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THE ROLE OF NATURAL KILLER CELLS IN THE IMMUNE RESPONSE TO HEPATITIS C VIRUS

TRINITY COLLEGE DUBLIN

A thesis submitted to the University of Dublin for the degree of Doctor of Philosophy

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Summary

Hepatitis C virus (HCV) infection remains a major clinical problem, particularly in the developing world where high cost makes treatment inaccessible to many. Those co-infected with HIV also face poorer prognosis and increased risk of chronic HCV infection. There is currently no vaccine publicly available for prevention of HCV infection. Several studies, including research from the Gardiner group, have suggested that NK cells play an important role in the immune response to HCV. Several NK cell genes have been identified as predictors of long-term prognosis in HCV infection. These include several genes encoding for NK cell Killer Immunoglobulin-like Receptors (KIR) as well as genes encoding their ligand, Human leukocyte antigen (HLA).

HLA-A*03, recognised by the KIR3DL2 receptor on NK cells, has previously been reported to be a protective allele in HCV infection. This study investigated the effect of HCV peptide variation and *KIR3DL2* allelic variation on the level of KIR3DL2 binding to the protective HLA-A*03 allotype. No significant binding of HCV peptide tetramers to KIR3DL2 was found, thus providing no evidence that KIR3DL2 plays a role in the protective effect of HLA-A*03. However, KIR3DL2 allelic variation resulted in varying levels of binding specificity to EBV/EBNA3A: HLA-A*03 restricted tetramer. From five variants tested, KIR3DL2*007 and KIR3DL2*009 allelic variants bound with lower frequency (50.1±13.2% and 66.9±4.9% tetramer positive cells respectively) in comparison with KIR3DL2*001 (87.9±1.0% tetramer positive cells). Further investigation is required to investigate whether differential peptide binding to individual KIR3DL2 alleles has a significant effect on the immune response to EBV infection. Analysis of $KIR3DL2^+$ NK cells from post renal transplant patients previously exposed to EBV showed no significant binding to EBV: HLA-A*03 peptide tetramer. Furthermore, no differences in KIR3DL2 expression were identified in those who resolved EBV infection compared with those who developed chronic high viral loads. However, NK cell expression of CD57 was found to be significantly reduced in those with chronic EBV infection compared with resolvers (65.7±14.2% and 44.6±14.7% $CD57^+$ NK cell respectively ($p=0.03$ CI=2.6, 39.5)). This suggests that this subset of $CD57^+$ NK cells may contribute to a protective immune response against EBV infection.

The impact of innate immune genes on the course of HCV infection was also investigated as part of this study. Genotyping analysis of a German Anti-D cohort of HCV infected patients revealed broadly similar results to what has been previously reported in the HCV

setting. The frequencies of both *KIR2DL3* and *HLA-C1* were not significantly associated with spontaneous resolution of infection as has been found in other cohorts of HCV infected patients. In contrast however, the frequency of *KIR2DS3* was found to be non-significantly decreased in those who developed chronic infection. This is a reverse in the significant trend previously identified in the Irish Anti-D cohort. This may suggest that *KIR2DS3* is a marker for a larger haplotype of innate immune genes which predispose to chronic HCV infection. Another NK cell receptor, which binds to HLA-C2 and is in linkage disequilibrium with *KIR2DS3* may be significantly associated with development of chronic infection in the Irish cohort. *KIR2DS3* may not mark the causative haplotype in the German cohort.

Despite significant efforts, a safe and effective vaccine against HCV infection has yet to be developed. We hypothesised that NK cells may also contribute to the immune response induced following HCV vaccine administration. In order to investigate this possibility, levels of NK cell activation and priming were examined in two cohorts of participants taking part in a clinical trial aiming to develop a novel vaccine for prevention of HCV infection. No evidence of *ex vivo* NK cell activation was reported in these participants with no significant changes in NK cell IFN-γ production or cytotoxicity following vaccine administration. Athough not significant, those vaccinated with a Chimp Adenoviral priming vector showed small increases in NK cell *ex vivo* functional responses following vaccine administration. Following 18 hour *in vitro* stimulation of PBMC with IL-12+IL-18 cytokine combination, individuals vaccinated with a Human Adenoviral priming vector showed a significant but short term attenuation in NK cell IFN-γ responses following vaccine administration. In contrast, cytotoxicity responses remained unchanged. Individuals vaccinated with Chimp Adenoviral priming vector showed no significant change in cytokine induced NK cell IFN-γ production post vaccination. However, cytotoxicity responses to prolonged cytokine stimulation were significantly increased in this cohort following vaccine administration.

The results described here add to the current literature examining the role of NK cells in the immune response to HCV. Further study is required in order to gain a greater understanding of the mechanisms which control NK cell activation and inhibition and the role of NK cells in human viral infection, including HCV.

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Ar scáth a chéile a mhaireann na daoine.

