. The cell suspension was then placed in the water bath at 37$_o$C for 20 minutes. The suspension was later centrifuged at 750g with the brake activated for 10mins and the supernatant containing DMSO was discarded. The cell pellet was then gently resuspended in 1ml of sRPMI/10%FCS.

A counting solution of 160μl of sRPMI to 40ul of 0.4% Trypan blue (1:4 Trypan blue: sRPMI) was then used. 190ul of counting solution and 10μl of PBMC suspension was added to a 500μl Eppendorf tube and mixed by pipetting. 10μl was loaded onto and improved Neubauer Haemocytometer and cover slip. Cells in two of the 4x4 squares were counted using manual tally counter. The viability was calculated as before by dividing the number of viable cells by the total number of cells (viable and trypan blue stained) and expressed as a percentage.

This technique of thawing consistently resulted in cell viability greater than 98%. Any thawed PBMCs below this threshold were not used for experimental assays.

2.6 T cell exhaustion phenotyping using flow cytometry

2.6.1 Principles

Four colour staining of PBMCs was performed to determine total CD3+ T cell population, CD8+ T cells and expression of exhaustion markers Programmed cell death protein 1 (PD-1) and Galectin-9 using flow cytometry. Frozen cells in liquid nitrogen were used for these assays. Thawing of these PBMCs, viability testing and counting was carried out using methodology described in section 2.6.6.

2.6.2 Flow cytometer characteristics

The coulter Epics XL MCL flow cytometer (Beckmann Coulter, High Wycombe, UK) was used for these experiments. It had a laser emitting light at 488nm wavelength with four fluorescent detector channels with a detection range of 200 to 800nm allowing the simultaneous detection of signals from 4 fluorescent dyes bound to the cells of interest. The fluorescent dyes that were compatible with emission detection wavelength of the detectors dictated which fluorescent-conjugated antibodies could be used for these experiments. Spectra for individual channels for the Epics XL flow cytometer are listed in Table 2.2.

Table 2. 2 Epics XL Flow cytometer channel wavelengths and compatible fluorochromes

Channel Name	Wavelength (nm)	Fluorescent dye emission detected
FL1	525	FITC
FL2	575	PE
FL3	620	PCeF710
FL4	675	PE-Cy5

2.6.3 FACS antibody panels selected for cell surface and intracellular staining

CD3+Lymphocytes were identified by using fluorochrome bound antibody CD3 PerCP -eFluor710 (eBiosciences 46-0037). CD8+ T cells were identified by CD8 FITC (BD 555366). Cells which stained positive for CD3 and negative for CD8 were identified as CD4 + T cells.

PD-1 PE fluorochrome bound antibody was used to determine expression of inhibitory receptor PD-1. Intracellular expression of Galectin 9 was determined using PE fluorochrome bound Galectin – 9 antibody.

Two separate antibody panels for staining was used for each case and control sample as in table 2.3.

All experiments were conducted using appropriate single colour compensation controls to identify and correct for spill over into other detectors. Fluorescence minus one (FMO) controls were used to determine the negative population to allow best gating for analysis.

Table 2. 3 List of flow cytometry antibodies used in each panel

Antibody	Fluorescent label	Dilution	Supplier
	Panel 1		
CD3	PCeF710	1:20	eBiosciences
CD8	FITC	1:20	BD
PD-1	PE	1:20	BD
Live/Dead far red		1:1000	Thermofisher
	Panel 2		
CD3	PCeF710	1:20	eBiosciences
CD8	FITC	1:20	BD
Galectin - 9	PE	1:20	BD
Live/Dead far red		1:1000	Thermofisher

2.6.4 Antibody titration

All antibodies were titrated with 3 concentrations (1:10, 1:20, 1:50) based around manufacturer's suggested concentration to find the lowest necessary concentration that could accurately detect the relevant cell population with high specificity.

2.6.5 Sample preparation for flow cytometric analysis

Initial optimisation experiments on PBMCs obtained from healthy controls found no difference in results between freshly isolated PBMCs and previously frozen and thawed PBMCs. PBMCs were thawed on the day of analysis as described in section 2.6.6 and viable cell count obtained. 1 x 10^5 PBMCs from each HCV treatment timepoint being studied was added to two wells in a 96 well V bottom plate; one well to be used for each panel. Same number of PBMCs from healthy controls was added into 9 wells to be used for compensation controls, Fluorescence minus one (FMO) controls and controls for each panel. 200μl FACS wash buffer (FWB) was added to each of the wells and centrifuged at 750g for 2 mins. Supernatant was then discarded and pellets were resuspended in 100μl of Live/Dead far red dye. This was allowed to incubate in the fridge for 30 mins. 100μl of FWB was added to each of the wells and centrifuged at 750g for 2 mins following which supernatant was discarded. All antibodies were prepared in concentration of 1:20 as per manufacturer recommendations. Antibodies for surface staining were diluted in FWB, while antibodies for intracellular staining were diluted in Perm/wash buffer.

2.6.6 Surface staining

Protocol in section 2.6.5 was followed by surface staining of PBMCs. CD3 PCeF710, CD8 FITC and PD-1 PE fluorochrome bound antibodies were added to the relevant samples at manufacturer recommended concentration of 1:20. Samples were then incubated in fridge in dark for 45 minutes. 100 μl FWB was the added to samples to wash any excess unbound antibody and centrifuged at 750g for 2 mins. Supernatant was discarded and 100μl of cytofix/cytoperm (BD) was added and mixed well with pipetting. This was incubated in the fridge for 15-20 minutes. Any excess was removed by adding FWB and centrifuging at 750 g for 2 minutes. Supernatant was discarded and pellets were resuspended in FWB for analyses on flow cytometer.

2.6.7 Intracellular staining of Galectin 9

Appropriate samples for panel 2 staining and FMO for panel 2 were transferred to a fresh 96 well plate and washed with 200μl of FWB by centrifugation at 750g. Supernatant was discarded and each pellet was re-suspended in Perm/wash solution (BD, Becton Dickinson and company) followed by incubation in dark in fridge at 4$_o$C for 15 minutes. This was centrifuged at 750g for 2 mins. Cells were then stained with intracellular antibody for Galectin-9 (made up in BD Perm/Wash) by resuspending the pellet in antibody mixture and incubated at 4$_o$C for 45 minutes. Excess antibody was removed by washing with FWB and centrifugation at 750g for 2 mins. Cells were then resuspended in 200μl FWB for analyses on flow cytometer.

2.6.8 Compensation and gating strategy

PBMCs were gated on forward and side scatter dot plots. Dead cells were excluded by Live/Dead far red staining. PD1 and Galectin 9 expressing cells were determined on CD8+ CD4+ cells within the live CD3+ gate as shown in Figure 2.1.

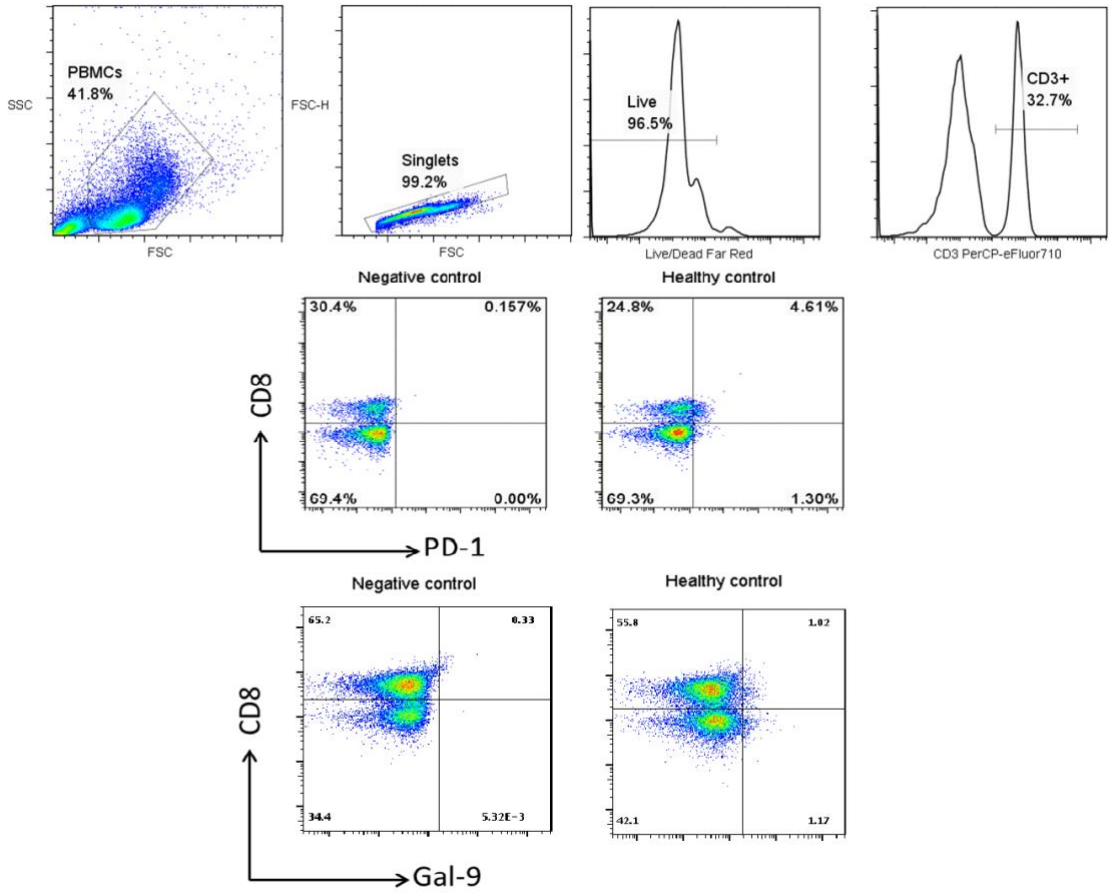

Figure 2. 1 FACS gating strategy

PBMCs were gated on forward and side scatter dotplots. Dead cells were excluded by Live/Dead far red staining. Live cells were gated for CD3. PD1 and Galectin 9 expressing cells were determined on live CD3 gate.

Fluorescence minus one negative control and a case of healthy controls is shown.

2.7 HCV specific T cell responses using Interferon-γ Enzyme-linked ImmunoSpot (ELISpot) assay

2.7.1 Principles of assay

ELISpot assay was used to determine individual T cell response in the form of IFN-γ production to determine reaction to specific antigen. The results are then represented as number of activated cells per million. In this thesis, CEFT peptide pool and Phytohaemagglutinin (PHA) were used as positive control antigens, while HCV overlapping peptides for genotype 1 and 3 were used to determine HCV specific response.

The ELISpot technique is summarised in Figure 2.2.

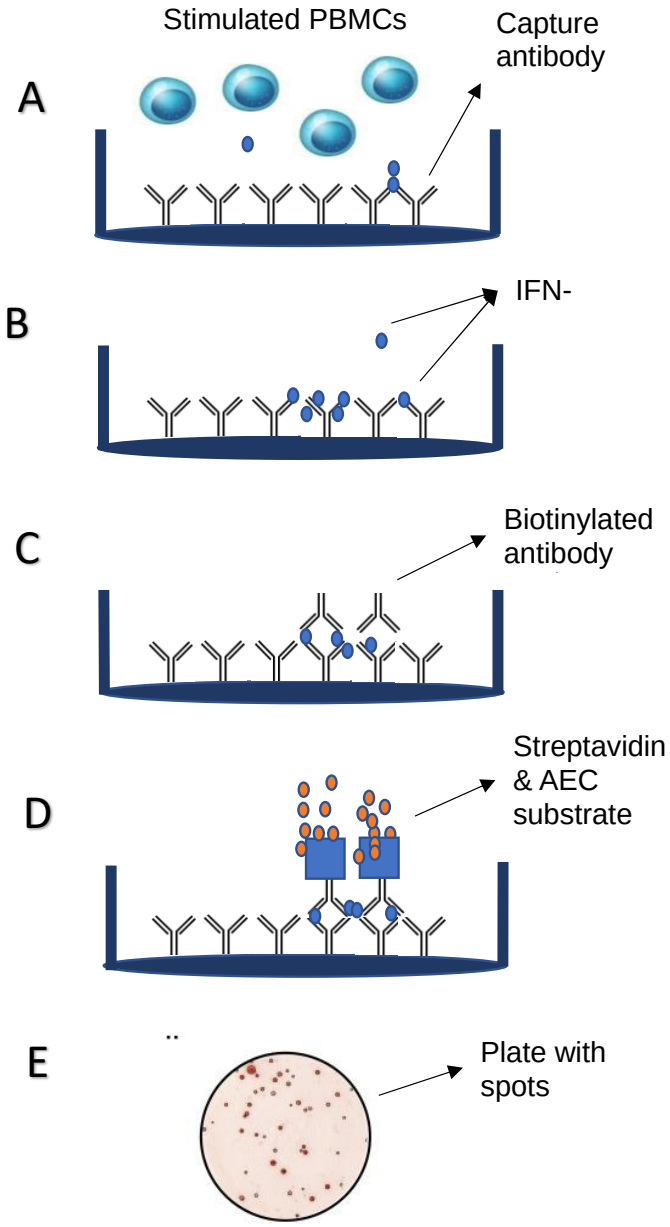

Figure 2. 2 Outline of ELISpot technique

(A) Wells of high membrane binding plate was coated with IFN-γ capture antibody and incubated with antigen exposed PBMCs **(B)** The plate was washed off cells, secreted IFN-γ remained bound to capture antibody **(C)** Incubation with biotinylated antibody forms sandwich complex with primary antibody-IFNγ complex **(D)** Streptavidin and chromogen allows colour development detecting bound IFN-γ **(E)** Spots as seen on plate and detected by ELISpot plate reader as spot forming unit

2.7.2 Antigens

Control Antigens

Pepmix CEFT peptide pool purchased from jpt (jpt peptide technologies, Germany) was used as positive control. This pool consists of 27 peptides, each corresponding to a defined HLA class I or II restricted T-cell epitope from Cytomegalovirus, Epstein-Barr virus, Influenza virus or Clostridium tetani. As majority of the individuals would have been exposed to these organisms, it was expected to generate a reactive T cell clone. It was stored at – 20^{o}C, reconstituted prior to use and diluted with PBS to achieve final concentration of 3μg/ml per 2 x 10^{5} PBMCs.

Phytohaemagglutinin (PHA) (Sigma-Aldrich, Dorset, UK) was used as a mitogen for T lymphocytes and served as a further positive control. It was used at a final concentration of 1μg/ml per 2 x 10^{5} PBMCs.

HCV overlapping peptides

The HCV overlapping peptides for HCV genotype 3a and genotype 1b were obtained from the NIH Biodefense and Emerging Infections Research Resources Repository (BEI resources, Virginia, USA). These consisted of recombinant overlapping peptide arrays spanning the entire genome of HCV genotype 3a and 1b. The lyophilised peptides were reconstituted in DMSO to a stock concentration of 20mg/ml to ensure small volume of DMSO used in cell culture. Subsequently, reconstituted peptide arrays corresponding to structural and non-structural regions were made up into peptide pools to be used as antigens in ELISpot

assays. Peptide pools were stored in aliquots at -20^{o}C and defrosted prior to use. Genotype 3a peptide pools were used for cases with genotype 3 infection and genotype 1b peptide pools were used for cases with genotype 1 infection. All peptide pools were used at a final concentration of 3μg/ml for each constituent peptide. Details of each peptide pool are shown in Table 2.3.

Table 2. 4 Composition of HCV overlapping peptide pools used for ELISpot assay

Corresponding HCV region	Number of peptides in pool	Length of individual peptides (amino acids)	Overlap of adjacent peptides (amino acids)
Genotype 3a			
Core	29	13 – 18	11 – 12
NS3	97	15 – 19	11 – 12
NS5A	72	13 – 19	11 – 12
NS5B	90	14 – 19	11 – 12
Genotype 1b			
Core	28	13 – 19	11 – 12
NS3	98	15 – 19	11 – 12
NS5A	71	13 – 19	11
NS5B	90	14 – 19	11

2.7.3 IFN-γ ELISpot assay kits

Human IFN-γ ELISpot assay kits were obtained from BD Biosciences (Batch no 5266669), including ELISpot plates, purified anti-human IFN-γ capture antibody, biotinylated anti-human IFN-γ detection antibody and streptavidin HRP.

2.7.4 Interferon-γ ELISpot technique

Previously frozen PBMCs were removed from liquid nitrogen, thawed and counted after cell viability assessment using protocol described on section 2.6.6.

Cells were re-suspended in sRPMI/10%AB serum to a concentration of 2×10^6/ml so 100μl suspension has 2×10^5 cells.

A master mix 100μl/well of HCV antigen in form of HCV overlapping peptide mix in a concentration of 6μg/ml was prepared by diluting in sRPMI/10% AB serum. Lectin and CEFT peptides served as positive controls to assess the validity of the assay and culture medium was used in the control wells as a negative control to determine background activity. 100μl of antigen mix was added to each relevant well in a round bottom 96 well plate. 100μl of medium alone (sRPMI/10%AB serum) was added to negative control wells. This was followed by addition of 100μl of PBMC suspension to each well. This allowed the final concentrations of antigen mix to be 1μg/ml for other antigens and 3μg/ml for HCV overlapping peptides. This plate was incubated for 20 hours at 37^oC at 5%CO_2. Replicates of 3 wells per antigen or peptide pool and negative control were used.

On the same day, 96-well high membrane binding plate (MAIPS4510, Millipore, UK) was prepared by adding IFN-γ primary capture antibody (BD Biosciences, Oxford, UK) in a concentration of 5μg/ml (1:200) after diluting in sterile PBS. 100μl per well was added to each well to ensure full coverage in a corresponding layout to that of the 96 well culture plate. This was then incubated in fridge at $4^{o}C$ overnight (16 hours).

On day 2, the primary capture antibody was flicked off from the high membrane binding plate which was then washed once with 200 μl/well of sRPMI/10%AB serum and then blocked for 2 hours at room temperature using 200 μl/well of sRPMI/10% AB serum. After 2 hours, the medium from ELISpot plate was flicked off and cultured PBMCs were transferred to the corresponding wells on the ELISpot plate. This was further incubated at $37^{o}C$ in a 5%CO_2 incubator for 24 hours.

On day 3, PBMCs were flicked off the ELISpot plate and the plate was blotted dry. Each well then washed twice using 200 μl/well of deionised water (diH_20) allowing each well to soak for 3 minutes at each step. Wash solution was flicked off and plate blotted briefly after each wash step. The plate was then washed three times by 200 μl/well PBS with 0.05% Tween 20 (Sigma-aldrich, Dorset, UK) allowing to soak for 1-2 minutes at each step. Wash buffer was discarded. 100μL of secondary detection IFN-γ antibody (BD Biosciences, UK) at a concentration of 2μg/ml (1:250 dilution) diluted in PBS/ 10% FCS was added to each well and further incubated at room temperature for 2 hrs. The addition of FCS was to avoid non-specific binding of the secondary antibody.

Secondary detection antibody was discarded after 2 hours. The plate was then washed 3 times with 0.05% Tween 20 in PBS using 200 μl/well allowing to soak for 1-2 minutes at each wash step. 100μL of Streptavidin- Horse Radish Peroxidase conjugate in PBS/10% FCS (concentration of 10μg/ml) was added to each well and the plate incubated for a further 1 hr at room temperature. Following this, the plate was washed four times with 0.05% Tween 20 in PBS using 200 μl/well at each step followed by washing twice with 200μl/well PBS alone, allowing to soak for 3 minutes at each step. Finally the plate was left to develop by adding 100 μl per well of amino-9- ethyl-carbazole (AEC) substrate (Sigma-Aldridge, Dorset, UK) and incubated in the dark at room temperature for 15 min. The reaction was stopped by immersing the plate in cold tap water. The plate was then thoroughly blotted dry and left in the dark for 3 hrs to develop fully. The individual spots in the wells were quantified using an ELISPOT reader (AID Diagnostika, Starssberg, Germany).

2.8 Stimulation of PBMCs with PRR ligands

2.8.1 Principles

PBMCs were cultured with Toll like receptor and RIG-I ligands to study cytokine response. Single TLR ligands used as a model for virus PAMPs to study cytokine responses. Ligands used were Polyinosinic-polycytidylic acid (Poly (1:C)), Poly (I:C) Lyovec complex and R848. This is outlined in Figure 2.3.

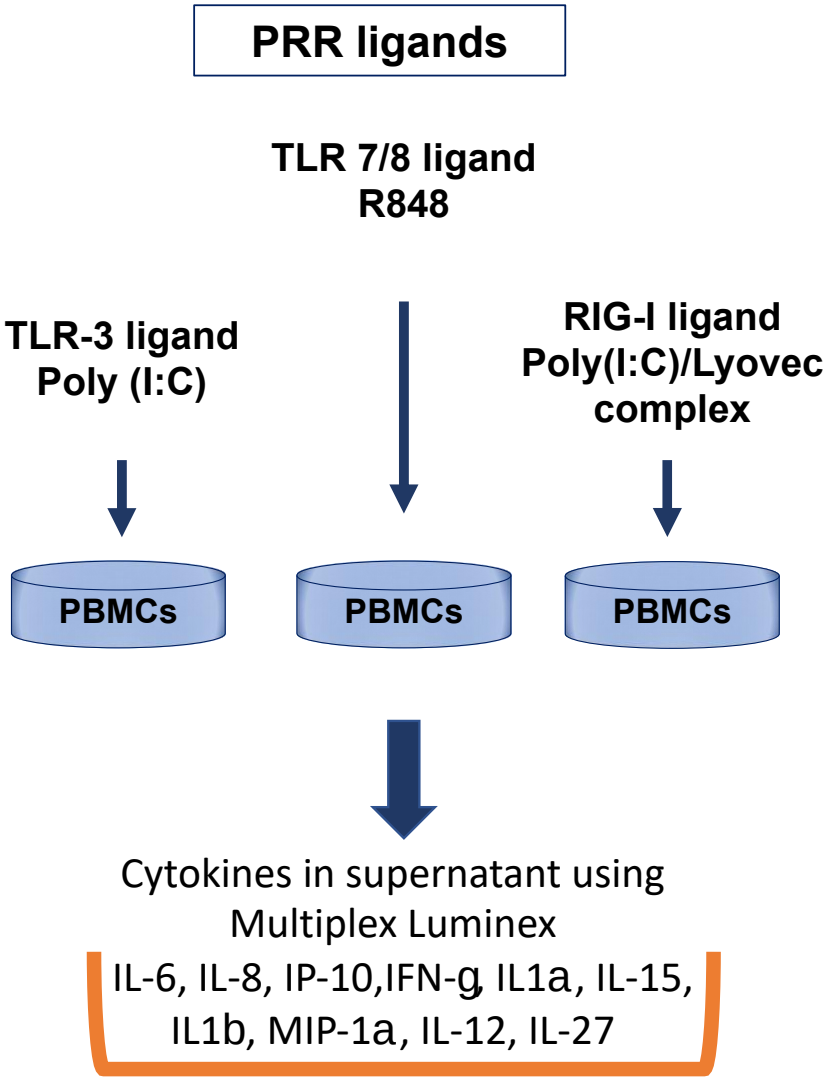

Figure 2. 3 Outline of PBMC stimulation experiments

2.8.2 Technique

Aliquots of PBMCs from patient group at selected timepoints were defrosted and the viability assessed using methodology described in Section 2.6.6. PBMCs were re-suspended in supplemented RPMI/10% FCS. The cell suspension was centrifuged at 750g for 10 minutes. Supernatant was discarded and remaining cell pellet was re-suspended in sRPMI/10% FCS. Cells were counted and 2 x 10^5 PBMCs in 200 μl of sRPMI/10% FCS were added to each well in a flat bottom 96 well culture plate. 20μg/ml Poly I:C and 2μg/ml R848 were added to appropriate experiment wells containing PBMCs. Prepared Lyovec/Poly I:C complexes were used. All experiments were performed in triplicates. Plates were incubated at 37^oC in 5% CO_2 for 16 hours. After 16hrs, plates were removed from the incubator, supernatant was aspirated into RNA-ase free Eppendorfs, and centrifuged at 750g for 5 minutes. Supernatant was aspirated from each Eppendorf, stored in 3 aliquots of 60μl each. These were frozen at -20 for assessment of cytokine secretion.

Table 2. 5 Drug concentration used for TLR3/7/8 and RIG-I stimulation on mixed PBMCs

PBMC stimulation drug	Final concentration
Poly (I:C)	20 μg/ml
R848	2 μg/ml
Poly (I:C)/Lyovec complex	6μg/ml poly(I:C) in 10μl Lyovec

2.8.3 Optimisation experiments for PBMC stimulation with PRR ligands

Different experimental conditions were considered when planning stimulation of PBMCs with Poly (I:C), R848 and Poly (I:C)/Lyovec complex and explored in a series of experiments using PBMCs from healthy controls to determine conditions for optimal results. This included:

(1) Number of PBMCs per well, including 10 x 10_3, 50 x 10_3,1 x 10_5 in 200μl sRPMI/10%FCS in each well in 96 well plate. 2 x 10_5 PBMCs were cultured with stimuli in 200μl sRPMI/10%FCS in each well in 96 well plate and 500μl sRPMI/10%FCS in each well in 24 well plate.

(2) Duration of incubation time including 6 hours, 16 hours and 24 hours.

(3) Two concentrations of PRR ligands were used to determine optimal final concentration. Poly (I:C) was used at 20μg/ml and 100μg/ml, R848 at 2μg/ml and 20μg/ml, Poly (I:C)/Lyovec complex at 1μg/ml, 3μg/ml and 6μg/ml.

(4) Exclusion of serial change in response over time in healthy controls. PBMCs obtained from same healthy control at 3 different time points at least 2 weeks apart did not show any difference in production of IL-6.

Output parameter used was measurement of IL-6 and IFN-α produced, using ELISA. ELISA technique used is described in detail in section 2.10.2.

2.9 Luminex magnetic multiplex cytokine bead assay

2.9.1 Principle

Cytokines in supernatant produced by PBMCs in culture with toll like receptor and RIG-I ligands (Poly (I:C), R848, and Poly (I:C)/Lyovec complex were analysed by magnetic multiplex cytokine bead assay using Luminex platform. This technique has advantage over ELISA in that it allows simultaneous measurement of multiple cytokines in a limited sample volume.

Analyte-specific antibodies are pre-coated into colour-coded microparticles. Microparticles, standards and samples are pipetted into wells. Immobilized antibodies bind the analyte of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE) is added to each well. A final wash removes unbound Streptavidin-PE, microparticles are resuspended in buffer and read using Luminex MAGPIX analyser.

A magnet in the analyser captures and holds the superparamagnetic microparticles in a monolayer. Two spectrally distinct Light Emitting Diodes (LEDs) illuminate the beads. One LED identifies the analyte that is being detected and the second LED determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound.

2.9.2 Reagents

Magnetic Luminex multiplex premixed multi-analyte kit including all reagents required was purchased from R&D systems (RD systems, USA)(Kit Lot number: 1372866, microparticle mix Lot number: 1372859, Biotin antibody mix lot number: 1372862). Luminex kit was used to analyse 10 cytokines including interleukin-6 (IL-6), Interleukin-8 (IL-8), Interferon-γ inducible protein-10 (IP-10), Interferon-γ (IFN-γ, Interleukin-1alpha (IL1-α), Interleukin-15 (IL-15), Interleukin-1β (IL1-β), (MIP-1α), Interleukin-12 (IL-12) and Interleukin-27 (IL-27).

2.9.3 Technique

Microparticle cocktail supplied was centrifuged for 30 seconds at 1000 x g prior to removing the cap, followed by gentle vortex to re-suspend the microparticles. The microparticle cocktail is diluted in a mixing bottle with the diluent provided. This microparticle cocktail was prepared within 30 minutes of use and care was taken to prevent exposure to light during handling.

For reconstituting the 4 standards provided, each is reconstituted in calibrator diluent with a volume specified for the kit by the manufacturer in certificate of analysis. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making further dilutions. This will result in 10 x concentrate of each standard cocktail resulting in four 10 x concentrated standards. 100 μl of each standard cocktail is combined with Calibrator Diluent to make a total volume of 1000μl as per manufacturer recommendations to make a single standard which contains 1 x concentration of all the analytes. This standard cocktail is used to

make a 3 fold serial dilution with Standard 1 serving as high standard and Calibrator Diluent as blank.

Cell culture supernatant samples previously stored at -20 C were defrosted for use in assay.

Microparticle cocktail was re-suspended by vortexing and 50 μl was added to each well of the microplate. 50 μl of standard or sample was added to appropriate wells and plate was securely covered with a foil plate sealer. Microplate was incubated at room temperature for 2 hours on a horizontal orbital microplate shaker set at 800 ± 50 rpm. A magnetic plate designed to accommodate the microplate was used to wash the plate. Magnet was applied to the bottom of the microplate, followed by removing the liquid, filling each well with 100 μl of wash buffer and removing the liquid again. Wash procedure was performed for a total of three times.

50 μl of diluted antibody cocktail was added to each well. The plate was securely covered with a foil plate sealer and incubated at room temperature on the horizontal orbital microplate shaker set at 800 ± 50 rpm. Microplate was then washed three times after applying magnet to the bottom of the plate and adding 100 μl of wash buffer and removing the liquid again.

50 μl of Streptavidin-PE was added to each well. The plate was then incubated at room temperature for 30 minutes after covering with a foil plate sealer on the shaker set at 800 ± 50 rpm. This was followed by washing the plate 3 times using 100 μl of wash buffer and magnet applied to the bottom of plate. Microparticles

were then re-suspended by adding 100 μl of wash buffer to each well and incubating on the shaker set at 800 ± 50 rpm for two minutes.

The plate was read on Luminex 200 platform within 90 minutes.

2.9.4 Instrument setting

Luminex 200 was used to read the plate. Microplate region for each analyte being measured was assigned as specified on the certificate of analysis. Further settings were: (1) 50 events per bead (2) Minimum events 0 (3) Flow rate fast at 60 μl/minute (4) Sample size 50 μl (5) Double discriminator gates set at approximately 8000 and 16,500 and (6) MFI was collected.

2.9.5 Calculation of results

Concentration of analytes in the sample was read from the standard curve generated. Standard concentrations for each analyte provided were by the manufacturer in the certificate of analysis and 3-fold dilutions for the remaining levels were calculated. Average of two duplicate readings was obtained. Standard curve was created for each analyte using computer software (χponent software solutions) capable of generating a five parameter logistic (5-PL) curve-fit.

2.10 ELISA for estimation of IL-6 and IFN-α

2.10.1 Principle

ELISA is used to measure an analyte protein, which is initially immobilized on microplate wells bound to a capture antibody, followed by use of another target protein specific enzyme labelled antibody. A substrate added later reacts with enzyme-antibody-analyte complex to produce signal measurable by light absorption.

IL-6 (Invitrogen) and IFN-α ELISA (Mabtech) assays were used to analyse presence of IL-6 and IFN-α in cell culture supernatant during optimisation of conditions for PBMC stimulation with TLR and RIG-I ligands.

2.10.2 Technique

High protein binding plate was coated with 100 μl/well of capture antibody diluted in PBS as per manufacturers recommendation and incubated overnight at 4^{o}C to allow binding process to take place. On day 2, the wells were emptied and plate was washed three times using ELISA wash buffer (PBS and 0.1% Tween 20) and blotted dry. The plate was blocked by adding 200 μl/well of 2% BSA in PBS and incubated at room temperature for one hour. Once blocked, all wells were washed three times and blotted dry on absorbent paper. Standard protein and supernatant samples were added to relevant wells in duplicate and plate was further incubated overnight at 4^{o}C. The following day, wells were emptied, washed three times and blotted followed by addition of biotinylated antibody

diluted in PBS as per manufacturer's recommendations. The plate was incubated at room temperature for 1 hour followed by three washes with PBS. Once dry, 100μl/well Avidin-HRP was added and incubated for 30 minutes at room temperature. A further cycle of three washes was carried out before adding 100μl/well of 3-ethylbenzthiazoline-6-sulfonic acid solution and allowed to develop for 15 minutes. The absorbance was read on a microplate reader with a 405nm filter (Tecan multiplate reader, Tecan Trading, AG Switzerland). A standard curve was plotted by use of known standard concentrations and mean absorbance values. Using linear regression, a best fit line was used for calculation of sample values.

2.11 Statistical analysis

GraphPad Prism version 8 (GraphPad Software, California, USA) and SPSS software version 9 (International Business Machines, IBM, New York) were used for data analysis.

Majority of the data analysed in this group was assumed to be non-parametrically distributed due to small sample size. Mann-Whitney U test was used for comparison of continuous variables between two groups, Wilcoxon-Signed Rank test was used for comparison of paired data. Student t test was used for data with parametric distribution.

Statistical significance was considered with a $p<0.05$ (*$p<0.05$, **$p<0.001$, ***$p<0.0001$).

