Molecular Mechanisms of Protection from Hepatitis C Infection

Paraskevi Mandalou

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MOLECULAR MECHANISMS OF PROTECTION FROM HEPATITIS C INFECTION

by

Paraskevi Mandalou

A thesis submitted to the University of Plymouth

in partial fulfilment for the degree of

DOCTOR OF MEDICINE

Peninsula Schools of Medicine and Dentistry

May 2018

Acknowledgements

Dedicated

To

My parents and brother

Author's declaration

As the author of this thesis I wish to declare the following:

-At no time during the registration for the Doctor of Medicine degree have I been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

-Work submitted for this research degree at Plymouth University will not form part of any other degree either at the University of Plymouth or at another establishment.

-This study was not a collaborative project and does not aim to a joint degree with another institution.

-Presentations at conferences:

• "A comparative gene expression study between individuals with apparent resistance, spontaneous clearance, or chronic infection from HCV".

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• "IL-27 levels are elevated in individuals exposed to who remain uninfected".

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Abstract

- Paraskevi Mandalou.

- Molecular mechanisms of protection from HCV infection.

Hepatitis C virus (HCV) infection is a major global health burden affecting 1-2% of the world's population. The majority of infected individuals will develop chronic infection and are at risk of cirrhosis and hepatocellular carcinoma. There is currently no preventative vaccine available for HCV. In the developed world, the highest HCV incidence and prevalence rate is amongst intravenous drug users (IDU). The duration, frequency of IDU, and sharing of drug injecting equipment contribute to particularly high rates of HCV infection in this population. Individuals at high risk of recurrent exposure to HCV infection from long term IDU have been recruited in Plymouth, UK, from 2003 onwards and if they remain negative for HCV infection are termed exposed uninfected (EU). Understanding the factors that prevent HCV infection in this cohort could give valuable insight into the mechanisms of natural resistance to HCV infection.

The EU cohort was previously shown to have characteristics attributable to the activation of both the adaptive and the innate arms of the immune system with no known dominant, immune or non- immune, mechanism of HCV protection. The aim of this thesis was to attempt and identify this mechanism and for that purpose a comparative transcriptional profile study was initially performed between 3 groups: EU, individuals who spontaneously cleared HCV infection (SR) and patients with chronic HCV infection (CHCV). Of the differentially regulated genes, the association with resistance to HCV was strongest for Interleukin-27 (IL-27) which was significantly upregulated in EU compared to the

2 other groups and C X C motif chemokine 7 (CXCL7) which was significantly upregulated in EU relative to the CHCV group. The CD28 mediated T-helper cell signalling pathway was significantly upregulated in SR relative to the 2 other groups.

We attempted to corroborate the above findings and we demonstrated that IL-27 is overexpressed in EU, compared to SR and CHCV. The possible role of IL-27 in natural protection from HCV infection remains to be elucidated and requires further study.

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Abbreviations

Ab	Antibody (ies)
ALT	Alanine aminotransferase
APC	Antigen presenting cells
APOBEC	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide –like cytidine deaminases
Apo-E	Apolipoprotein E
APS	Ammonium persulfate
β-ME	β-mercaptoethanol
BAD	BCL2-associated agonist of cell death
BCL2	B cell lymphoma
BCL-XL	B-cell lymphoma-extra large
BSA	Bovine serum albumin
cDNA	Complementary DNA
CHV	Hepatitis C virus
CLDN1	Claudin- 1
DMSO	Dimethylsulfoxide
CTAP	Connective tissue activating peptide

CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CVR	Centre of virus research
CXCL7	C X C motif chemokine 7
Da	Dalton
DAA	Direct-acting antiviral agents
DC	Dendritic cells
DMSO	Dimethylsulfoxide
EBI3	Epstein-Barr virus-induced gene 3
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-Linked ImmunoSpot
EMCV	Encephalomyelitis virus

EU	Exposed Uninfected
ER	Endoplasmic reticulum
FaSL	Fas ligand
FCS	Fetal calf serum
FRET	Fluorescence resonance energy transfer
GAG	Glycosaminoglycans
GSK3B	Glycogen synthase kinase 3 beta
Grb2	Growth factor receptor-bound protein 2
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HRP	Horseradish Peroxidase
IDU	Intravenous drug user(s)
IFN	Interferon
IkB	Inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells

IL	Interleukin
IRF	Interferon regulatory factor
ISG	Interferon stimulatory gene
ISRES	IFN-stimulated response elements
I-TAC	Interferon-inducible T-cell alpha chemoattractant
ITK	Interleukin-2 -inducible T cell kinase
JAK	Janus kinase
LAT	Linker for activation of T cells
Lck	Lymphocyte-specific protein tyrosine kinase
LTK	Leukocyte Receptor Tyrosine Kinase
MAPK	Mitogen-activated protein kinases
MAVS	Mitochondrial antiviral signalling
MDA5	Melanoma differentiation antigen 5
NDR	Nod-like receptors
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
Mtor	Mammalian target of rapamycin

MxA	Myxovirus protein
NAP-2	Neutrophil activating peptide-2
NFAT	Nuclear factor of activated T-cells
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKT	Natural killer T
NP-40	Nonidet-P40
NPC1L1	Niemann-Pick C1-like 1
qPCR	quantitative polymerase chain reaction
OCLN	Occludin
ORF	Open reading frame
PAMP	Pathogen recognition receptors
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCMD	Peninsula College of Medicine and Dentistry, Plymouth, UK
PDK-1	Phosphoinositide-dependent kinase 1

PH	Pleckstrin homology
PKCϴ	Protein kinase C-ϴ
PI3K	Phosphatidyl-inositol 3-kinase
PIP2	Phosphatidylinositol (3,4)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PPB	Platelet basic protein
PRR	Pathogen recognition receptors
PKB	Protein kinase B
RIG-I	Retinoic acid inducible gene-I
RIPA	RadioImmunoPrecipitation
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RPMI	Royal Park Memorial Institute
RT	Reverse transcription
RT-PCR	Reverse transcriptase polymerase chain reaction
PVDF	Polyvinylidene fluoride
SDS	Sodium dodecyl sulfate

SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine synthesis
SR	Spontaneous resolver
SRB-1	Scavenger receptor B1
s- RPMI	Supplemented RPMI
STAT	Signal transducers and activators of transcription
SVR	Sustained virological response
TC	Thrombocidin
TCR	T cell receptor
Th	T helper
TG	Thromboglobulin
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TRAF	TNF receptor associated factor
TrF	Transferrin receptor
TRAIL	TNF-related apoptosis-inducing ligand

TRIF	TIR (Toll/interleukin-1 receptor) domain-containing adaptor protein inducing interferon beta
Tris	Tris(hydroxymethyl)aminomethane
TYK	Tyrosine Kinase
TfR1	Transferrin receptor 1
Treg	Regulatory T cell
UTR	Untranslated regions
VLDL	Very low density lipoproteins
Vs	Versus
ZAP70	Zeta-chain-associated protein kinase 70

Chapter 1 Introduction

1.1 *Clinical and virological features of HCV infection*

1.1.1 Hepatitis C virus constitutes a significant health burden worldwide

Hepatitis C virus (HCV) is an RNA virus that primarily infects the liver, affects 130 to 150 million people worldwide and can cause both acute and chronic infection. Acute HCV infection is usually asymptomatic, and is very rarely associated with life-threatening disease. About 15-45% of the infected persons will spontaneously clear the virus within 6 months, whilst the remaining 55-85% will develop chronic HCV infection. Chronic HCV hepatitis often results in progression to liver fibrosis and ultimately cirrhosis, with the risk of developing liver failure and hepatocellular carcinoma. As a consequence, HCV is the most common indication for liver transplantation in developed countries (Thomas 2013). The standard of care for HCV infection has changed rapidly over the past couple of years and the new direct antiviral drugs can cure 90% of the people infected with HCV. However, due the high cost of these drugs, access to them is limited even in high income countries (WHO fact sheet No 164, July 2015).

The main strategies for prevention of HCV focus on reduction of the risk of exposure in high risk populations, since a vaccine is currently unavailable. Thus, ongoing research on understanding the immunological mechanisms of natural and treatment induced clearance of HCV is of importance to reach this aim.

This chapter will outline the virological and clinical features of HCV infection. Furthermore, it will focus on current understanding of the immunological factors

that determine the outcomes of HCV infection which might correlate with mechanisms of natural protection from HCV in the high risk population of intravenous drug users (IDU).

1.1.2 HCV virology

Hepatitis C virus (HCV) is a hepatotropic RNA virus of the genus Hepacivirus in the Flaviviridae family, originally cloned in 1989 as the causative agent of non-A, non-B hepatitis (Choo et al. 1989).

The HCV genome is a single-stranded RNA molecule. It contains a single open reading frame encoding a polyprotein of about 3,000 amino acids. The open reading frame is flanked by 5' and 3' untranslated regions (UTR) of 341 and approximately 230 nucleotides in length, respectively. Both 5' and 3' UTR bear highly conserved RNA structures essential for polyprotein translation and genome replication. The 5' UTR contains an internal ribosome 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 the endoplasmic reticulum membrane to yield 10 mature proteins. They are divided into structural (Core, E1, E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5) (Lindenbach & Rice 2013) (figure 1-1).

The structural proteins include the core (C), which forms the viral nucleocapsid, and the envelope glycoproteins E1 and E2. They are released by host-cell signal peptidases. The two envelope glycoproteins, E1 and E2, are thought to play pivotal roles at different steps of HCV replicative cycle (Bartosch et al. 2003). There is strong evidence that they are essential for host-cell entry, by binding to receptor(s) and inducing fusion with a host-cell membrane. Both E1

and E2 trans- membrane domains are composed of two short stretches of hydrophobic amino acids with the second hydrophobic stretch acting as an internal signal peptide for the downstream protein. After cleavage by a host signal peptidase, the signal-like sequence is reoriented toward the cytosol (Cocquerel et al. 2002). Hypervariable regions have been identified in the E2 envelope glycoprotein sequence. These 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).

The non structural proteins NS2 to NS5B are involved in polyprotein processing and viral replication. The proteolytic processing of NS polyprotein part is complex and requires two distinct proteinases: the NS2- NS3 zinc-dependent metalloproteinase, and the NS3 serine proteinase located in the N-terminal region of NS3. The NS2-NS3 proteinase appears to be dedicated solely to cleavage at the NS2/NS3 site that occurs rapidly and by a conformation-dependent, autocatalytic mechanism. The remaining NS proteins are released by the NS3 proteinase associated with its cofactor, NS4A. The C-terminal region of NS3 protein includes RNA helicase and NTPase activities, so it is important for organization of replicated RNA by unwinding of single and double stranded RNA (Brass et al. 2006). The roles of p2 and NS2 in viral replication remain unclear (Pavlović et al. 2003; Steinmann & Pietschmann 2010).

NS4B is an integral membrane protein, thought to play a role in the formation of the membranous web, which is necessary for the latter stages of viral replication and virion assembly (Egger et al. 2002). NS5A is a poly-phosphorylated protein involved in viral replication. NS5B is an RNA-dependent RNA polymerase which is important for HCV replication via the synthesis of a

complementary plus-strand RNA, from a complementary minus-strand RNA using the genome as template. Variability in sub genomic regions such as E1, NS4 and NS5 are responsible for the last HCV heterogeneity with 7 recognised distinct major genotypes with genetic variability of 30-50% and more than 100 sub-types (Simmonds 1995; Smith et al. 2014).

Figure 1-1 The content has been removed due to copyright restrictions.

1.1.3 HCV cell entry and life cycle

HCV particles are 50-80 nm that can be found in various forms in the serum of the infected host, including (i) virions bound to very low density lipoproteins (VLDL) or low density lipoproteins (LDL) which appear to represent the infectious fraction; (ii) virions bound to immunoglobulins; and (iii) free (Thomssen et al. 1993; André et al. 2002). The interaction with lipoproteins could contribute to the shielding of HCV glycoproteins from the host immune response and explain the poor detection or availability of HCV glycoproteins at the virion surface, but more importantly, they may 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; Lefèvre 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 suggest that apolipoprotein E (Apo-E), rather than HCV glycoproteins themselves, could be involved in this initial contact (Jiang et al. 2013). After the initial attachment to the cell surface, cell entry is facilitated by the coordinated action of at least four out six major cellular factors. They include SRB1, tetraspanin, CD81, tight-junction proteins claudin-1 (CLDN1), occludin (OCLN) and epidermal growth factor receptor (EGFR). CD81 and OCLN determine the tropism of HCV for human cells (Pileri 1998; Scarselli et al. 2002; Evans et al. 2007; Ploss et al. 2009). Finally, since the HCV virion is rich in cholesterol, the role of the cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) was investigated and NPC1L1 identified as an additional entry factor (Sainz et al. 2012). Transferrin receptor 1 (TfR1) has also been reported to be involved in HCV entry, however, the precise roles of these additional factors in HCV entry remain to be

determined (Martin & Uprichard 2013). HCV is known to be endocytosed by a clathrin-dependent process and after internalization, the virion is transported to endosomes, where fusion takes place (Blanchard et al. 2006) (figure 1-2).
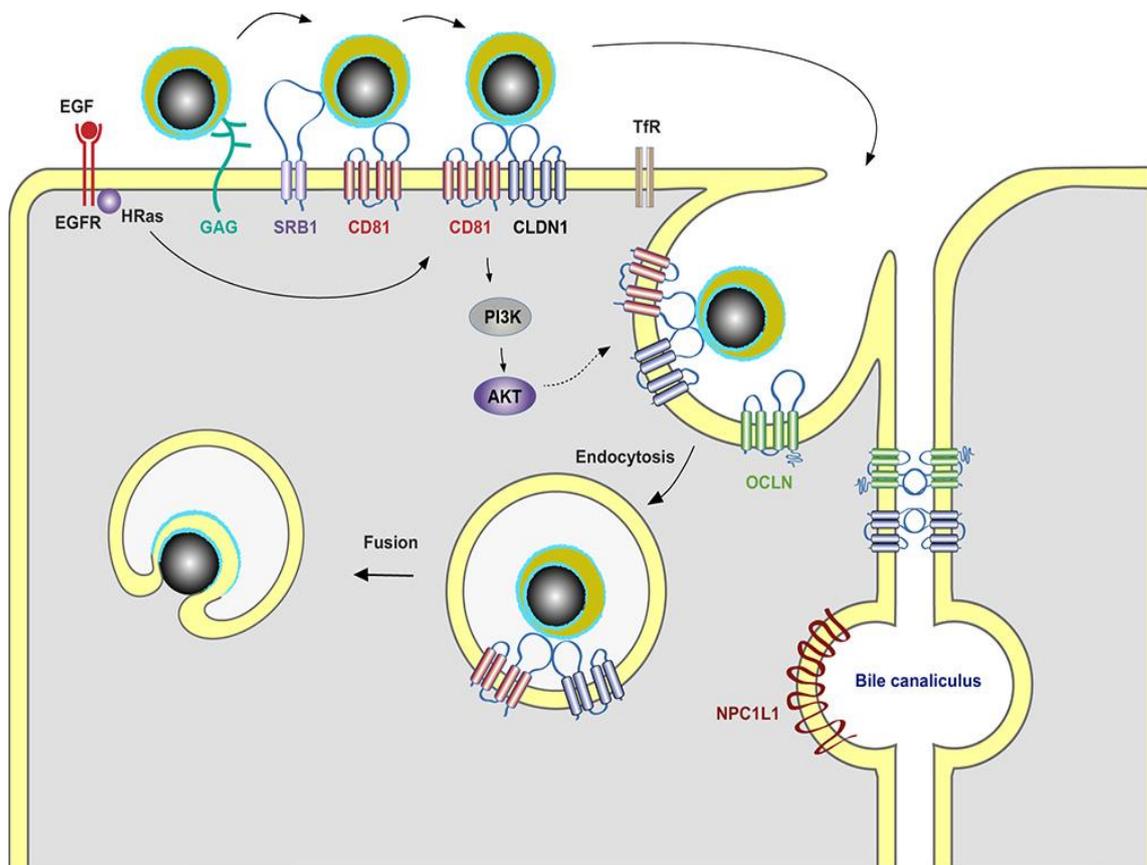
Figure 1-2 HCV entry.

The HCV virion is tightly associated with lipoproteins to form a complex particle that has been called lipoviroparticle. It initiates its life cycle by binding to glycosaminoglycans (GAGs) and scravenger receptor B1 (SRB1). Then the virus follows a complex multistep process, involving a series of specific cellular entry factors, which include SRB1, CD81, tight-junction proteins, claudin-1 (CLDN1) and occludin (OCLN), epidermal growth factor receptor (EGFR), transferrin receptor (TfR) and Niemann-Pick C1-like 1 (NPC1L1). After binding to several components of the host cell, HCV particle is internalized by clathrin-mediated endocytosis and fusion takes place in endosomes.

Adapted from Journal of Hepatology 2014 vol. 61 j S3–S13 S5.

https://doi.org/10.1016/j.jhep.2014.06.031

RNA translation is initiated with the help of cellular factors (Brodin et al. 2015). The 5 UTR contains an internal ribosomal entry site, which initiates translation of the HCV genome into a single polyprotein. Viral and host encoded proteases process the viral polyprotein into the 10 mature proteins (figure 1-1). After translation, the HCV proteins are associated with membranes derived from the endoplasmic reticulum. Together, NS3/4A, NS4B, NS5A, and NS5B constitute the viral proteins of the replication machinery, which replicates the positive sense RNA genome through a negative strand intermediate. To replicate its genome, HCV induces massive rearrangements of intracellular membranes to create in the cytoplasm a microenvironment, called the "membranous web". After assembly at the endoplasmic reticulum, the new particles are then released by budding at the cell membrane. HCV virion biogenesis is closely related to VLDL assembly pathway and there is a consensus about the involvement of apolipoprotein E (Apo-E) in HCV morphogenesis (Jiang & Luo 2009).

1.1.4 HCV transmission

HCV is a blood borne virus and early studies found a significant association between disease acquisition and a history six months prior to illness of blood transfusions, IDU, health care employment with frequent exposure to blood, personal contact with others who had hepatitis, multiple sexual partners or low socioeconomic status (Alter et al. 1982; Alter et al. 1989). Today, HCV is rarely transmitted by blood transfusion or transplantation of organs due to thorough screening of the blood supply for the presence of the virus and inactivation procedures that destroy blood borne viruses. These techniques have lowered

the risk of acquiring HCV via transfused blood products to 1 in 2 million (Stramer et al. 2004).

Injection drug use has been the principal mode of transmission of HCV since the 1970's. In comparison to other viral infections, HCV is the most prevalent in individuals who have injected for one year or less (Garfein et al. 1996).

The presence of tattoos has been independently associated with an increased risk of HCV infection. Other potential modes of unapparent blood contact include skin piercing and folk medicine (Ko et al. 1992; Kiyosawa et al. 1994).

Traditionally, in the healthcare setting, occupational needle stick injuries, use and reuse of non-sterile needles, syringes and haemodialysis can be routes of transmission of the virus (Lanphear et al. 1994; Frank et al. 2000). However, the overall risk of HCV acquisition in the modern health care settings is not higher than the rest of the population (Thorburn et al. 2001).

HCV is rarely transmitted by monogamous heterosexual intercourse, but the risk is increased in those with high risk sexual behaviour (0.4 to 1.8% per year), such as men who have sex with men or those with multiple sexual partners (Wyld et al. 1997). Vertical transmission is uncommon and it is estimated that 1150 pregnancies annually in the UK would involve a woman infected with HCV, leading to approximately 70 infected infants being born each year (Ades et al. 2000). Household contact HCV transmission has been reported but the incidence is low and overall in 20% of cases the route of transmission is unknown (MacDonald et al. 1996).

1.1.5 **HCV epidemiology**

According to a recent review of the global epidemiology and genotype distribution of HCV infection (Gower et al. 2014) the results are as follows:

• The total global prevalence of HCV is estimated to be 1.6% (1.3-2.1%), corresponding to 115 (92-149) million past viraemic infections.

• The majority of these infections, 104 (87-124) million, are among adults (defined as those older than 15 years old) with an anti-HCV infection rate of 2.0% (1.7-2.3%).

• The viraemic (RNA positive) prevalence is forecasted to be 1.1% (0.9-1.4%), corresponding to 80 (64-103) million viraemic infections.

•Most of these viraemic infections are among adults who account for 75 (62-89) million viraemic infections or a viraemic prevalence of 1.4% (1.2-1.7%).

• Globally, genotype 1 is the most common, accounting for 46% of all infections, followed by genotypes 3 (22%), and genotypes 2 and 4 (13% each). Subtype 1b accounts for 22% of all infections at the global level.

• There are significant variations across regions with genotype 1 dominating in Australasia, Europe, Latin America and North America (53-71% of all cases) and genotype 3 accounts for 40% of all infections in Asia.

• Genotype 4 is the most common (71%) in North and West Africa and the Middle East, but when Egypt is excluded, it accounts for 34%, while genotype 1 accounts for 46% of infections across the same region.

A map of global HCV genotype distribution is shown in figure 1-3.
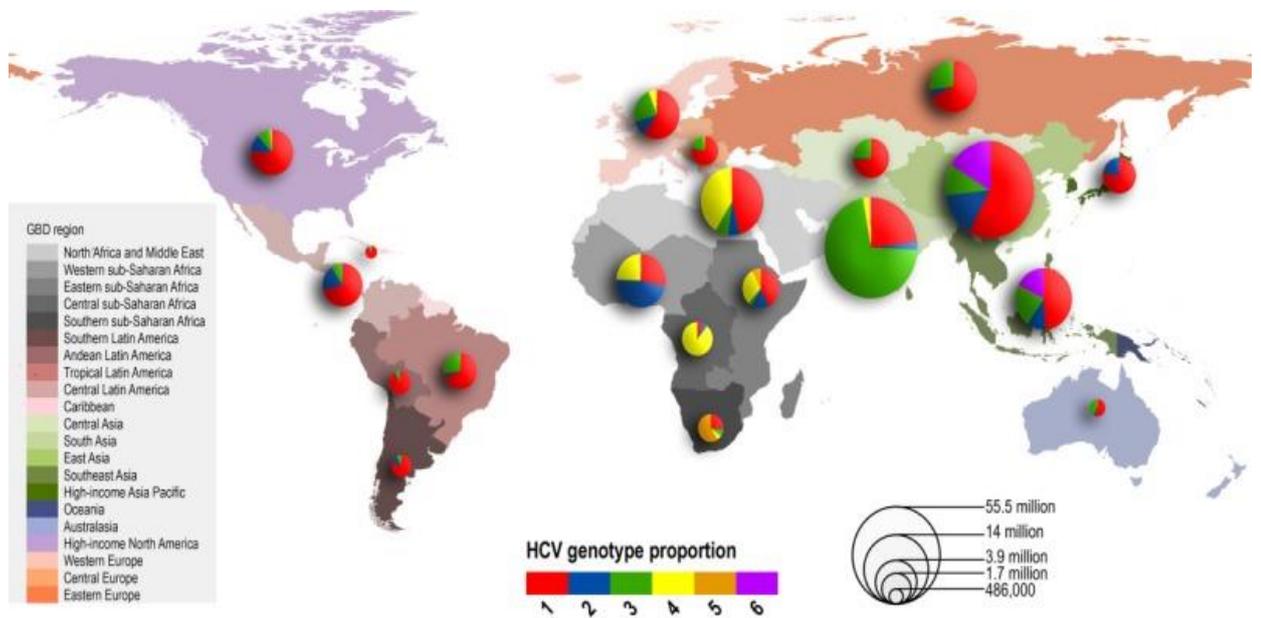
Figure 1-3 Map of global HCV genotype distribution.

Relative prevalence of each HCV genotype by geographical burden of disease region. The size of pie charts is proportional to the number of seroprevalent cases.

Adapted from Hepatology.2015 Jan 61(1):77-87.

https://doi.org/10.1002/hep.27259

In the UK a conservative estimate is that there 214.000 individuals chronically infected with HCV. Worldwide, the highest HCV incidence and prevalence rate is amongst IDU and it is estimated that 10 million active IDUs have been exposed to HCV and 8 million have chronic infection (Grebely & Dore 2011). Data from the Unlinked Anonymous Monitoring survey of people who inject drugs in the UK suggest that levels of infection in this group remain high in 2013 (50% in England, 32% in Northern Ireland and 47% in Wales).

Factors associated with HCV acquisition include recent initiation to injecting, unstable housing, female gender, ethnicity, survival sex work, frequent injecting cocaine use, imprisonment, having a partner who injects, injecting networks, requiring help injecting, and borrowing injecting equipment (Patrick et al. 2001; Miller et al. 2002; Roy et al. 2007; Shannon et al. 2010; Hellard et al. 2014).

The duration of IDU and frequency of drug use appear to influence the risk of transmission. Those with daily injecting, and injecting of crack/cocaine, compared to heroin, are at the highest risk of acquiring HCV. The highest risk of acquiring HCV is within the first 2 years of commencing IDU, with prevalence rates rising progressively to more than 90% in some reports in those who had injected for more than 10 years (Thomas et al. 1995; Garfein et al. 1996; Lorvick et al. 2001).

1.1.6 Natural history of HCV

The average time from HCV exposure to seroconversion is 5-12 weeks, whilst HCV RNA is detectable within 2 weeks (Puoti et al. 1992). This stage precedes the elevation of ALT by approximately 1-3 weeks. The acute infection is usually asymptomatic, however approximately one third of the affected individuals

might develop non -specific flu like symptoms. More specific symptoms of viral hepatitis can be encountered in a minority of individuals: jaundice, dark urine, anorexia, aversion to smoking among smokers and abdominal discomfort may occur. Physical findings are usually minimal, apart from jaundice in a third of patients. 15-40% of the affected individuals will clear the virus and the factors affecting this include host genetics, gender, mode of acquisition, the severity of the acute illness, presentation with jaundice, a poorly defined weak immune response, immunosuppression and HIV co-infection (Westbrook & Dusheiko 2014). Chronic HCV infection is defined by detectable HCV RNA in the serum of affected individuals for more than 6 months. Late spontaneous seroconversion can occur, but is rare and estimated at 0.5% per person per year (Watanabe et al. 2003). Those who clear the infection will have detectable HCV antibodies, however the titres tend to wane 18-20 years after the infection (Takaki et al. 2000).

Chronic HCV infection will lead to fibrosis and cirrhosis in 5-25% of the affected individuals within 20 years (Di Bisceglie 1998; Seeff 2002). Risk factors for progression to cirrhosis include age more than 40 years, male gender, alcohol consumption of more than 30g/day, human immunodeficiency virus (HIV) and /or hepatitis B virus (HBV) co-infection, severity of liver fibrosis at the time of diagnosis and the presence of co morbid conditions (Poynard et al. 1997; Lesens et al. 1999; Harris et al. 2001; Ryder et al. 2004). Once cirrhosis is established, the rates of decompensated chronic liver disease and hepatocellular carcinoma (HCC) are ~2%–4%/year and 1%–7%/year, respectively (Tsukuma et al. 1993; Fattovich et al. 1997).

1.2 Testing and treatment of HCV

Established chronic infection requires the detection of HCV antibodies and HCV RNA in the serum of the affected individuals. Spontaneous or treatment resolution of HCV infection is defined by the presence of HCV antibodies but absence of HCV RNA.

HCV antibodies are currently identified by 3rd generation enzyme immunonoassay (EIA) that detects antibody reactivity to core, NS3, NS4 and NS5 proteins of the virus. It has a sensitivity of 98.1% (95% confidence interval, 92.6 to 99.7) and specificity of 99.8% (95% confidence interval, 99.2 to 99.9). Due to high sensitivity and specificity of the detection assays, previous additional confirmatory tests used are now obsolete (Abdel-Hamid et al. 2002).

HCV RNA is currently detected by quantitative reverse transcriptase–polymerase chain reaction. Apart from diagnosing chronic HCV infection it is also useful in treatment monitoring. In order to achieve standardization across the available quantification assays, the viral load is currently expressed in international units (IU) /ml of serum and there is a conversion factor, which is assay dependent, that is used to calculate the corresponding viral copies/ml of serum. There are a number of commercially available highly sensitive and reliable assays for HCV RNA that are able to quantify HCV viral concentration at a level as low as 10-20 IU / ml (Cobas v2.0, Roche Molecular Systems). Up until 2011, recombinant pegylated-interferon-α and ribavirin were the standard of treatment for HCV infection. Successful outcome is defined as undetectable HCV RNA 6 months following completion of treatment and is termed 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 or 3 and was frequently associated with intolerable side effects (Fried et al. 2002; Hadziyannis et al. 2004).

The treatment of HCV has been revolutionised over the past 4 years with the introduction of a variety of drugs able to directly inhibit HCV viral proteins (direct-acting antiviral agents or DAA), by targeting NS5A, NS5B or NS3/4A and have resulted in cure rates of up to 95% after a 12 week treatment (Zeuzem et al. 2015). The first generation of DAA, the NS3/4A inhibitors, were co-administered with pegylated interferon and ribavirin, thereby adding to the side effect burden (Jacobson et al. 2011), whereas subsequent DAAs, used in combination, can be administered without pegylated interferon and are more efficacious with shorter duration of treatment. However, various polymorphisms in the targeted viral proteins (resistance-associated variants), identified, mainly in the new generation NS5A DAA, can reduce the SVR rates (Wells et al. 2015).

The main issue related to the use of DAA for HCV treatment is their cost. The incremental cost-effectiveness ratio ranges from $35,000 to $410,548 per quality-adjusted life-year gained, making affordability a huge limitation in their use. Taking into account that the population mainly affected by HCV is IDU (see 1.1.5), whose compliance to treatment is lower compared to non-IDU (Hellard et al. 2009), a preventative as well as a curative approach to HCV infection remains an important goal.

1.3 *The discovery of HCV*

1.3.1 Animal models

Chimpanzees, studies on which led to the discovery oh HCV virus, are the only animal models (Houghton 2009) that can be used to completely study HCV infection. They can be infected with isolates of the 6 epidemiologically important

genotypes and have innate and adaptive immune responses similar to those observed in infected humans (Bukh 2004).They are, however, rare and expensive.

T- and B-cell deficient mice (severe combined immunodeficiency mice), grafted with human hepatocytes, are the only small animals that can be robustly infected with HCV. However, because these mice are immune-deficient, they have impaired utility for studies of adaptive immunity (Mercer et al. 2001).

There have been reports of HCV infection in New and Old World monkeys, but most evidence indicates that these primates are not susceptible to HCV infection (Bukh et al. 2001). Tree shrews (Tupaia belangeri, non-rodent small mammals that are easy to maintain and reproduce) have been proposed as a model for HCV infection. Even though these animals can apparently be infected with HCV (Xie et al. 1998), they have not found widespread use. The low and variable infection rates and HCV titres are problematic. The reported development of persistent infection and evidence for chronic liver disease are, however, attractive features of this model.

1.3.2 In vitro models

Initial attempts to study the establishment of HCV infection used primary cells from humans and chimpanzees. Primary human foetal hepatocytes infected with HCV-containing sera detected the positive strand of virus but the replication was low (Iacovacci et al. 1997). Due to short passage life and contamination problems in primary hepatocytes, scientists tried to develop immortalized human hepatoma cell lines. Many cell lines supported HCV infection and replication in-vitro such as human T-lymphocyte cell lines, human fibroblast cells (VH3), peripheral blood mononuclear cells (PBMC) and

hepatocytes. A human hepatocyte cell line, PH5CH, which is immortalised with simian virus 40 large antigen, was extensively studied. Although found to be more susceptible to HCV infection than others, the system was still inefficient (Kato et al. 1996). Studies looking at hepatoma cell lines HepG2 and HuH-7 produced poor results in the past even though conditions were changed extensively to try to optimise the approach (Mizutani et al. 1996; Seipp et al. 1997).

The HCV replicon system replicates a modified HCV genome to high levels in human hepatoma (Huh-7) cells (Lohmann et al. 1999). Replicons are either sub-genomic (containing only the non-structural proteins for RNA replication) or genomic in length (contains the entire HCV genome). Both types of replicons contain the neomycin phosphotransferase gene for selection. The encephalomyelitis virus (EMCV) internal ribosomal entry site (IRES) sequence facilitates translation of the non-structural proteins in-vitro. All genes are driven by a T7 promoter. Following transcription with T7 RNA polymerase, replicon RNA is transfected into Huh-7 human hepatoma cells. Subsequent adaptive mutations, acquired by unknown mechanism, in NS3 and NS5A strongly increased RNA replication (Bartenschlager 2002).

Wakita developed a genotype 2a full length replicon (JFH-1) which was isolated from a Japanese patient with fulminant hepatitis. This HCV full length genome replicates efficiently and produces virus particle (HCVpp) in Huh- 7 (Wakita et al. 2005). Further refinements have led to the development of Huh7 derived cell lines (Huh7.5.1) which result in increase of the viral titre to 10^4–10^5 infectious units per ml of culture supernatant and these cell lines are highly permissive to JFH-1 virus infection. An important limitation of this replicon is that HCV

particles are based on genotype 2 which is not the dominant genotype in the world. Based on these findings, the infectious hepatitis C virus (HCV) cell culture system (HCVcc) was developed. It is a robust HCV cell culture infection system based on the HCV JFH-1 molecular clone and Huh-7-derived cell lines that allows the production of virus that can be efficiently propagated in tissue culture (Zhong et al. 2005).

HCV pseudo particles (HCV pp) were produced to study the early stages of viral life cycle. HCVpp were produced by transfecting the three vectors in Human embryo kidney cells (293T). The first vector encodes retroviral Gag and Pol proteins which are responsible for particle budding at the plasma membrane and RNA encapsidation. The second vector encodes a reporter protein (Luciferase). The third vector encodes HCV glycoproteins E1 and E2, which are necessary for viral tropism and fusion of HCV pp with the target cell membrane. 293T cells secrete virus pseudo particle (on average 10^5 particles/ml) which can be used to infect Huh 7 cells and infectivity is evaluated by quantification of the amount of luciferase expressed in Huh-7 cells (Bartosch et al. 2003). These virus like particles can be neutralized with monoclonal antibody against the viral glycoprotein E1, E2 and sera of HCV infected patient and are a powerful tool to identify inhibitors which block HCV entry (Hsu et al. 2003).

1.3.3 Evolving molecular methods for the study of HCV infection

The development of the HCVcc cell line mentioned above, allowed studying of the entire virus infectious cycle and its effect on cellular gene and protein expression.

