Natural Killer cell activation and evasion during chronic hepatitis C virus infection

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Summary

Hepatitis C virus (HCV) infects 3% of the global population and HCV-related liver inflammation is a major cause of liver failure and hepatocellular carcinoma. Current treatments are based upon long courses of interferon-α (IFNα) injections, which have significant side effects and are only effective in 40-80% of individuals depending on viral genotype. Natural killer (NK) cells are innate lymphocytes, which can kill virally infected cells and are stimulated by IFNα. To establish a chronic infection HCV must evade immune responses. I hypothesised that NK cells are important for the successful eradication of HCV and that chronic HCV infection impinges upon NK cell function to prevent viral clearance. I found that NK cell function was reduced in chronic HCV and correlated with the proportion of $NKp46^{+}$ NK cells *in vitro*. In keeping with these findings NKp46-rich intrahepatic NK cell populations were more activated and the proportion of these cells correlated with liver inflammation. During interferon-α treatment individuals who had the greatest increase in NK cell function in response to increasing stimulation had the fastest rate of viral clearance and were most likely to successfully clear the virus. Using a novel adenovirus vector expressing HCV proteins I have discovered that NS5B protein reduces NK cell cytotoxicity and cytokine production. Therefore, in this thesis I have described novel insights into the mechanisms of HCV immunoevasion, HCV-related disease pathogenesis with implications for viral eradication therapy.

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Abbreviations

a.a	amino acid
ADCC	Antibody Dependent Cell-mediated Cytotoxicty
ALT	Alanine aminotransferase
APC	antigen presenting cell
BAC	Bacterial Artificial Chromosome
BAT 3	HLA-B associated transcript 3
BCR	B-cell antigen receptor
bp	Base pair
BSA	bovine serum albumin
CAR	Coxsackie virus and adenovirus receptor
CBS	Central Biotechnology Services
CD	Cluster of differentiation
cDNA	complementary deoxyribonucleic acid
Cr	Chromium
DAP12	DNAX-activation protein 12
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPase	monodeoxyribonucleoside triphosphate hydrolase
DTT	dithiothreitol
EDTA	ethylenediaminetertraacetic acid
FCS	Fetal Calf Serum
FMO	Fidelity minus one
FNA	Fine needle aspiration
H&E	Haematolxylin and eosin
HAV	hepatitis A virus
HBV	hepatitis B virus
HCC	Hepatocellular carcinoma
HCMV	human cytomegalovirus
HCV	Hepatitis C virus
HDACi	Histone deactylase inhibitors
HF	human fibroblast
HFF	human foreskin fibroblast
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HRP	horseradish peroxidase
IFN	Interferon
IFNα	Interferon alpha
IL	Interleukin
ISGs	Interferon stimulated genes
ITAM	immunoreceptor tyrosine activation motif

IVDU	Intravenous drug use/user
JAK	Janus kinase
JFH-1	Japanese Fulminant Hepatitis -1
KIR	Killer cell immunoglobulin like receptor
LAMP-1	Lysosomal-associated membrane protein-1 (CD107a)
LB	Luria-Bertani media
LCMV	lymphocytic choriomeningitis virus
LLT1	Lectin-like transcript 1
MCMV	mouse cytomegalovirus
MFI	mean fluorescence intensity
MHC	Major Histocompatability Complex
MICA	MHC class I chain-related A
MICB	MHC class I chain-related B
MOI	multiplicity of infection
MxA	myxovirus resistance 1
NANBH	Non A, Non B Hepatitis
NCR	Natural cytotoxicity receptor
NK	Natural Killer cell
NP40	nonyl phenoxypolyethoxylethanol
NS	Non-structural
OAS-1	2-5 oligoadenylated synthase-1
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
PKR	double stranded RNA-dependent protein kinase
PMA	phorbol 12-myristate 13-acetate
pSTAT	phosphorylated signal transducer and activator of transcription
RNA	Ribonucleic acid
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute media
STAT	signal transducer and activator of transcription
SVR	sustained viraemic response
TAE	Tris, acetic acid and EDTA buffer
T_H1	T helper 1
T_H17	T helper 17
T_H2	T helper 2
TRAIL	Tumor necrosis factor-related apoptosis induced ligand
TRIS	tris(hydroxymethyl)aminomehtane
UHW	University Hospital of Wales
UTR	Untranslated region
VSV	vesicular stomatitis virus
SOCS-1	Suppressor of cytokine signalling-1

Chapter 1:

Introduction

1.1 Hepatitis C virus from syndrome to identification

In the 1940s it was recognised that hepatitis could be transmitted by enteric infection or by vertical transmission. These two infections were originally named "infectious hepatitis" and "homologous serum hepatitis" [1] and subsequently renamed type A and type B hepatitis respectively. In 1967 a DNA virus was identified as the causative agent of hepatitis B and was the first member of the Hepadnaviridae family of viruses.[2] Shortly after this discovery it was established that an acute Picornaviridae infection causes hepatitis A. These discoveries allowed the development of serological diagnosis of viral hepatitis. However, it was noted that hepatitis occurred following transfusion, injection of Factor VIII concentrate and on haemodialysis units in the absence of serological markers for hepatitis A or B viruses; this disease was named "non-A, non-B hepatitis" (NANBH).[3]

Unlike hepatitis A virus (HAV) infection NANBH established a chronic disease in a similar pattern to hepatitis B virus (HBV). Initially the disease was described as milder than HBV, with lower elevations in transaminases and bilirubin and a reduced incidence of jaundice. However, it was recognised that fulminant hepatic failure could occur with a 50% mortality rate.[4] Further evidence that NANBH was infectious was gathered from chimpanzee models in which a chronic transaminitis disease state, with similar histological changes to NANBH, was induced following inoculation of blood products from patients with NANBH. In the same study it was demonstrated that the infectious agent responsible was susceptible to lipid solvents and could pass through 80nm filters suggesting a small-enveloped virus.[5]

Plasma with a high NANBH infectious titre was derived from infecting chimpanzees with NANBH serum. Choo et al constructed a library of complementary DNA (cDNA) by

inserting nucleic acids from chimpanzee plasma, which was known to contain a relatively higher infectious titre, into a bacteriophage using random reverse transcriptase primers. The resulting library of 10^6 recombinant phage was screened with the serum of a chronic NANBH patient as a source of antibodies to reveal rare clones. A larger overlapping clone was then derived from the library. Failure to hybridise cDNA with human and chimpanzee DNA by Southern blot analyses, confirmed a non-host non-human clone. The cDNA clone hybridised with RNA from the infected liver confirming the presence of an RNA virus as the cause of NANBH.[6] At the time of discovery the causative virus was estimated to be 5000 to 100000 nucleotides with a single open reading frame (ORF) and was designated hepatitis C virus (HCV). The ability to clone HCV allowed the same group to coat micro-titre plates with the resulting polypeptide to develop an assay to measure serum antibody levels thus giving a diagnostic test for HCV.[7] The discovery of HCV identified a major human pathogen that causes significant global mortality and morbidity and forms a major focus of hepatology, virology and immunology.

1.2 Classification and structure of HCV

HCV forms the hepacivirus genus of the Flaviviridae family of viruses; the other genuses are flavivirus, which includes Dengue and Western Nile virus, pestiviruses and hepatitis G virus (Figure 1.1). More than 100 strains of HCV have been identified and the HCV has been grouped into 6 genotypes.

Flaviviridae are positive sense RNA viruses, thus the open reading frame of the virus can be directly translated into peptides by the host ribosomes without further modification. HCV has an RNA genome of approximately 9600 bases. The ORF consists of 9000 bases and encodes a single polypeptide which is then cleaved to form core protein, envelope proteins 1 & 2 (E1 & E2) and non-structural proteins p7, NS2, NS3, NS4 and NS5A and B (Figure 1.2). At either end of the ORF there are untranslated regions (UTR), which are required for viral replication. At the 5' UTR there is a clover-leaf shaped RNA structure that inserts into the host ribosome and binds to ribosomal RNA to initiate the viral translation.[8]

HCV is replicated by an RNA polymerase, which lacks a mechanism for screening for translation errors and thus, has an error rate of 1 in 10,000-100,000 base pairs copied.[9] This error rate combined with the high rate of virus production of 10^{12} virions per day [10] results in a wide diversity of RNA sequences or viral quasi-species found in the same individual. This diversity is more apparent between geographic areas has resulted in the six major genotypes of HCV being defined with 30-35% difference in the nucleotide sequences between the genotypes.[11]

Hepatitis G virus, genetically the most similar flavivirus to HCV, remains to be fully defined both phylogenetically and as a human pathogen.[12] Recently Stapleton and colleagues have proposed that hepatitis G forms a novel flavivirus genus of Pegivirus.[13] Pegiviruses are positive sense single strand RNA viruses, which appear to transiently infect horses and establish chronic infections in multiple rodent species.[12,14] At present the relationship between HCV and pegiviruses and the potential of pegiviruses to provide animal models of HCV remains to be fully established.

Figure 1.1. Flaviviridae phylogenic tree

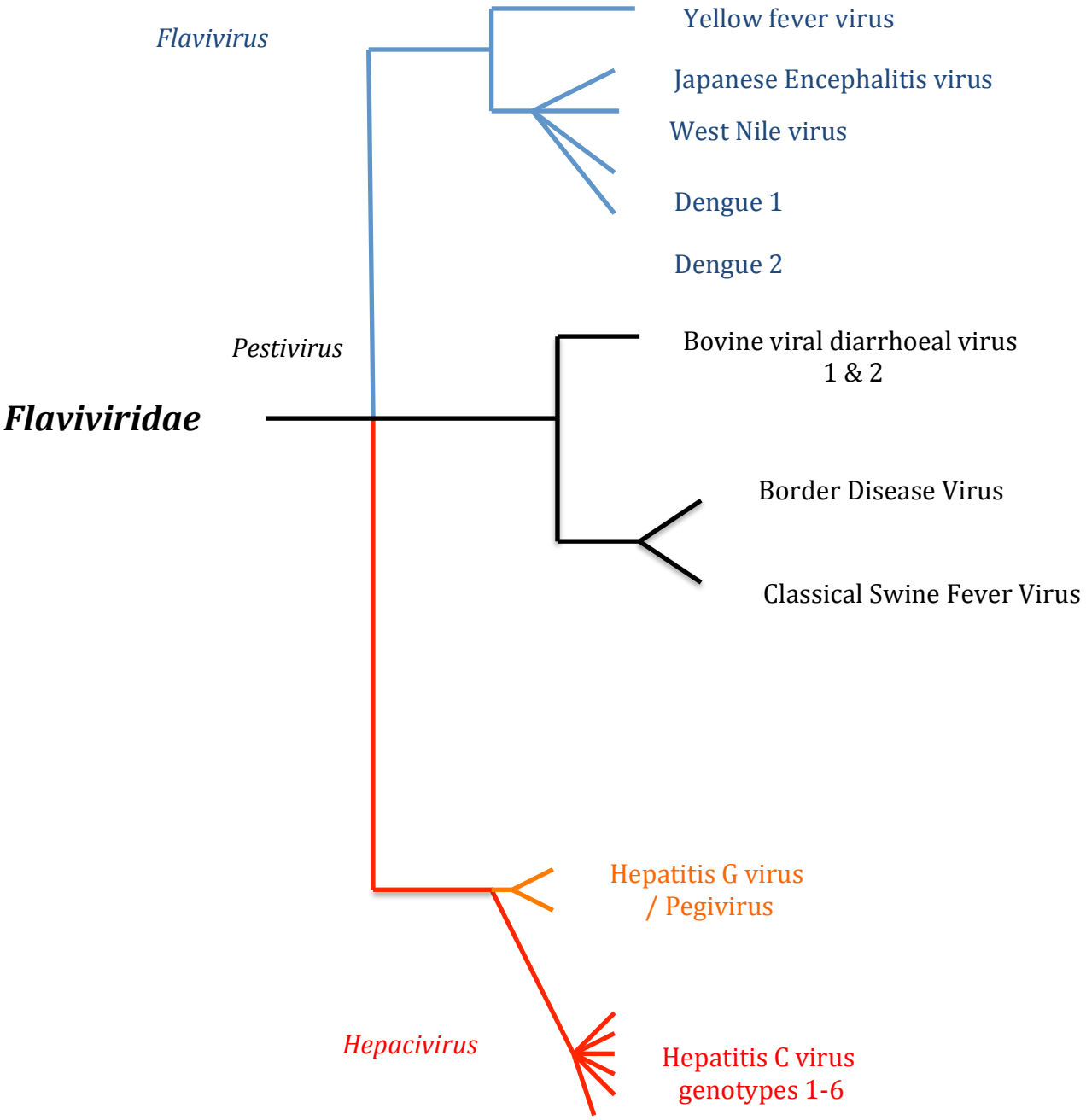
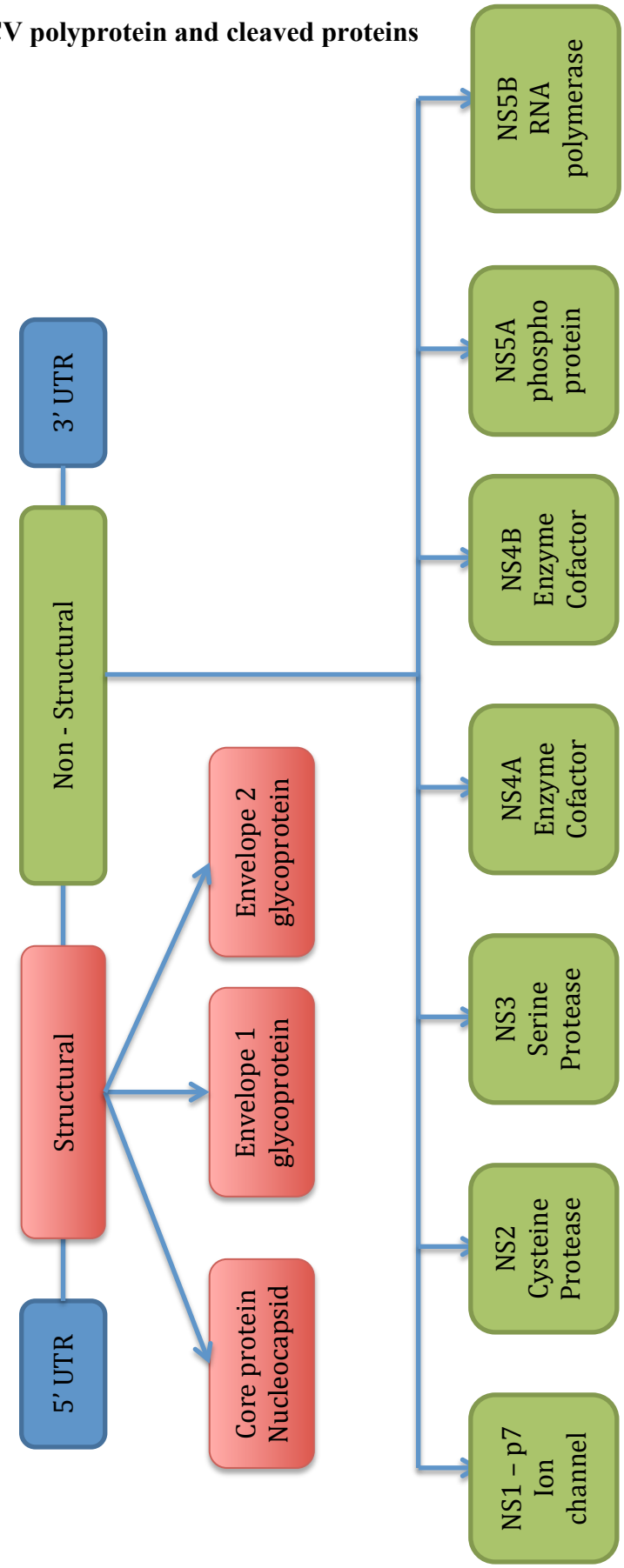


Figure 1.2 HCV polyprotein and cleaved proteins



1.3 Epidemiology of HCV infection

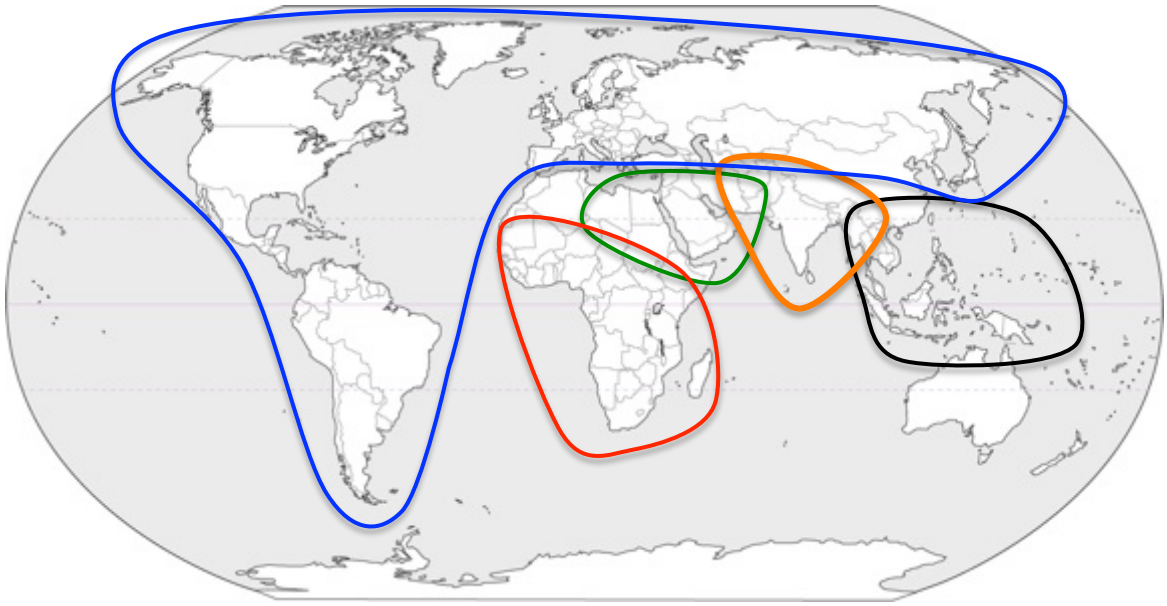
HCV genotypes are most commonly found originating from distinct geographical regions. Genotypes 1, 2 and 3 most commonly seen in Europe and North America, genotype 4 in the Middle East, genotype 5 in Africa and genotype 6 in Asia (Figure 1.3). World Health Organisation estimates of the prevalence of HCV infection are approximately 2-3% of the global population, representing 130 million individuals.[15,16] The global prevalence is estimated from nationally reported seroprevalence in blood donors and individuals with chronic liver disease and varices. There is marked variation between countries from a prevalence of less than 0.5-2% in the United States and Western Europe, 2-3% in Russia and Eastern Europe, 2.5-6.5% in India and Pakistan and the highest reported seroprevalence, 22%, is from Egypt.[15]

HCV transmission

HCV transmission is often associated with blood borne spread. In England, prior to the identification of HCV, 1 in 520,000 blood donations were HCV infected. However, after the introduction of blood product screening in 1991 the incidence of HCV contaminated blood products has reduced to an estimated 1 in 30 million transfusions.[17] Currently the primary mode of transmission in United States and Europe is intravenous drug use (IVDU). A prospective study of a British cohort found a rate of HCV infection of 41.8 per 100 IVDU years compared to 4.2 per 100 IVDU years for HIV infection.[18] Unsafe therapeutic injections remain a source of HCV outbreaks in lesser-developed countries and historically therapeutic injections have been a major source of HCV transmission. The most striking example of iatrogenic spread is from the Egypt; reuse of contaminated glass syringes during schistosomiasis treatment campaigns have resulted in a HCV seroprevalence of 22%.[19] Similarly, prior to 1990, haemodialysis was a major source of

iatrogenic HCV infection with transmission rates of 20-30% in Europe.[20] Perinatal spread of HCV is thought to be low and transmission is estimated to be 6% with an increased risk in mothers with a high viral load.[21] Sexual activity is also associated with low risk of HCV transmission and data from an Egyptian cross-sectional survey of seroprevalence suggested that 6% of transmissions were among spouses.[22] Occupational spread is thought to occur rarely and observed risk of transmission from a contaminated needle stick injury is less than 1%.[23]

Figure 1.3. Global distribution of HCV genotypes



Genotype 1 and to a lesser extent Genotype 2 (denoted by blue line) predominates in the Americas Europe and northern Asia.

Genotype 3 (denoted by orange line) originates from the Indian subcontinent although it is also prevalent within intravenous drug users in Europe and the United Sates.

Genotype 4 (denoted by green line) predominates in Egypt and the Middle East.

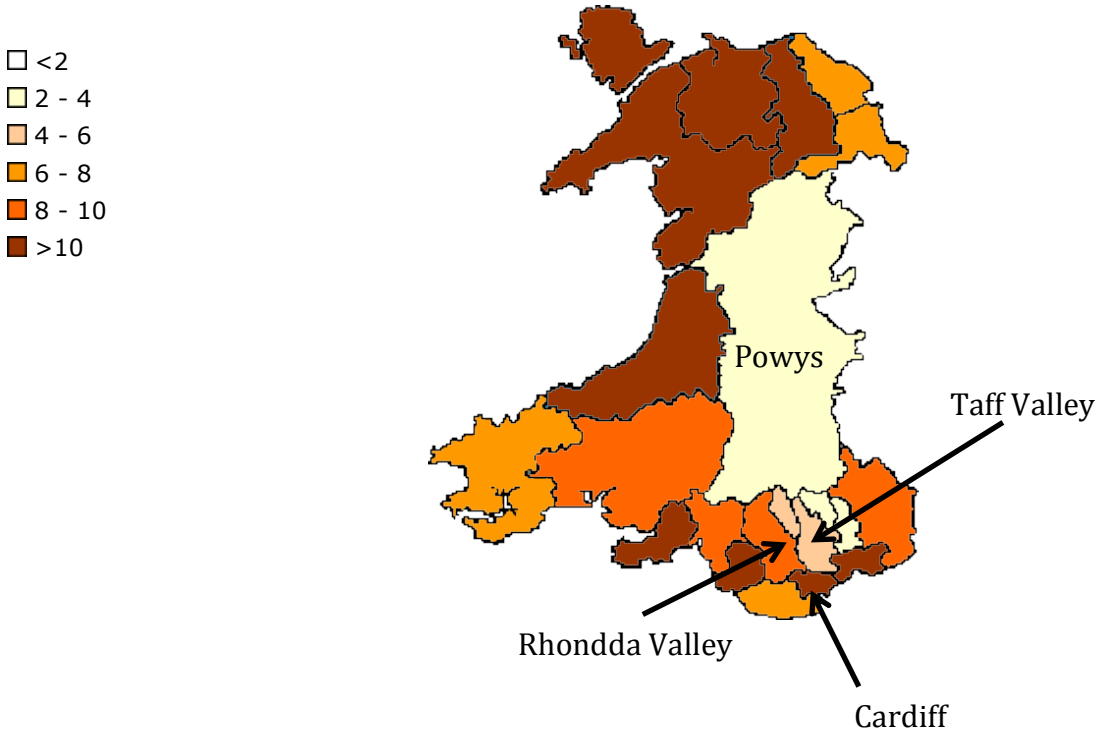
Genotype 5 (denoted by red line) predominates in Southern Africa

Genotype 6 (denoted by black line) predominates in the Far East

1.4 HCV in Wales

Antibody screening suggests that approximately 14,700 individuals (0.5% of the population) have been infected with HCV in Wales.[24] Public Health Wales estimates that approximately 12,000 individuals (0.4% of the population) have chronic HCV infection. The annual incidence of confirmed cases is 6-15 cases per 100,000 population between 2003 and 2013 with marked geographical variation (Figure 1.4).[24] Ninety per cent of chronic HCV infections in Wales are estimated to result from intravenous drug use. Within Cardiff it is estimated that two thirds of HCV infections are genotype 1 and the majority of the remainder are genotype 3. The geography of Wales appears to impact upon the distribution of HCV. Unsurprisingly rural Powys has the lowest rate of HCV in Wales. The Taff Valley has predominantly genotype 1 patients whilst the neighbouring Rhondda valley which lies parallel to the west has an almost exclusively genotype 3 HCV population. The incidence of these 2 genotypes represents an example of infection spread following the majors routes of communication (rail and road), which run north-south to Cardiff from both valleys with poor transport links over the central ridge.

Figure 1.4: HCV in Wales



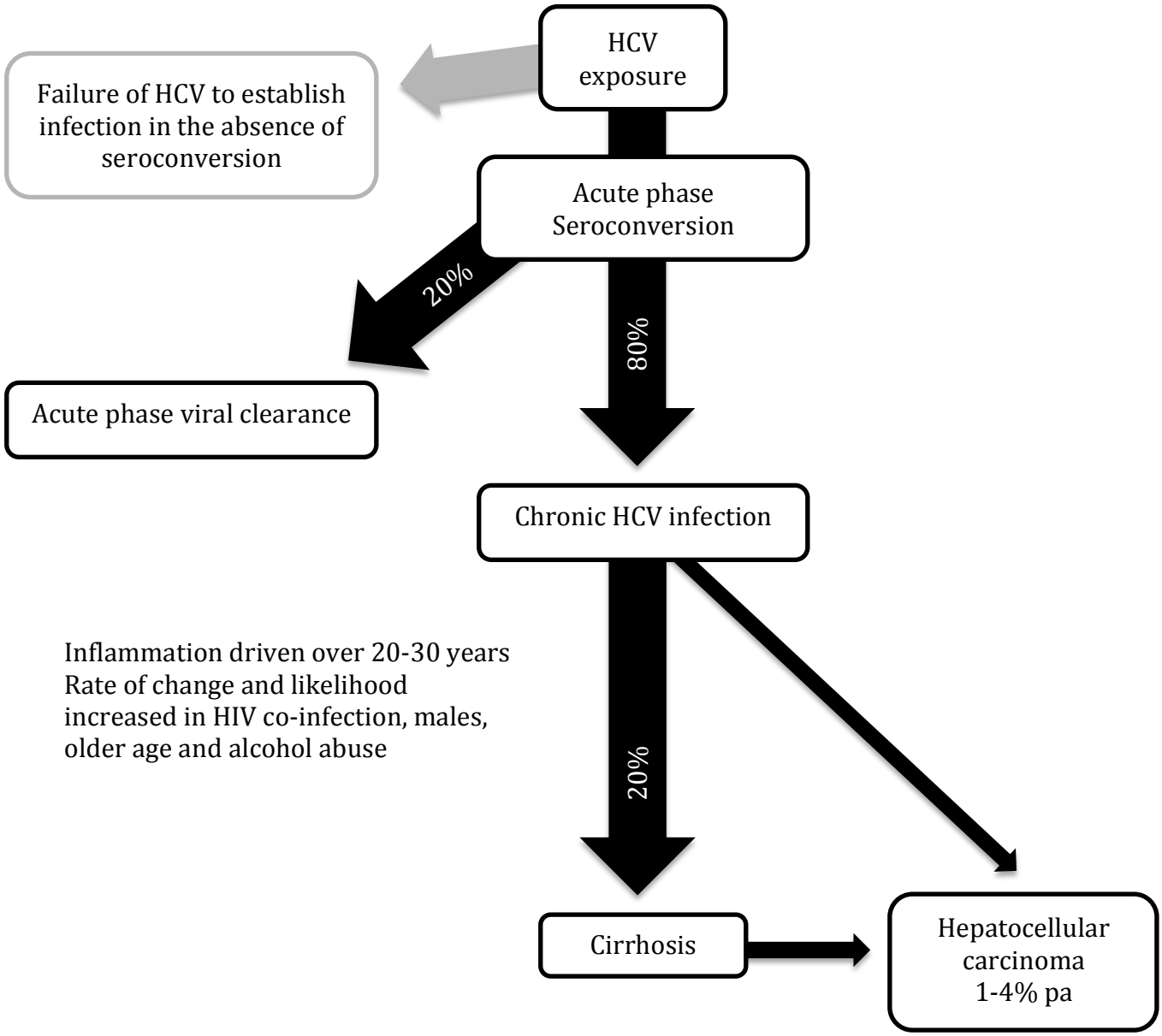
The average annuals of Hepatitis C in Wales between 2003 and 2013 per 100,000 population from Public Health Wales Health Protection Division. (www.wales.nhs.uk/sites3/page.cfm?orgid=457&pid=27973)

1.5 Natural history of HCV infection

HCV is a major cause of cirrhosis, liver failure and HCC. Acute HCV infection is usually asymptomatic and frequently not identified. Following transfusion related transmission, with inherently high inoculating viral loads, symptoms occur in 20-30% of individuals and include general malaise, anorexia, tiredness and jaundice. ALT levels can reach 10 times the upper limit of normal indicating significant hepatocyte necrosis 2-8 weeks following transfusion.[25] Persistent infection occurs in 70-80% of individuals in whom infection is confirmed by seroconversion (Figure 1.5).

Persistent chronic HCV infection is often silent for 20-30 years. During this time there may be mild elevations in serum transaminases. Diagnosis of HCV often occurs after raised transaminases are noted, leading to screening investigations for the potential causes including HCV, or by screening high-risk groups such as prisoners with an IVDU history. Of these individuals with chronic HCV infection the replication of virus within the liver is not directly cytopathic. However, in 20% of infections the chronic inflammatory response can result in advanced fibrosis and cirrhosis. HCC in chronic HCV infection results from the increased turn over of hepatocytes and hepatic inflammation and occurs at the rate of approximately 1% per year.[26] The standardised mortality rate of viral hepatitis related cirrhosis and its complications, including portal hypertension and liver failure, is approximately 10 times that of the general population in the first year after hospital admission. This mortality rate has remained unchanged in the UK between 1968 and 1999.[27] Factors that increase the risk of cirrhosis and liver failure are male sex, HIV / HBV co-infection and alcohol excess.

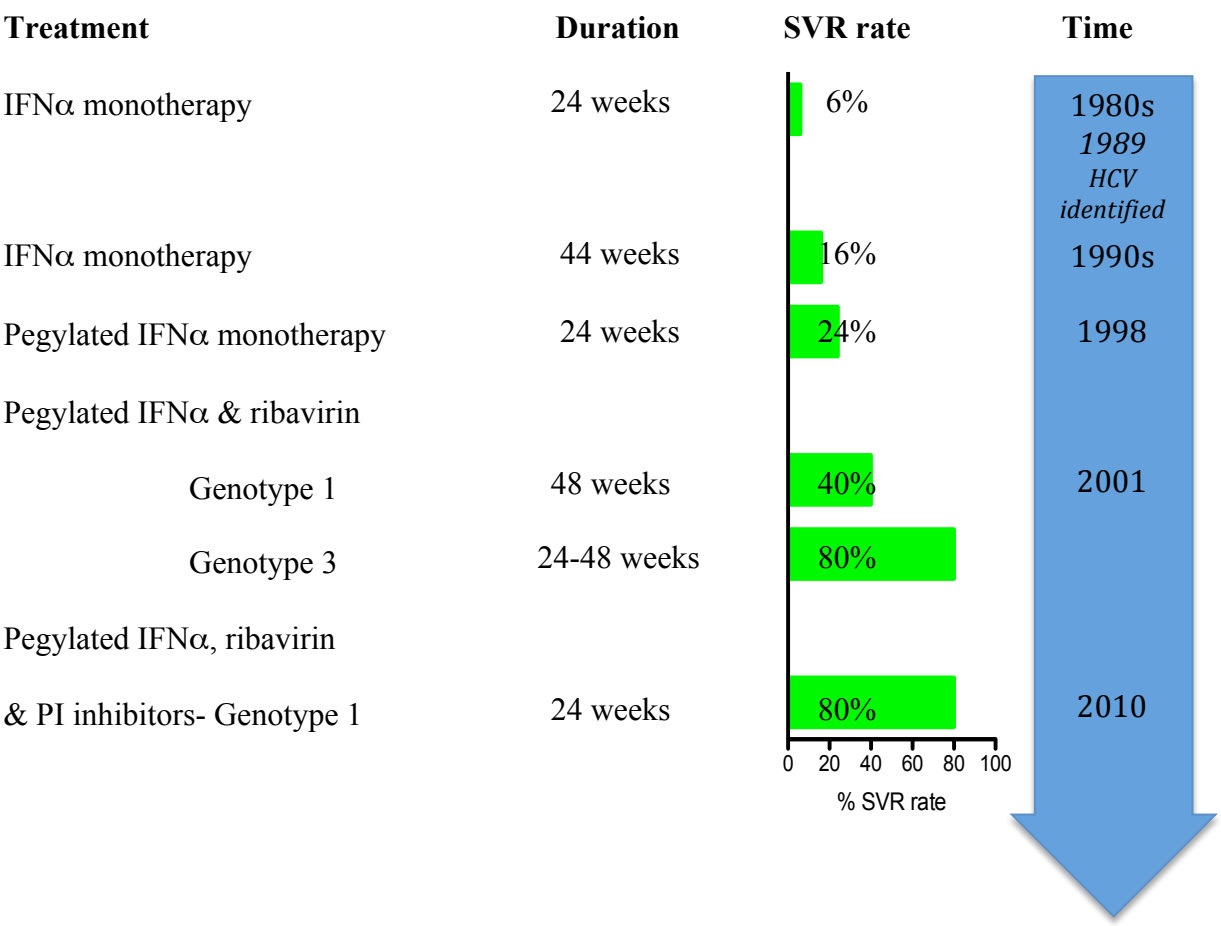
Figure 1.5: The natural history of HCV infection



1.6 HCV – current treatments & recent advances

The main therapeutic strategy in the management of HCV infection is to intervene during the chronic infection to avoid the development of established cirrhosis, liver failure or HCC. The aim of treatment is to successfully clear the virus from the body and is demonstrated by the undetectable viral PCR both at the end of treatment and 6 months following the completion of treatment. This is termed sustained viraemic response (SVR). Initially in the late 1980s IFNα was used to treat NANBH and HCV,[28] but with poor levels of success. The introduction of pegylated IFNα given weekly and the inclusion of ribavirin, a nucleoside inhibitor, has led to better treatment responses (Figure 1.6).[29] Approximately 40-50% of patients with genotype 1 and 80% of genotypes 2 and 3 infection successfully achieve SVR with treatment. Over the last decade new protease inhibitors (telaprevir and boceprevir) have been developed and found to increase treatment success in selected genotype 1 patients.[30,31] Early viraemic response, a 2log reduction in viral load at 12 weeks, is associated with SVR and a negative viral PCR allows reduction of the length of treatment.[32] Currently treatment regimens last 12-24 weeks for genotype 2 and 3 patients and 24-48 weeks for genotype 1 patients depending on the initial response to treatment. Standard IFNα and ribavirin treatment is complicated by side effects in up to 75% of patients including anaemia, rash, psychiatric disturbances (depression, psychosis), flu-like malaise, nausea and vomiting, autoimmune illness (Graves' disease, rheumatoid arthritis and vasculitits) and birth defects related to ribavirin. The addition of protease inhibitors markedly increases the risk of adverse reactions particularly anaemia and rash and increase the early discontinuation of treatment in 21% of patients compared to 11% of patients receiving IFNα and ribavirin alone.[30]

Figure 1.6: Advances in the treatment of chronic HCV



1.7 Assessing liver disease in chronic HCV infection

Liver biopsy remains the clinical gold standard to assess liver fibrosis and inflammation and can provide invaluable diagnostic information. However, there is a mortality and morbidity associated with liver biopsy underlined by the recent interest and development of biochemical and non-invasive markers (i.e. liver elastography) to assess chronic liver disease.[33] Percutaneous liver biopsy has a mortality rate of 1 in 10,000 and the most common complication is abdominal or right shoulder pain in 25% of patients.[34] Whilst the parenchyma of the liver has a relative paucity of nerve fibres, the liver capsule is well innervated and together with the skin requires infiltration with lignocaine local anaesthetic. In spite of this some patients may require simple (or occasionally opiate) analgesia. Other risks associated with liver biopsy include bleeding (blood transfusion rate of 1%), pneumothorax, perforation of the bowel and gallbladder and peritonitis.[34]

Inflammatory lymphocytes and polymorphs appear to drive many chronic liver diseases including viral hepatitis, autoimmune hepatitis and steatohepatitis. When studying these disorders it is recognised that peripheral blood lymphocytes may only reflect the function and phenotype of inflammatory cells within the liver. Fine needle aspiration (FNA) has been used as a research tool to sample intrahepatic lymphocytes but is not used clinically.[35]

1.8 Summary of outstanding clinical problems in the management of HCV

In spite of the huge improvements in knowledge of the pathophysiology of HCV infection and marked improvements in treatment of the chronic disease several key clinical problems remain:

- Recent advances in treatment result in SVR rates of 80%. However, 1 in 5 patients are unable to clear the chronic infection with treatment.
- It is not possible to accurately identify patients who will fail treatment.
- It is currently not possible to identify patients who are unlikely to develop the serious sequalae of cirrhosis and liver failure. This group may in fact benefit from an active monitoring programme rather than a long and difficult treatment, which is associated with serious complications
- Current treatments are expensive and a considerable associated morbidity and mortality.
- Anti-HCV vaccination remains elusive.
- The exact mechanisms of IFNα based treatment are unknown. It is not clear if those who achieve SVR are at risk of HCV re-infection.

These factors remain serious clinical problems and the basis of a broad body of ongoing research.

1.9 The innate and adaptive immune systems

Historically the immune system has been broadly divided into the innate and adaptive immune responses. In this paradigm the innate immune responses are non-specific to a variety of pathogens whilst the adaptive immune responses are specific to an antigen or group of antigens. Innate immune responses have a rapid onset and are able to control an infection whilst adaptive immune responses develop over an extended period of time. The significant advantage of the adaptive response is specific lasting protective memory responses that prevent the clinical illness of repeated infection. It is evident however, that this is a simplified model and there are complex and elegant interactions between these two components of the immune system with significant functional overlap (described in detail in Section 1.13). Effective innate and adaptive immune responses are interdependent and are both essential for the maintenance of health.

In the context of viral infections innate immune responses are characterised by cytokine and cellular responses. Interferons (IFN) are powerful anti-viral cytokines and have a direct anti-viral effect, induce apoptosis in infected cells and a broad range of responses in a variety of effector immune cells. The effector cells of the innate immune system include natural killer (NK) cells, which produce cytokines and can recognise and kill virally infected cells through release of cytotoxic granules. NK cells recognise infected cells through a series of receptors expressed on the cell surface. The control of the NK cell repertoire is described in detail below. Innate-like lymphocytes, which include natural killer T (NKT) cells and γδ T cells, have a limited repertoire of antigen receptors. These cells are able to respond to a series of pathogen related peptides and rapidly induce cytokine production.

Macrophages and dendritic cells form an important bridge between the innate and adaptive immune systems and are termed professional antigen presenting cells (APCs). APCs are rapidly activated during viral infections and are further stimulated by cytokines produced by the innate immune response. APCs are able to phagocytose virally infected cells and process viral antigens, which are presented to naïve T lymphocytes.

Naïve B lymphocytes possess immunoglobulin receptors (B-cell antigen receptor; BCR) that express highly specific binding sites to a huge range of chemical structures ranging from proteins and glycoproteins to viral particles and bacterial cells in the presence of co-stimulatory molecules. Upon binding to viral antigen the BCR initiates intracellular signalling pathways, which induce B cell proliferation and differentiation into antibody secreting cells and memory B cells. B cells may also act as APCs for naïve T cells as the bound antigen is processed and presented on the surface bound to MHC class II. Alternatively $CD4^+$ T effector cells may recognise the peptide:MHC class II complex and secrete cytokines that stimulate B cell proliferation.

T lymphocytes express antigen specific receptors (T-cell receptors; TCR) upon the cell surface. Naïve T lymphocytes require antigen to be presented upon MHC class I / II molecules by APCs to differentiate into two broad subtypes of T effector cells. $CD8^+$ cytotoxic T cells recognise antigen bound to MHC class I molecules and are able to kill virally infected cells bearing their cognate antigen. Helper T cells express the marker CD4, which binds to antigens presented upon MHC class II molecules, engage in coordinating the immune response. Within the $CD4^+$ T cell population there are a number of subsets that perform different functions. The phenotype and function of these subtypes are controlled by transcription factors, as described below, giving certain characteristics

of stable lineages. However, potential plasticity in the transcription factors allows some flexibility in $CD4^+$ T cell responses.[36] T_H1 cells express transcription factor T-bet and stimulate macrophages, $CD8^+$ and B cells through IFNγ and IL2 production. In addition to activation of these anti-viral effector cells IFNγ also has a direct antiviral role. T_H2 cells express Gata3 and secrete IL4, IL5 and IL13. IL4 production stimulates B cells to produce IgE, a critical factor in immunity to multicellular helminths. T_H17 cells express transcription factors STAT3 and $ROR\gamma_T$ and mediate host neutrophil responses to extracellular bacteria and fungi through IL17, IL6, IL1 and TNFα production. T_{Reg} cells express FoxP3 and control the immune response predominantly through IL10 and TGFβ to prevent immunopathology. The clonal expansion of activated adaptive immune cells, such as T and B cells, can result in memory to pathogen specific antigens.[37]

As discussed in detail below robust $CD4^+$ T cell adaptive immune responses are crucial to the successful spontaneous clearance of acute HCV infection. In chronic HCV infection and during the successful IFNα based treatment of HCV the role and relative importance of the innate and adaptive immune responses are less clear.

1.9 Interferons and viral infection

IFNα has been central to anti-HCV therapies since the 1980s, when it was proposed that a virus was the cause of NANBH. Issacs and Lindenmann first described IFN in 1957 as an inhibitor of influenza virus growth.[38] IFNs are cytokines that form a potent part of the immune armoury against viral infections and can be divided into type I (IFNα, β and ω), type II (IFNγ) and type III (IFNλ). Type I IFN are encoded on chromosome 9, there are over 13 recognised IFNα subtypes whilst IFNβ and IFNω each comprise a single subtype. Virally infected cells including endothelial cells, epithelial cells and fibroblasts release type I IFN, as do lymphocytes and plasmacytoid dendritic cells.[39] Lymphocytes may be activated by type I IFNs augmenting the immune response to viral infection.[40] IFNγ is produced by activated lymphocytes and serves to coordinate and promote inflammatory responses. In addition IFNγ has direct anti-viral effects and potentiates the effects of type I IFNs.[41] IFNλ acts in a similar manner to IFNα but primarily targets epithelial cells, which express its receptor.[42,43]

IFNα has a direct anti-viral role stimulating a cascade of gene transcription factors that interfere with viral replication and can induce apoptosis of infected cells.[44] IFNα binds to the ubiquitous cell surface type I IFN receptor and signals through the JAK-STAT pathway. The JAK-STAT pathway is a major signalling mechanism for a broad range of cytokines, including interferons, erythropoietin and growth hormone that direct cell growth, metabolism and immunoregulation.[45] Binding of IFNα to the type I receptor drives JAK and STAT phosphorylation resulting in the active pSTAT 1 molecules. pSTAT 1 traffics to the nucleus where it up regulates the expression of interferon stimulated genes (ISGs) which drive an antiviral state. The range of ISGs and their

functions have not been fully identified although the following proteins have been implicated as important in viral infections including HCV:

- myxovirus resistance 1 (MxA), a cytoplasmic protein that binds to cytoskeleton proteins, acts as a guanonsine triphosphatase and inhibits intracellular the transport of RNA viral capsids.[46,47]
- 2-5 oligoadenylate synthetase-1 (OAS-1) binds to double stranded RNA of viral origin, which it breaks down.[48,49]
- Double stranded RNA-dependent protein kinase (PKR) inhibits viral replication upon binding to double stranded RNA.[48]

The intrahepatic expression of ISGs during HCV infection varies between individuals. The initiation of IFNα treatment can induce varying relative increases of ISG expression to a maximal level. Individuals with low expression of ISGs at baseline in whom IFNα treatment induces marked up regulation of ISG expression are more likely to achieve SVR than those who have a higher expression at baseline line with a lower relative increase during treatment.[50,51]

IFNλ3 & -4 in HCV infection

Genome wide association studies (GWAS) are an analysis of genetic epidemiology that compares multiple genetic variations within a study population. Commonly a case-control design is used and the prevalence of susceptibility alleles, usually single nucleotide polymorphisms (SNPs), is compared.[52] In September 2009 an association was made between spontaneous- and treatment-induced HCV clearance and polymorphisms in the IFNλ gene. A SNP polymorphism (rs1279860) 3 kilobases upstream from the IFNλ3 gene, on Chromosome 19, was associated with spontaneous clearance of HCV infection.

C/C genotype (as opposed to T/T) was found to be favourable in European, Asians and Africans, although the C/C polymorphism was far more common in those of European and Asian ancestry.[53] The same polymorphism was found to be strongly associated with IFNα based treatment induced HCV clearance, providing a potential explanation for the higher SVR rates found in Europeans and Asians compared to those of African ancestry.[54] Further GWAS studies from Japan, Australia & Europe and Switzerland identified further SNP polymorphisms upstream from the IFNλ3 gene (rs809917 and rs4803217, G/G favourable compared to T/T in both) associated with SVR response to HCV treatment.[55-57] The exact mechanisms of how these polymorphisms impinged upon viral clearance were not immediately clear.

The HCV associated IFNλ3 SNP rs1279860 has been reported to be in strong linkage disequilibrium with a dinucleotide variant: ss469415590.[58] Located upstream from the IFNλ3, ss469415590 TT or ΔG variants result in a frame shift that creates a gene encoding the novel interferon designated IFNλ4. IFNλ4 induces expression of hepatoma cell ISGs *in vitro*. The ss469415590 variants were found in this study to be superior at predicting poor treatment outcome in individuals of African ancestry than the rs1279860 SNP. Therefore, expression of IFNλ4 provides a mechanism for the favourable associations of IFNλ3 polymorphisms and HCV clearance.

An alternative mechanism of favourable rs4803217 polymorphism activity may relate to the expression of myosin-encoding gene miRNAs by HCV infected hepatocytes. These miRNAs induce degradation of IFNλ3 mRNA thus reducing IFNλ3 expression. The miRNAs are able to bind to the unfavourable T/T, but not the beneficial G/G,

polymorphism. This mechanism of HCV immunoevasion provides potential therapeutic applications for novel miRNA therapy.[59]

1.11 Investigation of immune response to chronic HCV

Following the identification of HCV, it became evident that exposure to HCV virus does not result in seroconversion in all individuals. In 1994 the Irish Blood Transfusion service found that anti-Rhesus D immunoglobulin, used to prevent perinatal Rhesus isoimmunisation was contaminated with HCV by a single infected donor (from two separate donations). It is estimated that between 1880 and 6016 women were exposed to these samples.[60] Seven hundred and four women were HCV antibody positive. Of these, 390 were HCV RNA positive indicating a chronic infection and 314 were RNA negative indicating that they had spontaneously cleared the viral infection. The broad range of women estimated to have been exposed suggests that many women might have been exposed to HCV and yet did not develop an acute or chronic infection.[60] Similarly in a carefully detailed study of 93 health care workers exposed to HCV by accidental needle-stick injuries only 5 became infected and of these 1 spontaneously cleared the virus.[61] These studies suggest that i) exposed individuals may clear the infection by innate mechanisms. ii) infected subjects may clear the virus and the adaptive immune response may play a role.

Adaptive immune responses in HCV

The ability of $CD4^+$ T cells to infer lasting immunity to viral infections and coordinate $CD8^+$ effector T cell responses resulted in several early immunological studies focusing upon anti-HCV adaptive cellular responses.

Individuals who spontaneously clear acute HCV demonstrate robust $CD4^+$ and $CD8^+$ adaptive immune response to a broad range of HCV antigens (Table 1.1).[61-69] However, in three cohorts of individuals with acute HCV treated with IFNα $CD4^+$ and $CD8^+$ T cell responses were not associated with treatment outcome and the long-term $CD4^+$ T cell

response was diminished in treated cohorts compared to those who spontaneously cleared without treatment.[70,71]

Chronic HCV infection

The association between a robust adaptive immune response in acute HCV infection and spontaneous viral clearance has lead to in depth immunological investigation during the chronic phase, and IFNα based treatment, of HCV. These investigations have predominantly focused upon $CD4^+$ T cell proliferation and IFNγ ELISpot responses. Individuals who have spontaneously cleared HCV have increased $CD4^+$ T cell proliferation to HCV antigens, with increased IFNγ and reduced IL10 production compared to those with chronic HCV (Table 1.1).[62-65,67,72] Depletion of $CD25^+$ regulatory T cells has been associated with a further increase in $CD4^+$ T cell responses in a spontaneously cleared cohort but not in a chronic viraemic cohort. This suggests a failure of $CD4^+$ T cell priming in chronic HCV rather than regulatory T cell suppression of adaptive immunity.[72] In chronic untreated disease increasing magnitude of $CD4^+$ and $CD8^+$ T cell, and B cell responses, has been associated with both a more benign [65] and a more aggressive disease pattern.[73]

Viral impingement upon DC antigen-presenting function is a potential mechanism of impaired T cell responses in chronic HCV infection.[74] HCV surface glycoproteins E1 and E2 can bind to the activating receptor DC-SIGN upon dendritic cells facilitating viral internalisation into the non-lysosomal compartment.[75] The proportion of positive and the negative strand HCV RNA detected in peripheral blood DCs suggests only very low-levels of replication.[76,77] HCV replication does not however, appear to be required to impair DC function. Core and NS3 proteins inhibit DC allostimulatory functions, including reduced IL12 secretion, and induce IL10 thus inhibiting T cell proliferation.[78,79]

During IFNα based treatment of HCV robust $CD4^+$ T cell responses have been demonstrated in patients who achieved SVR.[80,81] However, further studies did not demonstrate a robust lasting adaptive immune responses associated with successful treatment outcome.[82,83] Viral genotype may impinge upon adaptive immune responses with genotype 1 patients having a reduced magnitude of $CD4^+$ T cell responses (measured by ELISpot) compared to genotype 2 and 3 infections, which are more amenable to treatment.[83] Robust early $CD8^+$ T cell responses have been associated with rapid viral decline in the first weeks of treatment.[84]

Initial studies were hampered by a relatively high cut off for PCR viral load detection assay (300-1000 IU/ml) thus rendering it difficult to confirm true viral clearance as opposed to viral suppression following treatment. Furthermore genotype 1 viral antigens are those that are available commercially but study patients have often been infected with a variety of genotypes, raising the possibility that immune responses have not been accurately measured.

Taken together these data suggest that whilst a failure to mount an effective $CD4^+$ and $CD8^+$ T cell response results in a chronic infection, a significant proportion of patients are able to effectively clear HCV with IFNα based treatment without establishing a lasting robust $CD4^+$ T cell response (summarised in Table 1.1). This raises the question of whether there is another cell type that has a significant effector function in the successful treatment of chronic HCV. NK cells represent a potential candidate as they are known to have an effective antiviral role and are stimulated by IFNα.

Table 1.1 Summary of reported $CD4^+$ & $CD8^+$ T cell responses in chronic HCV infection

		Spontaneously cleared vs. chronic HCV	SVR vs. Failed treatment
$CD4^+$ T cells			
Proliferation	↑	Boterelli 1993 Diepolder 1995 Lechmann 1996 Cramp 1999 Day 2002 Kamal 2004 Godkin 2001	Lasarte 1998 Cramp 2000
	Unchanged		Barnes 2002 Rahman 2003* Kaplan 2005 Lauer 2005*
IFNγ	↑	Cramp 1999 Godkin 2001 Day 2002 Kamal et al 2004	Cramp 2000 Rahman 2003*
	Unchanged		Barnes 2002 Wiegand 2004* Kaplan 2005 Lauer 2005*
IL10	Unchanged	Cramp 1999	
	↓	Godkin 2001	
IL2	↑		Lasarte 1998
$CD8^+$ T cells			
Proliferation	↑	Wedemeyer 2002	Pili 2007
	Unchanged		Lauer 2005*
IFNγ	↑	Missale 1996 Wedemeyer 2002	Pili 2007
	Unchanged		Lauer 2005*

*These studies investigated T cell responses during the IFNα treatment of *acute* HCV infection.