Chapter 3

Evolution of expression of T cell exhaustion markers during DAA treatment

3.1 Background

Virus specific CD8+ T cells play a central role in the outcome of acute HCV infection. This is evidenced by functional CD8+ T cell responses in spontaneous viral clearance (Rehermann, 2013). Failure of viral clearance leading to chronic infection is associated with T cell exhaustion and viral escape (Bengsch et al., 2010, Klenerman and Thimme, 2012). Most groups have reported an irreversible damage to the HCV specific T cell pool characterised by functional defects, reduced antiviral efficacy, and lack of full CD8+ T cell maturation that cannot be restored by pegylated interferon therapy (Abdel-Hakeem et al., 2010, Missale et al., 2012, Seigel et al., 2013). It has not been established whether this effect is due to chronic viral infection itself or direct inhibitory effects of interferon on lymphocytes (Barnes et al., 2002, Welsh et al., 2012). Recent advances in HCV

treatment that directly target viral replication has allowed the opportunity to analyse the nature of this loss of CD8+ T cell function more clearly.

Exhausted CD8+ T cells overexpress several inhibitory receptors and molecules including PD-1, TIM-3, CTLA-4, 2B4, in addition to changes in T cell receptor and cytoplasmic signalling pathways (Golden-Mason et al., 2009, Kasprowicz et al., 2008, Nakamoto et al., 2008, Radziewicz et al., 2007, Schlaphoff et al., 2011, Rutebemberwa et al., 2008).

Programmed cell death protein 1 (PD-1) is an immunoreceptor containing tyrosine-based inhibition motif expressed on activated T cells that mediates hyporesponsiveness (Radziewicz et al., 2007). Expression of inhibitory receptor PD-1 has been postulated to characterise a state of exhaustion of HCV specific CD8+ T cells in chronic HCV infection (Barber et al., 2006). High levels of PD-1 expression on CD8+ T cells has been identified in patients with chronic HCV infection (Radziewicz et al., 2007). Blockade of PD-1 signalling has shown to result in restoration of HCV specific CD8+ T cell responses (Radziewicz et al., 2007, Penna et al., 2007).

TIM-3 also mediates CD8+ T cell exhaustion along with other inhibitory receptors and is expressed at high levels in patients with chronic HCV infection (Mengshol et al., 2010). Galectin-9 is a ligand for TIM-3. TIM-3 is activated by Galectin 9, allowing TIM-3 to send inhibitory signals to T cell receptor. Binding of Galectin-9 to TIM-3 has shown to induce apoptosis of HCV specific CD8+ T cells (Mengshol et al., 2010).

The aim of this study was to analyse phenotypic changes in expression of exhaustion markers including PD-1 and Galectin 9 within the adaptive immune

compartments of PBMCs at baseline, during interferon free antiviral treatment and after achieving SVR, using flow cytometry.

3.2 Methods

A cohort of patients with chronic HCV infection undergoing treatment with varying interferon free regimens was recruited. PBMCs were isolated from blood samples obtained at baseline (week 0) and during treatment including treatment weeks 2, 4, and end of treatment. Relapse time point samples were acquired in cases of individuals who relapsed. Post treatment samples were obtained from cases achieving SVR at post treatment week 2 (SVR 2), post treatment week 4 (SVR4) and post treatment week 12 (SVR 12). Isolated PBMCs were frozen in liquid nitrogen and thawed prior to preparing for flow cytometry using methodology detailed in Chapter 2.

PBMCs were prepared by staining for CD3 and CD8 as well as for exhaustion markers, which include cell surface exhaustion marker PD-1 and intracellular Galectin-9 using fluorochrome bound antibodies. Staining for CD4 was not used due to the limitation of our flow cytometry equipment to four channels. However, CD3+CD8- cell population was assumed to be CD4+ T cells for the purpose of this study. Expression of PD1 and Galectin-9 was measured on total CD3+ cells, CD3+CD8-cells, and CD3+CD8+ cells.

3.3 Results

3.3.1 Baseline characteristics

6 cases relapsed amongst a total of 36 cases recruited. 1 out of 6 relapse cases received pegylated interferon alpha as part of treatment regimen and was excluded from further analysis. Amongst cases with sustained viral response, only 10 cases could be identified as matching with relapse group in baseline characteristics and the treatment regimen received, which were included in further experiments. Healthy controls were volunteers recruited from healthy laboratory staff members.

Baseline characteristics of study populations are summarised in table 3.1.

Table 3. 1 Baseline characteristics of subjects included in the study of T cell phenotype

		SVR cases (n=10)	Relapsers (n=5)	HC (n=10)
Gender	Male: Female	7:3	3:2	6:4
Age	Mean ±SD	45.9 ± 5.0	41.6 ± 8.2	37 ± 9
HCV genotype	1	4	1	
	3	6	4	
Fibrosis	F4	9	4	
	F0-F3	1	1	
Treatment regimen	Sofosbuvir +Ribavirin	3	2	
	Sofosbuvir + Ledipasvir + Ribavirin	1	2	
	Sofosbuvir + Daclatasvir	2	1	
	Sofosbuvir + Ledipasvir	4		
Relapse time point	Week 24 post treatment		1	
	Week 4 post treatment		4	

3.3.2 T cell expression of PD-1 and Galectin-9 is higher in cases with chronic hepatitis C infection

PD-1 and Galectin-9 expression in the total T cell population (defined as CD3+ cells), CD8+ T cells and CD4+ T cells were studied in all cases with chronic hepatitis C infection included in this study prior to commencing interferon free antiviral treatment for HCV and compared with healthy controls included in each experimental plan.

PD-1 expression on all T cell populations was significantly higher at baseline in cases with chronic HCV infection when compared with healthy controls. This includes cases who later achieved sustained viral response following interferon free antiviral therapy and cases which relapsed following treatment. This was observed on total T cell population identified as CD3+ T cells (p=0.0001), CD8+ T cells (p=0.0001) as well as CD3+CD8- T cells which act as a surrogate for CD4+ T cell population (p=0.001) (Figure 3.1).

Galectin-9 expression was also significantly higher in cases with chronic HCV infection in comparison with healthy controls at baseline (week 0) on total CD3+ T cells (p=0.02) and CD8+ T cells (p=0.01). There was no statistically significant difference in Galectin-9 expression on CD4+ T cells between healthy controls and patients with chronic HCV infection. (Figure 3.2).

These findings are consistent with the concept that chronic HCV infection aligns with signs of T cell exhaustion.

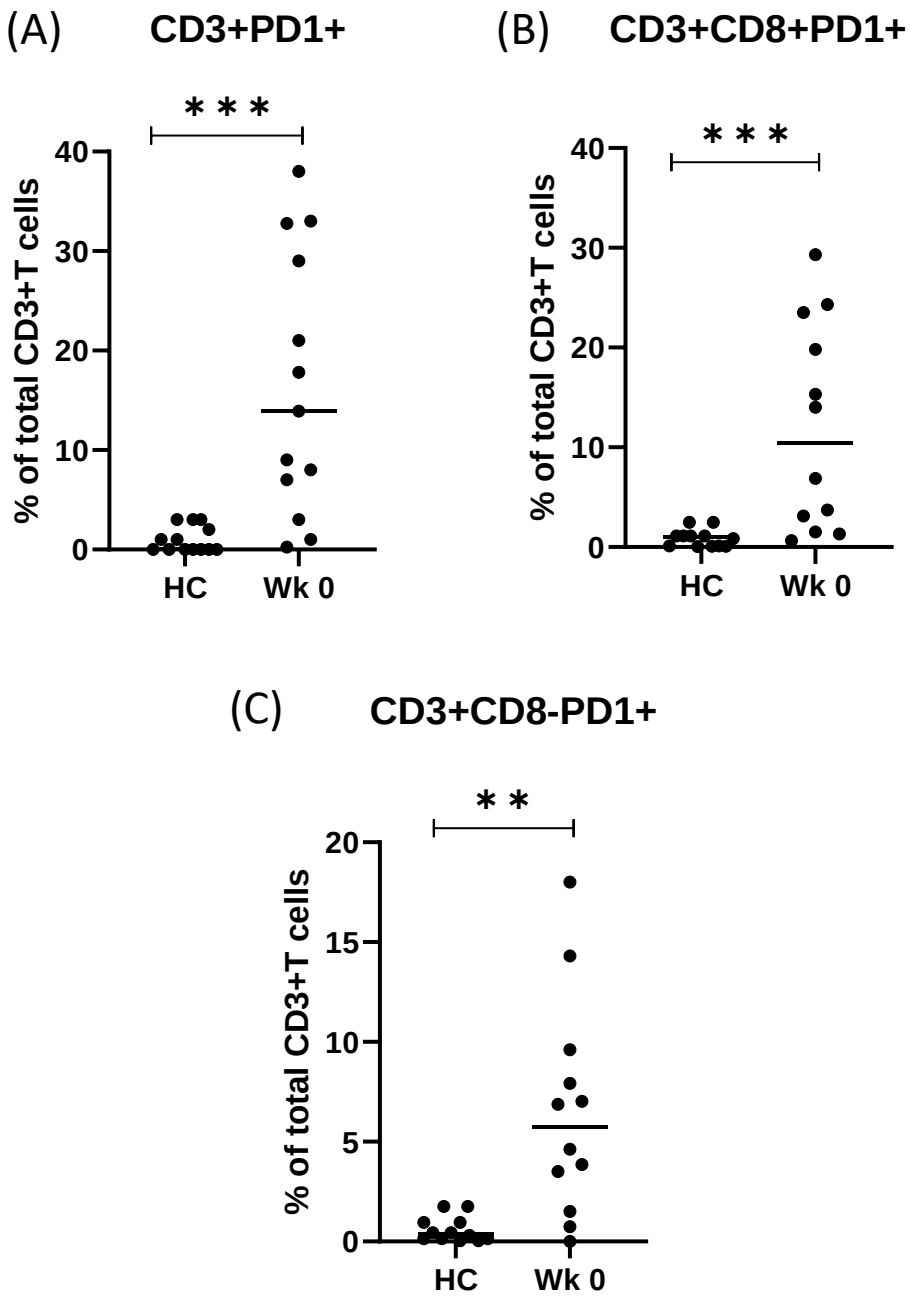

Figure 3. 1 Scatter plot of PD-1 expression on T cell populations

Study groups were Healthy controls (HC, n=12) and baseline samples (Wk0) from chronic HCV cases (n=12). Dots indicate percentage of T cells in each group expressing PD1 with **(A)** representing total CD3+ T cells, **(B)** showing CD8+ T cells and **(C)** representing CD4+ T cells expressing PD1. Horizontal lines represent group median. *p<0.05, **p<0.001, ***p<0.0001.

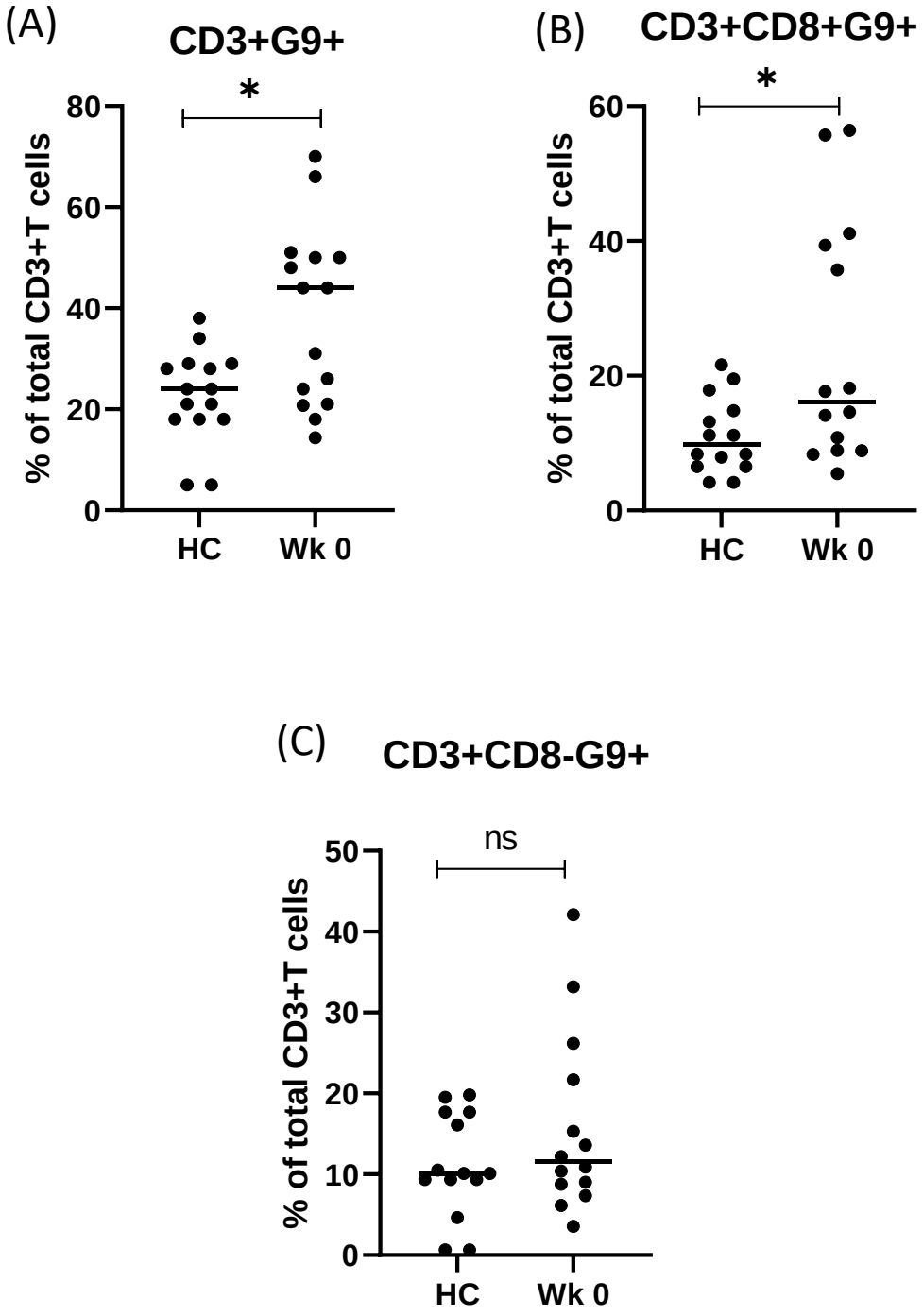

Figure 3. 2 Scatter plot of Galectin-9 expression on T cell populations

Study groups were Healthy controls (HC, n=15) and baseline samples (Wk0) from chronic HCV cases (n=15). Dots indicate percentage of T cells in each group expressing Galectin 9 with **(A)** representing total CD3+ T cells, **(B)** showing CD8+ T cells and **(C)** representing CD4+ T cells expressing Galectin-9. Horizontal lines represent group median. * $p<0.05$, ns not significant.

3.3.3 Decrease in PD-1 expression following SVR with interferon free therapy

PD-1 expression was studied in 8 cases achieving SVR following treatment with interferon free antiviral therapy. Baseline characteristics are described in section 3.2.1. PD-1 expression on T cell populations was quantified using flow cytometry at baseline (week 0) and 12 weeks after completing treatment (SVR12).

There was a significant decrease in expression of T cell exhaustion marker PD1 at SVR12 when compared to pre-treatment expression at week 0 in all cases except one case (SW517) where there was an increase in PD1 expression. This was seen in case of CD3+T cells ($p=0.02$), CD8+ T cells ($p=0.02$), and CD4+ T cells ($p=0.04$) defined as CD3+CD8-Tcells (Figure 3.3).

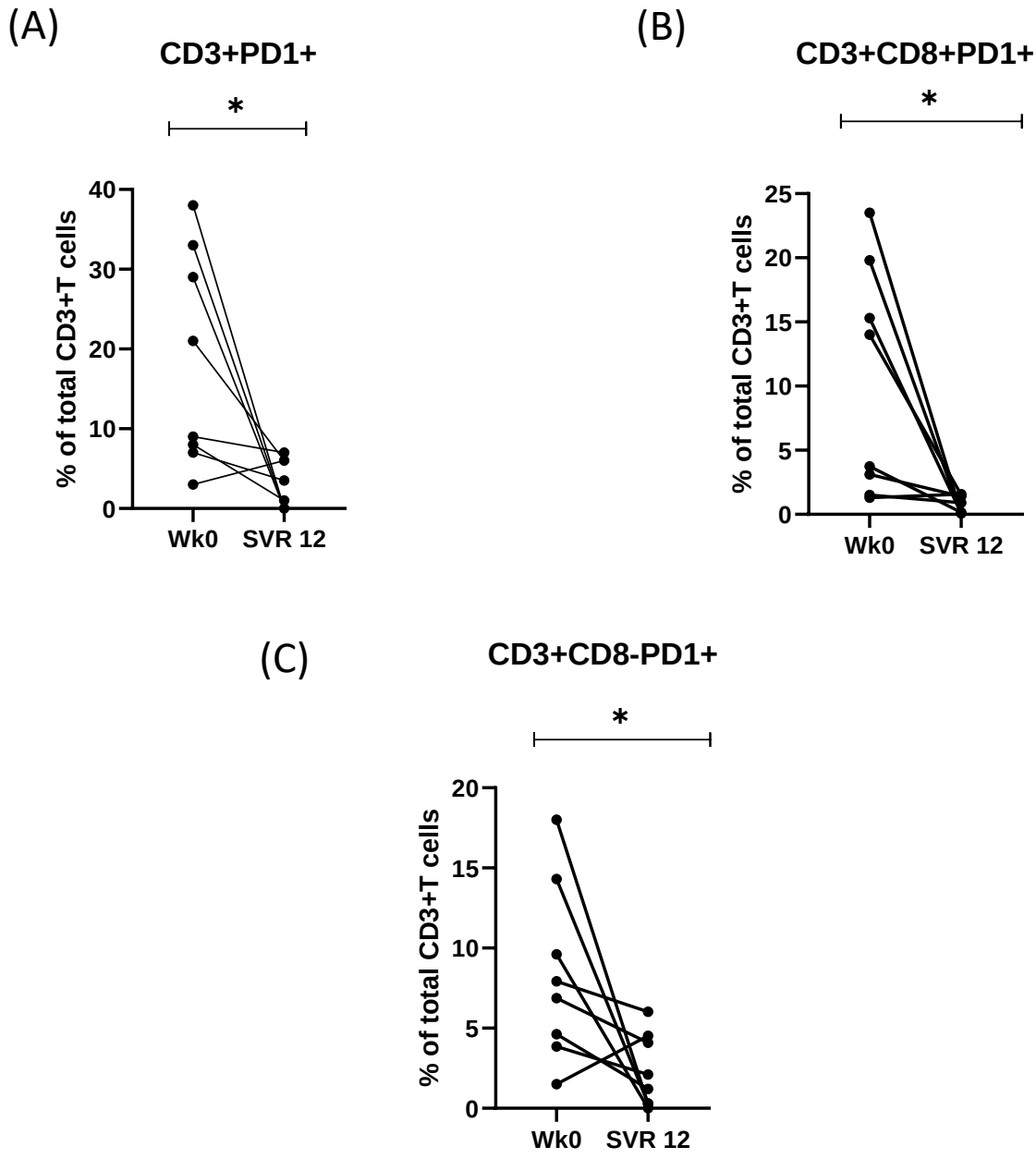

Figure 3. 3 Impact of antiviral therapy on T cell phenotype in CHCV cases achieving SVR (n=8)

Frequency of PD1 expressing T cells measured at baseline (week 0) and 12 weeks after completing interferon free treatment (SVR 12). **(A)** Compares PD1 expression on total CD3+ T cell population in each group, **(B)** represents PD1 expression on CD8+ T cells and **(C)** shows PD1 expression on CD4+ T cells (CD3+CD8-Tcells).* $p<0.05$.

3.3.4 Decrease in Galectin-9 expression following SVR with interferon free therapy

Galectin 9 expression on T cells was also studied in 9 cases achieving SVR with interferon free therapy. This decreased at SVR12 when compared to baseline on CD3+ T cells in all cases studied and the difference in mean reached statistical significance ($p=0.0002$). There was also a significant decline in Galectin 9 expression on CD3+CD8+ T cells on all except one case (SW517) where there was an increase in Galectin 9 expression. This case also showed higher PD-1 expression at SVR12 as explained in section 3.2.2. Six out of eight cases studied showed a decline in expression of Galectin 9 on CD3+CD8-T cells but this was not statistically significant. The remaining two cases showed a slight increase in Galectin 9 expression on only this T cell subpopulation (CD4 T cells) while there was a sharper decline in Galectin 9 expression on CD3+CD8+ T cells in these cases (Figure 3.4).

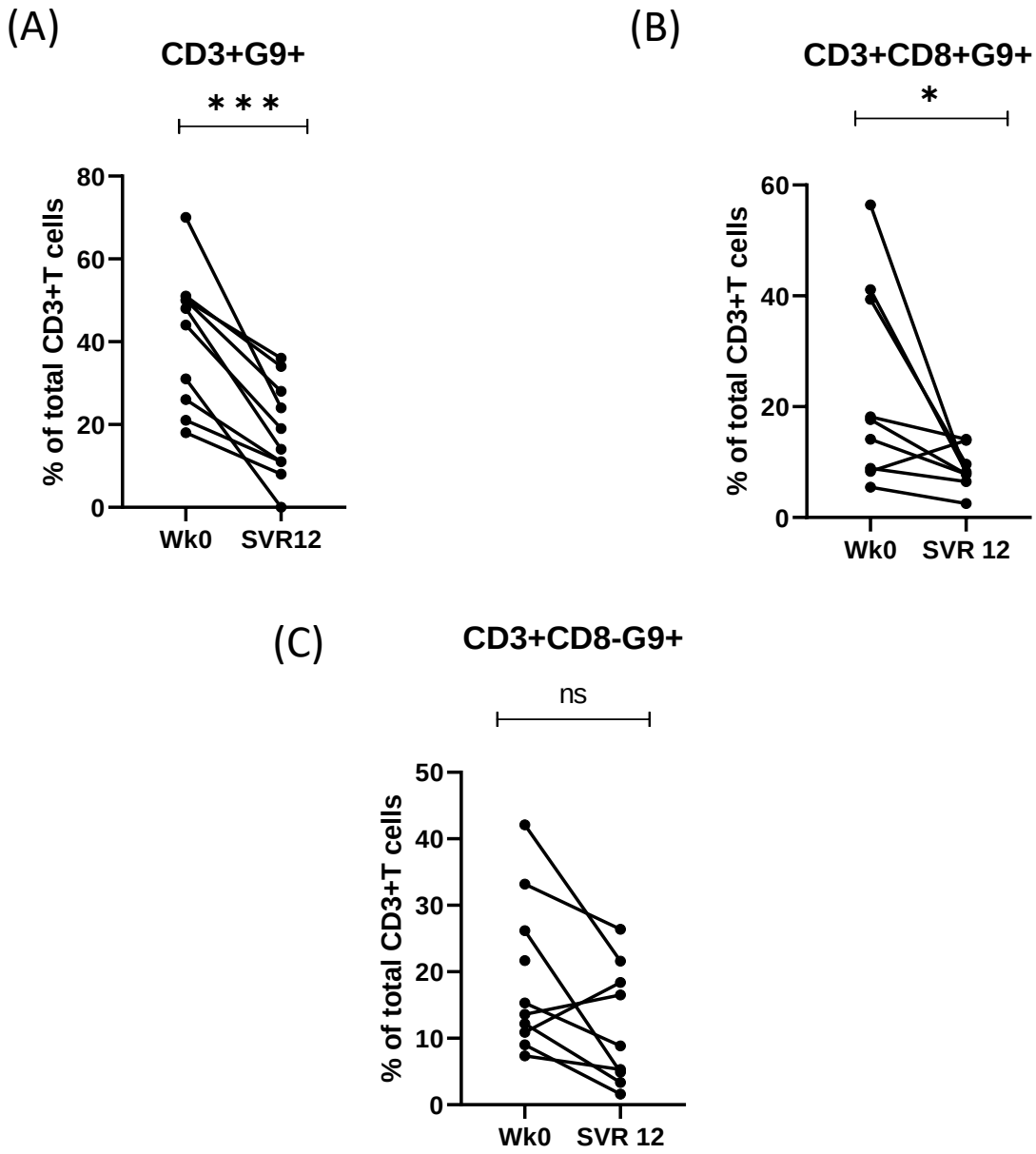

Figure 3. 4 Impact of antiviral therapy on T cell phenotype in CHCV cases achieving SVR (n=9)

Frequency of Galectin 9 expressing T cells measured at baseline (week 0) and 12 weeks after completing interferon free treatment (SVR 12). **(A)** Compares Galectin 9 expression on total CD3+ T cell population in each group, **(B)** represents Galectin 9 expression on CD8+ T cells and **(C)** shows Galectin 9 expression on CD4+ T cells (CD3+CD8-Tcells). *p<0.05, ***p<0.0001, ns not significant.

3.3.5 Baseline expression of PD-1 on T cells in Relapser cases

Following the finding that patients with chronic HCV have increased PD-1 expression relative to healthy controls, we sought to find differences in PD-1 expression after discriminating populations between those we could retrospectively determine were responders or relapsers. Expression of T cell exhaustion marker PD-1 was analysed at baseline (week 0) in five cases who relapsed following interferon free antiviral treatment and compared with cases achieving SVR (responders) and healthy controls. There was no significant difference in expression of PD1 on total CD3+ T cells prior to commencing treatment between cases which relapsed following treatment and those which achieved SVR (Figure 3.5C). There was a non-significant trend towards higher expression of PD-1 on CD8+ T cells while lower on CD4+ T cells in relapsers when compared with cases which achieved SVR, suggesting CD8+ T cells to be the main subtype with exhausted T cells (Figure 3.5 A, B).

Expression of PD-1 was significantly higher in relapsers when compared with healthy controls on total CD3+ T cells and CD8+ T cells (Figure 3.5 A, C). Although median PD-1 expression was numerically higher than healthy controls on CD4+ T cells, it was not statistically significant (Figure 3.5B).

Together, this data indicate that the difference between response and relapse is not related baseline PD1 expression.

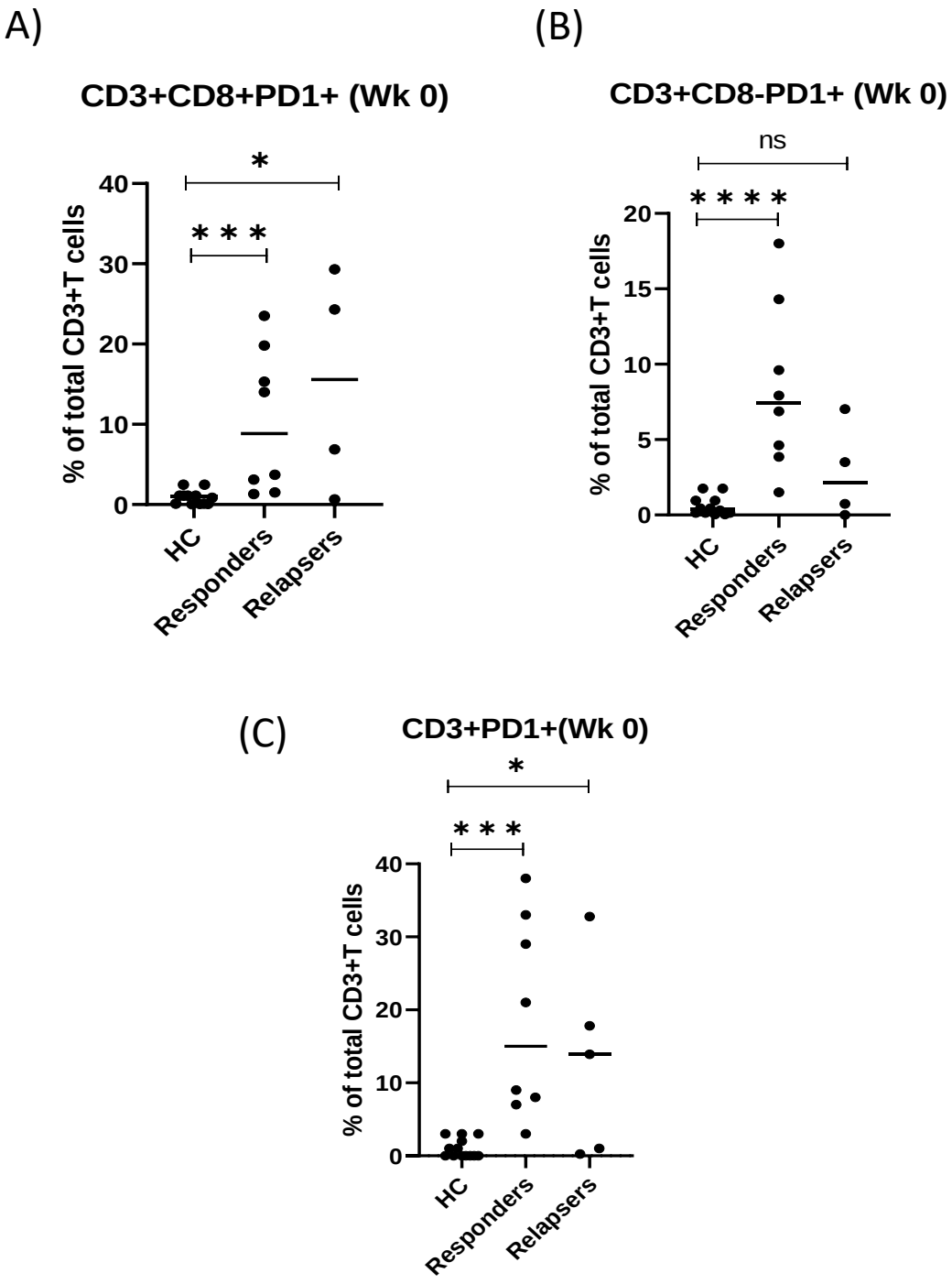

Figure 3. 5 Scatter plots of PD-1 expressing T cell populations in healthy controls, relapsers and treatment Responders

Study groups were Healthy controls (HC, n=13), baseline samples (Wk0) from chronic HCV cases achieving SVR (n=8) and cases which relapsed following interferon free treatment (n=5). Dots indicate percentage of T cells in each group with (A) showing PD1 expressing CD3+CD8+T cells, **(B)** showing PD1 expression on CD4+T cells (CD3+CD8-Tcells) and **(C)** showing PD1 expression on total CD3+ T cells. Horizontal line represents group median. *p<0.05, **p<0.001, ***p<0.0001, ns not significant.

3.3.6 Baseline expression of Galectin-9 on T cells in Relapser cases

Galectin-9 levels were elevated on CD8+ T cells and lower on CD4+ T cells in relapsers at baseline in comparison with responders (Figure 3.6 A, B). The frequency of Galectin-9 expressing cells amongst the total CD3+T cell population was lower in relapsers relative to this population in treatment responders (Figure 3.6 C).

CD8+ T cells also showed a significantly higher expression of Galectin-9 on relapsers when compared with healthy controls, while there was no difference in frequency of Galectin 9 expressing cells between two groups amongst CD4+ and total CD3+ T cell populations (Figure 3.6 A).

This data indicates that viral relapse associates with higher CD8+ T cell expression of Galectin-9 compared to sustained responders to therapy.