Traditionally differential gene expression in cells infected with HCV has been studied following microarray analyses of gene transcriptional profile. RNA is

extracted from a cell or tissue of interest and is then hybridized to the tethered probe DNA sequences corresponding to specific genes that have been affixed, in a known configuration, onto a solid matrix. This information is then captured and the comparison of hybridization patterns enables the identification of mRNAs that differ in abundance in two or more target samples. Issues with microarrays include cross-hybridization artefacts, poor quantification of genes with low expression and the need of prior knowledge of the target sequence.

RNA sequencing (RNA-Seq) is a relatively novel technique that will probably substitute microarray analyses in the future. In general, a population of RNA is converted to a library of cDNA fragments with adaptors attached to one or both ends. Each molecule, with or without amplification, is then sequenced in a high-throughput manner (Holt & Jones 2008) to obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing). Following sequencing, the resulting reads are either aligned to a reference genome or reference transcripts, or assembled de novo without the genomic sequence to produce a genome-scale transcription library that consists of both the transcriptional structure and/or level of expression for each gene. This makes RNA-Seq particularly attractive for non-model organisms with genomic sequences that are yet to be determined (Z. Wang et al. 2009).

Woodhouse et al have recently published data on the first full-genome RNA-Seq analysis in a host cell to analyse HCV infected and non-infected cells, and compared this to microarray and proteomic analyses. This combined approach has led to the identification of canonical pathways (see 3.2) and biological functions associated with HCV infection that have not been previously reported. These include pregnane X receptor/retinoic acid receptor activation as a

potential host antiviral response, and integrin-linked kinase signalling as an entry factor. Additionally, it was highlighted that HCV infection had a broad effect on cellular metabolism, leading to an increase in cellular cholesterol and free fatty acid levels, that is associated with a profound decrease in cellular glucose levels. The limitations of the technique are the requirements for large quantities of high-quality RNA, the current cost and requirement for specialist sequencing equipment, and management of a significant data load (Woodhouse et al. 2010). However, it allows a non- hypothesis driven transcriptional profiling study and could be useful in identifying the molecular mechanisms of natural protection from HCV in the future.

1.4   Outcome of HCV infection; Non immune determinants

Accurate studies of the time course for clearance of acute hepatitis C are difficult to carry out because of the silent onset of the acute disease. Studies to determine the rate of persistence are few and may be biased by the mode of ascertainment. They frequently involve the prospective study of symptomatic individuals who are more likely to clear the virus. Various host characteristics have been associated with spontaneous clearance of HCV (also mentioned in 1.1.6.) and the strongest association is with female gender (Alric et al. 2000; Grebely et al. 2014). Icteric and symptomatic clinical presentation as well as low peak viral titres also favour viral clearance (Villano et al. 1999). Ethnicity influences outcome as Caucasians are more likely to clear the virus compared to Afro Caribbeans (Villano et al. 1999; Piasecki et al. 2004). Viral co-infection with either HIV or HBV and immunosuppression favour HCV chronicity, possibly related to reduced CD4+ cell counts (Thomas et al. 2000; Grebely et al. 2014). The likelihood of spontaneous HCV resolution is, also, associated with several genetic factors, including IL28b inheritance and the DQB1∗0301 allele of the

major histocompatibility complex class (Alric et al. 1997; Cramp et al. 1998;

Thomas et al. 2009). Albeit the observation is lacking consensus (Santantonio

et al. 2003; Wietzke-Braun et al. 2007), it has been suggested that infection with

HCV genotypes 1 &3 favours spontaneous clearance (Lehmann et al. 2004;

Harris et al. 2007).

1.5   HCV outcomes; Immune determinants

Chronic HCV infection results from either the ability of the virus to escape the

host's immune responses or, conversely, from the inability of the host to mount

appropriate immune responses to clear the virus. The following chapters

discuss elements of the innate and adaptive immunity that may influence the

outcome of HCV infection.

1.5.1 **Innate immunity**

1.5.1.1 **HCV recognition and signalling pathways**

Innate immune responses are the first line of defence against viral infections.

During the viral replication process HCV is sensed as non-self by pathogen

recognition receptors (PRRs) in the host cell that identify and bind to pathogen

associated molecular pattern (PAMP) motifs within viral products, leading to

coordinated activation of the innate immune response. A variety of PRRs sense

viruses as foreign invaders within the host cell through specific PAMP

recognition to activate innate immune signalling. The RIG-I-like receptors

(RLRs), retinoic acid inducible gene-I (RIG-I) and melanoma differentiation

antigen 5 (MDA5), are cytosolic PRRs that sense RNA viruses. Toll-like

receptors (TLRs) mediate virus sensing from within endosomal compartments

to signal innate defences, while Nod-like receptors (NLRs) serve to sense

cytosolic viral products or viral metabolites to drive inflammatory responses.

HCV is recognized by RIG-I within hours of infection and activates downstream signalling prior to extensive viral protein synthesis (Loo et al. 2006). RIG-I signalling during HCV infection is initiated upon its binding of PAMP RNA that includes an exposed 5' triphosphate and the 3' non-translated region of the HCV genome RNA rich in poly U/UC ribonucleotides (Uzri & Gehrke 2009). HCV RNA binding induces a RIG-I conformational change that promotes oligomerisation and translocation from the cytosol into intracellular membranes. This process requires interactions with the chaperone protein 14-3-3ε and the E3 ubiquitin ligase TRIM25, which together with RIG-I comprise a translocon that facilitates the interaction of RIG-I with the mitochondrial antiviral signalling protein (Gack et al. 2007). The RIG-I/MAVS interaction promotes the formation of a MAVS signalosome that propagates activation of downstream effector molecules, including the transcription factors interferon regulatory factor (IRF-3) and NF-κB and a variety of pro inflammatory cytokines (Loo & Gale 2011).

TLR3 has also been implicated as a PRR that senses HCV, although its role in HCV detection and immunity is still not fully understood. TLR3 is an endosomal sensor of dsRNA expressed in a number of cell types within the liver, including hepatocytes and the liver resident macrophage Kupffer cells. TLR3 signals are transmitted through the adaptor protein TIR (Toll/interleukin-1 receptor) domain-containing adaptor protein inducing interferon beta (TRIF), which activates IRF-3 and NF-κB for the production of type I IFN, pro inflammatory cytokines, and chemokines, as well as for apoptotic signalling (Takeuchi & Akira 2009). Whereas synthetic dsRNA ligands of TLR3 can induce IRF-3 dependent signalling in cells expressing ectopic TLR3 within 24 hours, HCV infection triggers this response 3-4 days after the infection (N. Wang et al. 2009).

Protein kinase R (PKR) is a dsRNA binding protein PRR, whose kinase activity can lead to IFN production, mainly by suppressing host, but not HCV, mRNA translation (Arnaud et al. 2011). It is also known that PKR binding to HCV dsRNA activates a kinase-independent signal transduction cascade that drives induction of specific interferon inducible genes and IFN-β production by signalling through MAVS, TNF receptor associated factor 3 (TRAF3), IRFs, and NF-κB, all prior to RIG-I activation (Kumar et al. 1997; McAllister & Samuel 2009; Arnaud et al. 2011)

1.5.1.2 **Mechanisms of HCV evasion of innate immunity and the role of type I interferons**

Interferons are a family of cytokines grouped in 3 classes: type I, II and III. Type I interferons are encoded by a variety of genes, mainly including IFN-α, IFN-β, IFN-ε, IFN-κ. In humans, IFN-α and IFN-β fight viral infections either directly or indirectly by activation of the innate immune system. Type II IFN, mainly IFN-γ, is produced by NK cells and activated T-cells. Type III IFN include IFN-λ1 (IL-29), IFN-λ2 (IL-28α) and IFN-λ3 (IL-28β) and are also activated in the context of viral infections (Randall & Goodbourn 2008; Levy et al. 2011).

Type I interferons are produced by the liver both in acute and chronic HCV infection and IFN- α2 has been, up until recently, widely used in the treatment of HCV. They up regulate the major histocompatibility complex (MHC) class-I and class II expression and activate natural killer, dendritic and Kuppfer cells (Bigger et al. 2001; Rehermann 2013). Type I interferons bind the heterodimeric transmembrane receptor IFN-AR1/IFNA-R2 leading to activation of the receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which phosphorylate the signal transducers and

activators of transcription (STAT) 1 and 2. Following translocation to the nucleus they bind to interferon response factor 9 (IRF9) to form the interferon stimulatory gene factor 3 (ISGF3), which subsequently binds to IFN-stimulated response elements (ISRES) leading to transcription of ISGs. The ISG-encoded proteins inhibit, through a variety of mechanisms, viral transcription, translation and replication (Levy & Darnell 2002; Schoggins et al. 2011; MacMicking 2012; Stark & Darnell 2012).

Despite the fact that HCV induces the production of type I interferons, HCV manages to escape its immunomodulatory effects via a variety of mechanisms.

HCV core protein induces the synthesis of suppressor cytokine signaling 3 (SOCS3) which inhibits STAT 1 phosphorylation and suppresses JAK-STAT (Bode et al. 2003). The HCV NS3/4A protease cleaves MAVS and TRIF and leads to suppression of IFN production and the expression of interferon inducible genes (Li et al. 2005; Loo et al. 2006). NS5A inhibits the activation of PKR (Gale et al. 1997) and the interferon inducible gene 2-5 oligoadenylate synthetase (Taguchi et al. 2004). Thus whilst increased therapeutic levels of IFN-a can result in HCV clearance, albeit with high relapse rates (Pawlotsky 2011), endogenous IFN-a does not seem to influence the outcome of HCV infection (Bigger et al. 2001).

1.5.1.3 **The role of Dendritic cells in the outcome of HCV infection**
Dendritic cells (DC) are very efficient inducers and regulators of all immune responses. They are derived from bone marrow and they circulate in the peripheral blood as either mature or immature forms. Based on their origin, antigen presenting characteristics and function, DCs are differentiated in plasmatoid (PDC) and myeloid (MDC) (Kadowaki 2009). As immature cells they

express low levels of MHC Class I and II antigens but following maturation, after detection of pathogen or upon contact with pro-inflammatory cytokines, they increase the MHC expression and enhance their ability to present antigens to T cells, activate NK cells and produce IFN, therefore linking the innate with the adaptive immunity (Adema 2009). MDC express high levels of MHC Class II and are able to interact with T cells and produce high levels of IL-2 and IL-10, whereas PDC express high levels of MCH Class I and secrete type I IFN.

It has been suggested that the inability of individuals infected with HCV to clear the infection is associated with HCV induced DC functional impairment but the findings are controversial. A variety of DC defects have been reported in patients with HCV including reduced number of circulating DC, deficiency in co-stimulatory molecules, decreased T-cell stimulatory capacity, decreased production of IL-15 and over production of IL-10 (Kanto et al. 2004; Averill et al. 2007; Della Bella et al. 2007; Dolganiuc et al. 2008; Saito et al. 2008; Mengshol et al. 2009), however these findings have not been confirmed in all of the published studies (Rollier et al. 2003; Longman et al. 2004; Piccioli et al. 2005). Such discrepancies more likely represent difference in patient cohorts, assessment of non-human primate models of HCV infection, different experimental approaches (Szabo & Dolganiuc 2008).

1.5.1.4 **The role of Natural Killer cells in the outcome of HCV infection**
Natural killer (NK) cells account for 5-20% of the peripheral blood mononuclear cells but represent 30-50% of lymphocytes in the liver (Corado et al. 1997). They are able to directly lyse cells infected by viruses or tumours and unlike classic cytotoxic T cells they do not require the presence of MHC class I for target cell recognition (Kärre et al. 1986).

NK cells are rapidly activated by cytokine stimulation (IL-1, IL-10, IL-12, IL-15 and IL-18) and their function is amplified by IFN-α release by virally infected and dendritic cells (Reiter 1993; Corado et al. 1997). NK cell function is regulated by a combination of regulatory receptors with inhibitory or stimulatory effect which interact with MHC-I alleles and other ligands on a variety of cells with viral infections or tumours. The net balance of signals will determine the threshold at which NK cells will be activated. This receptor/ligand interaction provides a mechanism of self tolerance and inhibition of NK cell autoreactivity. Activating receptors include C- type lectin like NKG2D and CD94:NKG2C/E, natural cytotoxicity receptors NKp44, NKp30, NKp46, and CD16 (FC-γ-RIII) (Table1-1).

Function	Family	Receptor	Ligand
Activating	C-type lectin receptor	• NKG2D • CD94:NKG2C • CD94:NKG2E	• MIC-A/B, ULBPs • HLA-E
	Natural cytotoxicity receptor	• NKp30 • NKp44 • NKp46	• BAT-3, B7-H6, CMV pp65 • Viral haemagglutinin • Viral haemagglutinin
	Killer cell immunoglobulin receptor	• 3DS1	• HLA-Bw4
	Others	• CD16	• IgG
		• Toll-like receptors	• Pathogen-associated molecular patterns (PAMPs)
Inhibitory	Killer cell immunoglobulin receptor	• 2DL1 • 2DL2/3	• Group 2 HLA-C • Group 1 HLA-C
	C-type lectin receptor	• CD94:NKG2A	• HLA-E

Table 1-1 Key receptors for Natural Killer Cells.

Adapted from Gut 2011;60:268-278.

Key inhibitory receptors (KIR) are the killer cell immunoglobulin-like receptors and the CD94:NKG2A heterodimer. Once the signal from the activating receptors exceeds that of the inhibitory receptors their functions are initiated (Cheent & Khakoo 2009). Target cell killing is facilitated by degranulation of cytotoxic granules such as perforin and granzyme B and by the expression of death receptors Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL). Additionally NK cell activation leads to priming of the adaptive arm of the immune response by secretion of the Th1 cytokines IFN-γ and TNF-α (figure 1-4).

NK cell subsets include the NK-CD56^{dim} and NK-CD56^{bright} depending on the absence of the pan-lymphocytic CD3 and the presence of CD56 which is of no functional relevance. CD56^{dim} express a moderate level of CD56 and represent 90% of the circulating NK cells. CD56^{bright} express high levels of CD56 and are considered more immature cells that can potentially differentiate into mature. CD56bright NK cells under the influence of cytokines, especially IL-15 (Cooper et al. 2001). CD56dim express higher levels of CD16 and perforin and are more potent cytotoxic cells. The role of CD56bright is the production of cytokines at the site of inflammation but can also be cytotoxic through the expression of TRAIL (Stegmann et al. 2010). In the liver, compared to peripheral blood and spleen, NK cells exist in a hypo responsive state, are less cytotoxic and produce lower levels of INF-γ and higher levels of IL-10, features that might contribute to the development of chronic hepatotropic viral infections (Dunn et al. 2009). Additionally hepatic NK cells can contribute to reduction in liver fibrosis by inhibiting hepatic stellate cells (Baroni et al. 1996).

Figure 1-4 The content has been removed due to copyright restrictions.

Natural killer T cells (NKT) are a subset of lymphocytes that possess both NK markers and CD1d-restricted 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 the NKT cell TCR leads to rapid and copious secretion of Th1 and Th2 cytokines. Additionally, they express cytotoxic granules that contain granzyme and perforin and are, therefore, able to kill target cells (Cianferoni 2013).

The original implication of NK cells in the outcome of HCV infection was associated with the KIR genes and their human leucocyte antigen-C (HLA-C) ligands. KIR genes and their MHC class I ligands 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 group1 (HLA-C1) ligand directly influence resolution of hepatitis C virus (HCV) infection. This protective effect, however, was only evident in patients infected by low volume inoculum (S. Khakoo et al. 2004). Several studies have also shown increased expression of NKG2A on NK cells in chronic HCV infection (Jinushi et al. 2004; J Nattermann et al. 2006) causing reduction in NK cell cytotoxicity and mediated dendritic cell activation. NK cells are also inhibited by the heterodimeric receptor CD94:NKG2A, which has the oligomorphic MHC class I molecule HLA-E as is its ligand. An allelic variant of HLA-E, HLA-E^R was shown to be protective from chronic HCV infection genotypes 2&3 possibly due to its reduced affinity and less inhibitory potential (Strong et al. 2003; Schulte et al. 2009).

There is a definite role of NK cells in controlling acute HCV infection, even though due to the asymptomatic nature of the disease information of the NK cell

function at the early stages has been difficult to study. It has been demonstrated that the proportion of CD56^{dim} compared to CD56^{bright} increases and the levels of CD56^{bright} do not return to baseline until 1-3 months in individuals who spontaneously cleared HCV compared to healthy controls and patient who develop chronic HCV infection. The levels of activating receptor NKG2D are also elevated in the acute phase of the HCV infection as well as IFN-γ production and NK cell degranulation, especially in individuals who express HLA-C1 specific KIR receptors. Pelletier et al observed decreased expression of the inhibitory NKG2A receptor in NK cells in IDU who spontaneously clear HCV infection compared to those who develop chronic HCV and those who are exposed to the virus but remain uninfected. Additionally they were able to correlate NK with T-cell responses and show that NK cell degranulation correlated with the magnitude of HCV-specific T cells, suggesting a co ordinated adaptive and innate immune system activation in acute HCV infection (Pelletier et al. 2010).

The frequency, phenotype and function of NK cells have been easier to study in the context of chronic HCV infection. NK cell absolute number and percentage of total lymphocytic population are reduced in patients with chronic HCV infection compared to the healthy population, but this might be either a contributing factor or a consequence. Some studies support the latter showing reduced frequencies and depressed function of NK cells in chronic HCV infection which recover after treatment (Corado et al. 1997; Pár et al. 2002; Dessouki et al. 2010), whereas others demonstrated normal NK cell function in patients with chronic HCV infection (Morishima et al. 2006). A number of studies have shown a skewed subset distribution, with a relative increase of CD56^{bright} compared to CD56^{dim} in the peripheral circulation in chronic HCV compared to

spontaneous resolvers and healthy controls and this effect is not associated with sequestration of CD56^{dim} in the liver (Morishima et al. 2006; Bonorino et al. 2009). CD56- CD16+ is a NK cells subset that is defective in perforin expression, interaction with dendritic cells and production of IFN-γ and TNF-α. CD56$^-$ CD16$^+$ NK cells expand in many patients with chronic HCV infection and there is a skewing away from the CD56^{bright} – CD16$^+$ subset, which is the main cytotoxic subset of NK cells (Mavilio et al. 2006; Gonzalez et al. 2009). There's conflicting data with regards to alterations in NK cell receptor phenotypes but the most consistent finding is an increase in expression of NKG2A on NK cells in chronic HCV infection (J Nattermann et al. 2006; De Maria et al. 2007). Even though NK cell cytotoxicity is preserved in chronic HCV there seems to be a change in the cytokine profile that favours viral persistence. IFN-γ, which blocks HCV replication, production is reduced and IL-10 production from Th2 cells is increased contributing to maturation arrest of CD56^{bright} NK (Crotta et al. 2010; Dessouki et al. 2010). In chronic HCV infection the normal pattern of DC interaction with NK cells is altered and this can dampen the innate immune response to the virus. One of the main causes is reduced IL-15 production by dendritic cells, which is critical to NK cell maturation and activation. Reciprocally, NK cells of patients with chronic HCV infection have a reduced ability to activate DD, due to NK cell inhibition by the CD94:NKG2A receptor, the ligand of which (HLA-E) is upregulated in HCV. 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).

HCV has a number of strategies through which it can evade NK cell activity. HCV-core can upregulate HLA-E leading to leading to CD94:NKG2A mediated

inhibition of NK cells (Nattermann et al. 2005). Earlier studies had demonstrated the ability of HCV-E2 to reduce IFN-γ production by linking to CD-81 on NK cells. In these studies high concentration of plate bound HCV- E2 was used as opposed to complete infectious particles that became available later. Subsequent studies utilising the latter challenged this theory by showing that when HCV-E2 is part of a soluble infectious particle it is unable to bind to CD-81. Taking into account the fact that NK cells are activated in chronic HCV, this hypothesis in vivo is unlikely to be of significance. (Crotta et al. 2002; Yoon et al. 2009).

Our understanding of NK cell function in the context of HCV is still emerging, but there seems to be a difference in NK cell number, activation and cytokine profile between acute and chronic HCV. The small size of the cohorts with acute HCV that have been studied does not allow us to draw safe conclusions so further work is required in order to underpin the role of NK cells in natural protection from HCV.

1.5.2 **Adaptive immunity**

The adaptive immune response to HCV consists of an antibody and CD4+ and CD8+ HCV specific T cell responses. T cell responses appear within 7-10 weeks after detection of the virus and coincide with a reduction of viral load, indicating the important role of the adaptive response in viral clearance (F. Lechner et al. 2000; Thimme et al. 2001). Humoral responses are detectable 8-12 weeks after infection and even though all the infected individuals will develop antibodies this does not correlate with viral clearance (Chen et al. 1999).

Individuals who spontaneously resolve HCV infection develop broad, high amplitude CD4+ and CD+8 HCV specific T cell responses that are sustained

over time, whereas various mechanisms of T-cell failure will lead to viral persistence (Diepolder et al. 1996; Lechmann et al. 1996; Missale et al. 1996; Cramp et al. 1999; Thimme et al. 2002).

1.5.2.1 **The role T cell responses in successful outcome of HCV infection**

T cell activation requires two signals: peptide in the context of the major histocompatibility complex (MHC) interacting with the T cell receptor (TCR), and a co-stimulatory signal (Lafferty & Cunningham 1975). CD4+ (also known as T helper cells) & CD+8 T cells recognise antigens that are presented to TCR by the MHC class II & I molecules on the surface of antigen presenting cells (APC) respectively.

Naïve CD4+ T cells, following activation, and depending on the cytokine milieu of the microenvironment, further differentiate into a variety of lineages of effector cells. T helper 1 cells (Th1) produce IFN-γ and IFN-2 and leads to activation of macrophages, promote proliferation and activation of CD4+ and CD8+ cells and foster the development of cytotoxic lymphocytes (cytotoxic T lymphocytes (CTL) & NK cells) that are responsible for the cell-mediated immune response against viruses and tumour cells. Th2 cells secrete a variety of cytokines that promote B cell activation, proliferation and antibody production and limit Th1 response (Moser & Murphy 2000). CD8+T cells, in a cytokine dependent manner, are mainly cytolytic, however they can also secrete IFN-γ and TNF-α. In HCV infection, naïve Th and CD8+ cells are primed in the lymph nodes by dendritic cells and migrate to the liver where they destroy infected cells by cytolytic and non -cytolytic mechanisms (figure 1-5).
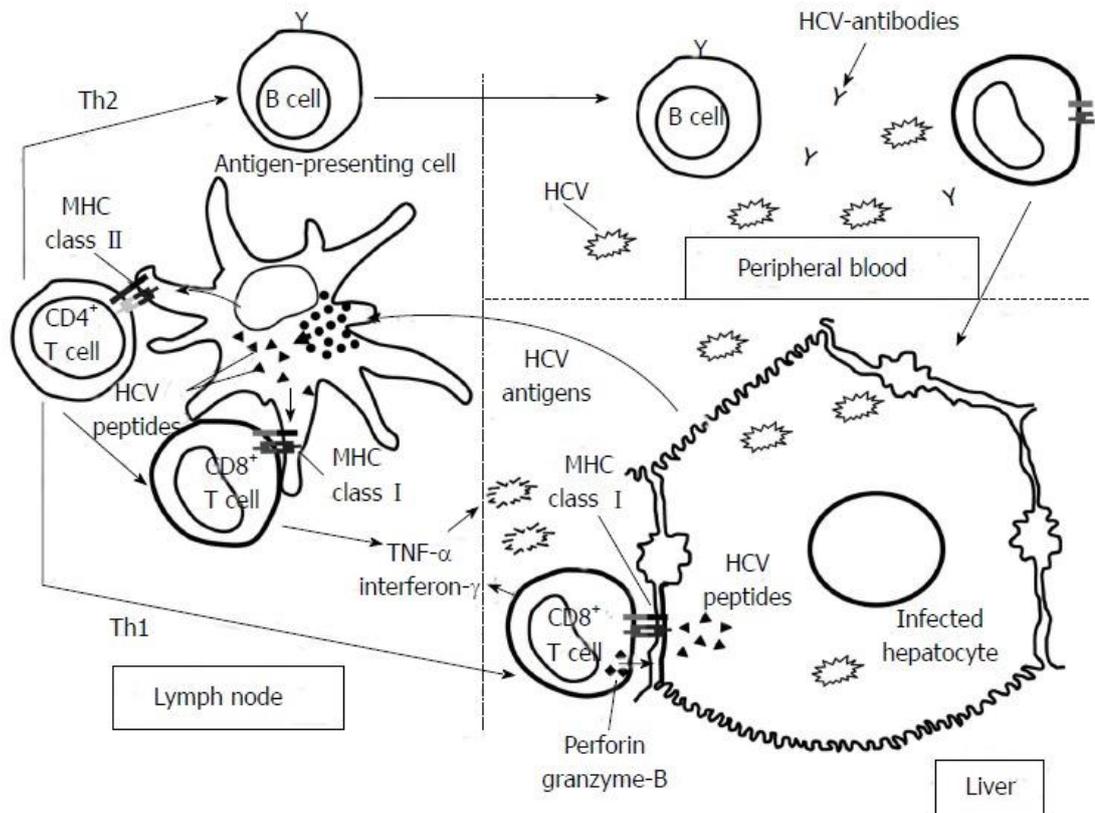
Figure 1-5 HCV specific adaptive immune response.

Naïve CD4+ and CD+8 cells are primed in the lymph nodes by antigen presenting dendritic cells following engagement of the TCR receptor with MHC class I or II. Once activated, they become effector cells and migrate to the infected liver tissue. Primed Th1 cells secrete Th1 cytokines and allow activation of specific cytotoxic T cells (CTL) therefore destroying cells in a non-cytolytic as well as a cytolytic manner (by secretion of perforin and granzyme-B). Th2 cells promote B cell proliferation and differentiation towards the production of HCV specific antigen.

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Studies in chimpanzees that had previously cleared HCV and were re challenged, showed that viral clearance was achieved within weeks and correlated with strong peripheral memory T cell responses and intrahepatic IFN-γ production (Bassett et al. 2001; Major et al. 2002).

Initial studies of acute HCV infection showed that rapid and strong CD4+ T cell responses are essential for HCV clearance (Diepolder et al. 1996; Missale et al. 1996). In order to be effective, these responses should also be targeted to multiple non structural HCV proteins, such as NS3, NS4 and NS5 and often target the same immunodominant epitopes in NS3 (Hoffmann et al. 1995; Diepolder et al. 1997). Additionally, CD4+ T cell responses need to be sustained over time, alternatively viraemia can recur and viral escape mutations in MHC class I epitopes can emerge (Gerlach et al. 1999; Grakoui 2003). Takaki et al had shown that effective CD4+ T cell responses persist for up to 2 decades after HCV clearance, whereas humoral responses decrease (Takaki et al. 2000). Subsequent studies elucidated the important role of CD+8 T cells in HCV clearance and showed that mounting vigorous and multispecific CD+8 T cell responses are important to achieve a self-limited course of the disease (Grüner et al. 2000; F Lechner et al. 2000). HCV specific CD8+ T cells have also been shown to inhibit the replication of HCV in the replicon model (Liu et al. 2003) Of importance though, is that at the onset of the disease HCV specific CD+8 cells emerge, but might not be always associated with a substantial decrease in viral load and are unable to produce IFN-γ, a property known as "stunned". Only in those whose CD+8 T cells recover and produce IFN-γ,at later stages of the disease, achieve viral clearance (F Lechner et al. 2000).

Both DC4+ and CD8+ T cell responses play a significant role in HCV clearance, but in comparison, CD4+ T cell responses are pivotal. Antibody mediated CD4+ T cell depletion in chimpanzees with previous HCV infection resulted in recurrence of viraemia despite the presence of strong intrahepatic CD+8 cell responses (Grakoui 2003). Additionally memory CD4+ T cells and to a lesser extent memory CD8+ cells are the subset of T cells that contribute to immunity (Takaki et al. 2000; Wertheimer et al. 2003).

1.5.2.2 **Failure of T cell responses to HCV infection**

Various mechanisms have been suggested to explain T cell failure to control HCV infection.

As mentioned in 1.5.2.1, several studies have demonstrated that weak, oligo specific, non virus specific and non sustained CD4+ and CD+8 T cell responses in the acute phase of infection lead to chronic viraemia. It is often difficult to differentiate between primary T cell failure and early T cell exhaustion following initial priming (Thimme et al. 2001) and the precise mechanisms are so far unclear. The data regarding impaired antigen presentation of dendritic cells are conflicting (Sarobe et al. 2002; Longman et al. 2004), but HCV has the ability to up-regulate negative co-stimulatory receptors on HCV specific CTL in order to provoke an anergic status. Up-regulation of programmed death-1 (PD-1) receptor that causes apoptosis has been suggested as a key molecule in this process and blockage of its signalling results in restoration of functional CD8+ T cell responses in chronic HCV infection (Golden-Mason et al. 2007; Radziewicz et al. 2007). On top of PD-1 there's a list of other negative co-stimulatory molecules (Golden-Mason et al. 2009; Nakamoto et al. 2009; Schlaphoff et al. 2011) (figure 1-6), blockage of which contributes to a restoration of a CD127+

phenotype in CD8+ T cells with positivity for IL-7. The latter are responsible for conferring protective CD8+ T cell immunity in chimpanzees (Grakoui 2003).

Viral escape mutations can also contribute to T cell response failure. As previously mentioned HCV RNA replicates by its RNA dependent polymerase that lacks proof reading and natural selection leads to evolution of variants resistant to cellular and humoral responses. In studies both in chimpanzees and humans it has been shown that substitution of amino acids that inhibit CD4+ and CD8+ T cell recognition is associated with the development of chronic HCV infection (Weiner et al. 1995; Chang et al. 1997; Tsai et al. 1998). However, viral escape occurs typically in the presence of a CTL response that is focused on a single viral epitope and this type of T cell response is unusual in acute HCV infection and the loss of a single epitope would probably not be sufficient for the survival of viral escape mutants (Neumann-Haefelin et al. 2005).

Regulatory T cells (Treg) are responsible for keeping a balance between the inflammatory response and viral control. In case of excessive host damage they can induce immune tolerance in viral epitopes. They express CD4+, CD25+ ,FoxP3+ and inhibit antigen presenting cell maturation and T-cell activation. In chronic HCV higher titres of Treg have been observed and in vitro depletion of CD25+ leads to enhanced responses of the HCV specific T cells (Boettler et al. 2005; Rushbrook et al. 2005) Interestingly, PD-1 mediated inhibition inhibits the expansion of Treg (Franceschini et al. 2009).

The main mechanisms through which HCV escapes T cell mediated immunity are summarised in figure 1-6.
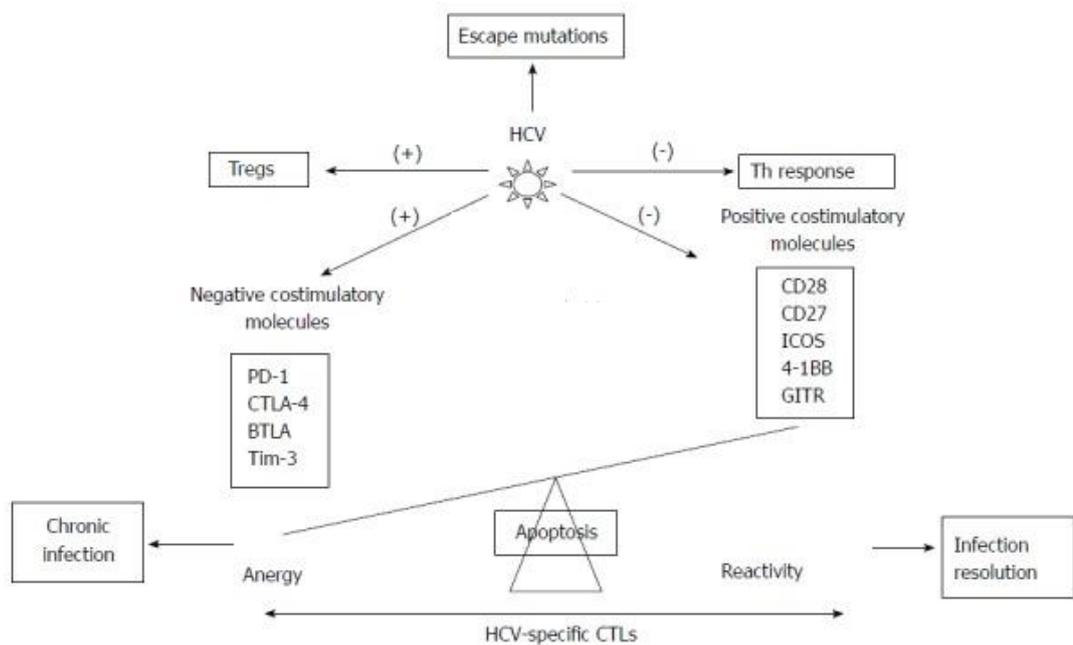
Figure 1-6 Hepatitis C virus strategies to escape from hepatitis C virus-specific cytotoxic T cells control.

Chronic HCV is mainly the result of an imbalance between negative and positive co stimulatory receptors on CTL, Treg and Th responses and the development of escape mutations. PD-1: Programmed cell death protein 1; CTLA-4: Cytotoxic T-lymphocyte antigen 4; BTLA: B- and T-lymphocyte attenuator; Tim-3: T-cell immunoglobulin domain and mucin domain 3; ICOS: Inducible T-cell Co stimulator; GITR: Glucocorticoid induced tumor necrosis factor receptor family related gene; (-) inhibition; (+) induction.

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1.5.2.3 The role of humoral immunity in the outcome of HCV infection

Earlier studies had shown that HCV specific antibodies appear between 8-12 weeks of infection, are of low titre, restricted to the IgG1 isotype and do not affect the outcome of the infection (Chen et al. 1999). Similarly, in chimpanzees who had cleared HCV and were re challenged with the same or different HCV strain, antibody responses did not seem to confer protection from re infection (Farci et al. 1992).