1.12 Natural Killer cells

Natural Killer (NK) cells are large granular lymphocytes and form part of the innate immune system. NK cells were first described in 1975 and derive their forceful name from the observed ability to destroy leukaemic cells without prior stimulation.[85,86] Kärre *et al*. first proposed the mechanism of NK cell activation was by identifying potential target cells which lack or have reduced expression of MHC class I: the missing-self hypothesis.[87] However, further observations demonstrated that NK cells could kill autologous MHC-matched tumour cells leading to a paradigm whereby NK cell activation is controlled by the balance of signals received from an array of inhibitory and activating receptors. Kärre compared this to attempts by the Swedish navy to enlist the general population in submarine surveillance during the Cold War. The navy felt the simplest approach was to publish pictures of the 3 types of Swedish submarine in service and requested to be notified if any other type of submarine was seen (missing-self or lack of inhibition activation). The tabloid press however, printed a wide range of pictures showing foreign submarines that could stray into Swedish waters; representing positive activation.[88]

NK cell maturation and subsets

NK cells are often defined phenotypically as lymphocytes expressing CD56, also known as neural cell adhesion molecule, a glycoprotein expressed on neural and skeletal muscle cells. In the peripheral blood NK cells make up 5-15% of the total lymphocyte population. CD56 expression varies on NK cells with approximately 10% of peripheral blood NK cells expressing high levels of CD56 ($CD56^{Bright}$ NK cells) and the remainder expressing lower levels ($CD56^{Dim}$ NK cells). $CD56^{Dim}$ NK cells are considered to represent a more mature subset that have greater cytotoxic potential and are able to release cytokines.[89]

$CD56^{Bright}$ NK cells are the first lymphocytes to reconstitute the peripheral blood compartment following haematopoietic stem cell transplantation.[90,91] The maturation of NK cells from $CD56^{Bright}$ to $CD56^{Dim}$ is associated with a stepwise reduction in NKG2A expression and an upregulation of Killer-cell Immunglobulin-like receptors (KIR).[92] Both of these NK cell receptors are inhibitory and further described below.

A further subset of $CD56^{-}$ $CD16^{+}$ (an FcγIII receptor described below) NK cells were initially described in HIV positive patients.[93] These cells are thought to represent approximately 5-10% of peripheral blood NK cells.[94] The exact role of $CD56^{-}$ NK cells remains unclear but they are thought to be more mature cells with reduced cytotoxic function and cytokine secretion but maintained chemokine secretion.[95]

Mechanisms of NK cell activation and killing

NK cells and cytotoxic T cells are thought to share mechanisms of activation and target cell killing. Cytotoxicity relies on formation of an immunological synapse and release of granules containing perforin, granzymes and Fas ligand.[96,97] NK cells demonstrate a hierarchical functional response to increasing stimulation. Low-level stimulation may induce leukocyte functional antigen (LFA)-1 mediated adhesion whilst stronger activation is required is required for degranulation and IFNγ production, reflecting an increased number of signalling cascades to induce these responses.[98] As in T cell activation LFA-1 signalling rapidly promotes convergence of cytotoxic granules to the NK cell microtubule organising centre.[99] Polarisation of lytic granules to the immunological synapse is dependent upon talin, WASP and CD3ζ activation, which are the result of poorly defined additional pathway signalling.[100] Release of Ca^{2+} into the cytoplasm from the endoplasmic reticulum triggers granule exocytosis. CD16 signalling can induce Ca^{2+}

release on its own and studies of knockout mice suggest a critical role for phosphatidylinositol specific phospholipase C-γ (PLC-γ) dependent inositol triphosphate in non-antibody directed cellular cytotoxicity.[101,102] PLC-γ enzymes are indispensable component of signal transduction complex and are also required for transcription of IFNγ and TNFα genes.[101]

NK activating receptors

NK cells can respond to activation through direct cellular cytotoxicity and the production of chemokines and inflammatory cytokines. NK cell activating receptors bind to ligands on host cells upon which proteins are up regulated in response to oncogenic transformation or viral infection (Table 1.2). To date not all the ligands for NK cell-activating receptors have been identified.

CD16 is an FcγIII receptor, which binds to the constant region of human antibodies and activates NK cells. Thus NK cells can recognise and be activated by targets that are antibody coated (using the constant region of the antibody).[103] Signalling occurs through the immunoreceptor tyrosine-based activation motif (ITAM). NK cell activation in this manner is termed Antibody-Dependent Cell-mediated Cytotoxicity (ADCC).

Table 1.2 NK cell activating and inhibitory receptors

Activating receptors	**Ligands**
CD16	Antibody Fc
DNAM-1 (CD226)	CD112, CD155
2B4	CD48
Natural Cytotoxicity Receptors	
NKp30	BAT3, B7H6, HCMV-pp65
NKp44	Viral haemagglutinin and neuraminidase mixed-lineage leukaemia 5 protein
NKp46	Viral haemagglutinin and neuraminidase heparin sulphate
C type lectin like receptors	
NKp80	Activation induced C-type lectin
NKG2D	MICA, MICB
CD94/NKG2C	HLA-E
Inhibitory receptors	**Ligands**
KLRG-1	E-cadherin
Killer Ig-like receptors	
KIR2DL/ KIR3DL/ KIR2DS	HLA-C /-A /-B
C type lectin like receptors	
CD94/NKG2A	HLA-E

Adapted from Viver *et al* 2011[104] and Jewett *et al* 2013.[105]

NKG2D is a C-type lectin-like transmembrane activating receptor that is expressed on NK and $CD8^+$ T cells. NKG2D is known to bind to two stress ligands MICA or MICB. These ligands are up regulated on cancer cells and virally infected cells.[106-108]

Natural Cytotoxicity Receptors (NCRs) are preferentially expressed on NK cells and are members of the immunoglobulin superfamily. The NCR activating receptors comprise NKp30, NKp44 and NKp46 and are associated with ITAM signalling. Phosphorylation of the NCR adaptor molecules, ITAMs, initiates the ZAP70 and SYK signalling pathways. These pathways lead to Ca^{2+} influx and cytoskeletal reorganisation resulting in cytotoxicity and IFNγ and TNFα production.[109,110] Each NCR Ig-like domain is formed as a sandwich-like structure of two facing β sheets held together by conserved disulphide bonds. The arrangement of disulphide bonds alters the lengths of surrounding loop regions potentially significantly altering the Ig-like domain's binding potential.[111] NKp46 is termed a C-like Ig receptor, which reflects a shorter a.a. residue between the disulphide bonds than that found in the NKp30 and NKp44, which are categorised as V-type Ig receptor (Figure 1.6).[110,111] The importance of NCRs is underlined by acute myeloid leukaemia cell resistance to NK cytotoxicity in individuals with low NCR expression.[112-114]

NKp46, the first NCR to be identified by the Moretta group,[115] is a 46kDa glycoprotein and consists of a single transmenbrane and two extracellular Ig-like domains. To signal the NKp46 transmembrane domain associates with CD3ζ and FcεRI (Figure 1.6). The NKp46 Ig-type domain dimerization is essential for NKp46 mediated NK cytotoxicity but is not found in NKp30 or NKp44.[116] The NKp46 Ig-like domains are held at 85° to each other by a hinge region, which has been proposed as a potential ligand binding site.[117,118]

NKp46 is the only NCR with an orthologue in mice (called NCR1) and other species,[110,117,119] the NKp46 gene is located upon chromosome 19.[120] This evolutionary conservation suggests NKp46 expression confers a significant host advantage of pathogen and tumour recognition. However, whilst heparin sulphate and viral haemagglutinins and neuraminidase have been identified as ligands for NKp46,[121-123] other ligands have yet to be identified.

NKp44 was the second NCR to be identified: the extracellular domain is a V-type Ig molecule and the intracellular domain associates with DAP12 for signalling (Figure 1.6A).[112] NKp44 is expressed at a low level unless NK cells are stimulated with interleukin-2 (IL-2).[124] NKp44 is coded by the class III region of the MHC locus, as is NKp30 with which it shares similar structural properties (Figure 1.6B). Recently mixed-lineage leukaemia-5 protein (MLL5) has been identified as a ligand for NKp44.[125] Blocking experiments suggest engagement of a single NCR can result in activation of the signalling cascades of other NCRs suggesting cross talk between NKp30, NKp44 and NKp46 during NK cell activation.[112,124]

NKp30 is a single V-type Ig-like protein with a short 5 a.a. stalk. The transmembrane domain associates with CD3ζ (Figure 1.6A).[126] Ligands for NKp30 have also remained elusive, it is known that tumour cells release nuclear factor HLA-B-associated transcript 3 (BAT3) that binds directly to NKp30 and results in NK cell cytotoxicity.[126,127] B7-H6 has been recognised as an activating ligand for NKp30 expressed on human K562 erythroblastic leukaemic cells.[128] Moreover, the ligands that NKp30 recognises appear to be distinct to NKp46 ligands as differing target cells show varying susceptibility to these activating molecules.[129] Following activation by mature DCs NK cells can kill immature

DCs in an NKp30-dependent manner, representing an important step in the development of a coordinated immune response.[130] Jarahian *et al*. demonstrated in a mouse poxvirus and human fibroblast model that ectromelia virus haemagglutinin serves as a ligand for NKp30 and, to a lesser extent, NKp46. In vaccinia infection viral haemagglutinin blocks NKp30 activation, through an unknown mechanism, whilst NKp46 activation is retained resulting in reduced NK cell activation.[121] These results demonstrate a viral immunoevasion mechanism for the NCR receptors.

NK inhibitory receptors

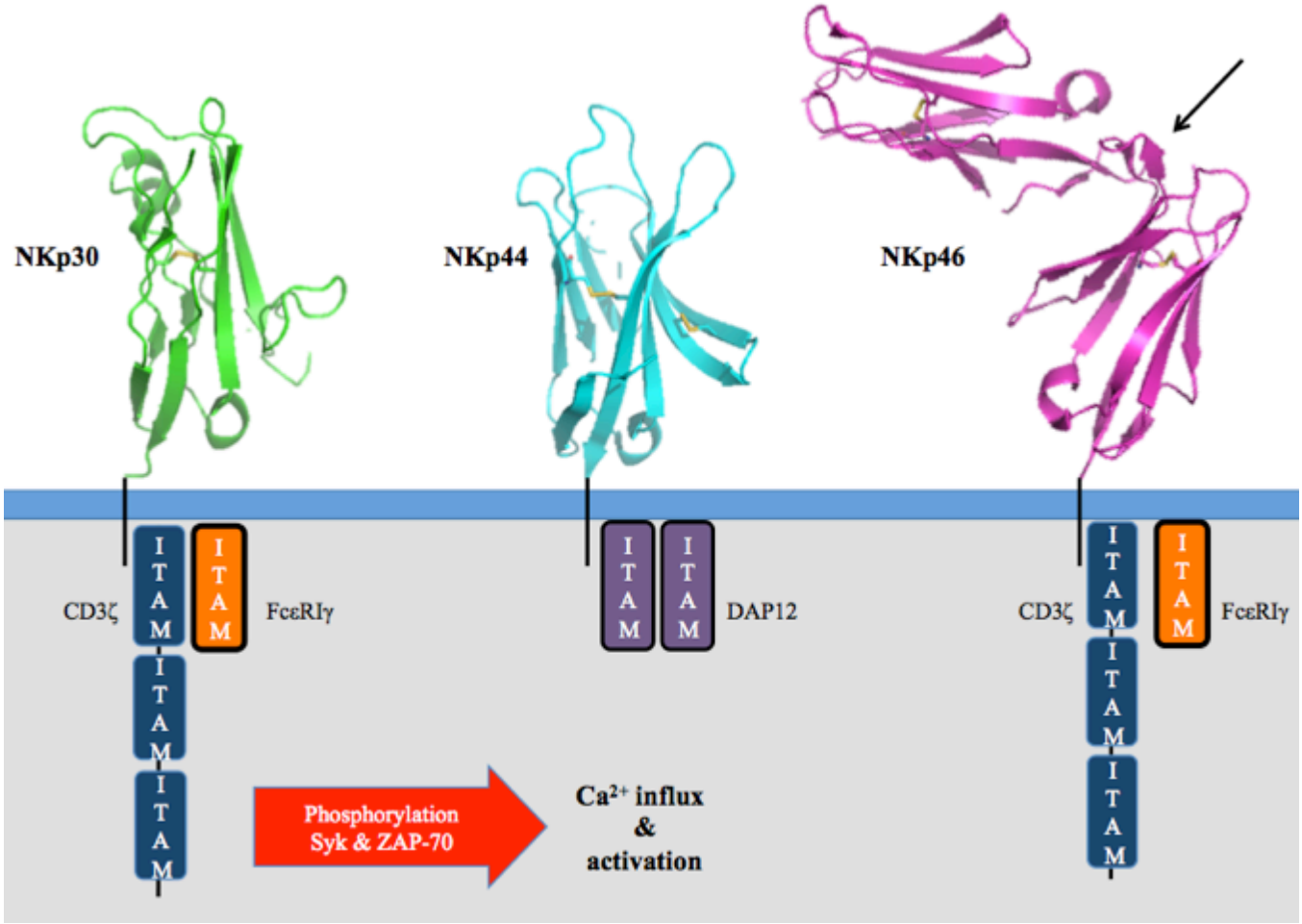
NK cells were first defined by their cytotoxic activity against tumour cells lacking MHC class I molecules. Thus the presence of MHC class I inhibits NK cells and loss of inhibition results in NK cell activation.[87] MHC class I molecules are part of the Human Leukocyte Antigen (HLA) complex and comprise six proteins encoded in a highly polymorphic region of chromosome 6. Of the HLA genes, HLA-A, -B and -C are polymorphic and HLA-E, -F and -G are conserved. NK cells recognise and are inhibited by HLA-A, -B and -C through Killer cell immunoglobulin-like receptors (KIRs) and HLA-E by NKG2A an inhibitory C-type lectin-like receptor.[131]

Development of the NK cell repertoire

The KIR locus, on Chromosome 19 encodes 13 different genes and there is marked polymorphisms within KIR genes.[132] Each NK cell expresses an individual repertoire of KIR with potential for differing receptor expression as well as number and combination of KIRs.[133] The expression of KIR genes can be induced by cell-to-cell contact signalling between NK progenitors and bone marrow-derived stromal cells.[134] It remains unclear, however, if tolerance of compatible HLA class I is induced at this stage of NK cell

development. The expression of endogenous KIR within NK clones is further dependent upon DNA methylation, which can silence KIR genes.[135] Exposure to CMV infection, but not other herpes viruses, may educate NK cell populations to induce stable expression of particular KIR genes, resulting in an increase in self-specific inhibitory KIR expression.[136] The impact of chronic HCV infection upon KIR expression independently to CMV infection remains to be elicited.[137]

Figure 1.7 NCR structure and signalling motifs
A)



The external domains of NKp30 (green) NKp44 (blue) and NKp46 (purple) with disulphide bonds are shown in yellow. The cell membrane (blue) and cytoplasm (grey) with the specific NCR intracellular signalling motifs: NKp30 & NKp46 - CD3ζ & FcεRIγ and NKp44 – DAP12. Arrow indicates NKp46 dimerization hinge joint. Adapted from Koch et al 2013.[110] Ribbon cartoons created by Dr Chris Holland, Cardiff University using PyMol software (SourceForge.net).

B) Overlay ribbon cartoon of NKp30 (green) and NKp44 (blue) demonstrating NK activating receptor homology.

1.13 NK cells and the adaptive immune response

Activated NK cells contribute to the development of a specific long-term adaptive immune response as well as providing an immediate innate immune response. Virus-specific $CD4^+$ T cells play an important role in the control or elimination of viral infections. The development of adaptive immune responses requires a longer period of time than those of the innate response. T cell priming may start within a few hours of infection if viral antigen penetrates the peripheral lymphoid compartment and appropriate co-stimulation is given to T cells.[138] However, in certain infections, such as HCV, T cell priming may be delayed by several weeks, the reasons for the delay being unclear.[61] After the virus specific $CD4^+$ T cells have received the necessary stimulation there is a short lag-phase before they undergo clonal expansion over 7-10 days.[139]

Natural Killer and Dendritic cell cross talk

NK cells can directly and indirectly augment the development of the adaptive immune response. NK cell release of IFNγ and proinflammatory cytokines in viral infections can promote antigen processing and presentation to T cells.[140] IFNγ release will polarise the inflammation towards a T_H1 cell responses.[141] In a mouse influenza model Ge et al. demonstrated that NK cell depletion reduces DC antigen presentation and limits the recruitment of both DC and T cells into the thoracic draining lymph nodes in an IFNγ-dependent manner.[142] DC stimulation may further promote NK activation through the release of inflammatory cytokines (IL-2, IL-12 and IL-18) by DCs.[141] This bidirectional stimulation between DCs and NK cells is the of MCMV immunoevasion. Mandaric et al have demonstrated that MCMV IL-10 suppresses NK-DC cross talk and therefore, reducing IL-12 expression which results in $CD4^+$ T cell suppression.[143]

In addition to stimulation of DCs NK cells can further promote a robust specific adaptive immune response through the lysis of immature DCs expressing MHC class I at lower levels than mature DCs in a NKp30 dependent manner.[130] This is a potential feedback mechanism to ensure a limited supply of DCs to prime a focused, co-ordinated immune response.

1.14 NK cell memory

Immunological memory describes the ability of certain cell to mount a greater, faster response following repeated antigen exposure. Classical antigen-specific memory responses mediated by B cells and T cells are the hallmark of adaptive immunity. Traditionally NK cells have been thought to belong solely to the innate immune system without adaptive immunological features such as memory. However, there is evidence, from mouse models that NK cells can demonstrate features of memory responses to highly diverse antigens.[144]

O'Leary et al. demonstrated that following initial contact exposure (sensitisation) T and B cell deficient mice can mount an antigen-specific hapten induced contact hypersensitivity response up to 4 months following initial exposure.[145] This memory was observed after adoptive transfer of NK cells to naïve mice. The NK cells, which demonstrated the hapten specific response, were Ly49C(+/-)I^+ and localised to the donors' livers but not spleens potentially reflecting functionally distinct NK cells within different tissues.

Mouse NK cells can acquire long-lived (up to 12 weeks) memory of MCMV, VSV, influenza A and HIV-1 following vaccination and rechallenge.[146,147] Previous exposure to influenza antigen conferred a survival benefit compared to naïve mice when inoculated a lethal dose of influenza A, however, the cognate antigen remained unclear. Again the memory NK cells localised to the donors' livers following adoptive transfer and expressed the chemokine CXCR6.[146] The ligand for CXCR6 is CXCL16, which is constitutively expressed within the liver sinusoidal endothelium. Antibody blockade of CXCR6 inhibited NK cell responses to haptens and viruses *in vivo* although CXCR6 blockade did not impinge upon hapten recognition *in vitro*. These data suggest CXCR6 is crucial for

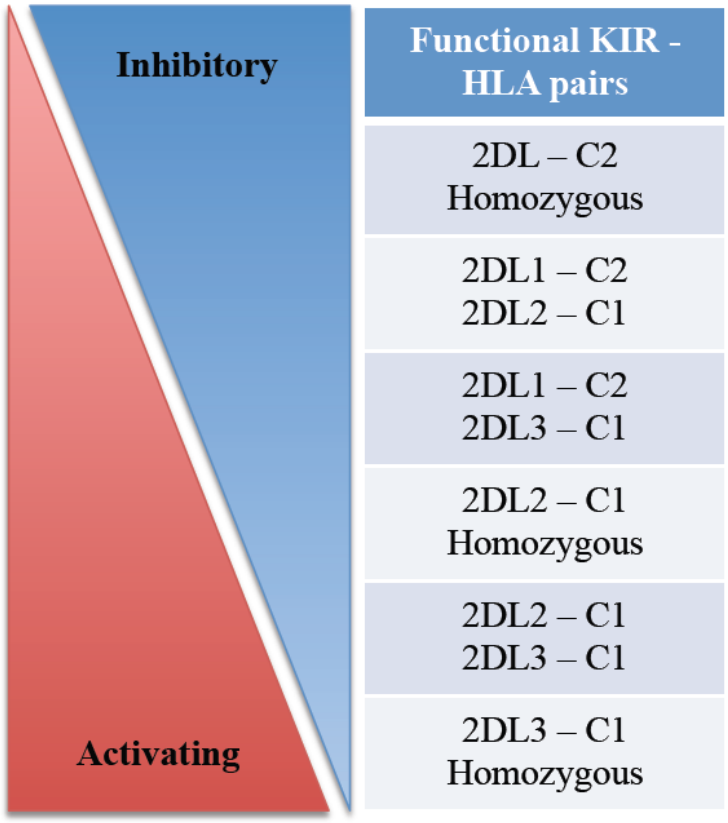
memory NK maintenance *in vivo* rather than function.[146] In a mouse model of MCMV infection, Sun and colleagues identified m157 as the antigen, which drove a memory population of Ly49H expressing memory NK cells.[147] Whether there are antigens that induce NK memory responses in human disease and if they are truly needed remains to be demonstrated.[148]

1.15 NK cell responses to HCV exposure & acute infection

There is a body of evidence suggesting that NK cells can play a protective role in preventing HCV viraemia and seroconversion following exposure. Golden-Mason et al compared a cohort of 25 intravenous drug users who were repeatedly exposed to HCV; 11 remained uninfected and 14 became infected over a median time course of 90 days. It was found that exposed but uninfected cohort of individuals had a higher proportion of $CD56^{Dim}$ cytotoxic NK cells, higher activating receptor NKp30 expression and *ex vivo* cytotoxic function than individuals who seroconverted following exposure.[149]

HLA-C molecules, the ligands for the inhibitory KIR receptors can be divided into C1, which binds to KIR2DL2 and KIR2DL3, and C2, which binds to KIR2DL1.[150] The relative binding strength of these HLA-C and KIR interactions vary as laid out below (Figure 1.7). The NK cell inhibitory receptor KIR2DL3 forms a weak bond with its cognate HLA-C ligand and can be overcome more easily than the other HLA-KIR combinations. This promotes activation of NK cells in individuals during viral infections. In a study of intravenous drug users exposed to HCV Knapp *et al*. demonstrated that KIR2DL3 homozygous individuals with the HLA-C1 were more likely to remain seronegative and aviraemic than other exposed individuals (odds ratio 3.1).[151]

Figure 1.8 HLA – KIR inhibitory interactions



HLA-C KIR interactions bind with varying strengths resulting in a activating/ inhibitory hierarchy. The KIR2DL3 – C1 genotype is protective against chronic HCV infection and confers greater treatment success rates. Adapted from P. Parham 2005.[150]

Acute HCV infection

The role of NK cells in acute HCV infection, confirmed by seroconversion remains to be fully elucidated. A genetic study of over a thousand anti-HCV antibody positive individuals shows that HLA-C1 allele and KIR2DL3 genotype confers protection against chronic HCV infection at low dose exposure but at high dose exposure the authors found no association. The authors concluded that the innate immune response was overwhelmed by the high dose of virus.[152] In a study of 22 individuals with acute HCV infection Amadei *et al*. reported an increase in expression of activating receptor NKG2D on NK cells and IFNγ in all individuals regardless of self-limiting infection or progression to a chronic infection. Subgroup analysis of HLA-C1 specific KIRs revealed that NK cells expressing these receptors demonstrated increased cytotoxicity, which was maximal in individuals who spontaneously cleared the infection.[153]

A further examination of peripheral blood NKs during acute HCV infection by Alter and colleagues indicated that the proportion of lymphocytes that are NK cells increases. The expression of the activating receptors NKp30 and NKp46 were reduced and NKG2A and CD94 were increased during acute HCV infection. Of note individuals who achieved viral clearance during the acute infection (i.e. became HCV antibody positive but HCV RNA negative) had lower expression of NKp30, NKp46 and the inhibitory receptor CD161 (which binds to LLT1 molecule) compared to those in whom a persistent infection was established. The authors concluded that NK cells with higher expression of NCRs may be trafficked to the liver resulting in a relative paucity in the peripheral blood. Furthermore lower expression of CD161 may result in a lower NK activation threshold and play a major role in the control of acute HCV infection.[154]

In conclusion acute hepatitis C virus infection is spontaneously cleared in a proportion of patients. The efficacious immune response may be augmented by NK cell cytotoxicity and IFNγ production.

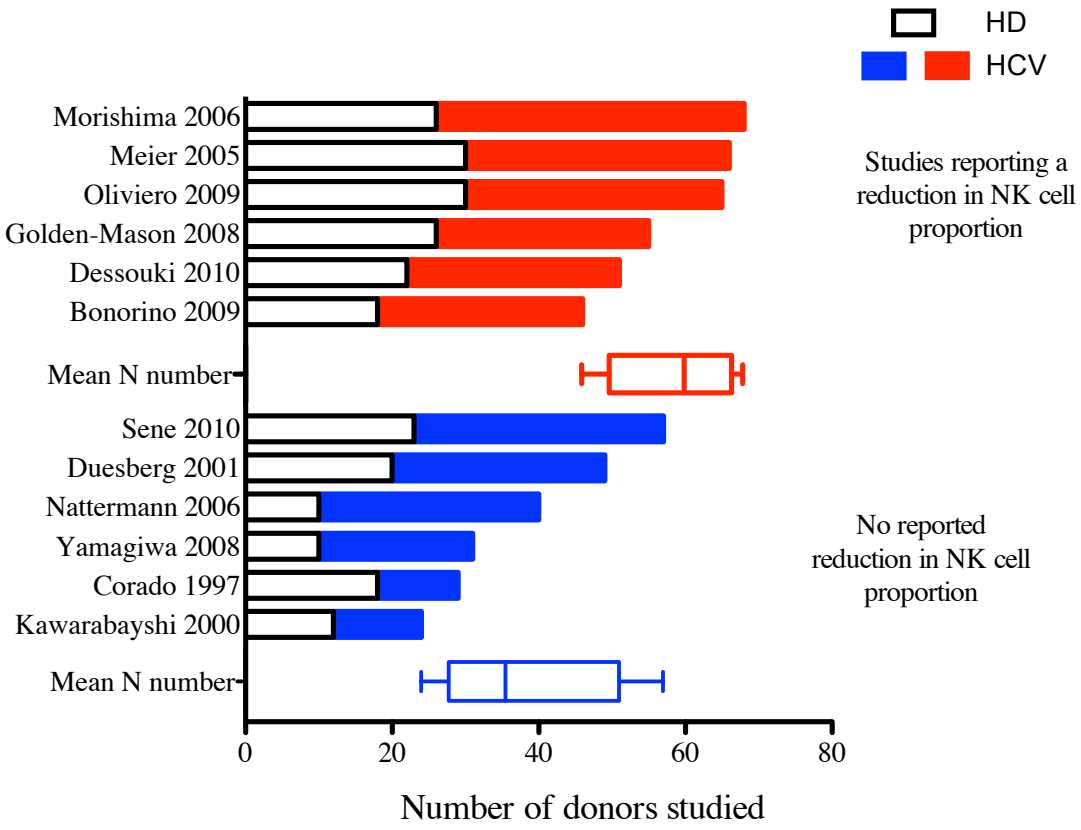
1.16 NK cells in chronic HCV infection

For over 15 years NK cells have been examined as a potential effector against HCV infection.[155] Whilst the findings can appear contradictory, possibly as a result of methodological issues and differences in patient populations, some consistent features of NK phenotype and function in chronic HCV infection have emerged from these studies (Tables 1.3 & 1.4). Most published findings focus upon peripheral blood NK cells rather than intrahepatic NK cells.

Twelve studies compared peripheral blood NK cells as a proportion of lymphocytes in chronic HCV infection and healthy donors. Six have reported no change [155-160] and six reported a reduction in peripheral blood NK cell proportion in the HCV cohort [149,161-165]. The six reporting a reduction tended to have a larger sample number within the HCV cohort (cohort range 28-42, mean 33) and healthy controls (range 18-30, mean 25) than those not reporting a difference (HCV cohort range 11-34, mean 22, healthy donor range 10-23, mean 15, Figure 1.9). These findings and the modest reported reductions in NK cell proportion in chronic HCV infection suggest that type II statistical errors (i.e. the null hypothesis is not rejected as the sample size is too small) may be contributing to conflicts within the literature.

There is an overall consensus that in chronic HCV infection the proportions of $CD56^{Bright}$ [161,163,164,166] and $NKG2A^{+}$ NK cells are increased.[158,163,165,167-170] However, there is no consensus in the literature regarding the expression of the NCRs, NKp30, NKp44 and NKp46 or NKG2D, these reports are summarised in Table 1.3.[158,159,162,165-167,171,172] These reports imply that varying technical factors, practical techniques and methods of analysis may contribute to conflicting reports of NK cell phenotype in chronic HCV infection.

Figure 1.9 The reported proportion of NK cells in chronic HCV



A) Studies with larger cohorts, shown in red, appear more likely to demonstrate a reduction in the proportion of lymphocytes that were NK cells (mean NK cell proportion Healthy donors 11.7% v HCV donors 7.7%).

Table 1.3 Summary of reported NK cell peripheral blood phenotype in chronic HCV infection compared to healthy donors

	Increase	No change	Decrease
%NK		Corado 1997 Kawarabayashi 2000 Duesberg 2001 Nattermann 2006 Yamagiwa 2008 Sene 2010	Meier 2005 Morishima 2006 Golden-Mason 2008 Bonorino 2009 Oliviero 2009 Dessouki 2010
$CD56^{Bright}$: $CD56^{Dim}$ ratio	Meier 2005 Morishima 2006 Bonorino 2009 Bozzano 2011	Jinushi 2004	
NKG2A	Jinushi 2004 Nattermann 2005 Nattermann 2006 Golden-Mason 2008 Ahlenstiel 2010	Bonorino 2009 Dessouki 2010	
NKG2D	Oliviero 2009	Nattermann 2006 Sene 2010*	Dessouki 2010 Yoon 2011
NKp30	De Maria 2007	Dessouki 2010	Nattermann 2006 Bozzano 2011 Yoon 2011
NKp44	Ahlenstiel 2010†		Nattermann 2007 De Maria 2007
NKp46	De Maria 2007 Ahlenstiel 2010†	Dessouki 2010	Nattermann 2006

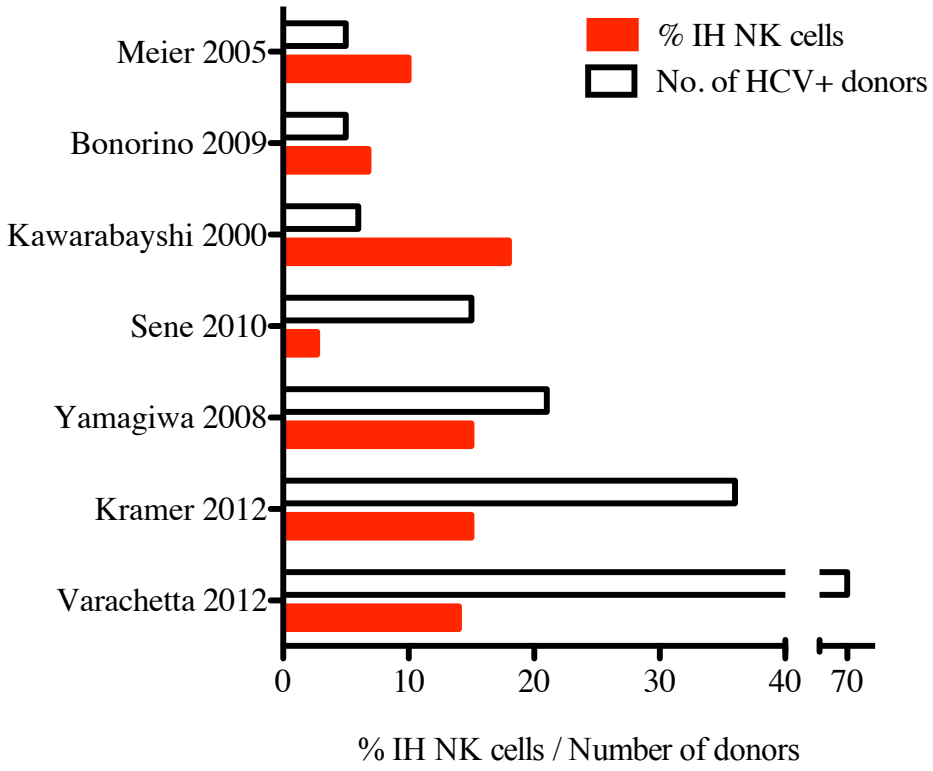
* NKG2D expression was reduced when measured by MFI

† NKp44 and NKp46 expression was unchanged when measured by MFI

1.17 Chronic HCV & Intrahepatic NK cells

In 1998 Norris *et al*. characterised the resident lymphocyte subsets in blood and liver biopsy samples taken from non-living donors at the time of donor organ retrieval.[173] This cohort consisted of 15 individuals who died of subarachnoid haemorrhage (7), road traffic accidents (7) and an individual who died of a brain tumour. It was reported that all donors had normal liver function tests and no known liver disease. The biopsies were mechanically disrupted stained with CD3 and CD56 monoclonal antibodies and examined by flow cytometry. It was demonstrated in this cohort that the mean intrahepatic NK cell proportion of lymphocytes was 30.6% (range 11.6-51.3) and 12% in the peripheral blood (range 1-22%). In chronic HCV infection lower proportions of intrahepatic NK cells have been reported, with a range of proportions of NK cells compared to total intrahepatic lymphocytes of 2.7 – 18% (Figure 1.10).[157,159-161,163,174,175]

Figure 1.10 Reported proportions of intrahepatic NK cells in chronic HCV



The proportion of intrahepatic NK cells reported by 7 studies (red bars mean= 11.6%) and the number of sample donors in each cohort (white bars. Proportion of NK cells and sample size are demonstrated on the same scale on the x axis)

1.18 Intrahepatic NK cell phenotype in chronic HCV infection

The phenotype of NK cell activating receptors determines NK cell function with higher expression of activating receptors conferring greater effector potential.[121,176] However, it is known that NK cell activating receptors can be down regulated on activated cells,[103] which would impact upon the overall phenotype of the NK cells within the setting of chronic inflammation. NK cell phenotype within the intrahepatic compartment of HCV infected individuals is not fully determined and, as highlighted above, reports are often based upon relatively small cohorts.

In the largest study to date of intrahepatic NK cells in chronic HCV, 70 HCV^{+} individuals were biopsied and a phenotypic analysis of each NCR activating receptor was analysed on a subsets of 13-20 individuals.[174] This study did not find an alteration in the proportion of cells expressing NCRs compared to non-HCV control biopsies. However, as outlined in the studies comparing peripheral blood samples, there is a conflict amongst studies over phenotypic changes (Table 1.4).[158,159,161,162,167] Krämer *et al* used MFI rather than proportions to measure NK cell phenotype and categorised NKp46 expression as $NKp46^{High}$ and $NKp46^{Dim}$ and $NKp46^{-}$ subgroups. There was a marked increase in $NKp46^{High}$ population within the intrahepatic compartment.[175]

Table 1.4 Summary of phenotypic changes upon intrahepatic NK cells in subjects with chronic HCV infection

Intraheptic NK cells v Peripheral blood NK cells from HCV^+ donors			
	Increase	**No change**	**Decrease**
% NK		Meier 2005	Yamagiwa 2008 Sene 2010 Bonorino 2009 **In cirrhosis –** Kawarabayashi 2000
$CD56^{Bright}$: $CD56^{Dim}$ ratio	Meier 2005		
NKG2A	Bonorino 2009		
NKG2D	Oliviero 2009 Sene 2010		
NKp30		Nattermann 2006	
NKp44		Nattermann 2006	
NKp46	Ahlenstiel 2010 Kramer 2012	Nattermann 2006	
HCV^+ donor Intrahepatic NK cells v non HCV donors			
NKp30		Varachetta 2012	Nattermann 2006
NKp44		Varachetta 2012	Nattermann 2006
NKp46		Varachetta 2012	Nattermann 2006

1.19 NK cell function in chronic HCV infection

Multiple studies have considered the impact of chronic HCV infection on NK cell function. The use of various experimental methodologies including i) varying stimulating cytokines ii) target cells iii) outcomes measured iv) time of stimulation in these studies which has lead to apparently conflicting results. Studies have reported increased, [162,165,167,168] reduced [155,159,161,166,171] and unaltered [156,158,162,164,168] NK function in chronic HCV infection compared to healthy controls (Table 1.5).

The majority of experiments utilised K562 erythroblastic leukaemic cells as targets, which lack MHC class I and therefore, induce a strong cytotoxic response. A broad range of stimulatory cytokines have been used to enhance NK cell function prior to exposure to targets. IFNα, the mainstay of treatment, has not been used to stimulate NK cells in HCV related studies. A broad pattern emerges when the length of time that NK cells remain in culture regardless of whether rested or stimulated, is considered: NK cytotoxic function is reduced in chronic HCV infection compared to healthy donors if shorter incubation times are employed but if cells are rested *ex vivo* then this impingement wanes (Table 1.5).[167]

Studies that rested or stimulated NK cells for 48 to 72 hours with a variety of cytokines did not demonstrate a significant difference in NK cell function in the HCV and healthy donors cohorts.[155,156,162,168] Furthermore, it has been noted that NK cells from healthy donors cultured in sera from HCV^+ donors for 72 hours reduces NK function compared to NK cells cultured in sera from HCV^- donors.[155] Together these studies suggest that NK cell cytotoxic function may be suppressed in chronic HCV infection, but this suppression may wane when NK cells are rested for 24 hours or more, indicating reversible inhibition.

NK cells produce IFNγ in response to stimulation. Again there appears to be a divergence in the literature regarding IFNγ production in chronic HCV infection compared to healthy donors. In experiments which stimulated NK cells using cytokines or PMA/ ionomycin in the *absence* of target cells, an increase in IFNγ production was found.[162,167,168] In the *presence* of target cells without cytokine stimulation NK cells from HCV^+ donors had relatively reduced IFNγ production.[159,165] These results again highlight how different experimental methods can produce conflicting results.

Table 1.5 Published reports of NK cell function in HCV compared to healthy donors

Outcome	Target cells	*Ex vivo* stimulation			Reported shift in NK function		
		0	4hrs	4-16hrs	>48hrs	Increase	No change	Decrease
Cytotoxicity CD107a/ ^{51}Cr release	HepG2	X						De Maria 2007
	C1RMICA	X						Sene 2010
	K562	X				Dessouki 2010	*Enriched NK only*- Morishima 2006	Corado 1997 Meier 2005 Sene 2010
			IL2					Corado 1997
				IL15				Meier 2005
				IL12 or 15				Ahlenstiel 2010*
					IL2		Corado 1997 Golden-Mason 2008	
					IL2+ IL12 or 21		Oliviero 2009	
					HD NK & HCV sera			Corado 1997
	P815				IL2 or IFNγ		Duesberg 2001	
					IL2+21	Oliviero 2009		
	Raji/ Daudi	X					De Maria 2007	
					IL2 or IFNγ		Duesberg 2001	
IFNγ production	No targets			PMA & ionomycin		Golden-Mason 2008		
				IL12+15or18		Ahlenstiel 2010		
				IL2+ IL12		Oliviero 2009		
	K562	X						Dessouki 2010 Sene 2010
	Anti-CD16 P815 anti-NKp30/46	X					De Maria 2007 Oliviero 2009	

X = no cytokine stimulation

* Ahlenstiel et al rested or stimulated NK cells for 14 hours with IL-12 or IL15 but did not elicit a reduction in CD107a externalisation. However, it was noted that in HCV infected individuals with raised ALT (>41 U/L), there was increased NK cytotoxic function compared to those with a normal ALT.

1.18 HCV NK - immunoevasion strategies

These studies suggest that HCV is effective at avoiding immune clearance to establish a chronic infection. HCV has been reported to impinge directly upon NK cells with reduced cytotoxicity and IFNγ capacity following exposure to hepatocyte cell lines infected with HCV *in vitro*.[172] Several studies have investigated potential mechanisms of HCV NK-immunoevasion (Figure 1.10) and they include:

i. HCV impingement upon hepatocyte IFNα signalling and ISG expression to alter NK cell activating ligands on the cell surface[177]. HCV core protein can act directly upon the IFNα-ISG pathway by selective degradation of STAT1.[178]

ii. HCV mediated up-regulation of inhibitory factors including SOCS-1, which inhibits JAK-tyrosine kinase and prevents STAT1 phosphorylation.[179,180]

iii. Upregulation of HLA-E on HCV-core protein^{+} hepatocytes (identified by liver biopsy immunohistochemistry). K562 cells transfected with $Core_{35-44}$ peptide have increased HLA-E expression reducing NK mediated cytolysis.[169]

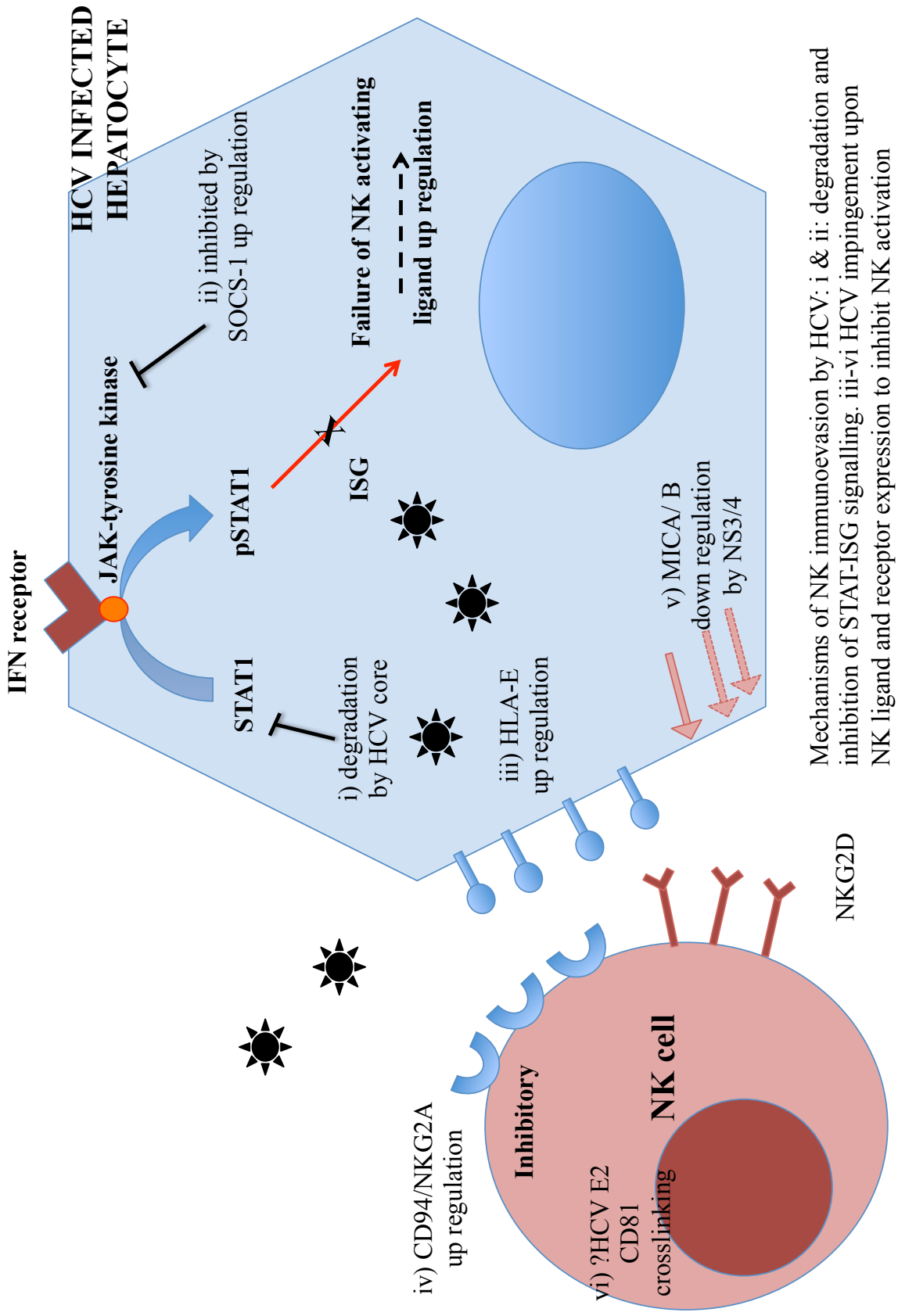
iv. Increased NK expression of CD94/NKG2A, the inhibitory receptor for HLA-E.[170]

v. NS3/4 protein down regulates NKG2D ligands MICA and MICB.[181]

vi. E2 protein binding to CD81, a B cell co-receptor, resulting in CD81 crosslinking, has an inhibitory effect upon NK cells within a plate-bound expression system.[182,183] However, infectious HCV virions, that solely express the structural proteins (core, E1 and E2), do not impinge upon NK cell function in a cell culture system.[184]

Any influences of other non-structural HCV protein upon NK cells have not been identified to date.

Figure 1.10 **HCV-NK immunoevasion**



1.20 NK cells during IFNα treatment of chronic HCV infection

Several studies have examined the effects of *in vivo* IFNα treatment for HCV infection upon peripheral blood NK cells. It has been reported that 24 hours following the initiation of IFNα treatment NK cells are driven towards increased cytotoxicity and reduced IFNγ production through increased pSTAT1 signalling and reduced pSTAT4 signalling.[180,185,186] Ahlenstiel et al measured NK degranulation by CD107a MFI following exposure to K562 target cells before and 6 hours after IFNα initiation. The rate of the first phase of viral load decline was associated with a greater ability to increase CD107a MFI in patients who cleared the virus compared to those who failed treatment. In the same study the expression of CD16 and TRAIL was measured on NK cells derived from liver biopsy specimens from a cohort of 6 patients pre-treatment and a separate cohort of 6 patients six hours after the initiation of IFNα treatment. The 6 patients who were biopsied after the initiation of treatment had higher intrahepatic expression of TRAIL and CD16 than those biopsied pre-treatment.[185] Furthermore the proportion of blood and intrahepatic NK cells is reported to increase in individuals who successfully clear HCV compared to those who have failed treatment.[160,165,168]

In summary chronic HCV remains a major global health problem in spite of major advances in medical therapy. NK cells are a major effector of the innate immune response to viral infection and can augment adaptive T cell responses. Advantageous NK cell phenotype and HLA type appears to provide a protective role to individuals who are exposed to HCV. In chronic disease the function and proportion of NK cells appears to be altered. However, there remain inconsistencies within the literature, and a marked paucity of data regarding the activity and phenotype of NK cells within the intrahepatic compartment during chronic HCV infection. There is also a lack of information regarding

the role that NK cells play during the successful clearance of chronic HCV infection with IFNα based therapies particularly within the intrahepatic compartment.

1.22 Hypothesis, objective & aims

The study described here aimed to explore NK cell function in chronic HCV infection and its relationship with expression of NK activating receptors in order to gain further insight into these areas of relatively limited information. For this reason, a detailed analysis of the phenotype and function of intrahepatic NK cells in chronic HCV infection and during IFNα treatment was conducted. Finally the ability of individual HCV proteins to impinge on NK cells to promote viral immunoevasion has been examined *ex vivo*.

Hypothesis

NK cell function is important for successful eradication of HCV. HCV infection compromises NK cell function through modulating expression of NK cell activating or inhibitory ligands by infected cells. This effect of HCV can prevent successful virus clearance in treated individuals.

Objective:

-Identify the effect of HCV infection on NK cell phenotype and function and identify correlates of successful treatment

Aims:

1. Is peripheral blood NK cell function impaired in patients infected with HCV? Do HCV genotypes with lower treatment response rates impinge on NK cell function?

2. Does intrahepatic NK cell activity reflect peripheral blood NK function? Is this altered with IFNα treatment?

3. Do individual HCV proteins derived from different genotypes cause an alteration of NK cell function when expressed in a target cell?

Chapter 2:

Materials & Methods

2.1 Patient Recruitment

Study approval

Cardiff and Vale Research Review Service reviewed and approved this study (reference number 10/cmc/4747). Ethical approval was granted by the South East Wales Research Ethics Committee (reference numbers 10/WSE02/45 and 04/WSE03/14).

Patient Recruitment

Patients were recruited from the hepatology and infectious diseases clinics at the University Hospital of Wales, Cardiff. The aim was to recruit a cohort of patients with chronic HCV, a cohort of non-viral chronic liver disease patients and a cohort of healthy donor controls.

Chronic HCV infection was defined as individuals who were identified as anti-HCV antibody positive and had positive HCV RNA, as measured by PCR, on two occasions at least 6 months apart. Inclusion criteria for the HCV cohort were: chronic HCV infection, over 18 years of age and capacity to provide informed consent. Individuals with HIV or HBV co-infection, less than 18 years old, pregnant or unable to provide consent were excluded.

Patients with chronic liver disease were considered for the study if they were over 18 years old and had capacity to consent to participation. Individuals with HIV infection were excluded. The cause of chronic liver disease did not affect eligibility to enrolment. Healthy donor controls were recruited from within Cardiff University.

Individuals identified by the medical and nursing teams in the clinic were invited to consider taking part in the trial and were provided with verbal and written information (Patient information sheet version 1.5 for HCV and chronic liver disease patients or Healthy donor

information sheet version 1.1). All patients participating in the trial were required to provide written consent prior to donating samples.

In total 35 patients with chronic HCV, 22 patients with non-viral chronic liver disease and 13 healthy donors were recruited (summarised in Table 2.1).

Table 2.1 Donor Characteristics

HCV donors

Patient	Age	Sex	Genotype/ Diagnosis	NI score	Fibrosis score	Viral Load	ALT	Treatment outcome	FNA	Degranulation assay
G1-01	31	F	1a	4	1	1.3×10^6	78	Failed	Yes	Yes
G1-03	57	F	1a	7	1	1.7×10^6	134	Failed	Yes	Yes
G1-05	45	M	1a NASH	8	5	6.5×10^5	110	Failed	Yes	Yes
G1-06	36	F	1	4	0	1.7×10^6	47	-	Yes	-
G1-08	62	M	1b	5	1	4.0×10^5	147	-	Yes	-
G1-11	42	M	1	4	3	7.0×10^5	47	-	Yes	Yes
G1-12	43	F	1A	3	0	2.3×10^6	26	-	Yes	-
G1-15	58	M	1	6	5	1.2×10^7	47	-	Yes	-
G1-17	52	M	1A	6	1	6.6×10^5	149	SVR	Yes	Yes
G1-18	62	F	1	5	2	6.5×10^5	57	Failed	Yes	Yes
G1-19	50	M	1	5	5	3.0×10^7	139	SVR	Yes	Yes
G1-21	53	M	1	3	0	3.6×10^6	33	-	Yes	-
G1-22	56	M	1	3	6	4.1×10^6	90	-	Yes	-
G1-23	37	F	1a NASH	6	5	24000	318		Yes	Yes
G1-24	53	M	1	3	0	3.6×10^6	33	-	Yes	-
G1-25	65	M	1a	4	5	3.2×10^6	90	-	Yes	-
G1-41	51	M	1 & 3	5	1	3.8×10^6	38	Failed	-	Yes
G1-42	27	M	1b	5	1	6.0×10^6	63	SVR	-	Yes
G3-01	44	M	3	-	-	5.5×10^5	66	SVR	-	Yes
G3-06	42	F	3a	0	4	2.0×10^6	80	SVR	-	Yes
G3-07	56	M	3a	6	6	2.3×10^6	35	SVR	Yes	Yes
G3-08	41	M	3a	2	1	4.4×10^5	87	-	Yes	-
G3-09	36	M	3a	6	2	2.4×10^6	332	SVR	Yes	Yes
G3-10	27	M	3	7	1	3100	29	SVR	Yes	Yes
G3-11	59	M	3	ND	ND	-	51	-	Yes	-
G3-12	49	M	3	3	2	1.6×10^5	174	-	Yes	-
G3-13	54	M	3	6	3	1900	179	-	Yes	-
G3-14	52	M	3	5	5	2.4×10^5	90	-	Yes	Yes
G3-40	58	M	3	4	6	3.1×10^5	53	Failed	-	Yes
G3-41	47	M	3	-	-	4.7×10^6	169	SVR	-	Yes
G3-42	39	M	3	5	2	1.6×10^6	136	SVR	-	Yes
G3-43	53	M	3	ND	ND	3.9×10^5	26	SVR	-	Yes
G3-44	67	F	3	3	1	2.2×10^5	174	SVR	-	Yes
G4-01	29	F	4	ND	ND	6.2×10^5	38	Failed	-	Yes
G5-01	46	F	5	ND	ND	7.6×10^6	52	SVR	-	Yes

Chronic Liver Disease donors

Patient	Age	Sex	Genotype/ Diagnosis	NI score	Fibrosis score	Viral Load	ALT	Treatment outcome	FNA	Degranulation assay
DD03	61	F	Deranged LFTs? Cause	0	0		89		Yes	
DD12	40	F	PBC	0	1		72		Yes	
DD13	49	F	NASH	0	5-6		25		Yes	
DD14	67	F	NASH	1	3-4		81		Yes	
DD15	52	F	Steatosis	0	1		53		Yes	
DD16	40	M	MTX use - NAD	0	0		17		Yes	
DD18	58	F	NASH	6-7	5-6		57		Yes	Yes
DD19	38	M	Steatosis	0	0		55		Yes	Yes
DD20	46	M	ALD HHC	1	1		73		Yes	Yes
DD21	69	M	GH	ND	ND		39		Yes	
DD22	53	M	ALD	0	0		34		Yes	
DD23	43	M	Steatosis	2	0		96		Yes	Yes
DD24	55	M	ALD HHC	2	5-6		46		Yes	
DD25	29	M	NASH	1	3		122		Yes	Yes
DD28	58	M	ALD HHC	2	5-6		39		Yes	Yes
DD29	55	F	PBC	2	3-4		59		Yes	Yes
DD30	42	F	PBC	2	3-4		179		Yes	Yes
DD32	55	M	HHC	1	4		23		Yes	Yes
DD33	55	M	PSC	2	1		54		Yes	Yes
DD34	51	F	PBC	2	5		151		Yes	
DD35	29	M	Wilsons				31		Yes	
DD36	58	M	MTX / NASH				64		Yes	

ALD- Alcoholic liver disease, HHC- hereditary haemochromatosis, NASH – Non alcoholic steatohepatitis, PBC- Primary biliary cirrhosis, MTX- methotrexate, LFTs – Liver function tests, GH- Granulomatous hepatitis, SVR – Sustained Viraemic Response, PSC- Primary Sclerosing Cholangitis, NAD – no abnormality detected, ND – not done

Healthy donors

13 healthy donors peripheral blood donated only

8 male, age range 24-45 (mean 28)

2.2 Patient samples & study protocol

Patients who entered the study were asked to donate blood samples with or without paired liver tissue samples.