List of Abbreviations

ADCC	Antibody dependent cellular cytotoxicity
AIDS	Acquired immune deficiency syndrome
ALT	Alanine transaminase
APC	Antigen Presenting Cell
CMV	Cytomegaloviruses
CTL	Cytotoxic T lymphocytes
DAA	Direct Acting Anti-virals
DC	Dendritic cell
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethlyenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent assay
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
gDNA	Genomic DNA
HCV	Hepatitis C virus
HIV	Human Immunodeficiency virus
HLA	Human leucocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif

ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer cell immunoglobulin like receptor
LILR	Leukocyte immunoglobulin like receptor
LRC	Leukocyte receptor cluster
MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptors
NK	Natural Killer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered aaline
PCR	Polymerase chain reaction
PE	Phycoerythrin
RNA	Ribonucleic acid
RPM	Revolutions per minute
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
SVR	Sustained Virological Response
TCR	T cell receptor
Th1	T helper cell type 1
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRAIL	TNF related apoptosis inducing ligand

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Chapter 1

Introduction

1.1. The human immune response

The primary role of the human immune response is to defend against potential pathogen invasion, preventing serious disease. Individuals with a compromised immune system are at high risk of developing serious and potentially fatal infections. In healthy individuals, most infections are eliminated by the immune system without any illness or major physical symptoms. The immune response to pathogen infection can be segregated into two components, the innate and adaptive immune response, and each play a distinct role in the prevention of serious disease (Parham, 2005).

The innate immune response is centered on the activation of immune cells with specialised effector functions, which are capable of quickly recognising and immediately responding to pathogen infection. Initial recognition of pathogen involves soluble proteins and cell surface receptors which can bind pathogen directly or bind to host cells which become altered following pathogen infection. In response to pathogen recognition, cells of the innate immune response are capable of recruiting specialised effector cells which can engulf bacteria, directly kill virus infected cells or attack protozoan parasites. Serum proteins called complement are also released which aid this cellular response, marking pathogens for destruction by effector cells of the immune system. Phagocytic cells, capable of engulfing microorganisms, are a central component of the innate immune system and include neutrophils, dendritic cells, monocytes and macrophages. Natural Killer (NK) cells are another important component of the innate immune response. These cells are capable of directly killing virally infected cells and secrete significant amounts of anti-viral cytokines which interfere with viral replication (Medzhitov, 2008).

Cells of the innate immune response also play a key role in the induction of the adaptive immune response which is specific to the invading pathogen and increases the effectiveness of the immune response against a particular pathogen. Activation of the adaptive immune system results in clonal selection, proliferation and maturation of B and T lymphocytes which specifically recognise a particular pathogen or pathogen derived peptide antigen. This clonal expansion includes the generation of long-lived memory cells, capable of mediating a more effective response to subsequent infections with the same pathogen. These cells are capable of inducing a potent immune response which is specifically directed against the invading organism. B and T cells possess specific cell surface receptors, consisting of immunoglobulins on B cells and T cells receptors (TCR)

on T cells. The genes which encode these receptors are cut, spliced and modified during lymphocyte development resulting in each lymphocyte expressing a single specific receptor. Importantly, B cell immunoglobulins can be secreted as soluble antibodies which have specialised effector functions. The immune response which is mediated by antibodies has been termed humoral immunity and is particularly effective at eliminating extracellular pathogens (reviewed in Iwasaki and Medzhitov, 2015).

The potency of this adaptive immune response, which is so effective against microbial infection, can result in adverse effects in the context of autoimmune disease and organ transplant rejection. Both situations are characterised by chronic inflammation resulting from immune recognition of normal components of healthy cells. The immune response can target healthy host cells resulting in autoimmunity. In addition, healthy donor cells can be targeted post-transplantation, resulting in the transplant rejection. Similar inappropriate immune activation can occur during pregnancy in the case where cells of the developing embryo are recognised as foreign by the immune system of the mother (Parham, 2005).

While antibodies secreted by B cells recognise antigens present on intact pathogens, the TCR binds to a specific pathogen derived peptide antigen which is complexed to a major-histocompatibility complex (MHC) molecule. These peptides result from the processing of pathogens or their protein products. The peptide: MHC complex is then transported to the surface of the cell where it can be recognised by T cell receptors on the surface of T lymphocytes. Each peptide: MHC complex forms a unique ligand for its T cell receptor. In humans the MHC is also termed Human Leukocyte Antigen (HLA) (Parham, 2005, van Kasteren et al., 2014).

MHC class I molecules present endogenous peptide antigens which can originate from pathogens which replicate intracellularly. Pathogens of this type include viruses and some bacteria. Almost all cell types are capable of signalling to the immune system through expression of MHC class I on the cell surface in the event of infection with such a pathogen. MHC class I complexes are recognised by a subgroup of T cells known as cytotoxic T lymphocytes (CTL) which are specialised at killing infected cells and are identified by their expression of the CD8 glycoprotein on their cell surface. $CD8^+$ CTL therefore play a significant role in the immune response to viral infection. This is most evident in individuals who lack functional CTL and are prone to persistent viral infection surface (Parham 2005, van Kasteren et al, 2014).