There is growing evidence that the early production of broad, neutralising antibodies may have a role in spontaneous clearance of infection when targeted on epitopes within the envelope glycoproteins E1 and E2 or the E1E2 heterodimer (Osburn et al. 2010; Giang et al. 2012; Osburn et al. 2014). It has also been suggested that spontaneous clearance of chronic HCV infection is associated with the appearance of neutralising antibodies and reversal of T cell exhaustion (Raghuraman et al. 2012). There is, also, limited data to suggest that individuals with apparent resistance to HCV infection can produce neutralizing anti-envelope antibodies in addition to adaptive humoral immune responses to HCV envelope proteins, 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 the hyper variable region of E2. Even though neutralisation of HCV infection by antibodies targeting E1 and E2 envelope glycoproteins is demonstrable in vitro (Pestka et al. 2007), in vivo this antibody response lags behind due to the development of emerging glycoprotein sequences and quasispecies (von Hahn et al. 2007). The virus has a variety of other strategies to evade humoral immune response the most

important of which include: (1) glycosylation shielding of epitopes targeted by neutralising antibodies (Helle et al. 2007), (2) lipid shielding of epitopes targeted by neutralising antibodies by being part of a lipoviroparticle (Nielsen et al. 2006), (3) induction of non neutralising virous specific antibodies interfering with neutralising antibodies (Zhang et al. 2007), (4) cell to cell spreading, therefore bypassing extracellular fluids and avoiding contact with the circulating antibodies.

In conclusion and to date, there is no robust evidence that HCV specific antibody responses can confer protection from the development of chronic infection or prevent re infection.

1.5.3 The role of cytokines in the outcome of HCV infection.

Cytokines are small soluble proteins that are secreted by the immune and other cells and act locally, in an autocrine or paracrine fashion, facilitating intercellular communication that will determine the nature of immune response. Although they play a significant role in viral clearance, they can also induce tissue damage (Steinke & Borish 2006).

More than 100 cytokines have been identified and can be roughly classified according to either main function or source, albeit strict classification is challenging as they frequently have multiple and overlapping actions and cells of origin.

1.5.3.1 T helper cytokines and HCV infection

HCV can prime naive CD4+ T lymphocytes to secrete either Th1 or Th2 cytokines.

Th1 mainly release IL-2, IL-12 and IFN-γ, which, as mentioned in detail in 1.4.2 leads to strong and polyclonal CD4+ and CD8+ T cells responses that are associated with HCV clearance. Release of IL-4, IL-5, IL-10, and IL-13 leads to a Th2 response that promotes B-cell activation and proliferation as well as production of HCV specific antibodies.

A Th1/Th2 skewed balance has been postulated to be contributing to the outcome of infection, including HCV, and has been studied by a few groups with conflicting data. A skewed Th1 phenotype has been shown in a few studies to be inversely associated with HCV viral load and progression to chronic infection (Cramp et al. 1999; Rosen et al. 2002). This observation has been made in both the peripheral and intrahepatic lymphocytes of patients with limited and chronic HCV infection (Sarih et al. 2000; Gramenzi et al. 2005; Bertoletti et al. 1997). Findings from other studies do not support this theory (Hempel et al. 2001; Sofian et al. 2012) and have even shown lower levels of IFN-γ in patients with chronic HCV versus (vs) normal controls (Osna et al. 1997; Abayli et al. 2003). It has also been suggested that a skewed Th1/Th2 response might only reflect the degree of inflammation and histological liver damage (Napoli et al. 1996). The discrepancy between these studies may be due to epidemiological and geographic variations such as small sample sizes, ethnic differences, comorbid conditions and composition of the study populations.

Findings regarding a skewed Th2 phenotype in chronic HCV viraemia are more consistent and have been described by a few (Osna et al. 1997; X G Fan et al. 1998; Chen et al. 2007). Furthermore, treatment with IFN-γ diminishes the Th2 cytokine response (Cacciarelli et al. 1996).

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 (Kadowaki et al. 2000; Kanto et al. 2004).

1.5.3.2 **Innate cytokines and HCV infection**

Type I interferons are the main innate cytokines released by hepatocytes early in HCV infection and the ability of HCV to evade their action has been described in 1.5.1.2.

Type III interferons (also known as IFN-λ) include IFN-λ1 (IL-29), IFN-λ2 (IL-28α) IFN-λ3 (IL-28β) and IFN-λ4 are also activated in the context of viral infections (Randall & Goodbourn 2008; Levy et al. 2011). Even though they share their antiviral effect with type I interferons, they are different in the receptor tissue expression and their activation pathway which contribute to a different effector profile. IFN-λ receptor consists of 2 subunits, the alpha-subunit IFN-λR1 and the beta-subunit IL10RB. The former is IFN-λ specific whereas the latter is shared with adaptive cytokines. Restricted expression of the IFNLR1 subunit, such as in liver tissue, leads to a tissue specific response to IFN-λ (Kotenko et al. 2003; Sommereyns et al. 2008; Miknis et al. 2010). IFN-λ initially binds to IFN-λR1, an action that induces the recruitment of IL-10RB and activation of the JAK-STAT pathway. The IFN-λ stimulated induction of ISG, is released in a slower but more sustained manner than the ones induced by type I interferons (Bolen et al. 2014). Humans chronically infected with HCV exhibit increased levels of IFN-λ expression (Dolganiuc et al. 2012). IFN-λ responses in HCV infection have been studied both in vivo and vitro and most of the observations indicate a clear correlation between IFN-λ induction and HCV attenuation (Doyle et al. 2006;

Marcello et al. 2006; Anggakusuma et al. 2015). Single nucleotide polymorphisms upstream of the IL28B gene are found in both treatment induced and spontaneous clearance of HCV (Doyle et al. 2006; Nattermann et al. 2011) and will be discussed in more detail in 1.5.5.

IL-6, a pro-inflammatory cytokine, is overexpressed and exhibits pleiotropic effects in patients with HCV infection (Malaguarnera et al. 1997). The receptor complex mediating the biological activities of IL-6 consists of the transmembrane glycoproteins gp80 and gp130. Receptors for IL-6 have been identified in many immune and non -immune cells, including hepatocytes. Upon ligation, signal transduction involves the activation of JAK/STAT and mitogen-activated protein kinases (MAPK) cascades (Kamimura et al. 2003). This results in production of IL-6 stimulated genes, the expression of which is important in apoptosis, cell differentiation, cell proliferation, cell recruitment and the acute phase response. 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 the immune response IL-6 attracts neutrophils, but subsequently switches from neutrophil to monocyte recruitment and skews their differentiation towards macrophages. Additionally, IL-6 is necessary for T cell recruitment and plays a crucial role in T and B cell differentiation and antibody production (Hirano et al. 1985; Chomarat et al. 2000; McLoughlin et al. 2005). IL-6 contributes to resistance from viruses and its dysregulation has been associated with chronic inflammatory conditions such as Crohn's disease and rheumatoid arthritis (Paludan 2001; Xia et al. 2015). IL-6 induces growth and proliferation of hepatocytes and has a protective role in liver injury (Xia et al. 2015). The levels of IL-6 have been associated with treatment outcomes of HCV (Malaguarnera et al. 1997; Ueyama et al. 2011) as well as the development of HCV related

liver fibrosis and HCC (Nakagawa et al. 2009; Giannitrapani et al. 2013).Various polymorphisms related to IL-6 gene have been reported to be associated with different outcomes of HCV infection and will be discussed in 1.5.5.

1.5.4 The role of chemokines in the outcome of HCV infection

An effective immune response to HCV infection requires, as discussed in 1.5.2, a vigorous intra hepatic activation of T cells. Due to the liver's tolerogenic environment, it has been postulated that the activation of T cells mainly occurs in secondary lymphoid tissues following activation of dendritic cells. T cells subsequently migrate into the liver in order to facilitate viral clearance under the influence of activated dendritic cells (Bowen et al. 2005).

Chemokines are chemoattractant cytokines that regulate the trafficking of leukocytes and their recruitment to sites of inflammation. They consist of 4 conserved cysteine residues that form 2 disulfide bonds, pairing the first with the third and the second with the fourth cysteines. The classification into C-X-C motif (CXC), C-C motif (CC), (X)-C motif ((X)C) and C-X3-C motif (CX3C) sub families is determined by the presence of one amino acid between the first 2 cysteines in the CXC group. According to function, chemokines can be sub divided in several other groups (Moser et al. 2004). Inflammatory chemokines facilitate recruitment of leukocytes to inflamed tissues, whereas homeostatic chemokines are constantly expressed in lymphoid organs and mediate migration of various cells, including lymphocytes. However, there's a degree of functional overlap amongst chemokines and some have dual function. Chemokines exert their effect by binding to seven-transmembrane spanning receptors and homeostatic chemokines receptors bind only one or two chemokines, whereas receptors that recruit cells to inflammatory sites often

have several ligands. A list of human chemokines that act on immune cells that

infiltrate the liver tissue (Heydtmann & Adams 2009) is shown in table 1-2.

Chemokine ligand	Chemokine receptors	Intrahepatic effector cells
CXCL9	CXCR3	NKT, CD4+, CD8+
CXCL10	CXCR3	NKT, CD4+, CD8+
CXCL11	CXCR3	NKT, CD4+, CD8+
CXCL12	CXCR4	NKT, CD4+, CD8+
CXCL16	CXCR6	NKT, NK, CD4+, CD8+
CCL2	CCR2	NK, CD4+, CD8+
CCL3	CCR1, CCR5	CD4+
CCL4	CCR2, CCR5	NK, CD4+, CD8+
CCL5	CCR1, CCR5	NKT, NK, CD4+, CD8+
CCL19	CCR7	CD8+
CCL20	CCR6	CD4+
CCL21	CCR7	CD8+
CCL22	CCR6	CD4+
CX3CL1	CX3CR1	CD4+

Table 1-2 List of chemokines that act on liver infiltrating immune cells.

Note: CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C motif) receptor; CXCL, chemokine (C-X-C motif) ligand; CXCR, chemokine (C-X-C motif) receptor; CX3CL, chemokine (C-X3-C motif) ligand; CX3CR, chemokine (C-X3-C motif) receptor; NK, natural killer; NKT, natural killer T cell.

Chemokines are excreted early in HCV infection and recruit immune cells that contain chemokine receptors such as neutrophils, monocytes, NK, NKT and DC to amplify secretion of type I interferons and ISG (figure 1-7). CXCL8, CXCL16, CXCL2 and CXCL3 are the main cytokines responsible for this effect. CXCL8 also increases the expression of the death-inducing receptor tumour necrosis factor–related apoptosis-inducing ligand R2 (TRAIL-R2) which renders hepatocytes sensitive to the effect of CTL (Dunn et al. 2007).

PDC in the liver secrete TNF-α, CCL3,CCL4, and CXCL10 and induce CCL2 secretion by other cell types that enhances leukocyte recruitment (Decalf et al. 2007). The expression of CXCR3 and its ligands, CXCL9, CXCL10 and CXCL1, is strongly associated with Th1 function. It has been demonstrated that successful HCV treatment is associated with an increase in CD8+ T cells and a reduction in CXCL9 and CXCL10. DC also increase the expression of chemokine 7 receptor, CCR7, and promote migration to lymph nodes. CCR7 is also expressed on naïve T and B cells, which, in the presence of CCL19 and CCL21 in lymphatics and lymph nodes respectively, subsequently interact with DC for immune activation (Förster et al. 2008).

CCR2 and CCR5 are characteristically found on memory T cells and CD+8 T cells expressing these receptors and are abundant in the liver (Kunkel et al. 2002)(Leroy et al. 2003). CXCL12 and CX3CL1 are expressed on inflamed bile ducts and their receptors are expressed in Th1 and NK and might help recruitment of these cells at the site of inflammation (Efsen et al. 2002; Wald et al. 2004).

Figure 1-7 The content has been removed due to copyright restrictions.

Even though the role of chemokines as potent leucocyte chemoattractant is significant, certain chemokines have been implicated in HCV evasion of the immune response. CXCL8 has been shown to inhibit IFN-α activity and suppress ISG (Polyak et al. 2001). Additionally, HCV induced secretion of CCL5 has been shown to attract immature DC cells to the liver which are unresponsive to CCR7 ligand as a result of HCV-E2 and CD81 interaction. This effect delays the activation of T cells and the establishment of an effective immune response (Jacob Nattermann et al. 2006). HCV-E2 antigen attracts CD8+ T cells that co express CCR5 and the inhibitory natural killer group 2A (NKG2A) receptor. The latter leads to their inactivation and this is another example of how HCV can evade chemokine mediated immune response (Nattermann et al. 2008).

In the context of chronic HCV infection, chemokine action will eventually lead to the accumulation of effector cells that cause liver damage without facilitating viral clearance. They contribute, through various mechanisms, to the accumulation of Th2 cytokines, that are pro fibrotic, suggesting an indirect role in fibrogenesis (Wynn 2008). However, they also have a direct role in liver fibrogenesis due to their effect on hepatic stellate cells. The latter secrete CCL2 that recruit CCR2+ macrophages and T cells that are associated with fibrogenesis (Marra et al. 1999). Hepatic stellate cells proliferate in response to chemokine effect, further contributing to liver scarring (Bonacchi et al. 2001). A few studies have also associated chemokines with the development of HCC in HCV mainly due to increased angiogenesis and migration of tumour cells (Akiba et al. 2001; Chu H1, Zhou H, Liu Y, Liu X, Hu Y 2007; Li et al. 2007).

1.5.5 The role of immunogenetics in the outcome of HCV infection

1.5.5.1 The role of major histocompatibility complex genes in the outcome of HCV infection

The major histocompatibility complex (MHC) genes regulate both innate and adaptive immunity in response to HCV infection. They are located in chromosome 6 and are amongst the most polymorphic in the human genome. The encoded human leucocyte antigens (HLA) class I and II form complexes with foreign peptides which they subsequently present to the TCR of T cells for recognition. HLA class II present HCV antigens to CD4+ T cells whereas HLA class I present HCV antigens to CD8+ T cells.

Various HLA polymorphisms have been demonstrated to influence the outcome of HCV infection and have been studied by a few groups. Taking into account limitations in study sizes and the presence of control groups the HLA alleles that are either strongly associated (OR>0.3) with HCV outcome or present in 2 or more studies (Kuniholm et al. 2010) are included in table 1-3.

HLA allele	Outcome of HCV infection
DQB1*0301	Clearance
DRB1*0101	Clearance
DRB1*0301	Persistence
DRB1*0401	Clearance
DRB1*1101	Clearance
DRB1*1501	Clearance
HLA-A*1101	Clearance
B*18	Persistence
B*27	Clearance
B*57	Clearance
Cw*0102	Clearance
Cw*04	Persistence

Table 1-3 HLA alleles and the outcome of HCV infection.

Association of HLA alleles with persistence or clearance of HCV infection.

The HLA alleles most frequently associated with spontaneous HCV clearance in the literature are DQB1*0301 and DRB1*1101 which might be positively influencing the presentation of immune-dominant HCV viral epitopes to T cells (Cramp et al. 1998; Minton et al. 1998; Yee 2004). This observation is consistent, irrespective of HCV genotype and ethnic background of study subjects (Alric et al. 2000; Yoon et al. 2005).

There is less data reporting polymorphisms of HLA class I, as opposed to class II, alleles in the outcome of HCV infection demonstrating both positive and negative associations with HCV clearance. Thio et al molecularly typed 231 individuals with well-documented clearance of HCV infection and 444 matched persistently infected individuals. HLA-A*1101, HLA-B* 57 and HLA-Cw*0102 were associated with viral clearance, whereas HLA-Cw*04 was associated with viral persistence (Thio et al. 2002). HLA-B*27 has been shown to be associated with viral clearance in females infected with genotype 1b from a single source (McKiernan et al. 2004). High resolution HLA class I and II genotyping was performed by Kuniholm et al in a large multi-racial cohort of US women with high prevalence of HCV and HIV infection. B*57 and CW*01 were associated with HCV clearance (Kuniholm et al. 2010). Interestingly, previously known associations of HLA polymorphisms such as DRB1*1101 were not observed in this study despite their high prior probability of association based on earlier reports, which may be related to host characteristics. There was no significant association between HLA alleles and persistence of HCV viraemia in this cohort, which is a finding previously reported by other groups (Verdon et al. 1994; Congia et al. 1996). However, it needs to be mentioned that despite the fact that the cohort of this study was large, it was restricted to specific ethnicities.

The role of NK cells in the outcome of HCV infection has been described in 1.5.1.4. NK cell function is regulated by a combination of regulatory receptors with inhibitory or stimulatory effect which interact with MHC-I alleles and other ligands on a variety of cells with viral infections or tumours. The net balance of signals will determine the threshold at which NK cells will be activated and contribute to HCV clearance. Khakoo et al studied polymorphisms of the KIR receptors of the NK cells, which are amongst the most polymorphic NK receptors, and their corresponding HLA in 685 individuals with chronic HCV and 352 who spontaneously resolved HCV infection. The KIR alleles examined included KIRs 2DL1, 2DL2, and 2DL3, of which the latter two are alleles of each other. These KIR alleles bind to HLA alleles, so HLA-C1 alleles bind KIR2DL2/2DL3 and the HLA-C2 alleles bind KIR2DL1. The former has the strongest and the latter the weakest inhibitory signal in NK cell activation. Homozygosity for the KIR2DL3 and HLA-C1 was associated with resolution of HCV infection and interestingly there was no association with the compound KIR2DL2/HLA-C1 genotype. This effect was, however, only shown in individuals that acquired HCV infection through small inoculums of HCV, such as high risk IDU, as opposed to individuals that acquired HCV through blood transfusion, suggesting a low threshold for HCV to evade this immune defence mechanism (Khakoo et al. 2004).

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

1.5.5.2 The role of cytokine gene polymorphisms in the outcome of HCV infection

Cytokine and chemokine gene polymorphisms can influence the level of their production and alter disease outcome. Several gene polymorphisms have been studied in association with HCV clearance, progression and response to treatment.

Il-10 is produced by macrophages, monocytes and T cells and promotes B cell activation, proliferation and antibody production and limits Th1 response. The responsible gene is in chromosome 1 and variations in the promoter region in relation to HCV outcome have been shown in a few studies. The G/G genotype (in position -1082) is known to be related to increased IL-10 production and is associated with a high risk of HCV persistence and resistance to interferon based therapy (Oleksyk et al. 2005; Paladino et al. 2006). However, this result might be gender or ethnicity specific and has not been confirmed in other studies (Constantini et al. 2002; Paladino et al. 2006).

IL-12 is important in generating Th1 responses which favour HCV clearance. Gene polymorphisms in the IL-12 promoter have been associated with different outcomes of HCV infection. The single nucleotide polymorphisms (SNP) -1188 A/A and A/C have been shown to be decreased and increased in spontaneous resolution of HCV infection respectively and the A/A genotype is more frequent in patients with chronic HCV infection (Yin et al. 2004; A. Houldsworth et al. 2005).

TNF-a gene polymorphisms have also been studied but, overall, failed to produce a robust association with any outcomes of HCV infection (Constantini et al. 2002).

Often, cytokine gene expression is a complex process and cannot be explained by a single SNP, and IL-6 gene polymorphisms are the prime example of this phenomenon. The IL-6 gene is located on chromosome 7 and more than 150 different gene polymorphisms have been identified. The presence of SNPs in the promoter region at position -174 with regards to the outcome of HCV infection has been extensively studied with controversial results. Two phenotypes have been characterised; the G/G and G/C which are associated with the high IL-6 circulating levels and the C/C which is associated with low circulating levels of IL-6. Barret et al compared various SNPs between individuals with spontaneous HCV clearance and patients with chronic HCV infection and found that the low IL-6 circulating levels phenotype was associated with SVR, whereas the presence of the G/G and G/C SNPs were associated with persistent infection which became more apparent when combined (Barrett et al. 2003). Nattermann et al studied IL-6 gene polymorphisms in a group of HCV and HIV co-infected patients compared to HCV and HIV mono- infected patients and healthy controls. He found that the high IL-6 circulating levels phenotype was associated with higher SVR rates compared to the CC genotype (Nattermann et al. 2007). Yee et al showed that SVR rates are associated with specific haplotypes, which are constructed by a combination of SNPs, but did not comment on whether there's correlation with the levels of IL-6 produced (Yee et al. 2009).

1.5.5.3 The role of chemokine gene polymorphisms in the outcome of HCV infection

The importance of CXCR3 and CXCR5 and their ligands in the pathogenesis of HCV has been described in detail in 1.5.4. The polymorphism −599del5 of the Interferon-inducible T-cell alpha chemoattractant (I-TAC) promoter, which is a Th1 chemoattractant and CXCR3 ligand, is more frequent in chronic HCV patients than normal controls (Helbig et al. 2005). Gene association studies have reported that CRCR5 or CXCL5 polymorphisms are associated with HCV chronicity but the data are controversial (Woitas et al. 2002). In a cohort of Irish women with blood transfusion associated HCV showed that a specific mutation was associated with SVR in those with specific HLA types (Goulding et al. 2005).

The immunogenetic studies with the most robust association with the outcomes of HCV are related to IL-28B polymorphisms. IL-28 (IFN-λ3) belongs to the IFN-λ family and their role in the pathogenesis of HCV has been discussed in 1.5.3.2. The gene for IL-28B is located on chromosome 19. Wide genome association studies, which enable studying of thousands of SNPs in the entire human genome with regards to a disease outcome, have shown consistent findings regarding the presence of certain polymorphisms in close proximity to the IL-28B gene and favourable outcome of HCV infection. Ge et al & Tanaka et al were the first to report such findings in patients receiving IFN-based treatment for HCV. In a large cohort of patients, at rs2979860, the CC genotype that favours HCV clearance was associated with a 2 fold increase in treatment response compared to TT genotype. The fact that the CC genotype is more common in Europeans vs Africans than the latter, explains the difference in response rates between patients of different ancestry (Ge et al. 2009). Two

further SNPs close to the IL-28B, rs129080275 and rs8099917 and on further fine mapping of the region 5 were more associated with SVR in a Japanese cohort (Tanaka et al. 2009). Subsequently, the rs2979860 polymorphisms were examined in a cohort of 388 patients who spontaneously cleared HCV infection and 620 patients with persistent infection. Strong association of the CC genotype with spontaneous clearance was shown, confirming the findings of the previous studies (Thomas et al. 2009).

1.6 HCV vaccines

To date, no prophylactic vaccine has been generated that is licenced for use in humans and this field of ongoing research.

The reasons that have made effective vaccination against HCV a huge challenge are related to a variety of factors that have been highlighted earlier in chapter 1 and will be summarised below. The biggest issue is the characteristics of the virus itself and its genetic variability. Due to the virus's error prone RNA polymerase, 7 HCV genotypes have been identified with 30-50% inter variability and over 100 subtypes. The HVR1 in E2 envelope glycoprotein offers the greater variability. Additionally, re infection with a different genotype after successful clearance is possible and the virus has multiple mechanisms through which it can evade immune response. Despite the discovery of neutralising antibodies especially targeted to confrontational epitopes on E2, humoral responses have not been shown to confer long term protection from HCV. The cardinal feature of effective immune response to HCV is rapid and polyclonal T cell production and activation but findings with regards to the factors that influence this outcome have been controversial. Additionally, the breadth and magnitude of T cell responses in patients who are protected

from HCV infection have also been shown in some patients who eventually develop chronic HCV. Even if these T cell responses are generated and are protective, they wane over time and have been shown to last up to 2 decades. Lastly there's no small animal model available for conducting HCV vaccine trials and the inclusion of chimpanzees in the studies is associated with ethical and financial constraints.

Current strategies in HCV vaccination include the use of recombinant proteins, synthetic peptides, DNA plasmids, live vectors, dendritic cells and prime-boost strategies (Garcia et al. 2014; Swadling et al. 2013).

The recombinant protein technique vaccines utilised the purified protein derived from genes encoding for HCV proteins isolated and cloned in yeasts, bacteria and mammalian cells. The benefit is that they do not contain pathogenic material and do not require cultivation of the organism. Despite some promising outcomes, quick decline of the produced antibody titres and technical challenges in the manufacture of recombinant E1E2 protein has hampered their use (Frey et al. 2010; Verstrepen et al. 2011).

Synthetic HCV peptides have been used to induce both T and B cell immunity. Due to the fact that they are HLA specific multiple epitope studies have been conducted and some showed to produce CD4+ and CD8+ HCV specific responses which were either weak or evident in only a percentage of the cases studied (Firbas et al. 2010; Huang et al. 2013).

DNA vaccines utilise the injection of recombinant plasmids that express HCV proteins and can induce a CTL response. Plasmids encoding HCV NS3/4a (ChronVac-c) have shown to be effective as therapeutic vaccines in mice and

humans, but clinical data on their effectiveness as prophylactic vaccines is yet to be reported (Ahlén et al. 2005; Alvarez-Lajonchere et al. 2009).

Viral vectors can express foreign antigens in mammalian cells, mimic the properties of a native virions, and they are non- pathogenic (Andreas Bråve et al. 2006). Alphaviruses, adenovirus, canary pox virus and paramyxovirus have been used for that purpose, to mention but a few (Lemmonier et al. 2002; Pancholi et al. 2003; Lin et al. 2008). It has been shown that viral vector vaccines can induce HCV specific T cell responses as well as neutralising antibodies (Elmowalid et al. 2007; Chmielewska et al. 2014). Adenoviruses are commonly used viral particles but a major limitation in its use is the fact that pre existing immunity can lead to its clearance before a response to the presented genes is elicited. Therapeutic trials of adenovirus based vaccines in chimpanzees induced protective T cell responses and a phase I trial in humans using an adenoviral vector to deliver NS3-NS5B proteins has produced promising results (Folgori et al. 2006; Barnes et al. 2012).

Dendritic cell vaccines studies have only been reported in mice. Their efficacy is highly dependent on antigen loading of DCs but they have been shown to be superior to DNA vaccines in mice that were immunised with NS5a protein (Yu et al. 2008). Multi epitope DC vaccines induce broad and strong T cell response in mice, but data on human trials on this type of vaccine have not yet been published.

As mentioned in 1.1., over the last few years HCV treatment has been revolutionised due to the development of direct antiviral agents that offer cure rates up to 95% in some genotypes. However, they pose a huge financial burden and are not affordable worldwide, and the issue of compliance and re

infection in high risk population, such as IDU is not addressed. Therefore, a preventative as opposed to a curative approach in HCV is still a pressing need that a vaccine would meet. The aim of a successful vaccine would be the production of lasting broad cellular and humoral immune responses with, possibly, the use of vectors that elicit an innate immune response that is key to the enhancement of adaptive immunity.

1.7 Protective immunity to HCV infection

1.7.1 Protection from HCV re infection

Initial studies in chimpanzees that developed recurrent episodes of acute hepatitis following repeated exposure to heterologous and homologous strains of the HCV virus, showed that re infection was associated with reduced periods on viraemia but there was no association with humoral immune responses (Farci et al. 1992; Prince et al. 1992). Later studies in the same models, showed that re infected animals had lower peak HCV RNA and alanine aminotransferase (ALT), IFN-γ and TNF-α (Bassett et al. 2001; Major et al. 2002). HCV clearance was associated with strong CD4+ and CD8+ T cell responses (Nascimbeni et al. 2003), a finding confirmed by CD4+ and CD8+ T cells depletion studies (Weiner et al. 1995; Grakoui 2003; Shoukry et al. 2003).

In humans, it has been suggested that individuals who have previously cleared HCV infection are less likely to develop a new infection. A cohort of 164 IDU who had no evidence of previous HCV infection and a cohort of 98 individuals who had been previously cleared HCV were compared for the incidence and persistence of HCV viraemia over a 2 year period. People previously infected, had half the chance of developing a new infection even after accounting for high risk behaviour (Mehta et al. 2002). Other studies in similar cohorts have failed to

show reduced HCV re infection rates but showed that spontaneous clearance rates were higher amongst individuals that had previously cleared HCV infection and was associated with broad T cell responses and generation of cross-reactive humoral responses (Micaleff et al. 2003; Osburn et al. 2010). Nevertheless, the previous data, albeit controversial, suggest that the immune system is able to generate immune responses to HCV virus that are at least partially protective and the constituents of the immune system that confer this protection warrant further study.

1.7.2 **Natural protection from HCV infection**

Established chronic HCV infection requires the presence of HCV antibodies and HCV RNA in the serum of the affected individuals. Spontaneous or treatment resolution of HCV infection is defined by the presence of HCV antibodies but absence of HCV RNA.

Several studies of natural HCV protection have focused on "seronegative immune" individuals, who despite being at risk of HCV infection have developed cellular immunity in the absence of established HCV infection (HCV ab and HCV RNA negative).

A study of uninfected spouses of patients chronically infected with HCV demonstrated that 20% exhibited CD4+ T cell proliferation in response to recombinant HCV proteins, particularly NS3, and this finding was consistent in subsequent testing a year later (Bronowicki et al. 1997). A similar percentage of seronegative family members of patients with chronic HCV infection were found to have low level CTL responses against multiple HCV epitopes (Scognamiglio et al. 1999). Immunological correlates of HCV clearance or resistance of the sexual partners of 52 health-care workers who developed HCV infection as a

result of a needlestick injury has been the subject of another study. They were followed up for 48 weeks following their partner's exposure and out of the 44 seronegative partners, 32% had detectable HCV specific cellular immune responses to recombinant proteins, of lower or higher magnitude, compared to the individuals in the cohort who spontaneously cleared or developed chronic HCV respectively (Kamal et al. 2004). In support of previous findings, HCV-specific CD4+ T cell responses were observed in 71% of children born from chronically HCV infected mothers. HCV specific DC4+ T cell proliferation was more frequent and vigorous in children than in their mothers and upon stimulation with HCV peptides lymphocytes from children produced lower levels of IL-10 compared to their mothers, observations that might contribute to the low level of vertical HCV transmission (Kamal et al. 2004).

As discussed in 1.1.5, incidence and prevalence of HCV is far higher in IDU compared to all other populations at risk and the duration of IDU and frequency of drug use appears to influence the risk of infection (Crofts et al. 1997; Villano et al. 1997). The highest risk of acquiring HCV is within the first 2 years of commencing IDU, with prevalence rates rising progressively to more than 90% in some reports in those who had injected for more than 10 years (Lorvick et al. 2001; Tseng et al. 2007). However, there's a small percentage of IDU who despite a long history of IDU, frequent sharing of IDU paraphernalia, and evidence of exposure to other blood borne viruses, remain HCV antibody and RNA negative (Thomas et al. 1995). Individuals with the latter characteristics have been studied by a few groups as their immune mechanisms of resistance to HCV infection would provide valuable insight into potentially protective immune responses to the virus, which is what work in this thesis intended to determine.

1.7.2.1 Natural protection from HCV infection in IDU and Exposed Uninfected (EU)

Freeman et al reported the presence of HCV specific T cell responses in 38 high risk EU recruited in Sydney, who had a median 7 year duration of injecting drug use, and of whom half reported sharing of injecting apparatus and half had serological markers of natural exposure to HBV. IFN-γ Enzyme-Linked ImmunoSpot (ELISPOT) responses were identified in 76% of EU and were associated with high risk behaviour. For 92% of the subjects, results of recombinant immunoblot assays demonstrated faint bands against non structural proteins (Freeman et al. 2004).

Mizukoshi et al studied 66 IDU, including patients with chronic HCV infection, SR and EU, who had been injecting drugs for more than 10 years in San Francisco. The 29 EU recruited in this study were, on average, 6.5 years younger , had a 15 year shorter duration of IDU and were predominantly male. IFN-γ ELISPOT responses, directed to multiple HCV proteins, were identified in 46% of the 28 EU identified in the cohort (Mizukoshi et al. 2008).

Zeremski et al studied a large cohort of IDU recruited in NewYork and selected 26 EU who were followed up for a median period of 2 years and had tested negative for HCV infection on multiple occasion during this period. Therefore individuals with episodes of brief viraemia without seroconversion or with rapid seroconversion could be identified and excluded from the study. 46% of EU had positive IFN-γ ELISPOT responses using 429 overlapping HCV peptides pooled in 21 mixes. Strong responses were noted for antigen combinations that correspond to the C-terminal of the HCV E1 glycoprotein and the N-terminal of the E2 glycoprotein as well as NS4B, NS5A and NS5B, suggesting definite

exposure and immune response to HCV. The comparison groups included SR

and healthy controls and no positive HCV specific T cell responses were

identified in the latter. The group also reported that high risk drug injecting

behaviour, such as frequency of IDU episode, sharing of drug injection

paraphernalia, assistance with injection from an individual more than 30 years

old and injection of crack positive correlated with detection of IFN-γ HCV T cell

responses in the EU studied (Zeremski et al. 2009).

 Thurairajah et.al characterised a cohort of IDU in 2005 in Plymouth, UK, with a

9.3 median duration of injecting drug use and frequent sharing of drug injection

equipment who remained HCV ab and HCV RNA negative. Recruitment of

these individuals has been on going until the current date and it is the cohort

studied in this thesis. They were termed exposed uninfected (EU) and were

recruited from a variety of sources, including a local prison and various needle

exchange and drug rehabilitation centres in Plymouth. Comparison groups

included healthy controls, patients with chronic HCV infection and SR. In the 40

EU studied, HCV specific T-cell ELISPOT responses were seen in 58% of the

cohort and they were weaker than those seen in SR making EU a distinct

group. Nonspecific IFN-γ production was eliminated by subtracting the count

obtained in negative controls and a response was deemed positive only if IFN-γ

production, was greater than the mean plus 2 standard deviations of healthy

controls. The responses included multiple epitopes and the strongest were seen

to non-structural antigens, NS3, NS4 and NS5, confirming immunological

response to a replicating virus (Thurairajah et al. 2008). Whether these

responses confer protection or represent a foot print of exposure to HCV is yet

to be determined. The same group later showed that initial T cell reactivity seen

in the EU cohort wanes over time following cessation of IDU. Since the HCV

inoculum in this mode of exposure is small, it is likely that priming of the positive responses via continuous exposure to HCV is required for their maintenance (Thurairajah et al. 2011).

The possibility of occult HCV infection, defined as infection in the absence of antibody or viremia (Castillo et al. 2004), or rapid loss of HCV antibody in the seronegative aviraemic cohorts, described above, has been considered by most of the groups. The duration and intensity of follow up with consistent lack of viraemia or seroconversion makes this hypothesis unlikely and typically HCV ab responses can last up to 2 decades following a single exposure as discussed in 1.5.2.1. As occult HCV infection requires the presence of HCV RNA positivity from liver tissue, performing liver biopsies in asymptomatic and healthy individuals is unethical and the risk involved unjustifiable. Furthermore it is debatable whether this entity actually exists, since no viral particles or viral proteins have been isolated from the cases described and there has been no evidence of transmission of HCV from patients described as having occult infection.