Liver biopsy

Patients undergoing liver biopsy were asked to consent to use of residual tissue following normal histological examination. Biopsy samples were immediately fixed in normal buffered formalin solution embedded in waxed and stained with haematolxylin and eosin (H&E) in the histopathology department, UHW, prior to examination by a consultant hisopathologist. Biopsy samples taken at UHW are scored for inflammation and fibrosis using the Ishak's necroinflammatory and fibrosis scoring system.[187] This is a semi-numerical scoring system designed to give an increasing numerical score (0-18) for worsening liver inflammation, based on a descriptive score of portal inflammation (0-4), periportal/ periseptal interface hepatitis (0-4), focal lytic necrosis/ apoptosis/ focal inflammation (0-4) and confluent necrosis (0-6). A fibrosis stage (0-6) is also made, stages 5 - 6 are indicative of established cirrhosis.

Fine Needle Aspiration (FNA)

To gain a sufficient lymphocyte research sample for flow cytometry it has been necessary to perform a second pass of the biopsy needle to utilise a separate biopsy core. This separate core then requires physical and enzymatic degradation to isolate lymphoctyes. The not insignificant clinical risks associated with liver biopsy prevent the use of this technique to monitor lymphocytes at frequent intervals. Fine needle aspiration (FNA) consists of inserting a small-bore needle into a compartment to aspirate a single cell suspension for cytological examination. FNA of the liver provides a lymphocyte sample that correlates strongly with that derived from a liver biopsy.[35] FNA has previously been successfully utilised to assess

intrahepatic lymphocytes [188] and acute rejection of renal transplants.[189] In addition the smaller safer size of the needle represent a potential method of repeatedly sampling lymphocytes from within the liver at multiple time points at a relatively high frequency.

I have used the following technique:

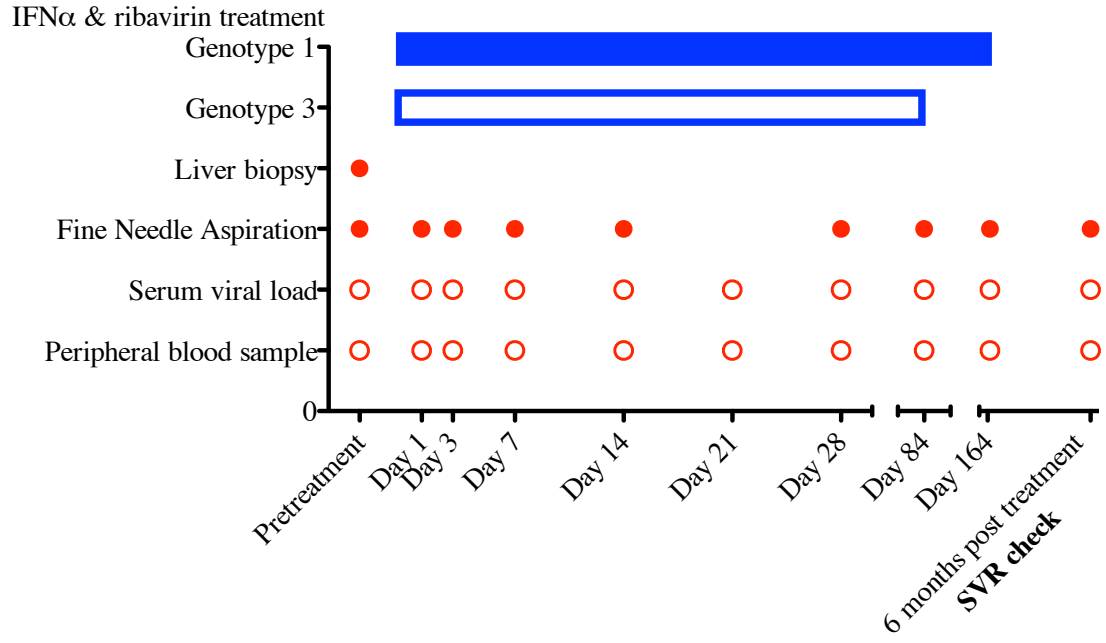
- Patient is in the supine position with the right hand behind the head. The abdomen is examined and a suitable site is marked in the right mid-axillary line approximately 2 intercostal spaces above the costal margin. The position of the liver is clinical confirmed by a dull percussion note in full expiration. In patients undergoing liver biopsy a liver ultrasound scan is performed within the last 6 months.
- The skin is cleaned with iodine solution.
- Up to 5ml of 2% lignocaine is infiltrated into the dermis and down to the hepatic capsule. The patient is asked to breath in and then exhale fully enabling lignocaine to be infiltrated into the liver capsule with the needle held 90° to the skin in all planes.
- A 22 gauge spinal needle, with an internal sylet, is then inserted into the intercostal space. The patient is then asked to inhale and exhale fully. The needle can then be inserted 2-3cm into the liver parenchyma from the capsule.
- The internal sylet is removed and a 10ml syringe filled with ice-cold RPMI 10% FCS is attached. Cells are then aspirated into the media as the needle is withdrawn to the liver capsule.
- The needle is then removed the sylet replaced and the needle is re-inserted for a second aspiration.
- The puncture site can then be dressed and the contents of the syringe are passed into a test tube containing RPMI 10% FCS on ice and then transported to the lab for immediate staining as described in Section 2.6 below.

It is important to note that these FNA samples are taken from the intracapsular space. As the liver is an extremely vascular organ it is likely that the needle may have passed through small sinusoids. If samples were heavily blood stained then they were discarded and the needle passed into fresh liver. (This occurred on less than 5 occasions during the study period.)

Following FNA sampling it is possible to then continue with a liver biopsy along the same tract of lignocaine anaesthetic. If the patient is not having a liver biopsy, is pain free and feels well they may be discharged without further follow up. This technique was applied to a cohort of 53 patients with 9 patients having FNA samples taken at multiple time points (Table 2.1).

Approximately 50ml of patient blood was drawn on each attendance. 4ml was placed in an EDTA test tube for serum separation and subsequent viral load analysis. The remainder of the sample was transferred to a 50ml test tube containing 250µl of unfractionated heparin.
Patient paired liver and peripheral blood samples were taken before treatment, on days 1, 3, 7, 14, months 1, 3 and 6 and 6 months following the completion of treatment. Additional blood and serum samples were taken on day 21 (Figure 2.1).

Figure 2.1 Study protocol schematic



Peripheral blood, serum (open circles) and liver samples (circles) were collected at multiple time points before, during and after treatment (blue bars).

2.3 Tissue culture

A full list of regents and suppliers is attached in Table 2.4 at the end of this chapter.

Media

Peripheral blood cells and K562 erythroblastic leukaemic cell line cells were washed and cultured in RPMI. RPMI is a phosphate rich media that utilises a bicarbonate buffering system for use in 5% carbon dioxide atmosphere. Huh7.5 hepatoma cells were washed and cultured in DMEM, which contains a higher concentration of vitamins, amino acids and glucose than RPMI.

RPMI and DMEM were supplemented with the following antibiotics; 100units/ml penicillin, 100μ/ml streptomycin and 2mM L-glutamine (Invitrogen). Further to this 10% heat inactivated FCS was added to maintain cells.

Target cell lines

K562 cells form a single cell suspension in RPMI 10% FCS and are incubated with 5% CO_2 at 37°C and the media was changed and cells split once they were confluent approximately every 36-48 hours. Huh7.5 cells are adherent cells, cultured in DMEM 10% FCS and grow more slowly than K562. Once the cells become confluent it is necessary to split them; the media was removed and the monolayer washed in PBS to remove FCS. Trypsin 0.05% was added and the cells incubated for 10 minutes the cells are loosened and the trypsin was neutralised with DMEM 10% FCS. The cells were then plated in fresh media at a lower concentration.

Cell freezing solution

Excess PBMC that were not used immediately were frozen for future use at -80°C or in liquid nitrogen in FCS supplemented with 10% DMSO.

Isolation of peripheral blood mononuclear cells

Whole blood was transferred into a 50ml test tube containing 250μl heparin. The fraction of mononuclear leukocytes, which contains T and B lymphocytes, NK cells and monocytes, were isolated by density gradient centrifugation by layering 25ml of blood onto 20ml lymphoprep and centrifuged for 20 minutes at 2000RPM with the brake off. Cells were aspirated washed twice in RPMI 10% FCS and counted with a haemocytometer. Cells were resuspended at 5 $x10^6$ /ml in PBS for immediate staining and excess cells were frozen for further experiments.

Purification of intrahepatic lymphocytes

The aspirate of FNA sampling was transferred to the lab on ice and PBS was added to the solution. Two techniques were compared to separate intrahepatic lymphocytes from aspirated debris. The methods are described below and the impact of these techniques was compared and the second was predominantly used as a higher lymphocyte yield was achieved with this method as outlined in Results Chapter 4, section 4.2:

Method 1. Initially samples were passed through a 70 μm cell strainer and centrifuged at 1600 RPM for 5 minutes. The pellet was resuspended in 5ml of RPMI 10% FCS and layered over Lymphoprep and centrifuged for 2000 RPM for 20 minutes. Although an interface was not evident the solution above the Lymphoprep layer was harvested and stained as described below.

Method 2. Samples were washed twice in PBS and plated for fluorochrome labelled monoclonal antibody staining. Cell surface markers were stained. Cellular debris was further

broken down during 0.5% saponin permeabilisation and removed by washing samples 3 times in perm buffer. Intracellular cytokines were then stained.

2.4 NK cell phenotyping and cytotoxicity assays

CD107a is a highly glycosylated protein that lines the membranes of cytolytic granules, upon degranulation granules bind to the cell membrane and as the contents are exocytosed CD107a is externalised and transiently expressed on the cell surface. Thus measurements of CD107a expression correlate strongly with target cell death as measured by ^{51}Cr release.[190]

Frozen PBMC samples were obtained from 23 chronic HCV patients pre-treatment, 13 healthy and 10 CLD donors (Table 2.1). Cells were thawed, washed and cultured overnight at 10^6/ml in RPMI-10% FBS in a 24 well plate with 1ml per well. The PBMC were incubated without stimulation or with 50 or 1000 IU/ml IFNα. PBMC were washed, counted and $5x10^5$ cells exposed to $2x10^5$ Huh7.5 or K562 target cells in DMEM-10% FBS or rested for 4 hours. During this time 5μl anti-CD107a antibody was added to each well (the details of antibodies used for flow cytometry are outlined in Table 2.2). To maximise staining of CD107a 1μL of Golgi stop (monensin) was added for the final 3 hours to inhibit intracellular protein transport preventing re-internalisation of CD107a from the cell surface. Non-adherent cells were washed and stained with Aqua live/dead stain and fluorochrome labelled monoclonal antibodies specific for CD3-APCH7, CD14-APCH7, CD19-APCH7 to exclude T cells, monocytes and B cells respectively. The phenotype of NK cell activating receptors was defined by CD16-FITC, NKG2D-APC, NKp30-Biotin, Streptavidin-PECy7, CD56-PerCP.Cy5 and NKp46-PB. Cells were washed, fixed with 2% paraformaldehyde solution and analysed by flow cytometry using a CyAn ADP flow cytometer (Beckman-Coulter).

NK target cell ligands

The expression of NK cell activating receptor ligands on Huh7.5 and K562 cells was analysed by flow cytometry using NKp30- and NKp46–human Fc fusion proteins with an

anti-human Fcγ-PE secondary antibody. The NKG2D ligands MICA, MICB and ULBP2, were analysed with mouse monoclonal antibodies and anti-mouse-AF647 secondary antibody.

Table 2.2 Antibodies used in flow cytometry assays

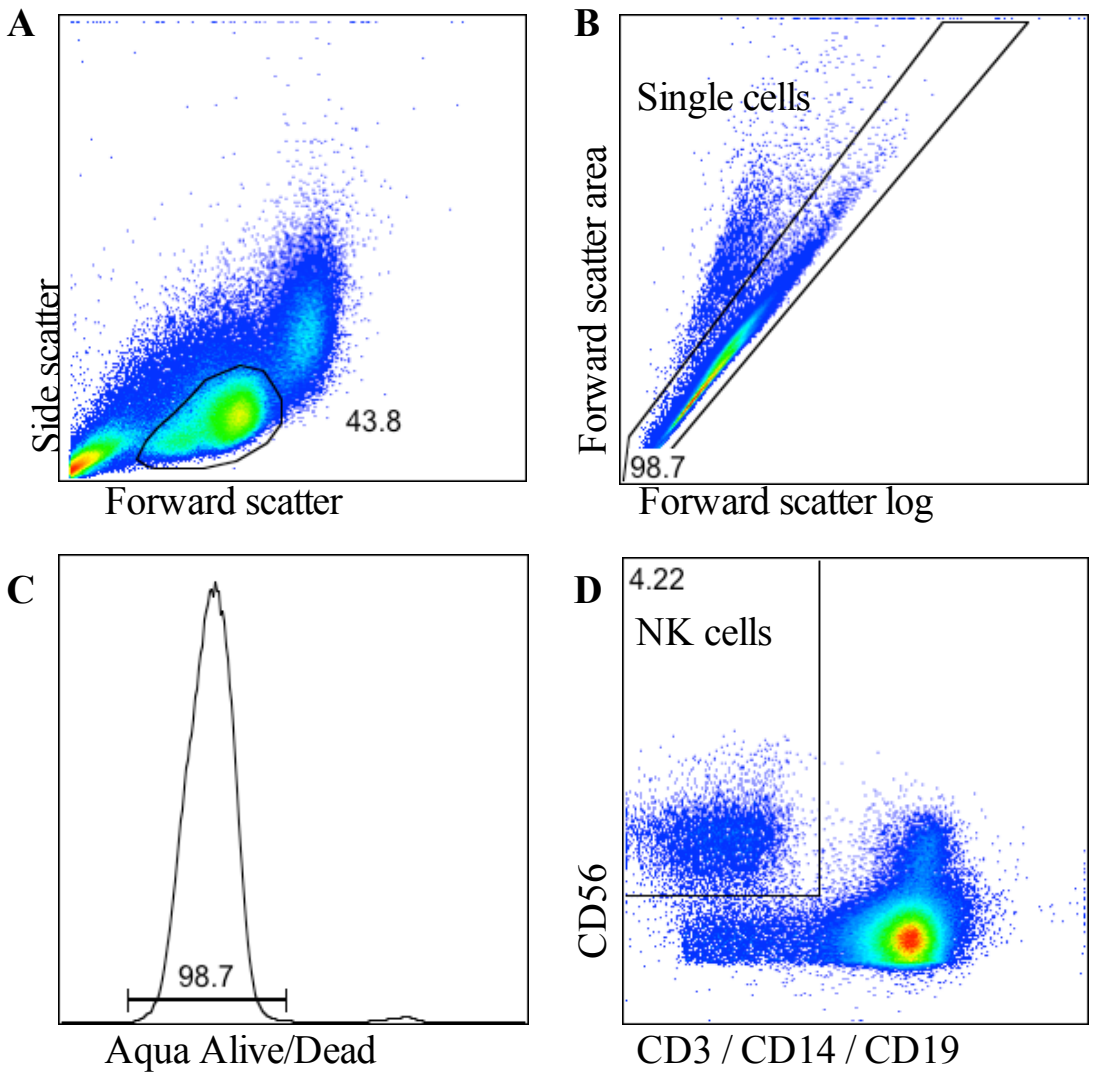
Antibody	Conjugate	Clone	Company	Dilution
Anti-Human IgG	PE	Polyconal	eBioscience	1:100
Anti-mouse IgG	AF647	polyclonal	Invitrogen	1:50
CD107a	FITC	H4A3	BD Pharmingen	1:40
CD107a	PE	H4A3	BD Pharmingen	1:40
CD14	APC.H7	MφP9	BD Pharmingen	1:50
CD16	FITC	VEP13	Miltenyi Biotec	1:50
CD16	PE	VEP13	Miltenyi Biotec	1:50
CD19	APC.H7	SJ25C1	BD Pharmingen	1:50
CD3	APC.H7	SK7	BD Pharmingen	1:50
CD56	PerCP-Cy5.5	HCD56	Biolegend	1:50
Granzyme B	APC	GB11	Invitrogen	1:100
IFNγ	e450	4S.B3	eBioscience	1:500
Ki-67	PE	Ki-67	Biolegend	1:50
Live/Dead	Aqua	-	Invitrogen	1:150
MICA	Mouse primary	AMO1	BAMOMAB	1:50
MICB	Mouse primary	BMO2	BAMOMAB	1:50
NKG2A	PE	131411	R&D Systems	1:100
NKG2D	APC	BAT221	Miltenyi Biotec	1:50
NKp30	Biotin	AF29-4D12	Miltenyi Biotec	1:50
NKp30-ligand	Fc fusion protein	-	R&D	1:50
NKp46	PB	9E2	Biolegend	1:25
NKp46-ligand	Fc Fusion protein	-	R&D	1:50
Streptavidin	PE-Cy7	-	eBioscience	1:500
ULBP2	Mouse primary	BUMO1	BAMOMAB	1:50

AF647	Alexa Fluor®647
APC	Allophycocyanin
APC.H7	Allophycocyanin-H7
e450	eFluor®450
FITC	Fluorescine-isothiocyanate
PB	Pacific Blue
PE	Phycoerythin
PE-Cy7	Phycoerythin-Cyanine 7
PerCP.Cy5.5	Peridinin chylorophyll-Cyanine 5.5

2.5 NK cell gating strategy

Isolated PBMC were stained with fluorochrome labelled monoclonal antibodies and amine reactive compound as described above. Lymphocytes were defined by granularity and size, cells with a proportional area to width were then selected as single cells (Figure 2.2). Dead cells were excluded by amine reactive compound and from the remainder (>97% of the lymphocyte gate) NK cells were defined as $CD56^{+}$ CD3, 14 and 19^{-} to exclude T cells, monocytes and B cells respectively. FMO stains were then used to create positive gates for markers of interest.

Figure 2.2 NK cell gating strategy

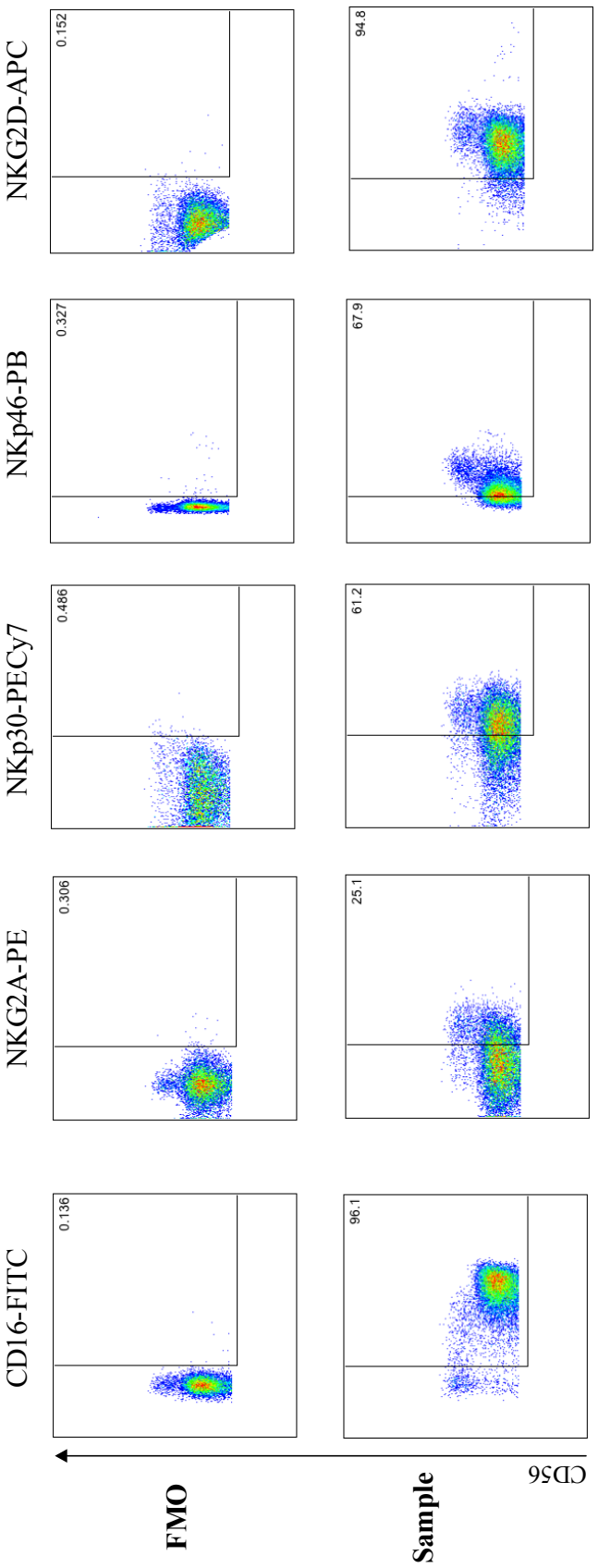


A) Lymphocyte gate Lymphocytes were defined by size and granularity on a forward and side scatter. **B) Single cells** Single cells were selected as events with proportional width and area. **C) Alive/ Dead stain** Aqua negative cells were selected as the alive population. **D) NK cells** NK cells were defined as $CD56^{+}$ CD3, 14 & 19^{-} to exclude T cells, B cells & monocytes.

FMO controls

FMO controls containing all the antibodies in the panel described above apart from the antibody of interest. FMOs are used to determine background fluorescence and any potential spill over from other fluorochromes into the channel of interest, which has not been fully compensated out, thus giving a cut-off level between positive and negative cells (Figure 2.3).[191,192] A major advantage of FMO stains compared to isotype control stains is that there is protection against potential variations in the flow cytometer over the long periods of time that the study was expected to take. Anti-mouse compensation beads were used to stain positive and negative controls and to create a compensation matrix during analysis. The data was analysed using FlowJo software (Ashland, Oregon, USA).

Figure 2.3 FMO controls and NK cell phenotype



NK cell phenotype was measured by the proportion of cells (%) with greater expression than the FMO control. Representative FACS plots showing FMO controls (top row) and pehnotypic samples for CD16, NKG2A, NKp30, NKp46 and NKG2D (bottom row). The quadrant contains the positive NK cell population with the percentage of positive cells in the top right corner.

2.6 Intrahepatic and peripheral blood NK phenotype and functional immunostaining

Intrahepatic lymphocytes were taken with paired peripheral blood. Samples were washed twice in PBS. All samples were stained with the following antibody panel: Aqua live/dead stain and anti- CD3, CD14, CD19, CD56 CD16, NKp30, NKp46, NKG2D and NKG2A (Table 2.2).

When samples contained larger numbers of lymphocytes it was possible to stain two separate panels of antibodies. In the second panel cells were also stained directly *ex vivo* without further stimulation with anti-CD107a. Cells were then permeabilised with saponin, fixed with paraformaldehyde (Fix/Perm) and stained with antibodies to Granzyme B, Ki67 and IFNγ. Cells were washed, fixed and analysed by flow cytometry as described above.

2.7 Immunohistochemistry

Liver biopsies were taken in the normal fashion and fixed immediately in normal Buffered Formalin Solution and sent to the histopathology department for clinical assessment. These fixed specimens were mounted on cover slips and were dewaxed with xylene and ethanol. Sections were then stained as follows:

i. Antigen retrieval was performed by heating specimens in TRIS EDTA.
ii. To prevent non-specific DAB substrate staining endogenous peroxidase activity was suppressed with Peroxidase Suppressor and non-specific antibody binding was blocked with 2.5% normal horse serum.
iii. Sections were stained overnight with goat anti-NKp46, mouse anti-CD56 and rabbit anti-CD3 antibodies diluted in 1% BSA.
iv. These antibodies were then detected with anti-goat, anti-mouse or anti-rabbit Immpress® reagents. The Immpress detection kit contains secondary antibodies conjugated to peroxidase micropolyers.
v. To visualise antibody bound cells DAB substrate was then applied.
vi. Sections were dehydrated with ethanol and xylene and then counterstained with haematoxylin and mounted with DPEX mounting medium.

Photomicrographs were taken using a NIKON microscope.

2.8 Viral Load Analysis

Serum samples were taken at multiple time points prior to and during IFNα treatment and frozen at -80°C. Samples were sent by batch to Lab21 (Cambridge UK) who quantified HCV RNA by rtPCR using Cobas Ampliprep/Cobas Taqman HCV test (Roche Diagnostic Systems). The lower limit of quantification was 30 copies per ml.

2.9 HCV protein expression using an adenovirus vector

Professor Gavin Wilkinson's group have developed an in-house recombinant adenovirus vector for the expression of individual human cytomegalovirus (HCMV) proteins.[193] I applied this technology to answer the question *"Do individual HCV proteins derived from different genotypes cause an alteration of NK cell function when expressed in a target cell?"*

2.10 HCV Gene selection

DNA copies of the Japanese Fulminant Hepatitis-1 (JFH-1) strain of genotype 2a (Core to NS5B, Genbank accession number AB047639) and Genotype 1b BB7 replicon (NS3-5B, Genbank accession number AJ238799) were kindly provided by Professor Peter Karayiannis (formerly of St Mary's Hospital, London). JFH-1 Core to E2 and Core to p7 (Figure 2.4) were expressed as polyproteins as E1 and E2 proteins lack signal peptides,[194] which ensure appropriate transfer and translation in the endoplasmic reticulum. NS2, 3, 4, 5, 5A and 5B proteins were expressed individually. The genes were cloned with 100 base pair (bp) primers containing approximately 80bp homology with the adenovirus genome depending on predicted binding properties (Table 2.3). Analysis of sequencing and primers were designed using CLC Main workbench 6.8 software (CLC bio, Aarhus, Denmark). The primers were constructed by Sigma-Aldrich (Dorset, UK) and samples sequenced in house by Central Biotechnology Services, School of Medicine, Cardiff University.

Figure 2.4 HCV proteins and polyproteins recominbeered into adenovirus vectors

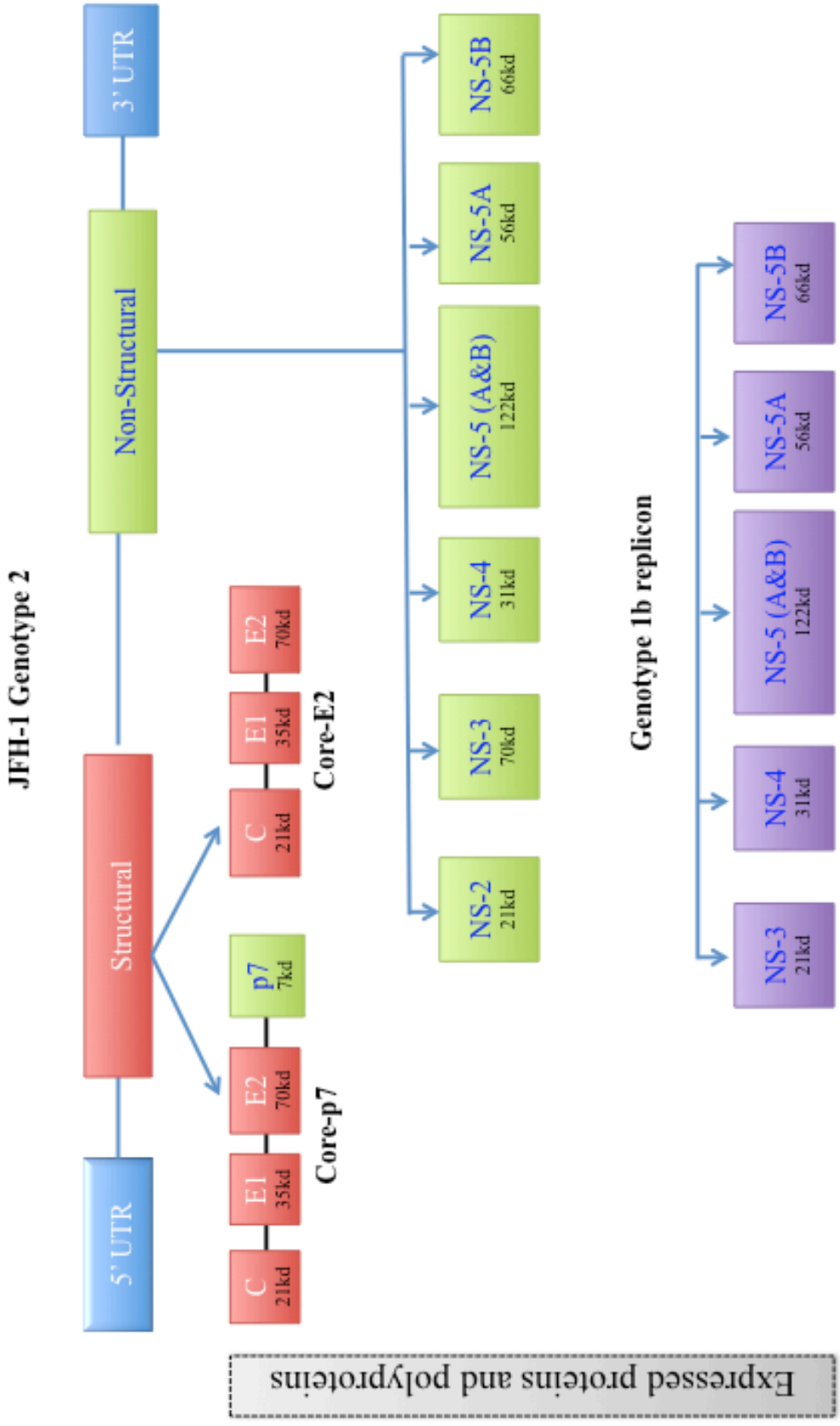
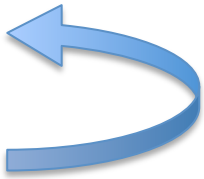


Table 2.3 Adenovirus vector expressed HCV proteins and primers

Protein (gene size)	Forward Primer	Reverse Primer
JFH-1		
Core-E2 (2.25kb)	5'-ATGAGCACAAAT CCTAAACCTCAA-3'	5'-TGCTTCGGC CTGGCCCAA-3'
Core-p7 (2.44kb)	5'-ATGAGCACAAAT CCTAAACCTCAA-3'	5'-ATGAGCACAAAT CCTAAACCTCAA-3'
NS2 (0.65kb)	5'-TATGACGCACC TGTGCACGGA-3'	5'-AAGGAGCTT CCACCCCTTG-3'
NS3 (1.89kb)	5'-GCTCCCATCA CTGCTTATGC-3'	5'-GGTCATGACC TCAAGGTCAG-3'
NS4 (0.95kb)	5'-AGCACGTG GGTCCTAGC-3'	5'-GCATGGGAT GGGGCAGTC-3'
NS5 (3.07kb)	5'-TCCGGATC CTGGCTCC-3'	5'-CCGAGCGGG GAGTAGGAA
NS5A (1.4kb)	5'-TCCGGATC CTGGCTCC-3'	5'-GCAGCACAC GGTGGTATC-3'
NS5B (1.77kb)	5'-ATGTCATACT CCTGGACCG-3'	5'-CCGAGCGGG GAGTAGGAA-3'
Replicon 1b		
NS3 (1.89kb)	5'-ATGGCGCCTA TTACGGCCTA-3'	5'-CGTGACGACC TCCAGGTCA-3'
NS4 (0.95kb)	5'-AGCACCTGG GTGCTGGTA-3'	5'-GCATGGCGTGGA GCAGTCCTCGTT-3'
NS5 (3.11kb)	5'-TCCGGCTCG TGGCTAAGA-3'	5'-TCGGTTGGGGA GTAGATAGATG-3'
NS5A (1.34kb)	5'-TCCGGCTCG TGGCTAAGA-3'	5'-GCAGCAGAC GACGTCCTCA-3'
NS5B (1.77kb)	5'-TCGATGTCCTA CACATGGACAG-3'	5'-TCGGTTGGGGA GTAGATAGATG-3'

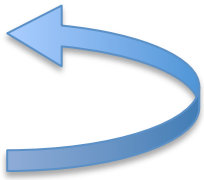
PCR products were amplified using Roche Expand Hi-Fi kit. Each 50µl reaction mix contained 1µl template, 4µl forward primer, 4µl reverse primer, 5µl Buffer 2 (10x concentrate, containing $MgCl_2$), 6µl 4mM dNTPase, 0.5µl Hi-Fi enzyme and 28.5µl double distilled water. Samples were synthesised using a T3 Thermocycler (Biometra Germany) with the following incubations:

1. 95°C 2 minutes
2. 95°C 30 seconds
3. 55°C 30seconds
4. 68°C 4 minutes 30 seconds



Repeat 9x cycles 2→4

5. 95°C 30 seconds
6. 55°C 30seconds
7. 68°C 4 minutes 30 seconds



Repeat 24x cycles 5→7

increasing by 20 seconds each cycle

8. 68°C 15 minutes

The PCR product was run on a 0.7% agarose gel and the bands of the correct gene size were excised and DNA purified using GFX columns according to the manufacturers instructions.

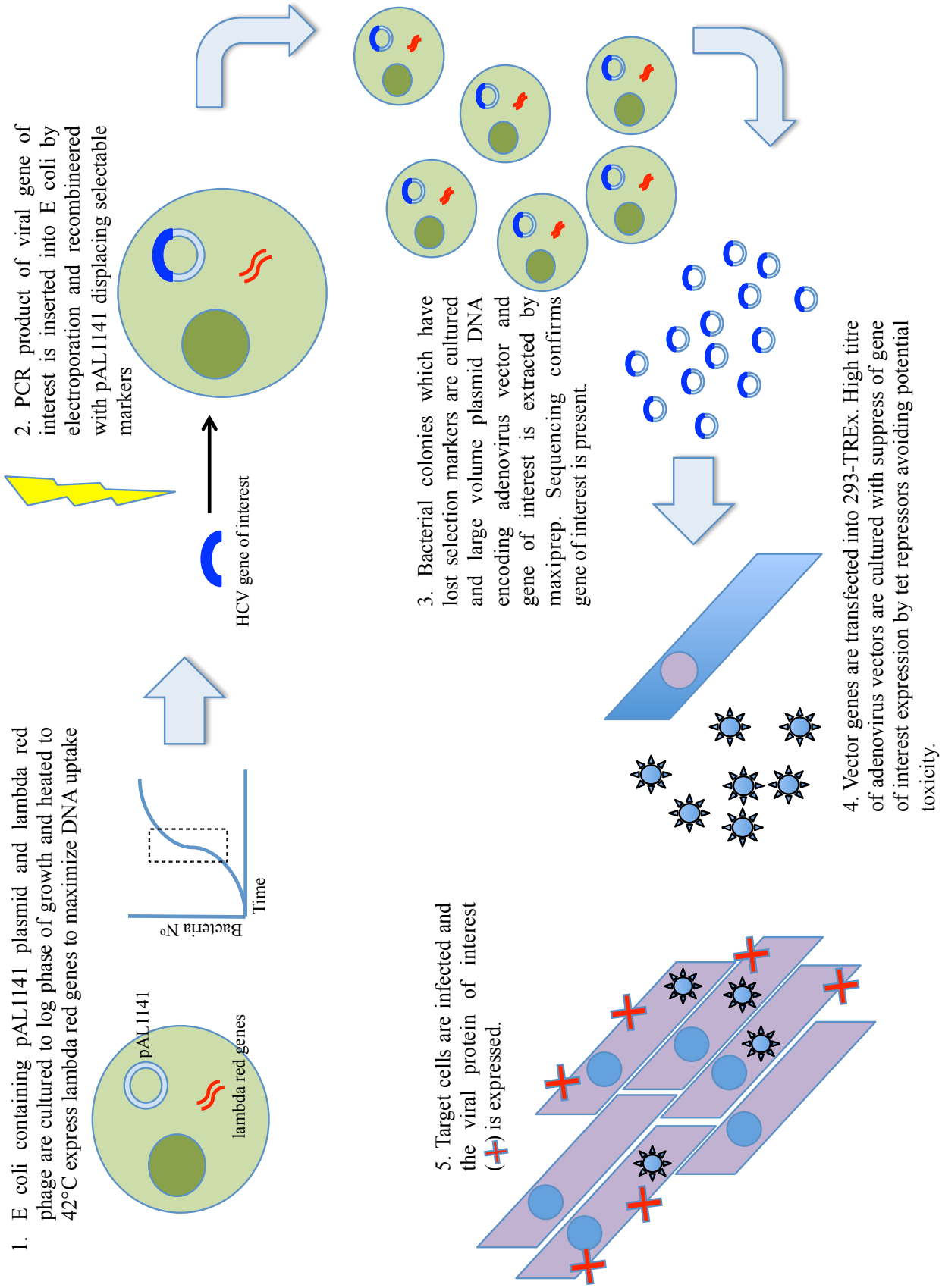
2.11 Advec expression system overview

The adenovirus vector recombineering system is laid out in detail below. In summary the principles are shown in Figure 2.5 and described as follows:

E. coli that contained a single copy of the plasmid pAL1141 and a defective bacteriophage, which encoded lambda red genes, were cultured. pAL1141 encoded i) bacterial artificial chromosome (BAC), ii) modified adenovirus genome, iii) selectable markers & iv) antibiotic resistance.

1. Bacteria were cultured overnight in LB media until log phase of growth was achieved, which is optimal for DNA uptake. Lambda red genes were expressed by heating the bacterial culture to 42°C for 15 minutes.
2. The viral genes of interest DNA were then mixed with the bacteria and inserted into the *E. coli* by electroporation. Recombination of the gene of interest into the plasmid displaced two selectable markers to allow isolation of clones containing recombineered plasmids.
3. The plasmid genome was then isolated by a maxiprep and the presence of the correct gene of interest was confirmed by sequencing.
4. The adenovirus vector was transfected into 293T-REx cells without further digestion. The insert expression suppressed by tetracycline (tet) repressors. Therefore, potentially toxic proteins of interest were not expressed whilst the virus was grown up in 293TREx.
5. Once a high viral titre was achieved the virus was extracted and can infect target cells in which the viral protein of interest was expressed.

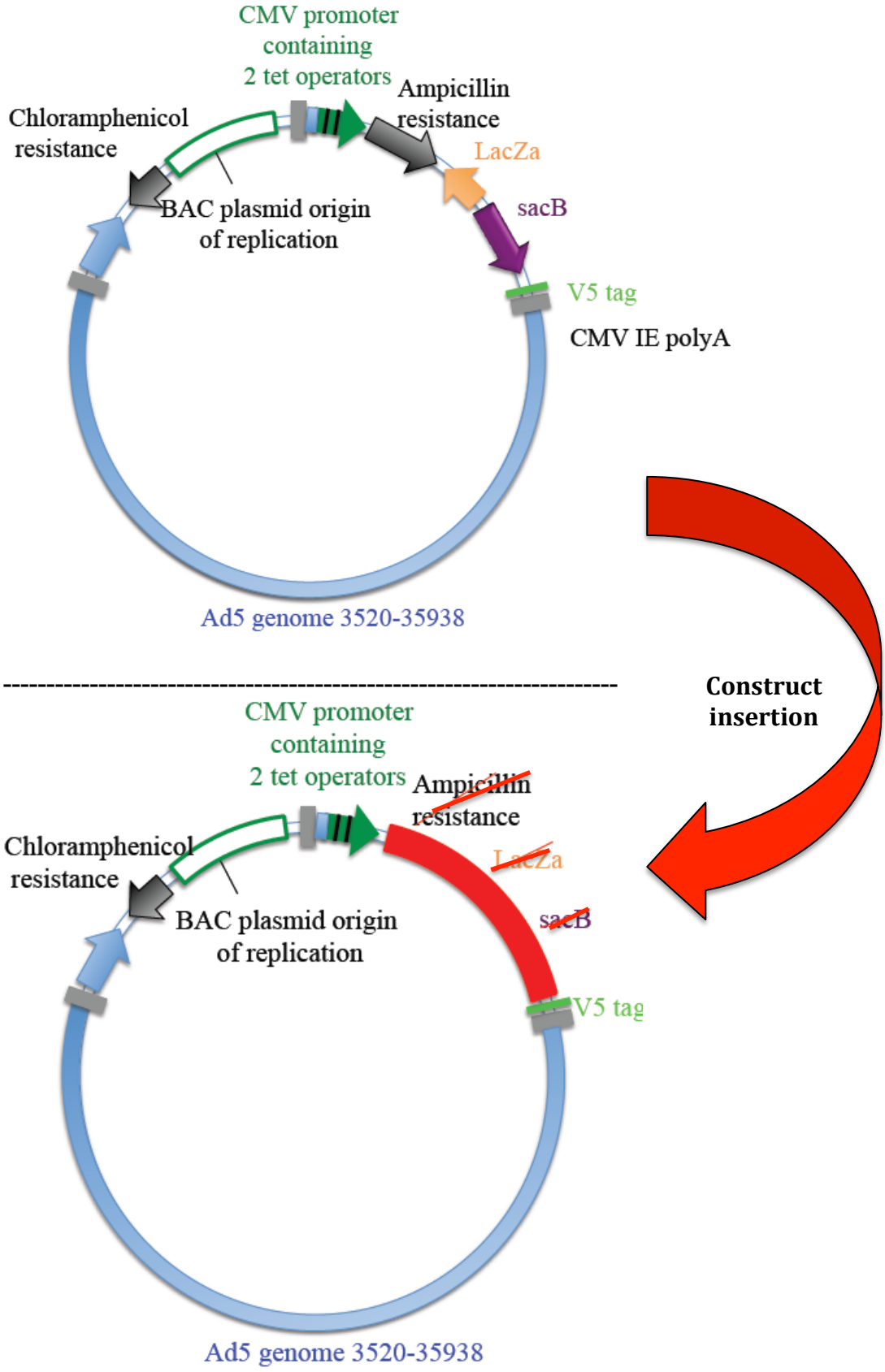
Figure 2.5 Advec expression system overview



2.12 pAL1141

In detail pAL1141 (Figure 2.6) contains the adenovirus vector genome with E1 and E3 regions deleted to prevent vector replication. The plasmid contains a HCMV promotor to induce transcription, chloramphenicol resistance gene, and a bacterial plasmid origin of replication. Following the HCMV promotor and before a V5 tag is a cassette, which encodes ampicillin resistance, LacZα, and SacB. This cassette is replaced during recombineering with the gene of interest. SacB encodes sucrose sensitivity rendering sucrose-based culture media toxic to bacteria. LacZα cleaves X-gal, an analogue of lactose, releasing 5-bromo-4-chloro-3-hydroxyindole marking a colony of bacteria containing this gene as blue on a culture plate.

Figure 2.6 Adenovirus vector in pAL1141 plasmid



2.13 Adenovector recombineering

pAL1141 bacteria were cultured overnight in 5ml LB media containing 12.5μg/ml chloramphenicol in a shaking incubator at 32°C. The culture was increased to 25ml LB containing chloramphenicol and cultured for ~3 hours. As bacterial cultures in a log phase of growth are optimal for DNA uptake. In LB media *E. coli* in a log phase of growth have an optical density$_{600}$ of approximately 0.6, this was measured using an Ultraspec 3000 (Pharmacia Biotech). Once this optical density was reached the culture was then transferred to 50ml test tubes and placed in a 42°C water bath for 15 minutes to induce the defective phage lambda red proteins. After cooling and removing supernatant the bacteria was transferred to a pre-cooled cuvette with 3μl of PCR product and stood on ice for 5 minutes before being electroporated at 2.5kV using a Biorad Micropulser. Bacteria were recovered in 1ml LB media and transferred to universal container with a total of 5ml LB to be cultured for a further 3 hours to ensure that all bacteria which have had the SacB gene replaced during recombineering lose any sucrose sensitive proteins.

Bacteria were then plated onto a salt deplete LB agar plate containing 1/1000 of 12.5mg/ml chloramphenicol, 1/500 of 40mg/ml X-gal and 1/500 of 100mM isopropyl β-D-1 thiogalactopyranoside to induce LacZa. Plates were incubated at 32°C for 48 hours until white and blue colonies appeared. Blue colonies had not lost LacZa through appropriate recombineering and with the gene of interest. Therefore, four white colonies were selected and cultured in 5ml LB media at 32°C overnight.

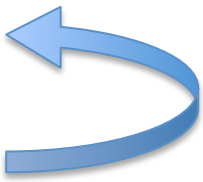
The plasmid DNA was isolated from colonies using Miniprep kits (Qiagen) according to manufacturer's instructions. Briefly following overnight culture the colonies were centrifuged at 4000RPM for 5 minutes and the pellet resuspended in 250μl P1 RNAase buffer. 250μl P2

lysis buffer was added and incubated for 5 minutes at room temperature. 250µl P3 neutralisation buffer was added and then centrifuged at 13000 RPM for 10 minutes. The supernatant containing non-nuclear DNA was transferred to a fresh tube and the DNA was precipitated with 250µl isopropanol and centrifuged at 13000RPM for 30 minutes at 4°C. The supernatant removed and the pellet was washed in 70% ethanol. The ethanol was aspirated and then the pellet was air dried before resuspension in distilled water.

Colonies bearing DNA inserts of the correct size were identified by BamHI restriction digest. 8µl of the DNA preparation was added to 1µl BamHI and 1µl Buffer E and incubated for 1 hour at 37°C before running on a 0.7% agarose gel.

To sequence the DNA forward and reverse primers were constructed by Sigma-Aldrich, designed to bind to the vector at i) the CMV promoter prior to the insert and ii) the CMV polyA following the V5 tag and iii) approximately every 750bp in between to ensure sequencing of the entire insert (Table 2.4). For each 10µl reaction 5ul of DNA was combined with 1µl primer and 4µl of BigDye 4.1 and run on a T3 Thermocycler with the following parameters:

1. 95°C 5 minutes
2. 95°C 30 seconds
3. 55°C 10 seconds
4. 60°C 4 minutes



Repeat 99x cycles 2→4

PCR reactions were cleaned through a Performa DTR column and the DNA was sequenced by CBS, Cardiff University.

Table 2.4 Adenovirus vector sequencing primers

Protein	Position	Sequence
CMV promoter		
Forward sequence for all constructs		5'-AATGTCGTAACAACTCCG-3'
CMV polyA		
Reverse sequence for all constructs		5'-ACCTGATGGTGATAAGAAG-3'
JFH		
Core-p7	1008	5'-TGCCCAGGTGAAGAATACC-3'
Core-p7	1402	5'-ATGATGATGAACTGGTCGC-3'
Core-p7	1820	5'-ACATCGAGGCTTTCCGGATA-3'
NS2		*Sequenced from promoter & polyA*
NS3	801	5'-GGACAAGCGGGGAGCATTG-3'
NS3	1302	5'-CATATGCGATGAATGCCAC-3'
NS4		*Sequenced from promoter & polyA*
NS5	618	5'-CGGCGCCAACATCTCTGG-3'
NS5	1419	5'-GAGGCCAGATTACCAACCG-3'
NS5	1882	5'-AGCCCCGAAGAGGAAAAGTT-3'
NS5	2704	5'-TGGGTAACACCATCACATGC-3'
Replicon 1b		
NS3	888	5'-CGCTGTGGGCATCTTTCGG-3'
NS3	1372	5'-GAGACGGCTGGAGCGCGA-3'
NS4		*Sequenced from promoter & polyA*
NS5	874	5'-CCGGCCCCCGAATTCTT-3'
NS5	1301	5'-TCCAAGCGGAGGAGGATGAG-3'
NS5	1775	5'-TCTGCTGCTCGATGTCCTAC-3'
NS5	2158	5'-TTGCTGGAAGACACTGAGAC-3'
NS5	2664	5'-ATGTTACTTGAAGGCCGCTG-3'

Once the fresh colonies with the correct inserts were identified an aliquot was frozen in 100% glycerol for later use. Plamsid DNA for transfection was isolated by maxiprep using NuceloBond BAC100 kit according to the manufacturer's instructions. In brief bacteria bearing the adenovirus vector of interest were cultured in 250ml LB media 1 in 1000 chloramphenicol overnight. The culture was then centrifuged at 6000RPM for 15 minutes at 4°C and the supernatant discarded. The pellet was resuspended in 24ml S1 RNAase buffer and 24ml S2 lysis buffer was added prior to incubation for 4 minutes at room temperature. 24ml ice-cold S3 neutralisation buffer was added and the mixture was incubated for 5 minutes on ice and then spun down at 6000RPM for 15 minutes. Filter columns were equilibrated with N2 buffer prior to adding the culture lysate. The column was washed twice with 18ml N3 wash buffer prior to elution with 15ml N5 buffer pre-warmed to 50°C. 11ml isopropanol was added to precipitate the DNA and the solution was centrifuged for 30 minutes at 15000RPM. The isopropanol was aspirated and the DNA pellet was washed in 70% ethanol prior to air-drying in a 37°C water bath. The DNA was resuspended in double distilled water and the concentration of DNA was measured using ND-1000 Nanodrop spectrophotometer.

2.14 Transfection & titration of adenovirus vectors

293 cells are derived from embryonic kidney fibroblasts which have been immortalised by integration of the adenovirus E1 gene region.[195] The adenovirus vector is deficient of the E1 region which ordinarily prevents viral replication and assembly, however, in 293 cells the adenovirus E1 promotes viral replication and assembly allowing expansion of the viral titre. In addition 293 T-REx cells stably express tetracycline repressor proteins. Hence, transfected cells with tet operators can have protein expression controlled. Adenovirus vectors were transfected into 293TREx cells by lipid micelle transfection (Effectene as specified in the manufacturers instructions). In brief 4μg of DNA was added to a final volume of 150μl Buffer EC. 8μl of enhancer was added and the sample vortexed for 30 seconds. 25μl of Effectene transfection agent was added and the sample mixed and then incubated at room temperature for 10 minutes. The sample was added to media and added to a confluent monolayer of 293TREx cells. Cells were cultured at 37°C to allow the virus to grow. Virus was harvested from cells by loosening cells with trypsin and resuspending in PBS. An equal volume of tetrachloroethylene was added to lyse the cells but leaving the capsulated virus intact. The emulsion was then separated by density gradient by centrifugation and the virus harvested in the less dense PBS layer.

Purified viruses were titrated using an accelerated plaque assay.[196] In brief 293TREx cells were seeded onto a 12 well plate and cultured after 24 hours serial dilutions of 10^{-4} and 10^{-5} of the virus stock was prepared and added to the wells. After 48 hours cells infected with virus were identified using goat anti-adenovirus antibody with an anti-goat horseradish peroxide secondary antibody and DAB metal concentrate. Visible cells were counted and virus titre calculated as plaque forming units (pfu)/ml.

Adenovirus vectors proteins were used to infect human foreskin fibroblasts (HFF) expressing the high affinity Coxsackie virus and adenovirus receptor (CAR).[197] Protein expression was confirmed by Western blot and immunostaining of cell lines.