In contrast, MHC class II molecules present peptides which are derived from extracellular pathogens that are taken up and processed by professional phagocytic APC of the immune system. MHC class II complexes, presented by these professional APC, are recognised by T helper (Th) cells which are specialised at further activating the cellular immune response. These T helper cells are characterised by expression of CD4 glycoprotein on the cell surface. Th1 $CD4^+$ T cells are potent activators of macrophages while Th2 $CD4^+$ cells are significant activators of the B cell response (Parham, 2005, van Kasteren et al., 2014).

1.2. Immune recognition of viral infection

The type of immune response which is induced is dependent on the class of MHC molecule through which antigen is being presented. Proteins derived from viruses and other intracellular pathogens are processed by a separate mechanism to those of extracellular origin. When a virus infects a cell it uses the host cell machinery to synthesise viral proteins which form infectious viral particles. In response, the host cell can initiate degradation of these viral proteins by the proteosome, a protein complex which is located in the cytosol and has protease activity. The resultant viral peptides are then transported to the endoplasmic reticulum (ER) with the aid of transporter associated with antigen processing (TAP) protein which is embedded in the membrane of the ER. In the ER, viral peptides are complexed with MHC class I. Several chaperone proteins are required for correct folding and peptide loading of MHC class I and the majority of viral peptides do not successfully form peptide MHC complexes and are removed from the ER. Newly formed peptide: MHC complexes are transported to the cell surface via the Golgi apparatus for recognition by the immune system (Parham, 2005, van Kasteren et al., 2014).

The function of the CTL is to kill infected cells in order to prevent the spread of infection to healthy cells. Mature, effector CTL contain lytic granules which contain cytotoxic proteins including perforins and granzymes. When activated, CTL travel to the site of infection where they recognise specific peptide:MHC complexes presented by infected cells. Interaction of the T cell receptor with its ligand results in the release of the lytic granules directly on to the surface of the infected cell. The specificity of this interaction allows for direct killing of target cell without damage to adjacent healthy tissue. The cytotoxic proteins in the lytic granules initiate the process of programmed cell death known as apoptosis following recognition by CTL. This tightly controlled process prevents

the release of potentially infectious viral particles from the infected cells. Perforins, released by the CTL, form large pores in the cell membrane of the target cell, allowing granzyme proteases, another group of cytotoxic proteins, to enter the cell and initiate apoptotic cell death. Receptor ligand interactions between the CTL and its target cell can also induce apoptosis of the target cell. The interaction of Fas ligand and TRAIL (expressed on activated CTL) with receptors on the surface of target cells mark the cell for destruction. CTL are also significant producers of anti-viral cytokines such as IFN-γ which inhibit viral replication, enhance antigen processing and activate phagocytic immune cells such as macrophages to aid removal of dying infected cells (Parham, 2005, van Kasteren et al., 2014).

MHC class I is recognised by the T cell receptor of $CD8^+$ CTL but is also recognised by cell surface receptors present on NK cells of the innate immune response. NK cells share many functional characteristics with CTL of the adaptive immune system. These include secretion of perforins and granzymes and expression of Fas ligand and TRAIL for the direct killing of target cells. NK cells when activated are also significant producers of anti-viral cytokines such as IFN-γ (Parham, 2005, van Kasteren et al, 2014).

1.3. NK cells and their importance in the immune system

The immune response to intracellular damage or infection involves complex interactions between host and the affected cell. NK cells are one of the main effector cell types of the innate immune response. NK cells are specialised at targeting and killing virus infected and cancerous cells and are critical in the innate immune defence against viruses and malignant transformation (Campbell et al. 2013). Resting NK cells circulate in the blood but following activation by cytokines, migrate into malignant or infected tissue. Their ability to rapidly induce a cytotoxic environment makes NK cells one of the most potent antiviral immune cells. They work to control viral infection through direct cytolysis of infected cells, indirect secretion of inflammatory mediating cytokines and chemokines, and also by activation of dendritic cells (DC) and antigen specific T cells (Lanier, 2008).

NK cells originate in the bone marrow and are derived from a common lymphoid progenitor shared with $CD8^+$ T cells. They account for up to 10% of the lymphocyte population in the blood, spleen and are even further enhanced in the liver (>30%). NK cells

can be divided into two subsets based on their expression of CD56. Approximately 10% of NK cells have a $CD56^{bright}$ phenotype and produce high amounts of cytokine in comparison with the more mature $CD56^{dim}$ subsets which show a greater capacity for cytotoxicity. Like $CD8^+$ T cells, NK cells become activated through antigen-specific receptors and by pro-inflammatory cytokines such as IL-12, IL-15, IL-18 and type I interferons. Activated NK cells release cytotoxic granules containing perforins and granzymes which lyse target cells in a similar mechanism to that used by $CD8^+$ T cells. Perforin is required for NK cell cytotoxicity while granzyme proteins induce apoptosis of target cells. NK cells also kill target cells through expression of Fas-L and TRAIL, known ligands for Tumour Necrosis Factor (TNF) receptors, capable of inducing apoptosis of tumour cells (Campbell and Hasegawa, 2015).