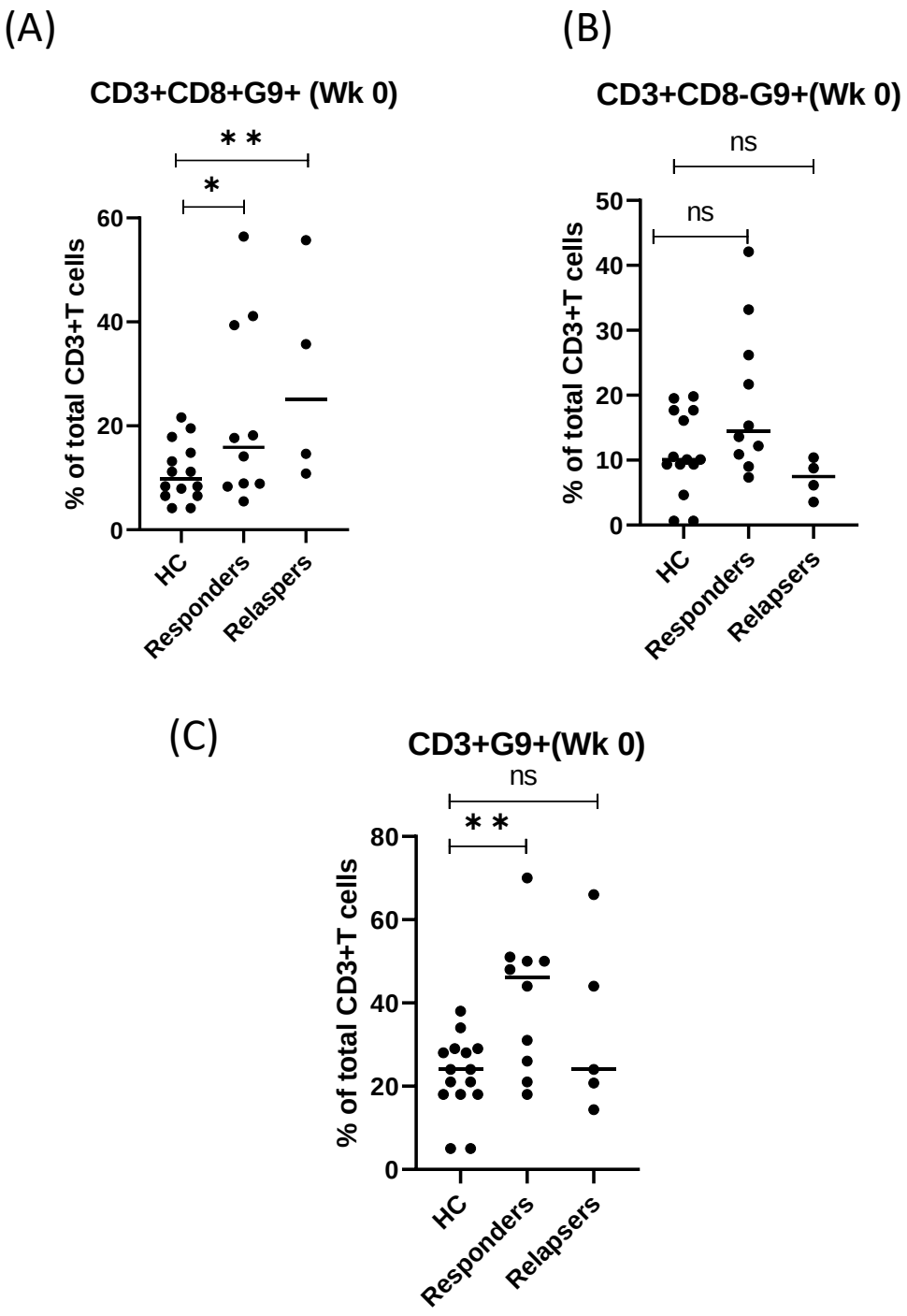

Figure 3. 6 Scatter plots of Galectin 9 expressing T cell populations in healthy controls, relapsers and treatment Responders

Study groups were Healthy controls (HC, n=15), baseline samples (Wk0) from chronic HCV cases achieving SVR (n=10) and cases which relapsed following interferon free treatment (n=5). Dots indicate percentage of T cells in each group with (A) showing Galectin-9 expressing CD3+CD8+T cells, **(B)** showing Galectin-9 expression on CD4+T cells (CD3+CD8-Tcells) and **(C)** showing Galectin-9 expression on total CD3+ T cells. Horizontal line represents group median. *p<0.05, **p<0.001, ns denotes not significant.

3.3.7 Changes in expression of T cell exhaustion markers during treatment were different between relapsers and treatment responders

Expression of PD1 and Galectin-9 on total CD3+ T cells, and CD8+, CD4+ subpopulations in relapsers and treatment responders was studied at treatment week 0, 2, 4, end of treatment (EOT), at relapse time point in relapsers, and post treatment in responders at weeks 2 (SVR 2), 4 (SVR 4) and 12 (SVR 12).

The frequencies of PD1+ and Galectin 9+ expressing T cells showed different trends in both groups. Surprisingly, relapser cases had a lower median PD-1 and Galectin-9 expression on total CD3+ T cells and subpopulation of CD3+CD8- T cells before initiating therapy (Figure 3.7 A, B, E, F)**.** However, expression of these exhaustion markers was higher at baseline on CD3+ CD8+ T cells throughout the treatment in case of PD-1 and at baseline in case of Galectin-9 (Figure 3.7 C, D).

There was no significant change in expression of these exhaustion markers on all cell types studied over time during treatment in case of relapsers. On the other hand, PD-1 and Galectin 9 expression showed a gradual decline during and after treatment towards lowest at week 12 post treatment (Figure 3.7).

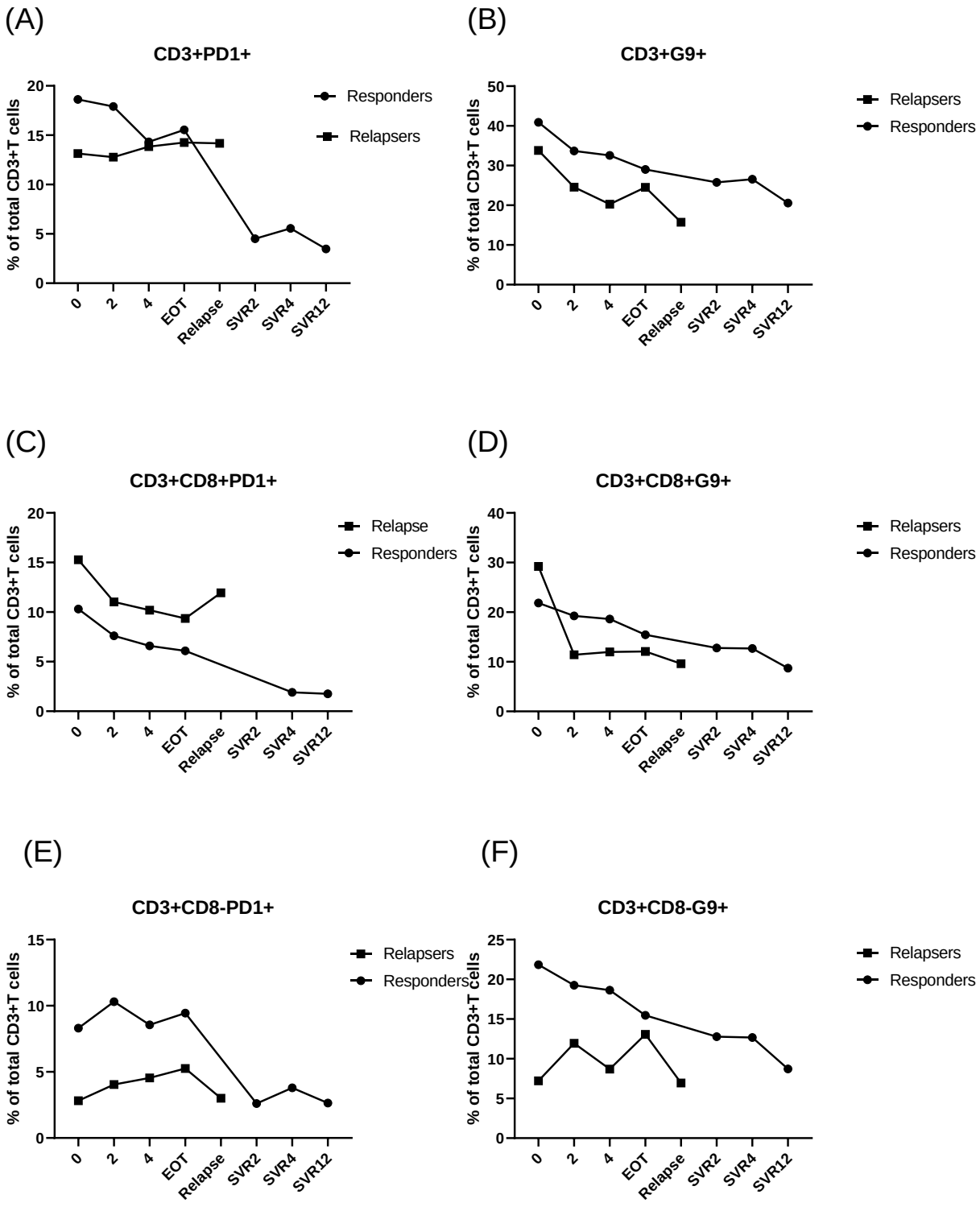

Figure 3. 7 Changes in PD1 and Galectin 9 expression on T cell populations in cases achieving SVR (Responders) and Relapsers during interferon free treatment

3.4 Discussion

This study has demonstrated that chronic HCV infection is characterised by increased expression of inhibitory receptors and checkpoint inhibitors including PD-1 and TIM-3 ligand Galectin-9 on T cells when compared with healthy controls. This is consistent with previous findings that a large fraction of HCV specific CD8+ T cells co-express inhibitory receptors including PD-1 (Bengsch et al., 2010) and TIM-3 ((Martin et al., 2014, Golden-Mason et al., 2009, Kasprowicz et al., 2008, Nakamoto et al., 2008, Radziewicz et al., 2007, Schlaphoff et al., 2011, Rutebemberwa et al., 2008).

Importantly, this study demonstrated that sustained virological response following interferon free treatment was associated with a significant reduction in expression of T cell exhaustion markers indicating restoration of T cell phenotype. Previous studies have demonstrated that successful treatment with pegylated type I interferon may rescue function of HCV specific CD8+ T cells, and thus prevent T cell exhaustion in acute HCV infection (Abdel-Hakeem et al., 2010, Badr et al., 2008). However, such immune restoration was not seen following treatment with pegylated interferon once chronic infection had developed as a result of ongoing recognition of viral antigen leading to T cell exhaustion. Indeed most groups have reported an irreversible damage to HCV-specific T cell pools, that are not restored by pegylated interferon therapy (Abdel-Hakeem et al., 2010, Missale et al., 2012, Seigel et al., 2013). However, since IFN also has direct inhibitory effects on lymphocytes, it has not been established whether this is due to direct effect of interferon therapy or long lasting exposure to viral replication. Results described in this chapter were able to establish at least phenotypic restoration of T cells

with loss/decline in exhausted T cells following treatment with interferon free direct acting antiviral treatment regimens. This finding was further explored by evaluating HCV specific T cell functional changes in response to interferon free therapy and will be covered in chapter 4.

There have been further recent reports of partial T cell restoration. Martin et al. reported a decrease in mean PD-1 expression on HCV specific CD8+ T cells at follow up following DAA treatment (Martin et al., 2014). Burchill et al reported reduction in expression of inhibitory molecule T cell immunoreceptor with immunoglobulin on all T lymphocytes and a partial reduction in PD-1 expression on HCV specific CD8+ T cells following DAA treatment (Burchill et al., 2015). Contrary to this, Zhang et al have recently reported no significant differences in PD-1 expression before and after treatment (Zhang et al., 2017). The reasons for this difference in results is not clear and larger studies may be warranted.

Previous studies have demonstrated that chronic activation of the adaptive immune responses in chronic HCV infection also predicts treatment failure with previous standard of care (pegylated interferon-alpha and Ribavirin). This is evidenced by increased pre-treatment expression of the programmed death (PD-1) molecule on lymphocytes of treatment non-responders (Golden-Mason et al., 2008).

Work described in this chapter studied PD-1 and Galectin-9 expression on T cells in treatment relapsers and compared with responders in order to identify any patterns predictive of treatment response between the two groups. There was no difference in expression of PD-1 on total CD3+ T cells between two groups while Galectin-9 expression was significantly higher in relapsers at baseline. Other co-

inhibitory exhaustion markers could not be studied due to limited cell numbers. It is possible that exhaustion in this cohort was augmented by other co-inhibitory receptors. There was however a difference in PD-1 and Galectin 9 expression on CD8+ and CD4+ T cells between the two groups. Relapsers had a higher PD-1 and Galectin-9 expression on CD8+ T cells while this was lower on CD4+ T cells in comparison with treatment responders. The reason and pathways behind this difference is unclear and merits further investigation. Exhausted CD8+ T cells are expected to result in loss of their effector function with lack of cytokine production and reduced cytotoxic activity, and may contribute to treatment failure in relapsers amongst patients treated with interferon free treatment. Restoration of T cell phenotype demonstrated in cases which achieved SVR and persistent expression of exhaustion markers on T cells who failed to achieve SVR points towards probable contribution of restoration of immune responses in achieving SVR following treatment with interferon free treatment regimens.

Regulation of CD8+ T cell responses is one of the core functions of CD4+ T cells. HCV specific CD4+T cell has shown to be a strong immunological factor in cases where there is spontaneous resolution of HCV infection following acute infection (Schulze Zur Wiesch et al., 2012, Chang et al., 2001). While the term exhaustion has been used to describe functionally impaired CD8+ T cell response, its use with regards to CD4+ T cells is less well established. Proliferation and secretion of IFN-γ are traits of CD4+ Th1 cells, and the expression of PD-1 on their surface as a sign of exhaustion in context of chronic hepatitis C infection is not well explored. In this context, significance of the findings in this chapter of lower PD-1 and Galectin-9 expression on CD4 T cells in relapse cases needs to be studied further. One explanation for lower PD-1 and Galectin-9 expression on CD4+ cells

could be from lack of exhaustion due to emergence of virus escape mutations not recognised by these cells, and hence loss of exhausted cells. However, this issue can only be addressed in cohorts where RNA sequence of inoculum HCV infection and T cell responses prior to treatment and at relapse are known and correlated. Secondly, this finding also confirms the concept that viral persistence is related to loss of CD8+ effector function in relapse related to interferon free treatment rather than CD4+T cell exhaustion.

Interestingly, contrary to observations in treatment responders, the frequency of exhaustion markers did not significantly change during the course of treatment in relapsers. On the other hand, there was continued longitudinal loss of exhaustion markers on T cells in treatment responders. Although this can be explained by loss of ongoing antigenic stimuli as a result of viral clearance in treatment responders and probable continued antigenic exposure preventing restoration of T cell exhaustion phenotype in treatment relapsers. However, in this cohort of relapsers, all cases developed low serum viral titres as determined by PCR during treatment, with a relapse demonstrated post treatment. Hence, a more likely plausible explanation would be the contribution of effector T cell restoration towards achieving viral clearance in the SVR group and lack of such restoration resulting in viral persistence despite interferon free treatment. Further studies will be required to establish CD8+ T cell exhaustion in this group is due to ongoing antigenic stimuli as a result of viral non-clearance or if this is the reason behind lack of response. Similarly, it is unclear whether restoration of T cell phenotype in SVR group is the cause or effect of viral clearance.

Chapter 4

HCV specific T cell responses during Direct Acting antiviral therapy

4.1 Background

Chronic hepatitis C infection is featured by an impaired HCV specific T cell response unable to control HCV replication (Larrubia et al., 2014, Wedemeyer et al., 2002). This response becomes exhausted due to long-lasting high antigenic burden (Larrubia et al., 2011, Penna et al., 2007) characterised by expression of negative co-inhibitory receptors, impaired proliferation, cytotoxicity and IFN-γ secretion (Bengsch et al., 2010, Wood et al., 2011). As discussed previously, restoration of specific T cell function was not detected in patients who achieved sustained virological response (SVR) with pegylated interferon based treatment (Humphreys et al., 2012). Also, therapeutic vaccines also fail to restore T cell immune responses in SVR patients implying persistent T cell exhaustion (Wieland and Thimme, 2016, Kelly et al., 2016). It remains to be fully established whether direct acting antiviral therapy in the absence of interferon is able re-establish HCV specific T cell function. Documentation of how successful DAA

therapy affects the immune compartment will allow understanding of the potential of effective T cell responses in the event of subsequent HCV challenges and guide development of preventive intervention i.e. vaccination to generate life-long sterilising immunity to HCV in high risk individuals.

There have been no reports to date studying the differences in HCV specific T cell activation between relapsers and responders to interferon free DAA treatment.

Study of T cell exhaustion markers during interferon free treatment described in Chapter 3 demonstrates restoration of T cell phenotype with a decline in expression of exhaustion markers following interferon free antiviral treatment. The aim of this study was to evaluate functional HCV specific T cell responses using overlapping HCV peptides before and after SVR with interferon free treatment regimens and compare with treatment relapsers to establish trends predictive of SVR and correlate these findings with trends in exhaustion marker expression reported in Chapter 3.

4.2 Methods

Previously isolated and frozen PBMCs in liquid nitrogen from healthy controls and cases with chronic hepatitis C infection undergoing interferon free treatment were thawed and used to perform IFN-γ ELISpot assays. 6 cases relapsed amongst a total of 36 cases recruited. 1 out of 5 relapsers received pegylated interferon alpha as part of treatment regimen and were excluded from further study. 10 cases which achieved SVR and were matching in baseline characteristics had

sufficient PBMCs available for inclusion in ELISpot assays. This is the same cohort of cases used to study expression of exhaustion markers described in chapter 3. PBMCs for all included cases with SVR were available at baseline, treatment week 0, 2, 4, end of treatment (EOT), post treatment weeks 2, 4 and 12. PBMCs for relapsers were available at baseline (week 0), treatment week 2, 4, EOT, relapse time point and 4 weeks post treatment. Most relapsers commenced second line treatment 4 weeks post relapse, hence further samples after completing treatment were not available. Healthy controls were recruited from healthy laboratory staff members.

HCV overlapping peptides for core, NS3, NS5A and NS5B were used as antigens. These polypeptide regions were chosen on basis of previously demonstrated dominant immunogenic epitopes. Overlapping peptides for HCV genotype 1 were used for cases with genotype 1 infection and overlapping peptides for genotype 3 were used for cases with genotype 3 infection.

Assay technique and materials used are detailed in materials and methods section 2.7.

4.3 Results

4.3.1 Study Groups

Baseline characteristics of study groups included in IFN-γ ELISpot assays are described in table 4.1.

Table 4. 1 Subjects included in study of HCV T cells responses to HCV overlapping peptides

		SVR cases (n=10)	Relapsers (n=5)	HC (n=10)
Gender	Male: Female	7:3	3:2	6:4
Age	Mean ±SD	45.9 ± 5.0	41.6 ± 8.2	37 ± 9
HCV genotype	1	4	1	
	3	6	4	
Fibrosis	F4	9	4	
	F0-F3	1	1	
Treatment regimen	Sofosbuvir +Ribavirin	3	2	
	Sofosbuvir + Ledispavir + Ribavirin	1	2	
	Sofosbuvir + Daclatasvir	2	1	
	Sofosbuvir + Ledispavir	4		
Relapse time point	Week 24 post treatment		1	
	Week 4 post treatment		4	

4.3.2 IFN-γ ELISpot responses in relapser cases are higher and more polygenic than treatment responders at baseline

Cases who relapsed had higher median HCV specific IFN-γ ELISpot T-cell responses at baseline prior to commencing antiviral treatment than those who achieved sustained viral response when cases with both genotype 1 and 3 infection were analysed. This was seen across all overlapping peptide pools studied including viral proteins core, NS3, NS5A and NS5B. This difference in ELISpot responses was statistically significant in case of NS3 and NS5A, with a clear trend observed in core and NS5B (Figure 4.1).

4 out of 5 relapsers had genotype 3 infection. The median HCV specific T cell responses of these cases when analysed separately at baseline were also higher when compared with treatment responders with HCV genotype 3 infection, although this difference did not reach statistical significance. This was seen in response to all overlapping peptide pools used including core, NS3, NS5A and NS5B (Figure 4.2). One patient in genotype 1 group relapsed, which showed similar quantitative differences on comparison with treatment responders, as seen in genotype 3 cases. However, findings were not amenable to find statistical significance due to a single case in genotype 1 relapser group.

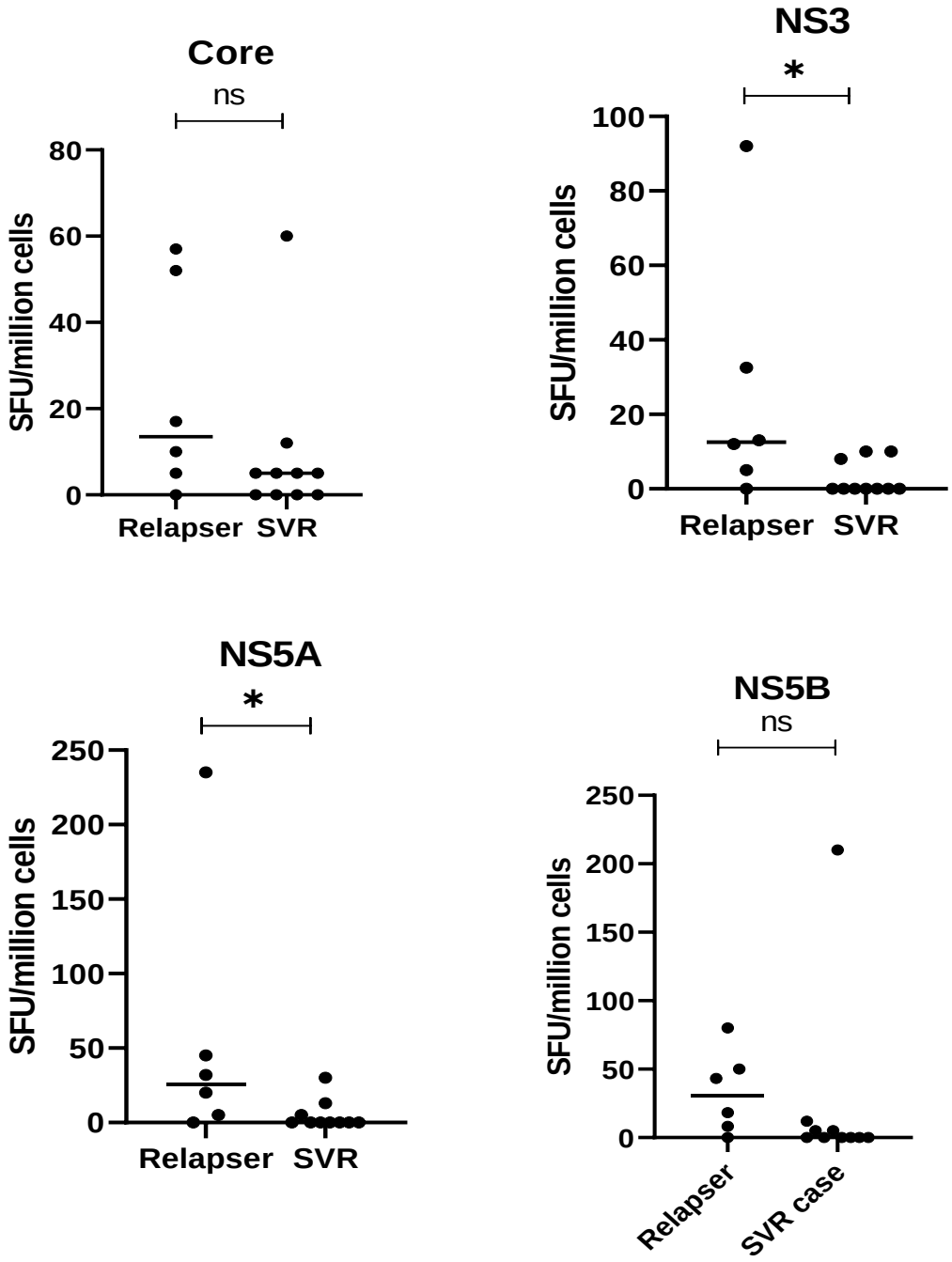

Figure 4. 1 Scatter plots of IFN- γ ELISpot responses to overlapping HCV peptides at baseline

IFN- γ ELISpot responses in relapsers (n= 5) and cases achieving SVR (n=10). Overlapping peptides for HCV core protein, Non-structural protein 3 (NS3), NS5A and NS5B were used. Y- axis shows number of spot forming units per million PBMCs. Horizontal line represents group median. * means $p<0.05$, ns denotes not significant.

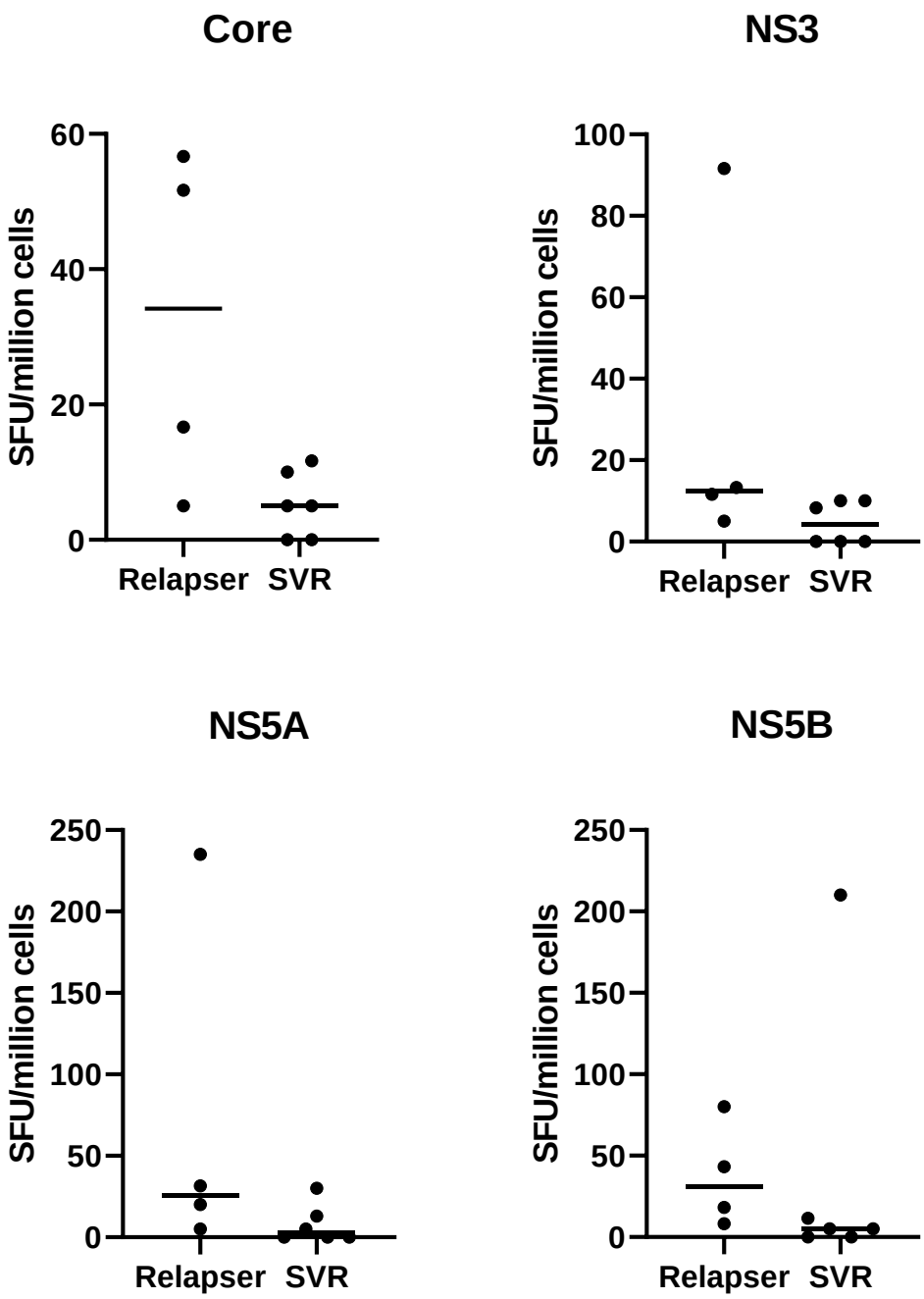

Figure 4. 2 Scatter plots of IFN- γ ELISpot responses to overlapping HCV peptides at baseline in Genotype 3 infection

IFN-γ ELISpot responses at baseline in relapsers (n= 4) and cases achieving SVR (n=6) with genotype 3 chronic hepatitis C infection. Overlapping peptides for HCV core protein, Non-structural protein 3 (NS3), NS5A and NS5B were used. Y axis shows number of spot forming units per million peripheral blood mononuclear cells (PBMCs). Horizontal line represents group median.

4.3.3 Pooled ELISpot responses in relapsers are higher than cases with SVR at baseline

The overall magnitude of HCV specific IFN-γ secretion could be assessed by pooling responses to all overlapping peptides. Pooled IFN-γ ELISpot T cell responses to all HCV peptide pools used including Core, NS3, NS5A and NS5B were significantly stronger in relapsers in comparison with cases which reached SVR (Responders). 4 out of 6 relapsers had genotype 3 infection. Pooled HCV specific ELISpot responses in this group of relapsers were also significantly higher than treatment responders with genotype 3 infection (Figure 4.3).

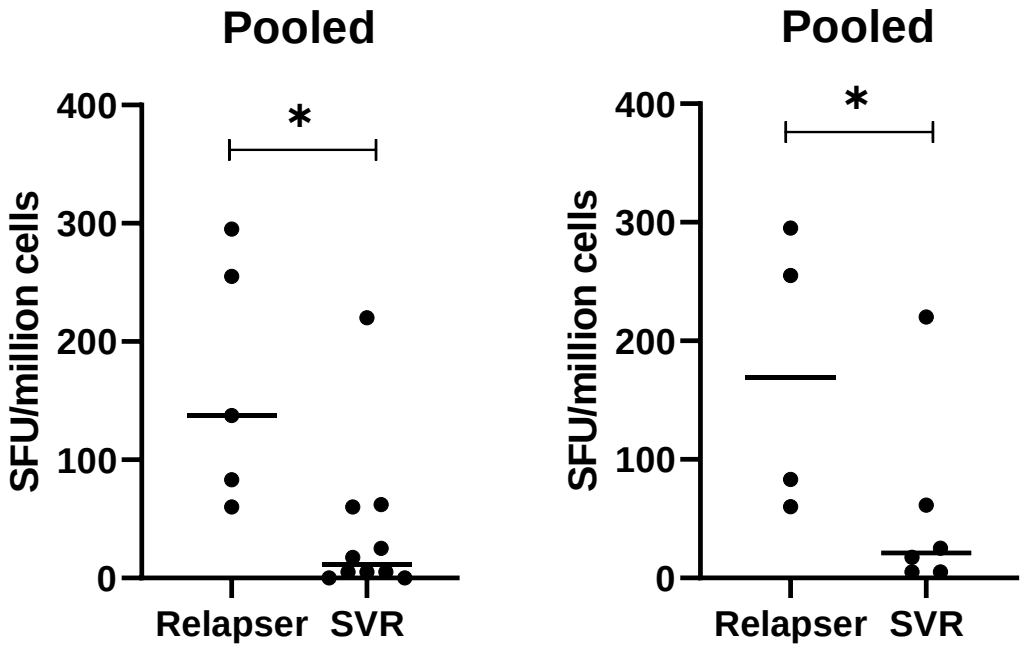

Figure 4. 3 Scatter plots of pooled IFN-γ ELISpot responses at baseline

(A) Represents chronic HCV cases with either genotype 1 or 3 infection. Study groups included relapsers (n=6) and cases with SVR following antiviral treatment (n=10). **(B)** represents cases with genotype 3 infection in both groups. Study groups included relapsers (n=4) and cases achieving SVR (n=6)

Y-axis represents number of spot forming units per million peripheral blood mononuclear cells (PBMCs). Dots indicate pooled response of each case to overlapping peptides. Short horizontal lines represent group median.

* means $p<0.05$.

4.3.4 IFN-γ ELISpot responses increase after successful treatment of chronic HCV

IFN-γ ELISpot T cell responses to overlapping HCV peptides for Core, NS3, NS5A and NS5B were examined at baseline and 12 weeks after completion of antiviral treatment in 8 cases which reached SVR following interferon free antiviral treatment. There was an increase in IFN-γ ELISpot T cell responses to all HCV overlapping peptides used individually and when pooled in all cases following successful treatment. This shows restoration of T cell function in these cases after attaining SVR with interferon free antiviral treatment (Figure 4.4).

In comparison to this, IFN-γ ELISpot responses in relapsers could only be assessed 4 weeks following relapse which demonstrated a decline in ELISpot responses to all overlapping peptides independently (Core, NS3, NS5A, NS5B) and on analysing pooled responses (Figure 4.5). No PBMCs were available at later time points in relapsers due to these patients commencing second line treatment.