Western blots

HFF CAR^+ cells were infected with adenovirus vectors with a multiplicity of infection (MOI) of 5 for 48 hours. The media was aspirated and 100µl NuPAGE buffer with 1 in 10 DTT was added. The cells were then scraped off and collected. The cell lysates were then heated to 100°C for 10 minutes prior to centrifugation at 13000RPM for 5 minutes. Samples were then loaded into precast NuPAGE Bis-Tris gels and run for 90 minutes at 150 volts. Gels were blotted onto Hybond ECL nitrocellulose membranes at 15 volts for 1½ hours. Membranes were blocked overnight with TRIS Tween (TRIS-T) 5% fat free milk at 4°C on a rocking platform. Membranes were washed in TRIS-T and then incubated with primary mouse anti-V5 and when required anti-JFH core, anti-NS3 and NS4 antibodies on a rocking platform at room temperature. Membranes were washed three times in TRIS-T and anti-mouse horseradish peroxidase antibody was added for 1 hour. Membranes were then incubated for 5 minutes with Super Signal West Pico chemiluminescent substrate and exposed for imaging as demonstrated in results Chapter 5.

Immunostaining of infected HFF cells

Cover slips were sterilised in 70% ethanol and washed with PBS. HF CAR cells were then seeded onto the coverslips in a 12 well plate. After 24 hours the HF CAR were infected with an MOI of 5. 48 hours later the media was removed and the cells fixed in 4% paraformaldehyde for 15 minutes at room temperature. After washing with PBS the cells were permeabilised with 0.5% NP40. Cover slips were washed in PBS and cells incubated with

primary mouse antibodies to V5, JFH core, NS3 and NS4 for 30 minutes at 37°C. The coverslips were washed and the coverslips incubated with anti-mouse AF488 antibodies for 30 minutes at 37°C. The coverslips were washed in PBS and allowed to dry prior to fixing in Dabco to glass slides for immunofluroscence.

The overall purpose of this adenovirus recombineering was to generate a series of reagents, which could be used to infect target cells with either individual or a combination of the HCV genes. This has enabled a series of experiments to be conducted examining whether HCV proteins could selectively impinge on NK cell functions using the methods described in section 2.4. The results of these experiments are described in chapter 5.

2.15 Statistical analysis

A comparison of phenotypic and functional markers was made using a student *t*-test assuming normal distribution of data. The kinetic parameters of viral clearance (rate constant k in day^{-1}) were calculated from a non-linear regression curve best fit ($y = y_0e^{-kx}$ + plateau; y_0 = viral concentration day 0, plateau = final viral concentration after treatment). Comparison of NK functional markers with activation markers and rate of viral clearance was by linear regression. Comparison of non-parametric data and NK markers was by Spearman's rho analysis. Software employed was Excel 2011 and GraphPad Prism 5.0.

Table 2.5 Reagents and suppliers

Reagent	Description / supplier
70µm cell strainer	Fischer Scientific Loughborough UK
Agarose gel (0.7%)	0.7% agarose powder (AGTC Bioproducts UK) in 1x TAE buffer (National diagnositics, Atlanta, GA)
Anti-adenovirus antibody	Goat origin Chemicon Millipore Watford UK
Anti-goat horseradish peroxidase	Abcam UK
Anti-JFH core antibody	Mouse anti JFH-1 core antibody, BioFront Technologies Tallahassee FL
Anti-mouse HRP antibody	Goat anti-mouse horseradish peroxidase antibody Millipore Watford UK
Anti-mouse/ -goat/ -rabbit Immpress	Vectorlabs Peterborough UK
Anti-NS3 antibody	Mouse monoclonal to HCV NS3 Abcam Cambridge UK
Anti-NS4 antibody	Mouse monoclonal to HCV NS4 Abcam Cambridge UK
Anti-V5	Mouse monoclonal antibody Invitrogen Paisley UK
BamHI	BamHI restriction digest enzyme Promega Southampton UK
BigDye 4.1	DNA sequening dye, ABI, Applied Biosystems Invitrogen Paisley UK
Chloramphenicol	Sigma-Aldrich, Gillingham UK
DAB	Vectorlabs, Peterborough UK
DAB metal concentrate	Thermo Scientific UK
Dabco	Dabco mounting agent, Sigma Aldrich Poole UK
DMEM 10%	DMEM (Invitrogen UK) 10% FCS, 250 units penicillin/streptomycin, 0.26mg/ml L-glutamine, 97mg.ml sodium pyruvate (all from Gibco UK)
DMSO	Sigma life sciences, Gillingham UK
DTT	Sigma-Aldrich
Effectene	Lipid micelle transfection kit Qiagen Manchester UK
Expand Hi-Fi	PCR enzyme buffer kit. Roche UK
Fix/Perm buffer	0.5% saponin permeabilisation paraformaldehyde fixation kit ebioscience Hatfield UK
GFX columns	PCR and Gel band purification kit. GE Healthcare Life Sciences Amersham UK
Golgi stop	Monensin, BD Biosciences Oxford UK
Heparin	unfractionated heparin- Wockhardt UK Ltd Wrexham
Hybond ECL	Nitrocellulose membrane for Western Blots GE Healthcare
IFNα	Roferon Roche UK
LB media	20mg/L of LB low salt broth (Melford Laboratories) in ddH_2O

Lymphoprep	Axis-Shield, Oslo Norway
Miniprep kit (Qiagen)	Plasmid DNA purification kit utilitsing gravity flow anion-exchange columns. Qiagen Manchester UK
Normal horse serum	Vectorlabs Peterborough
Nucelobond BAC100	Anion exchange column system for large scale purification of plasmid DNA, Macherey-Nagel, Germany
NuPAGE Bis-Tris gels	Precast Western blot gels Invitrogen Paisley UK
NuPAGE buffer	Invitrogen
PBS	Gibco UK
Performa DTR column	PCR product purification column, Edge Biosystems Gaithersburg MD
Plastic ware	All standard plastic ware for cell culture was purchased from Nunc, Greiner or Fisher UK
RPMI 10%	RPMI, 10% FCS, 250 units penicillin/streptomycin, 0.26mg/ml L-glutamine, 97mg.ml sodium pyruvate (all from Gibco UK)
SuperSignal West Pico	Enhanced chemiluminescent substrate for horseradish peroxidase Thermo Scientific Hemel Hempstead UK
Trypsin 0.05%	Gibco UK
Vacuette K_2 EDTA test tube	Greiner Bio-One, Stonehouse UK

Chapter 3:

Results –

Peripheral blood NK cells

&

chronic HCV infection

3.1 Peripheral blood NK phenotype & function in chronic HCV

NK cells are important in control of viral infections. As outlined in the introduction above there remains a lack of consensus as to NK phenotype in chronic HCV infection. Separate studies investigating activating receptors, such as NKp30, NKp46 and NKG2D have described variously reported as up-regulated,[162,167,171] down regulated,[158,166] or unchanged [159,165] in chronic HCV compared to healthy donors. There remains a similar lack of consensus surrounds the preservation or alteration of NK cell cytotoxic function in chronic HCV as laid out in Table 1.3.[156,162,164,165,167,168,186]

In this chapter the following questions are addressed

- *"Is peripheral blood NK cell function impaired in patients infected with HCV?"*
- *"Do HCV genotypes with lower rates of treatment response have an increased impact upon NK cell function?"*

In order to achieve this we recruited a cohort of 27 patients with chronic HCV infection, 13 healthy donors and 11 chronic liver disease (CLD) donors from the hepatology clinic. Of these patients 24 completed combined IFNα and ribavirin treatment; 7 failed to clear the virus and 17 achieved SVR and cleared the virus (Table 2.1 page 56). Serum samples were taken at multiple time points during treatment for viral load measurements and the rate of viral clearance was calculated for 18 patients. Pre-treatment PBMC were isolated by centrifuge density gradient and frozen until use. Thawed NK cells were rested or stimulated with IFNα overnight and then the frequency, phenotype and cytotoxic function of NK cells was assessed by flow cytometry.

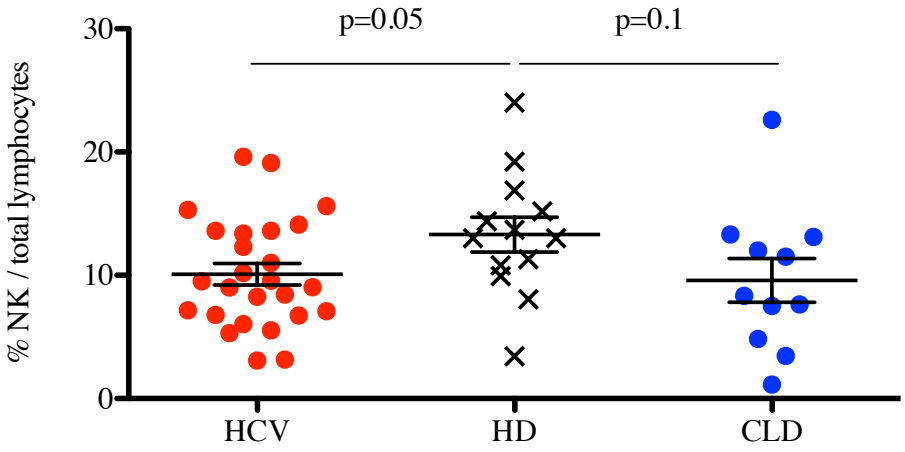
RESULTS

3.2 The proportion of peripheral NK cells is reduced in chronic HCV infection

The proportion of NK cells in the peripheral blood was lower in individuals with chronic HCV infection than healthy donors (mean 10.1% and 13.3% respectively p=0.05, Figure 3.1). Mean proportion of NK cells was also lower in the non-viral CLD cohort (mean 9.5% v HD p=0.1). This reduction compared to healthy donors was more marked for genotype 1 patients (mean 9.2 p=0.036) than genotype 3 patients (mean 10.3, p=0.13). There was no a significant reduction in the mean proportion of NK cells of HCV infected and CLD controls.

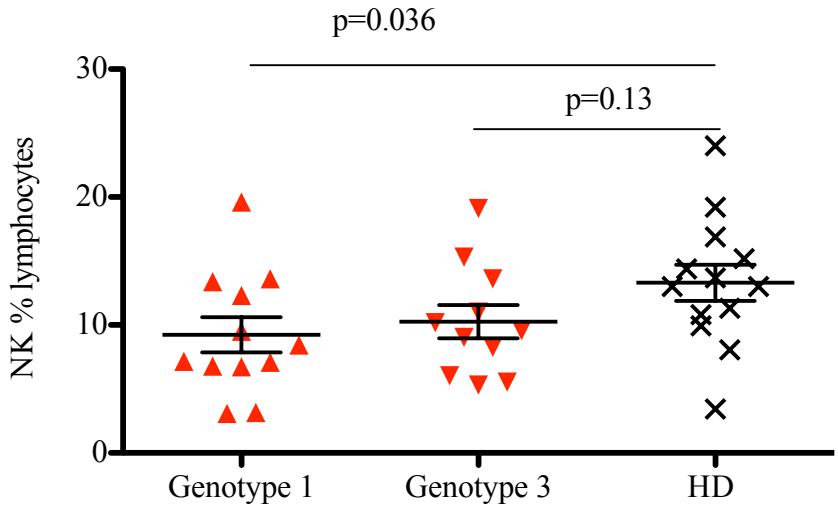
Figure 3.1 The proportion of peripheral NK cells in chronic HCV

A



NK cells as a proportion of peripheral blood lymphocytes in chronic HCV infection. There is a reduction in NK cell proportion in chronic HCV compared to healthy donors (HD). Individuals with non-viral chronic liver diseases also had a reduction in the proportion of NK cells but this did not statistical significance. Mean & SEM shown.

B) HCV genotype and peripheral blood NK cell proportion

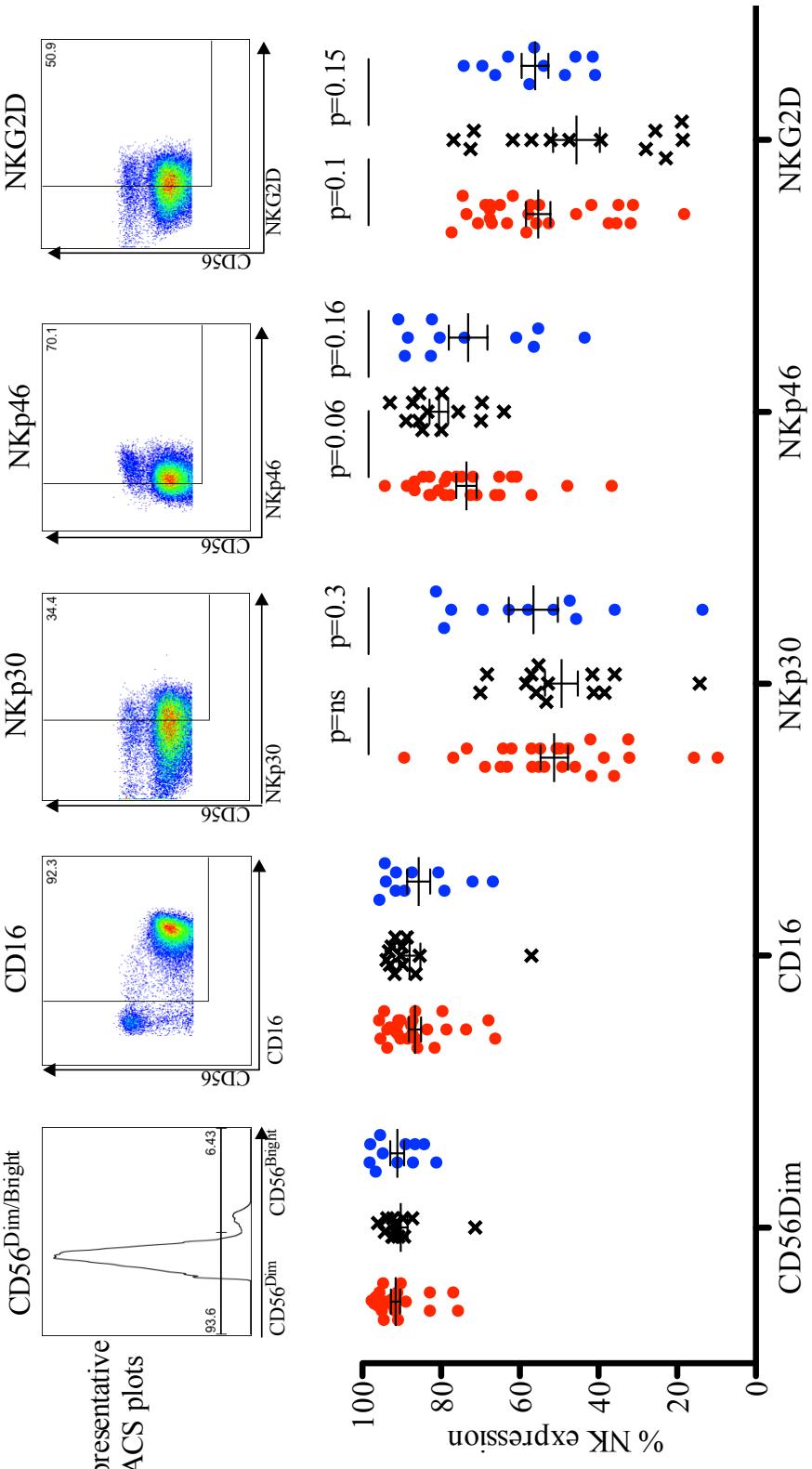


Patients with genotype 1 HCV have a more marked reduction in peripheral blood NK cell proportion than genotype 3 patients compared to healthy donors. Mean & SEM shown.

3.3 NK cell phenotype in chronic HCV

The phenotype of NK cells from chronic HCV, CLD and healthy donors was assessed by flow cytometry. There was no significant difference in the proportion of the $CD56^{Bright}$ and $CD56^{Dim}$ subsets or of the $CD16^{+}$ and $NKp30^{+}$ populations. There was a trend towards increased NKG2D expression in the HCV and CLD cohorts although this was non-significant reflecting the broad range of expression within the groups. There was a trend towards reduced NKp46 expression within the HCV cohort ($p=0.06$ Figure 3.2).

Figure 3.2 NK cell phenotype in chronic HCV infection



Top row: Representative FACS plots of NK cell gate demonstrating $CD56^{Dim}$, CD16, NKp30 and NKG2D expression on peripheral blood NK cells.

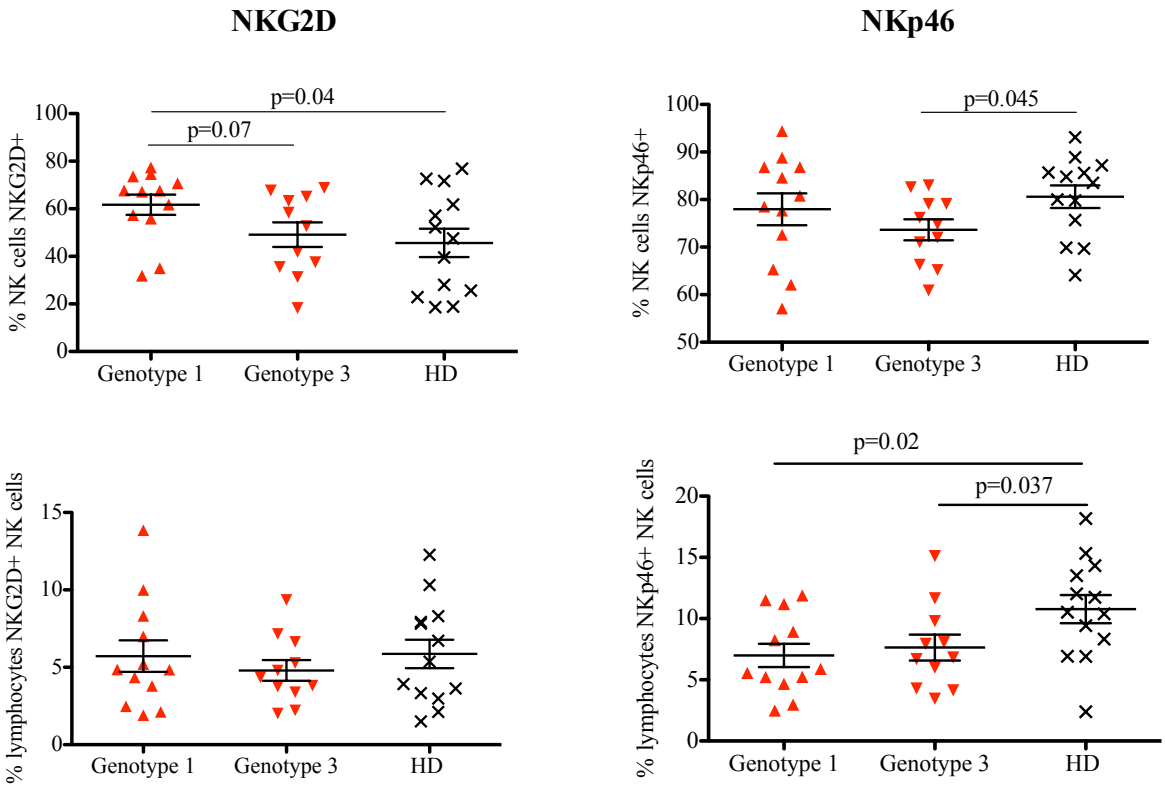
Bottom row: Peripheral blood NK cell phenotype of indivudals with chronic HCV infection (red, n=27), healthy donors (crosses, n=13) and non-viral chronic liver disease controls (blue, n=11). Mean and SEM shown, unpaired t-test

The expression of the activating receptors was compared in patients with genotype 1 or genotype 3 HCV infections. Of note genotype 1 patients had an increased $NKG2D^+$ proportion compared to healthy donors (p=0.04) and genotype 3 patients (p=0.07). Conversely individuals with genotype 3 infection had a lower proportion of $NKp46^+$ NK cells than healthy donors (p=0.045) whilst there was no significant difference between genotype 1 patients and healthy donors (Figure 3.3).

As a proportion of the total lymphocyte population, there was no difference between the 2 genotypes and healthy donors in terms of NKG2D expression (Figure 3.3). However, the proportion of $NKp46^+$ NK cells as a proportion of total lymphocytes was reduced in both the genotype 1 and genotype 3 patients compared to healthy donors (p=0.02 & p=0.037 respectively, Figure 3.3). This suggests that the host may respond to a reduction in the proportion of NK cells by increasing the proportion of $NKG2D^+$ NK cells. However, this is not the case for the other activating receptor NKp46.

It is tempting to speculate that these data suggest there are potentially two different mechanisms to achieve a reduction in the total NKp46 expression i) reduced $NKp46^+$ NK cells through reduction of total NK proportion, (genotype 1 patients), or ii) combination of reducing total NK cell proportion and the proportion of $NKp46^+$ NK cells (genotype 3 patients). There was no association between the proportion of $NKp30^+$ cells and genotype.

Figure 3.3 HCV genotype and NK cell activating receptor phenotype



Top row: NKG2D and NKp46 expression as a proportion of NK cells in genotype 1 and 3 patients. NKG2D expression is increased in genotype 1 and NKp30 expression is reduced in genotype 3 patients.

Bottom row: NKG2D and NKp46 expressing NK as a proportion of total lymphocytes. There is no difference in NKG2D expression between genotypes and healthy donors, however, both genotype 1 and genotype 3 patients demonstrate reduced NKp46 expression. Mean & SEM shown.

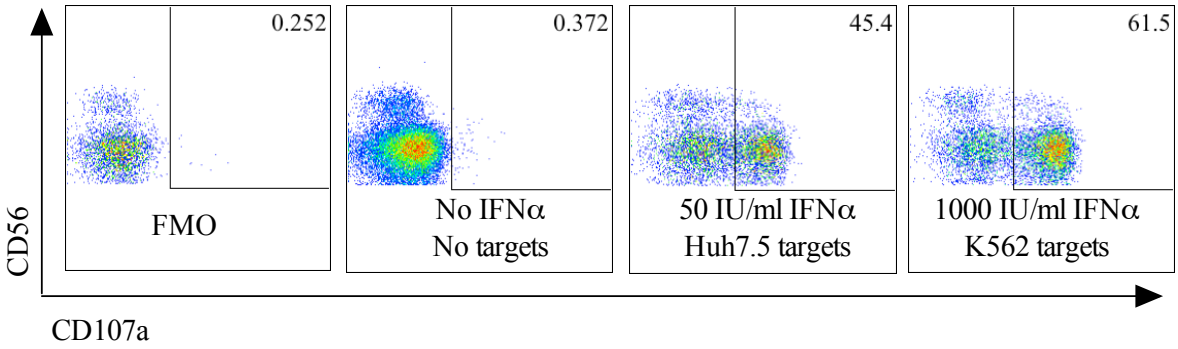
3.4 NK cell cytotoxic function in health and chronic HCV infection

CD107a is a glycoprotein that coats the internal surface of lymphocyte cytolytic granules the exact function of CD107a remains poorly defined.[198] CD107a is temporally exposed on the cell surface upon NK cell degranulation and CD107a externalisation has been shown to correlate strongly with target cell death.[190]

Thawed PBMC were rested or stimulated overnight with IFNα and NK cell function was measured by CD107a externalization in 4-hour degranulation assays. NK cell function was measured after a relatively low-level stimulation (using Huh7.5 human hepatoma cells target cells following overnight stimulation with 50 IU /ml IFN-α) and a high-level stimulation (using K562, MHC I deficient erythroblastic leukaemic cells, and 1000 IU /ml IFN-α, Figure 3.4A). Low level stimulation was used to replicate the chronic inflammatory state of the HCV infected liver and high-level stimulation was used to induce maximal NK cell activation as may be seen following the administration of high dose IFNα during the treatment of HCV.

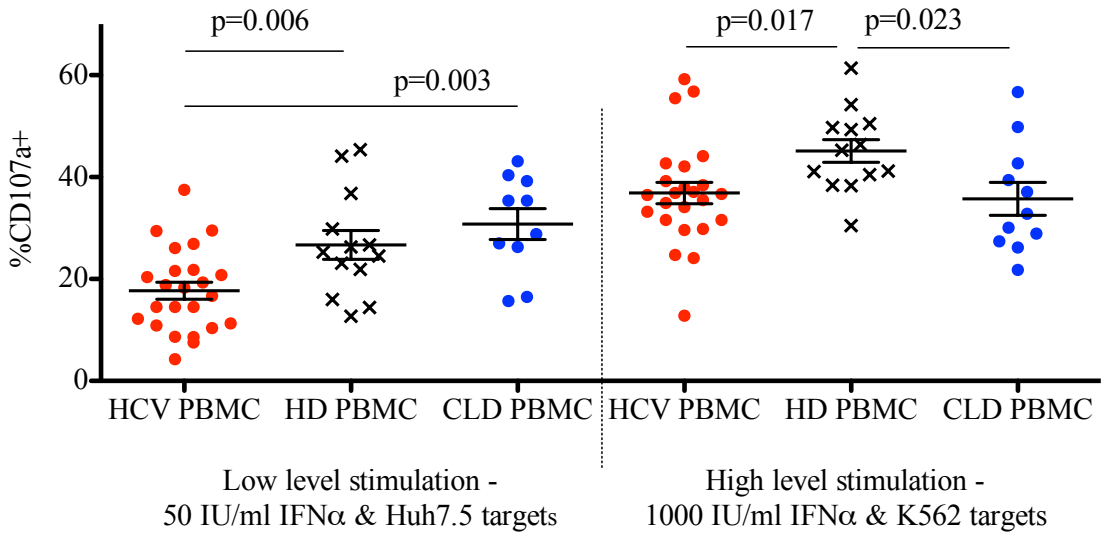
At low-level stimulation NK cell function was reduced in the HCV cohort (mean=17.7%) compared to the healthy (mean=26.7%, p=0.006) and CLD donors (mean=30.8%, p=0.003). At high-level stimulation patients with HCV infection had reduced CD107a externalization compared to healthy donors (mean=36.7 v 45.1% respectively p=0.017) but not compared to CLD patients (mean=35.7% p=n.s), CLD patients had significantly reduced level of CD107a externalization compared to healthy donors (p=0.023). These data indicate that NK cell cytotoxic function is impaired at low-level stimulation compared to health donor and CLD controls and both HCV and CLD patients have reduced cytotoxic function compared to healthy donors at maximal stimulation.

Figure 3.4 NK cell degranulation in chronic HCV infection
A. CD107a externalisation to increasing stimulation



Representative FACS plots from a healthy donor demonstrating increasing CD107a externalisation to increased stimulation.

B. NK cytotoxic function in chronic HCV infection



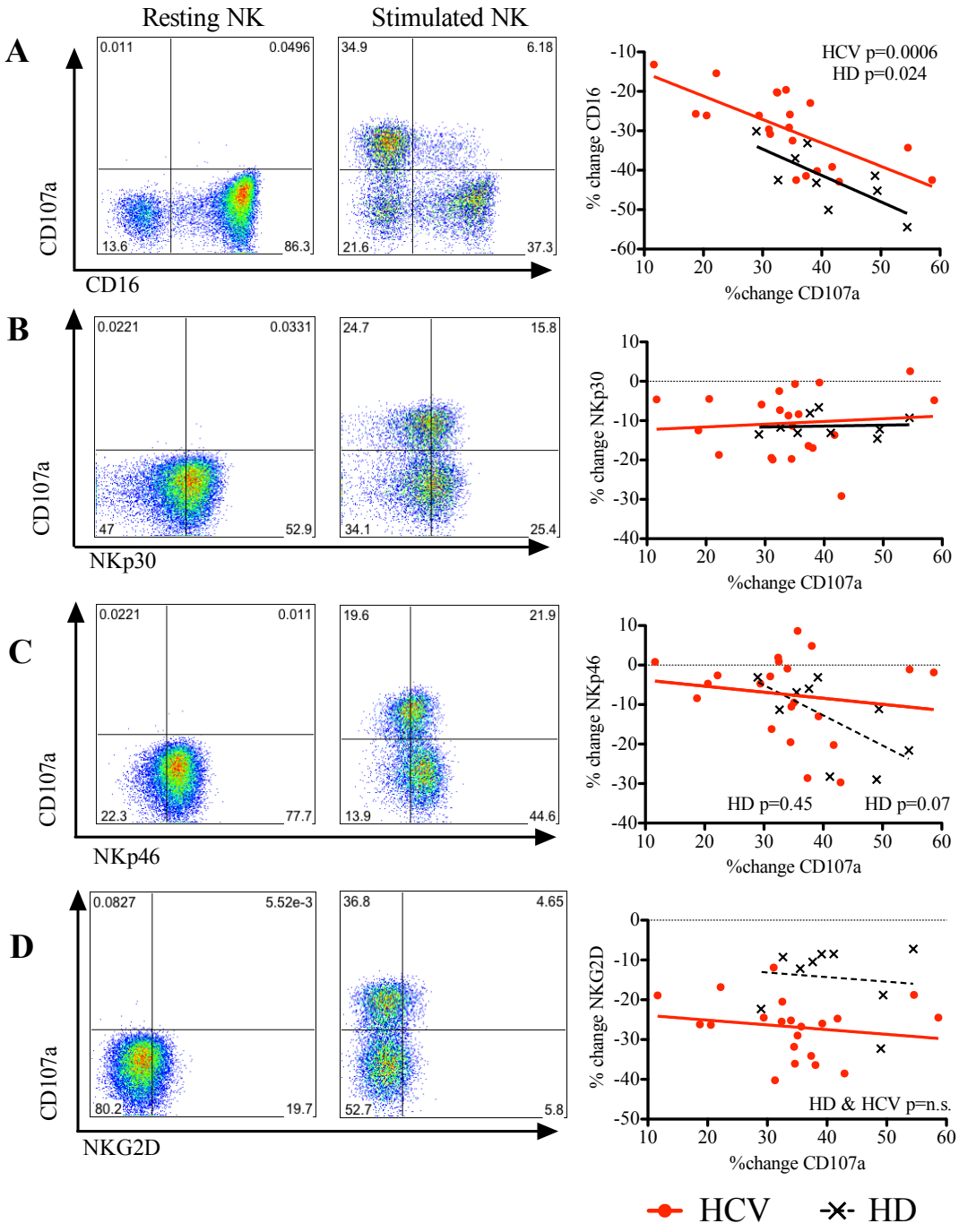
NK cell cytotoxic function is reduced in chronic HCV (red dots) compared to healthy donors (X) at low-level stimulation and high-level stimulation (p=0.006 &0.017 respectively).

3.6 NK cell phenotype and cytotoxic function

The relationship between stimulation-induced degranulation and stimulation-induced down regulation of NK receptors.

NK cell activating receptors are down regulated upon degranulation.[103] Figure 3.5 shows representative FACS plots of phenotypic changes following degranulation. CD16 expression is most markedly altered, with the majority of $CD107a^+$ cells not expressing CD16. CD16 down regulation correlates with increase in CD107a externalisation between unstimulated NK cells and NK cells have exposed to IFNα and K562 target cells in both the HCV and healthy donor cohorts ($p=0.0006$, $r^2=0.47$ & $p=0.024$, $r^2=0.54$ respectively). Marked changes were also seen in $NKp30^+$, $NKp46^+$ and $NKG2D^+$ NK cell populations although these shifts did not correlate with degranulation at high-level stimulation. There was a trend towards NKp46 down regulation and increasing CD107a externalisation in the HD ($p=0.07$, $r^2=0.4$) but not the HCV cohort (Figure 3.5).

Figure 3.5 NK cell degranulation and changes in activating receptor phenotype

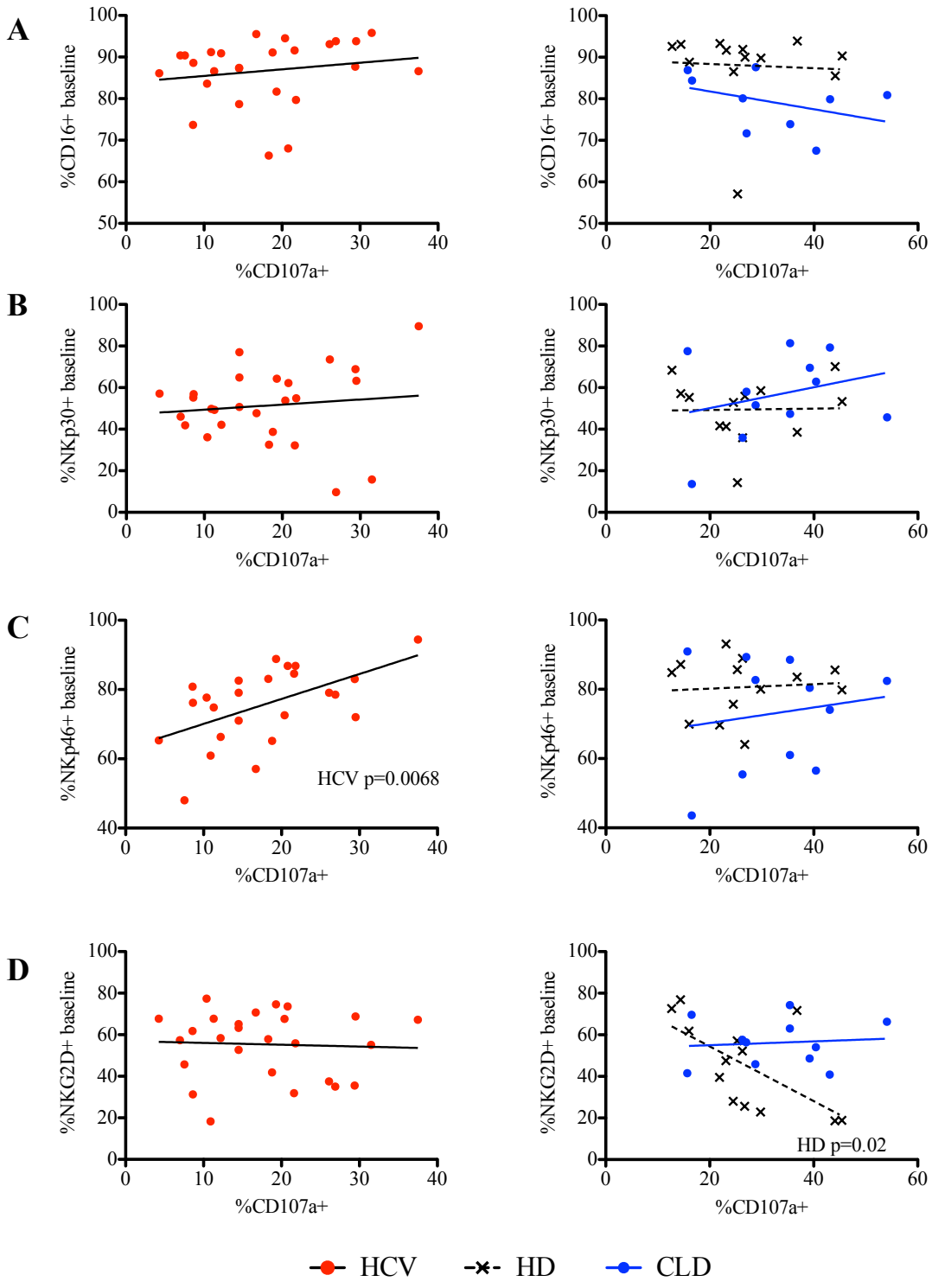


A) Representative FACS plots demonstrating CD107a & CD16 expression at baseline (left panel) and following high-level stimulation (centre panel). Degranulation and reduction in $CD16^+$ NK cell proportion in HCV and Healthy Donor cohorts (right panel).
B) $NKp30^+$ **C)** $NKp46^+$ **D)** $NKG2D^+$ NK cell proportions.

The relationship between stimulation-induced degranulation and baseline NK receptor expression

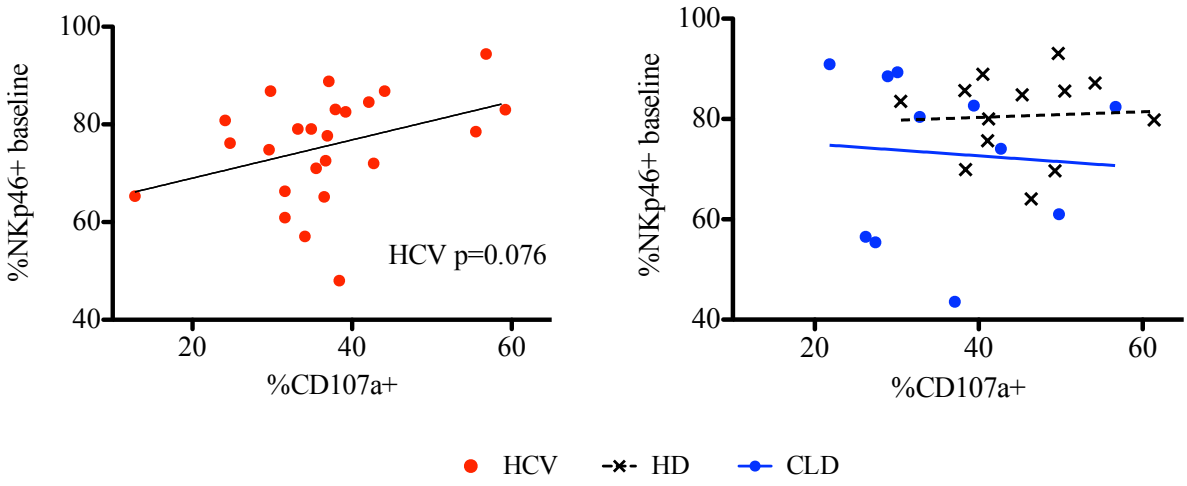
The proportion of NK cells expressing activating receptors on resting NK cells was therefore correlated with cytotoxic function following IFNα stimulation and exposure to target cell lines *in vitro.* At low-level stimulation the proportion of $NKp46^+$ cells correlated with NK cell degranulation in the HCV cohort ($p=0.0068$, $r^2=0.29$) but not in the HD or CLD cohorts (Figure 3.6). There was an inverse correlation between healthy donor $NKG2D^+$ NK cells and degranulation ($p=0.02$, $r^2=0.4$) but not in the HCV or CLD cohorts. $CD16^+$ and $NKp30^+$ proportions did not correlate with degranulation in the HCV, CLD or healthy cohorts (Figure 3.6). NK cell degranulation was measured following high-level stimulation (1000 IU/ml IFNα and K562 target cells). Under these conditions there was a trend towards NKp46 correlation and degranulation in the HCV cohort ($p=0.076$, $r^2=0.14$, Figure 3.7). There were no associations between CD16, NKp30 or NKG2D and degranulation in the HCV cohort or between degranulation and NK phenotype in the CLD or healthy donors at high-level stimulation, which induces maximal degranulation.

Figure 3.6 NKp46 correlates with degranulation in chronic HCV infection at low-level stimulation



A) CD16$^+$ NK cell proportion at baseline and degranulation following low-level stimulation with 50 IU/ml and exposure to Huh7.5 target cells in HCV cohort (left panel) and healthy donors (X) and CLD (blue dots, right panel). **B)** NKp30$^+$, **C)** NKp46$^+$ and **D)** NKG2D$^+$ NK cell proportions.

3.7 NKp46 and NK cell function at high-level stimulation



NKp46^{+} proportion at baseline and degranulation following high-level stimulation with 1000 IU/ml and exposure to K562 target cells in HCV cohort (left panel) and healthy donors (X) and CLD (blue dots, right panel).

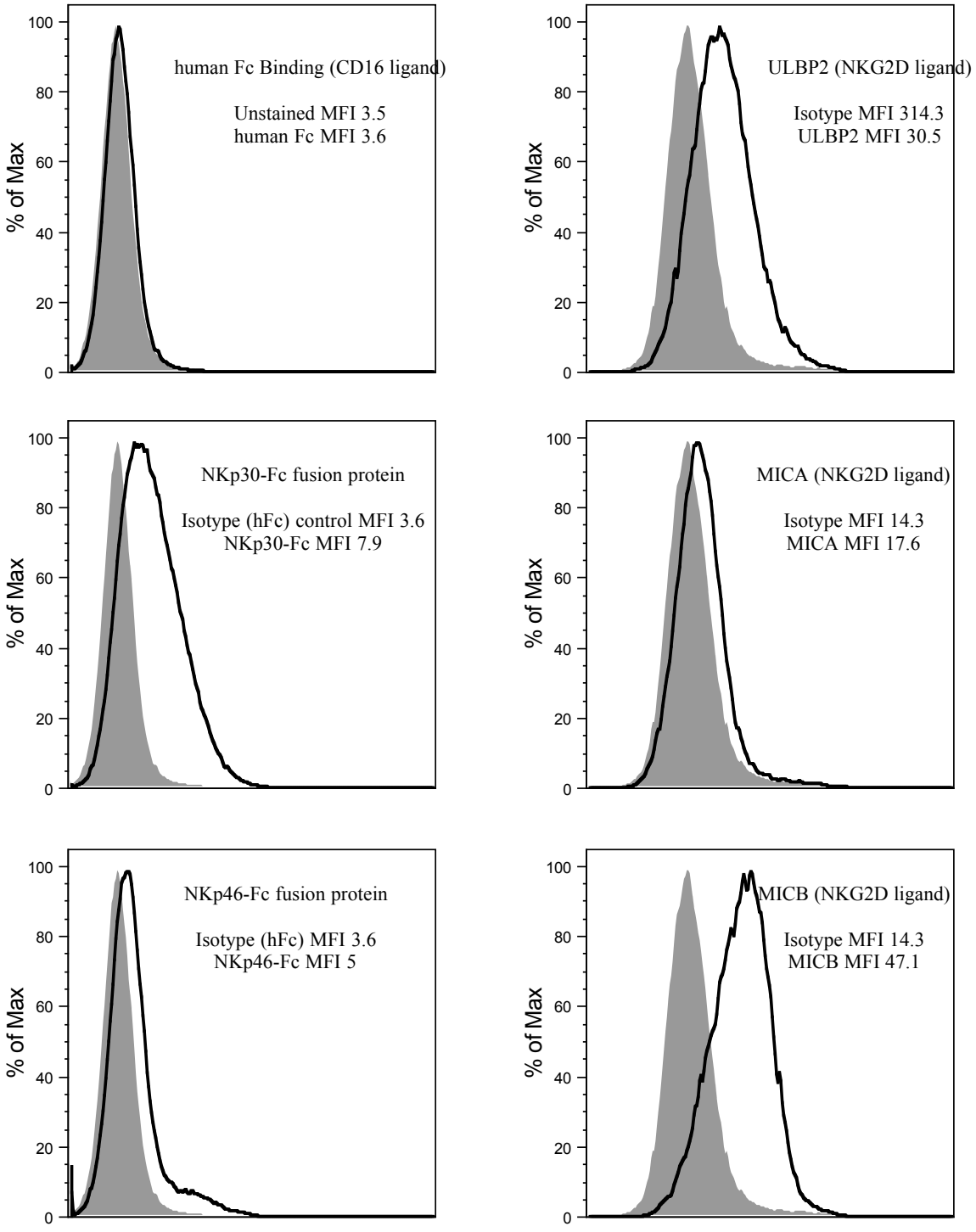
3.7 NK activating receptor ligand expression on target cells

The association between activating receptor and NK function is dependent upon the presence of the appropriate receptor ligand. The ligands for many NK receptors are unknown. CD16 is an Fc receptor on antibody-coated cells [103] and NKG2D binds to MICA, MICB and ULBP2 proteins.[106-108] However, only a handful of NCR ligands are known and these include influenza haemaglutinins, BAT 3 and B7-H6.[121,127,128] NKp30 and NKp46-Fc fusion proteins allow the identification of known and potentially unknown ligands expressed on the surface of target cells.

Huh7.5 and K562 target cells were stained with NKp30 and NKp46-Fc fusion proteins and monoclonal antibodies to MICA, MICB, ULBP2 and anti-human Fc antibody. NKp30 and NKG2D ligands were expressed at high levels on both Huh7.5 and K562 target cells (Figure 3.8A&B). The anti human Fc antibody did not bind to either target cell lines indicating that there was no ligand for CD16 expressed on the cell surfaces. NKp46 ligands were expressed at lower levels than NKp30 and NKG2D ligands but relatively better expressed on Huh7.5 than K562 cells (Figure 3.8). The relatively increased expression of NKp46 ligands presumably contributes to the fact that they form a superior target for NK cells that express high levels of NKp46 in HCV infection.

Figure 3.8 Huh7.5 & K562 NK activating receptor ligand expression

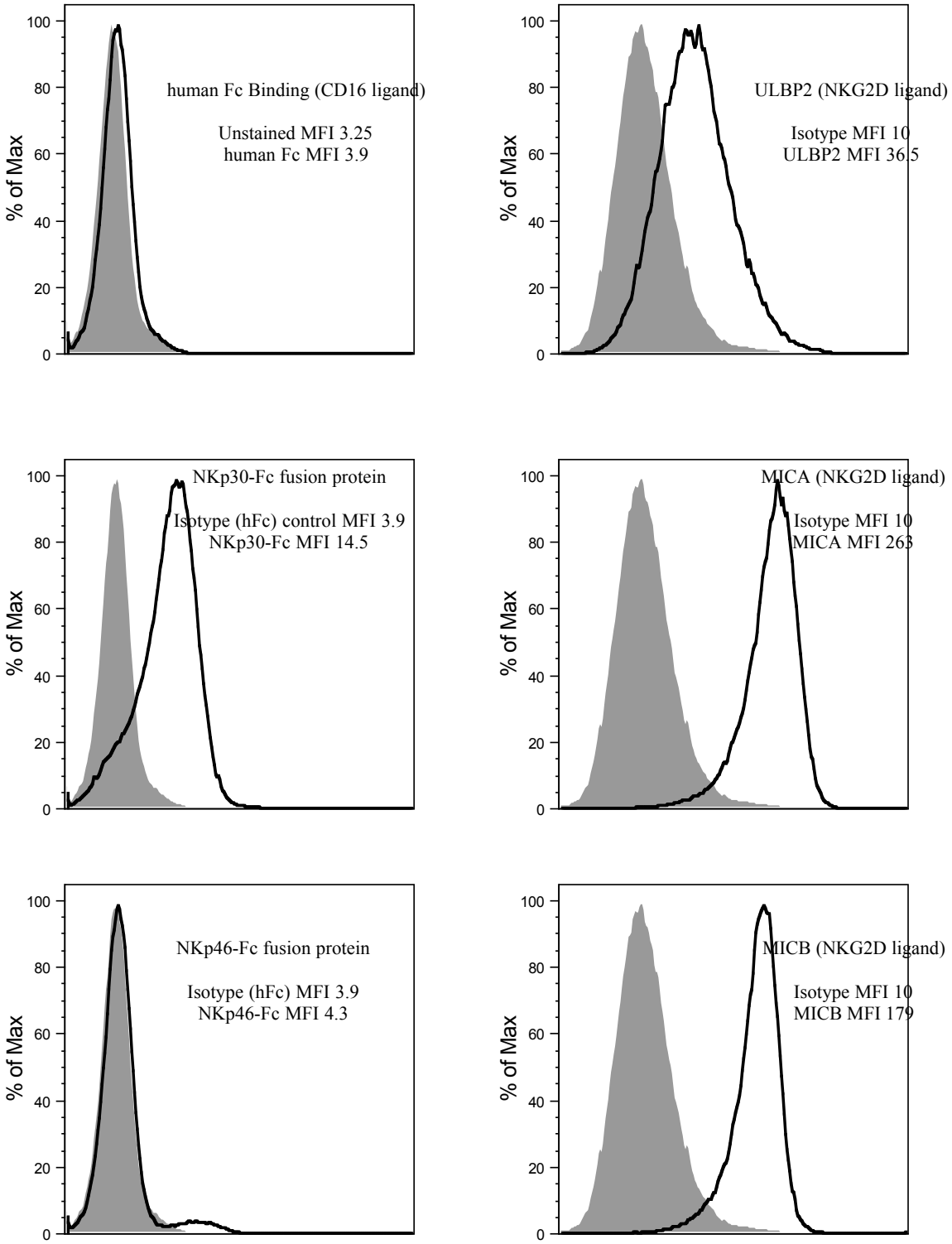
A Huh7.5 targets cells



Representative FACS histograms demonstrating NKp30-Fc, NKp46-Fc, MICA, MICB and UBLP2 staining on Huh7.5 target cells (solid grey = isotype control, black line = ligand of interest).

Figure 3.8 Huh7.5 & K562 NK activating receptor ligand expression

B K562 targets cells



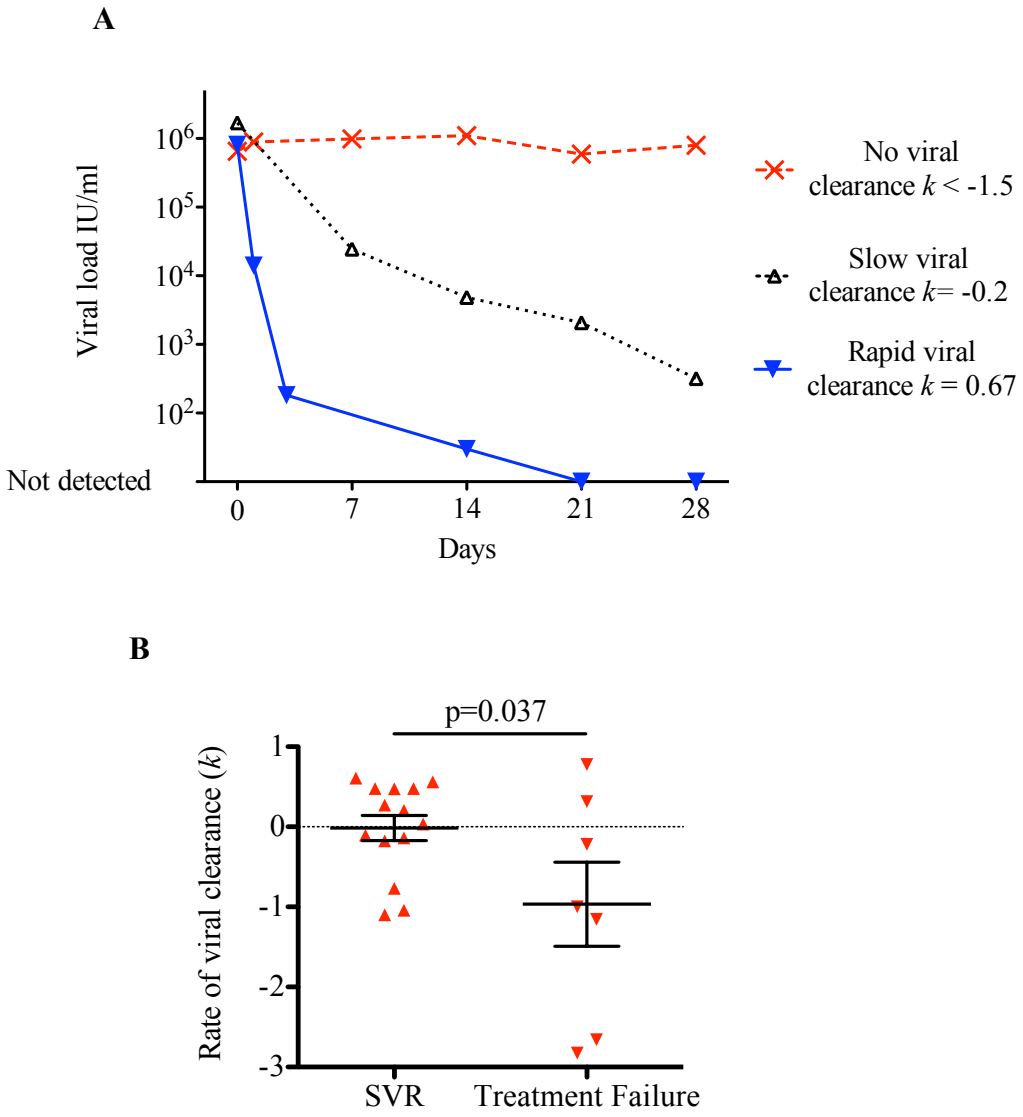
Representative FACS histograms demonstrating NKp30-Fc, NKp46-Fc, MICA, MICB and UBLP2 staining on K562 cells (solid grey = isotype control, black line = ligand of interest).

3.8 IFNα treatment of HCV infection – viral clearance and rate of viral control

The successful IFNα treatment of HCV, a sustained viraemic response (SVR), is defined by two undetectable viral loads as measured by PCR 6 months apart. It is known that rapid viral clearance is associated with successful treatment outcome, however, it is possible for individuals to respond to treatment slowly and go on to achieve SVR. IFNα treatment can impinge upon the immune system through multiple mechanisms. These varying mechanisms include stimulation of the innate and adaptive branches of the immune system as well as the direct impact of IFNα upon HCV and infected hepatocytes. The relative impact of these mechanisms is not fully understood and it is likely that these may vary between individuals. Varying clinical responses to IFNα treatment, including rapid viral clearance, relapsing responders and SVR with slow viral clearance, may reflect the predominance of a particular immune pattern of response within an individual. Therefore, rate of viral clearance may be a more subtle outcome measure to assess the role of different cell linages during IFNα treatment of HCV.