The main cytokines produced by activated NK cells are IFN-γ, TNF-α, IL-10, TNF-β and GM-CSF. Production of these soluble factors by NK cells early in the innate immune response influences the recruitment and function of other hematopoietic cells (Cooper et al., 2001). Production of cytokines also gives NK cells a regulatory function, allowing then to act in tandem with DC and neutrophils to regulate the subsequent adaptive immune response which is induced (Wehner et al., 2011). A small proportion of NK cells are resident in the lymph node where they interact with DC to promote IFN-γ responses by T cells (Martin-Fontecha et al. 2004). Significant numbers of tissue-resident NK cells are also found in the adult gut, liver, and endometrium as well as other sites in the body. These subpopulations may differ phenotypically and functionally from peripheral blood NK cells and provide a unique population of lymphocytes capable of providing the specific immunological requirements of that tissue. In the gastrointestinal tract, the immune system must tolerate a large number of commensal bacteria and dietary antigens while at the same time protecting against potentially pathogenic organisms. Similarly, the blood entering the liver is rich in nutrients and products of microbial degradation which have been transported from the gut and are processed and cleared in the liver. In order to prevent pathogenic immune responses to these microbial products, the liver must suppress both innate and adaptive immune responses to prevent unnecessary immune activation while retaining the ability to detect potentially harmful pathogens. Hepatic NK cells resident in the liver make up 30-50% of the lymphocyte population reflecting the importance of these cells in protecting against hepatotrophic infections and malignancy. In addition, NK cells resident in the uterus play an important role in supplying angiogenesis during placentation (Lysakova-Devine and O'Farrelly, 2014)

Unlike the elements of the adaptive immune system, NK cells provide an immediate response to damage or infection of cells without the need for prior sensitisation and are therefore central to the initial host immune response. NK cells can be rapidly activated to attack abnormal cells, particularly tumour or virus infected cells. Individuals who selectively lack NK cells have an increased susceptibility to tumours and to viral infection, particularly herpesviruses (Orange, 2006). Most persistent viruses have evolved mechanisms to reduce the impact of host immune defences allowing them to replicate and spread among healthy individuals. Many of these evasion strategies involve production of specific immune-evasion molecules to avoid detection by NK cells, providing further evidence of their important anti-viral function (Lanier, 2008, Lodoen and Lanier, 2005).

1.4. A balance of signals from a wide range of both inhibitory and activating receptors controls NK cell activation

The cell surface of NK cells comprises of activating and inhibitory receptors which are capable of recognising and binding their cognate ligands on target cells. NK cells can be divided into distinct subsets on the basis of cell surface expression of these receptors. The C-type lectin family of receptors includes the activating receptor NKG2D and the CD94-NKG2A/C/E heterodimer receptors. Another family of activating receptors which are expressed on NK cells include the natural cytotoxicity receptors (NCR). Important NK cell inhibitory receptor families include the Leukocyte Immunoglobulin-like Receptors (LIR) and the Killer Immunoglobulin-like Receptors (KIR). In addition, the Fc receptor CD16 is capable of inducing antibody mediated cellular cytotoxicity (ADCC) upon encountering target cells opsinised by antibody (Pegram et al., 2011).

These families of receptors recognise a wide variety of determinants on infected cells. These receptors recognise cellular stress ligands as well as MHC class I molecules which are capable of inducing NK cells functional responses. In order to maintain tolerance to healthy tissue, some of these receptors can also prevent NK cell activation. The balance of signals detected by the various NK receptors will determine the level of NK activation (Pegram et al., 2011, Lodoen and Lanier, 2005). Signalling of inhibitory receptors is mediated by immunoreceptor tyrosine-based inhibitory motifs (ITIM) which are found in the cytoplasmic tail of the receptor. Following interaction with ligand, ITIM are tyrosine phosphorylated and associated with Src-homology domain bearing tyrosine phosphatases

(SHP-1 and SHP-2) resulting in inhibition of the intracellular activation cascade. In contrast, activation receptors transduce signals through the association with molecules containing immunoreceptor tyrosine-based activating motifs (ITAM) (Pegram et al. 2011).

The CD94-NKG2A/C/E heterodimers recognise the non-classical HLA molecule HLA-E. The CD94-NKG2C and CD94-NKG2E receptors are activating receptors while CD94-NKG2A is an inhibitory receptor. The activating receptor NKG2D recognises a variety of stress induced ligands. These include MHC class I chain related gene (MIC)-A and MIC-B and ULBP1, ULBP2, ULBP3 and ULBP4. Expression of these stress ligands is induced in response to DNA damage upon malignant transformation and the NKG2D receptor has been found to have an important role in the NK cell response to cancer and the induction of CTL, Th1 and Th2 responses (Pegram et al., 2011).