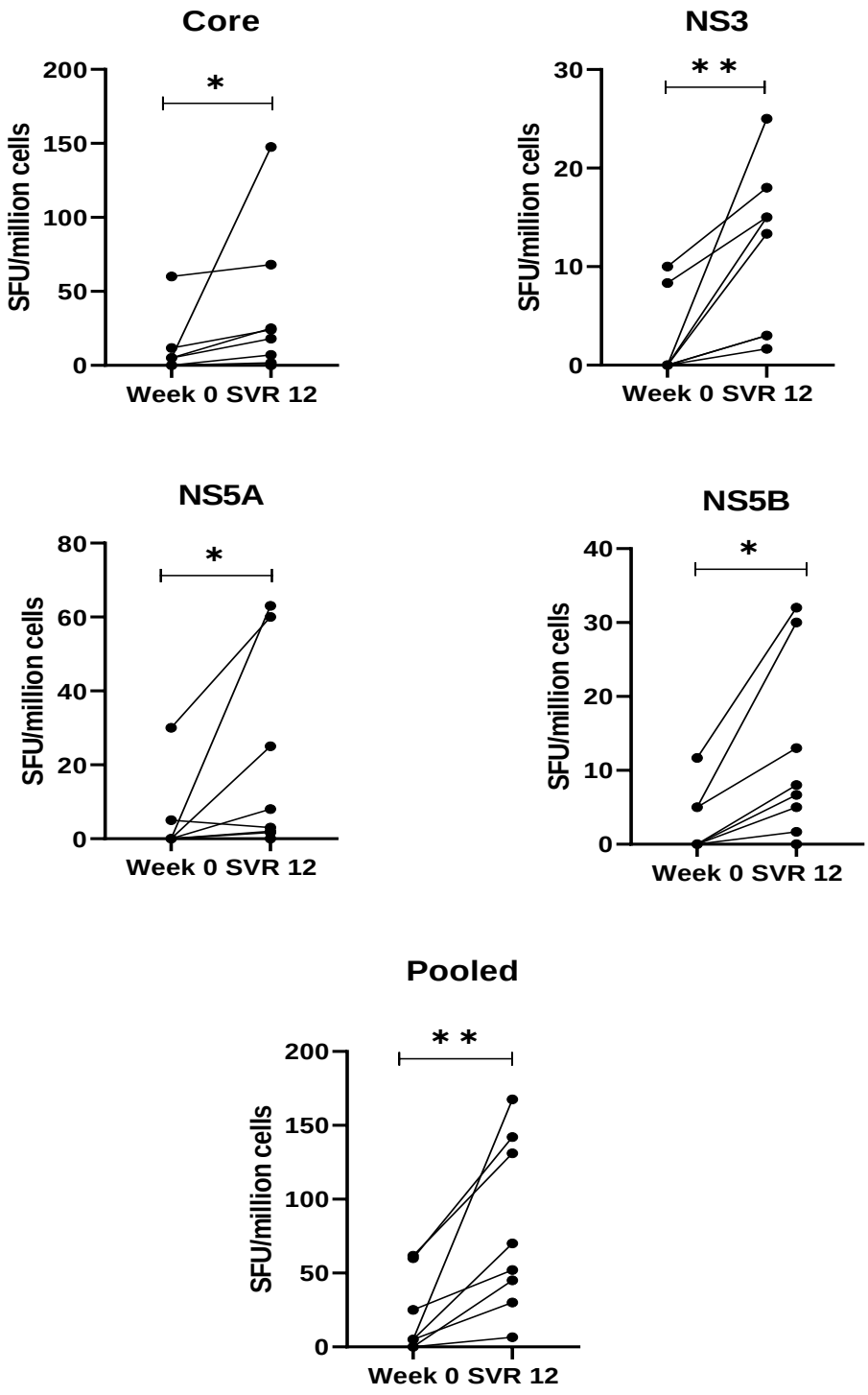

Figure 4. 4 Impact of antiviral treatment on IFN- γ T cell ELISpot responses in cases with SVR

Comparison of IFN-γ ELISpot T cell responses in cases who achieved SVR at week 0 and post treatment (n=8). This shows restoration of T cell function with increase in IFN-γ responses post successful treatment of chronic HCV with IFN free antiviral treatment. * denotes p<0.05, ** denotes p<0.01.

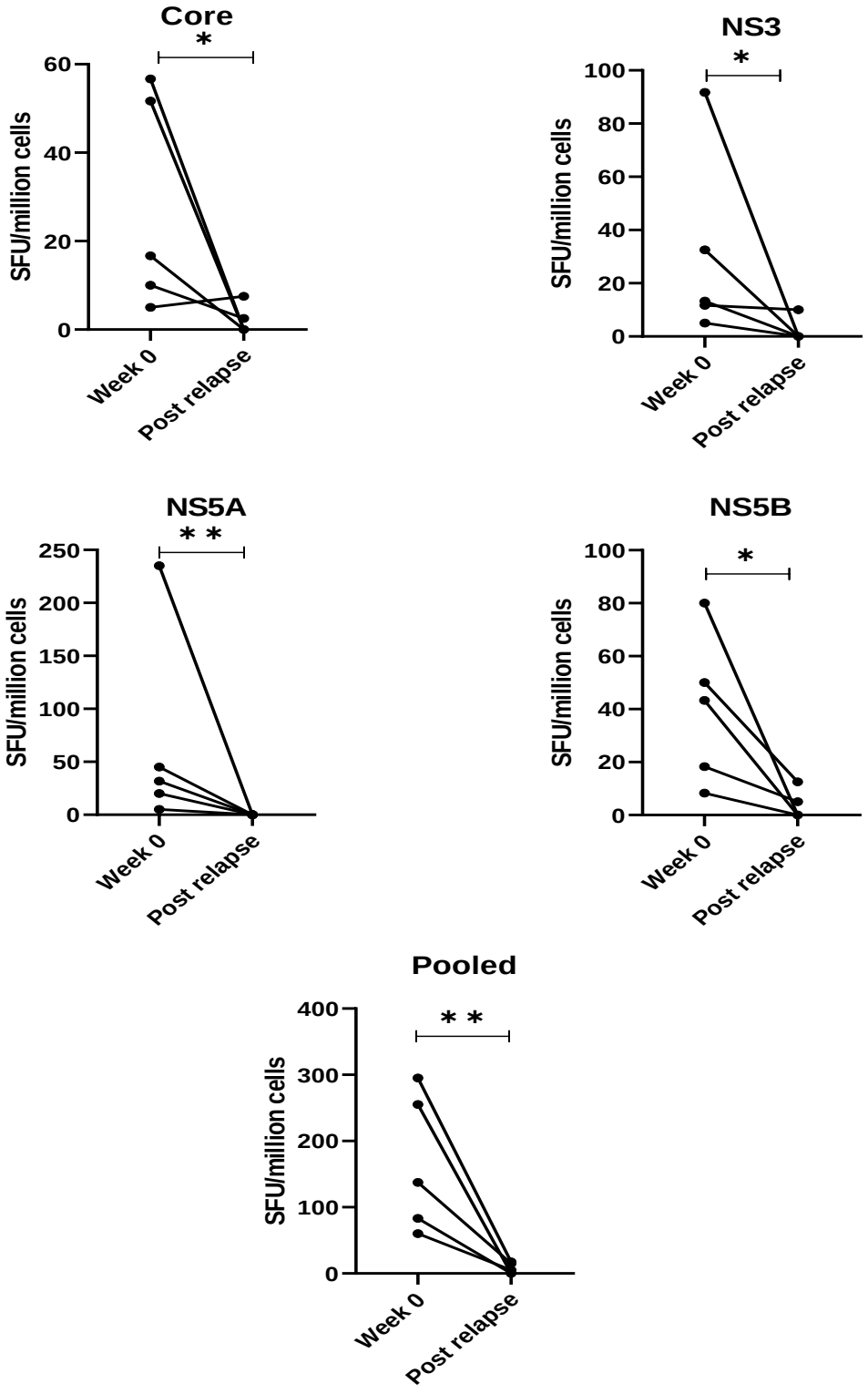

Figure 4. 5 Impact of antiviral treatment on IFN- γ T cell ELISpot responses in relapsers

Comparison of IFN-γ ELISpot responses in relapsers at week 0 and 4 weeks after relapse to HCV overlapping peptides. * denotes $p<0.05$, ** denotes $p<0.01$

4.3.5 Trend in IFN- γ T cell responses during treatment in relapsers is different from treatment responders

Trends in IFN- γ ELISpot response during treatment was studied in 5 relapsers who received interferon free treatment. All cases had higher baseline T cell response to all overlapping peptides separately and when pooled, which rapidly reduced to minimal at week 1/2 in all 5 cases. PBMC sample was available for 4 out of 5 cases at the time point of relapse. All of these cases showed an increase in IFN-γ T cell response to overlapping peptides for core protein and NS5B at relapse time point. This response was higher when responses to all overlapping peptides were pooled (Figure 4.6).

Post relapse PBMC sample was available for all 5 cases at week 4 post relapse. This showed a rapid reduction in T cell responses to minimal in all cases.

This is dissimilar to the trend observed in responders. IFN-γ ELISpot T cell responses were studied in 8 cases who received interferon free antiviral treatment for genotype 1 and 3 chronic hepatitis C infection. All cases showed significantly lower baseline IFN-γ ELISpot responses in comparison with relapsers. This gradually increased throughout the treatment, highest being 12 weeks post treatment (Figure 4.7).

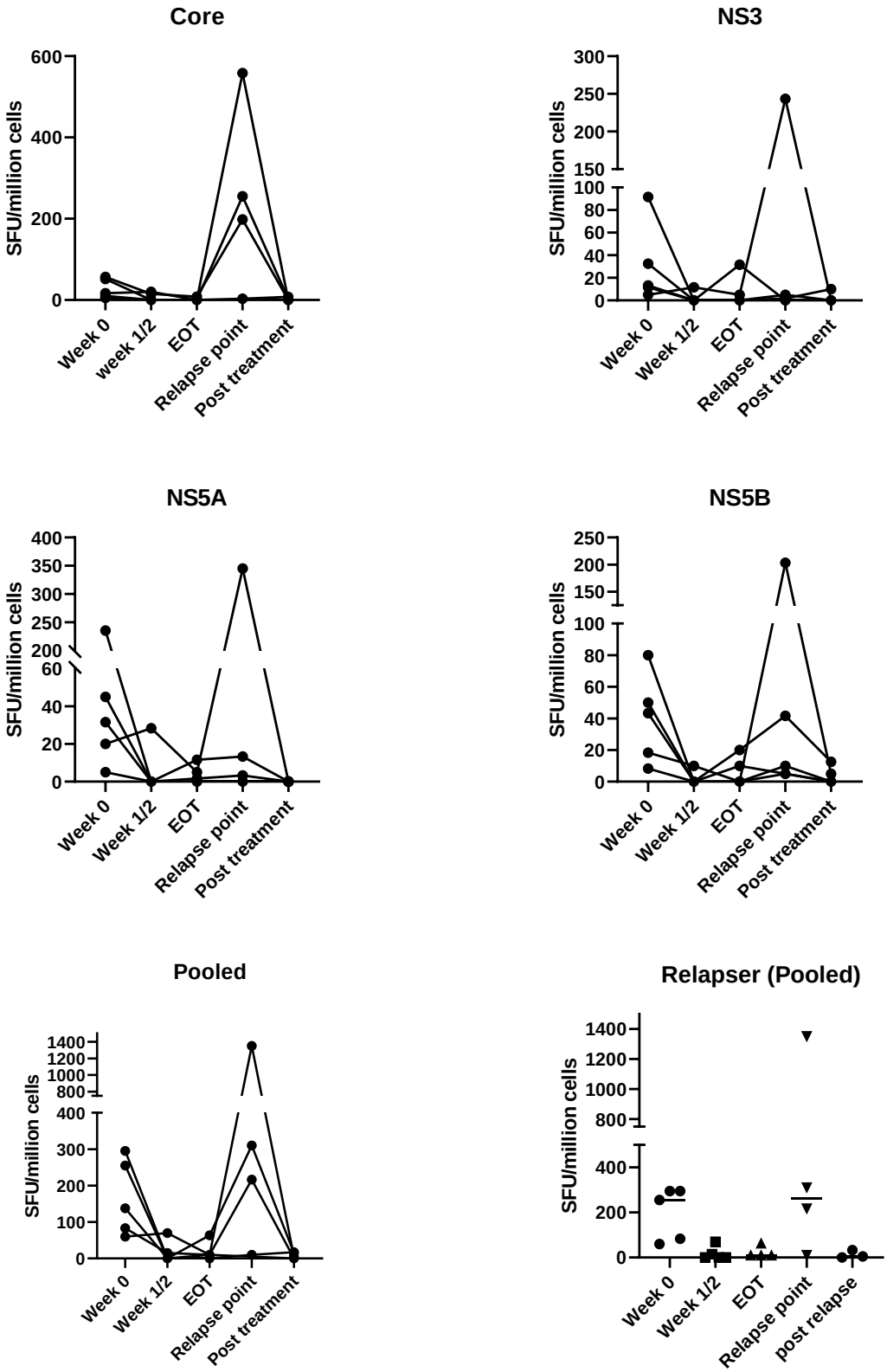

Figure 4. 6 Trends in T cell responses using IFN - γ ELISPOT assay in relapsers receiving IFN free treatment

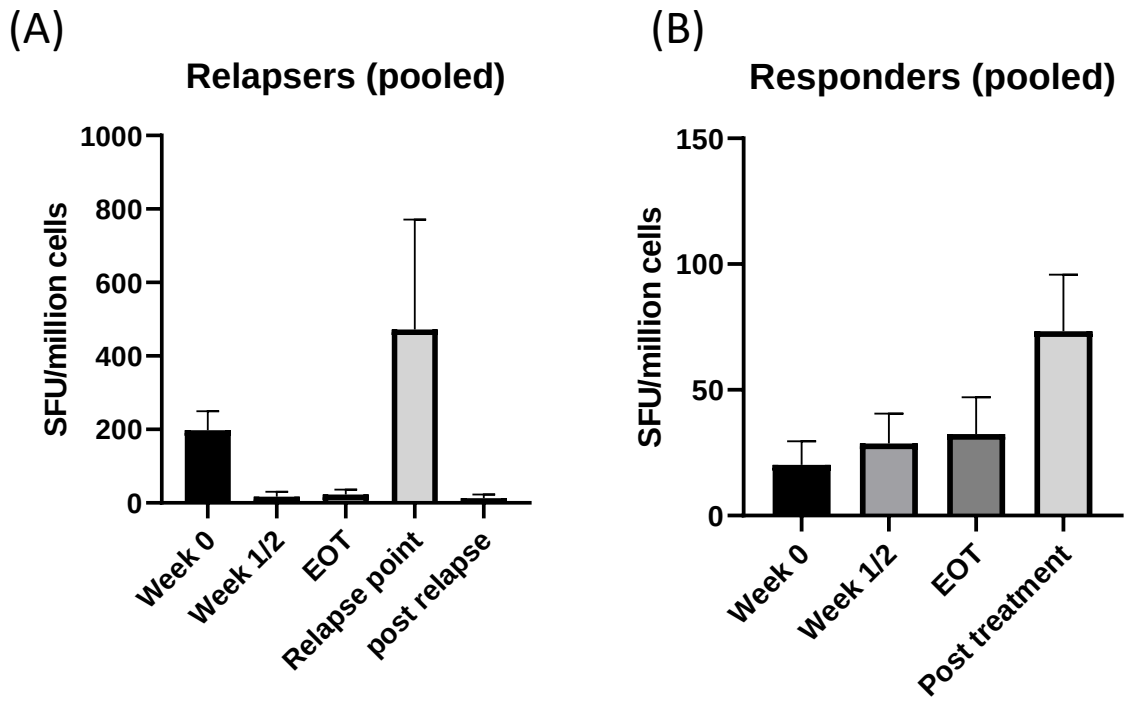

Figure 4. 7 Bar graphs comparing on treatment trend in pooled IFN-γ ELISpot T cell responses in relapsers and responders to pooled

Trends in pooled IFN-γ ELISpot T cell responses expressed as spot forming units (SFU) per million PBMCs against overlapping HCV peptides from core, NS3, NS5A and NS5B during and after interferon-free treatment.

(A) represents pooled IFN-γ ELISpot T cell responses in relapsers studied at treatment weeks 0, 1 or 2, end of treatment (EOT), relapse time point and 4 weeks post relapse. **(B)** represents pooled IFN-γ ELISpot T cell responses in responders studied at treatment weeks 0, 1 or 2, EOT and 12 weeks post treatment.

4.4 Discussion

In this study using a patient cohort of previously treatment naïve patients with chronic HCV genotype 1 and 3 who received interferon free antiviral treatment, it was found that there is restoration of HCV specific T cell function following successful viral clearance.

This was elucidated by significantly higher HCV specific IFN-γ ELISpot responses expressed as SFU/million 12 weeks post treatment (SVR 12) in comparison with baseline. T cell ELISpot responses were observed to gradually improve throughout the course of treatment when analysed at week 0, 2, 4, end of treatment and post treatment reaching maximum at SVR week 12. Recently, Martin et al also demonstrated that both the frequency of HCV specific CD8+ T cells in peripheral blood and their proliferative capacity in vitro increased in patients who responded to a combination DAA therapy but not in non-responders (Martin et al., 2014). A remaining question unanswered is whether this immunological recovery remains stable over time or perhaps there is enhanced restoration of immune function if studied over longer periods of time.

An interesting finding in this study was a distinct difference in baseline ELISpot responses between treatment responders and relapsers, and early during the course of treatment. IFN-γ ELISpot responses in relapser cases are of higher amplitude and multi-specific in comparison with treatment responders at baseline. This finding was not specific for viral genotype and was observed for both genotype 1 and 3. Furthermore, the magnitude of this difference was higher in response to pooled HCV peptides. Previously, similar strong and multi-specific CD4+ and CD8+ T cell responses are observed and thought to be essential in

spontaneous virus clearance following acute HCV infection (Diepolder et al., 1995, Cramp et al., 1999, Lechner et al., 2000, Thimme et al., 2001, Missale et al., 1996). Following clearance, memory responses to multiple HCV epitopes can persist beyond 10 years (Takaki et al., 2000).

A possible explanation of these findings could be the presence of viral escape mechanisms which led to viral persistence leading to chronic HCV infection in relapsers despite stronger and higher T cell responses to prototype virus, which also prevented viral clearance using direct acting antiviral drugs.

HCV exhibits high replicative fitness paired with a high error rate of its polymerase, and many dominant T cell epitopes are lost due to HCV sequence mutations. The first HCV mutations in T cell epitopes were demonstrated in HCV infected chimpanzees (Weiner et al., 1995b). Subsequent studies in humans led to the identification of HCV variant sequences encoding peptides with reduced MHC affinity; they had decreased T cell recognition or competed with T cell recognition of wild type peptide (Chang et al., 1997). A series of studies later showed that variant sequences were more frequently found in the presence of prototype T cell responses confirming the hypothesis of viral escape (Cox et al., 2005b, Ray et al., 2005, Seifert et al., 2004, Tester et al., 2005, Timm et al., 2004). The selection of HCV escape mutants occurs in the acute phase of infection (Callendret et al., 2011, Fernandez et al., 2004, Fuller et al., 2010). CD8+ T cells that target the wild type sequence of ‘escape regions’ have previously shown to display CD127 memory phenotype (Kasprowicz et al., 2010, Rutebemberwa et al., 2008). Such T cells respond well in in vitro assays upon stimulation with their wild type epitope but are ineffective in vivo as they do not recognise alternate variants.

Another novel finding of this study is the rapid reduction in ELISpot responses to antigenic stimulation by overlapping HCV peptides at week 2 in relapsers whilst T cell responses in SVR cases started to increase at this time point which continued to reach a maximum at 12 weeks post treatment. The reason behind this early decline in T cell response in relapsers whilst on antiviral therapy is unclear, but may predict cases which are likely to relapse. One plausible explanation could be that early improvement in T cell function contributes to viral clearance in addition to direct antiviral effect of these new therapeutic agents leading to high SVR rates observed with interferon free direct acting antiviral therapy.

Further questions need to be explored regarding the timing of recovery of effector T cell function and if it persists longer term and plays a role in preventing HCV infection or leads to early robust spontaneous clearance of HCV in case of re-infection. Further research interest would be to establish if further induction of functional T cell responses in treatment responders through vaccination could provide robust HCV protection in previously treated cases.

Chapter 5

Innate Immune responses during antiviral treatment for HCV infection

5.1 Background

Innate immune responses are crucial in the antimicrobial defence against invading pathogens in humans. Pathogen associated molecular patterns (PAMPs), such as bacterial endotoxins, viral nucleic acids and replicating intermediates are recognised by various pattern recognition receptors (PRRs) including Toll like receptors (TLRs) and cytoplasmic RIG-I like receptors. Virus sensing mechanisms are activated mostly by viral nucleic acids via endosomal TLR3, 7, 8 and 9 as well as through non-TLR cytoplasmic signalling RIG-I receptors. The activation of these receptors leads to the production of Type I and III interferons (IFNs), chemokines and proinflammatory cytokines.

Hepatitis C virus activates innate immune response by several mechanisms. Double stranded RNA (dsRNA) is recognised by two types of pattern recognition receptors, endosomal toll like receptors (Meier et al., 2007, Decalf et al., 2007,

Zhang et al., 2009, Wang et al., 2009) and cytoplasmic RNA helicases RIG-I and Md5 (Loo and Gale, 2011, Takeuchi and Akira, 2008). Activation of both leads to downstream signalling which differ in their initial steps but converge in the activation of transcription factors interferon regulatory factor 3 (IRF3), IRF7 and NF-κB. Activated IRF3 and NF-κB promotes transcription of type I and III interferons as well as pro-inflammatory cytokines and chemokines. These type I and III interferons are essential for defence against viruses which induce an antiviral state by transcription activation of a multitude of interferon stimulated genes (ISGs) (Zhang et al., 2009, Takeuchi and Akira, 2009).

Hepatitis C virus simultaneously has mechanisms to evade this immune response leading to chronic infection by impairing these signalling pathways through several mechanisms. Firstly, NS3/4A ablates innate immune signalling at TLR-3 and RIG-I receptors by inducing degradation of Toll/interleukin-1 receptor domain-containing adaptor-inducing IFN-β (TRIF) and MAVS involved in downward signalling at the two receptors respectively (Horner et al., 2011, Horner and Gale, 2009). NS3/4A also binds to TBK1 protein kinase to reduce TBK1-IRF3 interaction, thereby inhibiting IRF3 activation and IFN production (Otsuka et al., 2005). In addition, HCV NS5A binds with MyD88 to impair cytokine production in response to ligands for TLR2, TLR4, TLR7 and TLR9 (Abe et al., 2007). HCV lipoviral particles have been shown to directly interfere with TLR4 signalling in dendritic cells (DCs) while core protein suppresses TLR4 expression (Agaugue et al., 2007, Sato et al., 2007). Greater production of anti-inflammatory cytokine IL-10 by macrophages with TLR2 stimulation in chronic HCV infection has also been reported reported (Chang et al., 2007). It is agreed that these

mechanisms lead to evasion of an effective immune response and persistence of hepatitis C virus leading to chronic infection.

HCV proteins also inhibit JAK-STAT pathway involved in interferon mediated signalling (Horner and Gale, 2009, Bode et al., 2003). It is well established that patients with activated endogenous IFN system are poor responders to IFN alpha based therapies which has been the standard of care until recent development of direct acting antiviral drugs.

Interferon free treatment regimens combining direct acting antiviral drugs targeting HCV proteins, which play a role in evasion of immune system, have shown to achieve high treatment response rates of above 96% across all HCV genotypes. These viral protein targets are involved in viral replication, and efficacy of these drugs has been associated with direct inhibition of viral replication.

This study was carried out to determine if these newer interferon free treatment regimens have an effect in restoration of defective receptor mediated signalling of innate immune pathways and if this restoration contributes towards viral clearance. In addition, receptor mediated signalling was studied during treatment to determine any patterns predictive of treatment response.

5.2 Methods

PBMCs obtained from a cohort of 5 cases with chronic HCV infection undergoing interferon free antiviral treatment were used to study longitudinal changes in innate immune responses during and after treatment. All cases achieved SVR

following treatment. Baseline characteristics of these cases are described in Section 5.3.1.

PBMCs were obtained at baseline, treatment week 2(TW2), week 4 (TW4), week 8 (TW8), end of treatment (EOT), 2 weeks (SVR2), 4 weeks (SVR4), 12 weeks (SVR12) and 24 weeks post treatment (SVR24), and aliquots were frozen in liquid nitrogen. Sufficient PBMCs were only available for all cases included at week 0, 2, 4, end of treatment and 4 weeks post treatment (SVR4) for inclusion in this study. Hence, samples obtained at these timepoints were used to study cytokine production in response to TLR and RIG-I agonist stimulation. Aliquots of PBMCs from patient group at these timepoints were defrosted and viability assessed using methodology described in Section 2.5.6.

A range of single TLR ligands were used as a model for virus PAMPs to study cytokine responses. The following stimuli were used:

Polyinosinic-polycytidylic acid (Poly (1:C)), which is a synthetic analog of viral replication intermediate nucleic acid double stranded RNA (ds RNA). Poly (I:C) is recognised by Toll like receptor 3 (TLR 3).

Poly (I:C) Lyovec complex, which is a complex of Poly (I:C) and transfection reagent Lyovec. Poly (I:C) also stimulates strong innate immune response initiated by RIG-I like receptors, which are cytoplasmic. While Poly (I:C) alone is recognised by cell surface and endosomal TLR3, Poly (I:C)/Lyovec complex allows transfection of cell with Poly (I:C) thereby allowing activation of the cytoplasmic RIG-I receptor signalling pathway.

R848, which is imidazoquinoline compound, acts as a ligand for Toll like receptor 7 and 8, thereby activating immune cells expressing these receptors via the TLR7/TLR8-MyD88 dependent signalling pathway.

Secreted cytokines were measured before and after stimulating PBMCs with ligands described above at each time point studied. Mononuclear phagocyte produced inflammatory mediators including type III interferons, IFN-γ IL-6, IL-8, IL1-α, IL1-β, CCL3 and IL-12 were measured. Luminex multiple cytokine detection system was used to measure levels of secreted cytokines. ELISA for detecting type I interferons was planned but not performed due to limited PBMCs and hence supernatant availability.

Methodology used for pattern recognition receptor stimulation and Luminex multiple cytokine detection assays are described in detail in Sections 2.8 and 2.9.

5.3 Results

5.3.1 Baseline Characteristics

5 cases with chronic HCV infection who underwent interferon free antiviral treatment were included in the study of longitudinal changes in innate immune responses whilst on antiviral treatment. Their baseline characteristics are summarised in table 5.1.

Table 5. 1 Baseline characteristics of subjects included in the study of innate immune responses

		Cases (n=5)
Gender	Male : Female	3:2
Age (Mean ± SD)		41 ± 14
HCV Genotype	3	4
	1a	1
Cirrhosis	Present	2
	Absent	3
Treatment regimen	Sofosbuvir + Ribavirin	3
	Sofosbuvir + Daclatasvir+ Ribavirin	1
	Sofosbuvir + Daclatasvir	1
Treatment outcome	SVR	5

5.3.2 Baseline cytokine secretion by PBMCs in culture

PBMCs from cases with chronic HCV infection who reached SVR following interferon free antiviral treatment demonstrated production of cytokines in the absence of stimulatory drugs. The level of cytokine production was studied at treatment week (TW) 0, 2, 4, end of treatment (EOT) and 4 weeks post treatment and changed throughout these timepoints. There was an early rapid increase at week 2 in the levels of most pro-inflammatory cytokines studied including IL-8, IFN-γ, IL- 6, IL1-beta, IL1-alpha, and CCL3. There was an increase in IL28A levels at week 2 which peaked at treatment week 4. Following this peak there was a decline in production of these cytokines with lowest values at EOT. IFN-γ, IL-6, IL1alpha, IL1 beta, IL28A and IL12 all showed an increase in their levels 4 weeks after completion of treatment. Two cytokines showed a decrease in their levels at week 2 including IL28B and IL-12. (Figure 5.1).

DAA treatment regimens are associated with an early rapid decline in HCV viral load. Hence, above changes are probably the result of loss or decline in viral inhibition of immune pathways. However, it remains to be established whether this also contributes to viral clearance and sustaining this viral response.

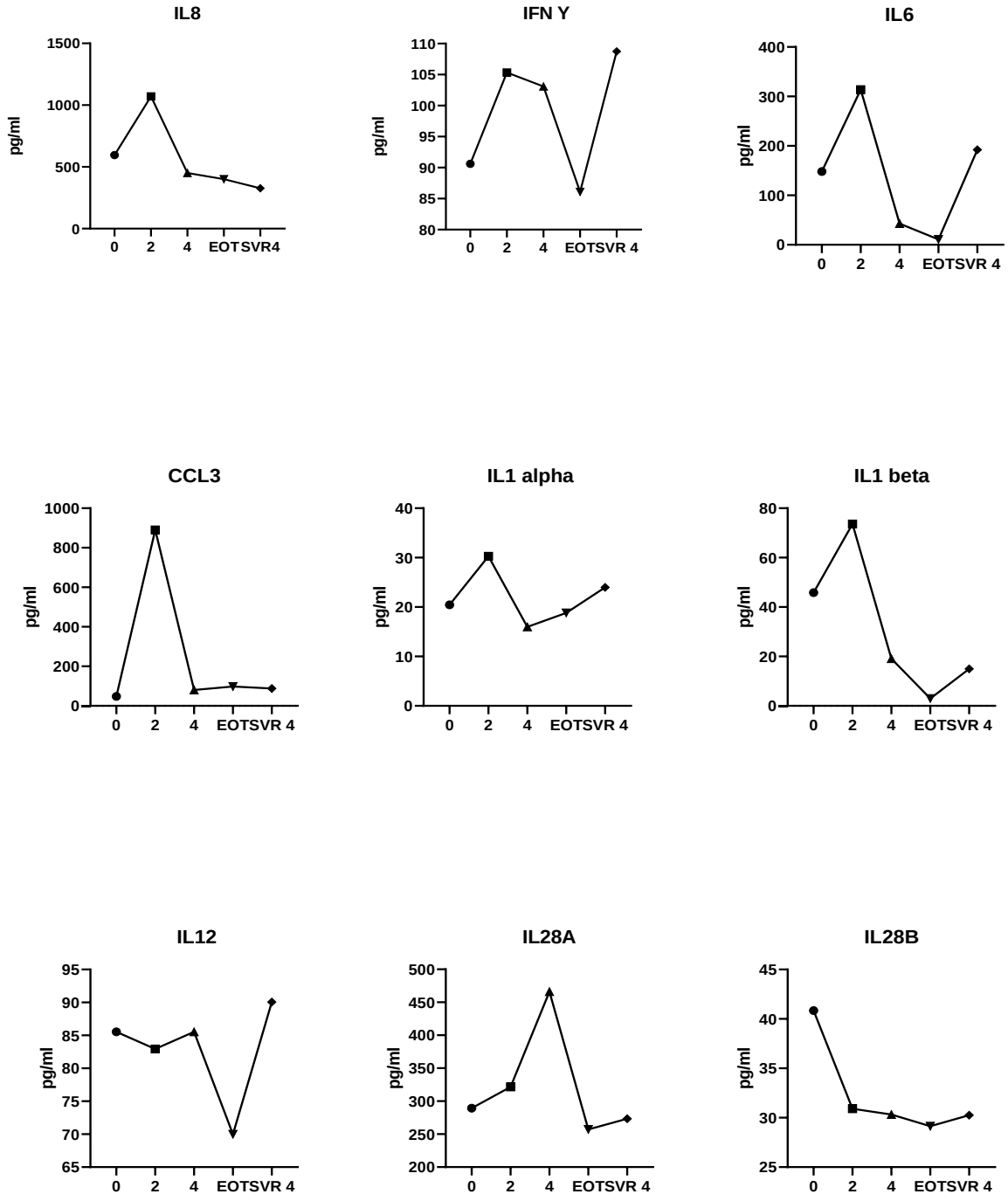

Figure 5. 1 Trends in pro-inflammatory and innate cytokine secretion by PBMCs in culture during interferon free direct acting antiviral treatment

Graphs show median cytokine levels in pg.ml at each time point during treatment. X-axis represents treatment time points: Week 0,2,4, end of treatment (EOT), 4 weeks post treatment (SVR4). Y-axis represents concentration in pg/ml of each cytokine.

5.3.3 TLR3 agonist induced secretion of cytokines

Supernatant collected following culture of PBMCs from individuals undergoing antiviral treatment was evaluated for secretion of IL-8, IFN-γ, IL-6, IL1-α, IL1-β, CCL3, IL-12, IL28A, IL28B before and after stimulation by TLR3 agonist at week 0 and 4 weeks post treatment. Percentage change in secretion of these cytokines by PBMCs following stimulation by TLR3 agonist was calculated and compared between the two timepoints.

Three different patterns of cytokine production were recognised in response to TLR3 ligand Poly (I:C), although there was no statistical difference in this small cohort of cases. There was higher percentage change in production of IFN-γ, IL28B and IL1 - alpha in response to TLR3 stimulation at week 0 in comparison with week 4 post treatment when there was comparatively lower response in case of IFN-γ and IL28B and no change in case of IL1-alpha (Figure 5.2). It is noteworthy that there may be delayed change or augmentation in responses beyond 4 weeks post treatment, not evaluated in this study.

In contrast to this, there were significantly higher responses in production of IL-6, IL-12, CCL3 and IL28A at week 4 post treatment in comparison with Week 0 where there was minimal change following stimulation (Figure 5.3 and 5.4). There was a similar increase in production of IL1-beta at week 4 post treatment although there was no significant change at week 0. This would be in keeping with at least partial restoration of TLR3 signalling four weeks post successful treatment.

There was no significant change in production of IL-8 following stimulation by TLR3 agonist either at baseline or week 4 post treatment (Figure 5.5).