To calculate the rate of viral clearance viral loads were measured in the serum of patients at multiple time points (day 0, 1, 3, 7, 14, 21, 28 and then three monthly). The decline in viral load was calculated as a non-linear regression k (day^{-1}) illustrated in Figure 3.9A. Patients who successfully cleared HCV with IFNα treatment had a higher rate of viral decline than those who failed treatment (mean= -0.01 k v -0.97 k, p=0.037 Figure 3.9B).

3.9 Rate of viral control during IFNα treatment

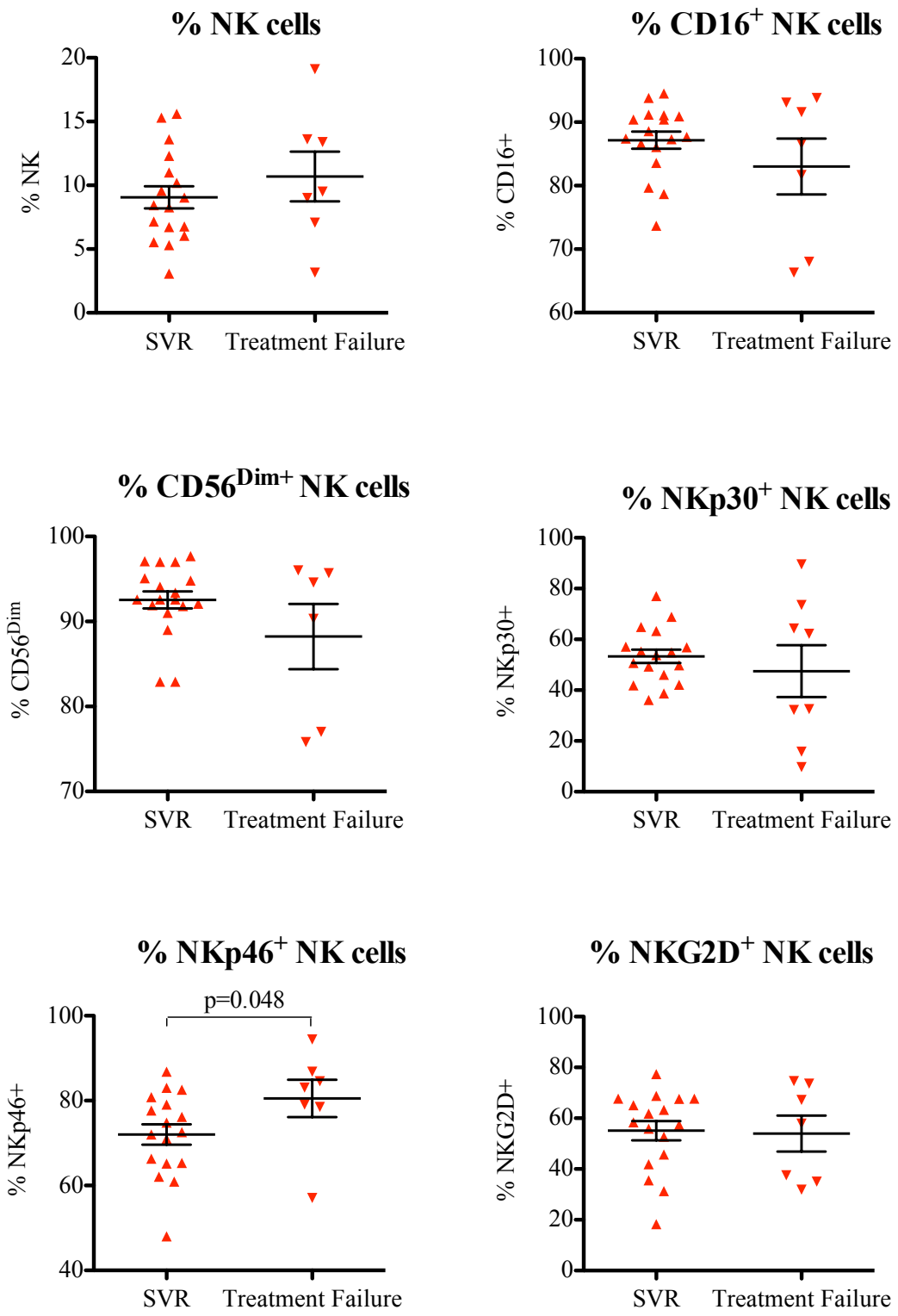


A) Representative rates of viral clearance for 3 subjects following initiation of IFNα treatment. Subject with no viral clearance did not decrease the viral load and had a low rate constant k < -1.5 (Red crosses). The subject with slow viral clearance achieved SVR but had a measurable viral load at 1 month and a rate constant of k = -0.2 (black triangles). The individual with rapid viral control had a rate constant of k = 0.67 (blue inverted triangles). **B**) Rate of viral clearance in patients who achieved SVR following IFNα treatment and those who failed treatment.

3.9 NK cell phenotype and IFNα treatment outcome in chronic HCV infection

Baseline NK cell phenotype was known for 24 patients who underwent IFNα and ribavirin treatment for HCV, of these 17 achieved SVR and 7 failed treatment (Table 2.1). The mean proportion of $NKp46^{+}$ NK cells was surprisingly higher in the treatment failure group than those patients who achieved SVR (mean= 72.0% v 80.5% respectively, p=0.048, Figure 3.10). There was no difference in proportion of NK cells or $CD56^{Dim}$ cells or CD16, NKp30 and NKG2D expression between the groups (Figure 3.10).

Figure 3.10 NK cell phenotype & treatment outcome

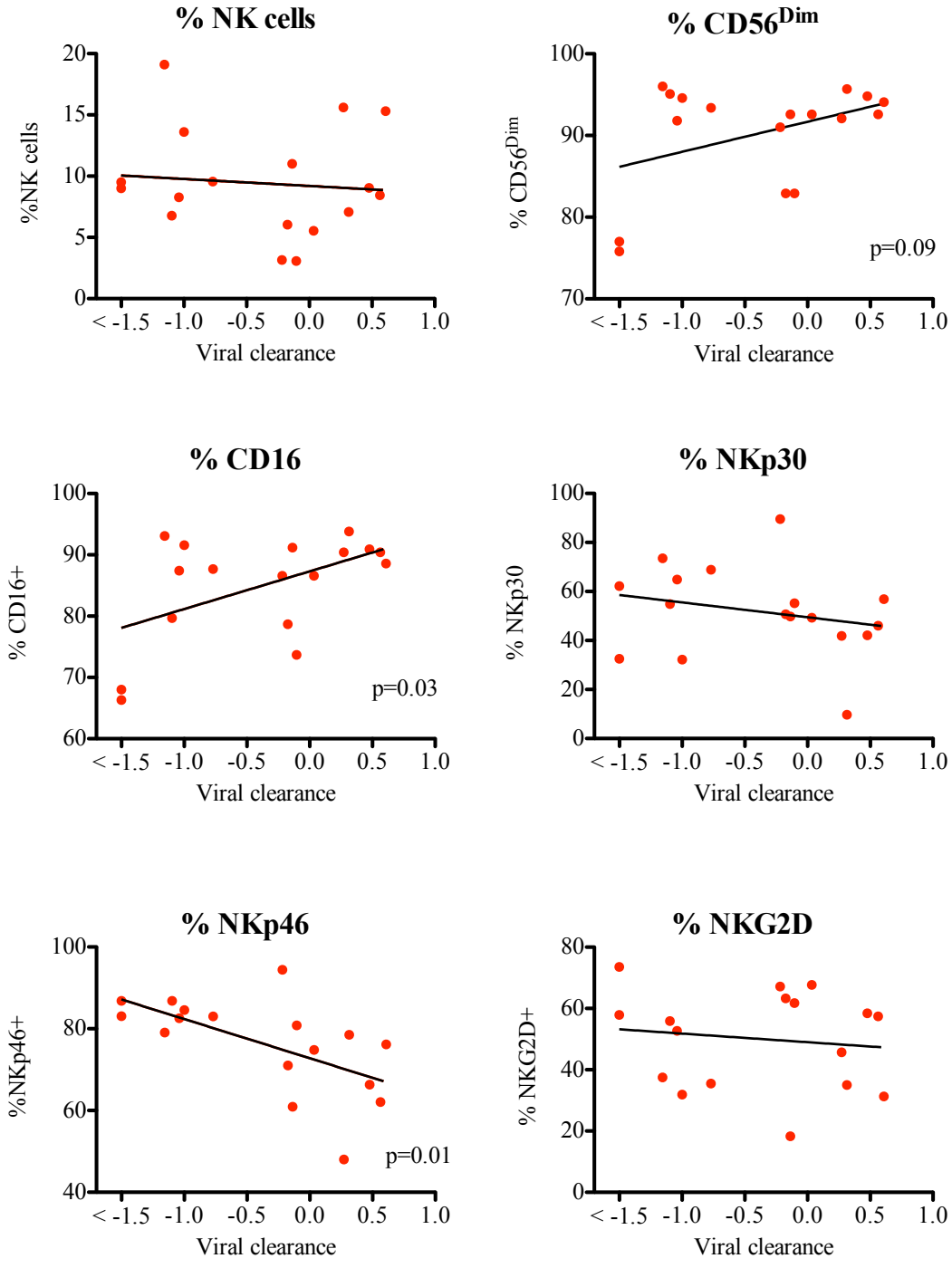


Viral clearance following IFNα and ribavirin treatment was associated with lower proportions of NKp46$^+$ NK cells at baseline but not NK cell, CD56^{Dim}, CD16$^+$, NKp30$^+$ or NKG2D$^+$ proportions. Mean & SEM shown.

Rate of viral control was calculated as described above and correlated with baseline NK cell phenotype. $CD16^+$ proportion at baseline correlated with rate of viral clearance (r^2=0.27, p=0.03). In keeping with this there was a trend towards an increased proportion of $CD56^{Dim}$ NK cells and increasing rate of viral clearance (r^2=0.17, p=0.097, Figure 3.11). $CD16^+$ $CD56^{Dim}$ NK cells have a greater cytotoxic potential than $CD16^-$ $CD56^{Bright}$ NK cells. It is not clear from these data if the correlation between CD16 and rate of viral clearance is a reflection of NK cytotoxic potential or the impact of CD16 on NK cell activation. It is interesting to note that in this cohort of 17 individuals the two patients who did not have any reduction in viral load following initiation of IFNα and ribavirin (k <-1.5) had markedly lower $CD16^+$ expression of 66% and 68% with a reduced $CD56^{Dim}$ proportion. If these individuals were excluded from the analysis statistical significance is lost and the r^2 value in both cases is less than 0.018.

Again surprisingly $NKp46^+$ NK cell proportion inversely correlated with rate of viral clearance (r^2= 0.36, p=0.01, Figure 3.11). The NK cell, $NKp30^+$ and $NKG2D^+$ proportion did not correlate with rate of viral clearance.

Figure 3.11 NK cell phenotype and rate of viral clearance during IFNα treatment

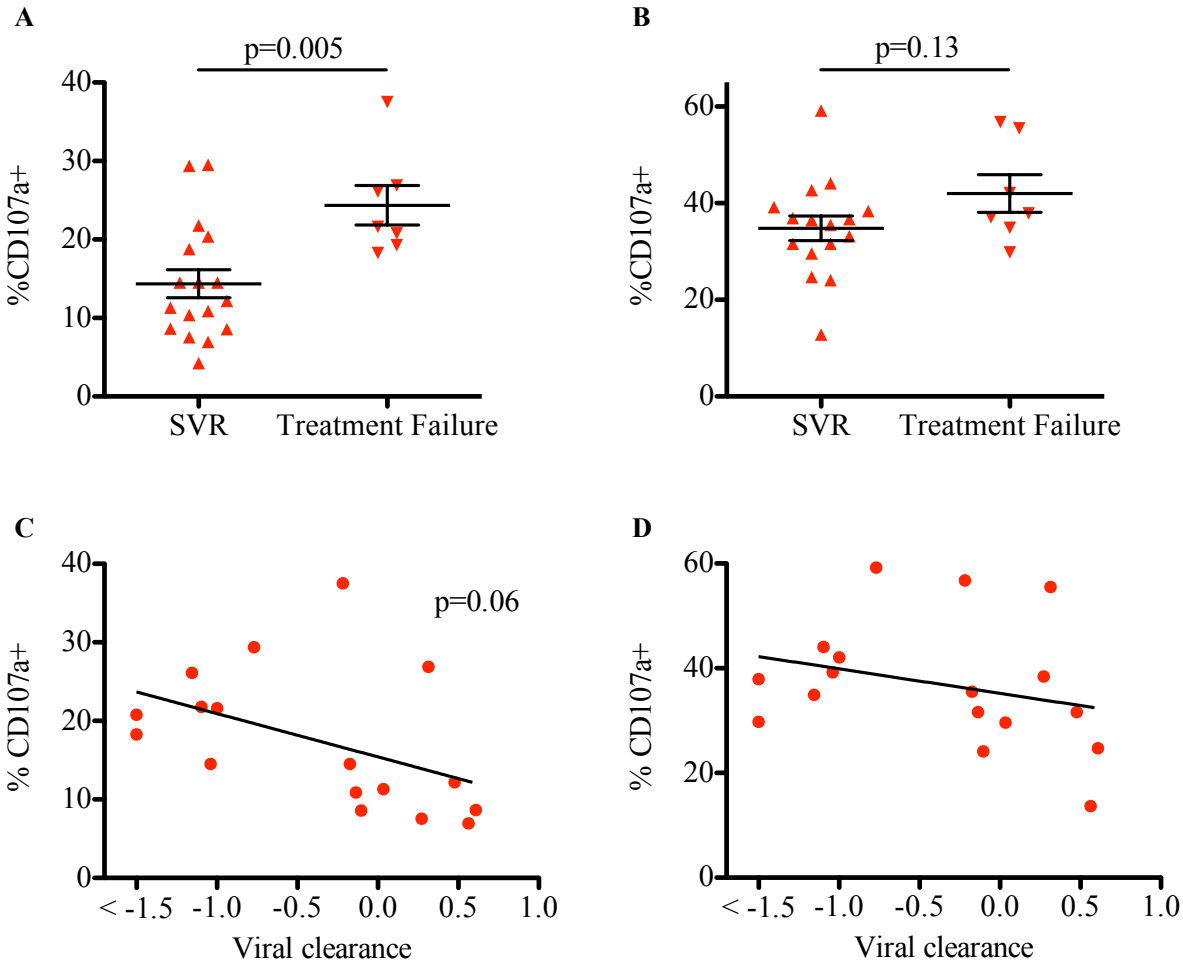


$CD16^+$ proportion weakly correlated with rate of viral clearance following initiation of IFNα treatment as measured by the rate constant *k*.. There was an inverse correlation between $NKp46^+$ proportion and rate of viral clearance but no association between proportion of NK, $NKp30^+$ or $NKG2D^+$ cells.

3.10 *In vitro* NK cytotoxic function & rate of viral clearance

Patients who successfully cleared HCV infection with IFNα and ribavirin treatment had a *lower* cytotoxic response to low-level stimulation (50 IU IFNα and Huh7.5 target cells) than patients who failed treatment (mean 14.4% v 24.4% p=0.005, Figure 3.12A). Following stimulation with 1000 IU IFNα and K562 target cells there was a non-significantly reduced cytotoxic response in the SVR group (mean 34.8% v 42.0%, p=0.13, Figure 3.12B). There was an inverse trend between rate of viral clearance and *in vitro* cytotoxic function at low-level stimulation (r^2 = 0.21, p=0.067, Figure 3.12C). Following maximum stimulation CD107a externalisation did not correlate with rate of viral clearance (Figure 3.12D).

Figure 3.12 NK cell cytotoxic function & response to treatment



A) NK cell CD107a externalisation following low stimulation (50 IU/ml IFNα and Huh7.5 target cells) in patients who achieved SVR and failed IFNα treatment.

B) CD107a externalisation following high level stimulation (1000 IU/ml IFNα and K562 target cells). Mean & SEM shown. **C)** Correlation of rate of viral clearance and CD107a externalisation low-level stimulation. **D)** Correlation of rate of viral clearance and CD107a externalisation following high-level stimulation.

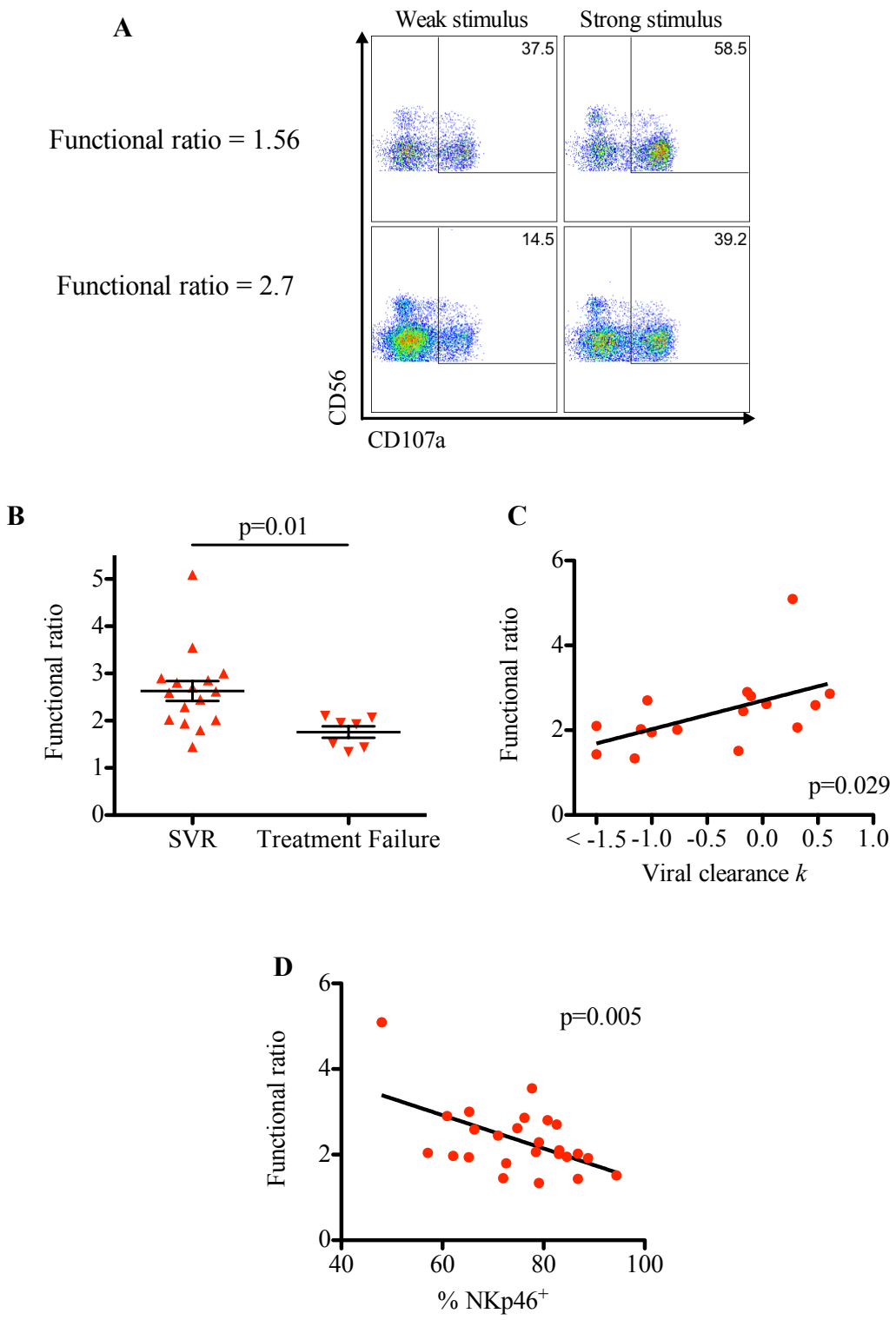
3.11 Functional ratio

It was surprising that patients with an SVR appeared to have populations of NK cells that were not as activated by IFNα compared to patients who failed treatment. However, a clear difference emerges when the relative changes in measured in each subject, using lower vs. high-level stimulation, are compared. I called this change functional ratio:

$$\text{Functional ratio} = \frac{\%\text{ NK cells expressing CD107a high-level stimulation}}{\%\text{ NK cells expressing CD107a low-level stimulation}}$$

FACS plots of CD107a externalisation at high and low stimulation from two representative individuals are shown in Figure 3.13A. Patients who achieved SVR had a higher functional ratio than patients who failed treatment (mean = 2.6 v 1.7, p=0.016, Figure 3.13B). Importantly the functional ratio correlated with rate of viral clearance (r^2=0.30, p=0.029, Figure 3.13C) and inversely correlated with NKp46 expression (r^2=0.29, p=0.005, Figure 3.13D). These data support the notion that the relative ability to increase NK cell function in response to IFNα stimulation contributes to more rapid viral clearance and treatment success, and the $NKp46^+$ proportion of the NK population seemed to be key.

Figure 3.13 Functional ratio and treatment outcome

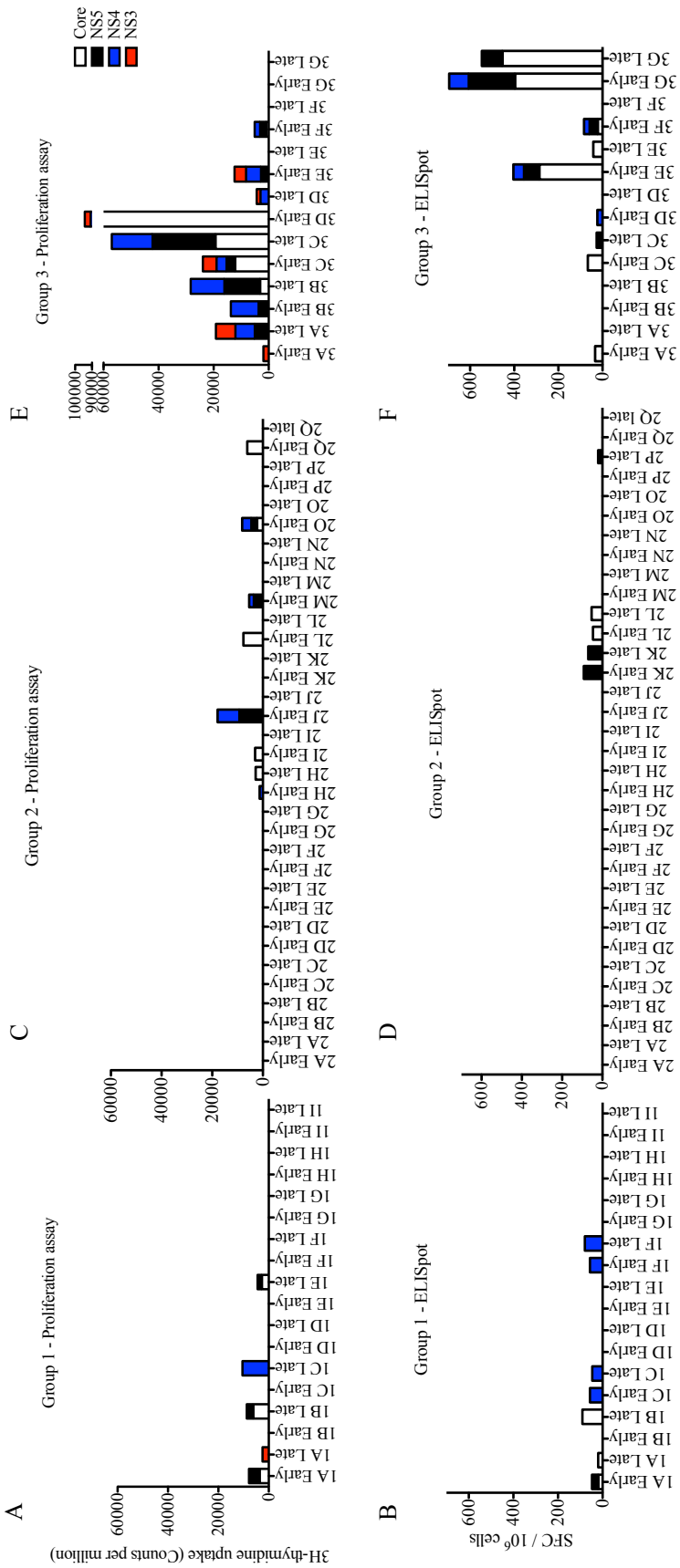


A) Representative FACS plots from two HCV infected individuals demonstrating low (top row) and high (bottom row) relative increase in cytotoxic function and functional ratio. B) Functional ratio and treatment outcome. Mean & SEM shown. C) Functional ratio and rate of viral clearance. D) Functional ratio and % NKp46^{+}.

3.12 HCV specific $CD4^+$ T cell and NK cell responses following IFNα treatment.

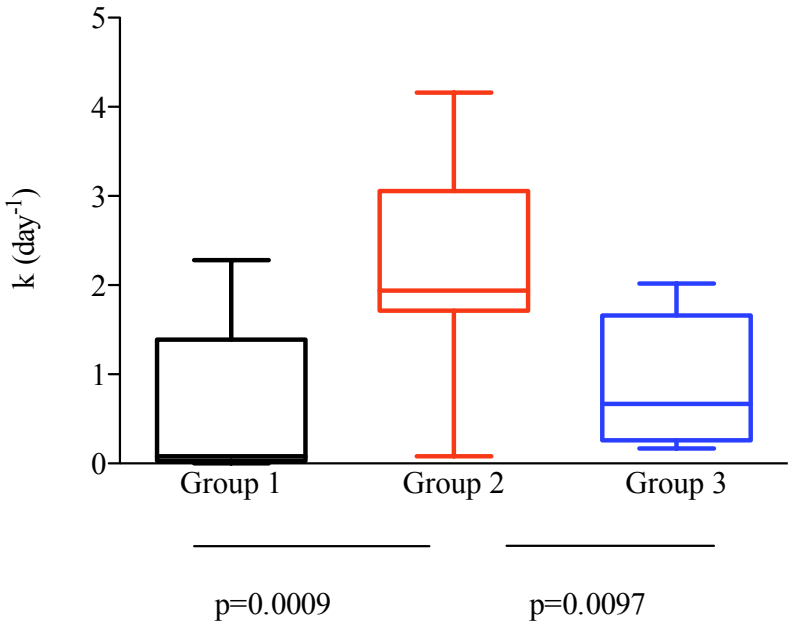
Previous research conducted by Dr Ian Rees (MD student in Godkin/Gallimore lab) investigated *ex vivo* IFNγ producing and cultured anti-viral $CD4^+$ T cell anti-HCV responses at multiple timepoints before and during IFNα treatment in a cohort of 33 patients.[199] Patients who failed treatment had absent or transient low-level $CD4^+$ T cell responses. Surprisingly of those patients who achieved an SVR (and the most rapid rate of viral decline) had absent or minimal anti-viral $CD4^+$ T cell responses. Patients with the most robust $CD4^+$ T cell HCV specific responses had lower rates of viral clearance ($p<0.0006$, Figure 3.14).

Figure 3.14 IFNα induced rapid control of hepatitis C virus results in SVR in the absence of robust $CD4^+$ T cell responses



Proliferation and ELISpot assays to HCV antigens; core NS3, NS4, NS5 were measured at multiple Early (days 2, 7, 14, 21 & 28) and Late (weeks 12, 24 & 48) time points. Group 1 (A&B) failed treament, Group 2 (C & D) successfully cleared HCV but with absent or minimal $CD4^+$ T cell responses and Group 3 (E & F) successfully cleared the virus with robust $CD4^+$ T cell responses. Work undertaken by Dr Ian Rees

3.14 G

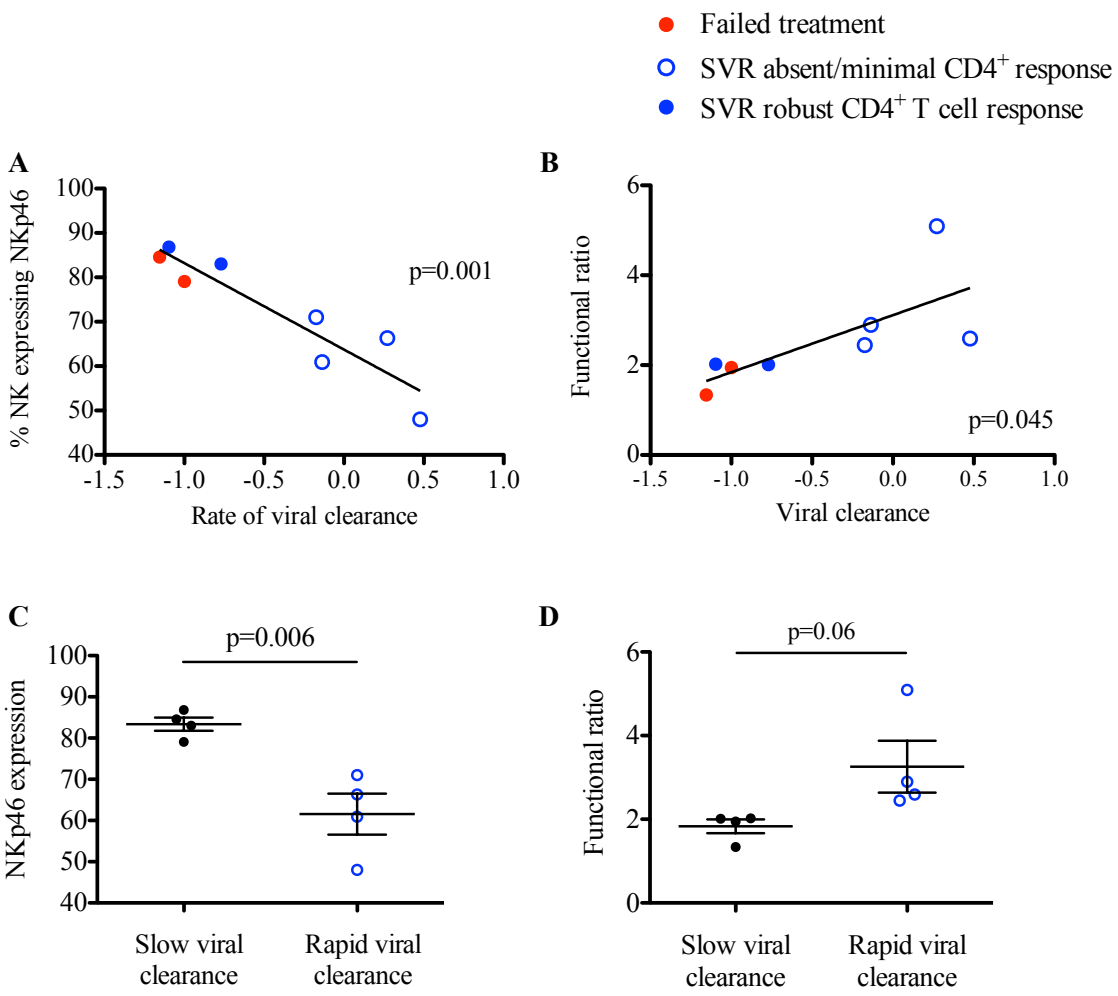


Hepatitis C viral clearance was measured by a rate constant of viral decline following initiation of IFNα and ribavirin treatment *k*. Group 1 comprised of those who failed to clear HCV on treatment. Group 2 achieved SVR in the absence of or with minimal CD4+ T cell responses. Group 3 achieved SVR with robust CD4+ T cell responses. (Work undertaken by Dr Ian Rees in [199])

Frozen PBMC were available from 8 of these patients for whom the $CD4^+$ T cell responses are known and have been included in the analysis of NK cells described in the early part of this chapter. In this sub-group 4 patients achieved SVR with a low/minimal $CD4^+$ T cell response, 2 patients achieved SVR with robust $CD4^+$ T cell responses and 2 failed treatment without a robust $CD4^+$ T cell response.

As in the larger cohort there was an inverse correlation between % $NKp46^+$ NK cells and rate of viral clearance (r^2= 0.85, p=0.001, Figure 3.15A) and functional ratio correlated with rate of viral clearance (r^2=0.51, p=0.045, Figure 3.15B) in this subset of 8 patients. However, these data indicate that there is segregation in NK phenotype and function in individuals who have rapid viral clearance and achieve SVR and those who clear the virus more slowly regardless of whether they achieve SVR or failed treatment. When the patients are grouped in this manner the rapid responders had lower proportion of $NKp46^+$ cells than individuals with slow viral decline (mean = 61.5 v 83% p=0.006, Figure 3.15C). In keeping with this pattern patients with rapid viral decline had greater functional ratio than those with slow viral decline regardless of whether the individual had a robust $CD4^+$ T cell response and achieved SVR or did not and failed treatment (mean = 3.2 v 1.8 p=0.06 Figure 3.15D). These data suggest that NK cell phenotype and function may dictate rate of viral clearance, rapid viral clearance may negate or prevent a $CD4^+$ T cell response from developing. Individuals who have low rates of viral clearance are dependent upon robust $CD4^+$ T cell responses to achieve SVR.

Figure 3.15 NK cells and CD4$^+$ T cell responses to IFNα treatment



A) NKp46$^+$ proportion and rate of viral clearance in patients who failed treatment and those who achieved SVR with absent/minimal or robust CD4$^+$ T cell responses. Demonstrating segregation between rapid clearers with absent/minimal CD4$^+$ T cell responses and slow clearers regardless of CD4$^+$ response and treatment outcome. B) Functional ratio and rate of viral clearance. C) NKp46$^+$ proportion in patients with rapid viral clearance and slow viral clearance. The slow viral clearance group contains 2 individuals who failed treatment and did not have CD4$^+$ T cell responses and 2 individuals who achieved SVR and had robust CD4$^+$ T cell responses. D) Functional ratio in patients with rapid and slow viral clearance. (Mean and SEM shown).

3.13 Discussion

NK cells play a role in controlling viral infections. Although the exact mechanism of IFNα treatment is unclear, it seems plausible that its ability to activate NK cells may be important. NK cells have been examined as potential effectors during IFNα treatment, however, there is a lack of consensus regarding their phenotype and function in the literature as outlined in the introduction. Some of these conflicts may result from type II statistical errors, methodological and variation in populations sampled. In keeping with the studies that have larger cohorts we have found that NK cells were reduced in chronic HCV infection, however, with a more marked reduction in genotype 1 compared to genotype 3 patients (Figure 3.1). HCV genotype appears also to alter NK cell phenotype with more markedly reduced $NKp46^{+}$ proportion in genotype 3 patients. Once the reduction in the proportion of NK cells is considered it is interesting to note that there is an equivalent reduction in $NKp46^{+}$ cells as a proportion of lymphocytes in genotype 1 and genotype 3 patients (Figure 3.3).

$NKp46^{+}$ proportion correlated with NK cell function in the HCV cohort but not in the healthy donors or CLD cohorts (Figure 3.6). The cytotoxic response was lower in chronic HCV infection. Two studies have recently described two subpopulations of NK cells defined by NKp46 expression on a per cell basis using mean fluorescence intensity (MFI) and termed these cells $NKp46^{High}$ and $NKp46^{Dim}$.[175,200] In these studies $NKp46^{High}$ cells had increased cytotoxic function and produced higher levels of IFNγ and TNFα than $NKp46^{Dim}$ cells and were able to able to inhibit HCV replication *in vitro*. I found that NKp46 expression in peripheral blood NK cells tended to form a single population rather than two discrete populations (representative FACS plot Figure 3.2). This may relate to

differing methodologies between the studies but the uniting message remains that NKp46 expression marks out NK cells with greater *in vitro* cytotoxic activity in chronic HCV.

In an *in vitro* model of fibroblast pox-virus infection Jarahian *et al* have reported NKp30 specifically induces greater NK cytotoxic function than NKp46. However, vacinnia virus haemaglutinin, either bound to the infected cell surface or as a soluble factor, is able to block NKp30-triggered NK cell activation.[121] NK cells therefore, have attenuated cytotoxic function and degranulated in an NKp46 dependent manner. A similar pattern of the failure of NKp30 activation as the primary activating receptor may occur in chronic HCV permitting NKp46 expression to dictate NK cell function in the HCV. It is possible that there is a race between host NKp30-dependent viral control and expression of HCV immunoevasive factors dictating if exposure progresses to acute infection. In a cohort of HCV exposed intravenous drug users high NKp30 expression has been associated with reduced levels of seroconversion compared to individuals with low NKp30 expression.[149]. These data suggest that a viral immunoevasion strategy may block a primary activating receptor (possibly NKp30) in chronic HCV infection passing the role of principle activating receptor to NKp46, which results in lower NK cytotoxic function.

Rapid viral control is a predictor of treatment success (Figure 3.9).[32] The findings described above support a role for NK cell cytotoxic function in the rapid early control of viral load during IFNα treatment of HCV. These data are in keeping with those of Ahlenstiel *et al*, who sampled peripheral blood NK cells at multiple time points before and during IFNα treatment allowing *in vivo* stimulation and measured NK cell function *in vitro.* Ahlenstiel and colleagues reported patients who achieved SVR had a significantly greater increase in $CD56^{Dim}$ NK cell CD107a MFI from baseline to 24 hours following

initiation of IFNα treatment compared to those who failed treatment.[185] In this study we have calculated a functional ratio to describe the relative increase in NK cell cytotoxic function, which correlates with rate of viral clearance and treatment success (Figures 3.13B & C). In addition at low-level stimulation there was an inverse correlation between NK cell function and rate of viral clearance (Figure 3.12). Previous studies of interferon-stimulated genes (ISG) in chronic HCV reported a higher rate of SVR in individuals with a low level of ISG expression at baseline and a marked increase in ISG expression following IFNα treatment compared to those with high baseline expression and a poor IFNα response.[50,51,201] In my study the proportion of NKp46$^+$ appears to drive NK cytotoxic function at low-level stimulation with high expression resulting in increased cytotoxic function at baseline but is associated with poor treatment outcome. Increased baseline NK cell cytotoxic function may increase selection pressure on HCV resulting in the expansion of more resistant quasi-species contributing to viral persistence during IFNα treatment.

Innate & adaptive immunity

As previously described NK cells can help to establish a successful adaptive immune response through the production of IFNγ and NK-DC cross talk.[141-143] However to mount a robust adaptive immune response appropriate antigen presentation is required and there has been increasing interest in innate immune impingement upon adaptive immunity through antigen clearance in mouse models.

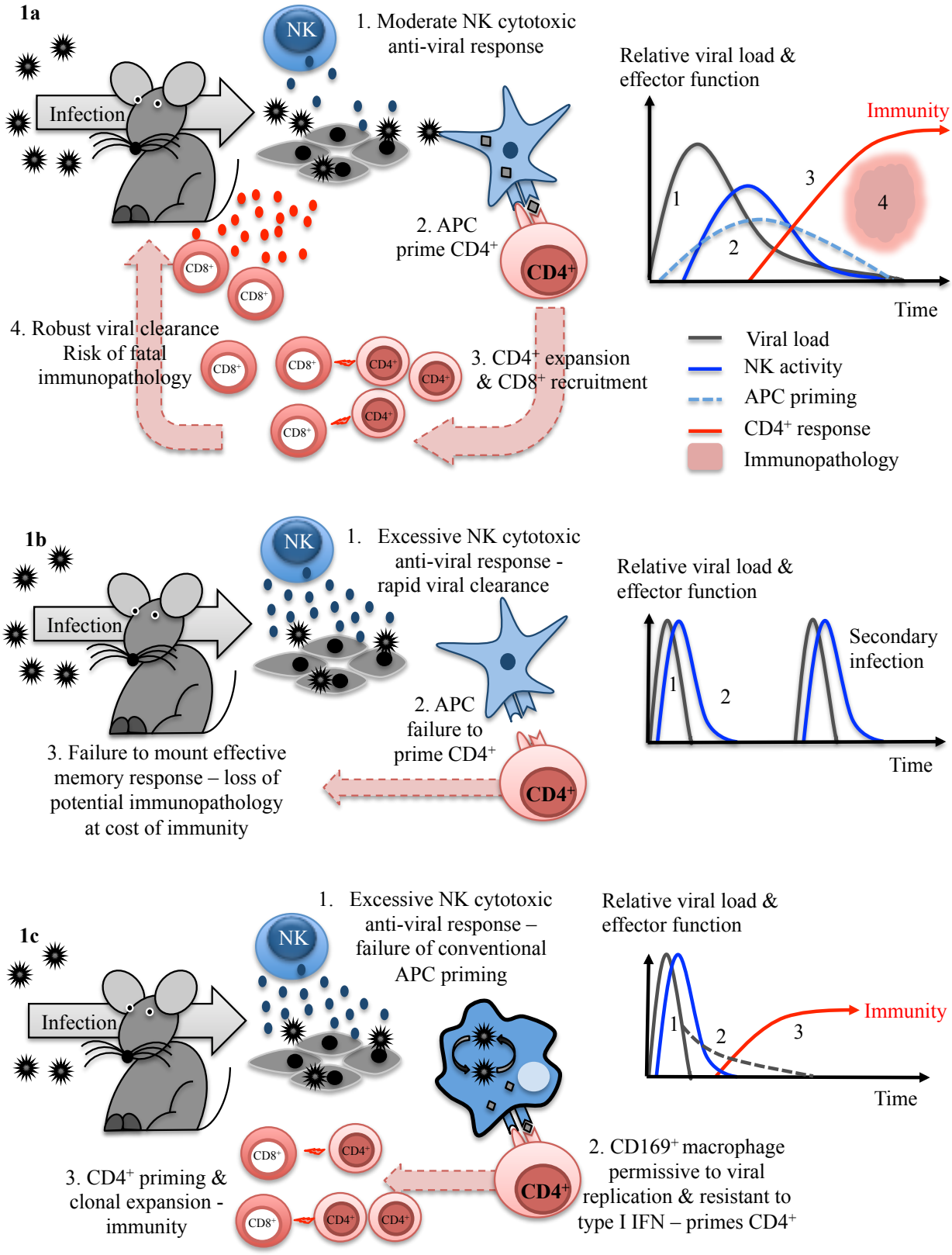
Honke *et al* described a mouse model of vesicular stomatitis virus (VSV) infection, which demonstrated the importance of an appropriate time period of antigen presentation for the development of CD4$^+$ T cell responses.[202] Robust type-I IFN innate responses effectively

cleared VSV from the majority of tissues. Rapid clearance of the virus prevents the development of the adaptive immune response. However, a subgroup of splenic $CD169^+$ macrophages, resistant to type-I IFN, remain permissive to on going VSV replication, thus allowing persistent antigen presentation and the development of adaptive immune responses. Following $CD169^+$ macrophage depletion VSV infection was again effectively cleared by the innate immune response but without the appropriate antigen exposure there was a failure to prime an adaptive immune response. This resulted in a loss of specific adaptive immunity rendering the mice vulnerable to reinfection.

Andrews *et al*. demonstrated that during MCMV infection depletion of NK cells increased *ex vivo* virus specific IFNγ production by $CD4^+$ and $CD8^+$ T cells. NK cells depleted infected dendritic cells in a perforin dependent manner reducing the length of antigen presentation and thus magnitude of the adaptive immune response. The diminished $CD4^+$ T cell response to the MCMV infection increased viral replication in the salivary glands thus providing a viral advantage.[203] Interestingly a compromised adaptive immune response to viral infections can also provide the host with a survival advantage. Lymphocytic choriomeningitis virus (LCMV) infects mice and can, depending on the viral load during the infection, induce a robust immune response resulting in severe immunopathology.[204] LCMV infected cells are resistant to NK cell lysis and immunopathology is $CD8^+$ T cell mediated. Waggoner *et al*. elegantly demonstrated that mice inoculated with a high LCMV viral load did not develop immunopathology as NK cells depleted virus–specific $CD4^+$ T cells allowing $CD8^+$ T cell exhaustion to occur. However, inoculation with moderate LCMV viral loads that fail to induce NK cell depletion of $CD4^+$ T cells resulting in on-going $CD8^+$ T cell activation and immunopathology (Figure 3.16).

The ability of NK cells to impinge on the development of adaptive immune responses has been corroborated by Narni-Maninelli et al. in mouse models of MCMV and ovalbumin expressing Listeria monocytogenes infection. Mice were bred with hyper-responsive NK cells, which failed to express NKp46 resulting in up regulation of the transcription factor *Helios*.[205] Mice with hyper-responsive NK cells rapidly controlled both viral and bacterial infections reducing antigen availability and therefore failed to mount effective $CD4^+$ and $CD8^+$ responses rendering the mice susceptible to reinfection. Furthermore NK cell activating receptor NKG2D has been implicated in perforin mediated killing of primed $CD8^+$ T cells thus moderating the adaptive immunity in both mouse virus and tumour models.[206,207]

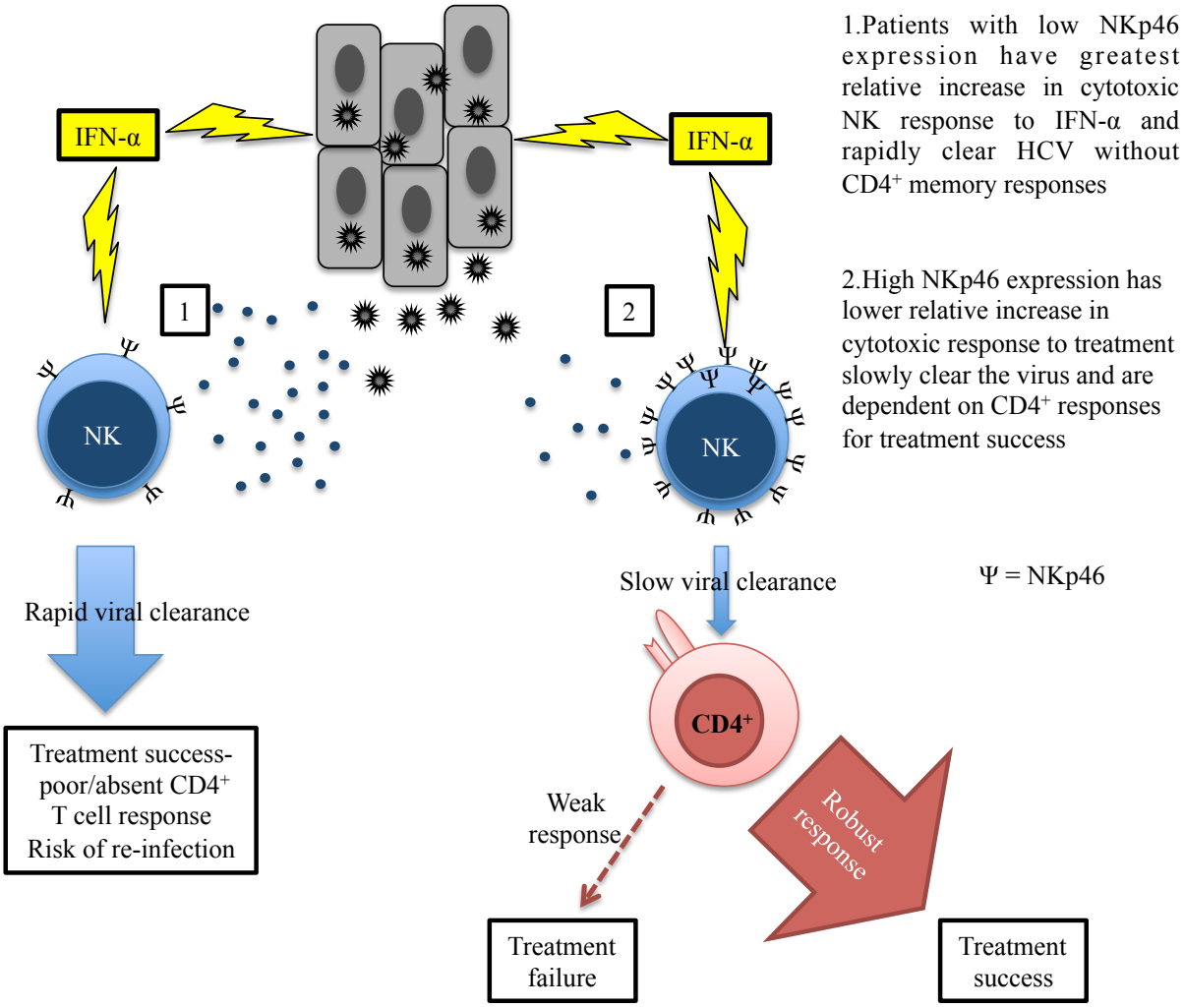
Figure 3.16 Models of immune response to viral pathogens and development of adaptive immunity. [208]



1a: A moderate NK cell response to viral infection allows APC priming of CD4+ T cells. The adaptive immune response may result in fatal immunopathology (Waggoner *et al*.). 1b: An excessive NK cytotoxic response may clear viral antigen without appropriate APC priming of CD4+ T cells leaving the host at risk of future infection (Andrews *et al*. & Narni-Mancinelli *et al*.). 1c: CD169+ macrophages, IFN-α resistant and permissive to viral replication, enable on-going priming of adaptive immune responses during rapid innate viral control (Honke *et al*.).

In human disease the relationship between antigen availability and adaptive immune priming is harder to demonstrate owing to restriction of tissue availability to predominantly the blood compartment. Previous work from the lab I worked in demonstrated that patients with the most rapid viral clearance actually have absent or minimal $CD4^+$ T cell responses.[199] This study suggests that innate immune responses may prevent the development of adaptive immunity in human infections, as has been demonstrated in mice, in an NKp46 dependent manner during treatment with IFNα (Figure 3.17). Alter et al found that individuals who spontaneously cleared HCV had lower expression of NKp46 and NKp30 than those developed a persistent infection.[154] When these findings are considered in the context of NK cells impingement upon adaptive responses one could hypothesise that lower levels of NK activating receptors may reduce the innate response allowing the propagation of a robust $CD4^+$ T cell response; the hallmark of successful spontaneous clearance of acute HCV infection.

Figure 3.17 NKp46$^+$ NK cells and CD4$^+$ T cell responses during IFNα treatment of chronic HCV



In conclusion I have demonstrated an association between $NKp46^+$ NK cells and the function of peripheral blood NK cells in chronic HCV. This relationship is particularly important during IFNα treatment as individuals with low $NKp46^+$ populations have the greatest potential to up regulate cytotoxic function and rapidly control viral load. This response is in essence a double-edged sword; rapid viral clearance is strongly associated with successful treatment outcome but also with a failure to mount a robust adaptive immune response. This potentially leaves the treated patient at risk of re-infection following further exposures. However, this opens intriguing questions regarding our ability to fine tune immune response to induce favourable adaptive immune responses and more pressingly for infected individuals – does a more active NK phenotype impact on disease progression?

Chapter 4:

Results –

Intrahepatic NK cells

&

chronic HCV infection

4.1 Intrahepatic NK cell phenotype and function in chronic HCV

The majority of immunological studies of chronic HCV infection have been restricted to clinical samples from the peripheral blood compartment rather than the site of infection: the intrahepatic compartment. The studies that have investigated NK cells from the intrahepatic compartment have reported a similar[161] or reduced[159,160,163] NK cell proportion compared to the peripheral blood. There is however, a lack of consensus regarding the phenotype of intrahepatic NK cells (summarised in Table 1.4).[158,161,163] These studies demonstrate that the peripheral blood offers a reflection of the intrahepatic compartment. However, the small sample sizes, limited phenotypic data and lack of functional analyses point to a hole in our knowledge of an important subset of lymphocytes in chronic HCV infection.