The natural cytotoxicity receptors (NCR) belong to the Ig receptor superfamily and include NKp30, NKp46 and NKp80 which are expressed on both activated and resting NK cells. The NKp44 receptor is upregulated on NK cells following IL-2 stimulation. Reported ligands for NKp46 and NKp44 include viral haemagglutinins and nuclear factor HLA-B associated transcript 3 which is released from tumour cells and binds NKp30. Both NKp46 and NKp30 have been shown to bind to heparin sulphate proteoglycans and NKp80 binds activation induced C-type lectin. The NCR have been found to have a significant role in the killing of tumour cells (Kruse et al., 2014).

A lot of progress has been made in determining how NK cells recognise their target cells using various receptors on their cell surface. Loss of MHC class I is a common mechanism which tumours and virus infected cells use to evade immune detection by CTL. In this situation, NK cells are capable of targeting for cytolysis, cells with downregulated expression of MHC class I molecules which are normally expressed on healthy cells. This is known as the 'missing self' hypothesis whereby the loss of target cell MHC class I removes the ligands for inhibitory NK cell receptors, resulting in the activation of NK cell effector function. In this model, the inhibitory signals mediated by MHC class I ligand interactions with inhibitory receptors control NK cell functionality (Ljunggen, 1990).

Like $CD8^+$ T cells, NK cells are educated during development through interaction of self-recognising inhibitory receptors (SRIR) with self MHC class I ligands expressed on normal healthy host cells. The level of NK cell responsiveness is dependent on the number of SRIR that they express. SRIR interactions with ligand promote NK cell education to mediate NK cell responses against abnormal cells which have lost expression of MHC. A

lack of self-ligands to interact with SRIR during development results in NK cell hypo-responsiveness or anergy (Anfossi et al., 2006, Campbell and Hasegawa, 2013). These anergic NK cells have the capability to be re-educated following adoptive transfer into an MHC sufficient environment. Anergic NK cells acquired the ability to produce effector cytokines. In contrast, previously functional NK cells lost their effector capabilities in an environment devoid of MHC molecules. This process is known as NK cell licensing and results in the generation of functional NK cells in the periphery, similar to the process of positive selection of developing T cells. These studies suggest that continuous engagement of inhibitory receptors with self MHC class I molecules is required in order to regulate NK cell responsiveness (Elliot et al., 2010).

1.5. Killer Cell Immunoglobulin-like receptors on NK cells

NK cells possess Killer Cell Immunoglobulin Receptors (KIR) which interact with HLA class I antigens. KIR are one of the most important families of receptors on human NK cells (Vilches and Parham, 2002). Different KIR receptors have the ability to activate or inhibit NK cells and their immune functions. Inhibitory KIR proteins are characterised by long cytoplasmic tails containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) which activate inhibitory signalling pathways, leading to the general shutdown of NK cell effector functions. Contrastingly, KIR proteins with short cytoplasmic tails function in activating NK cells via an association with the molecule DAP12. DAP12 contains immune tyrosine based activating motifs (ITAMs) and signalling through this molecule results in NK cell activation (Vilches and Parham, 2002). KIR genes are named according to the structure of the molecules that they encode. The first digit following the KIR prefix corresponds to the number of Ig-Like domains in the molecule, the domain is designated by the nomenclature of either 2D or 3D. This is followed by either 'L' or 'S' indicating a long or short cytoplasmic tail (Figure 1.1 (a)) (Vilches and Parham, 2002). Type 1 KIR2D receptors contain 2 extracellular domains with a D1 and a D2 conformation. Type II KIR2D receptors contain 2 extracellular domains with a D0 and D2. KIR3D receptors contain 3 extracellular domains (Figure 1.1.(a)).

The genes which encode for KIR proteins are highly variable and polymorphic. This has resulted in the generation of large family of KIR receptor proteins which differ only slightly in amino acid sequence. There are 16 KIR genes including 3 pseudogenes. All of

these KIR genes are located on chromosome 19q13.4 in a region known as the leukocyte receptor cluster (LRC) which also contains other members of the immunoglobulin superfamily including LILR receptor family (Figure 1.1.(b)) (Hsu et al., 2002). These different KIR receptors are highly similar which makes it difficult to identify these receptors in isolation. Furthermore, if a particular KIR gene is present in an individual, it is normally only expressed on some of their NK cells. This results in a large variety of subsets of NK cells within individual expressing varying combinations of KIR receptors. Individual donors will vary in the number of KIR receptor genes expressed – this can vary from 6 to 16 (Hsu et al., 2002, Uhrberg et al., 1997). Four KIR genes, including 1 pseudogene have been designated 'framework genes', which are common to all KIR haplotypes (*KIR2DL4, KIR3DL2, KIR3DL3* and *KIR3DP1*). These framework genes define two intervals within the haplotype, dividing it into centromeric and telomeric regions (Hsu et al., 2002) (Figure 1.1.(b)).