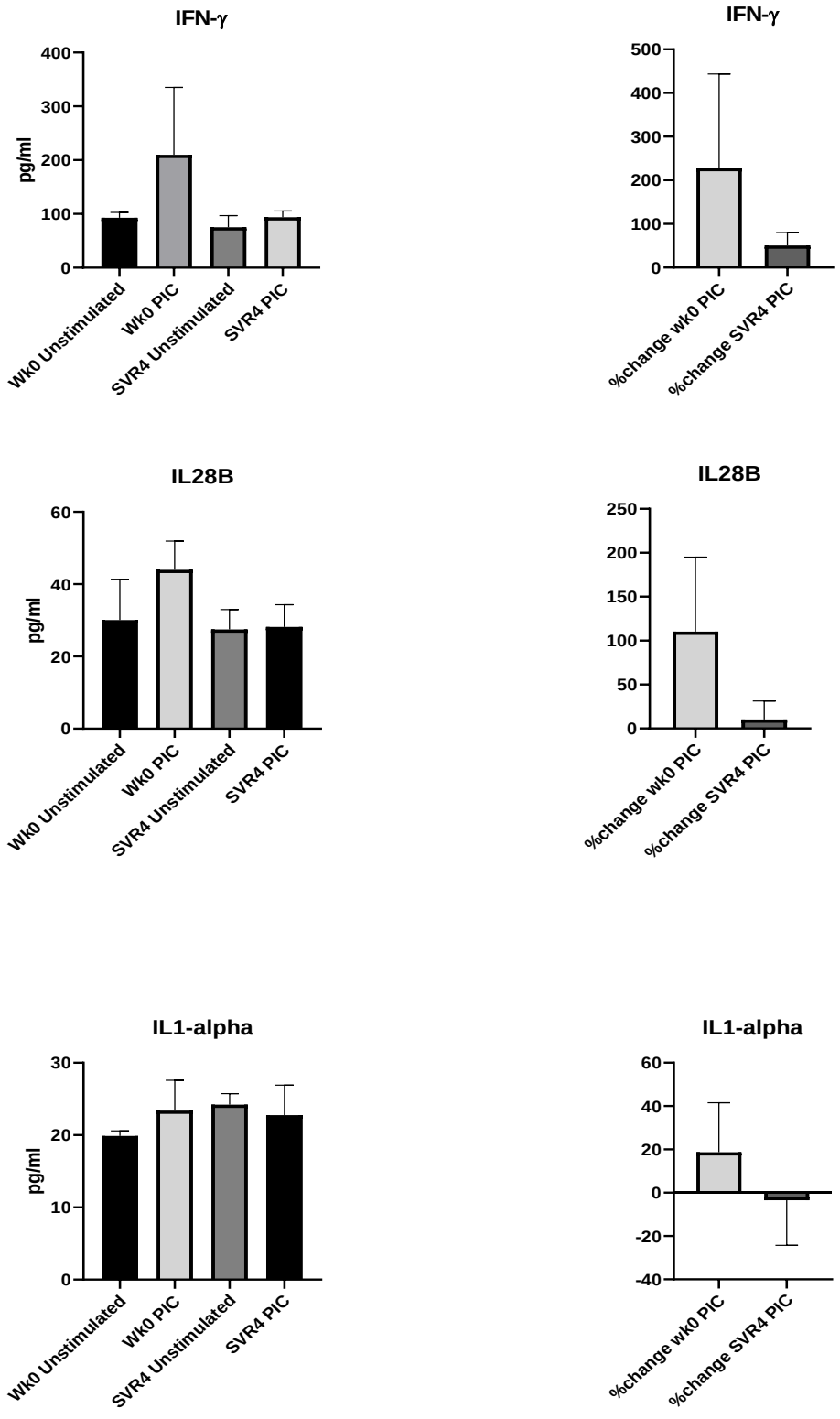

Figure 5. 2 Bar graph representing cytokine response to Poly (I:C) at baseline, 4 weeks post treatment (SVR4), and percentage change after stimulation by TLR3 agonist

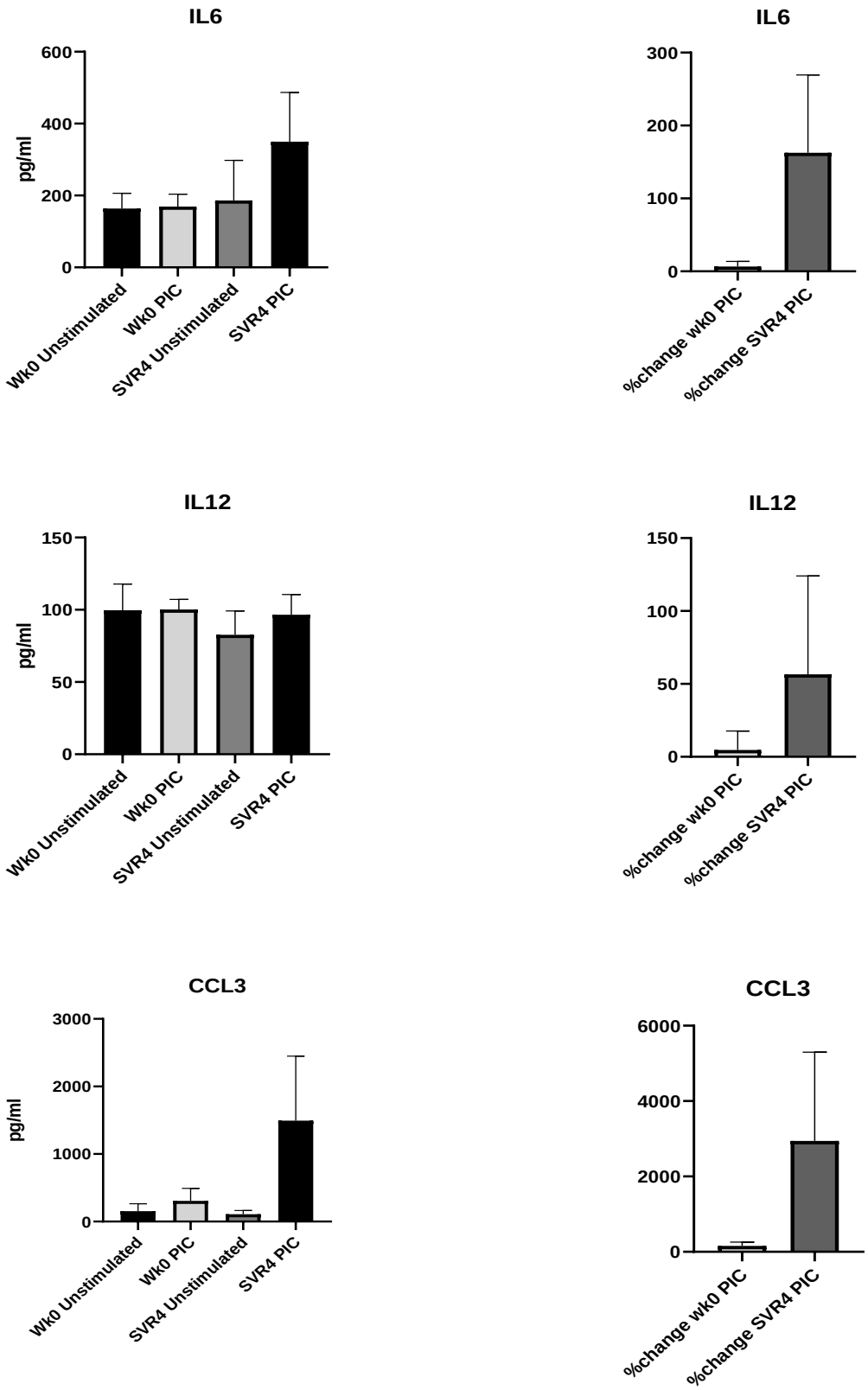

Figure 5. 3 Bar graph representing cytokine response to Poly (I:C) at baseline, 4 weeks post treatment (SVR4), and percentage change after stimulation by TLR3 agonist

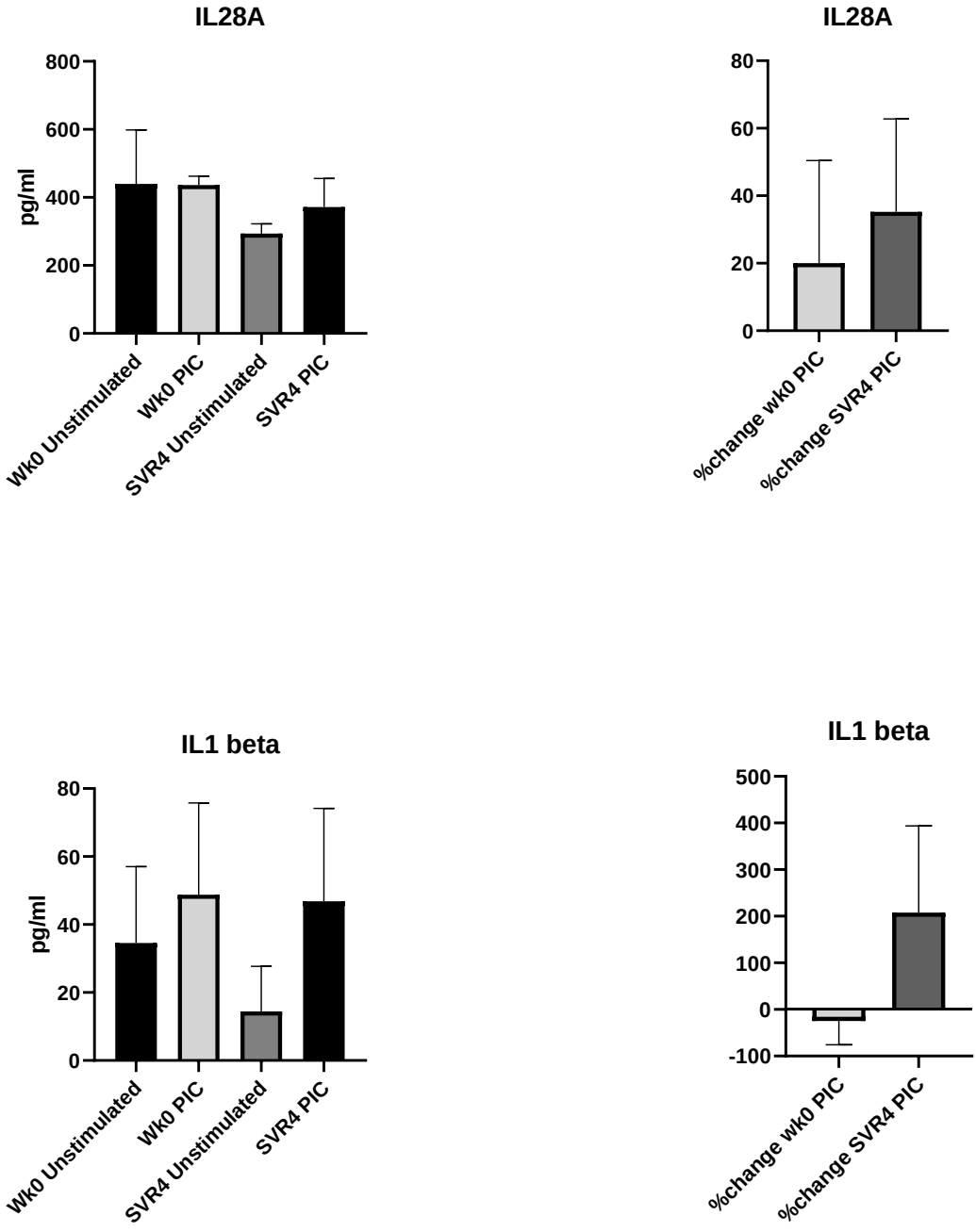

Figure 5. 4 Bar graph representing cytokine responses to Poly (I:C) at baseline, 4 weeks post treatment (SVR4), and percentage change after stimulation by TLR3 agonist.

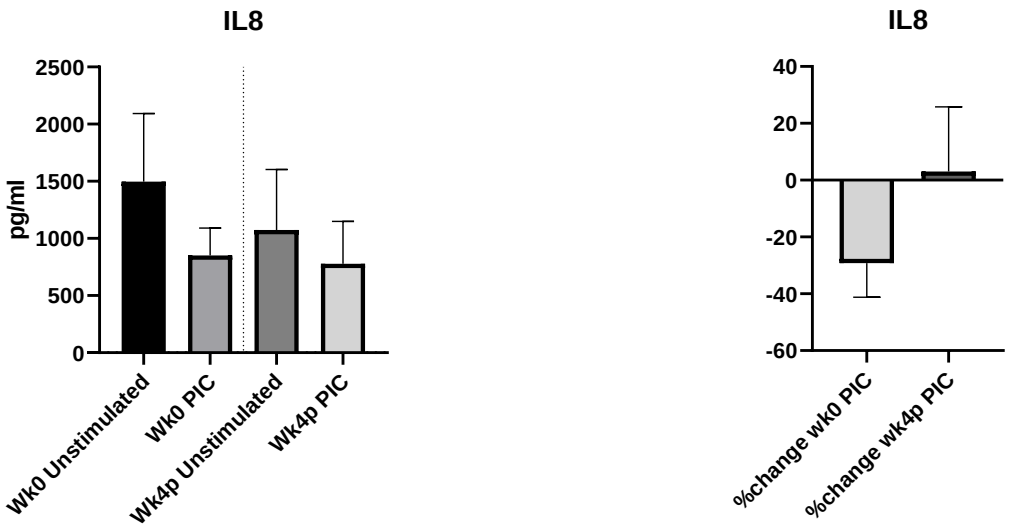

Figure 5. 5 Bar graph representing IL8 secretion in response to Poly (I:C) at baseline, 4 weeks post treatment (SVR4) and percentage change after stimulation by TLR3 agonist Poly (I:C).

5.3.4 TLR7/8 agonist induced cytokine secretion

Secretion of proinflammatory cytokines IFN-γ, IL-6, IL-8, IL-12, CCL3, IL1-alpha and IL1-beta induced by the TLR7/8 stimulant molecule R848 was higher at 4 weeks post treatment compared to baseline at week 0 (Figure 5.6). TLR7/8 ligand was also a potent stimulator causing augmentation in secretion of IL-6, CCL3, IL1-alpha, IL1-beta, as well as IL28A and IL28B at week 0, although the change in magnitude of response was higher at 4 weeks post treatment except in case of IL28A and IL28B (Figure 5.6). There was a slight increase in secretion of IL28A and IL28B at 4 weeks post treatment following stimulation of TLR7/8 but the magnitude of change was lower than week 0 (Figure 5.7). These findings were based on quantitative data, which did not reach statistical significance in this small cohort of cases.

Amongst PBMCs, TLR7/8 are predominantly expressed on pDCs. They are additionally expressed on NK cells and monocytes while mDCs express TLR8. These results would suggest at least partial restoration of innate signalling via TLR7/8 amongst these cells following HCV clearance. However, further study in a larger cohort of cases is required to validate these findings.

Further longitudinal studies beyond 4 weeks would establish either the stability or further augmentation of these responses at later time points.

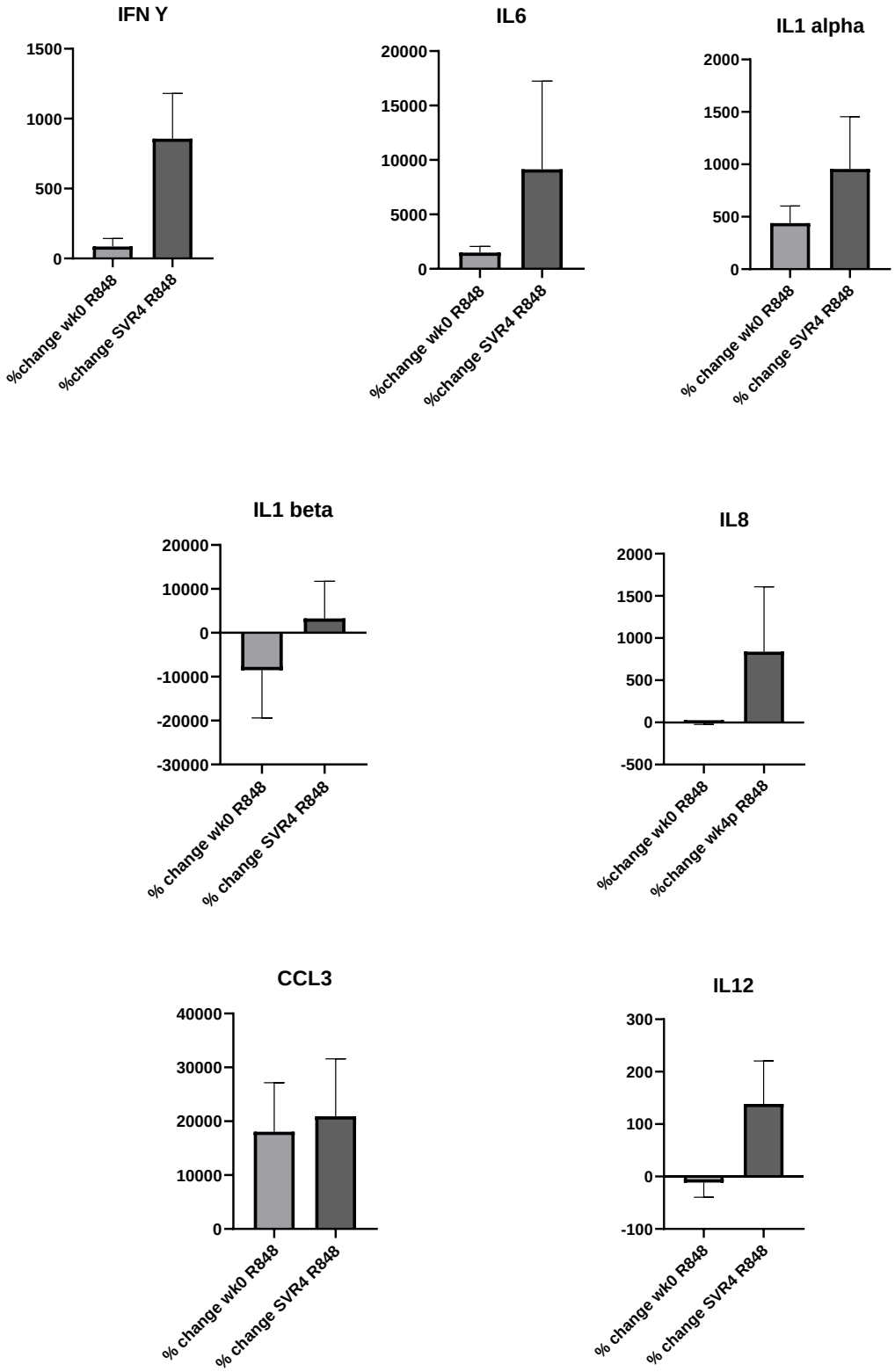

Figure 5. 6 Bar graphs representing percentage change in cytokine secretion in response to R848 (TLR7/8 ligand) at baseline and 4 weeks post treatment (SVR4)

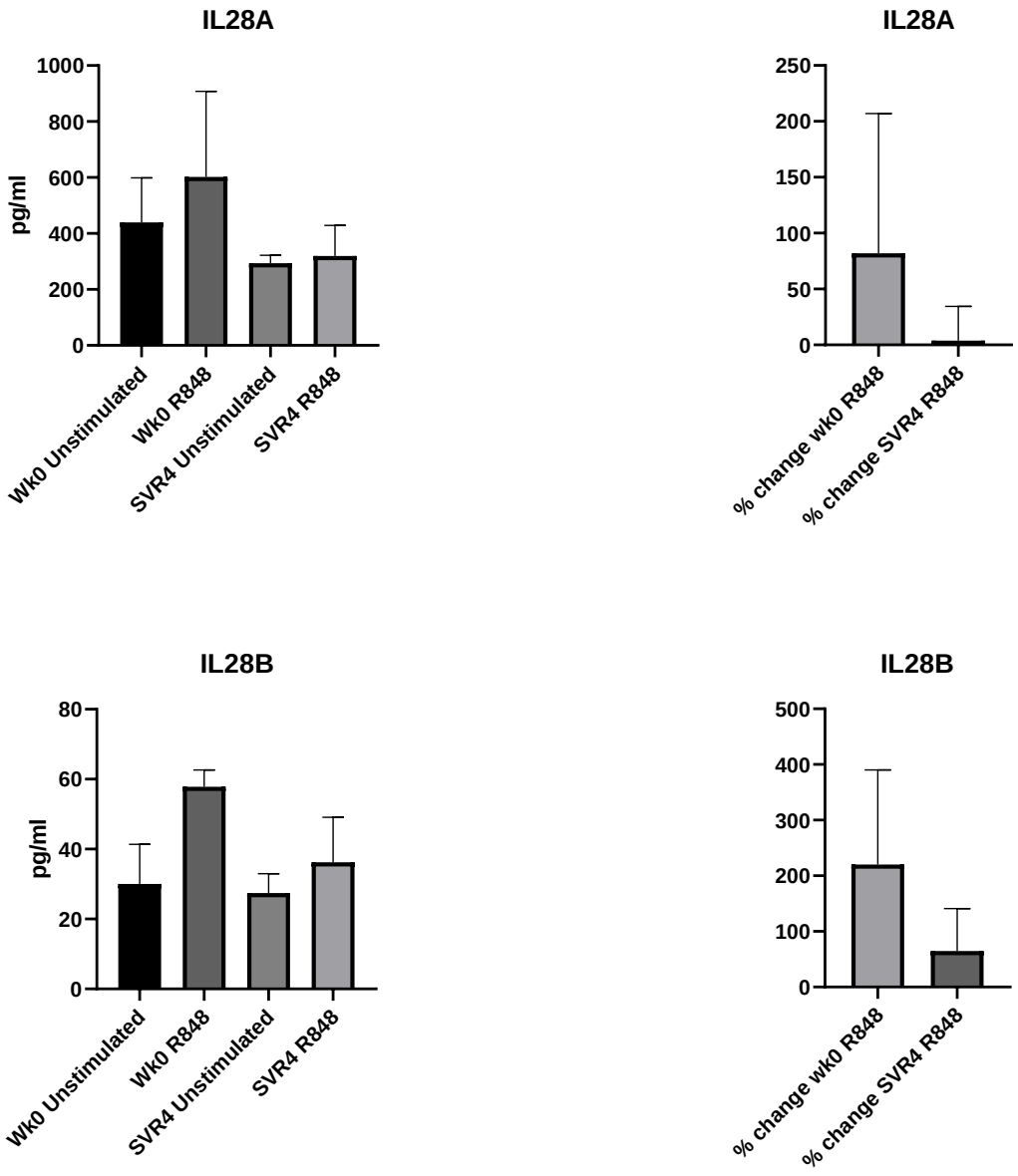

Figure 5. 7 Bar graphs representing type III interferon response to R848 (TLR7/8 ligand) at baseline and 4 weeks post treatment (SVR4).

5.3.5 RIG-I agonist induced cytokine secretion

Transfected Poly (I:C) used as RIG-I stimulant induced increased secretion of IFN-γ, IL-8, IL-6, IL-12, CCL3, IL1-alpha, IL1-beta and IL28A at 4 weeks post treatment after clearance of HCV compared to baseline at week 0 (Figure 5.8). This suggests restoration of innate immune signalling via the RIG-I pathway following viral clearance.

This effect was not observed in case of IL28B, where there was no change or decline in secretion both at baseline and week 4 post treatment (Figure 5.9).

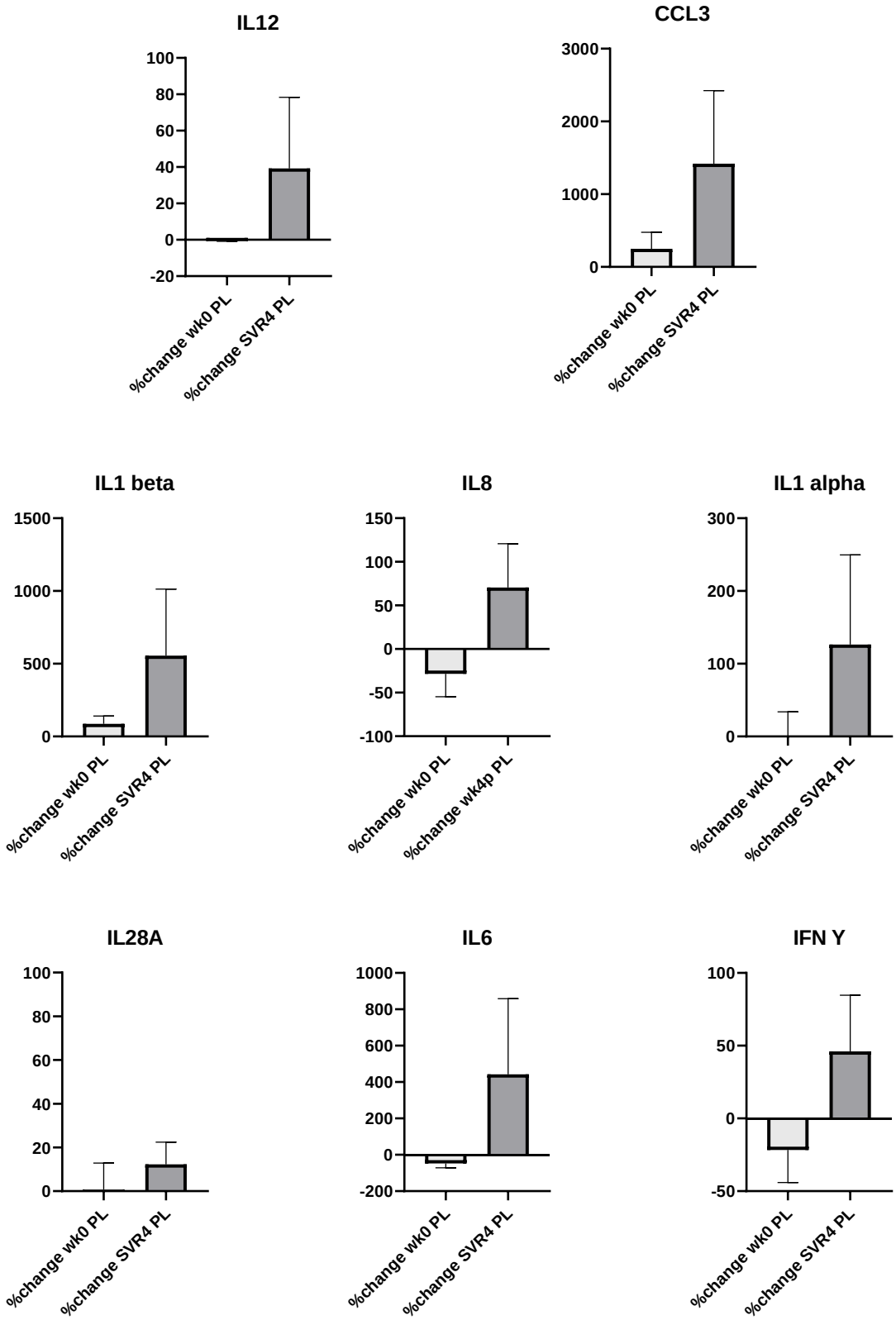

Figure 5. 8 Bar graphs representing percentage change in cytokine secretion in response to transfected Poly(I:C) activating RIG-I pathway at baseline and 4 weeks post treatment (SVR4)

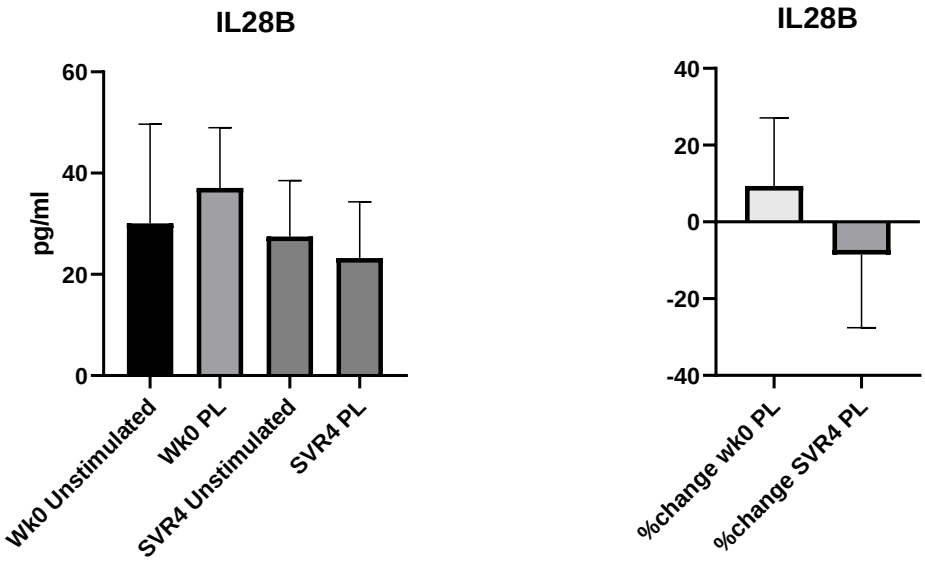

Figure 5. 9 Bar graphs representing type III interferon response to transfected Poly(I:C) at baseline and 4 weeks post treatment (SVR4).

5.4 Discussion

The work described in this chapter shows that there is an increase in secretion of pro-inflammatory cytokines very early after initiating therapy at week 2 in the absence of synthetic TLR receptor ligands during the course of interferon free antiviral treatment for chronic hepatitis C. This demonstrates that viral inhibition by use of antiviral drugs acting directly against viral proteins and inhibiting viral replication leads to early restoration of cytokine responses by removing the negative effect of viral proteins on innate immune signalling pathways described in section 1.12.4. It is possible that this early amplification of cytokine and chemokine responses contributes towards viral clearance with interferon free treatment regimens.

Interestingly, this effect was not seen in case of IL28B. Levels of IL28B continued to decline throughout treatment and remained low after completion of treatment. This is a probable response to rapid decrease in viral load leading to decrease in release of IL28B. IL28B along with other type III interferons have been shown to be upregulated following both in vitro and in vivo HCV infection. SNPs in or near IFNL3 gene encoding IL28B are known to be highly predictive of both antiviral success when using interferon alpha based regimens and spontaneous recovery in untreated patients. The favourable IFNL3 variant is associated with lower ISG expression in pre-treatment liver biopsies, allowing exogenous IFN alpha to rapidly induce an antiviral state. High levels of ISG induction in response to type III interferons has been linked to refractoriness to exogenous IFN alpha. Hence, a decline in IL28B with possible resultant lower induction of ISGs throughout treatment may represent viral clearance or indirectly contribute towards

effectiveness of treatment. A further evaluation of the IL28B genotype of the cases studied here will clarify if these had favourable IL28B genotype.

A study of similar responses in cases who failed to achieve sustained viral response with use of similar treatment regimens would allow to clarify differences, and the role of these responses in viral clearance.

Activation of NF-κB via TLR activation leads to gene transcription of a number of pro-inflammatory cytokines. This study demonstrated an increased release of these cytokines after viral clearance when compared with baseline response in keeping with the restoration of innate immune responses mediated by viral PAMP recognising toll like receptors and RIG-I. More specifically, this study demonstrates that there is restoration of innate immune responses generated by stimulation of TLR7/8 and RIG-I receptors after successful treatment of chronic HCV with interferon free therapy. This is shown by increased secretion of a range of pro-inflammatory cytokines using TLR7/8 and RIG-I ligands 4 weeks after successful antiviral treatment in comparison with pre-treatment levels at week 0. However, there was no increase in secretion of IL28A and IL28B demonstrated by stimulation of these PRR, which are important antiviral molecules suggesting that there is persistence of lack of some antiviral immune responses despite successful clearance of HCV. It is noteworthy that samples examined were obtained 4 weeks following completion of treatment, hence not evaluating delayed recovery of these responses.

Nonetheless, there was an increase in production of type II interferon IFN-γ in response to TLR7/8 and RIG stimulation. TLR7 and 8 are abundantly expressed on NK cells although predominant expression of TLR 7 is on pDCs. IFN-γ

secretion by NK cells is an early host defence against viral pathogens. It is well established that NK cell production of IFN-γ is suppressed in chronic HCV (Oliviero et al., 2009, Ahlenstiel et al, 2010). Increased secretion of IFN-γ in response to TLR7/8 stimulation suggests recovery of this response after viral clearance. Further study of TLR7/8 signalling in an isolated NK cell population will allow to confirm the source of IFN-γ.

pDCs are known to be inducers of antiviral state by producing abundant type I interferons following viral recognition by TLRs and RIG-I like receptors. We hoped to perform assays to evaluate Interferon alpha secretion in the supernatant following PRR stimulation; however these experiments could not be performed due to insufficient PBMCs and supernatant availability following the above experiments.

Another limitation of this study is the small size of the cohort. Although all cases showed comparable trends, findings were not amenable to find statistical significance although indicative trends were observed. Hence further study to evaluate such responses in a larger cohort is required to confirm or refute these initial findings.

In conclusion, this study demonstrates early restoration of innate immune responses generated by pattern recognition receptors involved in viral infection during treatment with interferon free antiviral treatment, which is further augmented following viral clearance. Further studies in a larger cohort of cases, over a longer period of time after treatment would help confirm or disprove these findings and establish if this improvement is stable or shows further augmentation

with time. Moreover, comparison with the relapse group will differentiate factors associated with viral clearance in treatment responders.

Chapter 6

Conclusion

6.1 Summary

The studies described in this thesis have allowed to elucidate changes in both innate and adaptive immune responses during antiviral treatment with interferon free direct acting anti-viral agents, as well as determine immune factors predictive of successful treatment response. This was demonstrated using two cohorts of patients undergoing interferon free antiviral treatment with one group developing sustained viral response following treatment, while patients in the second group relapsed. The experimental model used in this thesis was focussed on characterising phenotypic and functional T cell changes, and changes in innate immune signalling pathways in each group of patient, allowing comparisons.