In the study described in this Chapter, the following question was addressed:

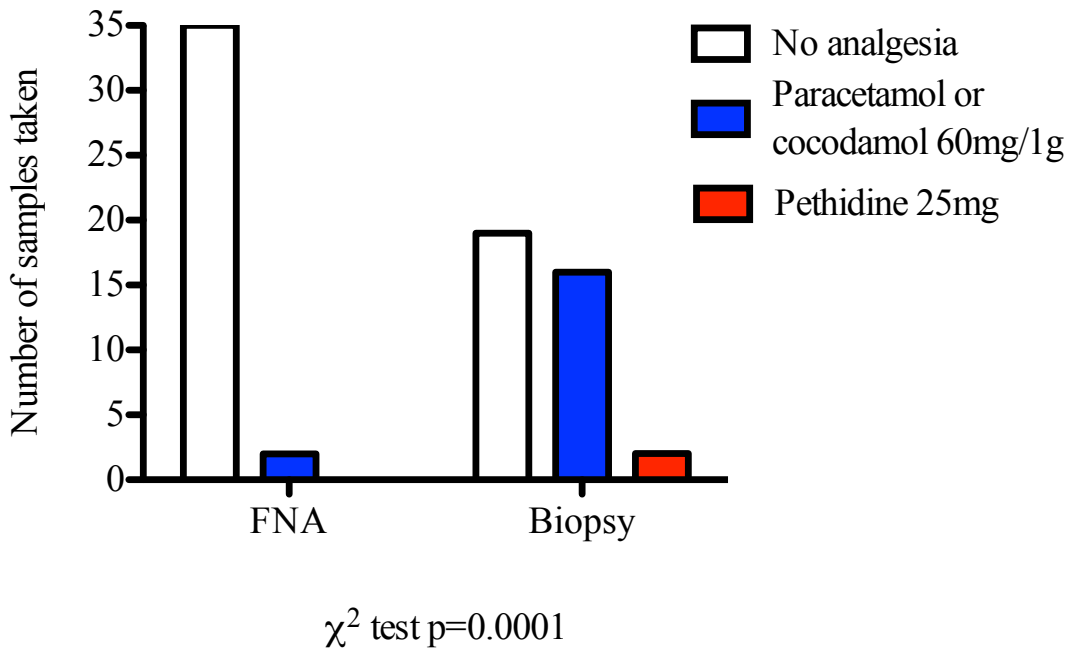
- "*Does intrahepatic NK cell activity reflect peripheral blood NK function and is this altered with IFNα treatment?"*

In order to address this I utilised FNA sampling of the liver to assess NK cell phenotype and markers of function in 46 patients with chronic HCV and non-viral chronic liver diseases. In a subset of 9 patients I performed repeated FNA sampling during IFNα treatment of chronic HCV infection (Table 2.1 page 56). FNA samples were taken with paired peripheral blood samples, transported on ice to the lab and phenotypic and functional markers immediately assessed by flow cytometry.

4.2 FNA sampling

To our knowledge this study represents the largest cohort of patients to undergo FNA sampling of the liver to retrieve intrahepatic lymphocytes for research purposes. However, a significant concern during the study design was the acceptability and safety of patients undergoing FNA sampling. The procedure was well tolerated and patients informally reported that the discomfort was approximately half way between a blood test and a liver biopsy. In this study a total of 81 FNA samples were taken. Following FNA sampling 42 patients proceeded to liver biopsies and 39 FNA samples were taken without biopsies (including 9 patients who underwent repeat FNA sampling). Following the 39 FNA procedures without biopsies 1 patient required 1g of paracetamol and another received 30mg of codeine and 500mg of paracetamol. In patients who proceeded to liver biopsy following FNA, 16 patients required cocodamol and 2 patients required intramuscular pethidine (X^2 test = 0.0001, Figure 4.1). Of note 9 of the 12 patients who were treated agreed to repeat FNA sampling with up to three samples taken during the first week. This indicates that the majority of patients found the procedure acceptable.

Figure 4.1 Analgesia requirements following FNA and liver biopsy



Analgesia requirement following FNA procedure alone and FNA and biopsy combined. Patients who had biopsy had a far higher requirement for opiate analgesia. A specialist nurse offered analgesia at 15 minute intervals for 1 hour then every 30 minutes.

Fine needle aspiration is a clinical technique that provides single cell cytology samples and as such is unable to provide histological information. The liver is an extremely vascular organ and aspiration of cells from within the liver capsule contained particules assumed to be cellular debris that prevented flow cytometry analysis (Figure 4.2A). A significant hurdle in the use of FNA is the need to isolate the intrahepatic lymphocytes from sample debris. Sample debris may effect immunohistochemistry and flow cytometry analysis. Two methods to isolate intrahepatic lymphocytes from the aspirate were compared. As described in "Materials and Methods" the first method required layering aspirate upon Ficoll and isolating lymphocytes by a density gradient. The second method utilised saponin permeabilisation and repeated washing to clear cell debris. In both cases cells were stained with monoclonal antibodies. Paired peripheral blood samples were taken and mononuclear cells were isolated by Ficoll density gradient. For both intrahepatic and peripheral blood samples the following flow cytometry gating strategy was used:

Single cells were selected using Forward scatter height and Forward scatter area gating. Lymphocytes were then gated based on forward and side scatter and dead cells were excluded with aqua live dead stain. Lymphocyte subsets (NK, NKT and T lymphocytes) were gated based upon expression of CD56 and CD3, CD14 and CD19 (Figure 4.2B-E).

Figure 4.2 Lymphocyte gating strategy for intrahepatic & peripheral blood samples

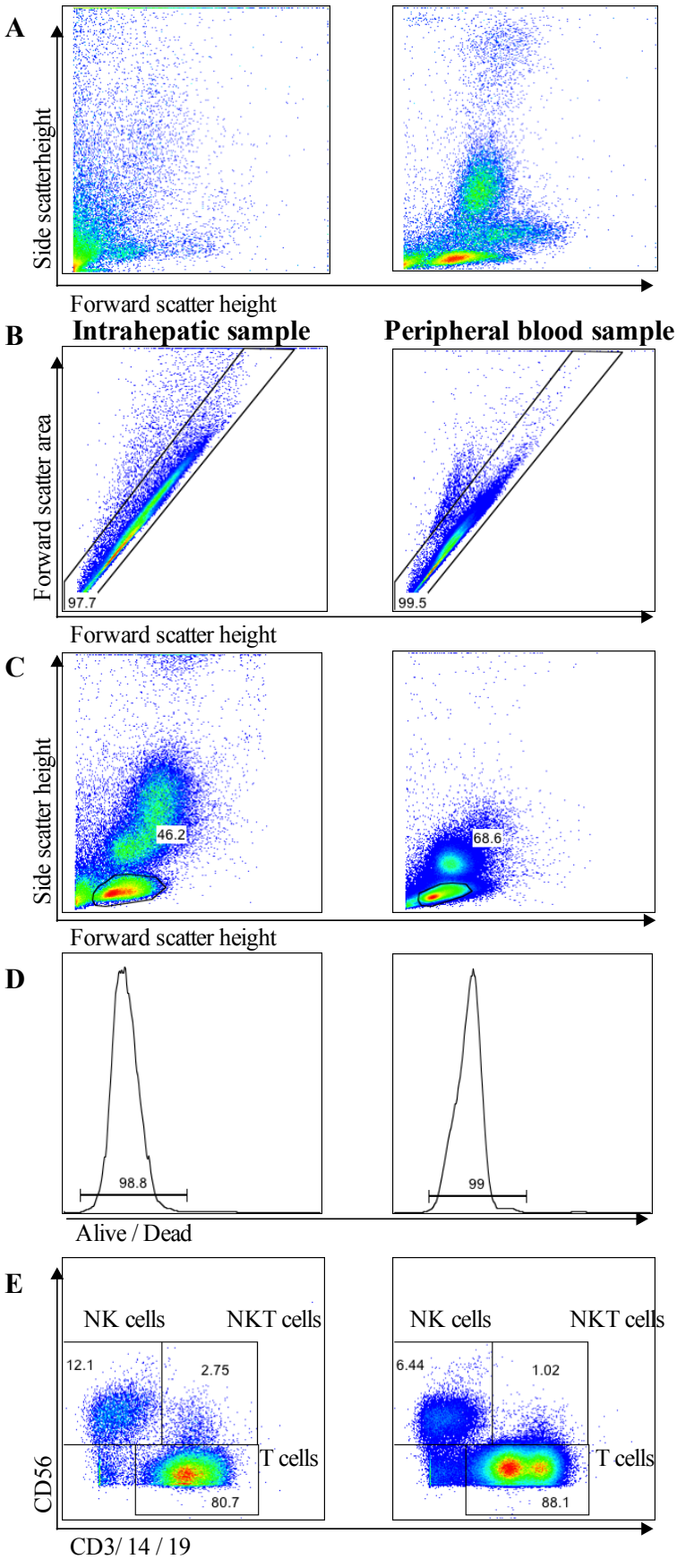


Figure 4.2 A) Left panel FNA sample demonstrating debris on forward and side scatter on unprocessed sample. Right panel forward and side scatter for sample for which debris had been removed by saponin permeabilisation.

B-E) Lymphocyte gating strategy for samples obtained by FNA of the liver (Left panels) and peripheral blood (Right panels). Isolated lymphocytes were stained with monoclonal antibodies and analysed by flow cytometry using the following gating strategy:

B) Single cells were selected based upon forward scatter area and height.

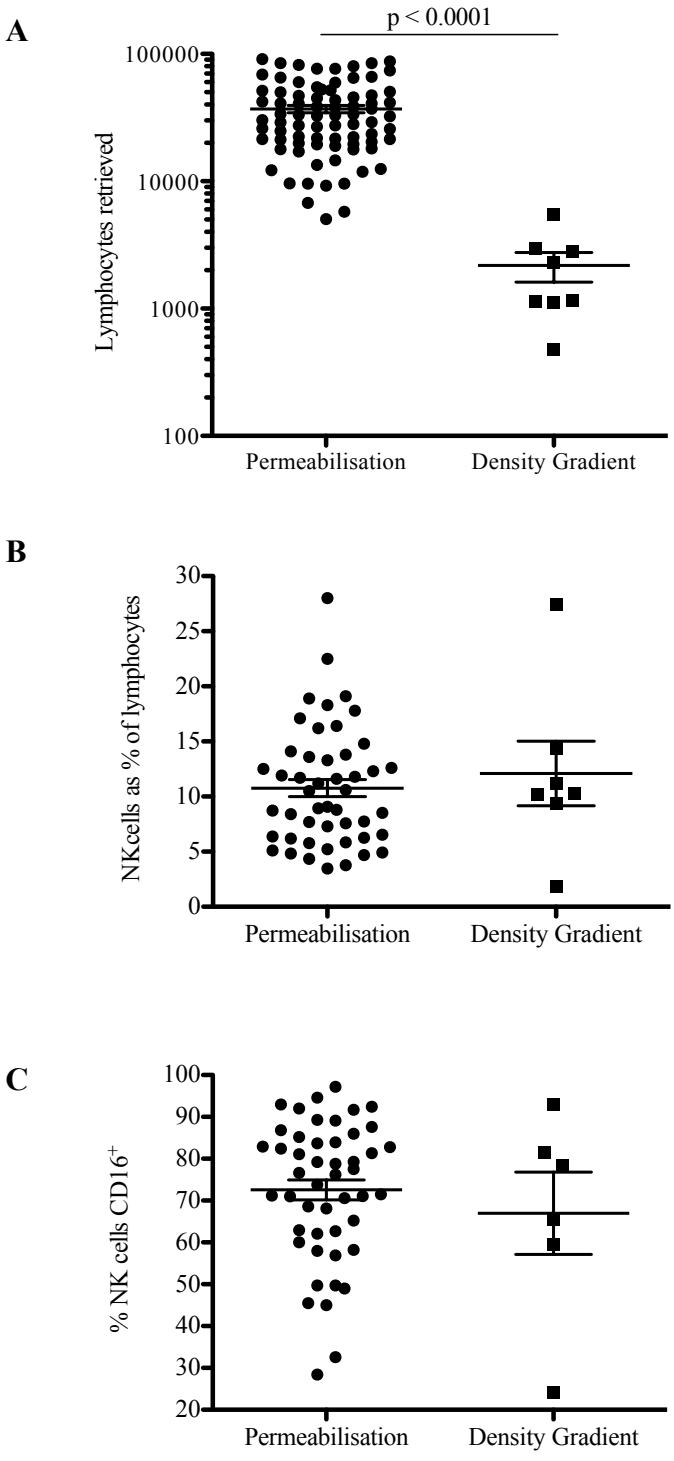
C) Lymphocyte gate was based upon forward and side scatter profile.

D) Dead cells were excluded using Aqua Live/Dead stain.

E) NK cells were identified by CD56 & CD3,14, 19 profile

It was rapidly evident that the number of intrahepatic lymphocytes retrieved was significantly higher using the permeabilisation method compared to a density gradient (mean lymphocytes = 36959 v 2183 respectively $p<0.0001$, Figure 4.3A). The proportion of these lymphocytes that were NK cells was similar with both methods (10.8% v 12.1% $p=0.7$, Figure 4.3B). With the caveat that very few cells were recovered by density gradient, I observed no significant difference in the phenotype of NK cells isolated using each method (e.g. CD16: 72.5% v 67.0% $p=0.46$, Figure 4.3C). Given the superior numbers of NK cells recovered, the permeabilisation method was subsequently routinely used.

Figure 4.3 Intrahepatic NK cell isolation by permeabilisation & density gradient

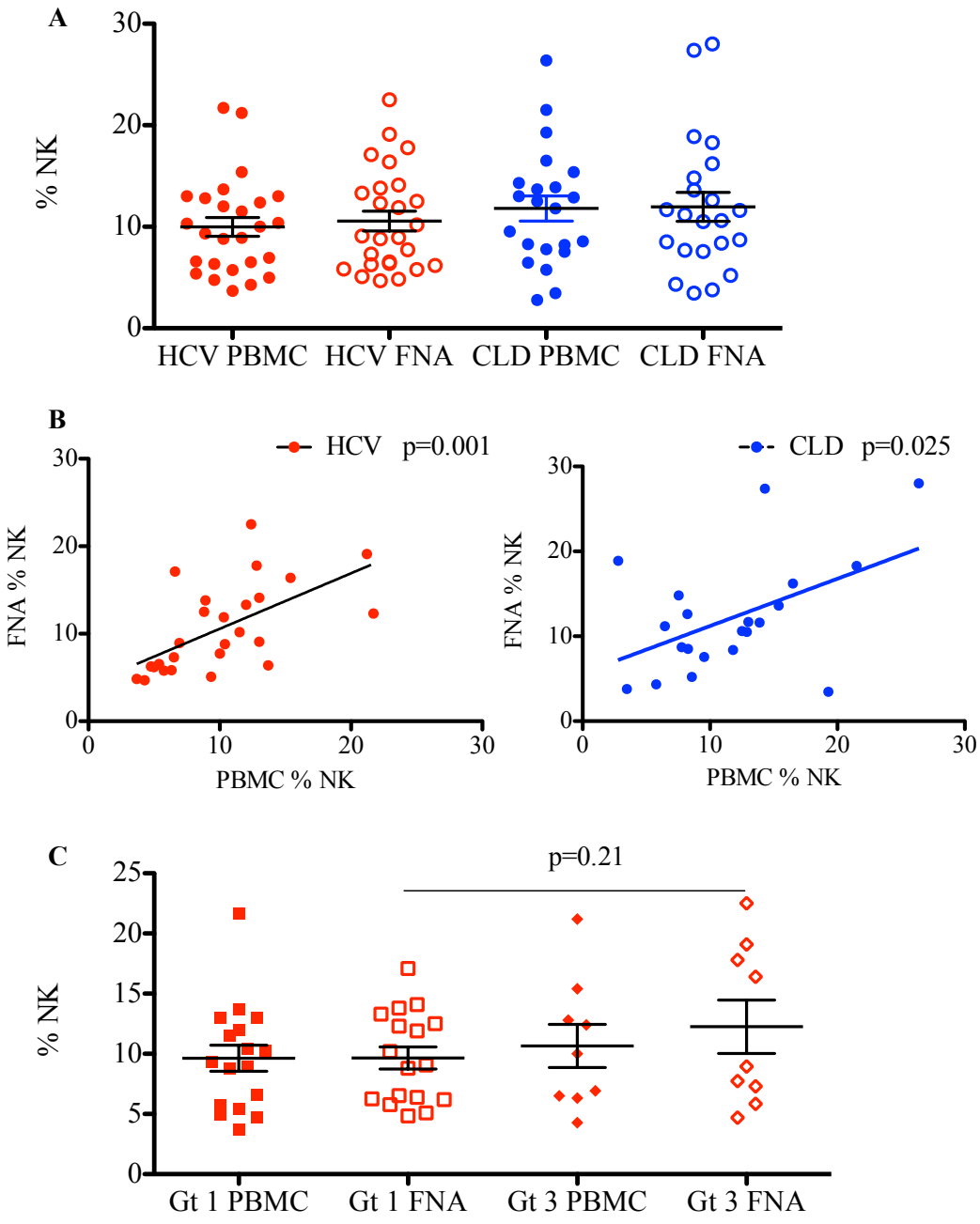


Intrahepatic lymphocytes were prepared by saponin permeabilisation (•) and washing to remove cellular debris or by density gradient purification over Ficoll (■). (A) Comparison of the total number of intrahepatic lymphocytes retrieved, (B) NK cells as a proportion of lymphocytes and (C) $CD16^+$ NK cells. Mean and SEM shown.

4.3 Intrahepatic NK cells in chronic HCV and non-viral chronic liver disease

In keeping with previous studies,[159-161,163] the frequencies of intrahepatic and peripheral blood NK cells were similar in the cohort of individuals chronically infected with HCV; the mean NK cell proportion was 9.9% in the peripheral blood and 10.6% in the FNA aspirate (p=0.51). Similarly, in the CLD cohort there were similar NK cell proportions in the peripheral blood and intrahepatic compartments (11.8% and 12.0% respectively p=0.9, Figure 4.4A, representative FACS plots Figure 4.2D). The proportion of intrahepatic and peripheral blood NK cells correlated in both the HCV and the CLD cohorts (HCV: r^2=0.36, p=0.001 and CLD: r^2=0.23, p=0.025, Figure 4.4B). The spread of intrahepatic NK cell proportions was greater in the CLD cohort compared to the HCV cohort (3.5-28% and 5-22.5% respectively). This spread is perhaps unsurprising given the range of pathologies within the CLD cohort (Table 4.1). There was no difference in the proportion of intrahepatic NK cells in patients infected with HCV genotype 1 compared to those with HCV genotype 3 infection (mean 9.7% and 12.3% respectively p=0.21, Figure 4.4C).

Figure 4.4 Intrahepatic NK cells as a proportion of lymphocytes



A) Intrahepatic and peripheral blood lymphocytes were stained with anti-CD56 and anti-CD3, 14 & 19 antibodies to analyses the proportion of NK cells in a HCV (n=24) and CLD cohort (n=22). There were no significant alterations in the proportions of NK cells in either cohort. B) The proportion of NK cells in the intrahepatic compartment correlated with the peripheral blood in both cohorts. Left panel: HCV r^2=0.36, p=0.001 and right panel: CLD r^2=0.23, p=0.025. D) There was difference in the proportion of NK cells in patients with genotype (GT) 1 or 3 infection. Mean & SEM shown.

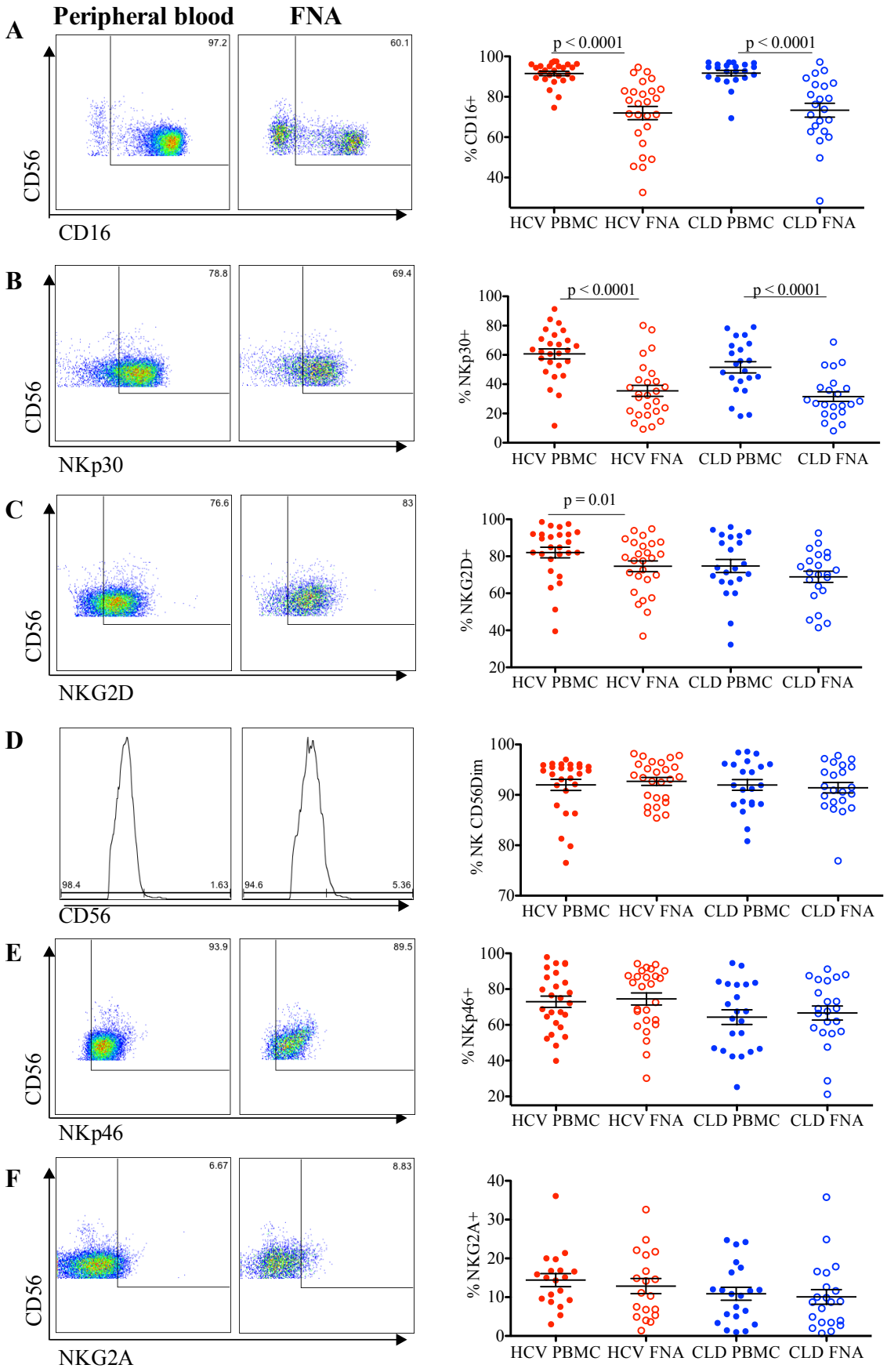
4.4 Intrahepatic NK cell phenotype

Although the proportion of NK cells was not altered between the peripheral blood and intrahepatic compartments we found a marked alteration in the phenotype of intrahepatic NK cells. The proportions of $CD16^+$, $NKp30^+$ and $NKG2D^+$ NK cells were reduced in the intrahepatic compartment in both the HCV and CLD cohorts (Figure 4.5 A-C, proportions in legend). For example the proportion of $CD16^+$ NK cells in the peripheral blood was 91.5% and 72.0% in the liver in the HCV cohort ($p<0.0001$) and 91.7% and 73.3% in the CLD cohort respectively, ($p<0.001$. Figure 4.5A). A potential explanation for the altered intrahepatic NK cell phenotype may have resulted from a decrease in proportion of the mature $CD56^{Dim}$ NK cell subset. However, there was no reduction in the proportion of intrahepatic $CD56^{Dim}$ NK cells in either cohort (Figure 4.5D). An alternative explanation for the altered phenotype is that CD16 and other NK cell activating receptors are reduced upon NK cell activation, as described in detail in Chapter 3.

There was no difference in the proportion of $NKp46^+$ or $NKG2A^+$ NK cells between the intrahepatic and peripheral blood compartments in either cohort (Figure 4.5E&F). There was no difference in intrahepatic proportions of $CD16^+$, $NKp30^+$, $NKp46^+$, $NKG2D^+$ and $NKG2A^+$ NK cells between genotype 1 and genotype 3 patients (data not shown).

These data demonstrate a distinct phenotype of intrahepatic NK cell population, confirming that a separate population of NK cells were sampled. The intrahepatic NK cell phenotype may reflect function within the chronically inflamed liver.

Figure 4.5 Intrahepatic NK cell phenotype (legend overleaf)



NK cell phenotype in the peripheral blood and intrahepatic compartments. Left panels demonstrate representative FACS plots. The proportion of activation marker^{+} cells are within the gated area and had higher expression than the FMO control. Right panels demonstrate HCV (blood●, FNA ○) & CLD (blood●, FNA ○) cohort phenotype.

A) CD16^{+} NK cells: HCV blood 91.5% v FNA 72.0%, p<0.001 & CLD 91.7% v 73.3% respectively, p<0.001.

B) NKp30^{+} NK cells: HCV blood 53.7% v FNA 27.7% p<0.001 & CLD 51.5% v 24.8% respectively, p<0.001.

C) NKG2D^{+} NK cells: HCV blood 82.0% v FNA 68.6%% p=0.01 & CLD 74.8% v 68.9% respectively, p=0.1.

D) CD56^{Dim} NK cells: HCV blood 92.0% v FNA 92.7% p=ns & CLD 92.0% v 91.4% respectively, p=ns.

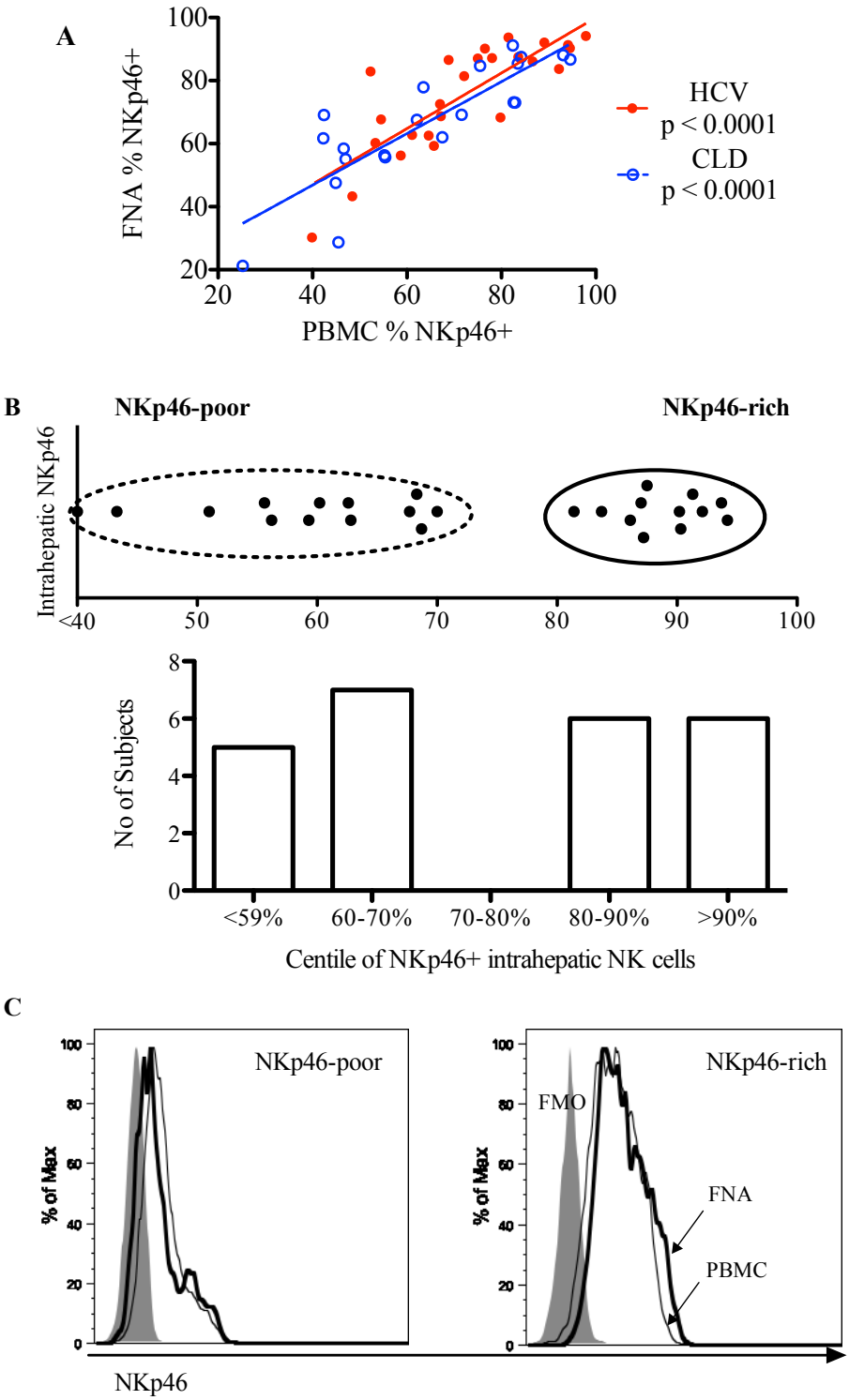
E) NKp46^{+} NK cells: HCV blood 73.0% v FNA 74.5% p=ns & CLD 64.4% v 66.7% respectively, p=ns.

F) NKG2A^{+} NK cells: HCV blood 14.4% v FNA 12.9% p=ns & CLD 10.9% v 10.1% respectively, p=ns.

Mean & SEM shown. Paired t-tests.

Conversely, NKp46, the activating receptor that was associated with *in vitro* NK cell activity in HCV infected donors, was not downregulated in the intrahepatic compartment. There was a strong correlation between intrahepatic and peripheral blood $NKp46^+$ proportion of NK cells (HCV: $p<0.0001$, $r^2=0.68$ & CLD: $p<0.0001$, $r^2=0.73$, Figure 4.6A). The data suggests two patterns of NK cell phenotype, characterised by the proportions of $NKp46^+$ cells, strikingly within the HCV (but to a lesser degree in the CLD) cohorts. Twelve individuals in the HCV group demonstrated an NKp46-rich NK cell population with greater than 80% of NK cells $NKp46^+$ whilst 14 patients were NKp46-poor with less than 70% $NKp46^+$ cells (Figure 4.6B & C). The significance of these two groups was examined as described in detail below.

Figure 4.6 Intrahepatic NKp46 expression



A) NKp46 expression has a strong correlation between the peripheral blood and the intrahepatic compartments. B) HCV infected subjects separated into those with NKp46-rich (>80%) and NKp46 poor (<70%) individuals. C) Representative FACS plots of NKp46-poor (left panel) and NKp46-rich (right panel) individuals. FNA sample: thick black line, peripheral blood: grey line, FMO: shaded area.

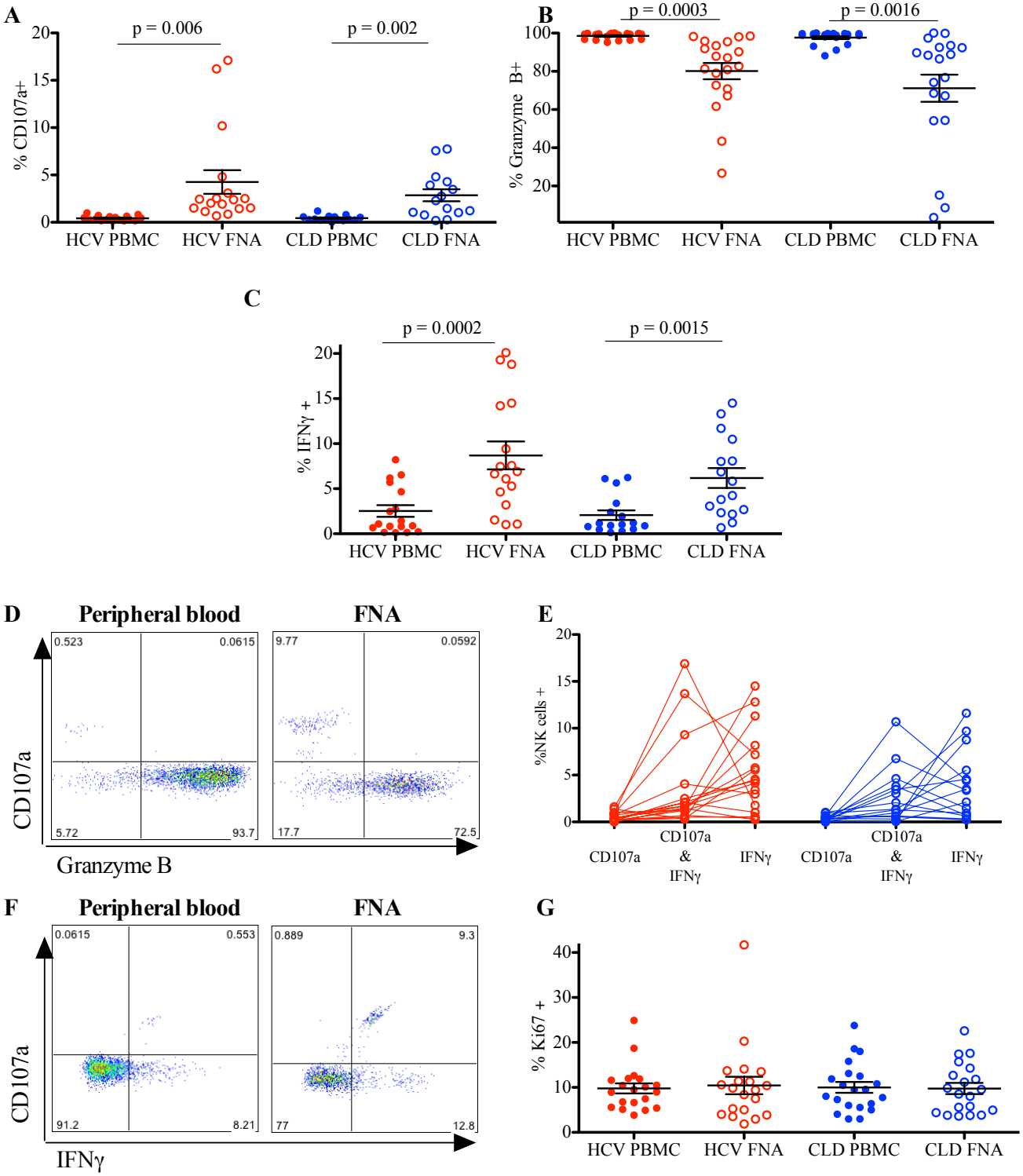
4.5 Intrahepatic NK cell function

The FNA sampling technique allowed aspirated lymphocytes to be assessed for functional markers directly *ex vivo*. In keeping with the altered phenotype of intrahepatic NK cells there was a distinct pattern of NK cell functional markers in the intrahepatic compartment. The proportions of $CD107a^+$ and $IFN\gamma^+$ NK cells were increased and the proportion of granzyme B^+ NK cells was reduced in the intrahepatic compartment compared to the peripheral blood in both cohorts. (Figure 4.7A-C). In addition $CD107a^+$ cells were granzyme B^- supporting the interpretation that intrahepatic NK cells are more likely to have recently degranulated (Figure 4.7D). Almost all $CD107a^+$ NK cells in the liver were $IFN\gamma^+$, although a significant proportion of activated NK cells were $IFN\gamma^+$ and $CD107a^-$ (Figure 4.7E&F). These data give an interesting insight into NK cell activation *in vivo.* Recent NK cell activation in the liver is in keeping with the altered CD16, NKp30 & NKG2D phenotype described above.

Ki67 is a marker of cell proliferation and is present during all active phases of the cell cycle. Proportions of $Ki67^+$ NK cells were similar in the peripheral blood and intrahepatic compartment in both the HCV and CLD cohorts (Figure 4.7G). There were no differences in levels of functional marker expression between patients infected with either genotype 1 & 3 virus in either the peripheral blood or the intrahepatic samples (data not shown).

In summary these data demonstrate that NK cells within the intrahepatic compartment have increased activity in terms of degranulation and IFNγ production, both of these factors are likely to contribute to inflammation in both HCV and other non-viral CLD.

Figure 4.7 NK cell functional markers



Intrahepatic NK cells demonstrate increased activity compared to the peripheral blood (HCV blood●, FNA ○ & CLD blood●, FNA ○).

A) Direct *ex vivo* proportion of NK cells CD107a+ without further stimulation in the intrahepatic and peripheral blood compartments. HCV: 4.3% v 0.4% respectively, p=0.006 & CLD: 2.8% v 0.4% p=0.001.

B) The proportion of NK cells containing intracellular Granzyme B in the intrahepatic and peripheral blood compartments. HCV: 98.5% v 80.2% respectively, p=0.0003 & CLD: 97.6% v 71.1% p=0.0016.

C) IFNγ$^+$ NK cells in the peripheral blood and intrahepatic compartments. HCV: 2.5% v 8.7%, respectively p=0.0002 & CLD: 0.94% v 3.8%, p=0.0015.

D) Representative FACS plot demonstrating NK cell CD107a and granzyme B staining from peripheral blood and intrahepatic samples.

E) The majority of activated NK cells were CD107a & IFNγ$^+$ or IFNγ$^+$ alone.

F) Representative FACS plot of NK cell CD107a and IFNγ staining in the liver and peripheral blood.

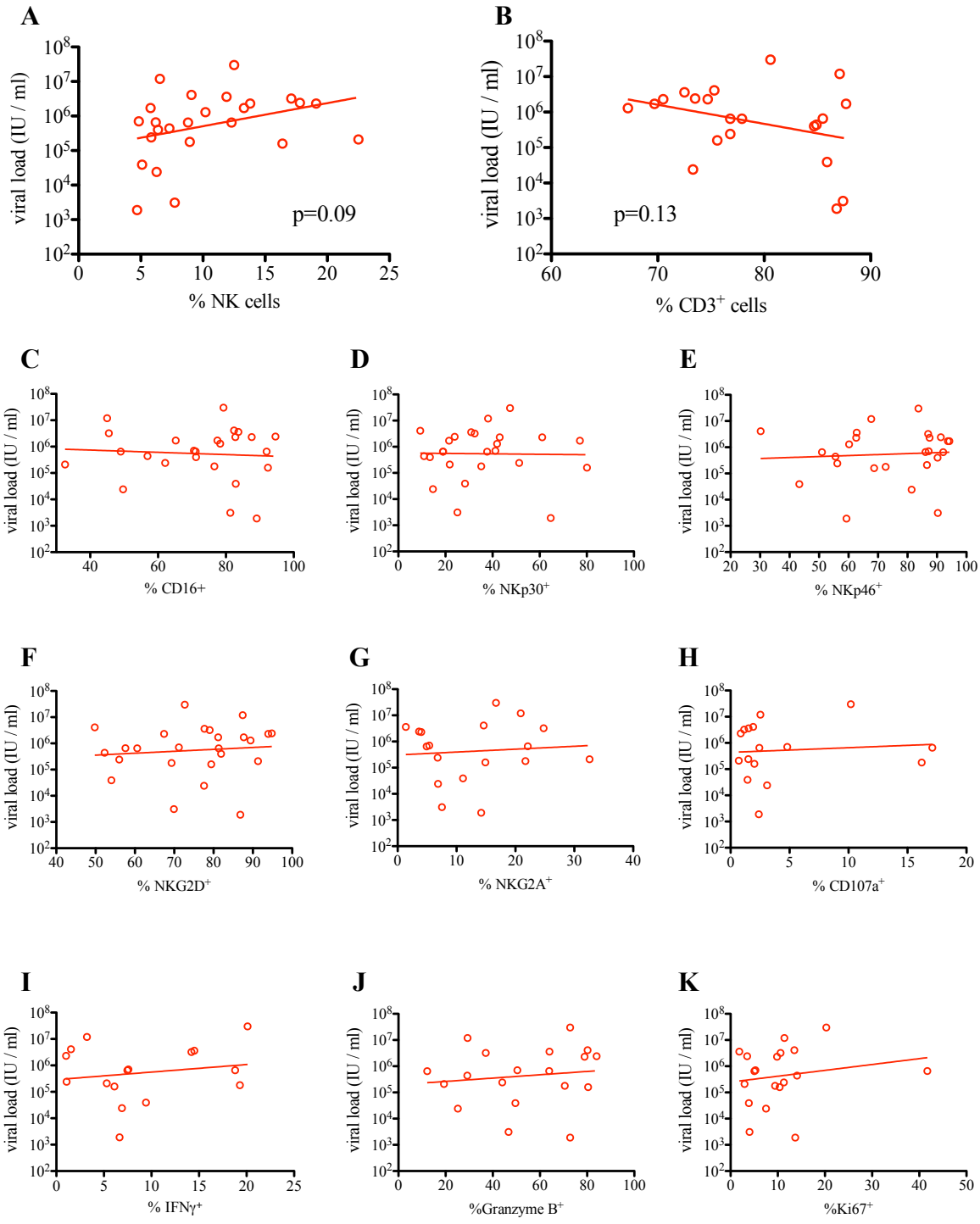
G) Ki67$^+$ NK cells in the peripheral blood and intrahepatic compartments of HCV and CLD cohorts.

Mean and SEM shown.

4.6 Intrahepatic NK cells and hepatitis C viral load

The increased functional activity of intrahepatic NK cells suggests that they may be active in control of virus during chronic HCV infection. There was no association between the viral load and the proportion of intrahepatic NK cells, T cells or NK cell phenotype as measured (Figure 4.8).

Figure 4.8 Viral load and NK cell phenotype & function in chronic HCV



A) Correlation of NK cells as a proportion of intrahepatic lymphocytes and viral load in chronic HCV r^2 0.1, p=0.09. B) Correlation of intrahepatic $CD3^+$ $CD56^-$ T cells and HCV viral load (r^2=0.1 p=0.13). Intrahepatic NK cell phenotype did not correlate with control of chronic HCV infection viral load; C) CD16. D) NKp30. E) NKp46. F) NKG2D. G NKG2A. Markers of intrahepatic NK cell function do not correlate with HCV viral load; H) CD107a. I) IFNγ. J) Granzyme B. K) Ki67

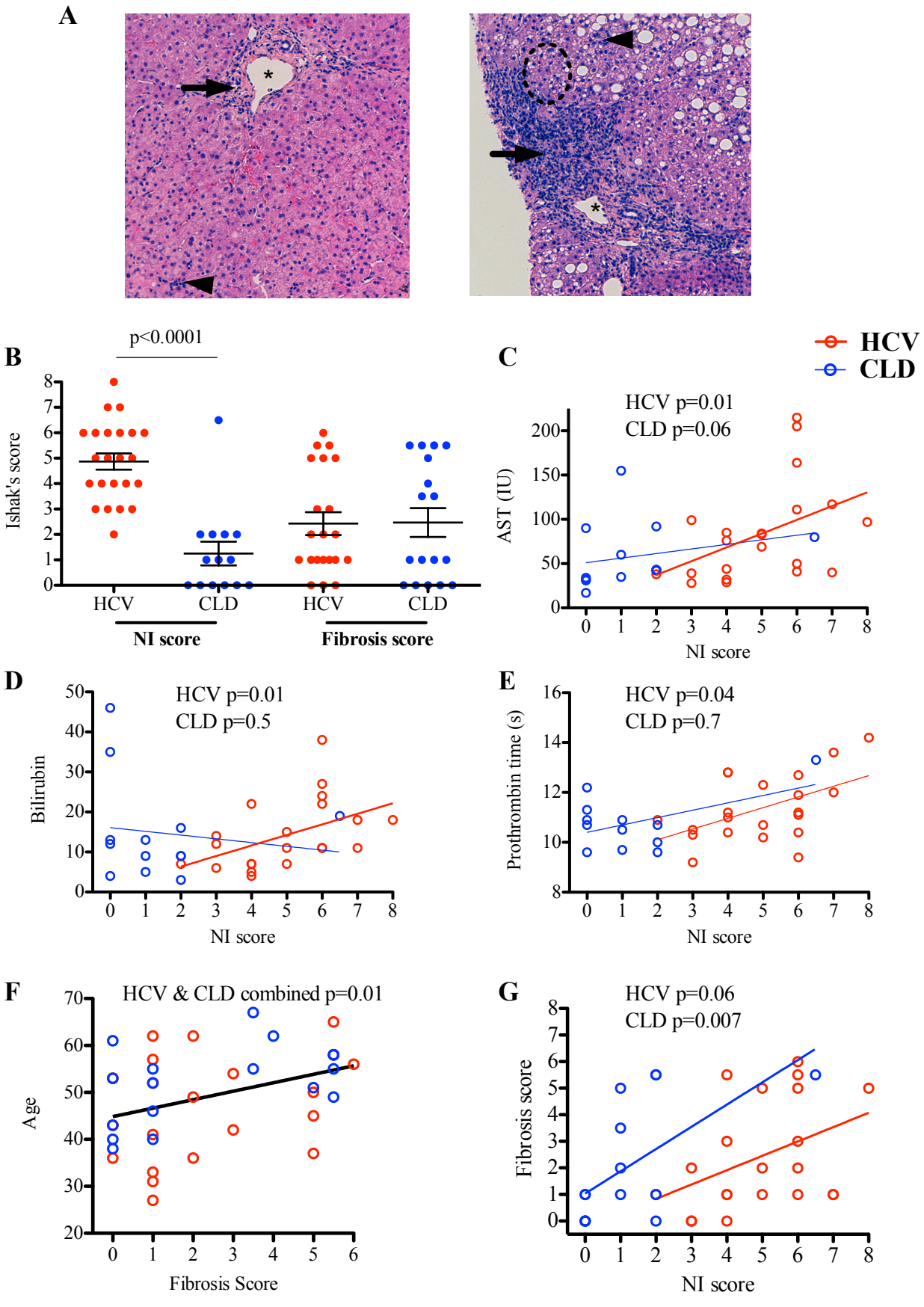
4.7 Markers of liver inflammation

HCV associated inflammation, rather than the viral load and viral replication *per se,* appears to drive liver fibrosis leading to liver failure and cirrhosis. In addition liver inflammation can result in hepatocellular carcinoma although HCV NS5A protein has also been reported to be independently oncogenic.[209] NK cells may contribute to the pathogenesis of HCV related disease via mechanisms of inflammation rather than direct control of the virus.

Liver biopsy specimens were available for 23 HCV-infected and 21 CLD donors (Table 2.1). A consultant histopathologist scored these specimens using the Ishak's necroinflammatory (NI) criteria for all HCV samples and 19 of the CLD samples. Representative specimens of low (NI score 3) high-grade (NI score 8) inflammation are shown in Figure 4.9A. Iskah's fibrosis score (0-6) was also used to stage liver fibrosis and cirrhosis. The NI score was significantly higher in the HCV cohort compared to the CLD cohort (4.9 v 1.7 respectively, $p<0.0001$ Figure 4.9B). There was no difference in the fibrosis score between the two cohorts. The marked difference in the NI score between the cohorts may reflect the variety of pathologies represented by the CLD cohort and how they result in the pathological changes to which the histopathologist applies the semi-numerical descriptive score (periportal inflammation, interface hepatitis, focal hepatitis and confluent necrosis). For example steatohepatitis may be more effectively scored by the Brunt criteria (Appendix I).[210] Within the HCV cohort NI score correlated with surrogate markers of hepatocyte death and dysfunction: increased serum levels of AST, bilirubin and prothrombin time (Figure 4.9C-E). When both the HCV and CLD cohorts were taken together fibrosis score correlated with age (Figure 4.9F). Necroinflammatory

score correlated with fibrosis score in the CLD cohort (Spearman's rho 0.66, p=0.007) and the HCV cohort (Spearman's rho 0.4, p=0.06, Figure 4.9G).

Figure 4.9 Necroinflammatory and Fibrosis scores



A) Representative hemaotoxylin and eosin (H&E) stain liver biopsies. Left panel - necroinflammatory score of 3 (* = portal tract, → = periportal inflammation, score=2. ◂ = focal hepatitis, score=1). Right panel - necroinflammatory score of 8, (periportal inflammation=3, interface hepatitis within broken circle=3, focal hepatitis=2).

B) Ishak's NI and fibrosis score in HCV (O) & CLD (O) cohorts. Mean & SEM, Mann Whitney U test. Six patients (2 HCV and 4 CLD) were scored as 5 to 6 out of 6 fibrosis by consultant histopathologist.

C) Serum AST and NI score, HCV; Spearman's Rho 0.53 p=0.01, CLD; Spearman's Rho 0.52 p=0.06.

D) Serum bilirubin & NI score, HCV; Spearman's Rho 0.53, p=0.01, CLD; Spearman's Rho -0.17 p=0.5.

E) Prothombin time and NI score, HCV; Spearman's Rho 0.45 p=0.04, CLD; -0.1 p=0.7.

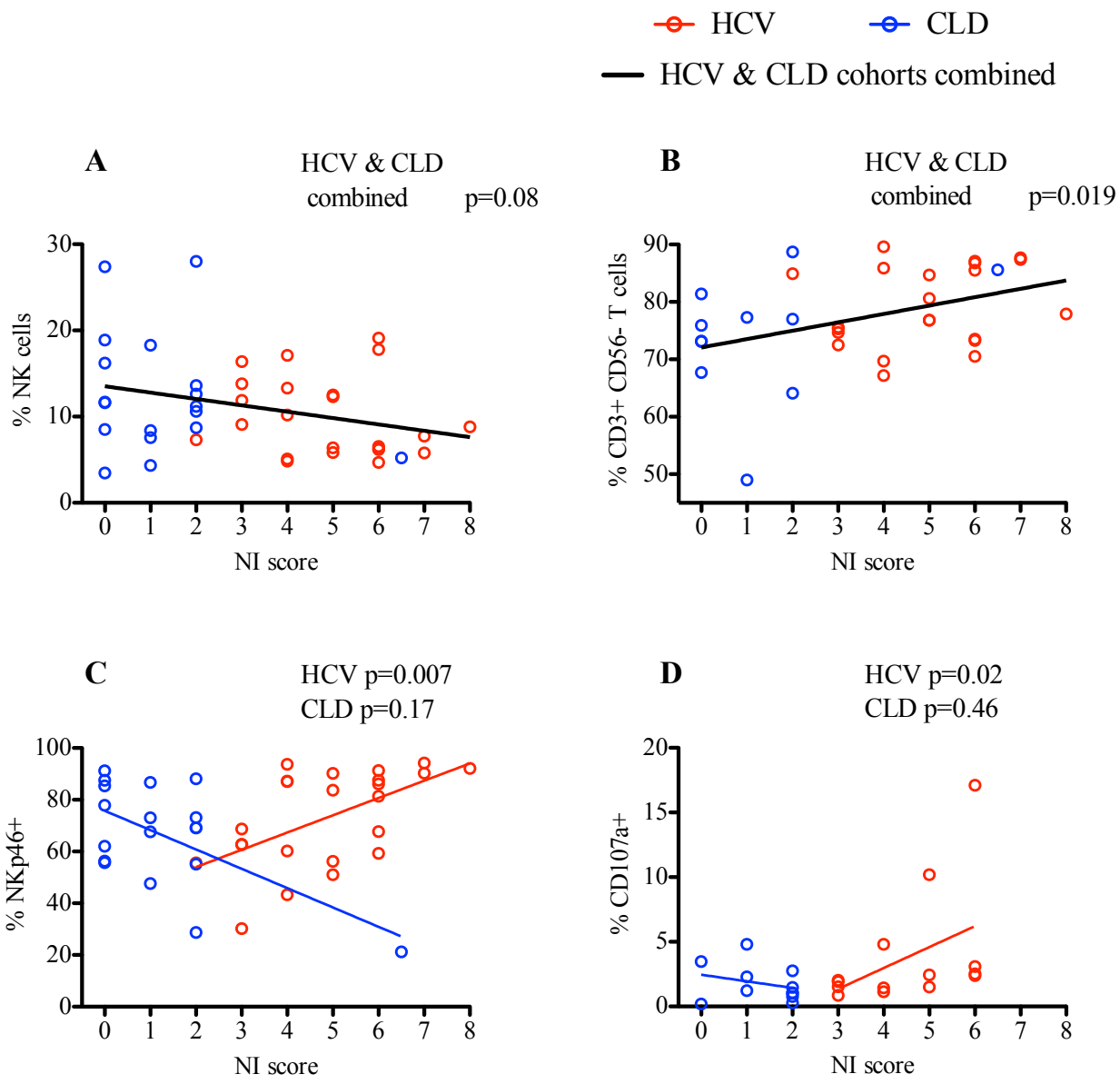
F) Age and Fibrosis score HCV and CLD cohorts combined; Spearman's rho 0.39 p=0.01.