1.7.2.2 Natural protection from HCV; what are the mechanisms?

HIV bears a homology with HCV virus, as they are both RNA viruses with similar immuno- pathogenic characteristics and frequency of escape mutations. Studies in HIV exposed but aviraemic individuals precede those related to HCV infection. HCV specific cellular immune responses have been identified in high risk aviraemic individuals, such as partners of HIV infected patients and babies born from infected mothers (Clerici et al. 1993; Langlade-Demoyen et al. 1994). The strongest evidence comes from a study of sex workers in Nairobi, who despite having frequently unprotected intercourse and working in areas

endemic for HIV remained uninfected. Furthermore the incidence of HIV-1 seroconversion decreased with increasing duration of exposure, which suggests either intrinsic lack of susceptibility to HIV infection or acquired immunity (Fowke et al. 1996). A variety of protective mechanisms have been suggested, including polarisation to Th1 phenotype (Clerici et al. 1992; Fowke et al. 2000), strong specific CTL responses (Rowland-Jones et al. 1993; R Kaul et al. 2001), the presence of specific HLA polymorphisms (MacDonald et al. 2000) and reduced expression of chemokines essential for viral entry (Alvarez et al. 1998). In a subsequent study by Kaul et al, studying the same cohort of Kenyan sex workers, 9% of the original cohort exhibited late seroconversion and that was associated with waning of the CTL responses following interruption of sex work, and this suggests that the responses require priming with frequent antigenic exposure (Rupert Kaul et al. 2001).

Similar to HIV, HCV specific T cell responses have been demonstrated in a number of seronegative and aviraemic cohorts, such as spouses or household contacts of individuals chronically infected with HCV, individuals with occupational exposure due to needle stick injuries, as well as IDU (Bronowicki et al. 1997; Freeman et al. 2004; Kamal et al. 2004; Al-Sherbiny et al. 2005; Mizukoshi et al. 2008; Thurairajah et al. 2008; Zeremski et al. 2009). As discussed in 1.7.2 and 1.7.2.1, the latter cohort is at higher risk of HCV exposure than the previous ones and the presence of specific T cell response could merely represent a marker of exposure as opposed to protective immunity to HCV infection.

Following on from the initial findings by Thurairajah et al, further studies of immune mechanisms of HCV protection in the Plymouth EU cohort, studied in

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this thesis, revealed further findings. Hegazy et al examined IL-12B

polymorphisms in EU vs healthy controls, SR and patients with chronic HCV

infection. IL-12, as previously mentioned is a Th1 cytokine and the variant C

allele of the 1188A/C polymorphism has been associated with enhanced IL-12

production (Seegers et al. 2002) .This study demonstrated that the CC

genotype is associated with higher levels of IL-12 in EU compared to both

healthy controls and individuals with chronic HCV. Associations with IL-28B

polymorphisms, strongly effecting HCV infection outcome, have not been

demonstrated in EU, but further distinguish them from SR (Knapp et al. 2011).

In parallel to the generation of the work presented in this thesis, and in

collaboration with the centre of virus research in Glasgow, UK, we investigated

the presence of anti-HCV-envelope antibody responses in EU, compared to

healthy controls and patients with chronic HCV infection. Purified IgG from sera

was tested by enzyme-linked immunosorbent assay (ELISA) for binding to

genotype 1a and 3a envelope glycoproteins E1E2 with further testing for IgG

and IgM reactivity against soluble E2. Virus-neutralizing activity was assessed

using an HCV pseudoparticle system. EU subjects demonstrated significantly

greater IgG and IgM reactivity to envelope glycoproteins than healthy controls

with IgG from a small proportion of those individuals additionally showing

significant neutralisation. This study is the first to describe humoral

immunological responses targeting the HCV envelope, important for viral

neutralization, in EU (Swann et al. 2016). Cellular or humoral immunity are,

however, not the only immunological mechanisms of protection demonstrated in

the EU cohort. Activation of cellular and humoral immunity lag behind activation

of innate immunity by weeks, and not all EU have exhibited cellular and humoral

responses in the aforementioned studies. Knapp et al presented results from

the study of 48 EU vs 257 patients with chronic HCV infection and demonstrated that the KIR2DL3/HLA-C1 compound genotype, associated with favourable outcomes to HCV infection, was found at a greater proportions in EU compared to patients with chronic HCV infection (Knapp et al. 2010). Warshow et al studied 22 EU individuals for the expression of a range of cytokines and chemokines, and compared them to 16 patients with chronic HCV, 16 SR and 10 healthy controls. The innate IL-6 and IL-8 cytokines were significantly upregulated in EU compared to comparison groups. Additionally, higher levels of TNF-α were seen in EU and the level of adaptive cytokines was no different between the comparison groups (Warshow et al. 2012).

Association of natural protection from HCV with non- immunological host factors has also been described.Claudin-1 is a co-receptor for HCV, required for late-stage binding of the virus. A whole gene association study was conducted by Bekker et al in IDU who had injected drugs for more than 10 years, compared to SR to examine whether CLDN1 genetic variants were associated with the risk of HCV infection or with viral clearance. The EU cases were largely recruited from an IDU cohort earlier studied and previously discussed in 1.7.2.1. (Zeremski et al. 2009). The presence or absence of specific claudin-1 haplotypes were associated with natural protection from HCV, whereas there was no association with SNP haplotypes and HCV clearance. The lack of functional correlation of these SNPs with claudin-1 production is a major limitation of this study (Bekker et al. 2010).

1.8 Aims

The conclusion from what has been earlier discussed regarding the mechanism that confers natural protection from HCV in well characterised cohorts of IDU at

high risk of exposure to the virus is that it is either multifactorial, involving activation of all aspects of the immune response, or/and driven by a single, immune or non-immune, factor which has not been identified yet.

The aim of this thesis was to attempt to provide an answer the above query. As an initial step, we undertook a transcriptional profile comparison study between EU, SR and patients with chronic HCV infection. Based on the findings of this analysis, we further characterised the aims of this thesis as described in detail in chapter 3.

Chapter 2 Materials and methods

2.1 Exposed Uninfected (EU) cohort

2.1.1 Ethical approval

Recruitment of this cohort commenced in 2003 and received approval by the local research ethics committee. The recruitment was ongoing until the completion of the studies described in this thesis with regular updates and feedback to the research ethics committee on the study's progression, short and long term aims, and possible completion dates. Up until the completion of my studies we had received written confirmation of renewal of ethical approval. The participants provided with verbal and written informed consent (Appendix 2), the signed hard copies of which, are kept at the John Bull building of Peninsula College of Medicine and Dentistry in Plymouth, UK.

2.1.2 Inclusion criteria

Exposed Uninfected to HCV (EU) individuals were identified based on their apparent resistance to HCV infection, despite a high probability to frequent exposure to HCV inoculums. As described in previous studies, in order to be included in the cohort, EU should fulfil the following criteria (Thurairajah et al. 2008; Warshow et al. 2012):

1. Age over 18

2. A history of previous and current injection drug use.

3. A history of sharing of drug injecting paraphernalia.

4. Negative HCV Ab and HCV RNA status as tested by commercially available assays, described in 1.2.

Recruitment of this cohort, and in line with the experiences of my predecessors, has been one of the most challenging projects I have co- ordinated so far in my career. By definition, EU individuals exhibit a chaotic lifestyle and drug injecting behaviour, not enabling a smooth process of recruitment and follow up. The use of a structured questionnaire, detailing the current and previous drug injection habits and risk factors for others routes of HCV transmission was fundamental in this process and is attached in Appendix 3.

2.1.3 Sources of EU recruitment

From 2003 until 2007, EU were recruited mainly from the local prisons (Dartmoor prison, near Plymouth, and Channings Wood prison, near Torbay, UK), needle exchange centres and long term drug rehabilitation centres in the urban areas of Plymouth, England (Thurairajah et al. 2008). From 2009 until the end of the work in this thesis, in order to allow identification of IDU who were still currently injecting and had contemporary on-going risk behaviour, EU recruitment included needle exchanges, drop-in homeless centres and short term homeless hostels, excluding prisons, where intermittent or temporary cessation of recent drug use would make quantification of risk behaviour challenging. For similar reasons, recruitment from long term rehabilitation centres was avoided.

2.1.4 EU identification

According to data available from the Sweep 7 public health report, the South West region is among the areas of high prevalence (3.29/1000) of injecting drug use in the 15-64 population in England.

Multiple methods were utilised in order to identify subjects suitable for inclusion in the EU cohort and are listed below:

-Addiction liaisons, social workers, pharmacists and staff working in needle exchange centres, were informed regarding the purpose of the study and the inclusion criteria. Information leaflets (Appendix 4) with details regarding the study were provided to all sites, as well as posters notifying of ongoing recruitment for the study. Additionally, a flyer containing the same information was included in needle exchange packs at various needle exchange centres. Both the poster and the flyer included the team's contact details.

- The blood borne virus community specialist nurse assisted in identification of suitable study subjects. She was responsible for testing high risk groups for blood borne viruses and providing vaccination for HBV in the community. She was contacted and visited regularly in order to identify individuals eligible for recruitment.

-Frequently during the week, recruitment centres were visited with an aim to identify any tenants or visitors that where eligible to take part in the study. Potential candidates would be then briefly interviewed and provided with the study information leaflet (Appendix 4) and the team's contact details. If agreeable to be recruited, an appointment would be made to meet at the "Harbour" centre in Plymouth, UK, at a mutually convenient place and time.

-Potential candidates also contacted the team directly from information provided by either staff at the recruitment sites, or on the posters and flyers. After conducting a brief interview, information regarding the study would be provided over the phone. Subsequently, an appointment would be made to meet at the "Harbour" centre in Plymouth, UK, at a mutually convenient place and time at which stage a hard copy of the study information leaflet would be provided.

-Social workers and drug liaisons from the recruitment centres would often notify the team of individuals suitable for recruitment and provided us with their contact details having obtained verbal consent for us to contact them. The rest of the process was as described above.

-A detailed presentation of the study's previous findings and future aims, with a view to boost EU recruitment, was delivered to local general practitioners, addiction liaisons and social workers at the Guildhall manor in Plymouth, UK, in 06/2014.

In order to arrange for a recruitment appointment with EU subjects, they would have to report current injecting drug use for at least a year's duration, along with a history of sharing drug injection paraphernalia, including syringes, spoons, filters, etc., during the brief screening interview. Some of them had a known negative HCV status, but they would always be tested for HCV antibodies at the time of recruitment. The same process would also apply if follow up recruitment time points were arranged with an individual.

2.1.5 EU recruitment

The recruitment appointment was always arranged at the Harbour rehabilitation centre in Plymouth, UK, which provides with a separate area with clinical facilities. EU subjects would then be provided with a written copy of the study's information, if not already provided, and were asked to complete a written consent from that can be found in Appendix 2. They would subsequently be asked to complete a confidential questionnaire containing detailed information about the duration and frequency of IDU, frequency and type of drug equipment sharing, and other risk behaviours associated with high risk of HCV exposure. The 2009 amendment of the questionnaire, used during the period of my

recruitment, can be found in Appendix 3. Information on significant additional co morbidities, as co infection with other hepatotropic viruses or malignancy was additionally sought for and documented if of relevance.

Venesection was subsequently performed and up to 40mls of blood was obtained and stored in EDTA, sodium- heparin and serum tubes (Fischer Scientific). The blood would be subsequently processed to be used in a variety of assays as described in chapters 3-6. The EU often had very poor peripheral venous access with pedal and femoral phlebotomy sites frequently used as opposed to the standard upper limbs sites. Issues regarding drug injection sites requiring medical attention were often identified and flagged up and information on prevention of HCV transmission via IDU route was always provided, as well as advice for cessation of IDU.

A unique sequential study number, starting with the prefix "SW" was assigned to the each of the individuals recruited to ensure confidentiality. The questionnaires, consent forms and blood samples were subsequently transferred to the John Bull building of Peninsula College of Medicine and Dentistry (PCMD), Plymouth, UK, where they were safely stored in the manual and electronic databases and the laboratory facilities respectively. Testing for HCV antibodies, with commercial assays described in 1.2 was performed at the laboratories of Derriford hospital, Plymouth, UK, and only the HCV ab negative individuals were included in the EU cohort. The positive HCV ab and/or HCV RNA individuals identified with similar drug injection characteristics were included in one of the comparison groups described in 2.1.7. Testing of hepatotropic viruses other than HCV was not covered by the ethics approval of

this study and was, therefore, not routinely performed, but individuals previously tested positive for HBV or HIV viruses were not recruited.

2.1.6 **EU demographics and characteristics**

During my studies I recruited 45 EU, but the subjects included in my study are a mixture of some of the cases I recruited, in addition to previously recruited EU since 2003, whose samples were stored as described in 2.1.5. The demographics of the EU, samples of whom were included in the experiments described in this thesis, are detailed separately in chapters 3-6 under the "Results" section. In total, samples of 23 EU were used in the studies of this thesis. In summary, they were all Caucasian, predominantly male, with no additional significant co morbidities and at high risk of HCV exposure. Based on their current IDU status at the time of recruitment, the frequency of intravenous drug use and sharing of drug use equipment, the demographics of the EU included in this study were comparable to the ones of EU included in other studies of this cohort (Thurairajah et al. 2008; Warshow et al. 2012; Swann et al. 2016). In terms of non IDU routes risks of transmission, the most frequent was tattoo/body piercing, found in 61%. Although, and as previously described, the risk of HCV transmission through sexual intercourse is low, 28.5% reported having unprotected intercourse with HCV positive individuals. HCV mucous membrane transmission routes including sharing of all non intravenous drug equipment was 100%.

2.2 Comparison groups

Three comparison groups were studied in this thesis:

    (1) IDU that spontaneously cleared HCV infection, termed SR (HCV ab positive, HCV RNA negative).

(2) IDU with chronic HCV infection, termed CHCV (HCV ab and RNA

positive).

(3) Healthy individuals/normal controls, with no history of IDU, termed NC

(HCV ab and RNA negative).

The first 2 groups were recruited via the process described for the EU cohort in 2.1. The third group consisted of aged matched individuals from staff of the PCMD and the outpatients' department of Derriford hospital who provided with verbal consent for inclusion in the study. As a non IDU group completion of questionnaire, assignment of a SW number and HCV testing was not deemed necessary. Approximately 40 ml of blood was obtained from each normal control in designated clinical areas which was safely stored in the PCMD laboratory and used for the experiments detailed in chapters 4-6.

Detailed demographics of the SR, CHCV and NC, samples of whom were used in the experiments of this thesis, can be found in the "Results" sections of chapters 3-6.

2.3   PBMC isolation, freezing and thawing

2.3.1   **Reagents and materials**

Royal Park Memorial Institute (RPMI) 1640 culture medium and sterile phosphate buffered saline (PBS) were purchased from Lonza Biowhittaker (Lonza, USA).

Supplemented RPMI 1640 (sRPMI) was made by the addition of the following to 500mls of RPMI 1640:

 (1) 12.5ml of 1M HEPES buffer (Lonza).

(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 ^{0}C and was made fresh every month.

Human AB serum and foetal calf serum (FCS) were purchased from PAA labs (Yeovil, UK). These were aliquoted, stored at -20ºC and thawed prior to use. Histopaque-1077 used for PBMC isolation and dimethylsulfoxide (DMSO) were purchased from Sigma.

The cell counting solution was made up of 1:50 1M acetic acid and 1:500 0.4% Trypan blue solution (Sigma) to a volume of 250mls with distilled water. Tryptan blue stains non -viable PBMC blue under light microscopy allowing for the distinction of viable to non-viable cells.

2.3.2 PBMC isolation from whole peripheral blood

15 ml of blood contained into sodium-heparin tubes was diluted in 1:1 with sterile PBS in a 50ml Polypropylene Falcon tube (Greiner Bio One, UK) and was layered on top of 15 ml histopaque-1077 (Sigma), using a Pasteur pipette (Fisher Scientific). Care was taken to avoid disrupting the surface interface of the histopaque. The tube was then centrifuged for 30 min at 750g with no brake applied (Heraeus labofuge centrifuge 400R, UK). The resultant centrifugation sample contained 3 layers, the top of which was serum which was discarded into 5% Virkon, whilst the second layer was isolated with a Pasteur pipette in to a 30ml universal container (Greiner Bio-One). The supernatant was then washed with 10ml of sRPMI medium and centrifuged at 750g for 10 min with the

brake activated. This process was repeated twice more. The pellet following the third wash was then re suspended in 1.5 ml of supplemented FCS and counted.

2.3.3 PBMC cell viability and counting

Freshly separated PBMCs were counted using a counting solution containing 1M Acetate and 0.4% trypan blue (as described in 2.3.1). The PBMC suspension was diluted to 1:20 with counting solution (475µL counting solution 25µl of PBMC cell suspension) and dispersed into the Neaubaeur haemocytometer filing chamber with cover slip for assessment of cell counting and viability. 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. 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.

2.3.4 Freezing and storage of freshly isolated PBMC

Freezing mix, consisting of 3 parts of RPMI and 2 parts DMSO was prepared at least 10 min before use. Roughly, 10^6 cells were re suspended in 1.5ml FBS and were divided into to 2 cryovials (Starstedt, UK), 0.75 ml each, with the addition of 250µl of the freezing mix to each tube. Stored cells were kept in -80 degrees freezer for 18-24 hours and then transferred to liquid nitrogen (BOC cryospeed/CRY/00809/APUK/0205/7.5 M).

2.3.5 Thawing of frozen PBMC stored in liquid nitrogen

The cryovial from liquid nitrogen, containing frozen PBMC, was transferred at room temperature, to a 37 ^{0}C water bath until there was a small piece of ice visible.10 ml of warm 20% FCS / sRPMI (2 parts FCS: 8 parts sRPMI), was

prepared in the interim and was instilled into the cells in a 30 ml universal container. Initially, 1 ml of warm medium was added, drop by drop, whilst the mixture was shaken. Subsequently 2 ml of the warn medium were added whilst the mixture was shaken and lastly 6 ml of the warm medium were subsequently added whilst the mixture was shaken. The final mixture was left in the water bath for 20 min and was centrifuged at 750g for 10 minutes with the brake activated. The pellet was then gently re suspended in 1ml of sRPMI/10% human AB serum. A counting solution of 160μl of sRPMI to 40μl of 0.4 % Trypan blue (1:4 Trypan blue: sRPMI) was then used. 190μl of counting solution and 10μl of PBMC suspension was added to a 500μl Eppendorf tube (Eppendorf UK, Stevenage, UK) and mixed by pipetting. 10μl were loaded onto a Neubauer haemocytometer with cover slip. Cells numbers and viability were expressed as described in 2.3.3. Acceptable cell viability was set at greater than 98% and any thawed PBMC alliquots that did not meet this target were not used for the experimental assays.

2.4 *Serum isolation from whole peripheral blood and thawing of stored serum*

Serum tubes containing whole peripheral blood were centrifuged at 1000g for 10 minutes with the brake applied (Heraeus labofuge centrifuge 400R, UK). The resultant supernatant was aspirated and aliquoted in 300 to 500μl of samples in Eppendorf tubes. The serum was stored in -20^0 C.

Thawing of stored serum was performed by placement of the serum containing Eppendorf tubes in a 37^0 C water bath. Samples from previous stages of recruitment, aliquoted in larger volumes, would then be re frozen after use, but

caution was exercised in avoiding repeated freezing-thawing cycles of the same sample.

2.5 *RNA extraction, purification and conversion to complimentary DNA (cDNA)*

2.5.1 **RNA extraction from PBMC**

This technique was based on the use of the TRI Reagent® solution (ThermoFischer Scientific, UK).

Thawing of PBMC frozen aliquots of up to 7×10^6 cells was performed as per 2.3.5. Subsequently the suspended mixture was centrifuged at 750g for 10 min and the isolated pellet was then transferred on ice where most of the next steps of this experiment were performed. RNAase free Eppendorf tubes and pipette tips were used throughout this process (ThermoFischer Scientific, UK).

1ml of the TRI Reagent was subsequently added on to cell pellet and pipetted until full lysis of the pellet was achieved. The cell- reagent mix was then transferred to a new Eppendorf tube and was incubated for 5 minutes. 100µ of chloroform were added and the mixture was shaken vigorously for 15 seconds and incubated for further 5 min. Following micro centrifugation at 11.500 rpm, at 4^0 C for 15 min, the aqueous (top) phase was transferred to a new Eppendorf tube, 50-100µl at a time, avoiding the interface layer. 500µl isopropanol was subsequently added to the RNA solution, the tube was inverted several times to allow thorough mixture and the mixture was incubated for 10 min. The supernatant would then be poured off and the pellet was washed by adding 1% ethanol and centrifuging at 9.500 rpm, at 4^0 C for 15 min. The supernatant would then be poured off and the tube was inverted to allow drying of pellet at

room temperature. The pellet was then suspended in 30-100μl of RNAse free water (ThermoFischer Scientific, UK) and stored in -80^0 C.

2.5.2 **Purification of RNA extracted from PBMC with the TRI Reagent®**

The RNA samples required for the microarray study, described in chapter 3, had to be of high quality, so RNA purification, extracted as described in 2.4.1, was performed with the use of "RNeasy® MinElute® Cleanup Kit" (Qiagen, Germany). The principal of this method, as advised by the manufacturer, combines the selective binding properties of a silica based membrane with the speed of micro spin technology. Guanidine thiocyanate–containing lysis buffer (RLT) and ethanol are added to the sample to promote selective binding of RNA to the silica membrane of the RNeasy MinElute spin column. The sample is then applied to the RNeasy MinElute spin column. RNA binds to the silica membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water. All RNA molecules, longer than 200 nucleotides, are purified and the procedure leads to an enrichment for mRNA since most RNAs <200, comprise 15–20% of total RNA, are selectively excluded.

RNA purification was performed as per manufacturer's protocol listed below:

- 4 volumes of ethanol (96–100%) were added to RPE buffer concentrate.

- 10 μl β-mercaptoethanol(β-ME) were added per 1 ml RLT buffer.

- Samples were adjusted to 100μl volume with RNase-free water (provided) and then 350 of RLT buffer was added.

- 250 μl of 96–100% ethanol was added to the diluted RNA, and the sample was then mixed well.

- The sample (700 µl) was transferred to an RNeasy MinElute spin column placed in a 2 ml collection tube (supplied). Following centrifugation for 15 s at >8000 x g (>10,000 rpm), the flow through was discarded.

- The RNeasy MinElute spin column was then placed in a new 2 ml collection tube (supplied) and 500 µl RPE buffer was added to the spin column. Centrifugation at for 15 s at >8000 x g (>10,000 rpm) was then performed and the flow through was discarded.

- 500 µl of 80% ethanol was then added to the RNeasy MinElute spin column. Following centrifugation for 2 min at >8000 x g (>10,000 rpm), the follow through and the collection tubes were discarded.

- The RNeasy MinElute spin column was then placed in a new 2 ml collection tube (supplied).Following centrifugation at full speed for 5 min, the follow through and the collection tunes were discarded.

- The RNeasy MinElute spin column was then placed in a new 1.5 ml collection tube (supplied). 14 µl RNase-free water were added to the centre of the spin column membrane. Following centrifugation at full speed for 1 min RNA was eluted at a total volume of approximately 10µl and the samples were stored in -80^0C.

2.5.2.1 **RNA quality control**

RNA concentration was measured by spectrophotometry absorbance at 260nm, using NanoDrop™ 2000 (Thermo Fisher Scientific) spectophotometry with the use of 1µl of RNA following calibration with 1µl of RNAse free water. The A260/A280 ratio of RNA is an indication of its purity and the optimal ratio is within the 1.8-2.2 range. The RNA samples sent for the microarray analysis

detailed in chapter 3 all had an A260/A280 ratio of more than 2.0. The highly

concentrated and purified RNA was also confirmed, commercially, by

microfluidic analysis using the Agilent 2100 bioanalyzer.

2.5.3 cDNA isolation from purified RNA extracted from PBMC

This experiment was performed with the use of the "High Capacity RNA-to-

CDNA Kit (ThermoFisher Scientifics, UK), as per manufacturer's protocol,

briefly listed below:

0.5 μg of total RNA, counted on Nanodrop 2000, as previously described, was

used per 20μL reaction.

-The kit components were allowed to thaw on ice.

-For every reverse transcription (RT) reaction, 10.0 μl of 2x RT buffer, 20x

enzyme mix, up to 9μl of RNA sample, and RNAase free water were added to a

total volume of 20μl. The samples were briefly centrifuged and incubated at 37^0

C for 60 min in a thermal cycler (G-Storm, labtech, G51, ThermoFischer

Scientific, UK).

-The samples were stored in -20^0 C.

2.6   Quantitative reverse transcriptase polymerase chain reaction (qPCR)

2.6.1 Reverse transcriptase polymerase chain reaction (RT-PCR)

IL-27 (Hs00377366_m1), PPBP (CXCL7) (Hs00234077_m1), IL-16

(Hs00189606_m1), PI3K (Hs00192399_m1), Akt1(Hs00920503_m1) , ZAP70

(Hs00896345_m1) and ribosomal protein S17 (Hs00734303_g1) commercially

available TaqMan® gene expression assays (Life technologies, Thermo Fischer

Scientific, UK) were used for qPCR, with the latter assay serving as an

endogenous control.

For each of the genes of study, samples and endogenous control were prepared in duplicates and subsequently transferred in a 96 well sealed reaction plate (Roche, Sussex, UK). The qPCR reaction mix contained 50ng of cDNA (2µl of the samples prepared in 2.4.3), 1µl of the TaqMan® gene expression assay, 10µl of the TaqMan® Gene Expression Master Mix (Applied Biosystems), and RNAse free water to a total volume of 20µl. 2 control wells, containing TaqMan® Gene Expression Master Mix and RNAse free water to a volume of 20µl were also prepared in every plate and served as negative controls.

The reaction plate was then placed in to LightCycler ® Real-Time PCR instrument (Roche) with the following instructions:

1. Hold at 50^0 C for 2 minutes.

2. Hold at 95^0 C for 10 minutes.

3. 40 PCR cycles. Each cycle run at 95^0 C for 15 seconds and 60^0 C for 1 minute.

PCR is a cyclic DNA amplification process allowing the amplification of a target DNA sequence that is too small for further examination, and follows the steps shown in figure 2-1. Theoretically, if optimal reaction conditions exist, every cycle of PCR process doubles the amount of the desired DNA sequences available, resulting in exponential product accumulation.
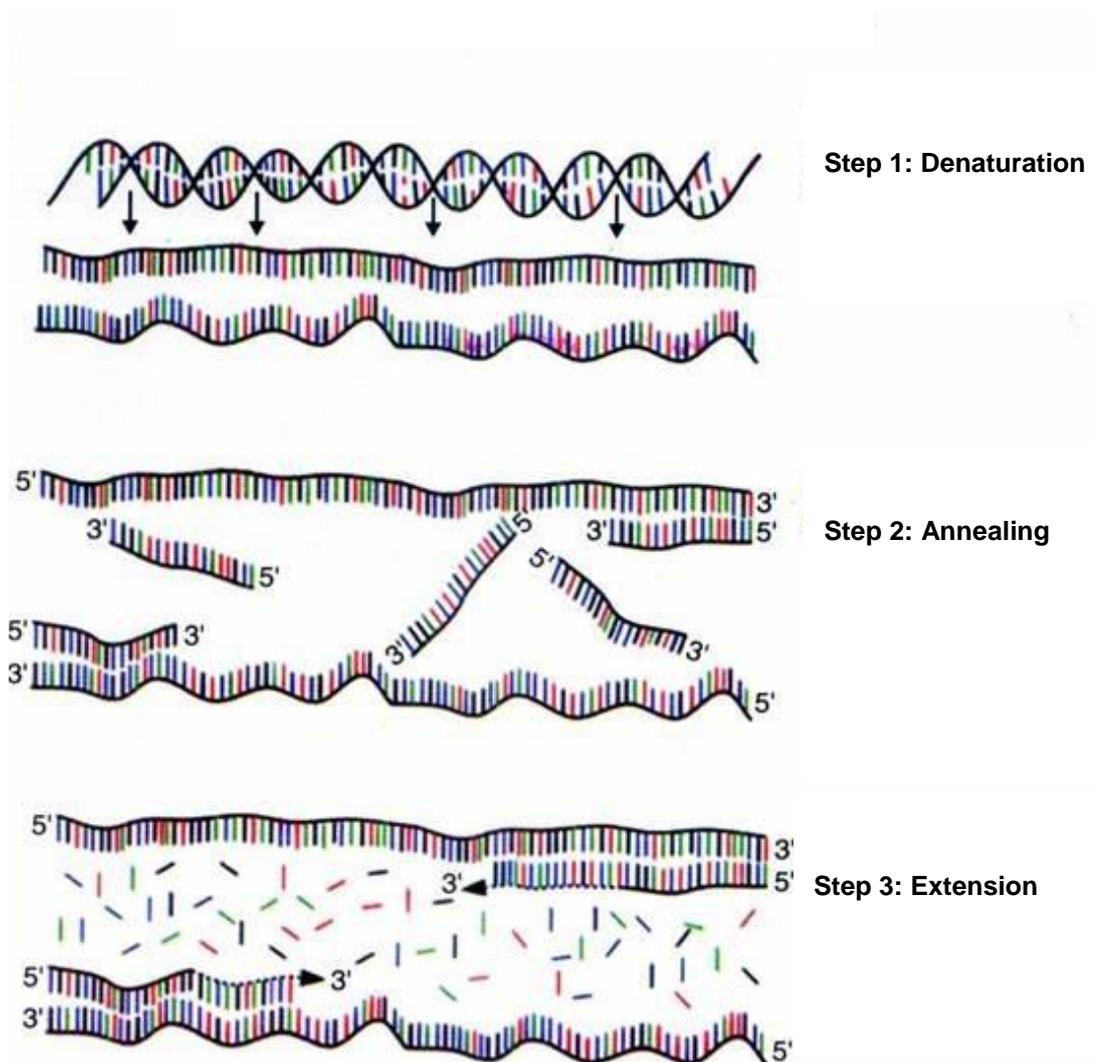
Figure 2-1 The PCR process.

During the first step DNA is separated, by heating, into 2 separate strands. PCR copies only a very specific sequence of the genetic code, targeted by the PCR primers, which are oligonucleotides that bind, or anneal, only to sequences on the complementary side (3' or 5') of the target DNA region. Two primers are used in step 2; one for each of the newly separated single DNA strands. The primers bind to the beginning of the sequence that will then be copied, marking off the sequence for step three. In the third phase, and beginning at the regions marked by the primers, nucleotides are added to the annealed primers by the DNA polymerase to create a new strand of DNA complementary to each of the single template strands. After completing the extension, two identical copies of the original DNA have been made.

Many real-time fluorescent PCR chemistries exist and one of the most widely used is the 5′ nuclease assay, conventionally referred to as TaqMan® or hydrolysis probes. The 5′ nuclease domain has the ability to degrade DNA bound to the template, downstream of DNA synthesis. A second key element in the 5′ nuclease assay is a phenomenon called fluorescence resonance energy transfer (FRET). In FRET, the emissions of a fluorescent dye can be strongly reduced by the presence of another dye, often called the quencher, in close proximity. The TaqMan® probe contains 2 components, a fluorescent reporter and a fluorescent quencher, in close proximity to each other. When the probe is intact, the quencher is close enough to the reporter to suppress fluorescent signal. During PCR, the 5′ nuclease activity of the polymerase cleaves the hydrolysis probe, separating the reporter and quencher. In the cleaved probe, the reporter is no longer quenched and can emit a fluorescence signal when excited. TaqMan® probes have higher specificity and reproducibility compared to other PCR fluorescent detection formats.

If enough molecules have been newly synthesized and detected by means of fluorescent dyes, amplification curves are visible and consist of an early background phase, the middle exponential and the late plateau phase. The PCR cycle at which the fluorescence signal first exceeds the background noise, represents the onset of the exponential phase of the curve and is termed crossing point (CP) or cycle threshold (CT). At this point approximately 10^{10} to 10^{12} amplified molecules have been produced and can be absolutely or relatively quantified.

Background noise in our study was automatically adjusted using the system's "second derivative maximum method" with no user input. The latter means that

the CT value was determined by identifying the first maximum of the second derivative of the amplification curve (Roche).

2.6.2 Relative quantification of the RT-PCR

There is a correlation between CT and nucleid acid concentration: the higher the concentration of the target nucleid acid in the starting material, the sooner a significant increase in the fluorescent signal will be observed, yielding a lower CT. Relative quantification allows the comparison of the levels of 2 different targets to a reference gene, known as endogenous control, which is found in constant copy numbers under all tested conditions. The relative amount of a target gene in an unknown sample can then be compared to another, using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). This method of relative quantification, assumes an optimal doubling of the target cDNA during each performed real-time PCR cycle. Expression differences were calculated as per the next formula:

1. ΔCT for each sample: CT sample (mean of 2 wells)-Ct endogenous control (mean of 2 wells).

2. The ΔCT mean of each group was calculated.

3. ΔΔCT of 2 groups: ΔCT mean group1-ΔCT mean group2.

4. $2^{-\Delta\Delta CT}$ expresses the difference in gene expression between two comparison groups as fold change.

The CT is determined from a log–linear plot of the PCR signal versus the cycle number. Any statistical presentation using the CT values should be avoided and leads to erroneous results according to Livac and Schmittgen. The only statistical parameter that can only be, possibly, calculated is the standard

deviation (SD) or the coefficient of variation (CV) of the 2^{-CT} values of the samples in order to examine intra sample variation.

2.7   Enzyme-linked immunosorbent assay (ELISA)

2.7.1   Principles of the assay

Sandwich ELISA assays were used for the detection and quantification of IL-27, CXCL7 and IL-16.

The sandwich ELISA quantifies antigens between two layers of antibodies (i.e. capture and detection antibody). The advantage over other forms of ELISA is higher sensitivity and the lack of need of sample purification. The principle for the assays used in this study is as follows: (1) Plate is coated with a capture antibody; (2) sample and standards of known concentration are added, and any antigen present binds to capture antibody; (3) biotinylated (containing biotin) detector antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody (streptavidin- horseradish peroxidase (HRP)) is added. Streptavidin binds to biotin and the conjugated HRP provides enzyme activity for detection using an appropriate substrate system; (5) Tetramethylbenzidine (TMB) substrate is added to detect HRP activity. (6) Stop solution(sulphuric acid) is lastly added that results in colour change from yellow to blue, the absorbance of which can be measured at 450 nm wavelength in a spectophotometer (figure 2-2)

Figure 2-2 The content has been removed due to copyright restrictions.

2.7.2 IL-27 Sandwich ELISA assay

The ab83695-IL-279 (Interleukin-27) Human ELISA Kit (Abcam, Cambridge, UK), was used for this assay, which was performed according to the manufacturer's instructions. Materials included in the kit, as well as reagents, and their preparation can be found at ab83695-IL-279 (Interleukin-27) Human ELISA Kit instruction manual, version 1, last updated 17 July 2013 (Abcam).