We elucidated that T cell responses in the form of cytokine production, in particular IFN-γ, showed a very early improvement at week 2 of treatment in cases which reached sustained virological response. This improvement continued throughout and after treatment to reach a maximum at 12 weeks post treatment. This corresponds to phenotypic changes in form of loss of T cell inhibitory receptors and molecules, including programmed death-1 (PD-1) and

Galectin-9 (ligand protein for TIM-3). Overall, this confirms restoration of both T-cell function and phenotype after successful treatment with interferon free antiviral therapy.

We also found a distinct difference in T cell responses between relapsers and treatment responders, both at baseline and during the course of treatment. Interestingly, relapsers have a multi-specific T cell response with high amplitude at baseline. This characteristic has previously been observed only in cases of spontaneous resolution of HCV infection, where such T cell responses were thought to be responsible for spontaneous clearance of the virus following acute infection (Diepolder et al., 1995, Lechner et al., 2000, Thimme et al., 2001, Missale et al., 1996). We have established presence of such responses in some cases with chronic HCV infection, who subsequently are poor responders to usually highly effective interferon free direct acting antiviral drugs. A likely explanation of this would be emergence of viral escape mutations leading to chronicity and subsequent lack of response to direct acting antiviral therapy despite the presence of prototype T cell responses. All relapse cases studied in this thesis showed an initial response to treatment, with later relapse within 24 weeks after treatment associated with a sudden surge in T cell responses. This may be due to the emergence of variant viral sequences allowing ongoing viral replication and preventing existing T cell responses to effectively clear the virus. However, this concept can only be confirmed where the RNA sequences of inoculum hepatitis C virus at the time of infection, prior to treatment and at the time of relapse are known.

Previous studies have demonstrated CD127 memory phenotype of CD8 T cells targeting wild type sequence rather than escape regions in cases of viral escape

mutations (Rutebemberwa et al., 2008, Kasprowicz et al., 2010). Further characterising the T cell pool at the time of relapse alongside viral sequencing will allow to establish the nature and target of these T cells.

This is the first study describing the above clear findings of distinctive T cell responses between the two groups which can be considered predictive of poor treatment response in a relapse group commencing antiviral treatment. In addition, it indicates an additional role of direct acting antiviral agents in restoration of immune responses further to their direct effect on viral replication as a mechanism towards viral clearance.

It would be interesting to see the changes in the composition of T cell compartments, and whether memory T cells persist and are able to proliferate and activate effector response in case of further antigenic stimulation and how far this restoration would influence the clinical course in case of re-infection.

The restoration of T cell function and loss of exhaustion markers are also of interest when considering the development of preventative strategies against acquiring hepatitis C virus infection such as vaccines utilising primed CD8 and CD4 T cells.

It remains to be determined if such restoration in immune responses remains stable over longer periods of time or if there are in fact enhanced changes with time.

We further evaluated the effects of interferon free treatment on innate immune signalling pathways mediated via pattern recognition receptors, in particular TLR3, 7, 8 and RIG-I. It is well established that the virus proteins inhibit such

signalling leading to viral persistence and lack of effectiveness of IFN-α based treatments.

Interestingly, similar to T cell responses of adaptive immune system, there was a rapid increase in secretion of pro-inflammatory cytokines even in absence of additional synthetic PRR ligand stimulation early at week 2 during the course of interferon free DAA treatment. It remains to be established whether this contributes to viral clearance or is a result of lack of inhibition of signalling pathways by viral proteins resulting from decreasing viral load due to inhibition of viral replication by this treatment regimen. Given IL28B levels, with its established antiviral role in HCV clearance, decreased during the course of treatment would suggest that above cytokine responses are a result of viral inhibition.

Another finding in this study is at least partial restoration of signalling pathways of innate immune system mediated by activation of Toll like receptors (in particular TLR3/7/8) and RIG-I in response to recognition of viral PAMPs. This was elucidated using TLR ligands including Poly (I:C) for TLR3, R848 for TLR7/8 and Poly (I:C)/Lyovec complex for RIG-I stimulation of PBMCs at baseline and 4 weeks after completing treatment. Change in pro-inflammatory and antiviral cytokine production was measured. We were able to demonstrate increased release of IFN-γ, IL-6, IL-1α, IL-1β, IL-8 CCL3, and IL-12 following stimulation with toll like receptor and RIG-I ligands in vitro, after viral clearance suggesting at least partial restoration of these immune pathways. Such response was not seen in IL28A and IL28B production. It is however noteworthy that this was assessed four weeks post treatment, hence not evaluating delayed recovery of these important antiviral cytokines.

Overall, the number of cases studied for evaluating innate immune responses was small, making difficult to draw any conclusive results. Further evaluation in a larger case cohort will allow to validate these findings.

Innate immune cell types responsible for increase secretion of pro-inflammatory cytokines could not be established in this study. However, RIG-I and TLR7/8 are abundantly expressed on NK cells, while TLR 7 is predominant on pDCs. Hence, these cell types are likely source of high IFN-γ production in response to R848 and Poly(I:C)/Lyovec complex. Although type-I interferon assays were planned to evaluate antiviral interferon-α secretion in supernatant following PRR stimulation, this could not be performed due insufficient sample available.

Additionally, innate immune responses mediated by pattern recognition receptors were studied using synthetic single PRR ligands as a model for PAMPS in this study. A more specific stimulation of PBMCs using hepatitis C derived stimuli such as cell culture model of HCV infection (HCVcc) may offer findings more characteristic of HCV infection.

The cohort of patients included in analysis of innate immune signalling pathways in this thesis comprised of those which achieved SVR, further studies to establish the course of innate immune responses in relapsers would be vital to establish factors related to treatment failure with direct acting antivirals. In addition, viral sequencing in both groups to determine the role and presence of viral escape mutations in treatment failure cohort (i.e. relapsers) and it's relation to immune responses may allow to further delineate genetic reasons behind differing outcomes. However, this may not be necessary in current era of highly effective direct acting antiviral agents with sustained viral response rate of almost 100%.

A few limitations of this study require mentioning. First, the cohort was relatively small, and although most cases showed comparable results, it is possible that biologically significant trends were missed.

In addition, other factors that profoundly affect HCV-specific T cell immunity such as host HLA type or the degree of underlying liver disease may also affect the degree of T cell restoration after DAA therapy and needs to be evaluated further.

It also remains to be determined if peripheral changes observed in this study are reflected in the hepatic compartment following DAA therapy.

The interferon free regimen used in this study in some cases included ribavirin and the effects of both interferon and ribavirin free DAA on immunity was not assessed.

It is important to note here that antiviral treatment has undergone extraordinarily rapid development since this study was carried out, leading to the availability of highly effective drugs with high barrier to resistance and achieving almost 100% SVR rates. This also led the World Health Organisation (WHO) to adopt the strategy with a target to eliminate viral hepatitis globally as a public health problem. This limits the practical role of understanding such immune responses in current and future clinical practice.

6.2 Suggested areas for future studies

An important question remaining unanswered is whether this immunological recovery demonstrated in innate and adaptive immune systems remains stable over time or perhaps there is enhanced restoration of immune function if studied over longer periods of time. This can be answered by similar studies with a longer follow up period.

As mentioned earlier, in the current era of highly effective DAAs with response rates of almost 100%, study of the role of immune responses in achieving successful treatment response may have limited clinical value as such. However, further studies to establish clinical implications and role of reconstituted adaptive immunity in preventing re-infection or early clearance of the virus (spontaneous resolution) in case of re-infection would be an important avenue to explore, as well as our understanding of T cell immunity.

Appendices

Appendix 1 Patient consent form

Consent Form
Version 4.0 09th June 2015

Plymouth Hospitals NHS
NHS Trust

PARTICIPANT **CONSENT FORM**

SOUTHWEST STUDY OF HCV INFECTION
CONSENT FORM

A study of immunological mechanisms of protection and guiding treatment

	The *participant* should complete the whole of this sheet himself/herself	(Please circle one)
1.	Have you read the *participant* information sheet? (Please take a copy home with you to keep)	YES/NO
2.	Have you had an opportunity to discuss this study and ask any questions?	YES/NO
3.	Have you had satisfactory answers to all of your questions?	YES/NO
4.	Have you received enough information about the study?	YES/NO
5.	Who has given you an explanation about the study? Dr /Mr/Ms …….	
6.	Do you understand that you are free to withdraw from the study: □ At any time? □ Without having to give reason? □ Without affecting your future medical care?	YES/NO
7.	Do you agree to your GP being informed?	YES/NO
8.	Have you had sufficient time to come to your decision?	YES/NO
9.	Do you agree to have some of your blood and/or urine kept and used *for future studies investigating mechanisms of resistance to HCV infection?*	YES/NO
10	Do you agree to take part in this study?	YES/NO

Participant

REC 1703

Plymouth Hospitals NHS
NHS Trust

Participant

Signed ……

Date ……

Name (BLOCK LETTERS) ……..

INVESTIGATOR
I have explained the study to the above participant and he/she has indicated his/her willingness to take part.

Signed ……

Date ……

Name (BLOCK LETTERS) ……..

Appendix 2 Healthy volunteer consent form

Consent Form
Version 4.0 09th June 2015

Plymouth Hospitals NHS
NHS Trust

PARTICIPANT CONSENT FORM

SOUTHWEST STUDY OF HCV INFECTION
CONSENT FORM FOR UNEXPOSED CONTROLS

Why are some people susceptible to hepatitis C and not others?

A study of innate and immunological mechanisms of protection

	The *participant* should complete the whole of this sheet himself/herself	(Please circle one)
1.	Have you read the *participant* information sheet? (Please take a copy home with you to keep)	YES/NO
2.	Have you had an opportunity to discuss this study and ask any questions?	YES/NO
3.	Have you had satisfactory answers to all of your questions?	YES/NO
4.	Have you received enough information about the study?	YES/NO
5.	Who has given you an explanation about the study? Dr /Mr/Ms …….	
6.	Do you understand that you are free to withdraw from the study: ☐ At any time? ☐ Without having to give reason? ☐ Without affecting your future medical care?	YES/NO
7.	Do you agree to your GP being informed?	YES/NO
8.	Have you had sufficient time to come to your decision?	YES/NO
9.	Do you agree to have some of your blood and/or urine kept and used *for future studies investigating mechanisms of resistance to HCV infection?*	YES/NO
10	Do you agree to take part in this study?	YES/NO

Participant

Signed ……

Date ……

Name (BLOCK LETTERS) ……..

INVESTIGATOR
I have explained the study to the above participant and he/she has indicated his/her willingness to take part.

Signed ……

Date ……

Name (BLOCK LETTERS) ……..

Appendix 3 Patient information sheet

PARTICIPANT INFORMATION SHEET

Why are some people susceptible to hepatitis C and not others?

What is the purpose of the study?
Hepatitis C virus (HCV) currently infects 200,000 to 400,000 people in the United Kingdom. The aim of this project is to identify immunological and / or inherited factors responsible for protection from HCV infection. There is still a large gap in our understanding of how this may happen, but information gained from this study has the potential to be of great importance in the development of new treatments and possibly design of a vaccine.

Why have I been chosen?
As part of this study we need to confirm that the immunological responses we are testing for are specific for hepatitis C and can only found in cases with exposure to hepatitis C virus or those who have hepatitis C. We will be studying immunological responses in cases with hepatitis C infection undergoing treatment.

Do I have to take part?
No. Participation is entirely voluntary and no reason need be given for refusing.

What will happen to me if I take part?

- We will ask your permission to take about 40 mls of your blood (two tablespoonfuls). This will only be taken when your routine blood samples are taken. You will not be asked to give blood sample at any other time specially for this study. Your blood will be used to study immune responses and genetic factors that may influence susceptibility to hepatitis C infection and response to treatment.

Are there any disadvantages in taking part in this study?

- There may be some soreness and bruising after having the blood sample taken. However, this blood sample will only be taken when taking samples for your care during treatment of hepatitis C.

Contact for further information
If you have any problems, concerns, or questions about this study, please contact Professor Matthew Cramp on 01752 432722.

Appendix 4 Healthy volunteer information sheet

PARTICIPANT INFORMATION SHEET

Why are some people susceptible to hepatitis C and not others?

For Non-Exposed Controls

What is the purpose of the study?
Hepatitis C virus (HCV) currently infects 200,000 to 400,000 people in the United Kingdom. The aim of this project is to identify immunological and / or inherited factors responsible for protection from HCV infection. There is still a large gap in our understanding of how this may happen, but information gained from this study has the potential to be of great importance in the development of new treatments and possibly design of a vaccine.

Why have I been chosen?
As part of this study we need to confirm that the immunological responses we are testing for are specific for hepatitis C and can only found in cases with a clear risk of exposure to hepatitis C virus. We will therefore be studying controls with no history of exposure to, or risk factors for, hepatitis C virus infection.

Do I have to take part?
No. Participation is entirely voluntary and no reason need be given for refusing.

What will happen to me if I take part?

- We will ask you to confirm that you have no risk factors for hepatitis C virus infection i.e. no history of hepatitis, transfusion of blood or blood products, drug use, and no family history of liver disease.
- We will ask your permission to take about 40 mls of your blood (two tablespoonfuls). Your blood will be used to study immune responses and genetic factors that may influence susceptibility to hepatitis C infection.

Are there any disadvantages in taking part in this study?

- There may be some soreness and bruising after having the blood sample taken.
- The immunological studies are not diagnostic tests for hepatitis C. Evidence of immunological recognition of hepatitis C proteins could indicate that you have been exposed to, or infected by, hepatitis C virus but could also occur due to cross–reactivity with other viral proteins. If we find evidence of reactivity to hepatitis C virus this will be discussed with you and appropriate diagnostic testing can be arranged.

Contact for further information

If you have any problems, concerns, or questions about this study, please contact Professor Matthew Cramp on 01752 432722.

References

1999. Hepatitis C--global prevalence (update). *Wkly Epidemiol Rec,* 74**,** 425-7.

2002. National Institutes of Health Consensus Development Conference Statement: Management of hepatitis C 2002 (June 10-12, 2002). *Gastroenterology,* 123**,** 2082-99.

2017. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. *Lancet Gastroenterol Hepatol,* 2**,** 161-176.

Abdel-Hakeem, M. S., Bedard, N., Badr, G., Ostrowski, M., Sekaly, R. P., Bruneau, J., Willems, B., Heathcote, E. J. & Shoukry, N. H. 2010. Comparison of immune restoration in early versus late alpha interferon therapy against hepatitis C virus. *J Virol,* 84**,** 10429-35.

Abe, T., Kaname, Y., Hamamoto, I., Tsuda, Y., Wen, X., Taguwa, S., Moriishi, K., Takeuchi, O., Kawai, T., Kanto, T., Hayashi, N., Akira, S. & Matsuura, Y. 2007. Hepatitis C virus nonstructural protein 5A modulates the toll-like receptor-MyD88-dependent signaling pathway in macrophage cell lines. *J Virol,* 81**,** 8953-66.

Abel, M., Sene, D., Pol, S., Bourliere, M., Poynard, T., Charlotte, F., Cacoub, P. & Caillat-Zucman, S. 2006. Intrahepatic virus-specific IL-10-producing CD8 T cells prevent liver damage during chronic hepatitis C virus infection. *Hepatology,* 44**,** 1607-16.

Accapezzato, D., Francavilla, V., Paroli, M., Casciaro, M., Chircu, L. V., Cividini, A., Abrignani, S., Mondelli, M. U. & Barnaba, V. 2004. Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. *J Clin Invest,* 113**,** 963-72.

Afdhal, N. H., McHutchison, J. G., Zeuzem, S., Mangia, A., Pawlotsky, J. M., Murray, J. S., Shianna, K. V., Tanaka, Y., Thomas, D. L., Booth, D. R. & Goldstein, D. B. 2011. Hepatitis C pharmacogenetics: state of the art in 2010. *Hepatology,* 53**,** 336-45.

Agaugue, S., Perrin-Cocon, L., Andre, P. & Lotteau, V. 2007. Hepatitis C lipo-Viro-particle from chronically infected patients interferes with TLR4 signaling in dendritic cell. *PLoS One,* 2**,** e330.

Ahlenstiel, G., Edlich, B., Hogdal, L. J., Rotman, Y., Noureddin, M., Feld, J. J., Holz, L. E., Titerence, R. H., Liang, T. J. & Rehermann, B. 2011. Early changes in natural killer cell function indicate virologic response to interferon therapy for hepatitis C. *Gastroenterology,* 141**,** 1231-9, 1239.e1-2.

Ahlenstiel, G., Titerence, R. H., Koh, C., Edlich, B., Feld, J. J., Rotman, Y., Ghany, M. G., Hoofnagle, J. H., Liang, T. J., Heller, T. & Rehermann, B. 2010. Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon-alfa-dependent manner. *Gastroenterology,* 138**,** 325-35.e1-2.

Albert, M. L., Decalf, J. & Pol, S. 2008. Plasmacytoid dendritic cells move down on the list of suspects: in search of the immune pathogenesis of chronic hepatitis C. *J Hepatol,* 49**,** 1069-78.

Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature,* 413**,** 732-8.

Alric, L., Fort, M., Izopet, J., Vinel, J. P., Bureau, C., Sandre, K., Charlet, J. P., Beraud, M., Abbal, M. & Duffaut, M. 2000. Study of host- and virus-related factors associated with spontaneous hepatitis C virus clearance. *Tissue Antigens,* 56**,** 154-8.

Alter, H. J., Purcell, R. H., Shih, J. W., Melpolder, J. C., Houghton, M., Choo, Q. L. & Kuo, G. 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *N Engl J Med,* 321**,** 1494-500.

Alter, M. J., Hadler, S. C., Judson, F. N., Mares, A., Alexander, W. J., Hu, P. Y., Miller, J. K., Moyer, L. A., Fields, H. A., Bradley, D. W. & et al. 1990. Risk factors for acute non-A, non-B hepatitis in the United States and association with hepatitis C virus infection. *Jama,* 264**,** 2231-5.

Andre, P., Komurian-Pradel, F., Deforges, S., Perret, M., Berland, J. L., Sodoyer, M., Pol, S., Brechot, C., Paranhos-Baccala, G. & Lotteau, V. 2002. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. *J Virol,* 76**,** 6919-28.

Apolinario, A., Majano, P. L., Alvarez-Perez, E., Saez, A., Lozano, C., Vargas, J. & Garcia-Monzon, C. 2002. Increased expression of T cell chemokines and their receptors in chronic hepatitis C: relationship with the histological activity of liver disease. *Am J Gastroenterol,* 97**,** 2861-70.

Asselah, T., Bieche, I., Narguet, S., Sabbagh, A., Laurendeau, I., Ripault, M. P., Boyer, N., Martinot-Peignoux, M., Valla, D., Vidaud, M. & Marcellin, P. 2008. Liver gene expression signature to predict response to pegylated interferon plus ribavirin combination therapy in patients with chronic hepatitis C. *Gut,* 57**,** 516-24.

Badr, G., Bedard, N., Abdel-Hakeem, M. S., Trautmann, L., Willems, B., Villeneuve, J. P., Haddad, E. K., Sekaly, R. P., Bruneau, J. & Shoukry, N. H. 2008. Early interferon therapy for hepatitis C virus infection rescues polyfunctional, long-lived CD8+ memory T cells. *J Virol,* 82**,** 10017-31.

Bain, C., Fatmi, A., Zoulim, F., Zarski, J. P., Trepo, C. & Inchauspe, G. 2001. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology,* 120**,** 512-24.

Barber, D. L., Wherry, E. J., Masopust, D., Zhu, B., Allison, J. P., Sharpe, A. H., Freeman, G. J. & Ahmed, R. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature,* 439**,** 682-7.

Barnes, E., Gelderblom, H. C., Humphreys, I., Semmo, N., Reesink, H. W., Beld, M. G., van Lier, R. A. & Klenerman, P. 2009. Cellular immune responses during high-dose interferon-alpha induction therapy for hepatitis C virus infection. *J Infect Dis,* 199**,** 819-28.

Barnes, E., Harcourt, G., Brown, D., Lucas, M., Phillips, R., Dusheiko, G. & Klenerman, P. 2002. The dynamics of T-lymphocyte responses during combination therapy for chronic hepatitis C virus infection. *Hepatology,* 36**,** 743-54.

Barnes, E., Salio, M., Cerundolo, V., Francesco, L., Pardoll, D., Klenerman, P. & Cox, A. 2008. Monocyte derived dendritic cells retain their functional capacity in patients following infection with hepatitis C virus. *J Viral Hepat,* 15**,** 219-28.

Barrett, S., Collins, M., Kenny, C., Ryan, E., Keane, C. O. & Crowe, J. 2003. Polymorphisms in tumour necrosis factor-alpha, transforming growth factor-beta, interleukin-10, interleukin-6, interferon-gamma, and outcome of hepatitis C virus infection. *J Med Virol,* 71**,** 212-8.

Bartenschlager, R. & Pietschmann, T. 2005. Efficient hepatitis C virus cell culture system: what a difference the host cell makes. *Proc Natl Acad Sci U S A,* 102**,** 9739-40.

Bartosch, B., Dubuisson, J. & Cosset, F. L. 2003a. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J Exp Med,* 197**,** 633-42.

Bartosch, B., Vitelli, A., Granier, C., Goujon, C., Dubuisson, J., Pascale, S., Scarselli, E., Cortese, R., Nicosia, A. & Cosset, F. L. 2003b. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *J Biol Chem,* 278**,** 41624-30.

Bengsch, B., Seigel, B., Ruhl, M., Timm, J., Kuntz, M., Blum, H. E., Pircher, H. & Thimme, R. 2010. Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. *PLoS Pathog,* 6**,** e1000947.

Bertoletti, A., D'Elios, M. M., Boni, C., De Carli, M., Zignego, A. L., Durazzo, M., Missale, G., Penna, A., Fiaccadori, F., Del Prete, G. & Ferrari, C. 1997. Different cytokine profiles of intraphepatic T cells in chronic hepatitis B and hepatitis C virus infections. *Gastroenterology,* 112**,** 193-9.

Bigger, C. B., Brasky, K. M. & Lanford, R. E. 2001. DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. *J Virol,* 75**,** 7059-66.

Bigger, C. B., Guerra, B., Brasky, K. M., Hubbard, G., Beard, M. R., Luxon, B. A., Lemon, S. M. & Lanford, R. E. 2004. Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees. *J Virol,* 78**,** 13779-92.

Biron, C. A. & Brossay, L. 2001. NK cells and NKT cells in innate defense against viral infections. *Curr Opin Immunol,* 13**,** 458-64.

Blackburn, S. D., Shin, H., Freeman, G. J. & Wherry, E. J. 2008. Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. *Proc Natl Acad Sci U S A,* 105**,** 15016-21.

Blattman, J. N., Wherry, E. J., Ha, S. J., van der Most, R. G. & Ahmed, R. 2009. Impact of epitope escape on PD-1 expression and CD8 T-cell exhaustion during chronic infection. *J Virol,* 83**,** 4386-94.

Blight, K. J., Kolykhalov, A. A. & Rice, C. M. 2000. Efficient initiation of HCV RNA replication in cell culture. *Science,* 290**,** 1972-4.

Bode, J. G., Ludwig, S., Ehrhardt, C., Albrecht, U., Erhardt, A., Schaper, F., Heinrich, P. C. & Haussinger, D. 2003. IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. *Faseb j,* 17**,** 488-90.

Boettler, T., Spangenberg, H. C., Neumann-Haefelin, C., Panther, E., Urbani, S., Ferrari, C., Blum, H. E., von Weizsacker, F. & Thimme, R. 2005. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. *J Virol,* 79**,** 7860-7.

Bowen, D. G. & Walker, C. M. 2005. Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature,* 436**,** 946-52.

Brass, V., Moradpour, D. & Blum, H. E. 2006. Molecular virology of hepatitis C virus (HCV): 2006 update. *Int J Med Sci,* 3**,** 29-34.

Brooks, D. G., Ha, S. J., Elsaesser, H., Sharpe, A. H., Freeman, G. J. & Oldstone, M. B. 2008. IL-10 and PD-L1 operate through distinct pathways to suppress T-cell activity during persistent viral infection. *Proc Natl Acad Sci U S A,* 105**,** 20428-33.

Brooks, D. G., Trifilo, M. J., Edelmann, K. H., Teyton, L., McGavern, D. B. & Oldstone, M. B. 2006. Interleukin-10 determines viral clearance or persistence in vivo. *Nat Med,* 12**,** 1301-9.

Bukh, J. 2004. A critical role for the chimpanzee model in the study of hepatitis C. *Hepatology,* 39**,** 1469-75.

Bukh, J., Apgar, C. L., Govindarajan, S., Emerson, S. U. & Purcell, R. H. 2001. Failure to infect rhesus monkeys with hepatitis C virus strains of genotypes 1a, 2a or 3a. *J Viral Hepat,* 8**,** 228-31.

Burchill, M. A., Golden-Mason, L., Wind-Rotolo, M. & Rosen, H. R. 2015. Memory re-differentiation and reduced lymphocyte activation in chronic HCV-infected patients receiving direct-acting antivirals. *J Viral Hepat,* 22**,** 983-91.

Cabrera, R., Tu, Z., Xu, Y., Firpi, R. J., Rosen, H. R., Liu, C. & Nelson, D. R. 2004. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology,* 40**,** 1062-71.

Callendret, B., Bukh, J., Eccleston, H. B., Heksch, R., Hasselschwert, D. L., Purcell, R. H., Hughes, A. L. & Walker, C. M. 2011. Transmission of clonal hepatitis C virus genomes reveals the dominant but transitory role of CD8(+) T cells in early viral evolution. *J Virol,* 85**,** 11833-45.

Castet, V., Fournier, C., Soulier, A., Brillet, R., Coste, J., Larrey, D., Dhumeaux, D., Maurel, P. & Pawlotsky, J. M. 2002. Alpha interferon inhibits hepatitis C virus replication in primary human hepatocytes infected in vitro. *J Virol,* 76**,** 8189-99.

Chang, K. M., Rehermann, B., McHutchison, J. G., Pasquinelli, C., Southwood, S., Sette, A. & Chisari, F. V. 1997. Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. *J Clin Invest,* 100**,** 2376-85.

Chang, K. M., Thimme, R., Melpolder, J. J., Oldach, D., Pemberton, J., Moorhead-Loudis, J., McHutchison, J. G., Alter, H. J. & Chisari, F. V. 2001. Differential CD4(+) and CD8(+) T-cell responsiveness in hepatitis C virus infection. *Hepatology,* 33**,** 267-76.

Chang, S., Dolganiuc, A. & Szabo, G. 2007. Toll-like receptors 1 and 6 are involved in TLR2-mediated macrophage activation by hepatitis C virus core and NS3 proteins. *J Leukoc Biol,* 82**,** 479-87.

Chen, L., Borozan, I., Feld, J., Sun, J., Tannis, L. L., Coltescu, C., Heathcote, J., Edwards, A. M. & McGilvray, I. D. 2005. Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. *Gastroenterology,* 128**,** 1437-44.

Chen, M., Sallberg, M., Sonnerborg, A., Weiland, O., Mattsson, L., Jin, L., Birkett, A., Peterson, D. & Milich, D. R. 1999. Limited humoral immunity in hepatitis C virus infection. *Gastroenterology,* 116**,** 135-43.

Chiaramonte, M., Stroffolini, T., Vian, A., Stazi, M. A., Floreani, A., Lorenzoni, U., Lobello, S., Farinati, F. & Naccarato, R. 1999. Rate of incidence of hepatocellular carcinoma in patients with compensated viral cirrhosis. *Cancer,* 85**,** 2132-7.

Chomarat, P., Banchereau, J., Davoust, J. & Palucka, A. K. 2000. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol,* 1**,** 510-4.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. & Houghton, M. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science,* 244**,** 359-62.

Choo, Q. L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, R., Barr, P. J. & et al. 1991. Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci U S A,* 88**,** 2451-5.

Colin, C., Lanoir, D., Touzet, S., Meyaud-Kraemer, L., Bailly, F. & Trepo, C. 2001. Sensitivity and specificity of third-generation hepatitis C virus antibody detection assays: an analysis of the literature. *J Viral Hepat,* 8**,** 87-95.

Constantini, P. K., Wawrzynowicz-Syczewska, M., Clare, M., Boron-Kaczmarska, A., McFarlane, I. G., Cramp, M. E. & Donaldson, P. T. 2002. Interleukin-1, interleukin-10 and tumour necrosis factor-alpha gene polymorphisms in hepatitis C virus infection: an investigation of the relationships with spontaneous viral clearance and response to alpha-interferon therapy. *Liver,* 22**,** 404-12.

Cooper, M. A., Fehniger, T. A. & Caligiuri, M. A. 2001. The biology of human natural killer-cell subsets. *Trends Immunol,* 22**,** 633-40.

Cooper, S., Erickson, A. L., Adams, E. J., Kansopon, J., Weiner, A. J., Chien, D. Y., Houghton, M., Parham, P. & Walker, C. M. 1999. Analysis of a successful immune response against hepatitis C virus. *Immunity,* 10**,** 439-49.

Corado, J., Toro, F., Rivera, H., Bianco, N. E., Deibis, L. & De Sanctis, J. B. 1997. Impairment of natural killer (NK) cytotoxic activity in hepatitis C virus (HCV) infection. *Clin Exp Immunol,* 109**,** 451-7.

Cox, A. L., Mosbruger, T., Lauer, G. M., Pardoll, D., Thomas, D. L. & Ray, S. C. 2005a. Comprehensive analyses of CD8+ T cell responses during longitudinal study of acute human hepatitis C. *Hepatology,* 42**,** 104-12.

Cox, A. L., Mosbruger, T., Mao, Q., Liu, Z., Wang, X. H., Yang, H. C., Sidney, J., Sette, A., Pardoll, D., Thomas, D. L. & Ray, S. C. 2005b. Cellular immune selection with hepatitis C virus persistence in humans. *J Exp Med,* 201**,** 1741-52.

Cramp, M. E., Carucci, P., Rossol, S., Chokshi, S., Maertens, G., Williams, R. & Naoumov, N. V. 1999. Hepatitis C virus (HCV) specific immune responses in anti-HCV positive patients without hepatitis C viraemia. *Gut,* 44**,** 424-9.

Cramp, M. E., Carucci, P., Underhill, J., Naoumov, N. V., Williams, R. & Donaldson, P. T. 1998. Association between HLA class II genotype and spontaneous clearance of hepatitis C viraemia. *J Hepatol,* 29**,** 207-13.

Crotta, S., Stilla, A., Wack, A., D'Andrea, A., Nuti, S., D'Oro, U., Mosca, M., Filliponi, F., Brunetto, R. M., Bonino, F., Abrignani, S. & Valiante, N. M. 2002. Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. *J Exp Med,* 195**,** 35-41.

Dao Thi, V. L., Granier, C., Zeisel, M. B., Guerin, M., Mancip, J., Granio, O., Penin, F., Lavillette, D., Bartenschlager, R., Baumert, T. F., Cosset, F. L. & Dreux, M. 2012. Characterization of hepatitis C virus particle subpopulations reveals multiple usage of the scavenger receptor BI for entry steps. *J Biol Chem,* 287**,** 31242-57.

Day, C. L., Lauer, G. M., Robbins, G. K., McGovern, B., Wurcel, A. G., Gandhi, R. T., Chung, R. T. & Walker, B. D. 2002. Broad specificity of virus-specific CD4+ T-helper-cell responses in resolved hepatitis C virus infection. *J Virol,* 76**,** 12584-95.

De Maria, A., Fogli, M., Mazza, S., Basso, M., Picciotto, A., Costa, P., Congia, S., Mingari, M. C. & Moretta, L. 2007. Increased natural cytotoxicity receptor expression and relevant IL-10 production in NK cells from chronically infected viremic HCV patients. *Eur J Immunol,* 37**,** 445-55.

Decalf, J., Fernandes, S., Longman, R., Ahloulay, M., Audat, F., Lefrerre, F., Rice, C. M., Pol, S. & Albert, M. L. 2007. Plasmacytoid dendritic cells initiate a complex chemokine and cytokine network and are a viable drug target in chronic HCV patients. *J Exp Med,* 204**,** 2423-37.

Diepolder, H. M., Gerlach, J. T., Zachoval, R., Hoffmann, R. M., Jung, M. C., Wierenga, E. A., Scholz, S., Santantonio, T., Houghton, M., Southwood, S., Sette, A. & Pape, G. R. 1997. Immunodominant CD4+ T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection. *J Virol,* 71**,** 6011-9.

Diepolder, H. M., Zachoval, R., Hoffmann, R. M., Jung, M. C., Gerlach, T. & Pape, G. R. 1996. The role of hepatitis C virus specific CD4+ T lymphocytes in acute and chronic hepatitis C. *J Mol Med (Berl),* 74**,** 583-8.

Diepolder, H. M., Zachoval, R., Hoffmann, R. M., Wierenga, E. A., Santantonio, T., Jung, M. C., Eichenlaub, D. & Pape, G. R. 1995. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet,* 346**,** 1006-7.

Dill, M. T., Makowska, Z., Duong, F. H. T., Merkofer, F., Filipowicz, M., Baumert, T. F., Tornillo, L., Terracciano, L. & Heim, M. H. 2012. Interferon-gamma-stimulated genes, but not USP18, are expressed in livers of patients with acute hepatitis C. *Gastroenterology,* 143**,** 777-786.e6.