G) Fibrosis score and NI score, HCV; 0.4 p=0.06 CLD; 0.66 p=0.007

4.8 Intrahepatic NK cells and liver inflammation

To assess the role of NK cells in driving HCV liver inflammation the NK cell proportion, phenotype and function were correlated with NI score. The proportion of intrahepatic NK cells of the HCV and CLD cohorts combined had an inverse trend with NI score, but as separate cohorts there were no associations (Spearman's rho = -0.27 p=0.08 Figure 4.10A). In the combined HCV and CLD cohorts there was a positive correlation between $CD3^+$ $CD56^-$ T lymphocytes and NI score (Spearman's rho = 0.4 p=0.019 Figure 4.10B). In the HCV cohort NI score correlated with the proportions of $NKp46^+$ and $CD107a^+$ NK cells (Spearman's rho 0.55, p=0.007 & Spearman's rho 0.6 p=0.02 respectively Figure 4.10C-D). These associations between NK cell activating receptor expression, degranulation and NI score indicate a potential mechanism of liver inflammation in chronic HCV infection. There were no associations between liver inflammation and NK phenotype and function in the CLD cohort.

Figure 4.10 Intrahepatic NK cell proportion, NKp46 & CD107a & NI score



Necroinflammatory score and intrahepatic NK cell phenotype and function in chronic HCV (O) & CLD (O). A) NI score and NK cells as a proportion of intrahepatic lymphocytes (HCV & CLD cohorts combined: Spearman's rho = -0.27 p=0.08).

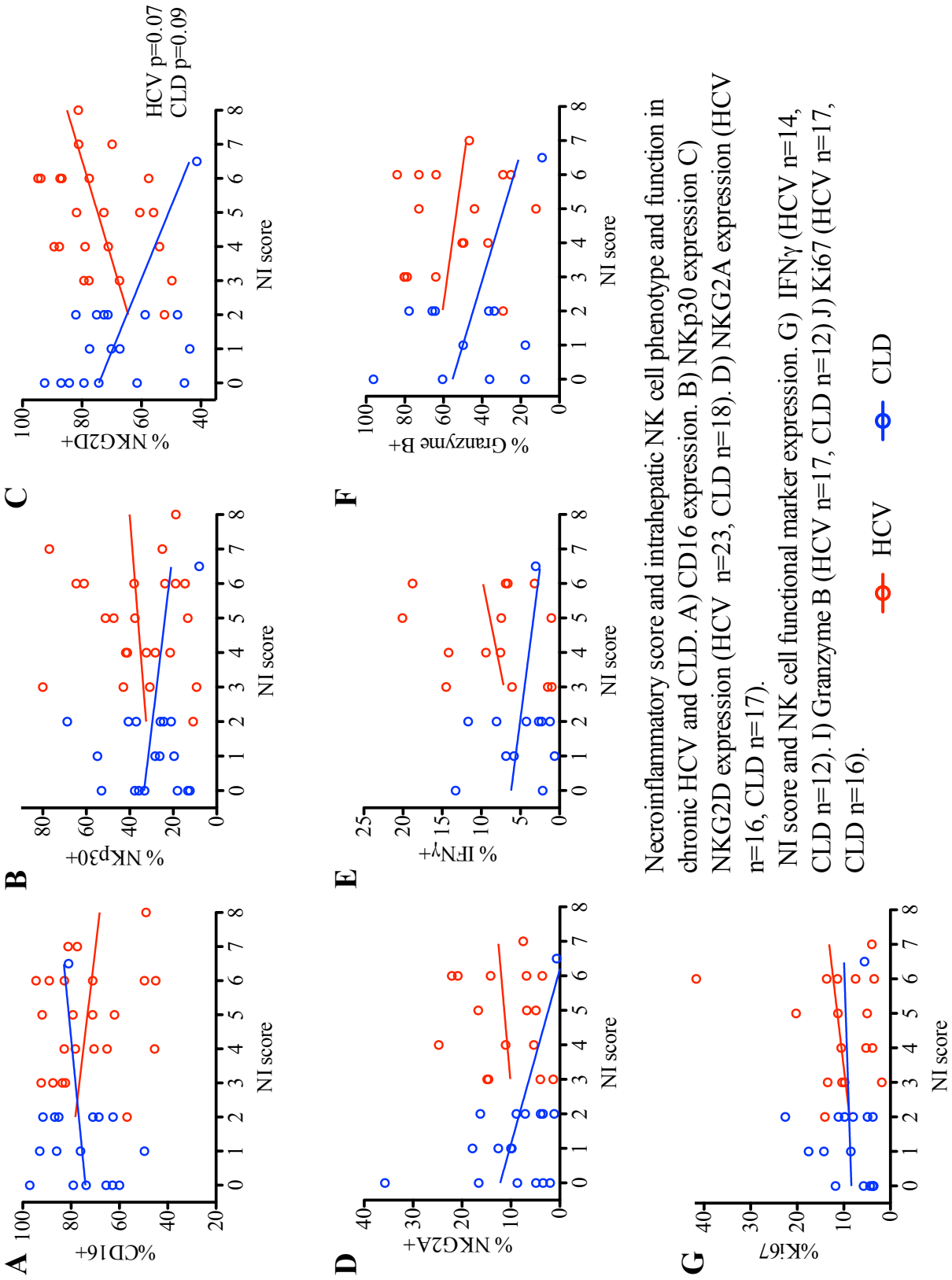
B) NI score and % NKp46^{+} NK cells. (HCV n=23, Spearman's rho 0.55, p=0.007 and CLD n= 18, -0.3, p=0.17 respectively.

C) NI score and % CD107a^{+} NK cells, HCV n=14, Spearman's rho 0.6 p=0.02 and CLD n=11, -0.25 p=0.46

There were no correlation between the proportions of $CD16^+$, $NKp30^+$, $NKG2D^+$, $NKG2A^+$, $IFN\gamma^+$, granzyme B^+ or $Ki67^+$ NK cells and NI score in either cohort (Figure 4.11A- G).

These data indicate a potential association between $NKp46^+$ NK cells and NK cell cytotoxic function and liver inflammation in chronic HCV, which is discussed in detail below.

Figure 4.11 Intrahepatic NK cell phenotype and function & NI score.

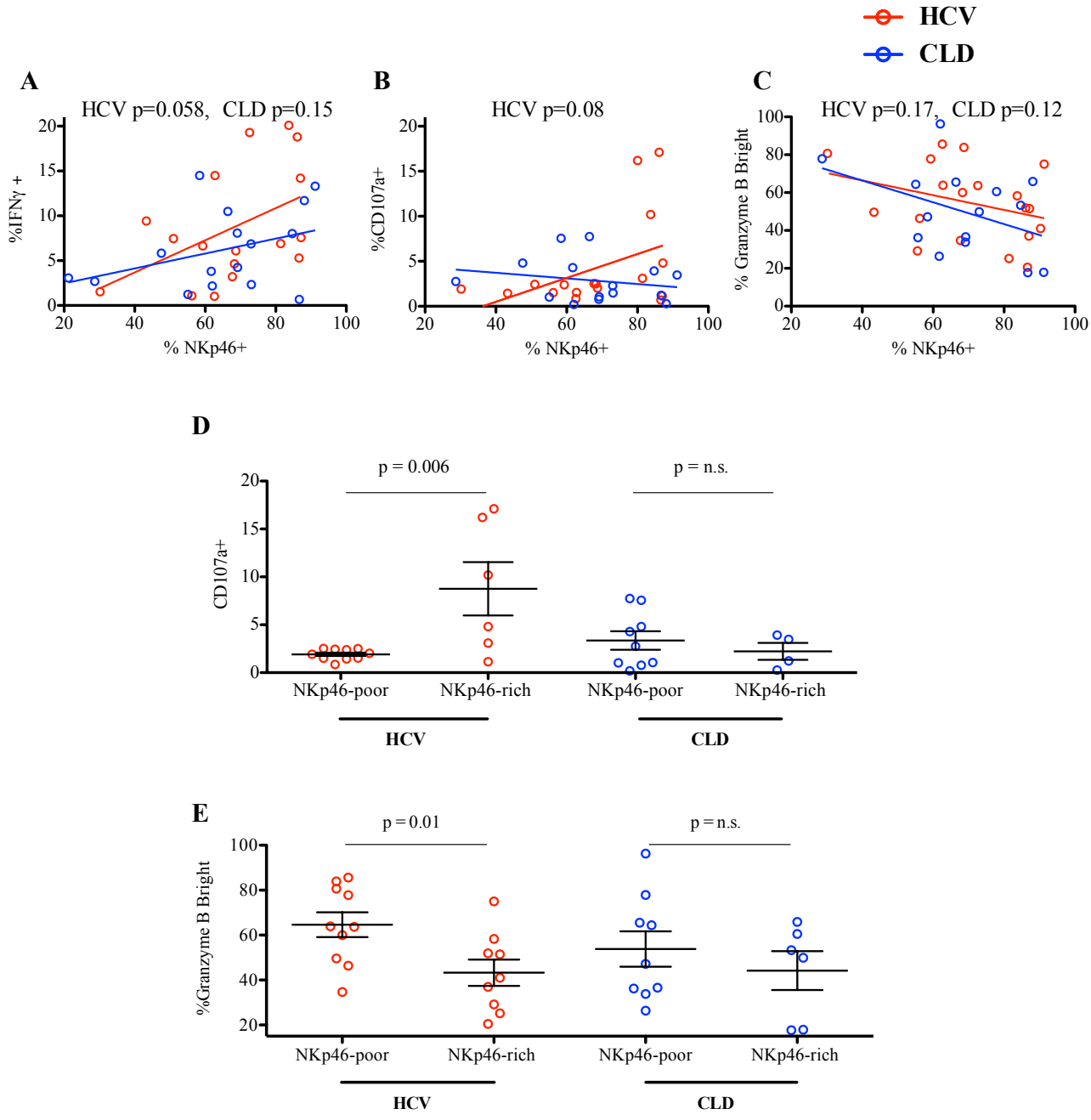


Necroinflammatory score and intrahepatic NK cell phenotype and function in chronic HCV and CLD. A) CD16 expression. B) NKp30 expression C) NKG2D expression (HCV n=23, CLD n=18). D) NKG2A expression (HCV n=16, CLD n=17). NI score and NK cell functional marker expression. G) IFNγ (HCV n=14, CLD n=12). I) Granzyme B (HCV n=17, CLD n=12) J) Ki67 (HCV n=17, CLD n=16).

4.9 Intrahepatic NKp46 expression and NK cell function

Ad hoc analysis of the proportion of intrahepatic NKp46$^+$ NK cells and NK cell function within the liver revealed an association between the proportions of IFNγ$^+$ and NKp46$^+$ NK cells in the HCV but not the CLD cohort (HCV r^2=0.22 p=0.058, Figure 4.12A). There was an association between NKp46$^+$ and CD107a$^+$ NK cells (HCV r^2=0.14, p=0.08) but not Granzyme B (Figure 4.12B-C). Individuals were separated into those with NKp46-rich (>80%) and NKp46-poor (<70%) intrahepatic NK populations as described above (Figure 4.6). There was increased in CD107a$^+$ NK cells in the NKp46-rich individuals in the HCV cohort (mean= 1.9% v 8.8%, p=0.006), but not in the CLD cohort (Figure 4.12D). In keeping with these data the proportion of Granzyme B^{Bright} NK cells was reduced in the NKp46-rich HCV$^+$ individuals but not the CLD cohort (HCV: mean= 64.6% v 43.3% p=0.01 Figure 4.12E). Together these data indicate that HCV$^+$ individuals with NKp46-rich intrahepatic NK cells have greater cytotoxic activity than those with NKp46-poor populations. Therefore, in the HCV cohort only, an increased proportion of NKp46$^+$ NK cells is associated with increased liver inflammation.

Figure 4.12 Intrahepatic NKp46 expression & NK cell function

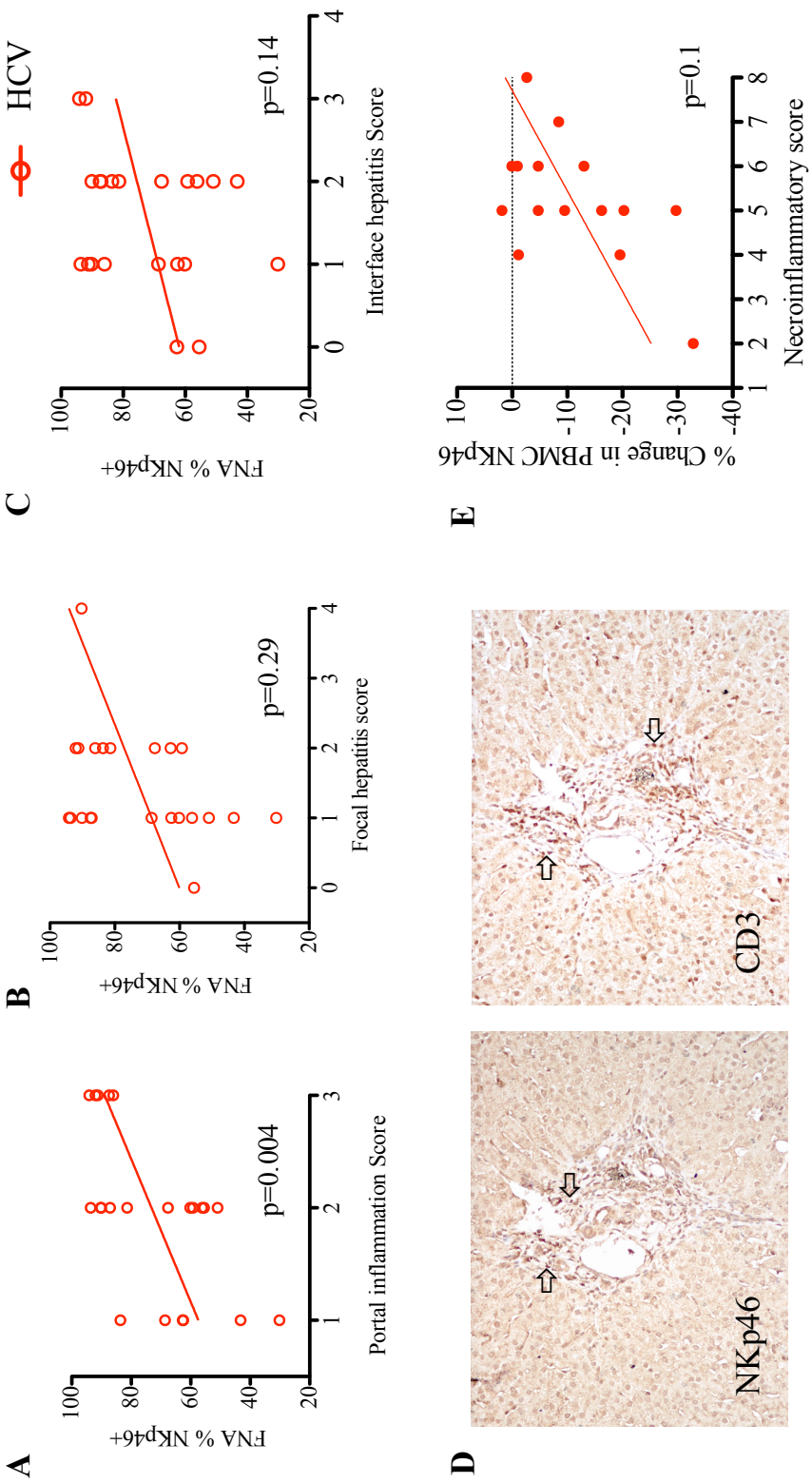


NKp46 is associated with increased NK cell IFNγ production and cytotoxicity. A) IFNγ$^+$ NK cells and % NKp46$^+$ intrahepatic NK cells in HCV and CLD cohorts (n=19 & 15 respectively). B) CD107a$^+$ and NKp46$^+$ NK cells (HCV n=17, r^2=0.14, p=0.13 & CLD n=15, r^2=0.05 p=0.44). C) Granzyme B Bright and NKp46$^+$ NK cells (HCV n=17 r^2=0.21 p=0.058 & CLD n=16 r^2=0.14, p=0.15). NKp46-rich and NKp46-poor intrahepatic NK cells have altered cytotoxic function in the HCV but not the CLD cohorts. D) CD107a (HCV n=17, CLD n=15) E) Granzyme B (HCV n=17 & CLD n=16). Mean and SEM shown.

Analysis of the proportion of $NKp46^+$ NK cells and the composite components of the NI score revealed an association between $\%NKp46^+$ NK cells and portal inflammation (Spearman's rho=0.59 p=0.004. Figure 4.13A). There was no association between NKp46 and interface hepatitis or focal hepatitis (Spearman's rho=0.23 p=0.29 and Spearman's rho=0.32 p=0.14 respectively. Figure 4.13B-C). No patient had a confluent necrosis greater than 0, this is in keeping with a clinically stable outpatient cohort (Appendix I). Immunohistological staining of HCV^+ biopsy samples demonstrated a relative enrichment of $NKp46^+$ cells in the periportal regions compared to within the liver parenchyma (Figure 4.13D).

Overall, these data strongly support the notion that *in situ* increased activation of NKp46-rich populations in the context of chronic HCV infection are a driving force in liver inflammation. NKp46 is down-regulated upon NK cell activation in healthy individuals (Figure 3.6C). However, in chronic HCV infection individuals with the highest levels of liver inflammation (NI score) fail to down-regulate NKp46 in *ex vivo* degranulation assays at maximal stimulation (Figure 4.13E).

Figure 4.13 NKp46 and liver inflammation



Proportion of intrahepatic NKp46$^+$ NK cells and components of the NI score. Intrahepatic NKp46$^+$ NK cells by flow cytometry and independent histological score of: A) portal inflammation, B) Focal hepatitis and C) interface hepatitis in chronic HCV (n=22). D) Left panel DAB immunohistology demonstrating representative NKp46$^+$ cells (Demonstrated by) enrichment around the portal tract. Right panel: DAB immunohistology demonstrating CD3$^+$ lymphocytes. E) Reduction in peripheral blood NKp46$^+$ NK cells after high level stimulation and degranulation to K562 target cells and necroinflammatory score in a HCV cohort (n=15).

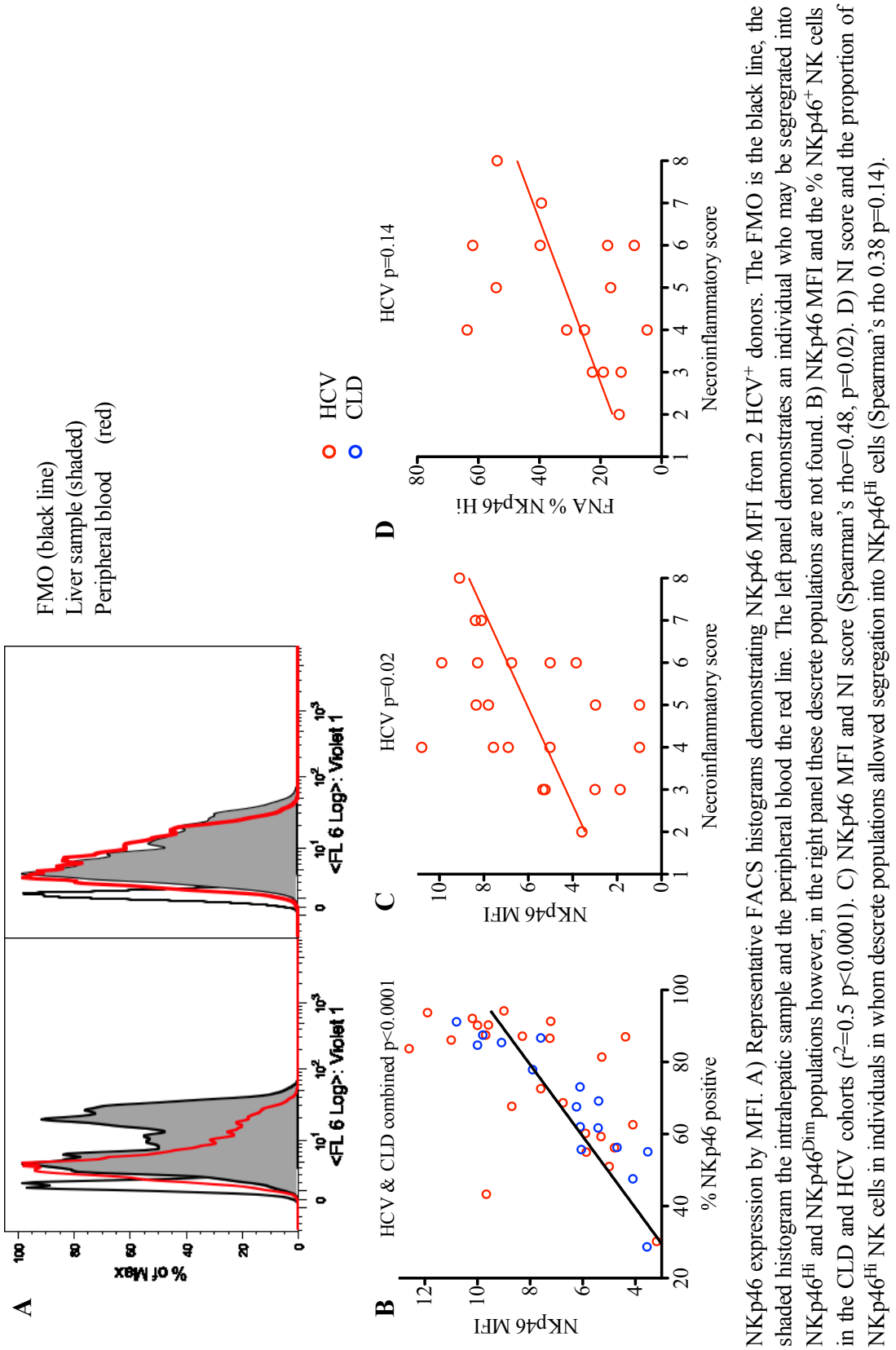
4.10 Measurement of NKp46: Proportion v MFI

It was recently reported that there is an association between NKp46 and *in vitro* HCV viral control.[175,200]. In these papers NKp46 expression was assessed by MFI; the fluorescent intensity of the labelled cells and therefore the density of NKp46 on the cell surface. NK cells were segregrated into $NKp46^{Hi}$ and $NKp46^{Dim}$ populations.

In light of these recent reports I attempted to further analyse my data using the MFI. However, I was unable to divide individual the NK cell populations into discrete $NKp46^{Hi}$ and $NKp46^{Dim}$ sub-groups; instead in the majority of cases a single NKp46 population was observed (Figure 4.14A). The inability to recapitulate these published findings may reflect use of different fluorochromes, anti-NKp46 antibody clones and flow cytometers. However, in my cohort, NKp46 MFI, proportion of $NKp46^{+}$ NK cells and NI score correlated with each other (Figure 4.14B-C). The proportion of distinct $NKp46^{Hi}$ NK cells, measured by MFI as described by Kramer et al, was associated with increasing NI score, in those HCV^{+} individuals in whom such a population was distinguishable (Figure 4.14D).

These data suggest that similar results are found with either methodology supporting the theme that NKp46 has a particular role in the activation of NK cells specifically in chronic HCV infection, as discussed below.

Figure 4.14 NKp46 measured by MFI and proportion

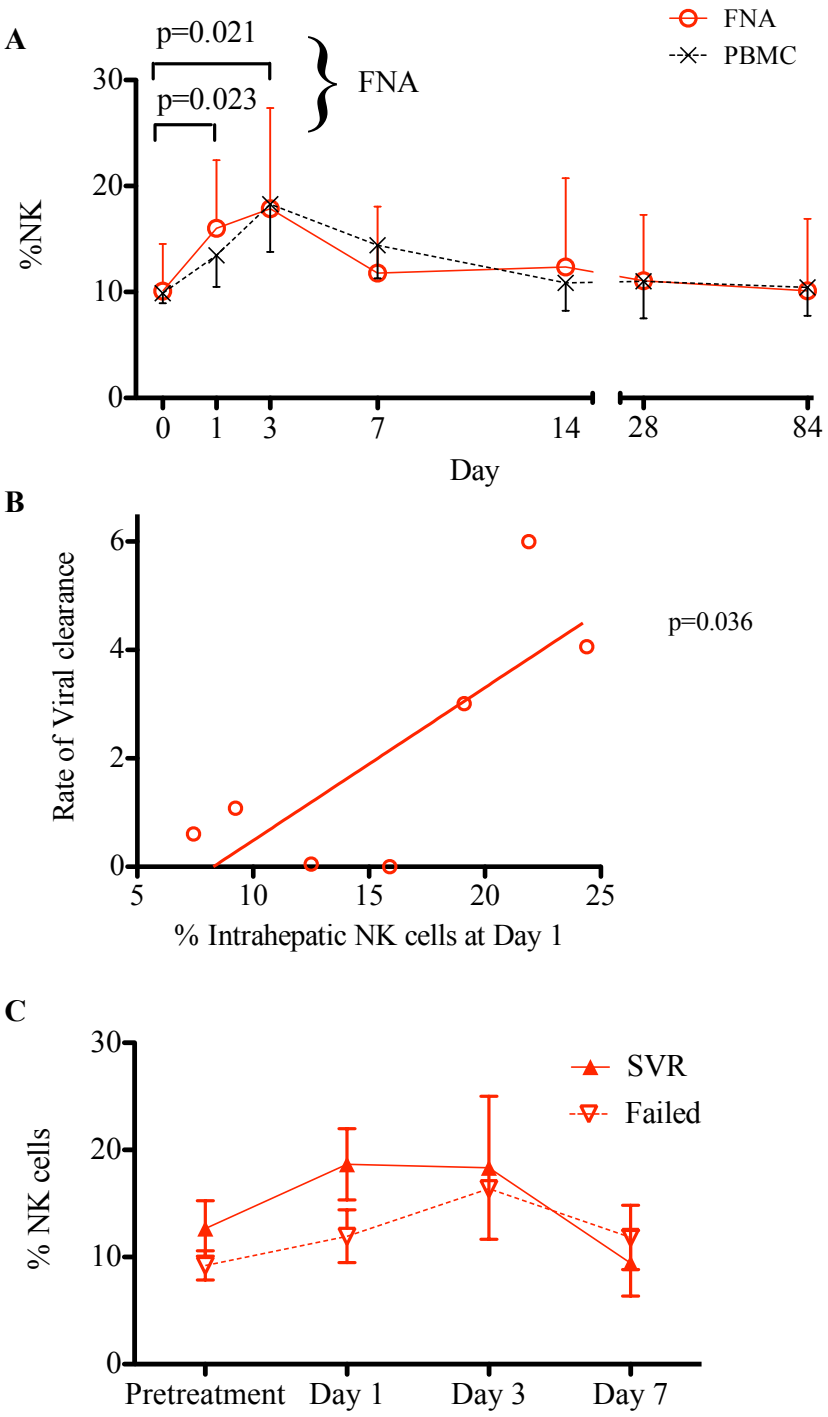


NKp46 expression by MFI. A) Representative FACS histograms demonstrating NKp46 MFI from 2 HCV^{+} donors. The FMO is the black line, the shaded histogram the intrahepatic sample and the peripheral blood the red line. The left panel demonstrates an individual who may be segregated into NKp46^{Hi} and NKp46^{Dim} populations however, in the right panel these descrete populations are not found. B) NKp46 MFI and the % NKp46^{+} NK cells in the CLD and HCV cohorts (r^2=0.5 p<0.0001). C) NKp46 MFI and NI score (Spearman's rho=0.48, p=0.02). D) NI score and the proportion of NKp46^{Hi} NK cells in individuals in whom descrete populations allowed segregation into NKp46^{Hi} cells (Spearman's rho 0.38 p=0.14).

4.11 Intrahepatic NK cells during IFNα treatment

I obtained serial samples from 9 HCV^+ patients with repeated FNA at multiple time points during the first 12 weeks of treatment. Of this cohort, seven patients had undetectable viral load by PCR at 12 weeks (and 6 of these eventually achieved an SVR) and two had no response to treatment. IFNα induced a significant increase in intrahepatic NK cells at days 1 and 3 but was not altered from baseline at later time points (p=0.008 repeated measures ANOVA). The intrahepatic NK cells approximately doubled over three days from the baseline mean of 10% (day 1 mean=16.0%, p=0.02; day 3 mean=17.8%, p=0.02; paired t-test Figure 4.15A). The proportion of intrahepatic NK cells at day 1 of treatment correlated with the rate of HCV viral clearance (r^2=0.61 p=0.036. Figure 4.15B). In keeping with this the 3 patients who failed treatment had a lower proportion of intrahepatic NK cells compared to those who achieved SVR at Day 1 (11.9% v 18.6% p=0.19). At Day 3 and Day 7 there was no difference in the proportion of intrahepatic NK cells in the treatment failure and SVR groups (Day 1 mean = 18.3 v 16.4% Day 3 mean =11.8 v 9.4% respectively p=0.6. Figure 4.15C). These data from this small cohort suggests that IFNα treatment increases the intrahepatic NK cell proportion and an earlier (Day 1) increase in NK cells is associated with rapid control of viral load and successful viral clearance.

Figure 4.15 Intrahepatic NK cells during IFNα based treatment of HCV



Variations in NK cells as % of lymphocytes during the first 12 weeks of IFNα treatment in the peripheral blood and intrahepatic compartments (n=9). A) Proportion of intrahepatic NK cells increases at Day 1 & Day 3 (Mean and SEM shown). B) The rate of viral clearance denoted by rate constant of viral decline (*k*) and NK cells as the proportion of intrahepatic lymphocytes. C) The proportion of intrahepatic NK cells in patients who achieved SVR (n=6) and those who failed treatment (n=3) over the first week of IFNα based treatment of HCV infection.

To further investigate the role of the intrahepatic NK cells during IFNα based treatment I conducted a detailed intrahepatic analysis of NK cell phenotype and functional markers within this cohort of patients.

In keeping with the changes in the proportion of NK cells at Days 1 and 3 there were permutations in proportions of NK phenotype and functional markers at these early time points within this cohort. At Day 1 there was a reduction in the proportions of $CD16^{+}$ (but not $CD56^{Dim}$ proportion) and granzyme B and an increase in the proportion of $CD107a^{+}$ NK cells (Figure 4.16A-D). By Day 3 these alternations in NK cell phenotype and function had returned to their baseline levels. The peripheral blood CD107a and granzyme B expression remained constant throughout treatment. The proportions of intrahepatic and peripheral blood $IFN\gamma^{+}$ NK cells increased over the 1st week of treatment (Figure 4.16E). Although the altered proportions of NK cells were not statistically significant individually taken together these data support the notion that intrahepatic NK cells have increased cytotoxic activity at Day 1, which is reduced at Day 3.

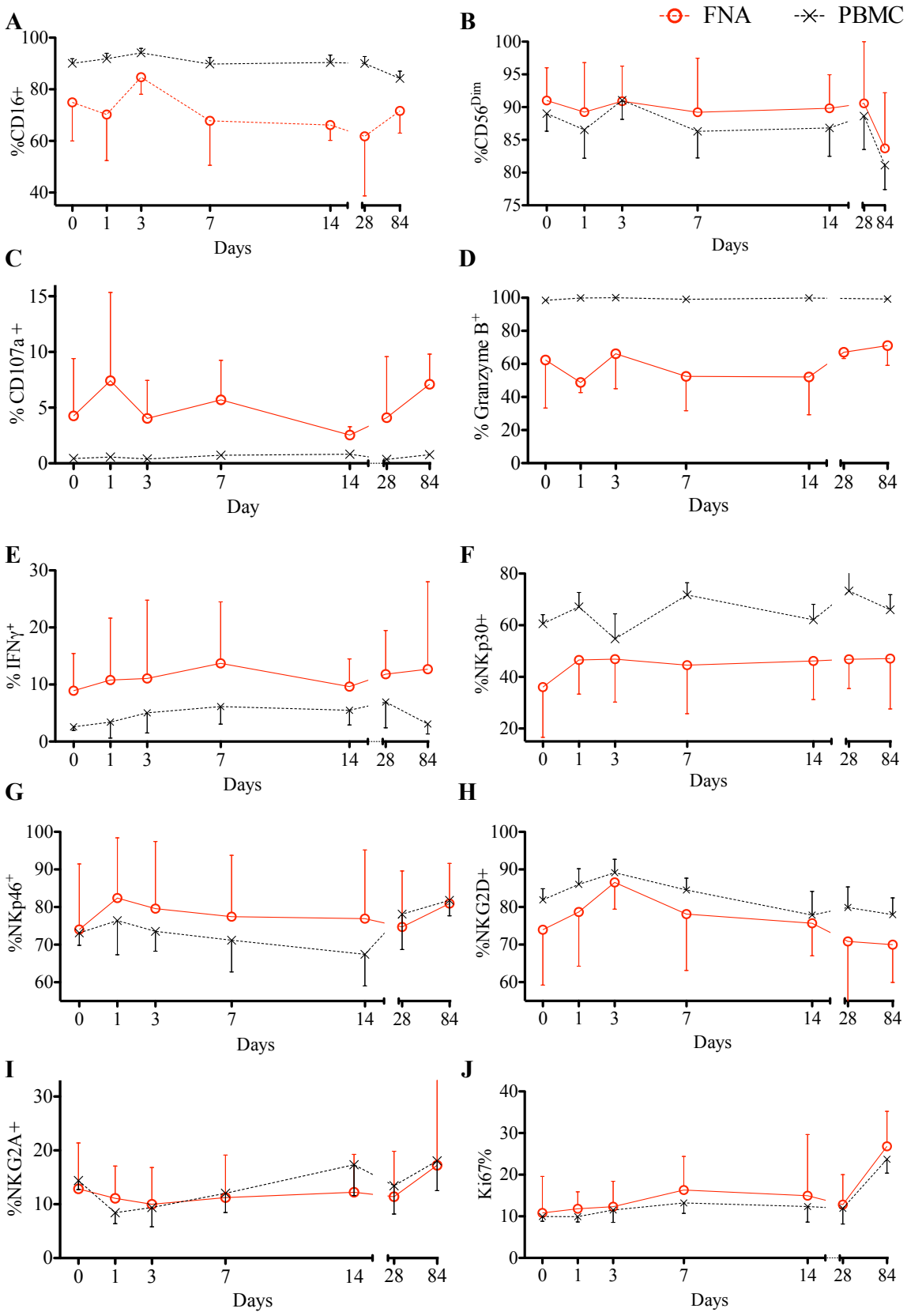
These data suggest that the proportion of NK cell cytotoxic function may be relatively more important than IFNγ production in the rapid control of HCV viral load during the first 24 hours of treatment when the greatest reductions in HCV viral load are seen (Figure 3.10A). However, IFNγ remains likely to be a major compontent of the anti-viral response and these data do not necessarily reflect the amount of IFNγ secreted by the total NK cell population.

There was an early increase in the proportions of intrahepatic $NKp30^{+}$, $NKp46^{+}$ and $NKG2D^{+}$ NK cells (Figure 4.16F-H). The proportion of peripheral blood $NKp46^{+}$ NK

cells was similar to the intrahepatic compartment pretreatment but did not increase to the same level as the intrahepatic NK cell population until Day 28. These data suggest that IFNα treatment induces increased proportions of NK cells activating receptors and that $NKp30^{+}$ and $NKp46^{+}$ NK cells may be more readily recruited to the liver during early IFNα treatment.

The proportions of $NKG2A^{+}$ and $Ki67^{+}$ NK cells were unaltered during the first month of IFNα treatment, however, these populations increased at Day 84 in both the peripheral blood and the intrahepatic compartment (Figure 4.16I-J). The increase in the proportions of $NKG2A^{+}$, $Ki67^{+}$ and the immature $CD56^{Bright}$ NK cells proportion at Day 84 (Figure 4.16B),[92] suggests an increase in the proportion of replicating and naïve NK cells as result of the long-term effects supraphysiological levels of IFNα from on lymphocyte production stimulation. This is in keeping with previous reports of increased Ki67 expression during IFNα based treatment of HBV.[211]

Figure 4.16 NK cell phenotype and function during IFNα treatment of HCV



Variations in NK cell phenotype and functional markers in the peripheral blood (X) and intrahepatic compartments (O) during the first 12 weeks of IFNα based treatment in a cohort of chronic HCV^+ patients (n=9).

A) The proportion of $CD16^+$ NK cells.

D) % $CD56^{Dim}$ NK cells.

C) % CD107a externalisation.

D) % Granzyme B^+.

E) % $IFN\gamma^+$.

F) % $NKp30^+$.

G) % $NKp46^+$

H) % $NKG2D^+$.

I) % $NKG2A^+$.

J) % $Ki67^+$

The changes in NK cell phenotype and functional markers overall were of a small magnitude, and there were no discernible differences in the changes measured prospectively in NK cell phenotype between the rapid responders and those who failed treatment in this cohort (n=3). A major hurdle to analysis of NK cell phenotype and functional data during this longitudinal study is the inability to distinguish between recent liver immigrants and the established intrahepatic NK cell population. Recent immigrants may not have had the opportunity to identify and respond to HCV infected cells and therefore potentially mask significant alterations in the resident NK cell population.

These data support the notion that NK cells have increased cytotoxic activity at Day 1. In conjunction with the increase in NK cells at Day 1 and the correlation between rate of viral control and proportion of intrahepatic NK cells at Day 1 these data imply that intrahepatic NK cells have an effective cytotoxic role in the control of viraemia during early IFNα treatment.

4.12 Discussion

In HCV infection chronic liver inflammation results in fibrosis, cirrhosis and increases the risk of hepatocellular carcinoma. There is some evidence that NK cells are an important component of the innate immune response to HCV.[163,166,167,175,186] However, until recently there has a paucity of data concerning intrahepatic NK cells in chronic HCV. In this study I investigated NK cell phenotype and function in both blood and liver of HCV-infected individuals.

NK cells were initially reported to account for 30-50% of lymphocytes within the intrahepatic compartment.[173] However, by flow cytometry, we found that this proportion was lower and strongly correlated with the proportion of NK cells in the peripheral blood (mean 10.5% and 9.6% respectively Figure 4.4). These data were similar to the proportions of $CD3^+$ and $NKp46^+$ lymphocytes upon immunohistological staining (Figure 4.12). The lower proportion of intrahepatic NK cells is in keeping with the findings of multiple studies, which report a mean intrahepatic NK cell proportion ranging from 2.7 – 18%.[157,159,160,163,174,175] There was an inverse trend between necroinflammatory score and NK cell proportion when the non-viral chronic liver disease and HCV^+ cohorts were combined and a positive correlation with $CD3^+$ $CD56^-$ T lymphocytes (Figure 4.10). These data are in keeping with a recent report by Varchetta and colleagues, which compared 70 HCV donors and 26 control donors with normal ALT undergoing elective cholecystectomy. In the HCV^+ donors the mean proportion of NK cells was lower than in the controls (14 vs. 24%) and there was a significant reduction in NK cells in individuals with increased histological activity index score.[174] Together these data support the notion that liver inflammation particularly in chronic HCV infection shifts the proportion of

intrahepatic lymphocytes from NK-enriched towards a similar proportion that is found in the peripheral blood.

Intrahepatic NK cells had a markedly altered phenotypic and functional profile suggestive of increased NK cell activity as well as confirming that the FNA technique sampled a separate compartment to the peripheral blood. HCV^+ individuals with a high proportion of $NKp46^+$ intrahepatic NK cells, termed NKp46-rich, have a more activated phenotype. In line with this, we found $NKp46^+$ proportion correlated with higher necroinflammatory scores. These data are further supported by the *in vitro* experiments described in Chapter 3, which demonstrated that the proportion of $NKp46^+$ NK cells is uniquely associated with NK cell function in chronic HCV.

It is possible that liver inflammation preferentially recruits $NKp46^+$ NK cells into the liver. If this were the case one would expect an enrichment of $NKp46^+$ NK cells in the intrahepatic compartment relative to the peripheral blood.

However, the following factors suggest this is not the case:

i. In both cohorts the proportion of $NKp46^+$ NK cells is similar across both compartments. There is a strong correlation between the proportion of $NKp46^+$ NK cells in the peripheral blood and intrahepatic compartments (Figure 4.6A).

ii. If $NKp46^+$ NK cells were preferentially recruited to the inflamed liver one may expect to see an relative increase in the proportion of $NKp46^+$ NK cells in the liver compared to the peripheral blood in individuals with high NI scores but this is not the case.

iii. In addition there appears to be a unique association between the activating receptor NKp46 and chronic HCV infection; high NI score is not associated with the proportion of $NKp46^{+}$ NK cells in CLD patients.

Therefore, these data suggest a causative relationship in HCV not a general association between inflammation and NKp46. This association compliments the results of in vitro experiments reported in Chapter 3.

Golden-Mason and colleagues purified $NKp46^{Hi}$ NK cells and demonstrated that these cells have greater *in vitro* anti-HCV activity; they also demonstrated that the unknown NKp46 ligand is present on the surface of hepatoma cells infected with JFH-1 strain of HCV.[200] Krämer and colleagues reported an association between NKp46 expression and NK cell function and in particular the ability to suppress *in vitro* viral replication. It was further reported that there was a non significant association between histological score and NKp46 expression. In that study there was a smaller sample size (n=17), a variation in methodologies as described above (Section 4.10) and use of Hepatitis Activity Index (HAI) scoring system (Appendix I).[175] The HAI scores are derived from assessment of piecemeal and lobular necrosis and do not score portal inflammation.[212] As the portal inflammation component of the Ishak's NI score correlated with the proportion of $NKp46^{+}$ NK cells in the cohort studied herein (Figure 4.13A), it is perhaps unsurprising that studies that use HAI scoring did not find an association between NKp46 and liver inflammation. Furthermore, Krämer and colleagues reported that the proportion of $NKp46^{Hi}$ intrahepatic NK cells was inversely correlated with viral load. However, in our cohort we did not find this pattern. Whilst it appears that NK cell phenotype is strongly

associated with liver inflammation the role of NK cells in suppressing viral load in the chronic disease state remains to be fully elucidated.

In healthy donors NK cell activating ligands are down regulated upon NK activation, however, in chronic HCV infection there appears to be a loss of this protective mechanism in some individuals (Figures 3.5 & 4.13). I hypothesize that NK cell populations that are persistently NKp46-rich may allow continuous engagement with putative activating ligands in vivo contributing to liver pathology in chronic HCV. These data suggest that the mechanism of HCV related immunopathology results from a failure of intrinsic homeostatic NK cell attenuation within the chronically infected liver.

In this cohort I found that the majority of $CD107a^+$ intrahepatic NK cells in the chronic state were $IFN\gamma^+$ (Figure 4.7) However, this is at odds with previous reports that in chronic HCV, NK cells are polarised towards cytotoxicity in a pSTAT1 dependent manner with reduced pSTAT4 related IFNγ production.[180,186] The differences in these reports and our data are likely to reflect variations of NK responses *in vitro* and *ex vivo.*

In the small cohort available I have demonstrated that there is a significant increase in intrahepatic NK cells during the first 72 hours of treatment. Importantly there appears to be an association between the proportion of intrahepatic NK cells and rate of viral clearance. These NK cells appear to have a more activated phenotype at 24 hours and this is in keeping with a previous report of increases in NK cell cytotoxicity at 24 hours and successful treatment outcome.[185] The first 24 hours is the time period of the greatest reduction in viral load in rapid responder patients who are well known to have an increased treatment success rate.[32] Whilst the data from this small cohort of 9 patients is

intriguing further work with a larger cohort of patients is required to determine whether there is a statistically significant increase in NK cell function and phenotype at the first 24 hours. Based on the current data to achieve a sufficiently powered cohort (power 0.8) to demonstrate a significant ($p<0.05$) increase in $CD107a^+$ NK cells at Day 1 a total cohort of 19 patients would have to be recruited ($\mu0=4.2$, $\mu1=7.5$ & $\sigma=5.1$, Statisical Solutions Power & Sample size calculator www.statisticalsolutions.net/pss_calc.php). Based on previous experience of recruitment this is achievable but would probably to take 20-24 months.

Importantly when the association between $NKp46^+$ NK cells and liver inflammation is considered in the context of the ability of these cells to respond to IFNα based treatment (Chapter 3.11) an important paradox emerges. Individuals who have the greatest need of treatment are those with high levels of liver inflammation as they are at the greatest risk of progression to liver failure, cirrhosis and HCC. However, these patients are likely to have NKp46-rich NK populations and are at the greatest risk of treatment failure. When this association is considered it the context of 30 years of IFNα based anti-HCV treatment it is likely that the identified HCV^+ population is under new selection pressures where treatment is available. In the United States there has been a striking increase in standardised mortality in HCV^+ individuals compared to HBV and HIV between 1998 and 2007.[213] The rise in mortality may in part be related to the removal, by IFNα therapy, of the low risk NKp46-poor patients from the HCV population shifting the phenotype of the population towards one at risk of increased mortality. These data demonstrate that there is potential for of measurement of $NKp46^+$ NK cell frequency as a biomarker to help predict treatment outcome. In particular patients who have high proportion of $NKp46^+$ NK cells may be best served by early treatment.

The association between NKp46 and liver inflammation may serve two purposes in the development of HCV treatment strategies as a therapeutic target. Reduction in NKp46 associated NK cell activity prior to IFNα treatment may 'rest' NK cells allowing a marked abrupt increase in the subsequent NK cell response. Secondly decreased NKp46 cytotoxicity may reduce inflammation as a long-term treatment goal in those unable to undergo IFNα treatment. To achieve a reduction in NKp46 activity either the HCV ligand would need to be blocked or the surface expression of NKp46 could be altered. Histone deacetylase inhibitors (HDACi) have been considered as anti-cancer agents as they directly arrest tumour growth and can increase tumour cell immunogenicity. HDACi include short chain fatty acids (phenylbutyrate and valproic acid) and derivatives of hydroxamic acid.[214] HDACi have been considered promising anti-leukaemia and lymphoma agents however, it has been reported that HDACi can impinge upon NK cell phenotype and function.[214,215] In particular expression of both NKp46 and NKG2D are down regulated and there is an associated reduction in NKp46- and NKG2D-cytotoxicity and IFNγ *in vitro*. Therefore, HDACi have potential to form an additional therapeutic option in the treatment of chronic HCV.

In conclusion I have demonstrated a separate intrahepatic NK cell population with an altered phenotype and increased activation. Importantly NKp46 again appears to have a unique and important role in the pathogenesis of HCV related liver disease. Increased $NKp46^{+}$ proportion increases NK cell activity resulting in damaging liver inflammation. During treatment IFNα stimulation has the potential to induce early NK cell activation, which may contribute to the rapid control of HCV viral load. Thus NKp46 presents itself

as a biomarker and a potential target for therapeutic intervention to reduce inflammation and increase the rates of therapeutic viral clearance.

Chapter 5:

Results –

Identification of NK Cell

Evasion Strategies

Employed by HCV

5.1 HCV and NK cell function

To establish a chronic infection it seems HCV must evade the anti-viral response by NK cells. As described in Chapter 3 and in keeping with previously published reports,[155,159,161] we have found that NK cell function is reduced in individuals with chronic HCV compared to controls. There has been considerable interest in HCV immunoevasion and previous studies have focused upon the impact of HCV on NK cells and NK cell lines as described in section 1.18. Possible mechanisms include impingement by core protein upon IFNα-ISG pathway;[178] up-regulation of NK inhibitory ligands;[169] and down-regulation of activating ligands upon infected cells.[181] However, the role of individual HCV proteins in NK cell impingement remains to be fully elicited. To address this, this part of the study focussed on evaluating whether non-structural HCV proteins derived from different viral genotypes alter NK cell function when expressed in a target cell.

Adenovirus vectors containing individual HCV proteins and structural polypeptides were generated. Individual proteins from two HCV constructs were expressed in this way: genotype 2a JFH-1 strain (GenBank accession no. AB047639) and the genotype 1b subgenomic replicon con 1 (Rep1b, accession no. AJ238799) containing proteins NS3-NS5B (Figure 2.4 page 76). These vectors were used to infect target cells (Human foreskin fibroblasts (HFF)) and thus to determine which, if any, HCV proteins affect *in vitro* NK cell cytotoxic responses.

5.2 HCV protein expression by adenovirus vectors.

HCV genes were cloned into adenovirus vectors as described in Materials and Methods. HCV protein expression from recombinant adenoviruses was confirmed by Western blot and immunofluorescence in HFF and HEK 293 cells.

Western blot

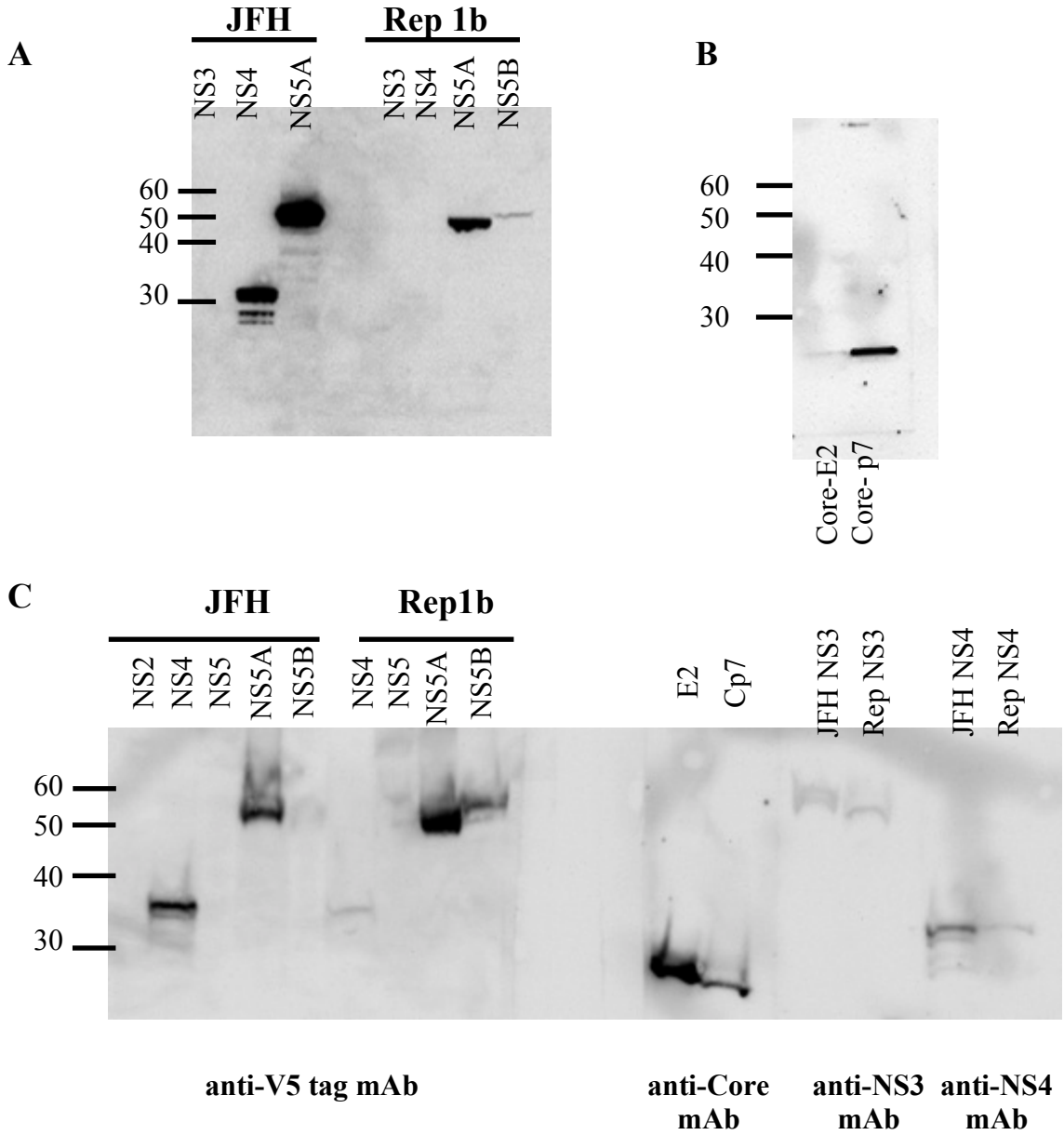
The adenovirus vector was designed with a V5 tag, which immediately followed the inserted gene, to allow identification of the expressed protein (Figure 2.6). The expression of JFH NS4, NS5A and Rep1b NS5A and NS5B was confirmed by Western blot with a monoclonal antibody to the V5 tag (Figure 5.1A). The remaining proteins were not identifiable in this way, perhaps due to low expression levels or post-translational autocleaving of the V5 tag. Repeated Western blot with anti-HCV core monoclonal antibody demonstrated bands consistent with a 22kd protein indicating that the core proteins, but not the remainder of the polyproteins, were expressed albeit with lower expression for Core-E2 than Core-p7 (Figure 5.1B). Core, E1, E2 and p7 are separated by signal peptides, the N and C termini of E1 and E2 are cleaved by signal peptidases and core protein is cleaved from E1 by signal peptide peptidase.[216-218] Thus these data suggest post translation processing of the core-p7 polypeptide. Unfortunately, glycoproteins E1 and E2 could not be similarly identified due to the lack of commercial reagents. Anti- NS3 and NS4 antibodies failed to demonstrate the presence of these proteins when HFF were infected.