All materials were equilibrated to room temperature before use. Immediately prior to use serial dilution of standards were prepared. Standard 1, contained, in tube1, had an IL-27 concentration of 1000 pg/ml. For the preparation of the rest of the standards 100µl of diluent buffer was initially added to tubes 2-6. Standard 2 was prepared by adding 100 µl of Standard 1 to tube 2. Standard 3 was prepared by adding 100 µl of Standard 2 to tube 3. Standard 4 was prepared by adding 100µL of Standard 3 to tube 4. Standard 5 was prepared by adding 100µl of standard 4 to tube 5 and standard 6 was prepared by adding 100µl of standard 5 to tube 6.

100µl of serum samples, standards and negative controls, consisting of diluting buffer, were added to an IL-27 pre-coated plate in duplicates. The plate was covered and incubated for 2 hours at room temperature. The liquid was subsequently aspirated from each well using a multi-channel pipette and 300µl of washing buffer. The liquid was aspirated from each well as before, and the same washing process was repeated twice more. 50µl of biotinylated anti-IL27 were, then, added to all wells and the plate was covered and incubated for 1 hour at room temperature. Washes were subsequently performed as described in previous step. 100µl of 1x streptavidin-HRP was added to all wells and the plate was covered and incubated for 30 minutes at room temperature.100µl

TMB were subsequently added into the wells and incubated in the dark for 10-20 minutes .100µ stop reagent were lastly added to the wells and the absorbance was immediately read at 450nm wavelength (Tecan Multiplate Reader, Switzerland).

The absorbance readings for each sample were then inserted into an Excel® 2010 sheet (Microsoft Office, USA). The mean blank absorbance was subtracted from the mean absorbance of the sample duplicates. A scatter plot of the standard values was created by plotting the mean absorbance (x axis) against the protein concentration (y axis). A trendline, the best fit linear curve through the points in the graph was then created, and was accepted only if the R^2 coefficient of determination value was above 0.97. Based on the linear regression model, an equation was produced that was used for calculation of each sample's mean concentration from mean absorbance of its duplicates minus mean blank absorbance.

2.7.3 CXCL7 Sandwich ELISA assay

The ab100613-CXCL7 Human Elisa Kit (Abcam) was used for this assay, which was performed according to the manufacturer's instructions. Materials included in the kit, as well as reagents, and their preparation can be found at ab83695-CXCL7 Human ELISA Kit instruction manual, version 2, last updated 09 September 2013 (Abcam).

All materials were equilibrated to room temperature before use. Immediately prior to use serial dilution of standards were prepared is a similar manner described for the production of the standards used in the IL-27 assay and described in 2.7.2.

Serum samples were diluted with dilution buffer to 1:1000 prior to use. 100µl of serum samples, standards and negative controls, consisting of dilution buffer, were added to a CXCL7 pre-coated plate in duplicates. The plate was covered well and incubated for 2.5 hours at room temperature. Following incubation the solution was then discarded and the wells were washed by adding 300µl of wash solution using a multi-channel pipette. The liquid was completely removed and the plate was then inverted and blotted against clean towels. This process was repeated another 3 times. 100µl of biotinylated CXCL7 were subsequently added to the wells and the plate was incubated for 1 hour at room temperature with gentle shaking. Following incubation the solution was discarded and washes were repeated as described above. 100 µl of 1X HRP-Streptavidin solution were added to each well and the plate was incubated for 45 minutes with gentle shaking. Following incubation the solution was discarded and the washes were repeated as per previous steps. 100µl of TBC substrate were added to each well and the plate was incubated for 30 minutes at room temperature in the dark with gentle shaking. 50µl of stop solution were lastly added to the wells was lastly added to the wells and the absorbance was immediately read at 450nm wavelength.

Calculation of the mean absorbance of the samples was performed as described for the IL-27 sandwich ELISA assay in 2.7.2 taking into account a serum dilution factor of 1000.

2.7.4 IL-16 Sandwich ELISA assay

The ab100555-IL-16 (Interleukin-16) Human Elisa Kit (Abcam) was used for this assay, which was performed according to the manufacturer's instructions. Materials included in the kit, as well as reagents, and their preparation can be

found at ab100555- IL-16 (Interleukin-16) Human Elisa Kit (Abcam) instruction manual, version 2, last updated 09 September 2013 (Abcam).

All materials were equilibrated to room temperature before use. Immediately prior to use serial dilution of standards were prepared is a similar manner described for the production of the standards used in the IL-27 and CXCL assays and described in 2.7.2.and 2.7.3.

100μl of serum samples, standards and negative controls, consisting of dilution buffer, were added to a IL-16 pre-coated plate in duplicates. The plate was covered well and incubated for 2.5 hours at room temperature. Following incubation the solution was then discarded and the wells were washed by adding 300μl of wash solution using a multi-channel pipette. The liquid was completely removed and the plate was then inverted and blotted against clean towels. This process was repeated another 3 times. 100μl of biotinylated IL-16 were subsequently added to the wells and the plate was incubated for 1 hour at room temperature with gentle shaking. Following incubation the solution was discarded and washes were repeated as described above. 100 μL of 1X HRP-Streptavidin solution were added to each well and the plate was incubated for 45 minutes with gentle shaking. Following incubation the solution was discarded and the washes were repeated as per previous steps. 100μl of TBC substrate were added to each well and the plate was incubated for 30 minutes at room temperature in the dark with gentle shaking. 50μl of stop solution were lastly added to the wells was lastly added to the wells and the absorbance was immediately read at 450nm wavelength.

Calculation of the mean absorbance of the samples was performed as described for the IL-27 sandwich ELISA assay in 2.7.2.

2.8   Western blotting

2.8.1   **Western blotting principles and workflow**

Western blotting, also known as immunoblotting, is a well-established and widely used technique for the detection and quantification of proteins. Western blotting protocols may vary from application to application; however they all follow some basic steps, also used in this thesis, and are listed below:

-Sample preparation.

-Gel electrophoresis.

-Transfer.

-Antibody probing.

-Detection.

-Imaging.

-Analysis.

The sample of interest must usually undergo some degree of preliminary treatment before continuing to separation by electrophoresis. Gel electrophoresis is applied to the sample for protein separation and the proteins are then immobilized on a membrane following electro transfer from the gel. Areas on the membrane where no binding of protein has occurred are blocked to prevent nonspecific binding of primary antibodies in the next step. The membrane is incubated with a primary antibody that specifically binds to the protein of interest. Unbound antibodies are removed by washing and a secondary antibody conjugated to an enzyme is used for detection. The

detected signal from the protein:antibody:antibody complex is proportional to the amount of protein on the membrane.

The most commonly used method for detection, also used in this study, is chemiluminescence, based on secondary antibodies conjugated with horseradish peroxidase enzyme. On the addition of a peroxide-based reagent, the enzyme catalyses the oxidation of luminol resulting in the emission of light. The light signal can be captured by exposure to X-ray film.

2.8.2 Sample preparation

2.8.2.1 Preparation of reagents and materials

-RadioImmunoPrecipitation (RIPA) buffer consisted of:

50 mM tris (hydroxymethyl) aminomethane (Tris) HCl pH 8.0, 150 mM NaCl 1% NP-40 (Nonidet-P40) 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8.0 (Sigma-Aldrich).

-1% Protease/Phosphatase inhibitor cocktail tablet –Halt ™(Pierce, Thermo Fischer Scientific).

2.8.2.2 Cell lysis and protein extraction

Detergent lysis is the most frequent method of choice for the lysis of mammalian cells. RIPA buffer contains the ionic detergent sodium deoxycholate as an active constituent and is particularly used for nuclear membrane disruption.

PBMCs were thawed and counted as per 2.3.5 and subsequently centrifuged at 750g for 10 minutes. For every cell pellet derived of 10^6 suspended cells, 500ul cold RIPA buffer was used for lysis with mix inhibitor cocktail added in straight prior to use. The lysate was incubated on ice for 5-15 minutes with periodical

pipetting and ultra -sonicated for 30 seconds at 25 kHerz. (Vibra-Cell™, Sonics). The lysate was centrifuged at 13.500g for 10 min to pellet cell debris and the supernartant was transferred in different Eppendorf tubes and stored at -20^0 C.

2.8.2.3 Protein quantification

Pierce® BCA Protein Assay Kit − Reducing Agent Compatible (Pierce, Thermo Scientific) was used for quantification of the extracted proteins. Standards, samples and blanks were prepared according to the manufacturer's instructions. The protein standards used (A-I) were prepared by serial dilutions of bovine serum albumin (BSA) at an initial concentration of 2000 µg/ml. The concentrations and dilution volumes can be found in table 2-1..

Standard name	Volume of Diluent (µl)	Volume (µl) and source of BSA	Concentration (µg/ml)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

Table 2-1 Preparation of diluted albumin (BSA) standards and their final concentration.

The standards were prepared from stock solutions and subsequently serial dilutions to the concentrations shown above.

10µl of the standards and unknown concentration samples in duplicates were loaded in a 96 well microplate (Pierce, Thermo Scientific) and 200µL of the working reagent were subsequently added to each well. The plate was thoroughly mixed on a plate shaker for 30 seconds and then incubated for 30 min at 37^0C. After incubation the plate was cooled to room temperature and was read at 562nm wavelength (Tecan Multiplate Reader). The absorbance readings for each sample were then inserted into an Excel® 2010 sheet (Microsoft Office, USA). The mean blank absorbance was subtracted from the mean absorbance of the sample duplicates. A scatter plot of the standard values was created by plotting the mean absorbance (x axis) against the protein concentration (y axis). A trendline, the best fit linear curve through the points in the graph was then created, and was accepted only if the R^2 coefficient of determination value was above 0.97. Based on the linear regression model, an equation was produced that was used for calculation of each sample's mean concentration from the mean absorbance of its duplicates minus mean blank absorbance.

2.8.3 Gel electrophoresis

2.8.3.1 Preparation of reagents and materials

-1.5 M Tris-HCL buffer contained: 12.11g Tris base, 80ml deionized H2O (diH2O) adjusted to pH 8.8 with HCL.

-0.5M Tris-HCL buffer contained: 6.06g Tris base adjusted to Ph 6.8 with HCL

-10XSDS running buffer contained: 30.30 g Tris base, 144.10 g glycine 10.00 g SDS and diH20 to 1 litre (Thermo Fischer Scientific).

Components	Volume for 10 ml resolving solution (2 mini gels)		
	For 8% gel	For 10% gel	For 12% gel
diH2O	4.73 ml	4.13 ml	3.43 ml
30% acrylamide/bisacrylamide	2.7 ml	3.3 ml	4.0 ml
1.5 M Tris-HCL, pH 8.8 with 0.4% SDS	2.5 ml	2.5 ml	2.5 ml
10% APS	60µl	60µl	60µl
Tetramethylethylenediamine (TEMED) (Thermo Fisher Scientific)	13µl	13µl	13µl

Table 2-2 Resolving gel preparation.

The components of the resolving gel are shown above.

Components	Volume: 5ml stacking gel solution (for 2 mini gels)
diH2O	3.0 ml
30% acrylamide/bisacrylamide	700µl
0.5 M Tris-HCL, Ph 6.8 with 0.45 SDS	1.25 ml
10% APS	25µl
TEMED	20µl

Table 2-3 Stacking gel preparation.

The components of the stacking gel are shown above.

2.8.3.2 **Method principal and protocol**

Electrophoresis is a separation technique based on the mobility of charged molecules in an electric field. It is used mainly for the analysis and purification of proteins and nucleic acids. Electrophoresis is carried out by loading a sample containing the protein of interest into a well in a porous matrix to which certain voltage is then applied. Differently sized, shaped, and charged molecules in the sample move through the matrix at different velocities. At the end of the separation, the molecules are detected as bands at different positions in the matrix. The matrix used in this study is gels made of polyacrylamide. The pore sizes in these gels are similar to the molecular radius of many proteins. As molecules are forced through the gel in an electric field, larger molecules are retarded by the gel more than smaller molecules. A typical gel consists of two sections of different densities, cast between two glass plates. The first section to be cast is known as the resolving or separating gel and the second as the stacking gel. The stacking gel facilitates rapid concentration of proteins in to a thin layered zone and they can be subsequently separated according to size in the resolving gel. The density (pore size) of the latter is an important factor affecting the separation profile of proteins and when separation of proteins of a wide molecular weight is required, higher density gels should be used. For the studies in this thesis, a 10% acrylamide gel concentration was used, targeting proteins with molecular weight between 14 to 205 kDa.

The glass casting stand and glass frame were assembled as per manufacturer's instructions (Mini-PROTEAN ® Tetra Cell, BIO-RAD, Watford, UK) and the resolving solution as described in table 2-2. The solution was then poured between the glass plates with a pipette leaving about ¼ of the space free for the stacking gel. The top of the resolving gel was covered with diH2O and the gel

was left for approximately 30 min to polymerize. When polymerization was complete a clear line appeared between the gel surface and the solution on top. The water was then discarded and the stacking gel (prepared as per table 2-3) inserted and the gel was then left to polymerize for approximately 60 minutes.

Two gel casts at a time were placed and secured in the Mini-PROTEAN ® Tetra Cell electrophoresis module (BIO-RAD) and were covered with 700ml of 10XSDS running buffer. The samples, prepared as described in 2.8.2.2 were thawed on ice and 20µl of each sample were transferred into an Eppendorf tube. 10µl of Laemmli buffer (Sigma-Aldrich), that breaks disulphide bonds, assists in protein separation according to size and serves as a dye front that runs ahead of the proteins, were subsequently added to each tube and the samples were heated in 70^0 C for 5 minutes in a heating plate (Dri-Block ® DB-2D, Techne). They were, then, micro centrifuged at 14000 rpm for 1 min. 10µl of rainbow molecular weight marker (Precision Plus Protein™ Dual Color Standards, BIO-RAD) and samples were subsequently loaded in to the wells and electrophoresis was performed at 200 Volt for 35 minutes.

2.8.4 Transfer

2.8.4.1 Preparation of reagents and materials

- 10x Transfer buffer contained: 30.3 g Tris base (Sigma-Aldrich), 144.1 g lysine and diH2O to 1 litre. Stored at 4^0 C.

2.8.5 Method principal and protocol

On completion of the separation of proteins by polyacrylamide gel electrophoresis, the next step is to transfer the proteins from the gel to a solid support membrane, usually made of either nitrocellulose (Amersham Protran Premium Sandwich 0.45µm nitrocellulose, GE health care life sciences, UK) or

or polyvinylidene fluoride (PVDF) (Hybond®-P polyvinylidene difluoride membranes, Sigma-Aldrich), both of which were used in the studies of this thesis. The main advantage of nitrocellulose membranes is the low background, whereas PVDF membranes have higher protein binding capacity and mechanical strength. PVDF membranes are highly hydrophobic and were pre wetted in 100% methanol for 1 minute before use to be compatible with aqueous solutions.

The proteins transferred from the gels were immobilized at their relative migration positions at the time point when the electric current of the gel run was stopped. The gel, membrane, blotting paper (Thick Blot Filter Paper, Precut, 7.5 x 10 cm, BIO-RAD) and electrodes were assembled in a "sandwich" so that proteins move from the negative charged anode, (where the gel is placed) to the positively charged cathode (where the membrane is placed). Essentially, this process results in proteins moving from gel, where they were initially captured, to the membrane in a pattern that perfectly mirrors their migration position in the gel. There are various modes of transfer, but the wet, used in the studies of this thesis, is ideal for large proteins and is important to obtain blots of the highest quality in terms of distinct, sharp bands. It requires full immersion of gel and membrane in cool transfer buffer and a constant current applied in the direction of the gel to the membrane.

Following completion of gel electrophoresis, the gel was removed from the casts and placed in to cool 1x transfer buffer where it was allowed to rest for 5-10 minutes. Subsequently, the gel/membrane/electrode "sandwich" was prepared and placed in the Mini Trans-Blot® Electrophoretic Transfer Cell apparatus (BioRad, Watford, UK), fully covered by cool 1x transfer buffer, along

with an addition of an icepack, with constant 350 m Ampere running conditions

for 1 hour (figure 2-3).

Figure 2-3 The content has been removed due to copyright restrictions.

2.8.6 **Antibody probing**

2.8.6.1 **Preparation of reagents and materials**

-Tris buffered saline (TBS) 10X contained: 24.23 g Trizma HCL, 80.06 g NACL, mixed in 800 ml diH2O. pH adjusted to 7.6 with pure HCL and topped up to 1 litre with diH2O.

-TBS-T contained: 100ml of TBS 10X +900ml diH2O + 1ml Tween®20 (Sigma-Aldrich).

-Semi skimmed milk (Tesco, UK) /Tween-20 contained: 5% dried milk in PBS and 0.1% Tween-20.

2.8.6.2 **Method principal and protocol**

Western blotting involves the immobilization of biomolecules on a membrane via hydrophobic interactions. As nonspecific binding of antibodies to the membrane can occur, it is important to "block" spaces not already occupied by proteins. The blocking agent used should have greater affinity for the membrane than the antibodies used and should fill unoccupied spaces on the membrane without interrupting binding of the proteins to the membrane.

Following the blocking step, the protein of interest can be detected using antibodies. The primary antibodies can be labelled or unlabelled, monoclonal or polyclonal, and the secondary antibody is usually alkaline phosphatase or horseradish peroxidase (HRP) labelled to allow detection of the protein:antibody:antibody complex. Optimisation of the incubation duration and temperature is important for sensitivity improvement with no increase in background. Sometimes it is necessary to detect more one than proteins on the same membrane, so stripping and re-probing might be required.

The blocking agents used in this thesis were dry semi skimmed milk (Tesco everyday value semi skimmed milk) and bovine serum albumin (BSA) (Sigma-Aldrich) when probing with antibodies to phosphorylated proteins.

The list of primary antibodies used and their basic characteristics are listed in table 2-4. b-aktin served as the endogenous (loading) control.

The secondary antibody was polyclonal anti-rabbit IgG (whole molecule)–Peroxidase antibody produced in goat (Sigma-Aldrich).

The membrane (prepared fresh on every Western Blot performed in the studies of this thesis, with no stripping or re-probing) was placed in a tray containing 1x TBS-T and 5% semi skimmed milk or 3% BSA and was incubated for either one hour at room temperature or overnight at 4^0C with gentle shaking. For the experiments including phosphorylated proteins the membranes were incubated in BSA and overnight at 4^0C with gentle shaking.

The membrane was briefly rinsed with 2 changes of TBS-T and was subsequently placed in a tray covered in TBS-T and various dilutions of primary antibodies. The membrane was then incubated either for 1 hour in room temperature or overnight at 4^0C with gentle shaking. Following incubation the membrane was washed 6 times in TBS-T for 5 minutes each, at room temperature and with gentle shaking. The membrane was then incubated in TBS-T with 1:20000 secondary antibody for 1 hour at room temperature with gentle shaking. Following incubation, washes were performed as per previous step.

Antibody	Molecular weight (kDa)	Polyclonal/ monoclonal	Cross- reactivity	Dilution
PI3K p110δ	110	Polyclonal	No	1:500
Pan Akt	60	Monoclonal	No	1:1000
Phospho-Akt(Ser 473)	60	Polyclonal	Yes	1:1000
Phospho-Akt (Thr 308)	60	Polyclonal	No	1:1000
b-aktin	40	Polyclonal	Yes	1:2000

Table 2-4 Primary antibodies used in Western blotting and their characteristics.

PI3K p110δ antibody was purchased from Santa Cruz biotechnology, USA; Pan-Akt, Phospho-Akt (Ser473) and Phospho-Akt (Thr308) antibodies were purchased from Cell Signaling Technology, USA; b-aktin antibody was purchased from Thermo Fisher Scientific, UK.

2.8.7 Detection and imaging

The most commonly used enzymatic detection system is chemiluminescence, also used in the studies of this thesis. The HRP-conjugated secondary antibody binds to the primary antibody, specifically bound to the target protein on the membrane. After the addition of a luminol peroxide detection reagent, the HRP enzyme catalyzes the oxidation of luminol in a multistep reaction. The reaction is accompanied by the emission of low intensity light at 428 nm but in the presence of certain chemicals, it is enhanced up to 1000-fold, making it easier to detect, and thus increasing the sensitivity of the reaction in a process known as enhanced chemiluminescence (ECL). One of the most effective enhancers is p-iodophenol; it increases HRP turnover rate and assists in the transfer of electrons from luminol to the enzyme. The intensity of signal is a result of the number of reacting enzyme molecules and is, therefore, proportional to the amount of antibody and protein on the blot. The light can be then captured either on an X-ray film or a charge-coupled device camera. X-ray films provide high sensitivity and flexible exposure times, needed for detection of very weak signals, but high-intensity signals tend to saturate making protein quantification challenging.

Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) was used for signal detection in the studies of this thesis. The solutions included in this assay were allowed to equilibrate in room temperature for 20 minutes. Luminol and peroxide were mixed in 1:1 working solution and a volume of 1ml/cm^2 membrane of detection reagent was prepared and used immediately. The membrane was drained from excess wash buffer and it was placed protein side up on a plastic wrap. The detection reagent was added onto the membrane until it was completely covered. The membrane was then

incubated for 5 minutes at room temperature. Following drainage of the excess detection reagent the membrane was placed in a fresh plastic wrap and in an autoradiography cassette (GE Healthcare Amersham™ Hypercassette™ Autoradiography Cassette, Fisher Scientific). X-ray film (GE Healthcare Amersham™ Hyperfilm™ ECL, Fisher Scientific0 was placed on top of the membrane, the cassette was closed and exposure time ranged from seconds to 30 minutes depending on the signal intensity. The film was then developed in an X-ray automatic processor (Xograph compact, UK).

2.8.8 Analysis

Detection of signals, in any mode including X-ray films, results in one or more visible protein bands on the membrane image. The molecular weight of the protein can be estimated by comparison with marker proteins and the amount of protein can be determined as this is related to band intensity. Quantification can be performed in a relative or absolute manner. Various factors affect this analysis and are briefly listed below:

-Sensitivity is the minimum amount of protein that can be detected using available detection systems. It can be affected by many factors, such as antibody quality, antibody concentration, and exposure times.

- The linear dynamic range is that over which signal intensity is proportional to the protein quantity on a blot, therefore allowing precise quantitation throughout that range. Excessive amounts of protein or high concentrations of antibodies, but also prolonged exposure times, can lead to saturated signals that are no longer proportional to protein concentration and can skew results.

-Signal stability. It is advantageous to use a detection system with high signal stability, as this will impact on the linear dynamic range. With a stable signal

detection reagent, the time window for reaching high sensitivity is longer. This allows multiple exposures and the possibility to detect weak bands that might be missed in a brief, single exposure.

-In-lane normalisation. The levels of the protein of interest should be normalized to an internal reference to allow corrections of loading errors or different protein concentrations in the samples used. The "endogenous control" protein is one that is expressed at a relatively constant rate and is required for the maintenance of basic cellular functions.

- The presence of a protein on a blot or gel gives rise to a signal of a certain intensity that is recorded and analysed. To properly quantitate the protein, however, it is essential to consider the specific signal, as well as background due to cross-reactivity or system-generated signals. A signal peak corresponds to a protein band and the volume under that peak, but above the background level, is directly proportional to the quantity of protein.

Various analysis softwares have been developed and can be used to absolutely or relatively quantify protein levels obtained from Western Blotting, taking into account the above factors. For the studies of this thesis Image J (1.50b; Java 1.8.0_60 (64 bit), which is an open source Java image processing program, has been used for protein quantification. The analysis process is briefly outlined below: following scanning of the films and loading onto the software, brightness and contrast were adjusted to allow accurate viewing of the bands. A rectangle was then drawn around a lane and subsequently the lanes were plotted and the protein concentrations re- presented as peak of a curve. Background noise was eliminated by drawing a line at the base of the peak. Once all peaks had been highlighted then the signal was analysed and the software provided with a

density value for each. The density of the signal was then expressed as a percentage of the total size of all of the highlighted peaks. The adjusted density for every peak, was calculated as the ratio of the percentage of the peak to the mean peak percentage. This process was repeated for both samples and corresponding loading controls. The adjusted relative density was calculated as the ratio of the relative densities of the target protein and their corresponding loading controls. The result represents expression fold change of the target protein of a sample compared to the mean expression level of the protein of all the samples tested. A calculation example is shown in table 2-5. The mean or median of the adjusted relative densities of the individuals in the groups tested were subsequently compared and analyzed.

Density	Percentage	Relative density	Adjusted relative density
Raw data	Sample density/total density of all samples	Percentage of sample/mean sample percentage	Relative density of sample/relative density of corresponding loading control
Density Sample1	Density sample 1/density sum of samples 1+2+3	Percentage 1/mean percentage of samples 1+2+3	Relative density 1/relative density of loading control 1
Density Sample 2	Density sample 2/density sum of samples 1+2+3	Percentage 2/mean percentage of samples 1+2+3	Relative density 2/relative density of loading control 2
Density Sample 3	Density sample 3/density sum of samples 1+2+3	Percentage 3/mean percentage of samples 1+2+3	Relative density 3/relative density of loading control 3

Table 2-5 Example of adjusted relative density calculation for a given sample.

The adjusted relative density, calculated as above, expresses a fold change of a sample's protein expression to the mean expression level of the protein in all the samples tested, normalised for loading errors.

2.9 Microarray data analysis

This method is described in detail in 3.2.

2.10 Statistical analysis

GraphPad Prism version 5 (GraphPad Software, California, USA) and Excel®
2010 (Microsoft Office, USA) were used for data analysis.

For continuous variables from more than 2 groups were compared using the
non-parametric Kruskall- Wallis test. Mann-Whitney U test was used for
comparison of 2 groups of continuous variables where appropriate. Coefficient
of determination (R^2) was used to denote the linear association between 2
variables with a cut off value of >0.97 for inclusion in the study.

Statistical significance was defined by p value <0.05.

Chapter 3 A comparative gene expression study between individuals with apparent resistance, spontaneous clearance, or chronic infection from HCV

3.1 Background

Incidence and prevalence rates of HCV can be as high as 90% amongst IDU populations (Lorvick et al. 2001). IDU cohorts at high risk of HCV exposure who remain HCV uninfected by conventional testing (HCV RNA and HCV ab negative) are well documented and are described in detail in 1.7.2.1 (Mizukoshi et al. 2008; Thurairajah et al. 2008; Zeremski et al. 2009). These individuals share certain demographic characteristics, as they regularly inject illicit drugs for long periods and share drug injecting paraphernalia, and are within the highest risk group for HCV exposure. Individuals within this very high risk group, who show no serological evidence of past or current infection, might have different mechanisms of viral resistance to individuals who have spontaneously cleared the virus or have become chronically infected and this is what the studies of this thesis were set out to determine.

As part of the earlier studies of the cohort in this thesis, Thurairajah et al, characterized a cohort of IDU in the urban areas of Plymouth, UK, who despite a median injection history of 9.3 years and significant sharing of drug injecting equipment remained negative for HCV Ab and HCV RNA when tested by commercial assays and they were termed exposed uninfected (EU). In these 40 EU, T-cell ELISPOT positive responses were seen in 58% (Thurairajah et al. 2008), which weaned off with cessation of IDU and were weaker than those

seen in SR, suggesting that they serve more as an immunological marker of exposure to HCV (Thurairajah et al. 2011). This initial cohort was followed up and maintained by continuous recruitment. Further studies on the immunological characteristics of the Plymouth EU have been discussed in 1.7.2.2. To summarise, early findings showed that they lack the IL28B polymorphism that favours HCV clearance (Knapp et al. 2011) but are homozygous for the 1188 C/C polymorphism of IL-12B that leads to enhanced production of IL-12 (Hegazy et al. 2008).However, subsequent work favours activation of the innate, as opposed to adaptive immune system, as the most likely mechanism of natural protection from HCV. Cytokine profiling demonstrated raised levels of the pro-inflammatory innate immune cytokines and chemokines IL-6 and IL-8 in the EU cohort (Warshow et al. 2012), who also overexpresses the KIR2DL3 polymorphism in combination with group 1 HLA-C allotypes suggestive of enhanced NK cell activity (Knapp et al. 2010) . Indicative of the diversity and complexity of mechanisms that appear to confer apparent resistance in EU is a recent finding that suggests the presence of humoral responses against envelope glycoproteins with neutralisation properties (Swann et al. 2016).

As evident by the summary of EU immunological characteristics discussed above, the exact and complete immunological mechanism of natural protection from HCV in the EU Plymouth cohort (recruitment of which is described in detail in 2.1) remains unclear and the aim of this study was to try and elucidate this, by comparing their transcriptional profile to individuals who have spontaneously cleared HCV (SR) or are chronically infected with HCV (CHCV).

3.2   Methods

Agilent GE1_107_Sep09 single-channel microarray was performed commercially at "Universitätsklinikum" in Regensburg, Germany, and the raw data were extracted in text files (Microsoft) using "Agilent Feature Extraction 10.7.3.1" (Agilent technologies). Quantile normalization and further statistical process of the extracted data was performed with the help of the biostatistics department of university of Plymouth, England, using "Limma in R, 2.13" software (Bioconductor). In total, 45000 probes were used, including positive and negative controls and included 41925 probe sets. The microarray was performed from RNA extracted (detailed method described in 2.4) from 6 EU, 6 SR and 6 CHCV. The differential gene expression between CHCV vs EU, CHCV vs SR and SR vs EU was expressed as a log2 fold change of the groups' mean, compared pairwise. Please note that the groups' comparison order is important for data interpretation in 3.3

The statistically significant (adjusted p value to account for the variables tested<0.05) differentially expressed genes from the 3 groups with a log2 fold change of more than 0.5 (i.e. more than 40% absolute fold change) were subsequently processed using "Ingenuity Pathway Analysis Spring 2013 release" (IPA) software(Qiagen), filtered for human species. IPA is a web-based software application for the analysis, integration, and interpretation of data derived from 'omics' experiments, such as microarrays, RNA sequencing, metabolomics, proteomics, and small scale experiments that generate gene and chemical lists. Data analysis and interpretation with IPA builds on the manually curated content of the Ingenuity Knowledge Base (Ingenuity, Qiagen) which is the one of the largest databases available worldwide. Various algorithms identify regulators, relationships, mechanisms, functions, and pathways relevant

to changes observed in an analysed dataset. The core analysis in IPA identifies relationships, mechanisms, functions, and pathways relevant to a dataset. Upstream regulator analysis identifies molecules, including transcription factors, which may be causing observed gene expression changes, whereas downstream effects analysis predicts downstream biological processes that are increased or decreased based on the analysed data (alternatively known as "heat maps"). Integrating results about potential regulators and effects, the regulator effects tool highlights connections to create hypotheses about upstream triggers responsible for downstream phenotypic or functional outcomes. Furthermore, IPA enables the interrogation of subnetworks and canonical pathways by selecting a molecule of interest, indicating up or down regulation, and simulating directional consequences on downstream molecules and the inferred activity upstream in the examined network or pathway. Networks are generated de novo based upon input genes, proteins, or chemicals. Canonical Pathways are generated prior to data input, based on the literature and do not change upon data input. The p value of a canonical pathway is determined by Fischer's exact test (IPA datasheet, Qiagen, see Appendix 1).

3.3 Results

3.3.1 Study subjects included in the microarray

Characteristic	EU (n=6)	SR (n=6)	CHCV (n=6)
Mean age	37.0	41.0	43.3
Male gender (%)	100.0	50.0	80.0
Serology (HCV Ab/HCV RNA)	-/-	+/-	+/+
HCV Genotype	N/A	N/A	3
Mean life time injecting episodes (rounded in hundreds)	4800	4400	2700
EU HCV specific T cell responses (%)	67	Non applicable	Non applicable
IDU at time of recruitment	Yes	Yes	Yes
Sharing needles (%)	66	50	66
Sharing drug injection equipment (%)	100	100	100
Additional co morbidities	No	No	No

Table 3-1 Demographics of the individuals included in the microarray.

Note: HCV specific T-cell responses in EU were discussed in 1.7.2.1, 1.7.2.2 and 3.1.

3.3.2 **Summary of microarray findings**

In total, 33,831 genes were studied for differential expression in CHCV, EU and SR. 1465 genes between the CHCV and EU group, 4377 between the CHCV and SR group and 4510 between the SR and EU group were significantly ($p < 0.05$) up or down regulated. From this point onwards and for the rest of this thesis, a positive log2 fold change of a gene expression between 2 groups, denotes upregulation in the first or down regulation in the second and a negative log2 fold change between 2 groups, denotes down regulation in the first or up regulation in the second. The total amount of genes exhibiting a positive or negative log2 fold change between CHCV vs EU, CHCV vs SR and SR vs EU are shown in figures 3-1 and 3-2. This summary is of no particular biological significance, but supported by the downstream effects analysis depicted in figures 3-3, 3-4 and 3-5, suggests that the majority of differentially expressed genes involve the SR group.
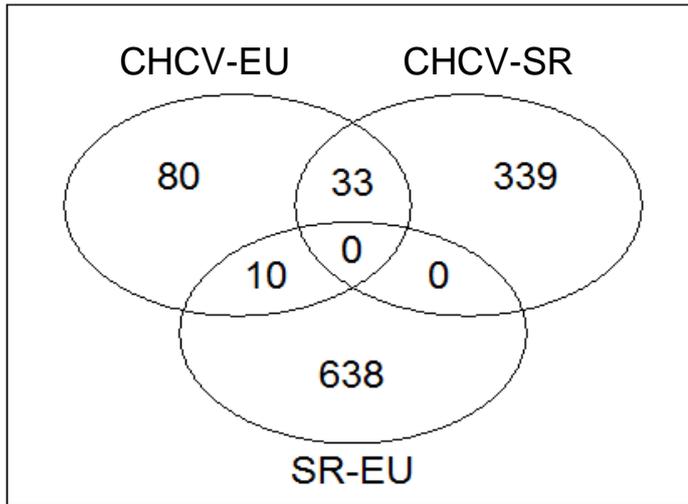
Figure 3-1 Venn diagram of the total amount of genes with a positive log2 fold change in the first versus second group in a pairwise comparison of EU, CHCV and SR.