Donnelly, R. P., Sheikh, F., Kotenko, S. V. & Dickensheets, H. 2004. The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10R2) chain. *J Leukoc Biol,* 76**,** 314-21.

Dreux, M., Garaigorta, U., Boyd, B., Decembre, E., Chung, J., Whitten-Bauer, C., Wieland, S. & Chisari, F. V. 2012. Short-range exosomal transfer of viral RNA from infected cells to plasmacytoid dendritic cells triggers innate immunity. *Cell Host Microbe,* 12**,** 558-70.

Duong, F. H., Trincucci, G., Boldanova, T., Calabrese, D., Campana, B., Krol, I., Durand, S. C., Heydmann, L., Zeisel, M. B., Baumert, T. F. & Heim, M. H. 2014. IFN-lambda receptor 1 expression is induced in chronic hepatitis C and correlates with the IFN-lambda3 genotype and with nonresponsiveness to IFN-alpha therapies. *J Exp Med,* 211**,** 857-68.

Durbin, J. E., Hackenmiller, R., Simon, M. C. & Levy, D. E. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell,* 84**,** 443-50.

Echeverria, I., Zabaleta, A., Silva, L., Diaz-Valdes, N., Riezu-Boj, J. I., Lasarte, J. J., Borras-Cuesta, F., Civeira, M. P., Prieto, J. & Sarobe, P. 2008. Monocyte-derived dendritic cells from HCV-infected patients transduced with an adenovirus expressing NS3 are functional when stimulated with the TLR3 ligand poly(I:C). *J Viral Hepat,* 15**,** 782-9.

Edlich, B., Ahlenstiel, G., Zabaleta Azpiroz, A., Stoltzfus, J., Noureddin, M., Serti, E., Feld, J. J., Liang, T. J., Rotman, Y. & Rehermann, B. 2012. Early changes in interferon signaling define natural killer cell response and refractoriness to interferon-based therapy of hepatitis C patients. *Hepatology,* 55**,** 39-48.

Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H. E., Moradpour, D. & Bienz, K. 2002. Expression of hepatitis C virus proteins induces distinct

membrane alterations including a candidate viral replication complex. *J Virol,* 76**,** 5974-84.

Ejrnaes, M., Filippi, C. M., Martinic, M. M., Ling, E. M., Togher, L. M., Crotty, S. & von Herrath, M. G. 2006. Resolution of a chronic viral infection after interleukin-10 receptor blockade. *J Exp Med,* 203**,** 2461-72.

Evans, M. J., von Hahn, T., Tscherne, D. M., Syder, A. J., Panis, M., Wolk, B., Hatziioannou, T., McKeating, J. A., Bieniasz, P. D. & Rice, C. M. 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature,* 446**,** 801-5.

Farci, P., Alter, H. J., Govindarajan, S., Wong, D. C., Engle, R., Lesniewski, R. R., Mushahwar, I. K., Desai, S. M., Miller, R. H., Ogata, N. & et al. 1992. Lack of protective immunity against reinfection with hepatitis C virus. *Science,* 258**,** 135-40.

Farci, P., Alter, H. J., Wong, D. C., Miller, R. H., Govindarajan, S., Engle, R., Shapiro, M. & Purcell, R. H. 1994. Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated in vitro neutralization. *Proc Natl Acad Sci U S A,* 91**,** 7792-6.

Fattovich, G., Giustina, G., Degos, F., Tremolada, F., Diodati, G., Almasio, P., Nevens, F., Solinas, A., Mura, D., Brouwer, J. T., Thomas, H., Njapoum, C., Casarin, C., Bonetti, P., Fuschi, P., Basho, J., Tocco, A., Bhalla, A., Galassini, R., Noventa, F., Schalm, S. W. & Realdi, G. 1997. Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients. *Gastroenterology,* 112**,** 463-72.

Fernandez, J., Taylor, D., Morhardt, D. R., Mihalik, K., Puig, M., Rice, C. M., Feinstone, S. M. & Major, M. E. 2004. Long-term persistence of infection in chimpanzees inoculated with an infectious hepatitis C virus clone is associated with a decrease in the viral amino acid substitution rate and low levels of heterogeneity. *J Virol,* 78**,** 9782-9.

Florentin, J., Aouar, B., Dental, C., Thumann, C., Firaguay, G., Gondois-Rey, F., Soumelis, V., Baumert, T. F., Nunes, J. A., Olive, D., Hirsch, I. & Stranska, R. 2012. HCV glycoprotein E2 is a novel BDCA-2 ligand and acts as an inhibitor of IFN production by plasmacytoid dendritic cells. *Blood,* 120**,** 4544-51.

Frank, C., Mohamed, M. K., Strickland, G. T., Lavanchy, D., Arthur, R. R., Magder, L. S., El Khoby, T., Abdel-Wahab, Y., Aly Ohn, E. S., Anwar, W.

& Sallam, I. 2000. The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. *Lancet,* 355**,** 887-91.

Fuller, M. J., Shoukry, N. H., Gushima, T., Bowen, D. G., Callendret, B., Campbell, K. J., Hasselschwert, D. L., Hughes, A. L. & Walker, C. M. 2010. Selection-driven immune escape is not a significant factor in the failure of CD4 T cell responses in persistent hepatitis C virus infection. *Hepatology,* 51**,** 378-87.

Gack, M. U., Shin, Y. C., Joo, C. H., Urano, T., Liang, C., Sun, L., Takeuchi, O., Akira, S., Chen, Z., Inoue, S. & Jung, J. U. 2007. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature,* 446**,** 916-920.

Gale, M. J., Jr., Korth, M. J., Tang, N. M., Tan, S. L., Hopkins, D. A., Dever, T. E., Polyak, S. J., Gretch, D. R. & Katze, M. G. 1997. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology,* 230**,** 217-27.

Garaigorta, U. & Chisari, F. V. 2009. Hepatitis C virus blocks interferon effector function by inducing protein kinase R phosphorylation. *Cell Host Microbe,* 6**,** 513-22.

Ge, D., Fellay, J., Thompson, A. J., Simon, J. S., Shianna, K. V., Urban, T. J., Heinzen, E. L., Qiu, P., Bertelsen, A. H., Muir, A. J., Sulkowski, M., McHutchison, J. G. & Goldstein, D. B. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature,* 461**,** 399-401.

Gerlach, J. T., Diepolder, H. M., Jung, M. C., Gruener, N. H., Schraut, W. W., Zachoval, R., Hoffmann, R., Schirren, C. A., Santantonio, T. & Pape, G. R. 1999. Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology,* 117**,** 933-41.

Giang, E., Dorner, M., Prentoe, J. C., Dreux, M., Evans, M. J., Bukh, J., Rice, C. M., Ploss, A., Burton, D. R. & Law, M. 2012. Human broadly neutralizing antibodies to the envelope glycoprotein complex of hepatitis C virus. *Proc Natl Acad Sci U S A,* 109**,** 6205-10.

Giannitrapani, L., Soresi, M., Balasus, D., Licata, A. & Montalto, G. 2013. Genetic association of interleukin-6 polymorphism (-174 G/C) with chronic liver diseases and hepatocellular carcinoma. *World J Gastroenterol,* 19**,** 2449-55.

Gitlin, L., Barchet, W., Gilfillan, S., Cella, M., Beutler, B., Flavell, R. A., Diamond, M. S. & Colonna, M. 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc Natl Acad Sci U S A,* 103**,** 8459-64.

Golden-Mason, L., Bambha, K. M., Cheng, L., Howell, C. D., Taylor, M. W., Clark, P. J., Afdhal, N. & Rosen, H. R. 2011. Natural killer inhibitory receptor expression associated with treatment failure and interleukin-28B genotype in patients with chronic hepatitis C. *Hepatology,* 54**,** 1559-69.

Golden-Mason, L., Cox, A. L., Randall, J. A., Cheng, L. & Rosen, H. R. 2010. Increased natural killer cell cytotoxicity and NKp30 expression protects against hepatitis C virus infection in high-risk individuals and inhibits replication in vitro. *Hepatology,* 52**,** 1581-9.

Golden-Mason, L., Klarquist, J., Wahed, A. S. & Rosen, H. R. 2008. Cutting edge: programmed death-1 expression is increased on immunocytes in chronic hepatitis C virus and predicts failure of response to antiviral therapy: race-dependent differences. *J Immunol,* 180**,** 3637-41.

Golden-Mason, L., Palmer, B., Klarquist, J., Mengshol, J. A., Castelblanco, N. & Rosen, H. R. 2007. Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. *J Virol,* 81**,** 9249-58.

Golden-Mason, L., Palmer, B. E., Kassam, N., Townshend-Bulson, L., Livingston, S., McMahon, B. J., Castelblanco, N., Kuchroo, V., Gretch, D. R. & Rosen, H. R. 2009. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol,* 83**,** 9122-30.

Golden-Mason, L., Stone, A. E., Bambha, K. M., Cheng, L. & Rosen, H. R. 2012. Race- and gender-related variation in natural killer p46 expression associated with differential anti-hepatitis C virus immunity. *Hepatology,* 56**,** 1214-22.

Goulding, C., McManus, R., Murphy, A., MacDonald, G., Barrett, S., Crowe, J., Hegarty, J., McKiernan, S. & Kelleher, D. 2005. The CCR5-delta32 mutation: impact on disease outcome in individuals with hepatitis C infection from a single source. *Gut,* 54**,** 1157-61.

Grakoui, A., Shoukry, N. H., Woollard, D. J., Han, J. H., Hanson, H. L., Ghrayeb, J., Murthy, K. K., Rice, C. M. & Walker, C. M. 2003. HCV persistence and

immune evasion in the absence of memory T cell help. *Science,* 302**,** 659-62.

Grebely, J., Conway, B., Raffa, J. D., Lai, C., Krajden, M. & Tyndall, M. W. 2006. Hepatitis C virus reinfection in injection drug users. *Hepatology,* 44**,** 1139-45.

Grebely, J., Page, K., Sacks-Davis, R., van der Loeff, M. S., Rice, T. M., Bruneau, J., Morris, M. D., Hajarizadeh, B., Amin, J., Cox, A. L., Kim, A. Y., McGovern, B. H., Schinkel, J., George, J., Shoukry, N. H., Lauer, G. M., Maher, L., Lloyd, A. R., Hellard, M., Dore, G. J. & Prins, M. 2014. The effects of female sex, viral genotype, and IL28B genotype on spontaneous clearance of acute hepatitis C virus infection. *Hepatology,* 59**,** 109-20.

Gruner, N. H., Gerlach, T. J., Jung, M. C., Diepolder, H. M., Schirren, C. A., Schraut, W. W., Hoffmann, R., Zachoval, R., Santantonio, T., Cucchiarini, M., Cerny, A. & Pape, G. R. 2000. Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. *J Infect Dis,* 181**,** 1528-36.

Helle, F., Goffard, A., Morel, V., Duverlie, G., McKeating, J., Keck, Z. Y., Foung, S., Penin, F., Dubuisson, J. & Voisset, C. 2007. The neutralizing activity of anti-hepatitis C virus antibodies is modulated by specific glycans on the E2 envelope protein. *J Virol,* 81**,** 8101-11.

Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K. & Akira, S. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol,* 3**,** 196-200.

Heydtmann, M. & Adams, D. H. 2009. Chemokines in the immunopathogenesis of hepatitis C infection. *Hepatology,* 49**,** 676-88.

Hoofnagle, J. H. 2002. Course and outcome of hepatitis C. *Hepatology,* 36**,** S21-9.

Hoofnagle, J. H., Mullen, K. D., Jones, D. B., Rustgi, V., Di Bisceglie, A., Peters, M., Waggoner, J. G., Park, Y. & Jones, E. A. 1986. Treatment of chronic non-A,non-B hepatitis with recombinant human alpha interferon. A preliminary report. *N Engl J Med,* 315**,** 1575-8.

Horner, S. M. & Gale, M., Jr. 2009. Intracellular innate immune cascades and interferon defenses that control hepatitis C virus. *J Interferon Cytokine Res,* 29**,** 489-98.

Horner, S. M., Liu, H. M., Park, H. S., Briley, J. & Gale, M., Jr. 2011. Mitochondrial-associated endoplasmic reticulum membranes (MAM) form innate immune synapses and are targeted by hepatitis C virus. *Proc Natl Acad Sci U S A,* 108**,** 14590-5.

Hornung, V., Guenthner-Biller, M., Bourquin, C., Ablasser, A., Schlee, M., Uematsu, S., Noronha, A., Manoharan, M., Akira, S., de Fougerolles, A., Endres, S. & Hartmann, G. 2005. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat Med,* 11**,** 263-70.

Hosel, M., Quasdorff, M., Wiegmann, K., Webb, D., Zedler, U., Broxtermann, M., Tedjokusumo, R., Esser, K., Arzberger, S., Kirschning, C. J., Langenkamp, A., Falk, C., Buning, H., Rose-John, S. & Protzer, U. 2009. Not interferon, but interleukin-6 controls early gene expression in hepatitis B virus infection. *Hepatology,* 50**,** 1773-82.

Houghton, M. 2009. The long and winding road leading to the identification of the hepatitis C virus. *J Hepatol,* 51**,** 939-48.

Houldsworth, A., Metzner, M., Rossol, S., Shaw, S., Kaminski, E., Demaine, A. G. & Cramp, M. E. 2005. Polymorphisms in the IL-12B gene and outcome of HCV infection. *J Interferon Cytokine Res,* 25**,** 271-6.

Huang, Y., Yang, H., Borg, B. B., Su, X., Rhodes, S. L., Yang, K., Tong, X., Tang, G., Howell, C. D., Rosen, H. R., Thio, C. L., Thomas, D. L., Alter, H. J., Sapp, R. K. & Liang, T. J. 2007. A functional SNP of interferon-gamma gene is important for interferon-alpha-induced and spontaneous recovery from hepatitis C virus infection. *Proc Natl Acad Sci U S A,* 104**,** 985-90.

Humphreys, I. S., von Delft, A., Brown, A., Hibbert, L., Collier, J. D., Foster, G. R., Rahman, M., Christian, A., Klenerman, P. & Barnes, E. 2012. HCV genotype-3a T cell immunity: specificity, function and impact of therapy. *Gut,* 61**,** 1589-99.

Jiang, J., Wu, X., Tang, H. & Luo, G. 2013. Apolipoprotein E mediates attachment of clinical hepatitis C virus to hepatocytes by binding to cell surface heparan sulfate proteoglycan receptors. *PLoS One,* 8**,** e67982.

Jinushi, M., Takehara, T., Tatsumi, T., Kanto, T., Miyagi, T., Suzuki, T., Kanazawa, Y., Hiramatsu, N. & Hayashi, N. 2004. Negative regulation of NK cell activities by inhibitory receptor CD94/NKG2A leads to altered NK cell-induced modulation of dendritic cell functions in chronic hepatitis C virus infection. *J Immunol,* 173**,** 6072-81.

Jo, J., Bengsch, B., Seigel, B., Rau, S. J., Schmidt, J., Bisse, E., Aichele, P., Aichele, U., Joeckel, L., Royer, C., Sa Ferreira, K., Borner, C., Baumert, T. F., Blum, H. E., Lohmann, V., Fischer, R. & Thimme, R. 2012. Low perforin expression of early differentiated HCV-specific CD8+ T cells limits their hepatotoxic potential. *J Hepatol,* 57**,** 9-16.

Judge, A. D., Sood, V., Shaw, J. R., Fang, D., McClintock, K. & MacLachlan, I. 2005. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat Biotechnol,* 23**,** 457-62.

Jurk, M., Heil, F., Vollmer, J., Schetter, C., Krieg, A. M., Wagner, H., Lipford, G. & Bauer, S. 2002. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nat Immunol,* 3**,** 499.

Kadowaki, N. 2009. The divergence and interplay between pDC and mDC in humans. *Front Biosci (Landmark Ed),* 14**,** 808-17.

Kanto, T., Hayashi, N., Takehara, T., Tatsumi, T., Kuzushita, N., Ito, A., Sasaki, Y., Kasahara, A. & Hori, M. 1999. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J Immunol,* 162**,** 5584-91.

Kanto, T., Inoue, M., Miyatake, H., Sato, A., Sakakibara, M., Yakushijin, T., Oki, C., Itose, I., Hiramatsu, N., Takehara, T., Kasahara, A. & Hayashi, N. 2004. Reduced numbers and impaired ability of myeloid and plasmacytoid dendritic cells to polarize T helper cells in chronic hepatitis C virus infection. *J Infect Dis,* 190**,** 1919-26.

Kared, H., Fabre, T., Bedard, N., Bruneau, J. & Shoukry, N. H. 2013. Galectin-9 and IL-21 mediate cross-regulation between Th17 and Treg cells during acute hepatitis C. *PLoS Pathog,* 9**,** e1003422.

Kasprowicz, V., Kang, Y. H., Lucas, M., Schulze zur Wiesch, J., Kuntzen, T., Fleming, V., Nolan, B. E., Longworth, S., Berical, A., Bengsch, B., Thimme, R., Lewis-Ximenez, L., Allen, T. M., Kim, A. Y., Klenerman, P. & Lauer, G. M. 2010. Hepatitis C virus (HCV) sequence variation induces an HCV-specific T-cell phenotype analogous to spontaneous resolution. *J Virol,* 84**,** 1656-63.

Kasprowicz, V., Schulze Zur Wiesch, J., Kuntzen, T., Nolan, B. E., Longworth, S., Berical, A., Blum, J., McMahon, C., Reyor, L. L., Elias, N., Kwok, W. W., McGovern, B. G., Freeman, G., Chung, R. T., Klenerman, P., Lewis-Ximenez, L., Walker, B. D., Allen, T. M., Kim, A. Y. & Lauer, G. M. 2008. High level of PD-1 expression on hepatitis C virus (HCV)-specific CD8+ and CD4+ T cells during acute HCV infection, irrespective of clinical outcome. *J Virol,* 82**,** 3154-60.

Kato, H., Sato, S., Yoneyama, M., Yamamoto, M., Uematsu, S., Matsui, K., Tsujimura, T., Takeda, K., Fujita, T., Takeuchi, O. & Akira, S. 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity,* 23**,** 19-28.

Kato, N. 2001. Molecular virology of hepatitis C virus. *Acta Med Okayama,* 55**,** 133-59.

Kelly, C., Swadling, L., Capone, S., Brown, A., Richardson, R., Halliday, J., von Delft, A., Oo, Y., Mutimer, D., Kurioka, A., Hartnell, F., Collier, J., Ammendola, V., Del Sorbo, M., Grazioli, F., Esposito, M. L., Di Marco, S., Siani, L., Traboni, C., Hill, A. V., Colloca, S., Nicosia, A., Cortese, R., Folgori, A., Klenerman, P. & Barnes, E. 2016. Chronic hepatitis C viral infection subverts vaccine-induced T-cell immunity in humans. *Hepatology,* 63**,** 1455-70.

Khakoo, S. I., Thio, C. L., Martin, M. P., Brooks, C. R., Gao, X., Astemborski, J., Cheng, J., Goedert, J. J., Vlahov, D., Hilgartner, M., Cox, S., Little, A. M., Alexander, G. J., Cramp, M. E., O'Brien, S. J., Rosenberg, W. M., Thomas, D. L. & Carrington, M. 2004. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science,* 305**,** 872-4.

Klenerman, P. & Thimme, R. 2012. T cell responses in hepatitis C: the good, the bad and the unconventional. *Gut,* 61**,** 1226-34.

Knapp, S., Warshow, U., Hegazy, D., Brackenbury, L., Guha, I. N., Fowell, A., Little, A. M., Alexander, G. J., Rosenberg, W. M., Cramp, M. E. & Khakoo, S. I. 2010. Consistent beneficial effects of killer cell immunoglobulin-like receptor 2DL3 and group 1 human leukocyte antigen-C following exposure to hepatitis C virus. *Hepatology,* 51**,** 1168-75.

Kotenko, S. V., Gallagher, G., Baurin, V. V., Lewis-Antes, A., Shen, M., Shah, N. K., Langer, J. A., Sheikh, F., Dickensheets, H. & Donnelly, R. P. 2003. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol,* 4**,** 69-77.

Kramer, B., Korner, C., Kebschull, M., Glassner, A., Eisenhardt, M., Nischalke, H. D., Alexander, M., Sauerbruch, T., Spengler, U. & Nattermann, J. 2012. Natural killer p46High expression defines a natural killer cell subset that is potentially involved in control of hepatitis C virus replication and modulation of liver fibrosis. *Hepatology,* 56**,** 1201-13.

Kulkarni, S., Martin, M. P. & Carrington, M. 2008. The Yin and Yang of HLA and KIR in human disease. *Semin Immunol,* 20**,** 343-52.

Lande, R. & Gilliet, M. 2010. Plasmacytoid dendritic cells: key players in the initiation and regulation of immune responses. *Ann N Y Acad Sci,* 1183**,** 89-103.

Larrubia, J. R., Benito-Martinez, S., Miquel, J., Calvino, M., Sanz-de-Villalobos, E., Gonzalez-Praetorius, A., Albertos, S., Garcia-Garzon, S., Lokhande, M. & Parra-Cid, T. 2011. Bim-mediated apoptosis and PD-1/PD-L1 pathway impair reactivity of PD1(+)/CD127(-) HCV-specific CD8(+) cells targeting the virus in chronic hepatitis C virus infection. *Cell Immunol,* 269**,** 104-14.

Larrubia, J. R., Moreno-Cubero, E., Lokhande, M. U., Garcia-Garzon, S., Lazaro, A., Miquel, J., Perna, C. & Sanz-de-Villalobos, E. 2014. Adaptive immune response during hepatitis C virus infection. *World J Gastroenterol,* 20**,** 3418-30.

Larsson, M., Babcock, E., Grakoui, A., Shoukry, N., Lauer, G., Rice, C., Walker, C. & Bhardwaj, N. 2004. Lack of phenotypic and functional impairment in dendritic cells from chimpanzees chronically infected with hepatitis C virus. *J Virol,* 78**,** 6151-61.

Lechmann, M., Ihlenfeldt, H. G., Braunschweiger, I., Giers, G., Jung, G., Matz, B., Kaiser, R., Sauerbruch, T. & Spengler, U. 1996. T- and B-cell responses to different hepatitis C virus antigens in patients with chronic hepatitis C infection and in healthy anti-hepatitis C virus--positive blood donors without viremia. *Hepatology,* 24**,** 790-5.

Lechner, F., Wong, D. K., Dunbar, P. R., Chapman, R., Chung, R. T., Dohrenwend, P., Robbins, G., Phillips, R., Klenerman, P. & Walker, B. D. 2000. Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med,* 191**,** 1499-512.

Lefevre, M., Felmlee, D. J., Parnot, M., Baumert, T. F. & Schuster, C. 2014. Syndecan 4 is involved in mediating HCV entry through interaction with lipoviral particle-associated apolipoprotein E. *PLoS One,* 9**,** e95550.

Levy, D. E. & Darnell, J. E., Jr. 2002. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol,* 3**,** 651-62.

Li, K., Foy, E., Ferreon, J. C., Nakamura, M., Ferreon, A. C., Ikeda, M., Ray, S. C., Gale, M., Jr. & Lemon, S. M. 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci U S A,* 102**,** 2992-7.

Li, W., Gomez, E. & Zhang, Z. 2007. Immunohistochemical expression of stromal cell-derived factor-1 (SDF-1) and CXCR4 ligand receptor system in hepatocellular carcinoma. *J Exp Clin Cancer Res,* 26**,** 527-33.

Lindenbach, B. D. & Rice, C. M. 2013. The ins and outs of hepatitis C virus entry and assembly. *Nat Rev Microbiol,* 11**,** 688-700.

Liu, C., Zhu, H., Tu, Z., Xu, Y. L. & Nelson, D. R. 2003. CD8+ T-cell interaction with HCV replicon cells: evidence for both cytokine- and cell-mediated antiviral activity. *Hepatology,* 37**,** 1335-42.

Lohmann, V., Korner, F., Dobierzewska, A. & Bartenschlager, R. 2001. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J Virol,* 75**,** 1437-49.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science,* 285**,** 110-3.

Longman, R. S., Talal, A. H., Jacobson, I. M., Albert, M. L. & Rice, C. M. 2004. Presence of functional dendritic cells in patients chronically infected with hepatitis C virus. *Blood,* 103**,** 1026-9.

Loo, Y. M. & Gale, M., Jr. 2011. Immune signaling by RIG-I-like receptors. *Immunity,* 34**,** 680-92.

Loo, Y. M., Owen, D. M., Li, K., Erickson, A. K., Johnson, C. L., Fish, P. M., Carney, D. S., Wang, T., Ishida, H., Yoneyama, M., Fujita, T., Saito, T., Lee, W. M., Hagedorn, C. H., Lau, D. T., Weinman, S. A., Lemon, S. M. & Gale, M., Jr. 2006. Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. *Proc Natl Acad Sci U S A,* 103**,** 6001-6.

MacDonald, M., Crofts, N. & Kaldor, J. 1996. Transmission of hepatitis C virus: rates, routes, and cofactors. *Epidemiol Rev,* 18**,** 137-48.

MacMicking, J. D. 2012. Interferon-inducible effector mechanisms in cell-autonomous immunity. *Nat Rev Immunol,* 12**,** 367-82.

Makowska, Z., Duong, F. H., Trincucci, G., Tough, D. F. & Heim, M. H. 2011. Interferon-beta and interferon-lambda signaling is not affected by interferon-induced refractoriness to interferon-alpha in vivo. *Hepatology,* 53**,** 1154-63.

Mandelboim, O., Lieberman, N., Lev, M., Paul, L., Arnon, T. I., Bushkin, Y., Davis, D. M., Strominger, J. L., Yewdell, J. W. & Porgador, A. 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature,* 409**,** 1055-60.

Mangia, A., Gentile, R., Cascavilla, I., Margaglione, M., Villani, M. R., Stella, F., Modola, G., Agostiano, V., Gaudiano, C. & Andriulli, A. 1999. HLA class II favors clearance of HCV infection and progression of the chronic liver damage. *J Hepatol,* 30**,** 984-9.

Marcello, T., Grakoui, A., Barba-Spaeth, G., Machlin, E. S., Kotenko, S. V., MacDonald, M. R. & Rice, C. M. 2006. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology,* 131**,** 1887-98.

Martin, B., Hennecke, N., Lohmann, V., Kayser, A., Neumann-Haefelin, C., Kukolj, G., Bocher, W. O. & Thimme, R. 2014. Restoration of HCV-specific CD8+ T cell function by interferon-free therapy. *J Hepatol,* 61**,** 538-43.

Martin, D. N. & Uprichard, S. L. 2013. Identification of transferrin receptor 1 as a hepatitis C virus entry factor. *Proc Natl Acad Sci U S A,* 110**,** 10777-82.

Matsumoto, M., Kikkawa, S., Kohase, M., Miyake, K. & Seya, T. 2002. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem Biophys Res Commun,* 293**,** 1364-9.

McKiernan, S. M., Hagan, R., Curry, M., McDonald, G. S., Kelly, A., Nolan, N., Walsh, A., Hegarty, J., Lawlor, E. & Kelleher, D. 2004. Distinct MHC class I and II alleles are associated with hepatitis C viral clearance, originating from a single source. *Hepatology,* 40**,** 108-14.

McLoughlin, R. M., Jenkins, B. J., Grail, D., Williams, A. S., Fielding, C. A., Parker, C. R., Ernst, M., Topley, N. & Jones, S. A. 2005. IL-6 trans-

signaling via STAT3 directs T cell infiltration in acute inflammation. *Proc Natl Acad Sci U S A,* 102**,** 9589-94.

McMahan, R. H., Golden-Mason, L., Nishimura, M. I., McMahon, B. J., Kemper, M., Allen, T. M., Gretch, D. R. & Rosen, H. R. 2010. Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity. *J Clin Invest,* 120**,** 4546-57.

Meier, A., Alter, G., Frahm, N., Sidhu, H., Li, B., Bagchi, A., Teigen, N., Streeck, H., Stellbrink, H. J., Hellman, J., van Lunzen, J. & Altfeld, M. 2007. MyD88-dependent immune activation mediated by human immunodeficiency virus type 1-encoded Toll-like receptor ligands. *J Virol,* 81**,** 8180-91.

Meissner, E. G., Kohli, A., Higgins, J., Lee, Y. J., Prokunina, O., Wu, D., Orr, C., Masur, H. & Kottilil, S. 2017. Rapid changes in peripheral lymphocyte concentrations during interferon-free treatment of chronic hepatitis C virus infection. *Hepatol Commun,* 1**,** 586-594.

Mengshol, J. A., Golden-Mason, L., Arikawa, T., Smith, M., Niki, T., McWilliams, R., Randall, J. A., McMahan, R., Zimmerman, M. A., Rangachari, M., Dobrinskikh, E., Busson, P., Polyak, S. J., Hirashima, M. & Rosen, H. R. 2010. A crucial role for Kupffer cell-derived galectin-9 in regulation of T cell immunity in hepatitis C infection. *PLoS One,* 5**,** e9504.

Micallef, J. M., Kaldor, J. M. & Dore, G. J. 2006. Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. *J Viral Hepat,* 13**,** 34-41.

Mihm, S., Frese, M., Meier, V., Wietzke-Braun, P., Scharf, J. G., Bartenschlager, R. & Ramadori, G. 2004. Interferon type I gene expression in chronic hepatitis C. *Lab Invest,* 84**,** 1148-59.

Minton, E. J., Smillie, D., Neal, K. R., Irving, W. L., Underwood, J. C. & James, V. 1998. Association between MHC class II alleles and clearance of circulating hepatitis C virus. Members of the Trent Hepatitis C Virus Study Group. *J Infect Dis,* 178**,** 39-44.

Missale, G., Bertoni, R., Lamonaca, V., Valli, A., Massari, M., Mori, C., Rumi, M. G., Houghton, M., Fiaccadori, F. & Ferrari, C. 1996. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. *J Clin Invest,* 98**,** 706-14.

Missale, G., Pilli, M., Zerbini, A., Penna, A., Ravanetti, L., Barili, V., Orlandini, A., Molinari, A., Fasano, M., Santantonio, T. & Ferrari, C. 2012. Lack of full CD8 functional restoration after antiviral treatment for acute and chronic hepatitis C virus infection. *Gut,* 61**,** 1076-84.

Miyagi, T., Takehara, T., Nishio, K., Shimizu, S., Kohga, K., Li, W., Tatsumi, T., Hiramatsu, N., Kanto, T. & Hayashi, N. 2010. Altered interferon-alpha-signaling in natural killer cells from patients with chronic hepatitis C virus infection. *J Hepatol,* 53**,** 424-30.

Miyamura, T., Saito, I., Katayama, T., Kikuchi, S., Tateda, A., Houghton, M., Choo, Q. L. & Kuo, G. 1990. Detection of antibody against antigen expressed by molecularly cloned hepatitis C virus cDNA: application to diagnosis and blood screening for posttransfusion hepatitis. *Proc Natl Acad Sci U S A,* 87**,** 983-7.

Morahan, G., Huang, D., Wu, M., Holt, B. J., White, G. P., Kendall, G. E., Sly, P. D. & Holt, P. G. 2002. Association of IL12B promoter polymorphism with severity of atopic and non-atopic asthma in children. *Lancet,* 360**,** 455-9.

Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel, R. M. & Aguet, M. 1994. Functional role of type I and type II interferons in antiviral defense. *Science,* 264**,** 1918-21.

Murakami, H., Akbar, S. M., Matsui, H., Horiike, N. & Onji, M. 2004. Decreased interferon-alpha production and impaired T helper 1 polarization by dendritic cells from patients with chronic hepatitis C. *Clin Exp Immunol,* 137**,** 559-65.

Najafi Fard, S., Schietroma, I., Corano Scheri, G., Giustini, N., Serafino, S., Cavallari, E. N., Pinacchio, C., De Girolamo, G., Ceccarelli, G., Scagnolari, C., Vullo, V. & d'Ettorre, G. 2018. Direct-acting antiviral therapy enhances total CD4+ and CD8+ T-cells responses, but does not alter T-cells activation among HCV mono-infected, and HCV/HIV-1 co-infected patients. *Clin Res Hepatol Gastroenterol,* 42**,** 319-329.

Nakagawa, H., Maeda, S., Yoshida, H., Tateishi, R., Masuzaki, R., Ohki, T., Hayakawa, Y., Kinoshita, H., Yamakado, M., Kato, N., Shiina, S. & Omata, M. 2009. Serum IL-6 levels and the risk for hepatocarcinogenesis in chronic hepatitis C patients: an analysis based on gender differences. *Int J Cancer,* 125**,** 2264-9.

Nakamoto, N., Cho, H., Shaked, A., Olthoff, K., Valiga, M. E., Kaminski, M., Gostick, E., Price, D. A., Freeman, G. J., Wherry, E. J. & Chang, K. M.

2009. Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade. *PLoS Pathog,* 5**,** e1000313.