The integration of adenovirus E1 gene in 293 HEK cells[195] enables expression of HCV proteins and competent recombinant adenovirus resulting in ongoing infection of cells. 293 HEK cells were infected with the recombinant adenoviruses for the purpose of

maximising HCV gene expression. JFH Core protein, NS3, 4, J5A and Rep1b NS3, 4, 5A and 5B were detectable by Western blot (Figure 5.1C) and expression of NS3 and NS4 proteins was confirmed by an anti-NS3 and anti-NS4 monoclonal antibody respectively. NS2 protein could not identified by Western blot using the V5 tag and no specific antibodies were available.

Thus, western blotting demonstrated expression of recombinant proteins JFH core, NS3, 4 and 5A and Rep1b NS3, 4, 5A and 5B. No expression of JFH NS2 was observed however in the case of JFH E1, E2 and p7, it was not possible to confirm whether this was simply due to cleavage of the V5 tag (Table 5.1).

Figure 5.1 HCV protein expression in HFF (A&B) and HEK 293 (C) cells by adenovirus vectors - Western blots

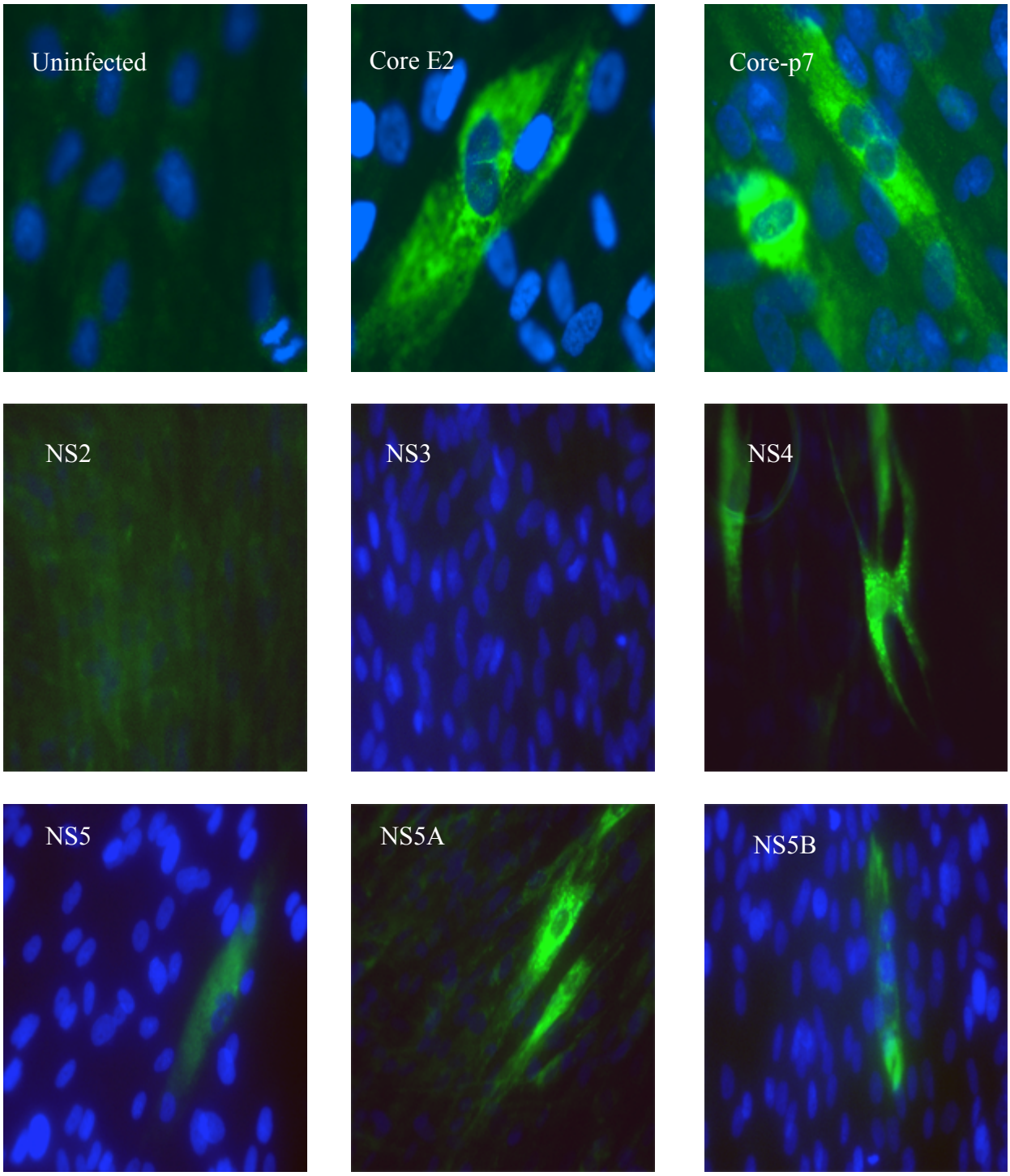


Western blot analysis HCV proteins expressed in HFF target cells with an MOI of 5. Molecular weight (in kDa) marker label on left of blots A) JFH NS4 & 5A and Rep 1b NS5A and NS5B were identified by monoclonal antibody to V5 tag. B) Core-E2 and Core-p7 analysis by Western blot using monoclonal antibodies to JFH Core protein revealed bands of approximately 22kD; the molecular weight of cleaved core protein. C) Western blot analysis with HEK 293 target cells to maximise protein expression. JFH NS4, NS5A & NS5B and Rep 1b NS4, NS5A and NS5B were detected with anti-V5 antibody. Core protein was detected with anti Core antibody. JFH and Rep1b NS3 and NS4 were detected with anti-NS3 and anti-NS4 antibodies respectively.

Immunofluorescence

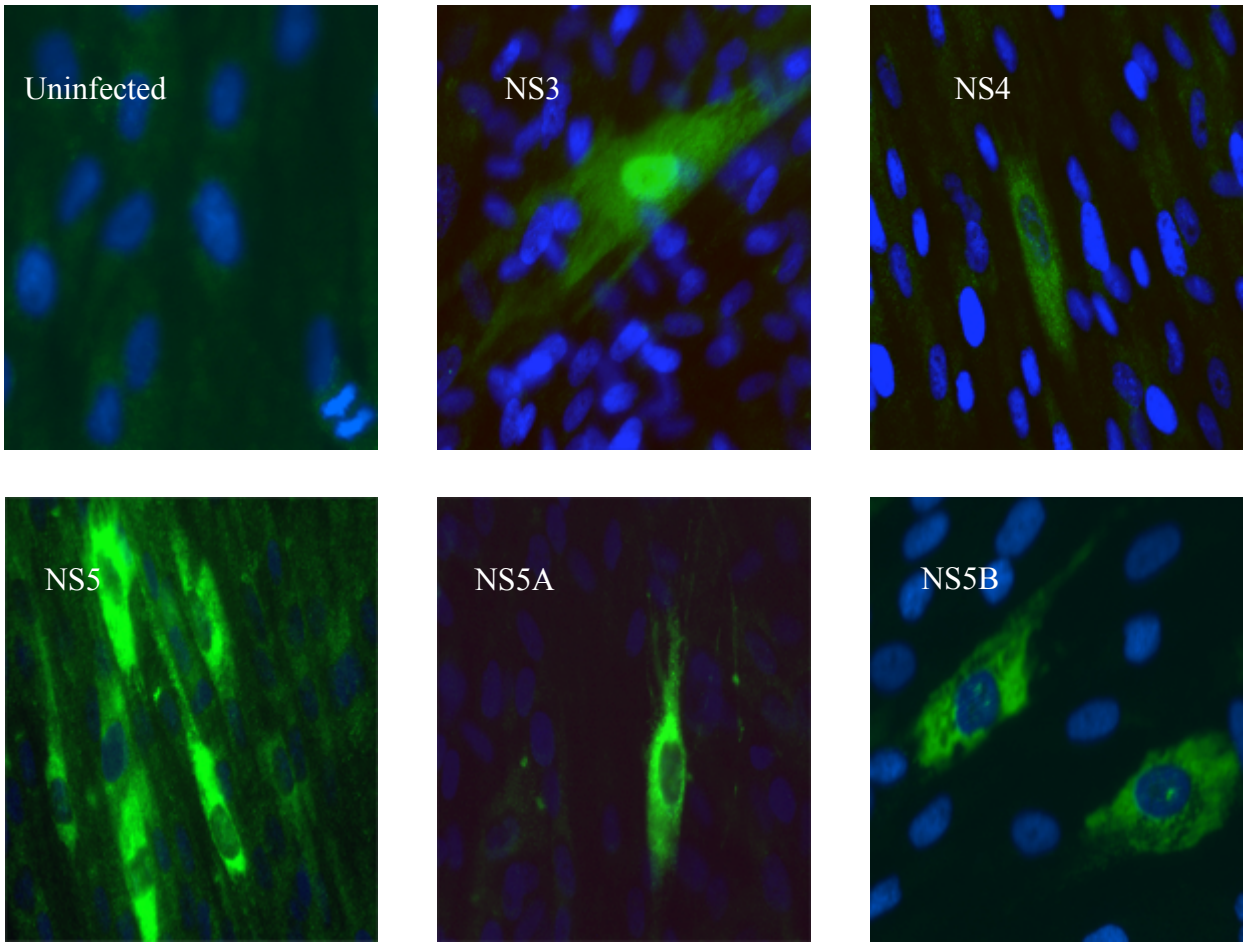
Protein expression was also examined by immunofluorescence. JFH NS4, NS5, NS5A & NS5B and Rep1b NS4, NS5, NS5A & NS5B expression was confirmed using the anti-V5 monoclonal antibody (JFH: Figure 5.2. Rep1b: Figure 5.3). Rep NS3 expression, but not JFH1 NS3, was detected using an anti-NS3 antibody (Figures 5.2 & 5.3). Cells infected with JFH core-E2 and core-p7 vectors were confirmed to be expressing core protein with an anti-core monoclonal antibody (Figure 5.2). NS2 protein expression could not be detected. Table 5.1 summarises HCV protein expression using the adenovirus vectors. There was concordance between Western blot and immunofluorescence detection of protein expression apart for the expression of JFH NS3 & 5 and Rep1b NS3 & 5. This may reflect levels of protein expression and/or the susceptibility of V5 tag cleavage during sample preparation for Western blot analysis.

Figure 5.2 JFH protein expression by immunofluorescence in HFF CAR$^+$ cells



HCV proteins NS4, 5, 5A and 5B were tagged with V5 protein and stained with mouse anti-V5 antibodies. Mouse anti-core and anti NS3 antibodies were used to stain Core-E2, Core-p7 and NS3 proteins. All cells were then stained with anti-mouse AF488 secondary antibody (green) and cell nuclei were stained with DAPI (blue).

Figure 5.3 Rep1b protein expression by immunofluorescence in HFF CAR$^+$ cells



Rep1b HCV proteins NS4, 5, 5A and 5B were tagged with V5 protein and stained with mouse anti-V5 antibodies. Mouse anti-NS3 antibodies were used to stain NS3 proteins. All cells were then stained with anti-mouse AF488 secondary antibody (green) and cell nuclei were stained with DAPI (blue).

Table 5.1 **Summary of HCV proteins expressed in HFF and HEK 293 cells using adenovirus vectors**

Protein		Western blot	Immunofluorescence
JFH-1 (Genotype 2)	Core E2*	✓	✓
	Core p7*	✓	✓
	NS2	✗	✗
	NS3	✓	✗
	NS4	✓	✓
	NS5	✗	✓
	NS5A	✓	✓
	NS5B	✓	✓
Rep1b (Genotype 1)	NS3	✓	✓
	NS4	✓	✓
	NS5	✗	✓
	NS5A	✓	✓
	NS5B	✓	✓

*Detected by anti-Core antibody protein size on Western blot suggests E1, E2 and p7 have been cleaved or possibly even not transcribed.

5.3 The impact of HCV protein expression on NK cell function.

In order to examine the impact of HCV protein expression upon NK function HFF were cultured for 24 hours and then infected with recombinant adenoviruses with a multiplicity of infection (MOI) of 5*. The HFF were then cultured for a further 48 hours to allow protein expression. 16 hours prior to the end of the culture period fresh PBMC were isolated from healthy donors and incubated overnight with 1000 IU/ml of IFNα. The PBMC were then cultured in the presence of the infected target cells and anti-CD107a antibody for 4 hours to assess the impact of HCV protein on NK cell function. PBMC were then stained with surface antibodies and fix-permeabilised and stained for IFNγ and TNFα and assessed by flow cytometry (gating strategy as demonstrated in Figure 2.2). The NK cell cytotoxic function and cytokine expression was compared between target cells expressing HCV protein and both negative and positive control target cells. Negative controls comprised HFF cells infected with an adenovirus vector containing an empty cassette. Positive control target cells comprised those infected with a vector containing CMV UL141 protein, known to markedly affect NK cell function through the down regulation of NK activating ligands CD112 (for DNAM-1 NK receptor) and CD155 (for DNAM-1 and CD96 NK receptors).[219,220] As reported in Chapter 3 the range of $CD107a^+$ NK cells following exposure to K562 target cells and 1000 IU/ml IFNα was 30-60% (Figure 3.4). Therefore, to determine whether HCV proteins impinge upon NK cell function the individual donor's response was expressed as a percentage of NK function when exposed to just control target cells. The cytotoxic and cytokine responses of 5 healthy donors were assessed in duplicate for each infected cell line.

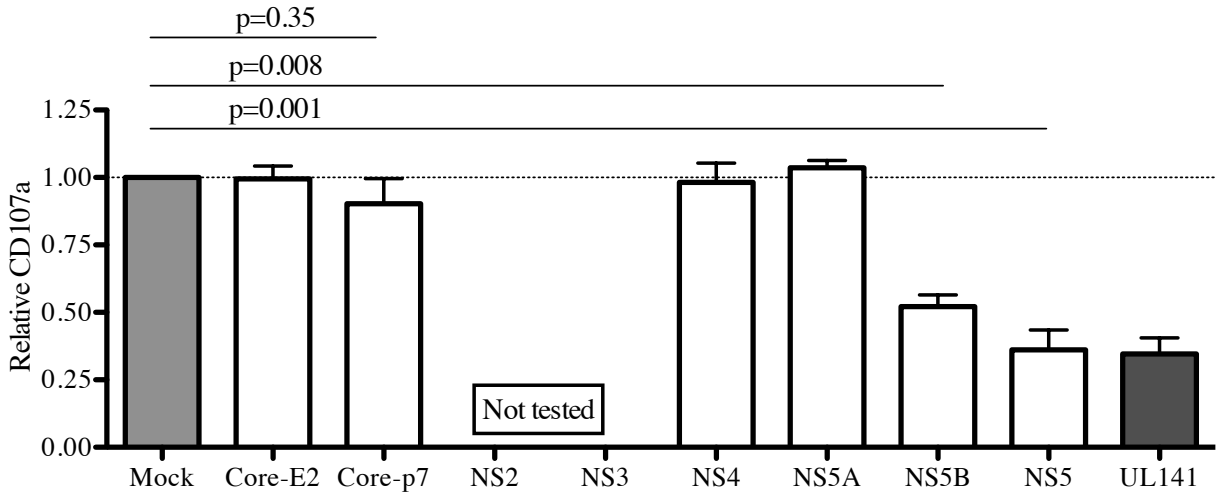
* MOI of 5 = 5 virions to 1 HFF cell

NK cell cytotoxic responses

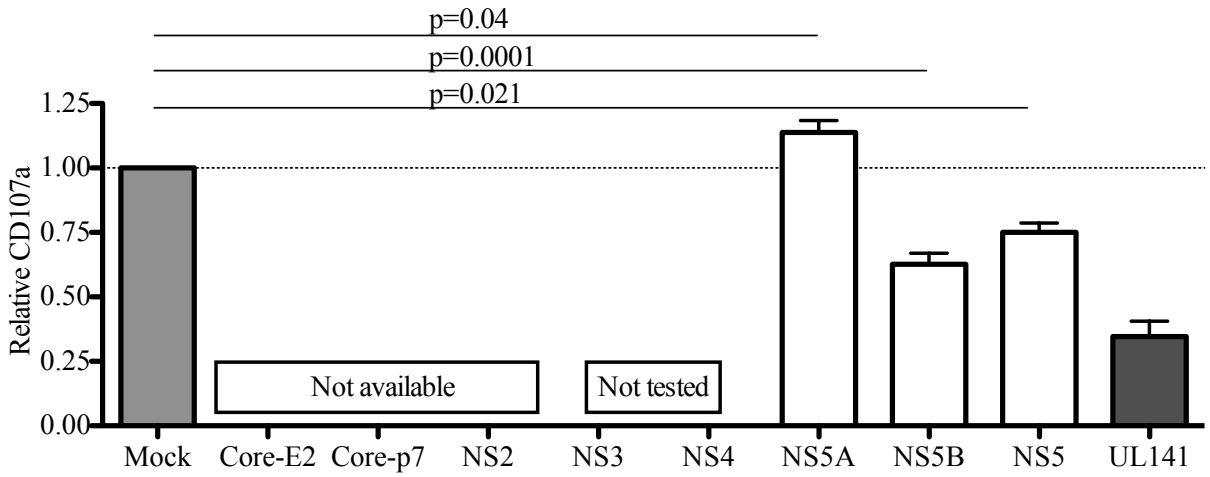
Analysis of the data revealed that JFH NS5 and NS5B proteins reduced NK cell function relative to negative control cells (36% p=0.001 and 52% p=0.0079 respectively). This reduction in NK cell cytotoxic function was similar to the effect of CMV UL141 (-34% degranulation). Expression of JFH Core-E2, Core-p7, NS4 and NS5A had no effect on NK cell function (Figure 5.4A). Rep 1b NS5 and NS5B proteins induced similar reductions in NK cell cytotoxic function (75% p=0.021 and 67% p=0.0001 respectively). Conversely Rep1b NS5A protein expression induced increased NK cell cytotoxicity (114% p=0.04) compared to infection control cells (Figure 5.4B). These data demonstrate NS5B is able to reduce NK cell cytotoxic function. The same effector function or an alternative mechanism may be induced by NS5 protein, when NS5B is expressed in conjunction with NS5A. NS5A, which is known to aid viral immunoevasion through IFNα signalling inhibition,[221] is unable to impinge directly upon NK cell function.

Figure 5.4 The impact of HCV protein expression on NK cell cytotoxic function

A) JFH-1 proteins



B) Rep1b proteins



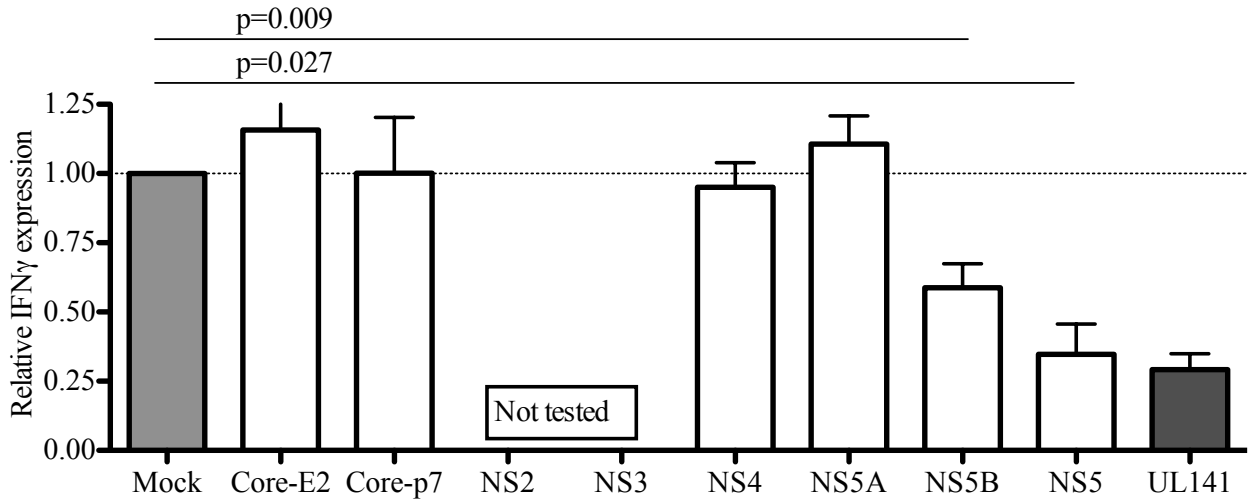
HCV proteins and polyproteins were expressed in HFF targets together with infection control (negative) and CMV UL141 (positive) control. % $CD107a^+$ NK cells induced by HFF infected with varying HCV proteins expressed relative to infection controls for 5 donors in duplicate in at least 2 separate experiments for each protein. Mean and SEM shown, paired t test, significant p values shown.

NK cell cytokine responses

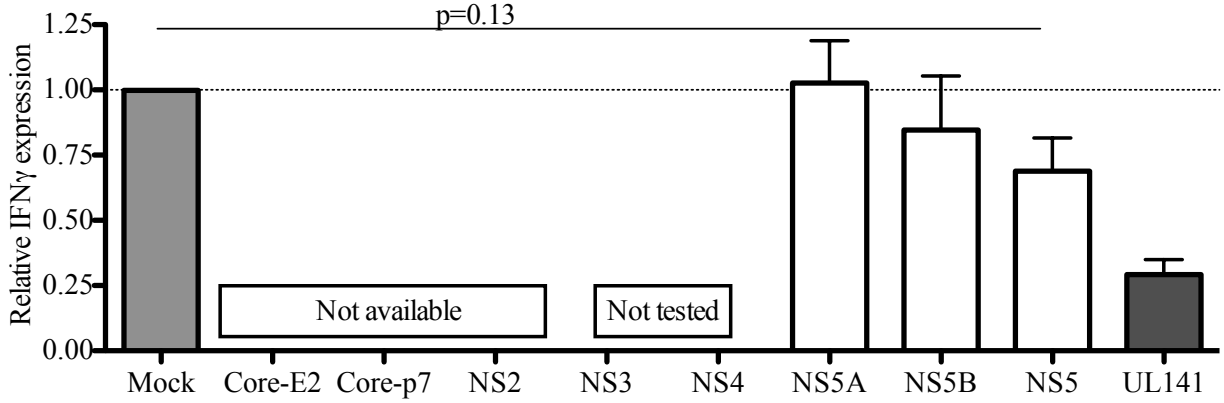
The impact on cytokine production (IFNγ and TNFα) by NK cells was also assessed. Expression of JFH NS5 and NS5B resulted in a significant decrease in the proportion of IFNγ- (34% p=0.027 and 58% p=0.009 respectively) and to a lesser extent, TNFα- (37%, p=0.0005 and 51% p=0.01 respectively) producing NK cells (Figure 5.5A-C). Following target HFF infection with Rep1b NS5 and NS5B there were smaller reductions in $IFN\gamma^+$ NK cells (86% p=0.5, 66% p=0.13 respectively, Figure 5.5B). Rep1b vectors expressing NS5 and NS5B induced modest reductions in the proportion of $TNF\alpha^+$ NK cells (71%, p=0.1 and 85% p=0.39 respectively, Figure 5.5D). There was a modest reduction in $TNF\alpha^+$ NK cells following target HFF infection with JFH NS4 expressing vectors (85% p=0.02). No effects were observed following expression of any of the other HCV proteins tested.

Figure 5.5 HCV protein impingement upon NK cell cytokine production

A) JFH-1 & IFNγ$^+$ NK cells

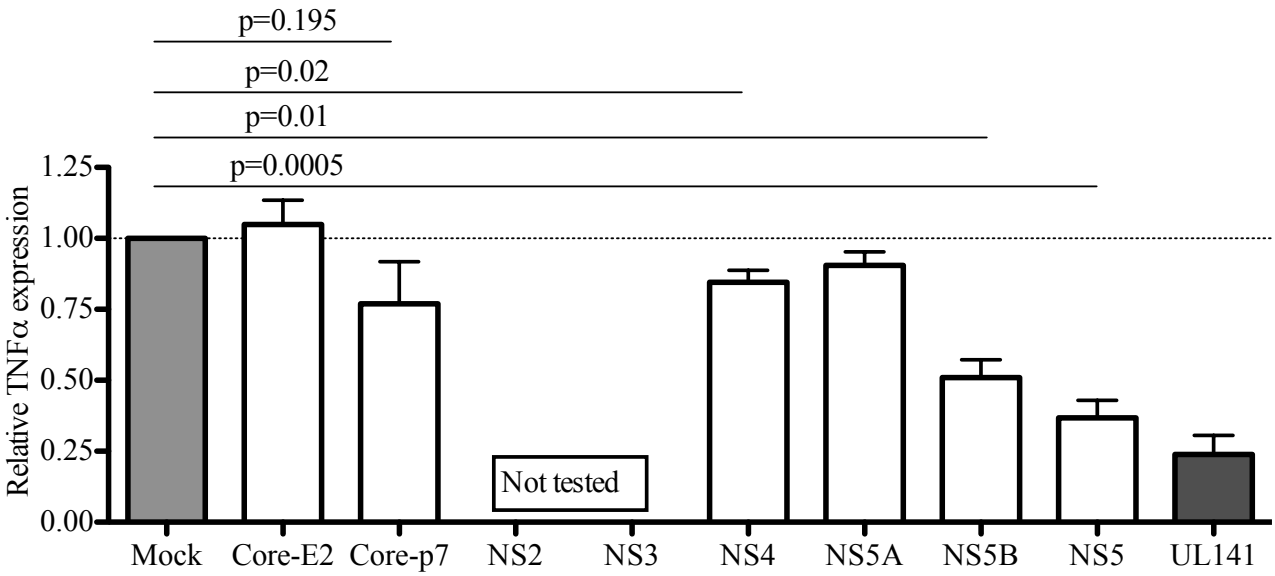


B) Rep1b & IFNγ$^+$ NK cells

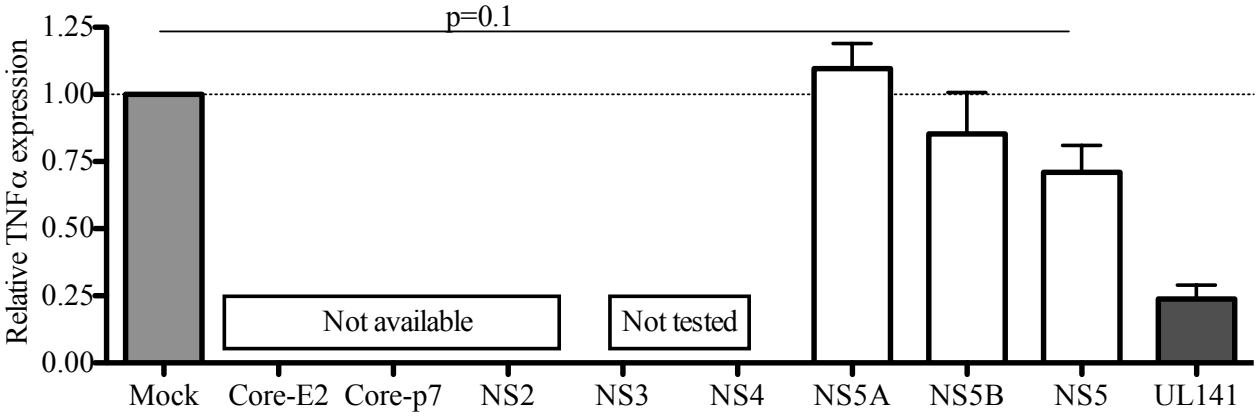


HCV proteins and polyproteins were expressed in HFF targets together with infection control (negative) and CMV UL141 (positive) control. % IFNγ$^+$ NK cells induced by infected targets relative to infection controls for 5 donors in duplicate in at least 2 separate experiments for each protein. Mean and SEM shown, paired t test, significant p values shown.

C) JFH-1 & TNFα$^+$ NK cells



D) Rep1b & TNFα$^+$ NK cells

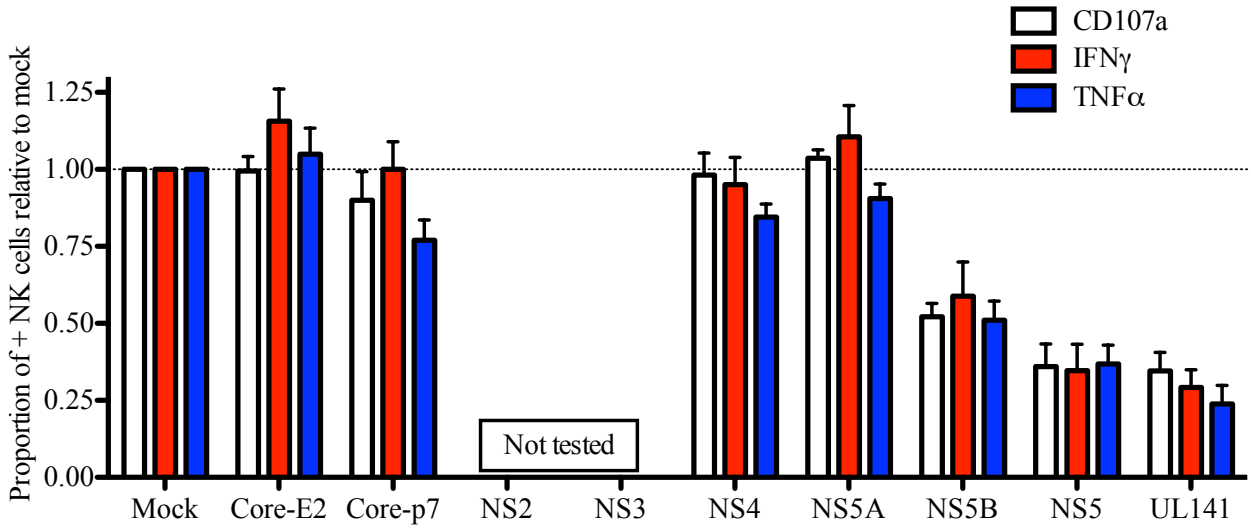


HCV proteins and polyproteins were expressed in HFF targets together with infection control (negative) and CMV UL141 (positive) control. % TNFα$^+$ NK cells induced by infected targets relative to infection controls for 5 donors in duplicate in at least 2 separate experiments for each protein. Mean and SEM shown, paired t test, significant p values shown.

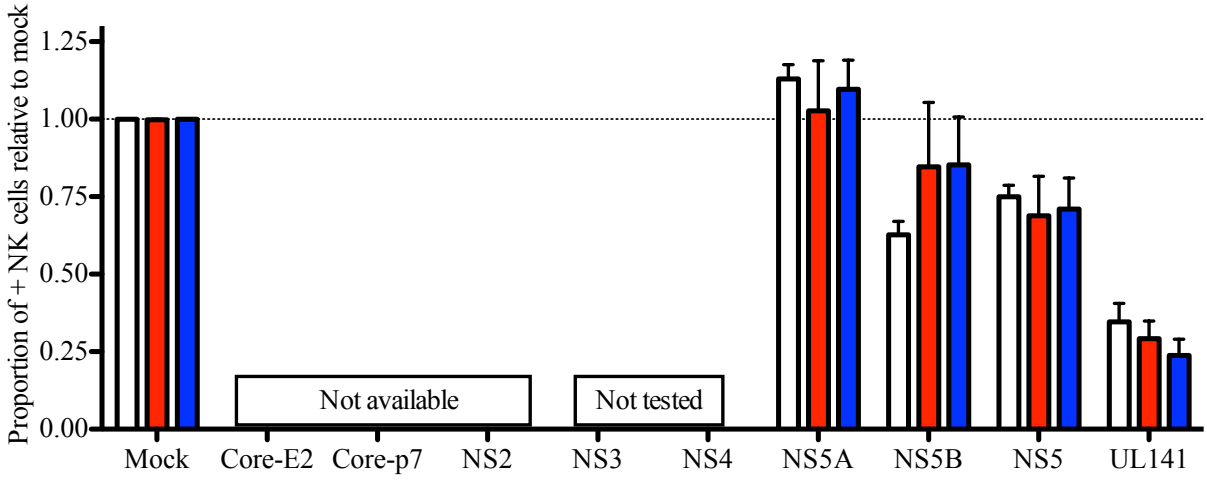
Overall, the data on cytotoxicity and cytokine production clearly point to NS5 and NS5B as proteins that have the greatest impact upon NK cell function for both the JFH and Rep1b strains of HCV (Figure 5.6A&B). JFH NS5 and NS5B impinge equally on both cytotoxicity and cytokine function. Similarly, Rep1b NS5 and NS5B both impinge on NK cytotoxicity but to a lesser extent on IFNγ and TNFα production with greater variability between individuals. These inter-genotype variations in the relative impingement upon NK cytotoxicity and cytokine production may contribute to the relative responsiveness of varying genotypes to IFNα based treatments.

Figure 5.6 Summary - NK cell response to HCV protein expression in target cells

A) JFH-1 protein expression & NK cell cytotoxicity and cytokine production



B) JFH-1 protein expression & NK cell cytotoxicity and cytokine production



HCV proteins and polyproteins were expressed in HFF targets together with infection control (negative) and CMV UL141 (positive) control. CD107a – white bars, IFNγ - red bars TNFα - blue bars. The proportion of positive NK cells relative to infection control cells for each protein for 5 donors in duplicate in at least 2 separate experiments for each protein.

5.4 Discussion

The data described in this Chapter demonstrate that HCV proteins have the ability to impinge upon NK cell function with NS5 and NS5B having the greatest effect. NS5B is one of the most variable regions of the HCV genome and genotype 2a and 1b strains of HCV have been reported to exhibit merely 64% homology.[222] Variability within NS5B has presumably conferred a survival advantage to the virus, and these results suggest one mechanism is viral immunoevasion of NK responses. A previous study has reported an inverse correlation between HCV viral load and NS5B mutation rate,[223] suggesting balanced selection between viability of viral protein and host immunoevasion.

The inhibition of both NK cell cytotoxic function and cytokine production may be an important step for HCV to establish a chronic infection. Inhibition of NK cell function may however, also benefit the host due to reduced liver inflammation and associated immunopathology as described in Chapter 4. Whilst potentially exciting, the data described in this Chapter, which revealed the inhibitory effects of NS5B, are preliminary. In particular, the impact of NS5B and NK cell function during chronic HCV infection and IFNα treatment remains to be elucidated. I have previously demonstrated that the proportion of $NKp46^{+}$ cells dictates NK function in chronic HCV. However, the experiments carried out to assess the effect of NS5B were performed with healthy donor samples where no such effect of NKp46 was observed (Figure 3.6C). Thus, in order to determine whether there is a link between the impact of NS5B on NK cell function and expression of NKp46, the experiments described in this Chapter should be repeated using blood obtained from individuals chronically infected with HCV.

The relationship NS5B impingement and NK cell function during IFNα treatment may be viewed as potentially beneficial or problematic to the host: NS5B mediated reduction in NK function in chronic disease that is subsequently overcome by IFNα could result in rapid viral decline during treatment. However, it is also possible that NS5B will remain a barrier to NK function during IFNα treatment. In this case, disabling the inhibitory effects of NS5B may represent a means through which the success of IFNα therapy can be improved.

Further experiments are clearly required to confirm and establish the mechanisms underpinning HCV protein impingement upon NK cell function: i) Assess the effect of IFNα titration upon NS5B impingement of NK function. ii) Assess the function of NK cells from HCV^+ donors with a range of NKp46 phenotypes to exposed to NS5B expressing target cells. iii) Assess impact of HCV proteins expressed in hepatoma cell lines on NK cell. iv) Determine whether viral proteins impinge directly or indirectly on NK cell function.

Whilst the experiments conducted thus far indicate a role for NS5 and NS5B, they do not rule out a role for other HCV proteins, which remain untested. These include NS2, NS3 and NS3/4 protease.

In conclusion, the preliminary data presented in this Chapter demonstrate that HCV proteins can down modulate NK cell cytotoxic and cytokine function in keeping with previous *ex vivo* NK functional data. These data provide insights into immunoevasive strategies utilised by HCV and how these same strategies may be beneficial to the

infected host. Furthermore, these data underline NS5 and NS5B proteins as important targets for anti-HCV therapies.

Chapter 6:

Discussion –

NK cell activation and evasion

during chronic HCV infection

In this thesis I have aimed to address the impact of chronic HCV infection upon NK cell phenotype and function. In addition I have sought to explore the roles that NK cells play in shaping the pathogenesis of HCV related illness and how this impacts on IFNα based treatment of HCV. Several key features of the role of NK cells in chronic HCV infection have emerged (summarised below and in Figure 6.1):

i) NK cell function is reduced in chronic HCV infection (Figure 3.4), these findings are in keeping with previously studies.[155,159,161,171] In an as yet unreported finding I have demonstrated that target cells that are infected with vectors expressing HCV NS5 and NS5B proteins interfere with the NK cytotoxic and cytokine response (Figure 5.6). The full implications and mechanism of this interaction are yet to be fully understood.

ii) Although NK cell function in chronic HCV is reduced I have demonstrated that it is dictated by NKp46 expression *in vitro,* an association not found in healthy donors or patients with non-viral chronic liver disease (Figure 3.7). This observation has been support by two further recent reports of a unique role for NKp46 in chronic HCV.[175,200]

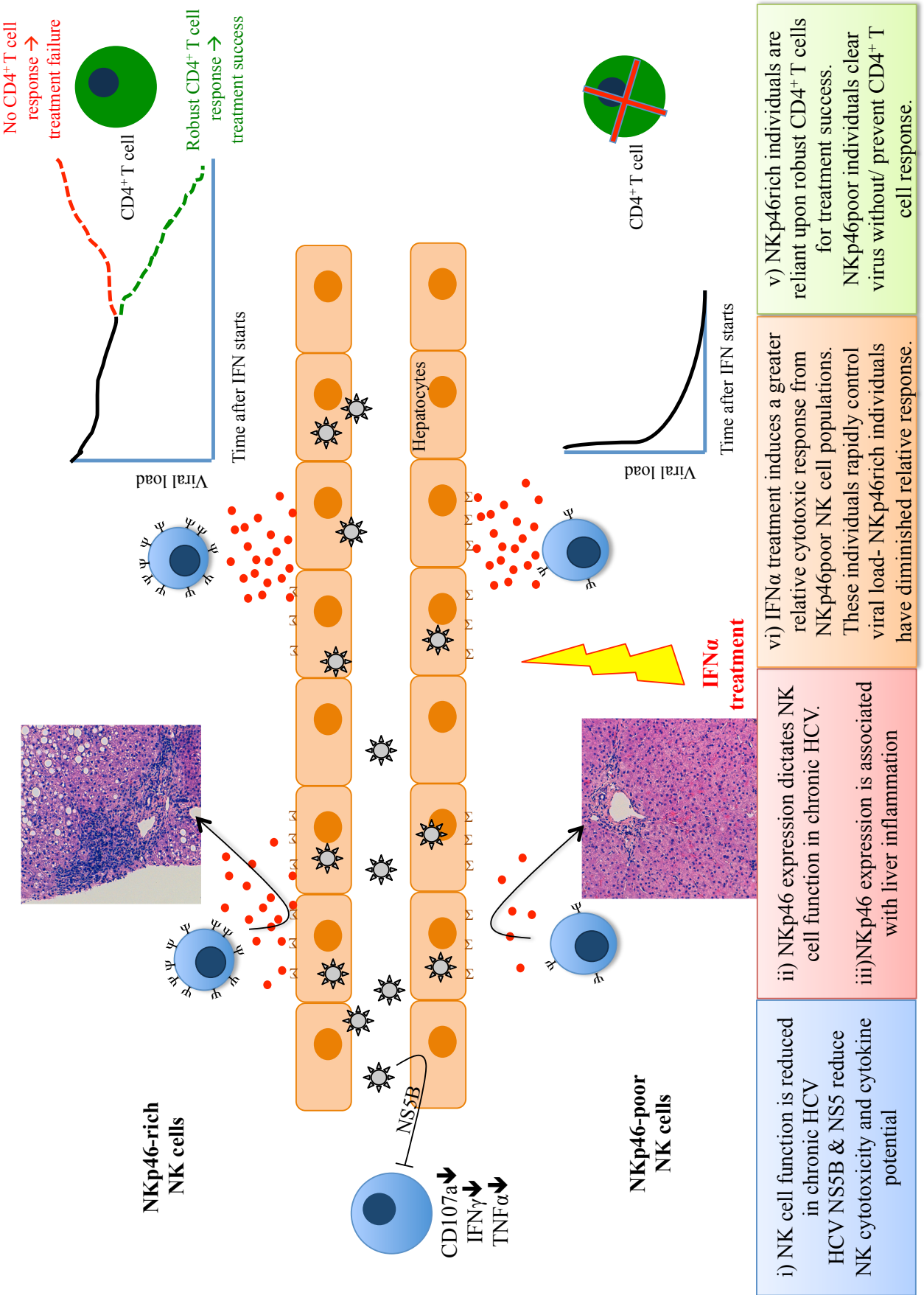
iii) I have demonstrated a strong association between NKp46, NK cell cytotoxicity and liver inflammation by FNA sampling of a large cohort of the liver HCV^+ patients (Figures 4.10-12). These findings are supported by those of Krämer and colleagues.[175] Importantly it has been demonstrated *in vitro* that hepatoma cell lines infected with JFH-1 HCV up regulated NKp46 ligand adding further evidence of NKp46 activation in chronic HCV.[200] A further study of a large cohort of HCV^+ individuals has demonstrated reduced intrahepatic NK cell cytotoxicity and IFNγ assays *ex vivo* compared to the peripheral blood.[224] This is keeping with the theme that NK cells within the liver are activated and

therefore have reduced cytotoxic potential when rechallenged *ex vivo* compared to the peripheral blood.

iv) There is a strong correlation between the marked relative increase in NK cytotoxicity *in vitro* and the rapid control of HCV viraemia during IFNα treatment (Figure 3.13). These data are supported by the near doubling in the proportion of intrahepatic NK cells and evidence of NK cytotoxic activity during very early IFNα treatment (Figures 4.15-16). These data corroborate the findings of Ahlenstiel *et al*.[167] Furthermore, the concept of relative response to IFNα is in keeping with previous findings of interferon-stimulated genes (ISG) in chronic HCV infection suggesting a common theme to treatment resistance.[50,51,201] Intriguingly NKp46 phenotype again impacts upon these findings as NKp46-poor individuals have the greatest increase in cytotoxic function and therefore most rapid viral clearance.

v) In keeping with evidence from multiple mouse models,[203,205-207] we have found that rapid viral clearance effectively abrogates the need and/ or the ability to mount an adaptive immune response by the rapid clearance of viral antigen. Importantly those individuals with NKp46-rich NK cell populations have the highest risk of liver inflammation. They are less likely to rapidly control viraemia by innate responses and appear reliant upon adaptive immune response to clear HCV. The failure to develop effective innate and $CD4^+$ T cell responses during IFNα treatment results in viral persistence (Figure 3.15).

Figure 6.1 NK cells and chronic HCV infection



A recurrent theme throughout this body of work is the importance of NKp46 as an activating receptor in chronic HCV infection. Individuals who are exposed to HCV but do not seroconvert are reported to have high expression of NKp30.[149] During chronic HCV infection NKp30 expression does not appear to dictate NK cell function. However, NK cell function is diminished in chronic HCV infection and correlates with NKp46 expression.[175,200] There are striking similarities between the loss of NK cell function and the relative increase in significance of NKp46 as the major activating ligand in chronic HCV and a report by Jarahian and colleagues of mouse pox-virus infections.[121] It was reported that usurpation of NKp30 as the primary NK activating molecule resulted in NKp46 associated NK cell activation. A similar scenario might occur in HCV infection – HCV proteins may block NKp30-activation. This could be tested using the adenovirus – expression system described in this thesis. In addition, the use of recombinant adenovirus to express individual HCV proteins, together with recombinant chimeric NKp46-Fc proteins, should allow screening for the HCV specific ligand of NKp46. The ligand could subsequently be captured and characterised using proteomic and biochemical methods. This would enable further examination of the interaction between NKp46 and a ligand of known important pathological sequelae.

Two striking factors mark NKp46 apart from other NCRs and NK cell activating receptors: i) the functional necessity for NKp46 dimerization providing a hinge region as a potential ligand binding site.[117,118] ii) it is the most conserved NK activating receptor across several mammal species.[110,119] Although NKp46 triggering may be less important for NK function in acute infections and have a lower activating potential compared to other NK activating receptors, the broad evolutionary conservation suggests that NKp46 expression may continue to confer a host advantage to a broad range viral pathogens.

The conserved nature of NKp46 has important implications in its consideration as a therapeutic target. As discussed in Chapter 4, the reduction of NKp46 expression may be a useful goal of anti-HCV associated inflammation treatment. However, further research is required to assess whether loss of such a widely expressed and conserved activating receptor has adverse outcomes particularly relating to failure of tumour recognition, especially HCC, and risk of viral infection. Further work leading on from this thesis could include treating primary NK cells and NK cell lines with HDACi to reduce NKp46 expression and assessing their cytotoxic and cytokine responses to HCV specific antigens and a variety of tumour and common viral infections including influenza. Following these experiments the impact of HDACi upon NKp46 phenotype and markers of liver inflammation could be assessed in longitudinal studies using FNA sampling.

NS5B inhibitors are currently under evaluation as potential therapies to prevent viral replication in addition to IFNα and ribavirin regimens or as part of IFNα free regimens. The data presented in Chapter 5 suggests that an additional outcome of NS5B inhibitor treatment may be restoration of NK cell function in chronic HCV in addition to reducing viral replication. A potential risk of using NS5B inhibitors in conjunction with IFNα stimulation is that the innate response from uninhibited NK cells will rapidly control viral antigen in all individuals preventing the development of an anti-HCV adaptive immune responses. If such individuals were re-exposed to HCV a lack of adaptive immune responses would place them at increased risk of a repeated chronic infection. Therefore, lower doses of IFNα or IFNα free regimens may actually increase the development of robust $CD4^+$ responses, which successfully clear HCV. 'Lead-in' or pre-treatment with NS5B inhibitors prior to IFNα based treatment may increase SVR rates purely through

increased NK cell response. In addition the reduction in viral replication by NS5B inhibitors may further reduce the potential for establishing IFNα associated immunoevasive quasi-species during HCV treatment. Further studies could assess the mechanisms of the interaction between NS5B and NK cell function *in vivo* by flow cytometry and microarray. In addition, to assess the longitudinal effect of this treatment upon intrahepatic NK cells using FNA sampling potential research could compare the effects of NS5B inhibition *in vivo* in conjunction with *in vitro* experiments.

At present two of the greatest pressures upon modern healthcare systems are the requirement to deliver personalised medicine, with reduced risk of adverse events and increased rates of successful outcomes, and the need to control healthcare spending. The data presented in this thesis suggests the potential use of NKp46 as a biomarker to direct treatment and as a novel target for treatment either to control chronic HCV-inflammation or to promote viral clearance. There is considerable excitement surrounding the possibility of IFNα free therapy for chronic HCV. However, if HCV NK immunoevasive mechanisms can be inhibited with single agent small molecular inhibitors then effective clearance may be achievable with lower doses of IFNα over shorter periods of time. There is a potentially significant economic advantage to shorter, low-dose IFNα based regimes. In addition whilst IFNα is a 'dirty' drug with a broad range of potential adverse events, these side effects are dose dependent, well-defined and monitoring protocols are established.

Conclusion

In conclusion, chronic HCV infection related inflammation results in fibrosis, cirrhosis and increases the risk of hepatocellular carcinoma. The ability to clear HCV with IFNα based treatments is unusual compared to other human chronic viral infections. There is increasing evidence that NK cells are an important component of the innate immune response to HCV and may be involved in the successful eradication of HCV.[163,166,167,175,186]

I have demonstrated that NK cells are closely associated with the pathogenesis of HCV related liver inflammation and impact upon IFNα based treatment of HCV. The mechanisms by which NK cells are inhibited by HCV and the ligands that activate NK cells in the chronic disease state remain unclear. However, initial work reported in this thesis indicates a role for the highly variable NS5B viral protein. This findings represents the starting point for a significant further body of research, which by expanding a critical area of HCV pathology and therapeutics will potentially alter HCV assessment and treatment on an individual patient basis.

Appendix I:

Histological scoring systems of liver inflammation and fibrosis

i) Ishak's Necroinflammatory score (Maximum score = 18) [187]

	Score
A) Periportal or periseptal interface hepatitis (piecemeal necrosis)	
Absent	**0**
Mild (focal few portal areas)	**1**
Mild/moderate (focal most portal areas)	**2**
Moderate (continuous around <50% of tracts or septa)	**3**
Severe (continuous around >50% of tracts or septa)	**4**
B) Confluent necrosis	
Absent	**0**
Focal confluent necrosis	**1**
Zone 3 necrosis in some areas	**2**
Zone 3 necrosis in most areas	**3**
Zone 3 necrosis & occasional portal central (P-C) bridging	**4**
Zone 3 necrosis and multiple P-C bridging	**5**
Panacinar or multiacinar necrosis	**6**
C) Focal (spotty) lytic necrosis, apoptosis and focal inflammation	
Absent	**0**
One focus or less per 10x objective	**1**
Two to four foci or less per 10x objective	**2**
Five to ten foci or less per 10x objective	**3**
More than ten foci per 10x objective	**4**
D) Portal inflammation	
None	**0**
Mild, some or all portal areas	**1**
Moderate, some or all portal areas	**2**
Moderate/marked all portal areas	**3**
Marked, all portal areas	**4**

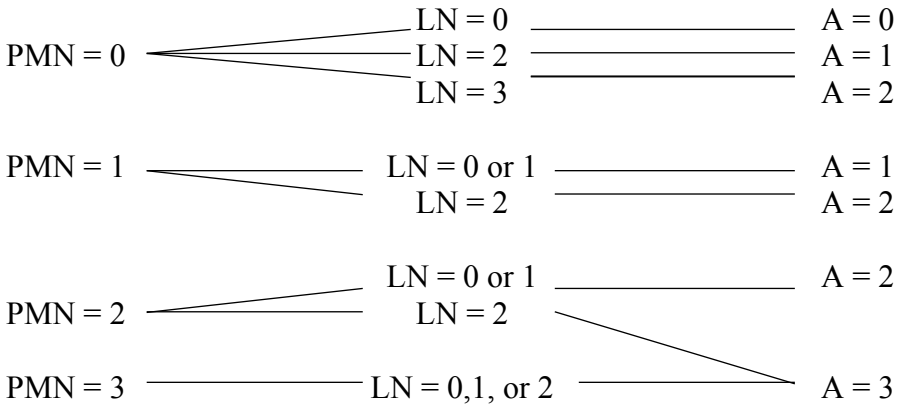
Ishak's fibrosis and cirrhosis staging criteria (Maximum score 6)

	Score
No fibrosis	**0**
Fibrosis expansion of some portal areas, with or without short fibrous septa	**1**
Fibrosis expansion of most portal areas, with or without short fibrous septa	**2**
Fibrosis expansion of most portal areas, with or without short fibrous septa with occasional portal to portal (P-P) bridging	**3**
Fibrosis expansion of portal areas, with marked bridging (P-P as well as portal to central (C-P)	**4**
Marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis	**5**
Cirrhosis, probable or definite	**6**

ii) Metavir Histological Activity Index [212]

	Score
Piecemeal Necrosis	
No	**0**
Mild	**1**
Moderate	**2**
Severe	**3**
Lobular Necrosis	
No or mild	**0**
Moderate	**1**
Severe	**2**

METAVIR Algorithm for evaluation of histological activity



iii) Brunt criteria for grading and stating non-alcoholic steatohepatitis [210]

			Inflammation	
	Steatosis	**Ballooning**	**Lobular**	**Portal**
Grade 1 mild	1-2	Minimal zone 3	L = 1-2	P = 0-1
Grade 2 moderate	2-3	Present zone 3	L = 1-2	P = 1-2
Grade 3 severe	2-3	Marked, predominantly zone 3	L = 3	P = 1-2

Steatosis: 1≤33%, 2= 33-66%, 3≥66%
Lobular inflammation: 0= absent; 1 <2 foci per x20 field; 2=2-4 foci per x20 field; 3= >4 foci per x20 field
Portal inflammation: 0= absent; 1= mild; 2= moderate; 3= severe

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