Note: CHCV-EU=CHCV vs EU, CHCV-SR=CHCV vs SR and SR-EU=SR vs EU. CHCV (patient with CHCV infection), SR (Spontaneous Resolver), EU (Exposed Uninfected).
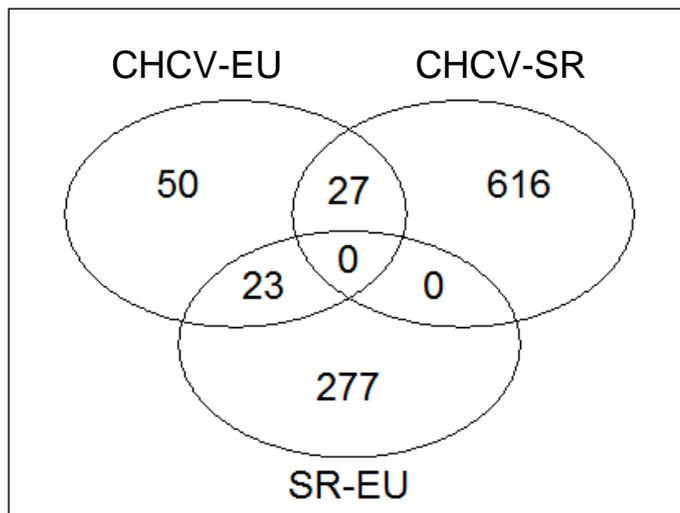
Figure 3-2 Venn diagram of the total amount of genes with a negative log2 fold change in the first versus the second group in a pairwise comparison of EU, CHCV and SR.

Note: CHCV-EU=CHCV vs EU, CHCV-SR=CHCV vs SR and SR-EU=SR vs EU. CHCV (patient with CHCV infection), SR (Spontaneous Resolver), EU(Exposed Uninfected).
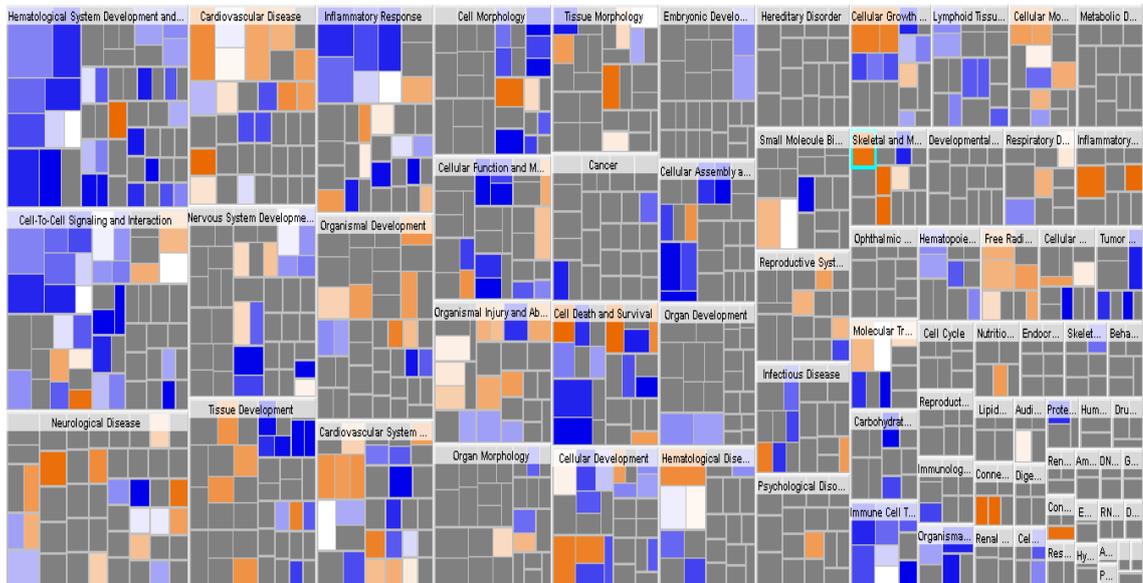
Figure 3-3 Downstream effects analysis ("heat map") of the CHCV vs EU groups.

Note colour coding: grey=no change, orange=upregulated in CHCV or downregulated in EU, blue=down regulated in CHCV or upregulated in EU.

Adapted,with licence, from IPA (Ingenuity, Qiagen).
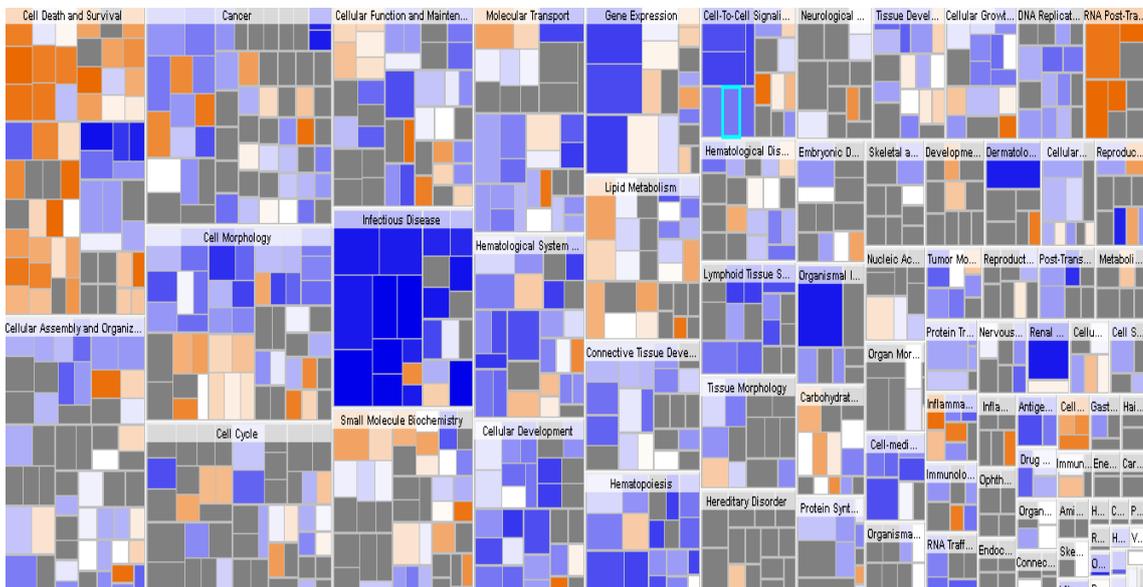
Figure 3-4 Downstream effects analysis ("heat map") of the CHCV vs SR groups.

Note colour coding: grey=no change, orange=upregulated in CHCV or downregulated in SR, blue=down regulated in CHCV or upregulated in SR.

Adapted, with licence, from IPA (Ingenuity, Qiagen).

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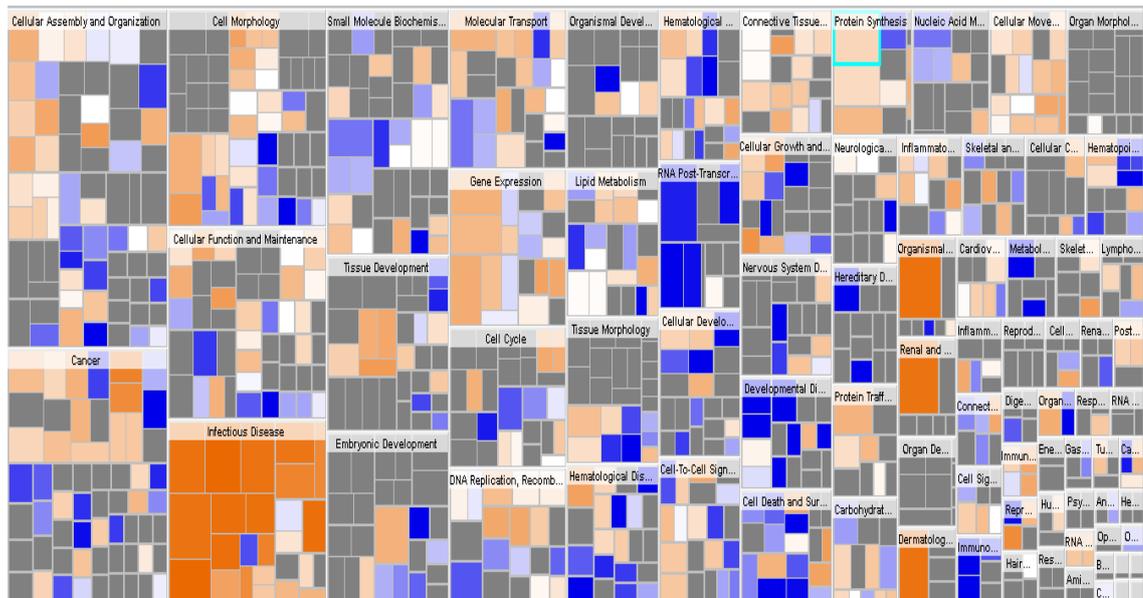
Figure 3-5 Downstream effects analysis ("heat map") of the SR vs EU groups.

Note colour coding: grey=no change, orange=upregulated in SR or downregulated in EU, blue=down regulated in SR or upregulated in EU.

Adapted, with licence, from IPA (Ingenuity, Qiagen).

3.3.3 **Microarray findings in EU**

As mentioned in 3.3.2, the differential gene expression was least prominent in the EU and CHCV groups, compared to SR and we could not readily identify any networks or canonical pathways in EU, relevant to HCV infection, that were up or down regulated in the EU group compared to CHCV or SR. We, therefore, looked for a list of genes that are upregulated in EU vs CHCV and SR or downregulated in CHCV and SR vs EU.18 genes fulfilled this criterion, one of which was Interleukin-27 (IL-27). It was also noted that the IL-17 (suppressed by IL-27, see 4.4.2) receptor activity (RA) was either upregulated in EU or down regulated in the two other groups and the IL-27 receptor activity was either upregulated in EU or down regulated in SR (table 3-2).Fluorescence intensity following normalisation, but with no further statistical analysis, for IL-27 between EU, CHCV and SR is shown in figure 3-6.

C X C motif chemokine 7 (CXCL7) was the gene that exhibited the highest log2 fold change between groups (EU vs CHCV) amongst any others identified in this microarray, and also exhibited a negative log2 fold change in CHCV vs SR (table 3-2). The log2 fold change of CXCL7 was higher in EU compared to CHCV and SR, albeit not more than 0.5 (table 3-2). Fluorescence intensity following normalisation, but with no further statistical analysis, for CXCL7 between EU, CHCV and SR is shown in figure 3-7.

Characteristic	Log2 fold change (and p value) in EU vs CHCV	Log2 fold change (and p value) in SR vs CHCV	Log2 fold change (and p value) in EU vs SR
IL-27	+0.947 (p value: 2.00E-02)	-0.796 (p value: 0.046)	+1.943 (p value: 1.72E-04)
IL-27 RA	No change	No change or <0.5	+0.38 (p value: 4.90E-02)
IL-17 RA	-0.504 (p value: 2.30E-02)	No change or <0.5	-0.796 (p value: 4.59E02)
CXCL7	+2.359 (p value: 6.04E-03)	+1.89 (p value: 1.283 E-03)	No change or <0.5 (p value 0.46)

Table 3-2 Log2 fold changes and p values in IL-27, IL-27 RA, IL-17 RA and CXCL7 between EU, CHCV and SR.

Note: CHCV (patient with CHCV infection), SR (Spontaneous resolver), EU (Exposed Uninfected). Cytokines listed in the table were selected according to log fold change between groups and biological relevance to the studies of this thesis, details of which can be found in chapters 4-6.
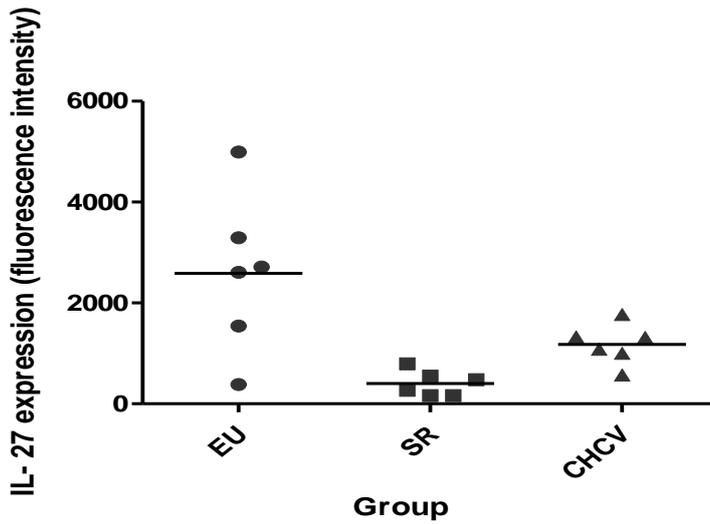
Figure 3-6 Fluorescence intensity of IL-27 between EU, CHCV and SR groups.

Note: Fluorescence intensity following normalisation but without further statistical analysis. CHCV (patient with CHCV infection), SR (spontaneous resolver), EU (Exposed Uninfected). The bars represent the group's mean.
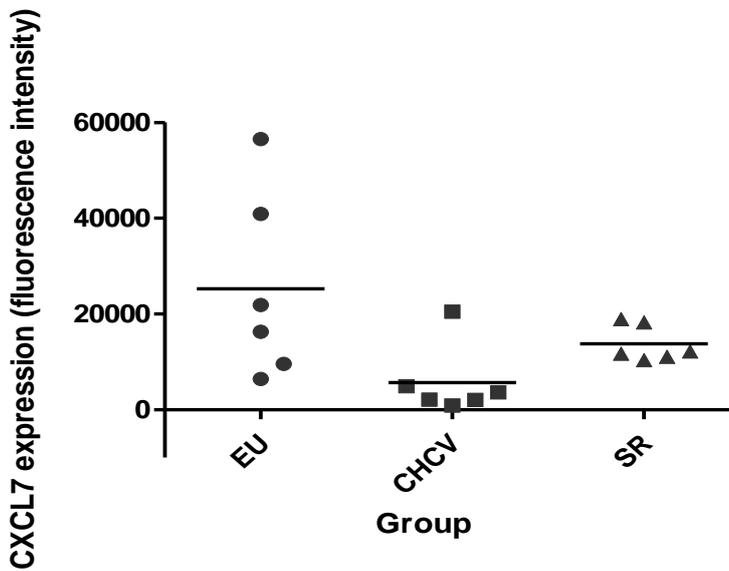
Figure 3-7 Fluorescence intensity of CXCL7 between EU, CHCV and SR.

Note: Fluorescence intensity following normalisation but without further statistical analysis. CHCV (patient with CHCV infection), SR (spontaneous resolver), EU (Exposed Uninfected). The bars represent the group's mean.

3.3.4 **Microarray findings in SR**

The vast majority of changes related to differential gene expression between EU vs CHCV, CHCV vs SR and SR vs EU involved the SR group (see 3.3.2). Canonical pathways related to activation of T lymphocytes were significantly over expressed in this groups compared to EU and CHCV. The CD28 mediated T cell signalling pathway (for detailed analysis see 6.1) was significantly upregulated in SR (upstream regulators affected >30%, p=2.77E-05). Out of the differentially expressed molecules that are important in the pathway's outcome, the CD28 activation dependant phosphatidyl-inositol 3-kinase (PI3K) and lymphocyte-specific protein tyrosine kinase (Lck), as well as the resulting activated Zeta-chain-associated protein kinase 70 (ZAP 70), were significantly upregulated in SR compared to EU and CHCV. Additionally, Akt1, which is one of the pathways outcomes, was significantly upregulated in SR compared to the 2 other groups.

Interleukin 16 (IL-16) log2 fold change was +0.752 (p value: 1.56E-02) in SR vs CHCV and +0.736 (p value: 2.72E-02) in SR vs EU, with no or less than 0.5 change between the CHCV vs EU group. Fluorescence intensity following normalisation, but with no further statistical analysis for IL-16 between EU, CHCV and SR is shown in figure 3-7.
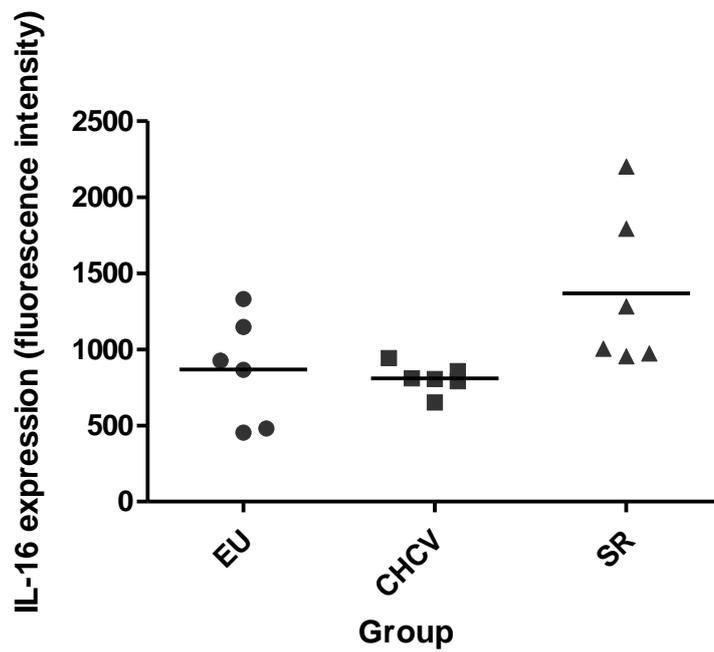
Figure 3-8 Fluorescence intensity of CXCL7 between EU, CHCV and SR.

Note: Fluorescence intensity following normalisation but without further statistical analysis. CHCV (patient with CHCV infection), SR (spontaneous resolver), EU (Exposed Uninfected). The bars represent the group's mean.

3.4 Discussion

In the literature, the cut-off log2 fold threshold for significantly differentiated gene expression is arbitrary, disease related, and usually between 1.5 to 2, depending on p value. Overall, the majority of Log2 fold changes in our study did not exceed -2 or +2 and lay within the -1.5 to +1.5 range, which was not a surprising observation as the microarray was performed from RNA isolated from blood peripheral mononuclear cells (PBMC) as opposed to hepatocytes. We, therefore, decided to review changes of any amplitude above the set cut off of 0.5, especially when involving transcription genes, that could be associated with protection or clearance of HCV infection.

In the EU group, we did not identify any upstream regulators, networks or canonical pathways associated with immunological protection from HCV, so we looked into all the genes whose expression is up regulated in EU vs CHCV and SR or downregulated in CHCV and SR vs EU. Out of the 18 that fulfilled this criterion the most significant, due to either log2 fold change (table 3-2) or relevant biological function (see 4.4.2 &4.4.3), was Interleukin-27 (IL-27), which was either upregulated in EU compared to SR and CHCV or downregulated in CHCV and SR compared to EU. It was also noted that the IL-17 (which is supressed by IL-27, see 4.4.2) receptor activity (RA) was either upregulated in EU or down regulated in the two other groups and the IL-27 receptor activity was either upregulated in EU or down regulated in SR (table 3-2), suggesting IL-27 activity.

CXCL7 was the gene with the highest log2 fold change amongst groups (EU vs CHCV) and it was shown to be either upregulated in EU and SR vs CHCV or down regulated in CHCV vs EU and SR. The differential expression between

EU and SR was lower than the set threshold of 0.5. CXCL7 has diverse biological functions (see 4.4.4), so we assumed that it might of relevance to the purpose of this study.

The SR was not the cohort our work initially focused on, but we could not ignore that it was involved in the vast majority of differential gene expression amongst the 3 groups. These changes mainly affected the activation of T lymphocytes which is a known mechanism of HCV clearance (see 5.1). We identified that the CD28 signalling system in T lymphocytes, a co-stimulator of T-cell activation (see 6.1) is either up regulated in SR compared to EU and CHCV, which could be of biological significance, or down regulated in EU and CHCV compared to SR.

IL-16 is an antiviral cytokine (see 5.4.1) which, according to the microarray findings could be either up regulated in SR compared to EU and CHCV or down regulated in EU and CHCV compared to SR.

Based on the above, we have attempted to corroborate the microarray findings by investigating as follows:

-To study whether IL-27 and CXCL7 are over expressed in EU compared to CHCV and SR.

-To study whether IL-16 is over expressed in SR compared to EU and CHCV.

-To study whether the CD28 signalling system in T lymphocytes is over expressed in SR compared to EU and CHCV.

Chapter 4 IL -27 and CXCL7 expression in Exposed Uninfected

4.1 Background

Cytokines and chemokines are redundant secreted proteins with growth, differentiation and activation functions that are central in regulating and determining the nature of immune responses to viral infections (Steinke & Borish 2006).

It is well established that the presence of anti HCV-Th1 cell responses favours HCV clearance (Diepolder et al. 1996; Missale et al. 1996; Cramp et al. 1999; Thimme et al. 2001), as opposed to anti HCV-Th2 responses that are associated with viral persistence (X. G. Fan et al. 1998; Lechmann et al. 1999; Gramenzi et al. 2005). Th1 cells primarily secrete IFN-γ, IL-2 and IL-12, whereas Th2 cells primarily secrete IL-4, IL-5, IL-10 and IL-13.

The activation of the innate immune system may result in IL-6, IL-12 TNF-α, IFN-α and IFN-λ secretion and NK cell function is, in general, amplified greatly by IFN-α released by dendritic and virally infected cells. IFN-α, as described in 1.5.1.2 inhibits HCV replication via the induction of genes encoding antiviral proteins, such as double-stranded RNA-dependent kinase, 2', 5'oligoadenylate synthetase and MxA (Pestka et al. 1987; Samuel 2001; Langer et al. 2004; Galligan et al. 2006) and had been the mainstay of treatment of CHCV infection up until fairly recently.

Various polymorphisms in cytokine genes have been associated, in a variety of studies, with favourable outcome of HCV infection either spontaneously or after

treatment, including IL-12B (Yin et al. 2004; Annwyne Houldsworth et al. 2005), IL-10 (Mangia et al. 2004) and IL-28B (IFN-λ3) (Ge et al. 2009; Tanaka et al. 2009; Thomas et al. 2009). In a recent smaller study it has been suggested that the SNP c.-964A>G (rs153109) of the IL-27p28 gene is present in non responders and relapsers compared to patients who achieved SVR after treatment (Zicca et al. 2014).

As mentioned in 3.1, in our EU cohort HCV specific IFN-γ ELISPOT responses have been identified in up to 50% of the subjects, but of weaker strength compared to those of individuals who spontaneously clear HCV and tend to wane only months after cessation of IDU. (Thurairajah et al. 2008). It has also been shown that the 1128 CC polymorphism of IL-12B gene is overexpressed in EU compared to healthy controls and patients with CHCV infection (Hegazy et al. 2008). However, in a wide cytokine profiling study, the two cytokines that appeared to be raised in EU compared to healthy controls, SR and patients with CHCV infection were the innate IL-6 and IL-8 as opposed to the adaptive cytokines. IFN-α was also elevated in EU and all groups exposed to HCV compared to healthy controls. Our previous findings indicate that the mechanism of natural resistance to our EU cohort is complex and might involve components of both the innate and adaptive immunity.

The aim of the following study was to ascertain whether Interleukin-27 (IL-27) cytokine and C X C motif chemokine 7 (CXCL7) with various immunomodulatory functions, are overexpressed in individuals with natural resistance to HCV (EU), compared to patients with chronic HCV infection (CHCV), individuals who have spontaneously cleared HCV (SR) and normal controls with no risk factors of HCV exposure (NC).

4.2 Methods

The micro array results for IL-27 and CXCL7 were validated in two steps both for IL-27 and CXCL7. In the first step, relative gene expression in the individuals included in the micro array (demographics described in chapter 3) was measured by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). Comparative basic relative quantification was performed in each group and the differential gene expression between 2 groups was calculated using the $2^{-\Delta\Delta CT}$ method of the mean ΔCT of each group. Coefficient variation (CV) of the $2^{-\Delta CT}$ values within a group was calculated as an indicator of intra sample variability. Details on the recruitment of the pre mentioned groups, the laboratory technique and the analysis methods are described in detail in chapter 2.

In the second step, IL-27 and CXCL7 concentration was measured by sandwich Enzyme linked-immunosorbent assay (ELISA), detailed description of which can be found in chapter 2 .Levels in EU (HCV Ab and HCV RNA negative) were compared with levels found in (1) patients who spontaneously resolved HCV infection (HCV Ab positive and HCV RNA negative) ; (2) treatment-naïve chronic HCV patients (HCV Ab positive and HCV RNA positive); (3) normal controls with no risk factors of HCV exposure that ,due to resource constraints ,were only included in the IL-27 study. The details of recruitment of these groups are discussed in chapter 2.

4.3 Results

4.3.1 Study subjects for quantitative reverse transcriptase polymerase chain reaction (qPCR)

Quantitative reverse transcriptase polymerase chain reaction for IL-27 and CXCL7 RNA concentration was performed in the 18 individuals included in the micro array study, details of which can be found in chapter 3. They consisted of 6 EU, 6 SR and 6 treatment naïve patients with CHCV. The demographics for the group in this part are summarised in Table 3.1.

4.3.2 Study subjects for cytokine and chemokine testing

IL-27 concentration was measured in 49 individuals in total, consisting of 13 EU, 14 SR, 14 treatment naïve patients with CHCV infection of various genotypes and 8 normal controls with no risk factors of HCV exposure. The demographics for the group in this part is summarised in Table 4.1.

CXCL7 concentration was measured in 24 individuals in total, including 8 EU, 8 SR and 8 treatment naïve patients with CHCV infection of various genotypes. The demographics for the group in this part is summarised in Table 4.2.

Characteristic	EU (n=13)	SR (n=14)	CHCV (n=14)	NC (n=8)
Mean age	31.0	41.6	36.0	33.75
Male gender (%)	84.6	61.5	80.0	62.5
Serology (HCV Ab/HCV RNA)	-/-	+/-	+/+	-/-
HCV Genotype	N/A	N/A	Any	N/A
Mean life time injecting episodes (rounded in hundreds)	4200	4200	1800	0
IDU at time of recruitment	Yes	Yes	Yes	No
Sharing needles (%)	61	57	57	0
Sharing any drug injection equipment	100	100	100	0
Additional co morbidities	No	No	No	No

Table 4-1 Demographics of individuals included in the IL-27 study.

Note: n=number; N/A; non-applicable; EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection; NC=normal control.

Characteristic	EU(n=8)	SR(n=8)	CHCV(n=8)
Mean age	30.6	38.3	34.1
Male gender (%)	75.0	62.5	87.5
Serology (HCV Ab/HCV RNA)	-/-	+/-	+/+
HCV Genotype	N/A	N/A	Any
Life time injecting episodes (rounded in hundreds)	3300	3200	2200
IDU at time of recruitment	Yes	Yes	Yes
Sharing needles (%)	75	50	50
Sharing any drug injection equipment (%)	100	100	100
Additional co morbidities	No	No	No

Table 4-2 Demographics of the individuals included in the CXCL7 study.

Note: n=number; N/A=non -applicable; EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection.

4.3.3 Quantitative reverse transcriptase polymerase chain reaction results for IL-27 and CXCL7 were comparable to the micro array results
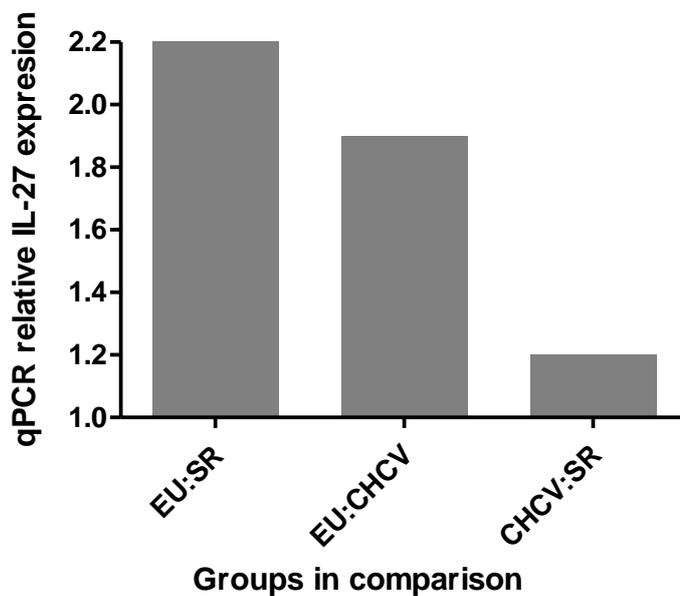
Figure 4-1 IL-27 qPCR in comparison groups.

Note: y axis represents absolute fold change. EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection. The bars represent IL-27 expression fold change between the mean of 2 groups. Coefficient variation (CV) of the $2^{-\Delta CT}$ values within each group was 16.1% for EU, 10.2% for SR and 13.3% for CHCV.
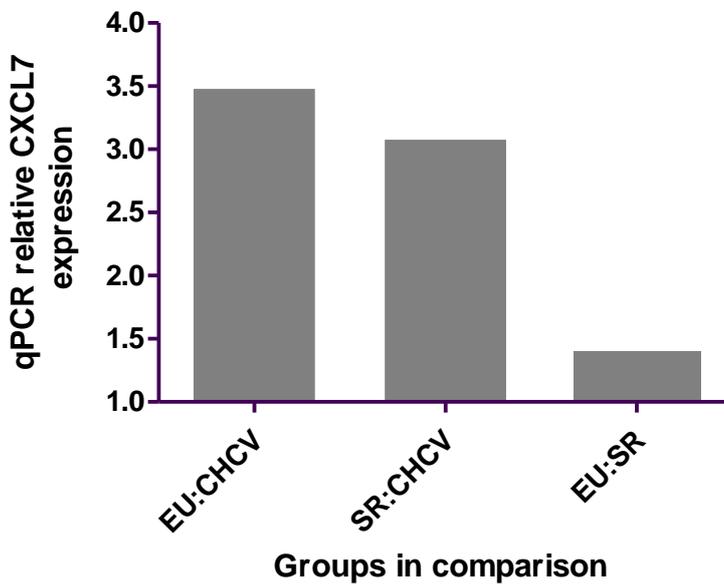
Figure 4-2 CXCL7 qPCR in comparison groups.

Note: y axis represents absolute fold change. EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection. The bars represent CXCL7 expression fold change between the mean of 2 groups. Coefficient variation (CV) of the $2^{-\Delta CT}$ values within each group was 17.2% for EU, 14.2% for SR and 15.3% for CHCV.

4.3.4 IL-27 concentration is elevated in EU compared to all other groups

IL-27 was elevated in 9 out of the 13 (69.2%) EU tested, of whom only 2 were included in the micro array study. The cut off value of a positive result was arbitrary set above the lowest concentration above 0 pg/ml seen in any group (4.38pg/ml). The maximum concentration measured in the EU group was 643.77pg/ml. Out of the rest groups, 4 individuals in total (1 SR, 3 CHCV and 1 NC) were found to have elevated IL-27 levels with a maximum concentration of 39.39 pg/ml.

The difference in the median concentration of IL-27 in the EU group (Figure 4.3) was statistically significant compared to all other groups (Kruskal-Wallis; p=0.005).

Figure 4-3 Scatterplot of IL-27 in EU, CHCV, SR and NC.

Note: Broken y axis. Horizontal line represents the median IL-27 concentration of the group. EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection.

4.3.5 CXCL7 concentration is elevated in EU compared to patients with CHCV infection

The median CXCL7 concentration was higher in the EU group, compared to the SR and CHCV ones (Figure 4.4), however the difference reached statistical significance only between the EU and CHCV group (Mann -Whitney; p=0.028).
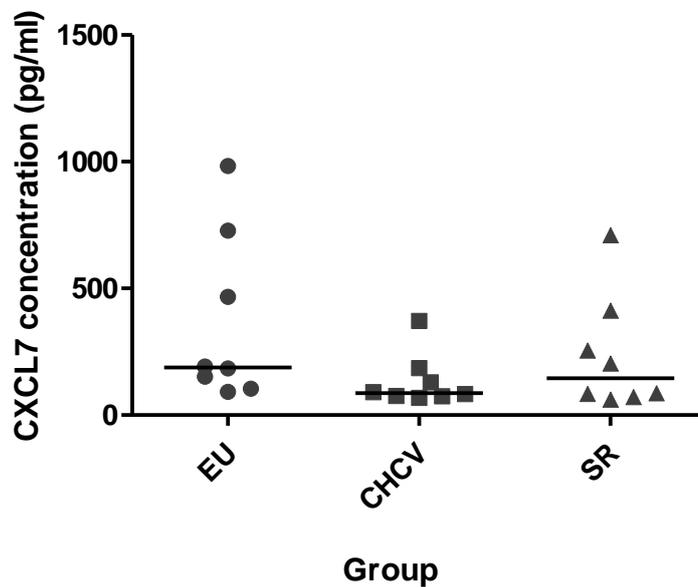
Figure 4-4 Scatterplot of CXCL7 concentration in EU, CHCV and SR.

Note: horizontal line represents the median CXCL7 concentration of the group. EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection.

4.4 Discussion

4.4.1 Prologue

IL-27 appeared to be over secreted in the serum of individuals with apparent resistance to HCV in comparison with patients with chronic CHCV infection, individuals who spontaneously cleared HCV and normal controls with no risk factors of HCV exposure.

CXCL7 appears to be over excreted in the serum of individuals with apparent resistance to HCV compared to patients with chronic CHCV infection, but its levels are not significantly different between the former group and individuals who have spontaneously cleared HCV.

4.4.2 IL-27 overview

IL-27 is a member of the IL-12 family cytokines that consists of p28 and Epstein-Barr virus-induced gene 3 (EBI3) (Pflanz et al. 2002; Kastelein et al. 2007). The p28 chain is related to a subunit of IL-12 (IL-12p35) and has a classical cytokine structure, while the EBI3 is related with IL-12p40 and structurally resembles the soluble IL-6 receptor α chain. Thus, it is recognised that there is a high degree of subunit promiscuity between these cytokines and it was originally thought that they would have similar function. However, whilst there is a degree of overlap in certain aspects of their bioactivity, there are also many distinct roles for each individual cytokine of this superfamily. Activated antigen presenting cells are the main source of IL-27, however, other cell types including endothelial cells, neutrophils, NKT cells and astrocytes can also produce IL-27(Smits et al. 2004; Sonobe et al. 2005; Fujita et al. 2009). Many TLR signalling pathways induce IL-27 expression including TLR2, TLR3, TLR4 and TLR9 and at the transcriptional level, binding sites for NF-kB and interferon

regulatory factor 3 (IRF3) have been identified in the p28 promoter (Pflanz et al. 2002; Schuetze et al. 2005; Wirtz et al. 2005; Liu et al. 2007; Molle et al. 2007). IL-27 binds to its receptor (IL-27R), which is composed of ligand-specific chain, IL-27 receptor α chain (IL-27Rα), and of gp130, a signal-transducing molecule shared with other cytokines, IL-6, IL-11, oncostatin M, and leukaemia inhibitory factor. IL-27 is capable of binding to IL-27Rα in the absence of gp130; however, the co-expression of both receptor subunits is required to induce signal (Pflanz et al. 2004; Kastelein et al. 2007). IL-27R is expressed on a range of cell types including T cells, monocytes, DC, mast cells, hepatocytes, endothelial cells, neurons, B and NK cells(Pflanz et al. 2004; Bender et al. 2009) and upon ligand binding phosphorylation of the signal transducers and activators of transcription protein(STAT) -1, -2, -3, -4, or -5 occurs (Hibbert et al. 2003; Kamiya et al. 2004; Batten & Ghilardi 2007).