Individuals can be segregated into groups based on KIR genotype. Individuals are found to resolve into 2 broad KIR haplotypes (A and B) based on KIR gene content (Uhrberg et al., 1997, Martin et al., 2000). The A haplotype is generally non-variable in its composition, consisting of the framework genes plus *KIR2DL1*, *KIR2DL3*, *KIR2DS4* and *KIR3DL1*. It has only one short tailed activating member (KIR2DS4). The B haplotype is defined by the presence of one or more of the genes encoding activating KIR such as *KIR2DS1/2/3/5*, *KIR3DS1* and the genes encoding the inhibitory KIR *KIR2DL5A/B* and *KIR2DL2* (Uhrberg et al., 1997). B haplotypes have previously been shown to be more prevalent in Non-Caucasian populations such as Australia Aborigines and Asian Indians. Among Caucasian populations, approximately 55% of the population have an A haplotype and 30% will have a homozygous AA haplotype (Uhrberg et al., 1997).

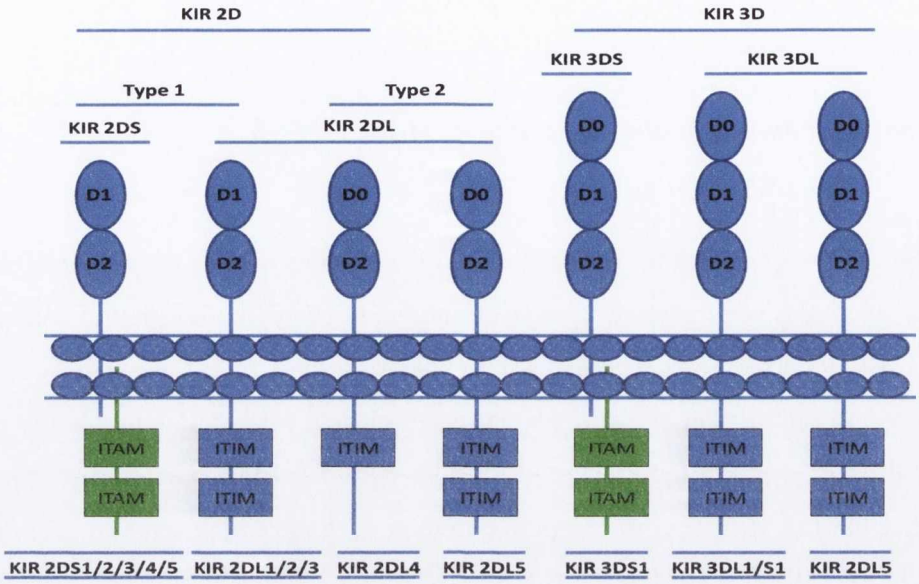
KIR gene content can vary between individuals, one haplotype can exhibit from as few as 8 to as many as 14 KIR genes. KIR gene copy number is also variable with at least 2 KIR genes known to be present multiple times on the same haplotype. Genetic polymorphism is the largest contributor to KIR gene diversity, multiple alleles for each KIR locus have been identified and it is likely that more remain to be identified. The benefit of such diversity in KIR genes is the protential for diversification of the immune response in the face of rapidly changing pathogens (Hsu et al. 2002).

HLA molecules provide the ligands for the KIR receptors. In order for a functional interaction to occur, receptor and ligand must be expressed within the same individual.

HLA class I genes are found on chromosome 6,which is unlinked to the KIR genes on chromosome 19 and the genes for HLA and KIR therefore segregate independently. Despite this, there is increasing evidence that HLA and KIR may be co-evolving. There is also evidence that natural selection has an active role in NK cell receptor evolution and differentiation and is involved in driving the evolution of these genes.

The inhibitory receptors KIR2DL1/2/3 recognise HLA-C ligands. HLA allotypes are divided into two ligand groups based on a polymorphism at position 80 in the alpha helix: group 1 HLA molecules (HLA-C1) contain an asparagine at this position while group 2 (HLA-C2) molecules contain a lysine. KIR2DL1 binds HLA-C2 allotypes and KIR2DL2/3 recognise HLA-C1 but can also bind with low affinity to some HLA-C2 allotypes. KIR3DL1 is known to bind to HLA-A and HLA-B allotypes with Bw4 motifs while KIR3DL2 binds to HLA-A*03 and HLA-A*011. Interestingly, the binding of inhibitory KIR to their HLA ligand has been shown to be peptide dependent. The strength of the KIR: HLA interaction can vary and has a consequential effect on the level of NK cell function. There is increasing evidence that this can affect disease pathogenesis following pathogen infection. The KIR2DL3:HLA-C1 and KIR2DL1:HLA-C2 interaction appears to be stronger that then between KIR2DL2 and HLA-C1. Genetic polymorphism in both KIR and HLA genes also impacts on the strength of the KIR:HLA interaction. KIR allelic variants vary in the level of NK cell surface expression and in the proportion of receptor positive NK cells. In general, genotypes that lead to increased NK cell activation appear to be beneficial in viral infections including HIV and HCV but may increase risk of autoimmune diseases (Kulkarni et al., 2008, Vilches and Parham, 2002).