Nakamoto, N., Kaplan, D. E., Coleclough, J., Li, Y., Valiga, M. E., Kaminski, M., Shaked, A., Olthoff, K., Gostick, E., Price, D. A., Freeman, G. J., Wherry, E. J. & Chang, K. M. 2008. Functional restoration of HCV-specific CD8 T cells by PD-1 blockade is defined by PD-1 expression and compartmentalization. *Gastroenterology,* 134**,** 1927-37, 1937.e1-2.

Nattermann, J., Nischalke, H. D., Hofmeister, V., Ahlenstiel, G., Zimmermann, H., Leifeld, L., Weiss, E. H., Sauerbruch, T. & Spengler, U. 2005. The HLA-A2 restricted T cell epitope HCV core 35-44 stabilizes HLA-E expression and inhibits cytolysis mediated by natural killer cells. *Am J Pathol,* 166**,** 443-53.

Nattermann, J., Sherzada, R., Iwan, A., Bogen, D., Niederle, I. M., Schulte, D., Mertens, E., Nischalke, H. D., Kramer, B., Sauerbruch, T., Leifeld, L. & Spengler, U. 2008. Hepatitis C virus-induced secretion of inflammatory chemokines preferentially recruits NKG2A+CD8+ T cells. *J Infect Dis,* 198**,** 213-7.

Nattermann, J., Vogel, M., Berg, T., Danta, M., Axel, B., Mayr, C., Bruno, R., Tural, C., Klausen, G., Clotet, B., Lutz, T., Grunhage, F., Rausch, M., Nischalke, H. D., Schewe, K., Bienek, B., Haerter, G., Sauerbruch, T., Rockstroh, J. K. & Spengler, U. 2007. Effect of the interleukin-6 C174G gene polymorphism on treatment of acute and chronic hepatitis C in human immunodeficiency virus coinfected patients. *Hepatology,* 46**,** 1016-25.

Nattermann, J., Zimmermann, H., Iwan, A., von Lilienfeld-Toal, M., Leifeld, L., Nischalke, H. D., Langhans, B., Sauerbruch, T. & Spengler, U. 2006. Hepatitis C virus E2 and CD81 interaction may be associated with altered trafficking of dendritic cells in chronic hepatitis C. *Hepatology,* 44**,** 945-54.

Neumann-Haefelin, C., Blum, H. E., Chisari, F. V. & Thimme, R. 2005. T cell response in hepatitis C virus infection. *J Clin Virol,* 32**,** 75-85.

Neumann-Haefelin, C., McKiernan, S., Ward, S., Viazov, S., Spangenberg, H. C., Killinger, T., Baumert, T. F., Nazarova, N., Sheridan, I., Pybus, O., von Weizsacker, F., Roggendorf, M., Kelleher, D., Klenerman, P., Blum, H. E. & Thimme, R. 2006. Dominant influence of an HLA-B27 restricted CD8+ T cell response in mediating HCV clearance and evolution. *Hepatology,* 43**,** 563-72.

Neumann-Haefelin, C. & Thimme, R. 2013. Adaptive immune responses in hepatitis C virus infection. *Curr Top Microbiol Immunol,* 369**,** 243-62.

Nielsen, S. U., Bassendine, M. F., Burt, A. D., Martin, C., Pumeechockchai, W. & Toms, G. L. 2006. Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. *J Virol,* 80**,** 2418-28.

Oleksyk, T. K., Thio, C. L., Truelove, A. L., Goedert, J. J., Donfield, S. M., Kirk, G. D., Thomas, D. L., O'Brien, S. J. & Smith, M. W. 2005. Single nucleotide polymorphisms and haplotypes in the IL10 region associated with HCV clearance. *Genes Immun,* 6**,** 347-57.

Oliviero, B., Mele, D., Degasperi, E., Aghemo, A., Cremonesi, E., Rumi, M. G., Tinelli, C., Varchetta, S., Mantovani, S., Colombo, M. & Mondelli, M. U. 2013. Natural killer cell dynamic profile is associated with treatment outcome in patients with chronic HCV infection. *J Hepatol,* 59**,** 38-44.

Oliviero, B., Varchetta, S., Paudice, E., Michelone, G., Zaramella, M., Mavilio, D., De Filippi, F., Bruno, S. & Mondelli, M. U. 2009. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. *Gastroenterology,* 137**,** 1151-60, 1160.e1-7.

Osburn, W. O., Snider, A. E., Wells, B. L., Latanich, R., Bailey, J. R., Thomas, D. L., Cox, A. L. & Ray, S. C. 2014. Clearance of hepatitis C infection is associated with the early appearance of broad neutralizing antibody responses. *Hepatology,* 59**,** 2140-51.

Osna, N., Silonova, G., Vilgert, N., Hagina, E., Kuse, V., Giedraitis, V., Zvirbliene, A., Mauricas, M. & Sochnev, A. 1997. Chronic hepatitis C: T-helper1/T-helper2 imbalance could cause virus persistence in peripheral blood. *Scand J Clin Lab Invest,* 57**,** 703-10.

Otsuka, M., Kato, N., Moriyama, M., Taniguchi, H., Wang, Y., Dharel, N., Kawabe, T. & Omata, M. 2005. Interaction between the HCV NS3 protein and the host TBK1 protein leads to inhibition of cellular antiviral responses. *Hepatology,* 41**,** 1004-12.

Paladino, N., Fainboim, H., Theiler, G., Schroder, T., Munoz, A. E., Flores, A. C., Galdame, O. & Fainboim, L. 2006. Gender susceptibility to chronic hepatitis C virus infection associated with interleukin 10 promoter polymorphism. *J Virol,* 80**,** 9144-50.

Park, H., Serti, E., Eke, O., Muchmore, B., Prokunina-Olsson, L., Capone, S., Folgori, A. & Rehermann, B. 2012. IL-29 is the dominant type III interferon produced by hepatocytes during acute hepatitis C virus infection. *Hepatology,* 56**,** 2060-70.

Parry, R. V., Chemnitz, J. M., Frauwirth, K. A., Lanfranco, A. R., Braunstein, I., Kobayashi, S. V., Linsley, P. S., Thompson, C. B. & Riley, J. L. 2005. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol,* 25**,** 9543-53.

Patsoukis, N., Brown, J., Petkova, V., Liu, F., Li, L. & Boussiotis, V. A. 2012. Selective effects of PD-1 on Akt and Ras pathways regulate molecular components of the cell cycle and inhibit T cell proliferation. *Sci Signal,* 5**,** ra46.

Pavlovic, D., Neville, D. C., Argaud, O., Blumberg, B., Dwek, R. A., Fischer, W. B. & Zitzmann, N. 2003. The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. *Proc Natl Acad Sci U S A,* 100**,** 6104-8.

Penna, A., Missale, G., Lamonaca, V., Pilli, M., Mori, C., Zanelli, P., Cavalli, A., Elia, G. & Ferrari, C. 2002. Intrahepatic and circulating HLA class II-restricted, hepatitis C virus-specific T cells: functional characterization in patients with chronic hepatitis C. *Hepatology,* 35**,** 1225-36.

Penna, A., Pilli, M., Zerbini, A., Orlandini, A., Mezzadri, S., Sacchelli, L., Missale, G. & Ferrari, C. 2007. Dysfunction and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. *Hepatology,* 45**,** 588-601.

Pestka, J. M., Zeisel, M. B., Blaser, E., Schurmann, P., Bartosch, B., Cosset, F. L., Patel, A. H., Meisel, H., Baumert, J., Viazov, S., Rispeter, K., Blum, H. E., Roggendorf, M. & Baumert, T. F. 2007. Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc Natl Acad Sci U S A,* 104**,** 6025-30.

Piasecki, B. A., Lewis, J. D., Reddy, K. R., Bellamy, S. L., Porter, S. B., Weinrieb, R. M., Stieritz, D. D. & Chang, K. M. 2004. Influence of alcohol use, race, and viral coinfections on spontaneous HCV clearance in a US veteran population. *Hepatology,* 40**,** 892-9.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., Houghton, M., Rosa, D., Grandi, G. & Abrignani, S. 1998. Binding of hepatitis C virus to CD81. *Science,* 282**,** 938-41.

Ploss, A., Evans, M. J., Gaysinskaya, V. A., Panis, M., You, H., de Jong, Y. P. & Rice, C. M. 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature,* 457**,** 882-6.

Polyak, S. J., Khabar, K. S., Paschal, D. M., Ezelle, H. J., Duverlie, G., Barber, G. N., Levy, D. E., Mukaida, N. & Gretch, D. R. 2001. Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. *J Virol,* 75**,** 6095-106.

Radziewicz, H., Ibegbu, C. C., Fernandez, M. L., Workowski, K. A., Obideen, K., Wehbi, M., Hanson, H. L., Steinberg, J. P., Masopust, D., Wherry, E. J., Altman, J. D., Rouse, B. T., Freeman, G. J., Ahmed, R. & Grakoui, A. 2007. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol,* 81**,** 2545-53.

Radziewicz, H., Ibegbu, C. C., Hon, H., Osborn, M. K., Obideen, K., Wehbi, M., Freeman, G. J., Lennox, J. L., Workowski, K. A., Hanson, H. L. & Grakoui, A. 2008. Impaired hepatitis C virus (HCV)-specific effector CD8+ T cells undergo massive apoptosis in the peripheral blood during acute HCV infection and in the liver during the chronic phase of infection. *J Virol,* 82**,** 9808-22.

Raghuraman, S., Park, H., Osburn, W. O., Winkelstein, E., Edlin, B. R. & Rehermann, B. 2012. Spontaneous clearance of chronic hepatitis C virus infection is associated with appearance of neutralizing antibodies and reversal of T-cell exhaustion. *J Infect Dis,* 205**,** 763-71.

Ray, S. C., Fanning, L., Wang, X. H., Netski, D. M., Kenny-Walsh, E. & Thomas, D. L. 2005. Divergent and convergent evolution after a common-source outbreak of hepatitis C virus. *J Exp Med,* 201**,** 1753-9.

Rehermann, B. 2009. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. *J Clin Invest,* 119**,** 1745-54.

Rehermann, B. 2013. Pathogenesis of chronic viral hepatitis: differential roles of T cells and NK cells. *Nat Med,* 19**,** 859-68.

Reichard, O., Norkrans, G., Fryden, A., Braconier, J. H., Sonnerborg, A. & Weiland, O. 1998. Randomised, double-blind, placebo-controlled trial of interferon alpha-2b with and without ribavirin for chronic hepatitis C. The Swedish Study Group. *Lancet,* 351**,** 83-7.

Ren, Y., Poon, R. T., Tsui, H. T., Chen, W. H., Li, Z., Lau, C., Yu, W. C. & Fan, S. T. 2003. Interleukin-8 serum levels in patients with hepatocellular carcinoma: correlations with clinicopathological features and prognosis. *Clin Cancer Res,* 9**,** 5996-6001.

Rolfe, K. J., Curran, M. D., Alexander, G. J., Woodall, T., Andrews, N. & Harris, H. E. 2011. Spontaneous loss of hepatitis C virus RNA from serum is associated with genotype 1 and younger age at exposure. *J Med Virol,* 83**,** 1338-44.

Rosen, H. R. 2013. Emerging concepts in immunity to hepatitis C virus infection. *J Clin Invest,* 123**,** 4121-30.

Rushbrook, S. M., Ward, S. M., Unitt, E., Vowler, S. L., Lucas, M., Klenerman, P. & Alexander, G. J. 2005. Regulatory T cells suppress in vitro proliferation of virus-specific CD8+ T cells during persistent hepatitis C virus infection. *J Virol,* 79**,** 7852-9.

Rutebemberwa, A., Ray, S. C., Astemborski, J., Levine, J., Liu, L., Dowd, K. A., Clute, S., Wang, C., Korman, A., Sette, A., Sidney, J., Pardoll, D. M. & Cox, A. L. 2008. High-programmed death-1 levels on hepatitis C virus-specific T cells during acute infection are associated with viral persistence and require preservation of cognate antigen during chronic infection. *J Immunol,* 181**,** 8215-25.

Sainz, B., Jr., Barretto, N., Martin, D. N., Hiraga, N., Imamura, M., Hussain, S., Marsh, K. A., Yu, X., Chayama, K., Alrefai, W. A. & Uprichard, S. L. 2012. Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. *Nat Med,* 18**,** 281-5.

Saldanha, J., Lelie, N. & Heath, A. 1999. Establishment of the first international standard for nucleic acid amplification technology (NAT) assays for HCV RNA. WHO Collaborative Study Group. *Vox Sang,* 76**,** 149-58.

Santantonio, T., Sinisi, E., Guastadisegni, A., Casalino, C., Mazzola, M., Gentile, A., Leandro, G. & Pastore, G. 2003. Natural course of acute hepatitis C: a long-term prospective study. *Dig Liver Dis,* 35**,** 104-13.

Sarasin-Filipowicz, M., Oakeley, E. J., Duong, F. H., Christen, V., Terracciano, L., Filipowicz, W. & Heim, M. H. 2008. Interferon signaling and treatment outcome in chronic hepatitis C. *Proc Natl Acad Sci U S A,* 105**,** 7034-9.

Sarasin-Filipowicz, M., Wang, X., Yan, M., Duong, F. H., Poli, V., Hilton, D. J., Zhang, D. E. & Heim, M. H. 2009. Alpha interferon induces long-lasting refractoriness of JAK-STAT signaling in the mouse liver through induction of USP18/UBP43. *Mol Cell Biol,* 29**,** 4841-51.

Sarobe, P., Lasarte, J. J., Casares, N., Lopez-Diaz de Cerio, A., Baixeras, E., Labarga, P., Garcia, N., Borras-Cuesta, F. & Prieto, J. 2002. Abnormal priming of CD4(+) T cells by dendritic cells expressing hepatitis C virus core and E1 proteins. *J Virol,* 76**,** 5062-70.

Sato, K., Ishikawa, T., Okumura, A., Yamauchi, T., Sato, S., Ayada, M., Matsumoto, E., Hotta, N., Oohashi, T., Fukuzawa, Y. & Kakumu, S. 2007. Expression of Toll-like receptors in chronic hepatitis C virus infection. *J Gastroenterol Hepatol,* 22**,** 1627-32.

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R. M., Acali, S., Filocamo, G., Traboni, C., Nicosia, A., Cortese, R. & Vitelli, A. 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *Embo j,* 21**,** 5017-25.

Schlaphoff, V., Lunemann, S., Suneetha, P. V., Jaroszewicz, J., Grabowski, J., Dietz, J., Helfritz, F., Bektas, H., Sarrazin, C., Manns, M. P., Cornberg, M. & Wedemeyer, H. 2011. Dual function of the NK cell receptor 2B4 (CD244) in the regulation of HCV-specific CD8+ T cells. *PLoS Pathog,* 7**,** e1002045.

Schoggins, J. W., Wilson, S. J., Panis, M., Murphy, M. Y., Jones, C. T., Bieniasz, P. & Rice, C. M. 2011. A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature,* 472**,** 481-5.

Schulze Zur Wiesch, J., Ciuffreda, D., Lewis-Ximenez, L., Kasprowicz, V., Nolan, B. E., Streeck, H., Aneja, J., Reyor, L. L., Allen, T. M., Lohse, A. W., McGovern, B., Chung, R. T., Kwok, W. W., Kim, A. Y. & Lauer, G. M. 2012. Broadly directed virus-specific CD4+ T cell responses are primed during acute hepatitis C infection, but rapidly disappear from human blood with viral persistence. *J Exp Med,* 209**,** 61-75.

Seeff, L. B. 2002. Natural history of chronic hepatitis C. *Hepatology,* 36**,** S35-46.

Seegers, D., Zwiers, A., Strober, W., Pena, A. S. & Bouma, G. 2002. A TaqI polymorphism in the 3'UTR of the IL-12 p40 gene correlates with increased IL-12 secretion. *Genes Immun,* 3**,** 419-23.

Seifert, U., Liermann, H., Racanelli, V., Halenius, A., Wiese, M., Wedemeyer, H., Ruppert, T., Rispeter, K., Henklein, P., Sijts, A., Hengel, H., Kloetzel, P. M. & Rehermann, B. 2004. Hepatitis C virus mutation affects proteasomal epitope processing. *J Clin Invest,* 114**,** 250-9.

Seigel, B., Bengsch, B., Lohmann, V., Bartenschlager, R., Blum, H. E. & Thimme, R. 2013. Factors that determine the antiviral efficacy of HCV-specific CD8(+) T cells ex vivo. *Gastroenterology,* 144**,** 426-36.

Semmo, N., Lucas, M., Krashias, G., Lauer, G., Chapel, H. & Klenerman, P. 2006. Maintenance of HCV-specific T-cell responses in antibody-deficient patients a decade after early therapy. *Blood,* 107**,** 4570-1.

Serti, E., Chepa-Lotrea, X., Kim, Y. J., Keane, M., Fryzek, N., Liang, T. J., Ghany, M. & Rehermann, B. 2015. Successful Interferon-Free Therapy of Chronic Hepatitis C Virus Infection Normalizes Natural Killer Cell Function. *Gastroenterology,* 149**,** 190-200.e2.

Shi, Q., Jiang, J. & Luo, G. 2013. Syndecan-1 serves as the major receptor for attachment of hepatitis C virus to the surfaces of hepatocytes. *J Virol,* 87**,** 6866-75.

Shin, E. C., Park, S. H., Demino, M., Nascimbeni, M., Mihalik, K., Major, M., Veerapu, N. S., Heller, T., Feinstone, S. M., Rice, C. M. & Rehermann, B. 2011. Delayed induction, not impaired recruitment, of specific CD8(+) T cells causes the late onset of acute hepatitis C. *Gastroenterology,* 141**,** 686-95, 695.e1.

Shoukry, N. H., Grakoui, A., Houghton, M., Chien, D. Y., Ghrayeb, J., Reimann, K. A. & Walker, C. M. 2003. Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. *J Exp Med,* 197**,** 1645-55.

Simmonds, P. 1995. Variability of hepatitis C virus. *Hepatology,* 21**,** 570-83.

Sivori, S., Pende, D., Bottino, C., Marcenaro, E., Pessino, A., Biassoni, R., Moretta, L. & Moretta, A. 1999. NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. *Eur J Immunol,* 29**,** 1656-66.

Smith, D. B., Bukh, J., Kuiken, C., Muerhoff, A. S., Rice, C. M., Stapleton, J. T. & Simmonds, P. 2014. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. *Hepatology,* 59**,** 318-27.

Sofian, M., Aghakhani, A., Farazi, A. A., Banifazl, M., Eslamifar, A., Rashidi, N., Khadem Sadegh, A. & Ramezani, A. 2012. Serum profile of T helper 1 and T helper 2 cytokines in hepatitis C virus infected patients. *Hepat Mon,* 12**,** e6156.

Stark, G. R. & Darnell, J. E., Jr. 2012. The JAK-STAT pathway at twenty. *Immunity,* 36**,** 503-14.

Steinmann, E. & Pietschmann, T. 2010. Hepatitis C virus p7-a viroporin crucial for virus assembly and an emerging target for antiviral therapy. *Viruses,* 2**,** 2078-95.

Stetson, D. B. & Medzhitov, R. 2006. Type I interferons in host defense. *Immunity,* 25**,** 373-81.

Stone, A. E., Giugliano, S., Schnell, G., Cheng, L., Leahy, K. F., Golden-Mason, L., Gale, M., Jr. & Rosen, H. R. 2013. Hepatitis C virus pathogen associated molecular pattern (PAMP) triggers production of lambda-interferons by human plasmacytoid dendritic cells. *PLoS Pathog,* 9**,** e1003316.

Su, A. I., Pezacki, J. P., Wodicka, L., Brideau, A. D., Supekova, L., Thimme, R., Wieland, S., Bukh, J., Purcell, R. H., Schultz, P. G. & Chisari, F. V. 2002. Genomic analysis of the host response to hepatitis C virus infection. *Proc Natl Acad Sci U S A,* 99**,** 15669-74.

Swann, R. E., Mandalou, P., Robinson, M. W., Ow, M. M., Foung, S. K., McLauchlan, J., Patel, A. H. & Cramp, M. E. 2016. Anti-envelope antibody responses in individuals at high risk of hepatitis C virus who resist infection. *J Viral Hepat,* 23**,** 873-880.

Takahashi, K., Asabe, S., Wieland, S., Garaigorta, U., Gastaminza, P., Isogawa, M. & Chisari, F. V. 2010. Plasmacytoid dendritic cells sense hepatitis C virus-infected cells, produce interferon, and inhibit infection. *Proc Natl Acad Sci U S A,* 107**,** 7431-6.

Takaki, A., Wiese, M., Maertens, G., Depla, E., Seifert, U., Liebetrau, A., Miller, J. L., Manns, M. P. & Rehermann, B. 2000. Cellular immune responses

persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med,* 6**,** 578-82.

Takeuchi, O. & Akira, S. 2008. MDA5/RIG-I and virus recognition. *Curr Opin Immunol,* 20**,** 17-22.

Takeuchi, O. & Akira, S. 2009. Innate immunity to virus infection. *Immunol Rev,* 227**,** 75-86.

Taylor, D. R., Shi, S. T., Romano, P. R., Barber, G. N. & Lai, M. M. 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science,* 285**,** 107-10.

Tester, I., Smyk-Pearson, S., Wang, P., Wertheimer, A., Yao, E., Lewinsohn, D. M., Tavis, J. E. & Rosen, H. R. 2005. Immune evasion versus recovery after acute hepatitis C virus infection from a shared source. *J Exp Med,* 201**,** 1725-31.

Thimme, R., Bukh, J., Spangenberg, H. C., Wieland, S., Pemberton, J., Steiger, C., Govindarajan, S., Purcell, R. H. & Chisari, F. V. 2002. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci U S A,* 99**,** 15661-8.

Thimme, R., Oldach, D., Chang, K. M., Steiger, C., Ray, S. C. & Chisari, F. V. 2001. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med,* 194**,** 1395-406.

Thio, C. L., Gao, X., Goedert, J. J., Vlahov, D., Nelson, K. E., Hilgartner, M. W., O'Brien, S. J., Karacki, P., Astemborski, J., Carrington, M. & Thomas, D. L. 2002. HLA-Cw*04 and hepatitis C virus persistence. *J Virol,* 76**,** 4792-7.

Thomas, D. L., Astemborski, J., Rai, R. M., Anania, F. A., Schaeffer, M., Galai, N., Nolt, K., Nelson, K. E., Strathdee, S. A., Johnson, L., Laeyendecker, O., Boitnott, J., Wilson, L. E. & Vlahov, D. 2000. The natural history of hepatitis C virus infection: host, viral, and environmental factors. *Jama,* 284**,** 450-6.

Thomas, E., Gonzalez, V. D., Li, Q., Modi, A. A., Chen, W., Noureddin, M., Rotman, Y. & Liang, T. J. 2012. HCV infection induces a unique hepatic innate immune response associated with robust production of type III interferons. *Gastroenterology,* 142**,** 978-88.

Thomssen, R., Bonk, S. & Thiele, A. 1993. Density heterogeneities of hepatitis C virus in human sera due to the binding of beta-lipoproteins and immunoglobulins. *Med Microbiol Immunol,* 182**,** 329-34.

Thursz, M. & Fontanet, A. 2014. HCV transmission in industrialized countries and resource-constrained areas. *Nat Rev Gastroenterol Hepatol,* 11**,** 28-35.

Timm, J., Lauer, G. M., Kavanagh, D. G., Sheridan, I., Kim, A. Y., Lucas, M., Pillay, T., Ouchi, K., Reyor, L. L., Schulze zur Wiesch, J., Gandhi, R. T., Chung, R. T., Bhardwaj, N., Klenerman, P., Walker, B. D. & Allen, T. M. 2004. CD8 epitope escape and reversion in acute HCV infection. *J Exp Med,* 200**,** 1593-604.

Tinoco, R., Alcalde, V., Yang, Y., Sauer, K. & Zuniga, E. I. 2009. Cell-intrinsic transforming growth factor-beta signaling mediates virus-specific CD8+ T cell deletion and viral persistence in vivo. *Immunity,* 31**,** 145-57.

Tsai, S. L., Chen, Y. M., Chen, M. H., Huang, C. Y., Sheen, I. S., Yeh, C. T., Huang, J. H., Kuo, G. C. & Liaw, Y. F. 1998. Hepatitis C virus variants circumventing cytotoxic T lymphocyte activity as a mechanism of chronicity. *Gastroenterology,* 115**,** 954-65.

Ueyama, M., Nakagawa, M., Sakamoto, N., Onozuka, I., Funaoka, Y., Watanabe, T., Nitta, S., Kiyohashi, K., Kitazume, A., Murakawa, M., Nishimura-Sakurai, Y., Sekine-Osajima, Y., Itsui, Y., Azuma, S., Kakinuma, S. & Watanabe, M. 2011. Serum interleukin-6 levels correlate with resistance to treatment of chronic hepatitis C infection with pegylated-interferon-alpha2b plus ribavirin. *Antivir Ther,* 16**,** 1081-91.

Ulsenheimer, A., Lucas, M., Seth, N. P., Tilman Gerlach, J., Gruener, N. H., Loughry, A., Pape, G. R., Wucherpfennig, K. W., Diepolder, H. M. & Klenerman, P. 2006. Transient immunological control during acute hepatitis C virus infection: ex vivo analysis of helper T-cell responses. *J Viral Hepat,* 13**,** 708-14.

Urban, T. J., Thompson, A. J., Bradrick, S. S., Fellay, J., Schuppan, D., Cronin, K. D., Hong, L., McKenzie, A., Patel, K., Shianna, K. V., McHutchison, J. G., Goldstein, D. B. & Afdhal, N. 2010. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. *Hepatology,* 52**,** 1888-96.

Urbani, S., Amadei, B., Fisicaro, P., Tola, D., Orlandini, A., Sacchelli, L., Mori, C., Missale, G. & Ferrari, C. 2006. Outcome of acute hepatitis C is related to

virus-specific CD4 function and maturation of antiviral memory CD8 responses. *Hepatology,* 44**,** 126-39.

Uzri, D. & Gehrke, L. 2009. Nucleotide sequences and modifications that determine RIG-I/RNA binding and signaling activities. *J Virol,* 83**,** 4174-84.

Vejbaesya, S., Songsivilai, S., Tanwandee, T., Rachaibun, S., Chantangpol, R. & Dharakul, T. 2000. HLA association with hepatitis C virus infection. *Hum Immunol,* 61**,** 348-53.

Villano, S. A., Vlahov, D., Nelson, K. E., Cohn, S. & Thomas, D. L. 1999. Persistence of viremia and the importance of long-term follow-up after acute hepatitis C infection. *Hepatology,* 29**,** 908-14.

von Hahn, T., Yoon, J. C., Alter, H., Rice, C. M., Rehermann, B., Balfe, P. & McKeating, J. A. 2007. Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology,* 132**,** 667-78.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G., Mizokami, M., Bartenschlager, R. & Liang, T. J. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med,* 11**,** 791-6.

Wang, N., Liang, Y., Devaraj, S., Wang, J., Lemon, S. M. & Li, K. 2009. Toll-like receptor 3 mediates establishment of an antiviral state against hepatitis C virus in hepatoma cells. *J Virol,* 83**,** 9824-34.

Wedemeyer, H., He, X. S., Nascimbeni, M., Davis, A. R., Greenberg, H. B., Hoofnagle, J. H., Liang, T. J., Alter, H. & Rehermann, B. 2002. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol,* 169**,** 3447-58.

Weiner, A., Erickson, A. L., Kansopon, J., Crawford, K., Muchmore, E., Hughes, A. L., Houghton, M. & Walker, C. M. 1995a. Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. *Proc Natl Acad Sci U S A,* 92**,** 2755-9.

Weiner, A. J., Christopherson, C., Hall, J. E., Bonino, F., Saracco, G., Brunetto, M. R., Crawford, K., Marion, C. D., Crawford, K. A., Venkatakrishna, S. & et al. 1991. Sequence variation in hepatitis C viral isolates. *J Hepatol,* 13 Suppl 4**,** S6-14.

Weiner, A. J., Erickson, A. L., Kansopon, J., Crawford, K., Muchmore, E., Houghton, M. & Walker, C. M. 1995b. Association of cytotoxic T lymphocyte (CTL) escape mutations with persistent hepatitis C virus (HCV) infection. *Princess Takamatsu Symp,* 25**,** 227-35.

Welsh, R. M., Bahl, K., Marshall, H. D. & Urban, S. L. 2012. Type 1 interferons and antiviral CD8 T-cell responses. *PLoS Pathog,* 8**,** e1002352.

Wieland, D. & Thimme, R. 2016. Vaccine-induced hepatitis C virus-specific CD8+ T cells do not always help. *Hepatology,* 63**,** 1411-4.

Woitas, R. P., Ahlenstiel, G., Iwan, A., Rockstroh, J. K., Brackmann, H. H., Kupfer, B., Matz, B., Offergeld, R., Sauerbruch, T. & Spengler, U. 2002. Frequency of the HIV-protective CC chemokine receptor 5-Delta32/Delta32 genotype is increased in hepatitis C. *Gastroenterology,* 122**,** 1721-8.

Woltman, A. M., Boonstra, A. & Janssen, H. L. 2010. Dendritic cells in chronic viral hepatitis B and C: victims or guardian angels? *Gut,* 59**,** 115-25.

Wood, N. A., Linn, M. L. & Bowen, D. G. 2011. Exhausted or just sleeping: awakening virus-specific responses in chronic hepatitis C virus infection. *Hepatology,* 54**,** 1879-82.

Wyld, R., Robertson, J. R., Brettle, R. P., Mellor, J., Prescott, L. & Simmonds, P. 1997. Absence of hepatitis C virus transmission but frequent transmission of HIV-1 from sexual contact with doubly-infected individuals. *J Infect,* 35**,** 163-6.

Xia, C., Liu, Y., Chen, Z. & Zheng, M. 2015. Involvement of Interleukin 6 in Hepatitis B Viral Infection. *Cell Physiol Biochem,* 37**,** 677-86.

Xie, Z. C., Riezu-Boj, J. I., Lasarte, J. J., Guillen, J., Su, J. H., Civeira, M. P. & Prieto, J. 1998. Transmission of hepatitis C virus infection to tree shrews. *Virology,* 244**,** 513-20.

Yee, L. J. 2004. Host genetic determinants in hepatitis C virus infection. *Genes Immun,* 5**,** 237-45.

Yee, L. J., Im, K., Borg, B., Yang, H. & Liang, T. J. 2009. Interleukin-6 haplotypes and the response to therapy of chronic hepatitis C virus infection. *Genes Immun,* 10**,** 365-72.

Yenigun, A. & Durupinar, B. 2002. Decreased frequency of the HLA-DRB1*11 allele in patients with chronic hepatitis C virus infection. *J Virol,* 76**,** 1787-9.

Yin, L. M., Zhu, W. F., Wei, L., Xu, X. Y., Sun, D. G., Wang, Y. B., Fan, W. M., Yu, M., Tian, X. L., Wang, Q. X., Gao, Y. & Zhuang, H. 2004. Association of interleukin-12 p40 gene 3'-untranslated region polymorphism and outcome of HCV infection. *World J Gastroenterol,* 10**,** 2330-3.

Yoon, J. C., Shiina, M., Ahlenstiel, G. & Rehermann, B. 2009. Natural killer cell function is intact after direct exposure to infectious hepatitis C virions. *Hepatology,* 49**,** 12-21.

Yoon, S. K., Han, J. Y., Pyo, C. W., Yang, J. M., Jang, J. W., Kim, C. W., Chang, U. I., Bae, S. H., Choi, J. Y., Chung, K. W., Sun, H. S., Choi, H. B. & Kim, T. G. 2005. Association between human leukocytes antigen alleles and chronic hepatitis C virus infection in the Korean population. *Liver Int,* 25**,** 1122-7.

Zeuzem, S., Feinman, S. V., Rasenack, J., Heathcote, E. J., Lai, M. Y., Gane, E., O'Grady, J., Reichen, J., Diago, M., Lin, A., Hoffman, J. & Brunda, M. J. 2000. Peginterferon alfa-2a in patients with chronic hepatitis C. *N Engl J Med,* 343**,** 1666-72.

Zhang, C., Hua, R., Cui, Y., Wang, S., Yan, H., Li, D., Zhang, Y., Tu, Z., Hao, P., Chen, X., Zhong, J., Niu, J. & Jin, X. 2017. Comprehensive mapping of antigen specific T cell responses in hepatitis C virus infected patients with or without spontaneous viral clearance. *PLoS One,* 12**,** e0171217.

Zhang, T., Lin, R. T., Li, Y., Douglas, S. D., Maxcey, C., Ho, C., Lai, J. P., Wang, Y. J., Wan, Q. & Ho, W. Z. 2005. Hepatitis C virus inhibits intracellular interferon alpha expression in human hepatic cell lines. *Hepatology,* 42**,** 819-27.

Zhang, Y. L., Guo, Y. J., Bin, L. & Sun, S. H. 2009. Hepatitis C virus single-stranded RNA induces innate immunity via Toll-like receptor 7. *J Hepatol,* 51**,** 29-38.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & Chisari, F. V. 2005. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A,* 102**,** 9294-9.