Much of the research on IL-27 has focused on its function in adaptive immunity and in particular, its effect on Th cells. In conjunction with IL-12 and via the induction of the transcription factor T-bet, it induces proliferation and development of naïve Th1, increasing IFN-γ production (Pflanz et al. 2002; Hibbert et al. 2003). In the later phases of inflammatory response it can also exert an inhibitory effect on Th1 activation (Yoshimura et al. 2006) and suppress INF-γ production. The initial boost and later dampen of this inflammatory response can be partially be explained by the expansion of IL-10 (a potent anti-inflammatory cytokine) producing Th1 cells by IL-27 (Fitzgerald et al. 2007; Stumhofer et al. 2007). In contrast to the paradoxical effects of IL-27 on Th1 responses, the inhibitory effect of IL-27 on other Th cell subsets is clearer. IL-27 abrogates Th2 and Th17 development by blocking the transcription factors GATA-3 and RORγt/RORα in a STAT-1 mediated manner

(Yoshimura et al. 2006; El-behi et al. 2009; Diveu et al. 2009). Various groups have, also, demonstrated that IL-27 suppresses the development of Treg cells (Neufert et al. 2007; Huber et al. 2008).

IL-27 has also exhibited enhanced NK cell cytotoxicity, mainly in the context of various cancers (Matsui et al. 2009; Chen et al. 2012), but additionally it has been shown to increase NK survival , albeit not cytotoxicity, in normal controls (Laroni et al. 2011).

4.4.3 IL-27 in viral infection

IL-27 has shown antiviral properties against a range of viruses and has, extensively, been explored as a potential therapeutic adjunct for the treatment of HIV. It has been shown to inhibit HIV in vitro in PBMCs, macrophages, dendritic cells and CD+4 cells via a variety of mechanisms. It, primarily, upregulates the expression of IFN- inducible genes both in an IFN-α dependent and independent manner. Additionally, it modulates host restriction factors such as the APOBEC proteins and inhibits HIV in macrophages by down -regulation of a b-spectrin gene that disrupts HIV viral cycle .(Fakruddin et al. 2007; Imamichi et al. 2008; Greenwell-Wild et al. 2009; Chen et al. 2013; Dai et al. 2013). Due to the inhibitory effects of IL-27 on HIV viral replication, there has been emerging interest on the possible role of this cytokine in the treatment of chronic HCV and HBV infections. Existing, albeit limited, evidence suggests that HCV and HBV can be suppressed in vitro with IL-27 acting as a type I IFN (Frank et al. 2010; Cao et al. 2014). Elevated levels of Treg cells, which IL-27 suppresses, are associated with HCV viral persistence and antibody depletion of these cells are associated with an in vitro enhancement of functional HCV-specific CD8+ T cell responses (Cabrera et al. 2004).

4.4.4 CXCL7 overview

CXCL7 (synonym to platelet basic protein; PPB) belongs to the family of CXC – ELR$^+$ chemokines. By post translational cleavage, it gives rise to connective tissue-activating peptide-III (CTAP-III), neutrophil activating peptide-2 (NAP-2), β-thromboglobulin (β-TG) and two variants of thrombocidin (TC-1 &TC-2) (figure 4-5). Peptidases, some of which are derived from monocytes and granulocytes play a role in the post-transcriptional processing of CXCL7 (Castor et al. 1983; Walz & Baggiolini 1989; Brandt et al. 1991; Car et al. 1991; Krijgsveld et al. 2000). CXCL7 is known as a major granular protein of platelets, but it can also be produced by inflammatory cells and cancers of different origin (El-Gedaily et al. 2004; Cunningham et al. 2010; Grépin et al. 2014; Desurmont et al. 2015). CXCL7 G protein coupled receptors CXCR1 and CXCR2 are expressed on neutrophils, monocytes, lymphocytes, macrophages, NK, endothelial and tumour cells (Chuntharapai et al. 1994; Gerszten et al. 1999; Wang et al. 2006; Ginestier et al. 2010; Unver et al. 2015). CXCL7and its derivatives exhibit diverse biological functions that have mainly been studied in the setting of bacterial infections and tumours. Please note that in the literature the term CXCL7 has many synonyms, but for the purpose of this thesis it denotes platelet basic protein (PBP) and all its isoforms.
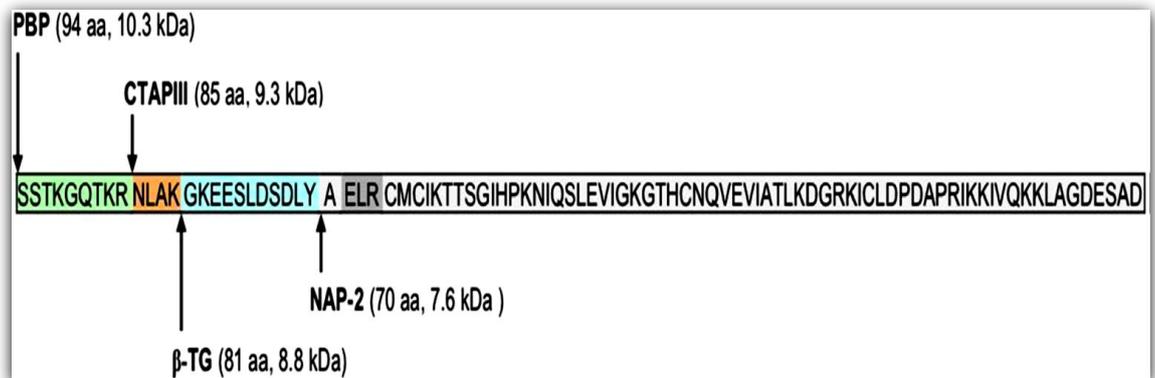
Figure 4-5 Amino acid sequences of CXCL7 derivatives.

CXCL7(PBP) ,by post translational cleavage, gives rise to connective tissue-activating peptide-III (CTAP-III), β-thromboglobulin (β-TG) and neutrophil activating peptide-2 (NAP-2).

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Most of the isoforms, but primarily NAP2 are potent neutrophil chemoattractants (Walz et al. 1989) and exhibit bacteriocidal and antifungal properties (Tang et al. 2002; González-Cortés et al. 2012). CXCL7 has also be shown to induce macrophage chemotaxis and activate histamine release from basophils (Proudfoot et al. 1997; Unver et al. 2015).

CTPA-III stimulates angiogenesis (Strieter et al. 1995) as well as DNA, proteoglycan and glucosaminoglycan synthesis, playing an important role in the homeostasis of the extracellular matrix (ECM) (Castor et al. 1983). The ECM represents a non-cellular component in tissues and organs that is primarily composed of water, proteins, and proteoglycans. It forms an intricate network that provides a physical scaffold for cells as well as structural support, strength and elasticity in all tissues and organs (Frantz et al. 2010). Besides its mechanical and biochemical properties, it helps maintain hydration and homeostasis and by interacting with cell-surface receptors and matrix components it regulates cell differentiation, adhesion, proliferation, migration, and survival (Hynes 2009; Järveläinen et al. 2009) .The ECM also binds and secretes growth factors and cytokines that drive morphogenesis, cell function, and metabolism . Thus, the ECM creates a complex microenvironment that is particularly dynamic in nature and which undergoes continuous remodelling not only during development but also throughout differentiation and wound healing. Accordingly, well-coordinated regulation of ECM remodelling is essential to maintain homeostasis and to prevent disease onset and progression(Daley et al. 2008). In HCV infection, the initial virion entry onto hepatocytes is mediated by binding to glycosaminoglycans (see 1.1.3).

Additionally, CXCL7 inhibits megacaryopoeisis, has heparinise activity and can induce adhesion of endothelial progenitor cells after vascular injury (Han et al. 1990; Hoogewerf et al. 1995; Hristov et al. 2007).

4.4.5 **Conclusions**

Based on the above, higher levels of endogenous IL-27 in our EU cohort could be a contributing factor to their natural resistance to HCV and a plausible explanation of the difference in its levels noted between the groups. It remains to be elucidated which arm on the immune system it augments in our cohort, but based on previous findings related to EU immunological characteristics, it is possible that it functions primarily as a type I IFN and activator of NK cells, albeit additional activation of the Th1 and CD28+ cells by IL-27 cannot be excluded. Further work could focus on identifying the level of expression of IFN-inducible genes in our cohort compared with normal controls and patients with CHCV, NK cell cytotoxicity assays in CHCV in the presence of IL27, Treg levels in EU compared to CHCV and other control groups, as well the presence of IL-27 SNPs in EU that are associated with favourable outcome of CHCV infection, such as rs153109. Detailed suggestions for further studies can be found in chapter 7.

The possibility that the result is a consequence of trauma or infection, other than HCV, has been considered. However, given the fact that none of the subjects included had known additional co morbidities and with the exception of normal controls all subjects shared common risk factors in terms of IDU, this hypothesis is considered unlikely.

In terms of the findings related to CXCL7, due to limited resources and the technical challenges of the ELISA assays, we were not able to expand our

study in more than 8 individuals from each group and include normal controls. Therefore, there is always a chance that the lack of statistical significance is related to the sample sizes. However, given the diverse functions of CXCL7, its elevated concentration in the serum of EU vs CHCV, could merely reflect a difference in the frequency and timing of IDU of the individuals included. In order to make a safe conclusion we need to measure concentration of CXCL7 in more individuals including normal controls.

Chapter 5 IL-16 expression in Spontaneous Resolvers

5.1 Background

The adaptive immune response to HCV consists of an antibody and CD4+ and CD8+ HCV specific T cell responses and is discussed in detail in 1.5.2. T cell responses appear within 7-10 weeks after detection of the virus and coincide with a reduction of viral load, indicating the vital role of the adaptive response in viral clearance (F. Lechner et al. 2000; Thimme et al. 2001).

CD4+ cell responses are critical to both the generation and maintenance of immunity to viruses, as they produce cytokines that promote CD8+ cell cytolytic function and trigger the production of antibodies. In both chimpanzee and human models, clearance of HCV is associated with a rapid, strong, polyclonal and sustained HCV specific CD4+ T-cell responses and inability to mount these, results in chronic viraemia (Diepolder et al. 1996; Lechmann et al. 1996; Missale et al. 1996; Tsai et al. 1997; Shata et al. 2002; Thimme et al. 2002). Responses directed against multiple HCV proteins of Th1 (IFN-γ) type were found to persist for decades after spontaneous clearance of HCV (Takaki et al. 2000) and their loss within the first months of infection is associated with relapse of viraemia (Gerlach et al. 1999). As mentioned in various other paragraphs of this thesis, to date, in the EU cohort, there is no robust evidence that adaptive immunity contributes significantly to their natural resistance to HCV and previously shown HCV specific IFN-γ responses are weaker than those seen in SR.

The hypothesis tested was that endogenous IL-16, a potent CD4+ cell chemoattractant, is overexpressed in individuals that spontaneously clear HCV (SR) compared to patients with chronic HCV infection (CHCV) and individuals with natural resistance to HCV (EU).

5.2 Methods

IL-16 relative expression was measured by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) in (1) patients who spontaneously resolved HCV infection (HCV Ab positive and HCV RNA negative), ;(2) treatment-naïve chronic HCV patients (HCV Ab positive and HCV RNA positive), and; (3) individuals with apparent resistance to HCV (HCV ab and HCV RNA negative). Comparative basic relative quantification was performed in each group and the differential gene expression between 2 groups was calculated using the $2^{-\Delta\Delta CT}$ method of the mean $2^{-\Delta CT}$ of each group. Coefficient variation of the $2^{-\Delta CT}$ values within a group was calculated as an indicator of intra sample variability. Details on the recruitment of the pre mentioned groups, the laboratory technique and the analysis methods are described in detail in chapter 2.

IL-16 concentration was measured, in subjects of the groups mentioned above, by sandwich enzyme linked-immunosorbant assay (ELISA), detailed description of which can be found in chapter 2 .7.4.

5.3   *Results*

5.3.1   **Study subjects for quantitative reverse transcriptase polymerase chain reaction and cytokine testing**

Quantitative reverse transcriptase polymerase chain reaction for IL-16 was performed in 18 individuals in total, consisting of 6 EU, 6 SR and 6 CHCV. Due to limitations related to cell availability, we were only able to include 4 (2 EU and 2 SR) of those subjects included in the micro array. The demographics for this part of the study are summarised in table 5.1.

IL-16 concentration was measured in the serum of 26 individuals in total, consisting of 10 EU, 10 SR and 6 patients with CHCV of various genotypes. The demographics for this part of the study are summarised in table 5.2.

Characteristic	EU (n=6)	SR(n=6)	CVCH(n=6)
Mean age	32.7	40.2	38.0
Male gender %	83.3	66.7	83.3
Serology (HCV Ab/HCV RNA)	-/-	+/-	+/+
Mean life time injecting episodes (rounded in hundreds)	4800	4600	2000
HCV genotype	N/A	N/A	Any
IDU at time of recruitment	Yes	Yes	Yes
Sharing needles (%)	50	50	50
Sharing any drug injection equipment (%)	100	100	100
Additional co morbidities	No	No	No

Table 5-1 Demographics of subjects included in IL-16 q-PCR study.

Note: n=number; N/A=non -applicable; EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection.

Characteristic	EU(n=10)	SR(n=10)	CHCV(n=6)
Mean age	35.6	41.5	39.2
Male gender %	80.0	60.0	83.3
Serology (HCV Ab/HCV RNA)	-/-	+/-	+/+
Mean life time injecting episodes (rounded in hundreds)	4900	4800	2900
HCV genotype	N/A	N/A	Any
IDU at time of recruitment	Yes	Yes	Yes
Sharing needles (%)	50	40	50
Sharing any drug injection equipment (%)	100	100	100
Additional co morbidities	No	No	No

Table 5-2 Demographics of individuals included in IL-16 ELISA study.

Note: n=number; N/A=non -applicable; EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection.

5.3.2 Quantitative reverse transcriptase polymerase chain reaction results for IL-16 show no difference in gene expression between the groups
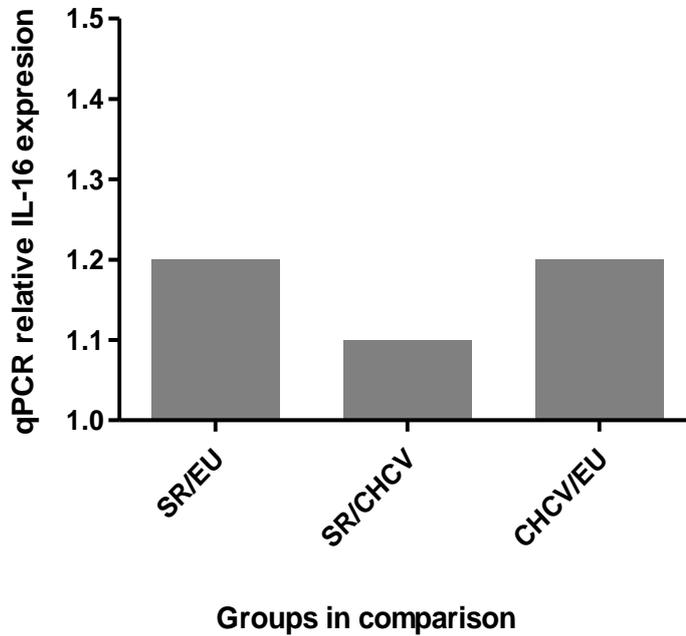
Figure 5-1 IL-16 qPCR in comparison groups.

Note: y axis expresses absolute fold change. EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV. The bars represent IL-16 expression fold change between the mean of 2 groups. Coefficient variation (CV) of the $2^{-\Delta CT}$ values within each group was 13.6% for EU, 24.2% for SR and 5.1% for CHCV.

5.3.3 IL-16 levels are not different between SR, EU and CHCV

There was no statistically significant difference in the median of IL-16

concentration (Figure 5.4) between the groups in comparison (Kruskal-Wallis).
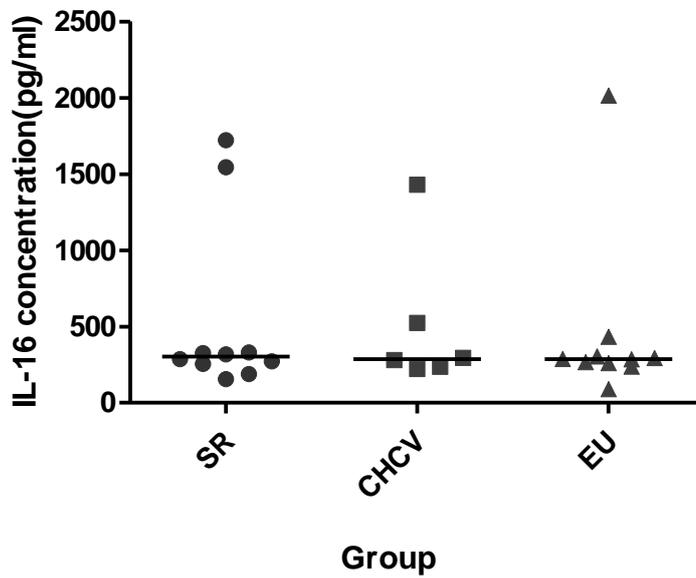
Figure 5-2 Scatterplot of IL-16 concentration in SR, CHCV and EU.

Note: horizontal line represents the median concentration of the group.
EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic
HCV.

5.4 Discussion

5.4.1 IL-16 overview

IL-16 is generated in lymphocytes and bronchial cells as pro-IL-16, which is
enzymatically cleaved by caspase 3, (which plays an essential role in cell
apoptosis), at a serine residue (S^{511}) after stimulation with T cell mitogens or IL-
9, respectively, resulting in the secretion of a peptide consisting of the carboxy-
terminal 121 amino acids (Baier et al. 1997; Zhang et al. 1998). Although there
might be a correlation between cell apoptosis and IL-16 release, the exact
mechanism has not been elucidated yet (Wu et al. 1999). CD4 serves as a
signal-transducing receptor for IL-16 and is required for mediating IL-16
functions (Liu et al. 1999; Nicoll et al. 1999). Sources of IL-16 include epithelial
cells, mast cells, lymphocytes, macrophages, synovial fibroblasts, and
eosinophils. IL-16 mRNA is constitutively expressed in both CD4+ and CD8+
cells, however, synthesis is induced in T lymphocytes upon exposure to antigen
or mitogen (Laberge et al. 1995; Kaser et al. 1999; Wu et al. 1999).

A variety of CD4+ target cells for IL-16 stimulation have been identified.
Although initially characterized as a chemoattractant specifically for CD4+ T
cells , it was later determined that IL-16 is also a potent chemoattractant for all
peripheral immune cells expressing CD4, including CD4+ monocytes ,
eosinophils and dendritic cells (Berman et al. 1985; Cruikshank et al. 1987;
Rand et al. 1991; Kaser et al. 1999). In addition to cell migration, IL-16 is a
competent growth factor. Stimulation with IL-16 results in cell cycle progression
in CD4$^+$ T lymphocytes and in combination with other cytokines it may help to
influence Th1 cell development (Cruikshank et al. 1987; Parada et al. 1998).
Although functions for IL-16 have largely been attributable only to the secreted

form, the vast majority of detectable IL-16 protein in lymphoid cell lines exists intracellurarly, as pro-IL16, but its role has not been fully elucidated yet. (Cruikshank et al. 2000).

5.4.2 IL-16 in viral infection

One of the most intriguing functions identified for IL-16 is as suppressor of HIV-1 (Berman et al. 1985; Idziorek et al. 1998). Although both IL-16 and HIV-1 use CD4 as a receptor, studies have shown that they do not share a common binding site. The mechanism of HIV suppression by IL-16, therefore, does not involve steric inhibition of viral binding to CD4 as is the case with several CC chemokines (i.e., RANTES, macrophage inflammatory protein (MIP-1a), and MIP-1 beta(Liu et al. 1999). The inhibitory effect is direct and a result of the suppression of HIV-1 promoter activity (Maciaszek et al. 1997) and viral mRNA expression (Zhou et al. 1997). In dendritic cells, IL-16 reportedly not only inhibits viral replication, but also prevents viral entry when added to cell cultures during the infection period (Truong et al. 1999). Additionally, it has been shown that IL-16 can prime CD4+ T cells for IL-2 responsiveness, and therefore may be a useful adjunct to IL-2 therapy for therapeutic conditions resulting in CD4+ T cell depletion (Parada et al. 1998).Serum IL-16 levels are increased in subjects with non-progressive HIV infection compared to patients with AIDS (Scala et al. 1997) and rise dramatically in HIV infected individuals after treatment (Bisset et al. 1997).

There's little association, so far, of IL-16 to HCV infection outcomes but it has been suggested that allograft TNFbeta and IL16 polymorphisms influence HCV recurrence and severity after liver transplantation (Kimball et al. 2006).

5.4.3 **Conclusion**

We hypothesized that elevated endogenous levels of IL-16 might contribute to spontaneous clearance of HCV. Admittedly, the log fold change in IL-16 gene expression between SR vs CHCV and SR vs EU groups, based on the micro array results (see 3.3), was of lower amplitude compared to the cytokines and chemokines discussed in chapter 4. However, given the biological functions of IL-16 outlined earlier, we felt that any change, especially if directed to a transcriptional level, might be relevant.

In the cases we studied, IL-16 levels have not been shown to be different between EU, SR and CHCV groups either at the transcriptional or translational level. Therefore, this hypothesis based on the microarray findings, whilst plausible has not been supported by the confirmatory tests. This could be due to the relatively small sample size or because IL-16 is not a major factor contributing to spontaneous clearance of HCV infection.

Chapter 6 The CD28 signalling in T helper cells pathway expression in Spontaneous Resolvers

6.1 Background

Adaptive immunity and in particular CD4+ T cell activation is essential for clearance of HCV as mentioned in paragraph 5.1.

T cell activation requires two signals: peptide in the context of MHC interacting with TCR, and a co-stimulatory signal (Lafferty & Cunningham 1975). Engagement of the TCR by peptide loaded MHC in the absence of other signals is insufficient to activate the T cell, and may render it unresponsive to further antigenic stimulation, a condition termed anergy (Quill & Schwartz 1987; Mueller et al. 1989).

One of the most well studied co-stimulatory molecules expressed by T cells is called CD28 (Rudd & Schneider 2003; Miller et al. 2009; Paterson et al. 2009; Sharpe 2009). In humans, CD28 is expressed on approximately 80% ofCD4+T cells and 50% of CD8+ T cells (Lee et al. 1990) and ligation on the former has profound and diverse consequences. CD28 signalling is essential for multiple facets of CD4+ T cell activation, including proliferation, survival, glucose metabolism and migration (Harding et al. 1992; Boise et al. 1995; Frauwirth et al. 2002; Marelli-Berg et al. 2007) .CD28 signalling increases the sensitivity of the T cell to antigen receptor engagement and increases the production of IL-2 by 50-fold (Damle et al. 1988; Lindstein et al. 1989).

The extracellular domain of CD28 binds to B7 proteins using a MYPPPY motif and this interaction initiates the costimulatory signal transduction cascade.CD28 has a highly conserved, relatively short cytoplasmic tail, consisting of 4 amino

acids, that has no intrinsic enzymatic activity. However, several motifs have been identified including: four tyrosine residues, four serine and two threonine residues, two PxxP motifs, and two lysine residues each of which may be important in function (Freeman et al. 1989; Rudd & Schneider 2003; Rudd et al. 2009). Ligation of CD28 to CD80 or CD86 expressed on APC, initiates signal transduction cascades dependent on specific association of proteins with its cytoplasmic tail. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antagonizes this effect, by providing a negative co-stimulatory signal.

PI3K, one of the enzymes in this pathway that CD28 mainly activates, is composed of p85 and p110 subunits. P85 lacks PI3K activity and acts as an adapter, coupling p110 to activated protein tyrosine kinase. There are various isoforms of p110 with p110δ expression specific to white blood cells. The proximal YMNM motif of CD28 binds directly to p85 subunit of phosphatidyl-inositol 3-kinase (PI3K) (Pagès et al. 1994) and initiates the major PI3K dependent signalling pathway, whereas the distal, PYAP motif binds to and activates a variety of other proteins, amongst them the lymphocyte-specific protein tyrosine kinase (Lck), interleukin-2-inducible T cell kinase (Itk),filamin-A, proto-oncogene tyrosine-protein kinase Fyn and growth factor receptor-bound protein 2 (Grb2) (August & Dupont 1994; King et al. 1997; Okkenhaug et al. 2001; Salojin et al. 1999) initiating the adaptive immune response (figure 6-1).

Figure 6-1 The content has been removed due to copyright restrictions.

Upon activation, PI3K induces the production of the D3-lipids, phosphatidylinositol (3,4)-bisphosphate (PIP2) and phosphatidylinositol (3,4,5)-triphosphate (PIP3) where PIP3 is the main TCR/CD28 induced D3-lipid. (Sasaki 2000). These D3-lipids recruit pleckstrin homology (PH) domain containing proteins including phosphoinositide-dependent kinase 1 (PDK1), protein kinase B (PKB)/Akt and possibly guanine-nucleotide exchange factor Vav. (Parry et al. 1997; Costello et al. 2002; Harriague & Bismuth 2002).PKD1 mediated phosphorylation of PKB/Akt at Threonine 308 leads to a regulation of downstream targets (figure6-2), that increase the transcriptional regulation of the nuclear factor of activated T-cells (NFAT) and activates nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB). PKB /Akt is also phosphorylated at Ser 473, the regulation of which is not fully understood, but may be influenced by auto phosphorylation following Thr308 phosphorylation.(Vanhaesebroeck & ALessi 2000; Osaki et al. 2004; Hemmings & Restuccia 2012).

Figure 6-2 The content has been removed due to copyright restrictions.

Grb2 can also bind to the proximal motif may subsequently bind Vav, activation of which leads to cytoskeletal re- arrangement and through mitogen-activated protein kinases (MAPK) activation, to the induction of formation of the activator protein-1 transcription (AP-1) complex (Collins et al. 1997; Su et al. 1994). CD28 can also activate ZAP70, that phosphorylates Vav and can lead to AP-1 complex formation in a Grb2 independent manner (Salojin et al. 1999). Vav also binds to the linker for activation of T cells (LAT) that ultimately leads to calcineurin and protein kinase C-θ (PCKΘ) activation. Calcineurin activation allows the nuclear translocation of NFAT and PKCθ activation leads to the formation of a multi-protein complex that induces NF-κB transcriptional activation (Fraser et al. 1991).

The IL-2 promoter has binding elements for members of the NFAT, NF-κB, and AP-1 (c-fos/c-Jun), therefore CD-28 activation leads to increased gene transcription. There is also evidence that it contributes to post-transcriptional stabilization of mRNA by inactivating specific AUUA sequences in the 3′ untranslated region that destabilise the message (Lindstein et al. 1989; Granelli-Piperno & Nolan 1991; Bohjanen et al. 1992; Shapiro et al. 1997). Further to the role of CD28 signalling in enhancement of IL-2 production, it enhances T-cell survival (Boise et al. 1995; Noel et al. 1996; Radvanyi et al. 1996) and regulates T-cell subtype development and differentiation (mainly Th2) (King et al. 1995; Rulifson et al. 1997).

We hypothesized that the CD28 signalling pathway in T lymphocytes is overexpressed in individuals who spontaneously cleared HCV infection (SR), compared to patients with chronic hepatitis C infection (CHCV) and individuals with natural resistance to HCV (EU).

6.2   Methods

PI3K, Akt1, and ZAP70 relative expression were measured by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) in (1) patients who spontaneously resolved HCV infection (HCV Ab positive and HCV RNA negative); (2) treatment-naïve chronic HCV patients (HCV Ab positive and HCV RNA positive); and (3) individuals with apparent resistance to HCV (HCV ab and HCV RNA negative). Comparative basic relative quantification was performed in each group and the differential gene expression between 2 groups was calculated using the $2^{-\Delta\Delta CT}$ method by the mean $2^{-\Delta CT}$ of each group. Coefficient variation of the $2^{-\Delta CT}$ values within a group was calculated as an indicator of intra sample variability. Details on the recruitment of the 3 groups, laboratory technique and the analysis methods are described in detail in 2.6.2.

PI3K p110δ, Pan Akt and Phospho-Akt (pSer473 &pThr308) protein levels were compared between individuals in each of the above groups using Western blot and semi quantification analysis using "ImageJ" software. The results are expressed as the mean of the adjusted relative density of each group. The methods are described in detail in chapter 2.

6.3 Results

6.3.1 Study subjects for quantitative reverse transcriptase polymerase chain reaction and Western Blot analysis

Quantitative reverse transcriptase polymerase chain reaction for PI3K, Akt1 and ZAP70 was performed in a total number of 18 individuals, including 6 EU, 6SR and 6 CHCV-genotype 3, none of whom were included in the micro array. The demographics of the individuals included in the Akt1, PI3K and ZAP70 studies are summarised in table 6.1.

Western blot analysis and protein quantification for PI3K p110δ, Pan Akt and Phospho-Akt (pThr308) was performed in the same individuals listed above. Due to limitations in cell availability, Phospho-Akt (pSer473) Western Blot analysis and protein quantification studies were performed in similar groups, but some of the individuals included were different to the ones described in table 6.1, so their demographics are summarised in table 6.2.

Characteristic	EU(n=6)	SR(n=6)	CHCV(n=6)
Mean age	30.8	41.3	37.4
Male gender (%)	83.3	50	66.7
Serology (HCV Ab/HCV RNA)	-/-	+/-	+/+
Mean life time injecting episodes (rounded in hundreds)	4900	4700	3900
HCV Genotype	N/A	N/A	3
IDU at time of recruitment	Yes	Yes	Yes
Sharing needles (%)	83	50	67
Sharing any drug injection equipment (%)	100	100	100
Additional co morbidities	No	No	No

Table 6-1 Demographics of individuals included in Akt1, PI3K and ZAP70 q-PCR and phospho-Akt (pThr308) and PI3K p110δ, Pan Akt Western Blots.

Note: n=number; N/A; non-applicable; EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection.

Characteristic	EU(n=6)	SR(n=6)	CHCV(n=6)
Mean age	34.1	45.2	36.5
Male gender (%)	83.3	50	66.7
Serology (HCV Ab/HCV RNA)	-/-	+/-	+/+
Mean life time injecting episodes (rounded in hundreds)	5100	4200	3900
HCV Genotype	N/A	N/A	3
IDU at time of recruitment	Yes	Yes	Yes
Sharing needles (%)	66	50	66
Sharing any drug injection equipment (%)	100	100	100
Additional co comorbidities	No	No	No

Table 6-2 Demographics of individuals included in phospho-Akt (pSer473) Western Blot.

Note: Note: n=number; N/A; non-applicable; EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection.

6.3.2 Quantitative reverse transcriptase polymerase chain reaction results for PI3K, Akt1 and ZAP70 show no significant difference between the groups

Relative PI3K, Akt1 (Akt serine/threonine kinase 1) & ZAP70 gene expression was not elevated in individuals who spontaneously cleared HCV (SR) compared to treatment naïve patients with CHCV –genotype 3 (CHCV) and individuals with natural resistance to HCV (EU). Further supported by the Western blot study results discussed in 6.3.3., it was felt that CD86, CD80 and CD28 q-PCR were not required for completion of this part of the study.
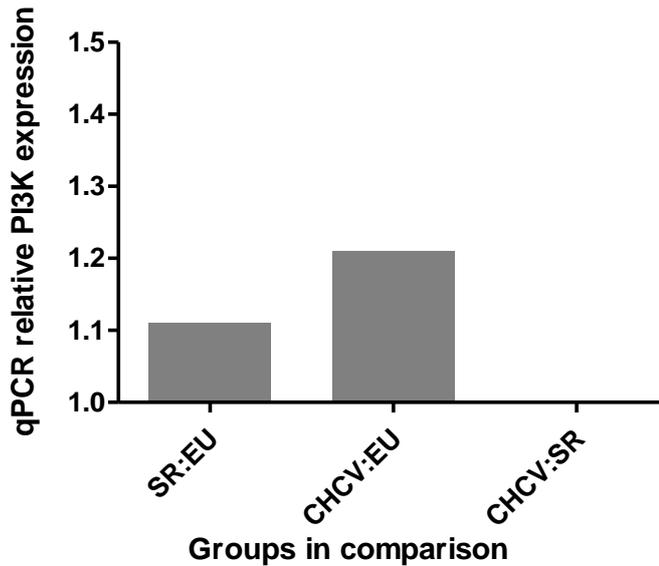
Figure 6-3 PI3K qPCR in comparison groups.

Note: y axis represents absolute fold change. EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection. The bars represent PI3K expression fold change between the mean of 2 groups. Coefficient variation (CV) of the $2^{-\Delta CT}$ values within each group was 9.1% for EU, 6.2% for SR and 9.3% for CHCV.
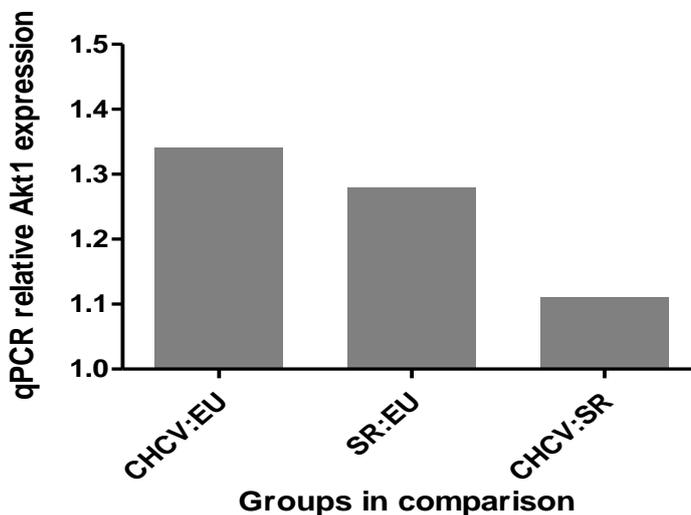
Figure 6-4 Akt1 qPCR in comparison groups.