(a)



(b)

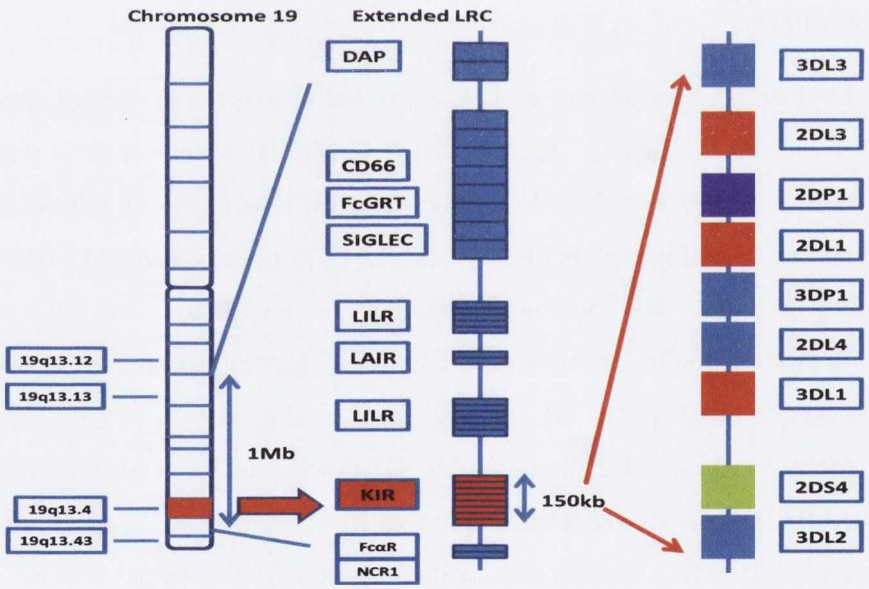


Figure 1.1. The genes which encode for KIR receptors are found within the extended leukocyte receptor complex of chromosome 19. Figure 1.1.(a) The structure of 2 and 3 Ig-like domain KIR proteins are shown. Adaptor molecules such as DAP12, containing immune tyrosine-based activating motifs (ITAM) which associate with activating KIR, are shown in green. Inhibitory KIR containing immune tyrosine based inhibitory motif (ITIM) are shown in blue. Figure 1.1. (b) illustrates the extended Leukocyte Receptor complex (LRC) on chromosome 19 (19q13.4). KIR genes are encoded within a 150kb segment of the extended LRC (1Mb). A representative KIR haplotype is also shown with framework genes in blue,

inhibitory KIR in red, activating KIR in green. The pseudogene 2DP1 is shown in purple (adapted from www.ebi.ac.uk/ipd/kir/introduction.html)

1.6. Leukocyte Immunoglobulin-like receptors (LILR) on NK cells

The LILR family of receptors are a family of 13 genes which encode inhibitory and activating receptors, which are expressed by immune cells of both myeloid and lymphoid lineage. LILR receptors are encoded within the leukocyte receptor complex (LRC) on chromosome 19 which also encodes several other closely related Ig superfamily proteins, including the KIR receptor family (Wende, 1999). Group A LILR receptors are stimulatory in function, pairing with the Fc receptor common γ chain which contains ITAMs. This group includes LILRA1, LILRA2, LILRA4, ILT8 and LIR9m/ILT11. Group B receptors of this family (LILRB1 - B5) are inhibitory, containing two ITIM-like sequences within the cytoplasmic region, and recruits SHP-1 resulting cellular inhibition. In this way, the LILRB family employ similar signalling pathways to the KIR receptors on NK cells (Kuroki et al., 2005).

The LILR bind both classical and non-classical MHC class I molecules. Non-classical HLA class I molecules, such as HLA-E, HLA-F and HLA-G associate with β_2m but perform distinct functions and show less polymorphism in comparison with classical HLA class I. In contrast, T cell receptors (TCRs) and KIR recognise subsets of MHC class I via their α1 and α2 domains. The broad recognition patterns for LILRB1 and LILRB2, indicate a different method of binding. Four separate Ig domains, designated D1-D4, make up the extracellular section of LILRB1. The primary ligand binding sites have been reported to be contained within the D1 and D2 domains. The D1 domain of LILRB1 associates with the α3 region of MHC, while its D2 domain engages β_2m. Both these regions are conserved among HLA-class I molecules resulting in this receptor having broad binding specificity independent of peptide (Chapman et al., 1999).