Note: y axis represents absolute fold change. EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection. The bars represent Akt1 expression fold change between the mean of 2 groups. Coefficient variation (CV) of the $2^{-\Delta CT}$ values within each group was 16.8% for EU, 9.2% for SR and 6.3% for CHCV.
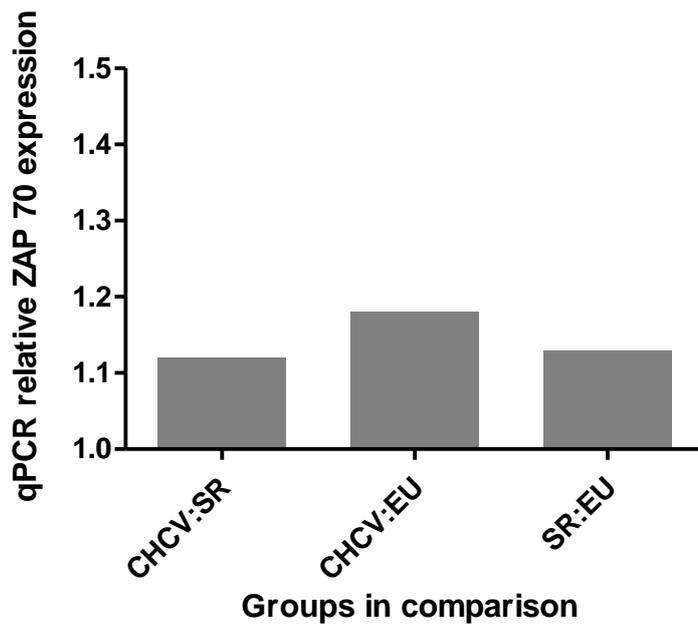
Figure 6-5 ZAP70 qPCR in comparison groups.

Note: y axis represents absolute fold change. EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection. The bars represent ZAP70 expression fold change between the mean of 2 groups. Coefficient variation (CV) of the $2^{-\Delta CT}$ values within each group was 4.8% for EU, 5.6% for SR and 11.8% for CHCV.

6.3.3 Protein semi-quantification shows that PI3K p110δ, Pan Akt, Phospho-Akt (pSer473 &pThr308) levels are not elevated in SR compared to both EU and CHCV

PI3K p110δ, Pan Akt, Phospho-Akt (pSer&pThr308) levels are not elevated in individuals who spontaneously cleared HCV (SR) compared to treatment naïve patients with chronic hepatitis C (CHCV) –genotype 3 (CHCV) and individuals with natural resistance to HCV (EU). Due to findings that do not confirm our initial hypothesis, ZAP70 Western blot was not performed. The mean level of proteins studied seemed to be higher in the CHCV group compared to the others, but not all group comparisons reached statistical significance.

EU1   EU2   EU3 EU4 EU5 EU6 CHCV1 CHCV2 CHCV3

CHCV4 CHC5   CHCV6 SR1 SR2 SR3 SR4 SR5 SR6

Figure 6-6 Loading control for PI3K p110δ, Pan Akt and Phospho-Akt (pThr308) Western blots.

Note: EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection.

EU1   EU2   EU3   EU4   EU5   EU6 CHCV1 CHCV2 CHCV3
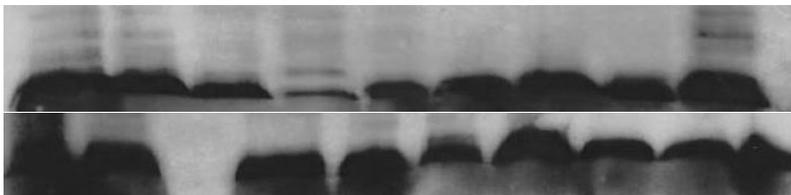
CHCV4 CHCV5   CHCV6   SR1   SR2   SR3   SR4 SR5   SR6

Figure 6-7 Pan-Akt Western blot.

Note: EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection.

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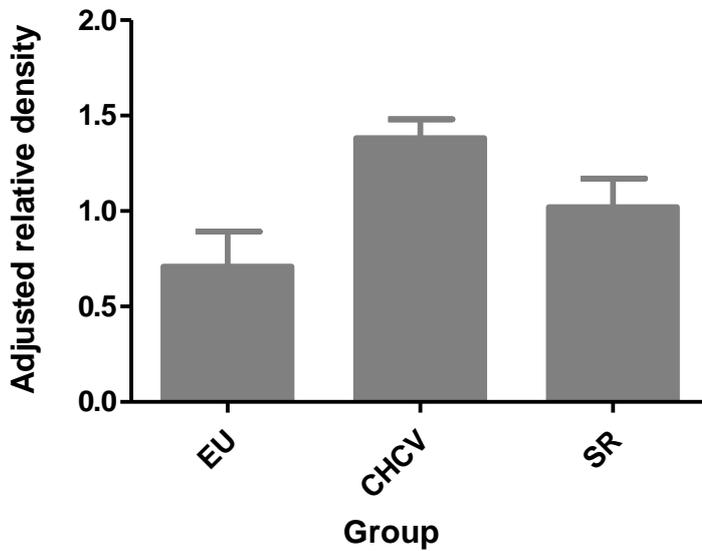
Figure 6-8 Pan-Akt adjusted mean relative density between EU, CHCV and SR.

The mean adjusted relative density for Pan-Akt, corresponding to the mean amount of Pan-Akt in each group, was higher in in patients with CHCV infection (CHCV), compared to individuals who are exposed to HCV but remain uninfected (EU) or have spontaneously cleared HCV (SR); Kruskal-Wallis, p=0.03.The EU group has the lowest levels of Pan-Akt. The bars represent standard error of the mean.
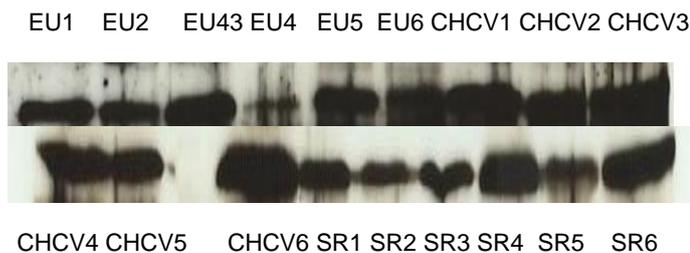
Figure 6-9 PI3K-p110δ Western blot.

 Note: EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection.
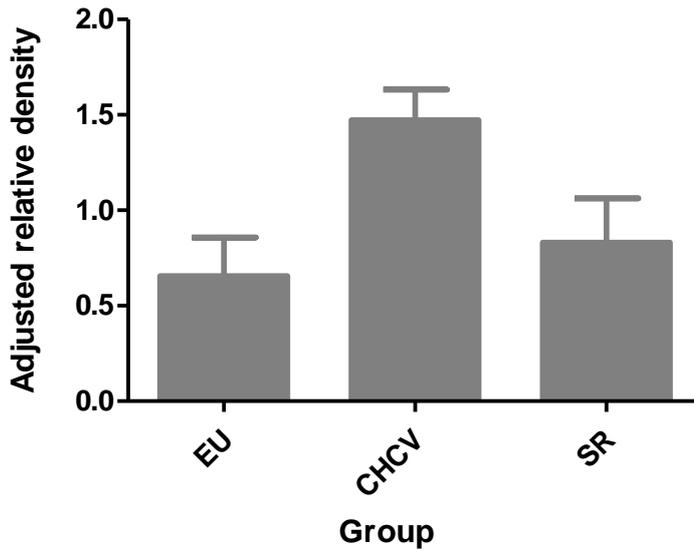
Figure 6-10 PI3K-p110δ mean adjusted relative density between EU, CHCV and SR.

The mean adjusted relative density, corresponding to the mean amount of PI3K in each group is not elevated in individuals who are exposed to HCV but remain uninfected (EU), compared to patients with CHCV infection or individuals who are exposed to HCV but remain uninfected (EU); Compared pairwise, CHCV have statistically significant higher median PI3K levels compared to EU only ;Mann-Whitney, p=0.02 The EU group has the lowest levels of PI3K-p110δ. The bars represent standard error of the mean.
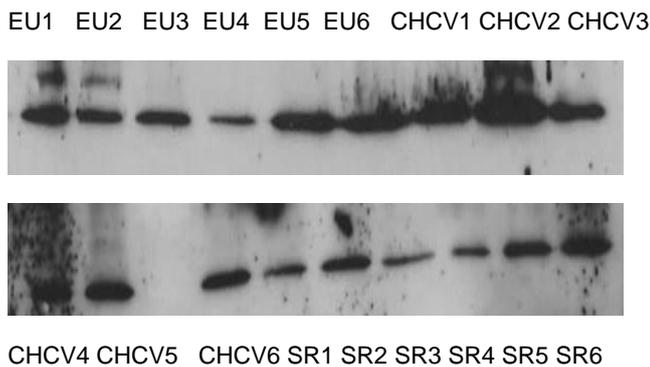
Figure 6-11 Phospho-Akt (pThr308) Western blot.

Note: EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection.
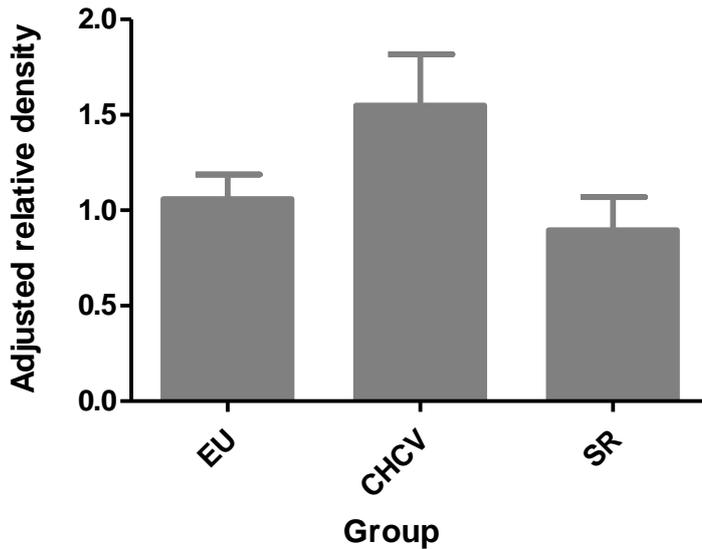
Figure 6-12 Phospho-Akt (pThr308) mean adjusted relative density between EU, CHCV and SR.

The mean adjusted relative density of phospho -Akt (pThr308), corresponding to the mean amount of phosphor-Akt (pThr308) in each group, is not higher in individuals who spontaneously cleared HCV infection (SR), compared to individuals who are exposed to HCV but remain uninfected (EU) or patients with chronic hepatitis C infection (CHCV). The median level of phospho -Akt (pThr308) is higher in the CHCV group compared to EU and SR but this difference did not reach statistical significance (Mann-Whitney; p=0.23 and p=0.31 respectively). The bars represent standard error of the mean.

Figure 6-13 Loading control for Phospho-Akt (pSer473).

Note: EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection.

CHCV4 CHCV5   CHCV6 SR7 SR8 SR3 SR4 SR5 SR6

EU6   EU7   EU8   EU1 EU2 EU3 CHCV1 CHCH2 CHCV7
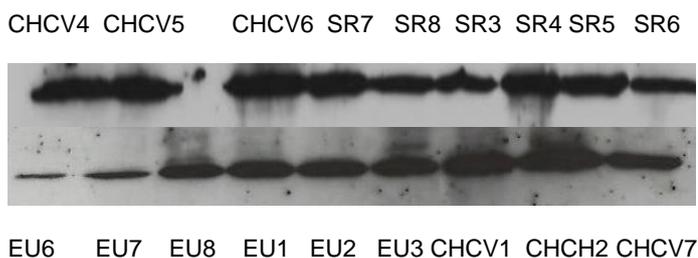
Figure 6-14 Phospho-Akt (pSer473) Western blot.

Note: EU=exposed uninfected; SR=spontaneous resolver; CHCV=patient with chronic HCV infection.
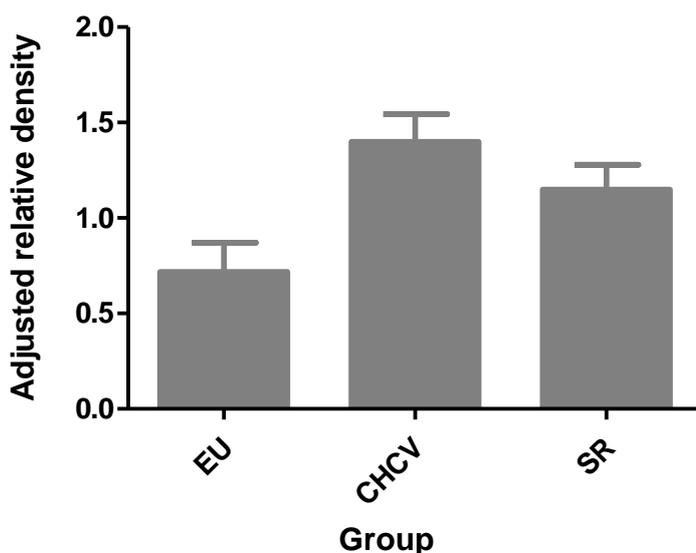
Figure 6-15 Phospho-Akt (pSer 473) mean adjusted relative density between EU, CHCV and SR.

The mean adjusted relative density of phospho-Akt (pSer473), corresponding to the mean amount of phosphor-Akt (pSer-473) in each group, is not higher higher in individuals who have spontaneously cleared HCV (SR), compared to individuals who are exposed to HCV but remain uninfected (EU) or patients with chronic HCV infection. The EU group has the lowest levels of Phospho-Akt (p Ser473). The CHCV group has higher median levels of phospho-Akt (pSer473) compared to the 2 other groups, but this observation was statistically significant only between the EU and HCV group; Mann-Whitney, p=0.02). The bars represent standard error of the mean.

6.4 Discussion

As mentioned in 6.1, the CD 28 signalling system in T helper lymphocytes is one of the most important and well studied T-cell stimulatory pathways, activation of which leads to enhanced production of IL-2 and T-cell survival. CTLA-4 antagonizes this effect and down regulates T-cell function by providing a negative co- stimulatory effect. Certain CTLA-4 SNPs have been associated with persistent HCV viraemia (Danilovic et al. 2012) and response to treatment with ribavirin and interferon (Yee et al. 2003). Gender-dependent association of CTLA-4 SNPs are, also, associated with spontaneous resolution of HCV infection (Schott et al. 2007). As a result, and based on the micro array findings, we hypothesized that CD28 signalling system in T helper lymphocytes is up regulated in individuals who spontaneously cleared HCV compared to patients with chronic HCV infection and individuals with apparent resistance to HCV. The q-PCR and western blot results are not supportive of this hypothesis and that could be explained due to a variety of factors, mainly related to host variability. However, the gene and protein expression of the studied molecules is, by and large, lower in EU cohort compared to the others, suggesting that activation of the adaptive immune system is unlikely to be the only mechanism of natural protection from HCV and distinguishes them further from SR.

Due to constraints related to cell availability, none of the individuals included in the micro array took part in these studies. There is significant inter individual CD4+ cell variability in the healthy general population, most marked for cytokine transcripts, that follows patterns more complex than Th cell partition and includes 39 genetic loci(Ye et al. 2014).Studies of the immune system in monozygotic twins, have shown that homeostatic cytokine responses are largely heritable, whereas most other immune cell responses are highly non-

heritable (Brodin et al. 2015) .A single non inheritable factor can affect more than 50% of all immune cell subsets and serum proteins with Cytomegalovirus (CMV) infection having the broadest influence and affecting more than 10% of T cells (Sylwester 2005).Additionally, highly variable factors in an individual, such as stress, are negatively related to the number of T helper cells at a given time (Herbert & Cohen 1993). It is, therefore, possible, that the suggested hypothesis in this chapter is only true for the individuals that were included in the micro array.

The major limitation in this study, related to the available cells of the individuals included, is that it did not allow separation of T cell subsets from PBMCs, so we have, essentially, measured the total (B and T lymphocyte) Akt, Phospho-Akt (pThr308 &pSer473) and PI3K that can lead to an erroneous result. The nature of the cohorts recruited, described in detail in 2.1, is such that obtaining large number of cells from each individual is difficult so that further detailed study will always be very challenging. As a result we felt that the experimental conditions could not be, realistically, further optimized and refined within the time available for this thesis.

Chapter 7 Discussion

7.1 Summary

The studies of this thesis were aimed to further elucidate the immune mechanisms of natural protection from HCV infection. The work in this thesis focused on studying the transcriptional gene profile of a well characterised cohort of IDU with apparent resistance to HCV infection (EU), whose immune mechanisms of HCV protection remain, to date, unclear. This aim notwithstanding, we also attempted to validate findings in a comparison group, individuals who spontaneously cleared HCV infection (SR), whose mechanisms of viral clearance have been better characterised. This attempt would not only provide new insight into the mechanisms of spontaneous clearance of HCV infection, but also highlight similarities or differences between EU and SR. The latter is important to further differentiate the EU from the SR group in addition to what has been previously described.

The EU have, so far, demonstrated immune characteristics involving the activation of both the innate and the adaptive arms of the immune system that can contribute to protection from HCV infection. We, therefore, performed a microarray analysis on RNA extracted from PBMC of EU, SR and patients with chronic HCV infection of the same genotype, all of whom were active IDU at the time of recruitment. The changes in differential gene expression were less prominent in EU compared to SR and CHCV, which is expected given the fact that EU are healthy individuals with no evidence of previous or established HCV infection. Since the RNA was extracted from PBMC as opposed to hepatocytes, we appreciated changes of any magnitude, if of relevance to the aims of the study.

We found that IL-27 is overexpressed in EU, compared to SR, CHCV and normal controls. This pleiotropic cytokine has a wide range of actions in both the innate and the adaptive immune system and could serve as a link between the two. However, its role in natural protection from HCV infection is yet to be elucidated.

The gene with the highest fold change between the EU and the other groups was CXCL7 that could contribute, amongst other things, to the prevention of viral entry prevention. Albeit the levels of CXCL7 were elevated in EU compared to SR and CHCV, the result reached statistical significance only between the EU and CHCV groups. Limitations in resources and technical challenges in the assays used, allowed testing of a limited number of subjects which might have affected the outcome.

The change in the differential expression in the genes tested in SR, were of lower magnitude but could be closely linked to HCV clearance. IL-16, a cytokine with proven direct antiviral properties, and the CD28 T cell co stimulatory signalling system did not seem to be overexpressed in SR compared to EU and HCV. Study design and resource availability could, again, have affected the outcome and these hypotheses cannot be safely rejected.

This is the first study that has examined the transcriptional profile of individuals with apparent resistance to HCV infection and has yielded interesting findings that can trigger further studies in immune factors related to protection from HCV infection. Interestingly, it has failed to demonstrate differential expression in genes related to previous findings regarding the immune characteristics of EU, which, again, highlights limitations in the sensitivity of an assay performed on PBMC, as opposed to hepatocyte, RNA.

As the studies in this thesis, as well as previous studies on the EU cohort, were performed in a cross sectional design, it is difficult to ascertain whether any changes are activated in a transient or temporary manner. Taking into account the difficulties in obtaining longitudinal samples from a large number of the same EU individuals, this question will probably remain unanswered.

Given the fact that HCV has the ability to evade immune response in a variety of ways, it is highly unlikely that a single protective strategy is responsible for conferring resistance and future work should focus on how genetic, innate and adaptive immune factors combine to provide a protective phenotype.

7.2   Areas of future work in immune mechanisms of HCV protection

The work of this thesis has focused primarily on a cohort, characterised since 2005, of IDU whose susceptibility to HCV infection is low. It was initially shown, that this cohort had an immunological footprint of exposure to HCV infection but no evidence of established HCV infection as evidenced by the presence of weak, HCV specific, T cell responses, but the absence of HCV RNA and ab positivity. On- going recruitment of EU individuals was based on identification of demographic factors, comparable to the ones so far described, that pose an IDU at high risk of HCV exposure. These factors are, however, self- reported and the need for further characterisation of this cohort, based on unique genetic and functional attributes, is of paramount importance and has, also, been previously suggested. The main issue arising from the current risk stratification is mislabelling an SR, with loss of HCV ab reactivity over time, as an EU. It is well established, and also suggested in the studies of this thesis, that the mechanisms of spontaneous resolution of HCV infection in SR are related to the activation of the adaptive immune system, so the presence of low level HCV

specific T cell reactivity, lack of HCV ab reactivity and favourable IL-28B related polymorphisms in EU could distinguish them from this group.

Resistance in this cohort is not absolute and can be overcome by the inoculum size. Therefore obtaining blood samples from individuals before and after they become infected would provide a valuable control group. As previously mentioned in this thesis, recruitment of these individuals and follow up is an extremely challenging process and in order to achieve longitudinal follow up recruitment has to be uninterrupted and in close liaison with the services that these individuals access at needle exchanges, drug rehabilitation centres and drop in centres.

IL-27 has been shown to be overexpressed in the EU cohort, a finding validated both at transcriptional and translational level. As previously discussed, IL-27 can act as a type I IFN in the context of viral infection which would be a plausible mechanism of action in the EU group. IFN-a inducible genes were not upregulated in EU compared to other groups based on the microarray analysis but this is an area that requires further study. Enhancement of NK cell cytotoxicity, that has previously been shown to contribute to the cohort's viral resistance, is another possible role of IL-27 in immune protection from HCV. Elevated levels of Treg cells, which IL-27 suppresses, are associated with HCV viral persistence and antibody depletion of these cells are associated with an in vitro enhancement of functional HCV-specific CD8+ T cell responses, so comparison of Treg levels in EU vs CHCV vs SR would be beneficial. As IL-27 primarily enhances Th1 development and function, measurement of HCV specific T cell responses in all the EU individuals would further elucidate the role of adaptive immunity in protection from HCV infection. Studying the

absence of the SNP c.-964A>G (rs153109) of the IL-27p28 gene in EU, which is associated with treatment relapse or non -response, would also be informative.

CXCL7 levels need to be measured in larger numbers of EU, CHCV and SR in order to reach a conclusion about its role, if any, in HCV protective immunity.

This study aimed to determine the correlation of genetic and functional factors in protection from HCV and this work should be ongoing. New developments, such as RNA-Seq technology that is more sensitive to microarray and provides information regarding the transcriptome in a non hypothesis driven manner may be informative in the EU cohort. The effect of environmental factors in functional correlates should also be taken into consideration and continued testing over time might provide the answer to this question.

Hepatitis C virus is a global health burden and continued study of the EU cohort will possibly provide valuable insight in protective mechanisms that will aid the development of a preventative vaccine as well as the identification of new therapeutic targets in the future.

Appendices

8.1 IPA factsheet

IPA – Ingenuity Pathway Analysis from *QIAGEN* (USA)

Date: last updated August 2016

For the interpretation of ´Omics (Proteomics & Genomics) data.

(IPA is updated on a weekly basis and has four releases per year.).

The "IPA-Core Analysis" quickly explores (in circa 7 minutes) relationships, mechanism, functions, and pathways which are relevant for a dataset. The regulator analysis surfaces molecules as Bio-Profilers identify molecules which are causally necessary to a disease or phenotype. Observations of upstream- and downstream effects of biological processes in IPA, support to create new scientific analyses of genes.

Special graphical features and evaluated literature:

- The Ingenuity Knowledge Base database contains around 5.5 million findings.

(context-based results, spring 2016) e.g. for specifies, diseases, mutation types and relationships.

-Findings from 3600 journals (abstracts) had been reviewed and included; more than 300 known full text journals were curated manually, including tables and figures. Publications were included from 1954 till today.

- "Core Analysis": In a first step Identifiers (like genes, proteins and RNA sequences) are generated as genes. Furthermore each gene is supplemented with a description, location, family and related drugs.

- In a second step IPA determines the p-value(s), (means probability-value) of the most known canonical pathways, up- and downstream regulators (down= arrow in red, up= arrow in green) and diseases/biological functions.

- **Graphic representation**:

Features & tools of the pathways visualize direct (designed as line) or indirect (designed as broken line) interactions or the increase or decrease of biological functions with different colors and create an interactive gene view.

Species-specific identifiers for:

- Mammalia: Human, Mouse and Rat.

- New: Dog, Zebrafish, Arabidopsis, Nematode, Fruitfly.

Choice of reference sets of the "IPA-Core Analysis":

- Ingenuity Knowledge Base (Endogenous Chemicals only).

- Ingenuity Knowledge Base (Genes only).

- (User Dataset).

- Affymetrix.

- Agilent.

- Code Link.

- Illumina.

- Life Technologies (Applied Biosystems).

Other Analysis options to Core Analysis:

- Tox Analysis.

- Metabolomics Analysis.

- Biomarker Filter.

- Filter Dataset.

- microRNA Target Filter.

- IsoProfiler Beta.

Formats for data-analysis:

- A dataset can be quickly uploaded as excel-file or text-file.

- Data can be explored tab delimited as text-format or as excel-file.

- A summary sheet of the analysis can be explored as pdf.

- The generated images can be produced in jpg-format which is available for print in 300/600 dpi as for publications or for presentations in 96 dpi; in case of publication please cite IPA!.

General upload raw dataset:

- Only one column may be designated as the ID column. A gene/protein ID is a unique public or vendor identifier that represents a gene or protein. IPA takes items found in the Gene/Protein ID column and attempts to map them to genes that exist in the Ingenuity Pathways Knowledge Base.

- Observation 1 to (…) is matched to the one and same ID; so that each observation must have the same number, type and order of expression value columns.

- A maximum of 20 observations in a single file may be uploaded into IPA.

- Only one header raw is allowed (except for metadata rows).

- "Networks" can only be composed in case of scores.

Markup languages for uploading the Users imported pathway workflows:

- SBML (all versions and levels).

- BioPax (all versions and levels).

- SIF.

- XGMML.

- PSI-M.

The IPA-user can import his own created pathways. Furthermore the user can also modify his pathways by adding molecules or generating new pathways' interactions. The graphic representation tool allows it to visualize these connections.

With the "compare"-Function the user can compare results of different analysis and visualize as list intersections.

E. Schlagberger – Scientific Information Services f. t. Biomedical-Section of the Max-Planck Society, April 2016.

<u>Why are some people susceptible to hepatitis C and not others?</u> A study of innate and immunological mechanisms of protection.

The participant should complete the whole of this sheet himself/herself.

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 of the study?
    …………………………………………………………………………………
    ....

6. Do you understand that you are free to withdraw from the study:
- At any time?
- Without having to give a reason?
- Without affecting your future medical care or drug treatment
    program?
    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 having some of your blood taken and
    kept so we can study factors affecting susceptibility to
    infection
    with hepatitis C virus?   YES/NO

10. Do you agree to take part in this study?
    YES/NO

Surname …………………………………………………………………………

First Name …………………………………………………………………………

Address …………………………………………………………………………..

…………………………………………………………………………………..

D.o.B. …………………………………. Hospital Number ………………………..

Participant

Signature …………………………………………………………………………

Name (CAPITAL LETTERS) …………………………………………………..

Date …………………………………………………………………………

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

Investigator

Signature …………………………………………………………………………

Name (CAPITAL LETTERS) …………………………………………………..

Date …………………………………………………………………………

Plymouth Hospitals **NHS**

NHS Trust

<u>Why are some people susceptible to hepatitis C and not others?</u>

A study of innate and immunological mechanisms of protection.

Questionnaire

Date: Trial No:

Initials: Date of Birth: Age:

1.   How old were you when you first used drugs IV? ………………………..

2.   Did anyone else teach/ help you with your first injection?   YES/NO

3.   Roughly how old was the person who injected for you?.........................

4.   How long were you being injected before you could to do it yourself?

………………………………………………………………………………....

5.   How many years have you been/were you injecting drugs? ……………

6.   At the most, how often were you injecting during that time?

Less than once a month	□	About once a month	□
2 – 3 times a month	□	About once a week	□
2 – 3 times a week	□	Most days	□

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| 1 – 3 times a day | □ | 4 – 6 times a day | □ |
| More than 6 times a day | □ | | |

7.   Which drug(s) did you mainly inject?

Heroin	□
Crack	□
Cocaine	□
Amphetamines (speed)	□

Other……………………………………………………………………………

8.   Do you still use drugs IV?   YES/NO

If not, when did you stop? ……………………………………………..

If yes, how often do you inject at the moment?

Less than once a month	□	About once a month	□
2 – 3 times a month	□	About once a week	□
2 – 3 times a week	□	Most days	□
1 – 3 times a day	□	4 – 6 times a day	□
More than 6 times a day	□		

9.   Are you on a maintenance script? (methadone)
        YES/NO

10.   Which drugs have you injected in the last 3 months?

Heroin	□
Crack	□
Cocaine	□
Amphetamines (speed)	□

Other……………………………………………………………………………

11.   Do/have you ever injected with others around?   YES/NO

If yes, roughly how often do you inject around others?

Every time   □

About half the time   □

Most times   □

Rarely   □

12. Where is the most common place you inject with others?

Home   □

Friend's house   □

Shooting gallery/ Sorter house   □

Outside/ Public areas   □

Other place   □

Please specify……………………………………………

13. Have you ever shared a needle (pin) with anyone else (using it either before OR after them)?   YES/NO

If yes, roughly how many times?

Once   □   Rarely   □

About half the time   □   Most times   □

Every time   □

If yes, when was the last time? …………………………………………..

14. Have you ever shared a syringe (barrel) with anyone else (using it either before OR after them)?   YES/NO

If yes, roughly how many times?

Once   □   Rarely   □

About half the time ☐   Most times   ☐

Every time   ☐

If yes, when was the last time? …………………………………………..

15.   Have you ever shared a spoon/ water container/ filter with

anyone else (using it either before OR after them)?   YES/NO

If yes, roughly how many times?

Once   ☐   Rarely   ☐

About half the time ☐   Most times   ☐

Every time   ☐

If yes, when was the last time? …………………………………………

16.   Have you ever shared ANY injecting items with someone you

KNOW has hepatitis C (using it before OR after them)?   YES/NO

If yes, what did you share? …………………………………………….

If yes, roughly how many times have you shared with someone you know has hepatitis C?

Once   ☐   2 – 10 times ☐

More than 10 times ☐   Every time   ☐

When was the last time?..

17.   Have you ever snorted drugs?   YES/NO

If yes, did you share the straw/banknote etc with anyone else

(using it either before OR after them)?   YES/NO

18.   Have you ever shared a crack pipe with anyone else

(using it before OR after them)?   YES/NO

19.   Have you ever received a blood transfusion or blood products?
                                                  YES/NO/DON'T KNOW

If yes, in which year/years? …………………………………………………

20.   Do you have tattoos?   YES/NO

If yes, how many? …………………………………………………………

If yes, where did you get these done?

Professional parlour   □   Friend did it   □

Did it yourself   □   In Prison   □

Other (please state) …..

21.   Have you ever had any part of your body pierced?   YES/NO

If yes, how many? …………………………………………………………

If yes, where did you get this done?

Professional parlour   □   Friend did it   □

Did it yourself   □   In Prison   □

Other (please state) …..

22.   Have you ever had acupuncture?   YES/NO

If yes, where? …………………………………………………………………

23.   Do you know if anyone you've ever had sex with has/had

hepatitis C?   YES/NO/DON'T KNOW

If yes, did you use a condom?   YES/NO

24.   Does anyone else that you live with inject drugs?   YES/NO

25.   Have you ever been in Prison?   YES/NO

Thank you.

Study Title

<u>Why are some people susceptible to hepatitis C and not others.</u>

What is the purpose of the study?
Hepatitis C virus (HCV) currently infects 200,00 to 400,000 people in the United Kingdom. Many people have become infected through injection drug use and up to 75% of people who have injected drugs for 6 months or more will have hepatitis C virus infection. However, some people who have injected drugs for years or shared injecting equipment with people known to have hepatitis C do not become infected and test negative for both antibody and virus. It is these people we are interested in for this study. We wish to understand what can make some people resistant to infection with hepatitis C virus.

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?
You have been potentially exposed to hepatitis C through injection drug use, however your results show no sign of hepatitis C virus infection. It may be that your immune system has protected you against hepatitis C virus, or it may be that you have inherited some factor that makes you resistant to this infection.

We wish to study your blood for immune responses and genetic factors that may have protected you. We are hoping to study about 50 patients in a similar situation.

Who is organising the study?
Dr Matthew Cramp, Consultant Hepatologist and Honorary Senior Lecturer in Medicine is running this research project with the help of NHS Research and Development money at Derriford Hospital, Plymouth. The research will take place at Derriford Hospital, Plymouth and at Plymouth Postgraduate Medical School.

What will happen to me if I take part?
If you agree to take part in our study

- We will ask you to fill in a questionnaire detailing your injection drug usage to assess the duration and degree of your exposure to hepatitis C virus infection.
- We will ask your permission to take about 40 mls of your blood (two tablespoonfulls). Your blood will be tested for hepatitis C once again and will be used to study immune responses and genetic factors that may have protected you from infection.
- With your permission, we will store some of your blood for testing in the future when additional genetic factors likely to influence susceptibility to hepatitis C have been identified.
- **We may ask you to return at specific time intervals in the future for further blood tests.**

Are there any disadvantages in taking part in this study?

There may be some soreness and bruising after having the blood sample taken.

We will be testing you on several occasions for any evidence of hepatitis C virus infection and it is possible that you will be found to have hepatitis C virus infection by highly sensitive modern tests. If this is the case then you will be informed of the result and you will be referred to our hepatitis clinic for further information and assessment.

What are the possible benefits of taking part?
There are no direct benefits to you from participating in this study. However, information learnt from you may help towards our understanding of this disease and will be a step closer towards developing a vaccine for hepatitis C.

Is my doctor being paid for including me in the study?
No.

Are there any restrictions on what I might eat or do?
No.

What if something goes wrong?
If taking part in this study harms you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action. Regardless of this, if you have any cause to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.

Confidentiality – who will know I am taking part in the study?
The information will be shared with doctors and nurses involved in the study only. The questionnaire you fill in will be identified by a number only and will be kept securely.

GP Notification
With your permission your GP will be informed that you are taking part in this study.

What will happen to the results of the study?
The results of this study will be presented during national and international specialist meetings. Results will be published in national and international peer review journals. No information identifying you as an individual will be published or presented.

Contact for further information
If you have any problems, concerns, complaints or other questions about this study you should Dr. Matthew Cramp on 01752 792725. Alternatively, you may contact the Consumer Affairs Department, Derriford Hospital on 01752 792648.

Thank you for taking time to consider entering this study.

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