Members of the LILR family of receptors play an important role as antigen receptors, costimulatory proteins, adhesion molecules and immunomodulatory receptors. Due to these wide-ranging functions, this family has been reported to influence signalling pathways of both the innate and adaptive immune response. The LILRB1 and LILRB2 inhibitory receptors are the most characterised of this receptor family which recognise MHC class I. Functions and ligands for most of this family have yet to be identified.

Ligation of LILRB-1 has been shown to inhibit NK cell activation. Like the KIR, LILRB-1 is more highly expressed by the cytotoxic $CD56^{dim}$ subset than the $CD56^{bright}$ subset (Brown et al., 2004). Like the KIR receptors, LILRB1 has been found to be highly polymorphic, not only in nucleotide sequence but also in the number of loci. Like the KIR receptor family, it has been suggested that functional polymorphisms of LILR genes may result in differential immune responses between individuals and variation in susceptibility to diseases (Kuroki et al., 2005).

1.7. The role of NK cells in the immune response to viral infection

NK cells have the ability to interact directly with virally infected cells. The integration of signals from both inhibitory and activating receptors will determine whether or not the NK cell becomes activated. The NK cell response to viruses is based on the absence of inhibitory interactions between NK cell inhibitory receptors and self MHC molecules normally expressed on healthy cells. This results in NK cell activation through engagement of activating receptors. A key mechanism of viral immune evasion is the down-regulation of expression of HLA molecules in virally infected cells to avoid T cell recognition. NK cells are capable of recognising the loss of inhibitory signals leading to the induction of NK cell activation and providing an alternative mechanism of viral recognition. Many viruses have developed specific mechanisms to inhibit NK cell function, highlighting the importance of these cells in the anti-viral immune response (Hamerman et al., 2005). NK cells have been reported to contribute to protection against HIV, HCV and other viruses.

NK cells may act as an important regulator in HIV infection by preventing HIV from infecting T cells by inducing T cell apoptosis, thereby preventing infection of the host. In addition, the presence of genes for the activating receptor KIR3DS1 and its putative ligand $HLA\text{-}Bw4^{80I}$ has been associated with slower progression to AIDS, lower viral load and protection against opportunistic infections (Martin et al., 2002, Pelak et al., 2011). In addition, $KIR3DS1^{+}$ NK cells (but not $KIR3DS1^{-}$ NK cells) are capable of inhibiting HIV-1 replication in cells expressing its HLA-Bw4 ligand (Alter et al., 2007). This data suggests that NK cells identify and target HIV infected cells through KIR3DS1:HLA-Bw4 interactions. KIR3DS1 expressing NK cells have also been associated with early, robust increases in NK cell IFN-γ production and cytotoxicity in treatment naive individuals

(Long et al. 2008). In addition, KIR3DS1 homozygosity has been found to be significantly increased in HIV exposed seronegative intravenous drug users (Boulet et al., 2008). In contrast, the *KIR2DS2* gene has been associated with faster progression to AIDS (Gaudieri et al., 2005). Haplotype B KIR genes, including *KIR2DS2*, *KIR2DS3* and *KIR3DS1* have also been associated with lower $CD4^+$ T cell counts in HIV^+ individuals (Jennes et al., 2011).

NK cells are also important in herpes virus infection. Individuals deficient in NK cells are particularly susceptible to this type of infection (Biron et al., 1989). Mouse and human cytomegaloviruses (CMVs) encode multiple gene products that down-regulate ligands for NKG2D (Sjolin et al., 2002), indicating a role for this NK cell receptor in the anti-viral immune response. In addition, CMV also encode several proteins that interfere with MHC class I expression in order to evade T cell mediated attack but which may make infected cells more susceptible to NK cell recognition via their KIR receptors (Lin et al., 2007). It has been reported that activating KIR are protective against CMV re-activation during T cell replete stem cell transplantation (Cook et al., 2006). Furthermore, the proportion of $KIR2DS2^+$, $KIR2DS4^+$ and $KIR3DS1^+$ NK cells have also been reported to be expanded in a subset of CMV^+ individuals (Béziat et al., 2013).

KIR2DS1 and KIR2DS3 have also been associated with fatal outcome in Ebola virus infection. The frequency of carriers of both these genes was increased in patient cohorts with fatal outcome of Ebola infections, compared with survivors, contacts and healthy controls (Wauquier et al., 2010).

1.8. NK cell phenotype and function is modulated by previous history of exposure to viruses

NK cells constitute the third major lineage of lymphocytes which also includes B and T cells of the adaptive immune system (Di Santo, 2006). NK cells are quite similar to $CD8^+$ T-cells in terms of their effector function. Both cell types secrete cytokines such as IFN-γ as well as producing perforins and granzymes which perforate the plasma membrane of the target cell and trigger apoptosis. Both cells types also interact closely with dendritic cells (Krzewski et al., 2012). For this reason, NK cells are said to function as the interface between the innate and adaptive immune system (Jobim and Jobim, 2008). There is increasing evidence from studies in both mice and humans that NK cells share some of the

attributes traditionally associated with the adaptive immune system. During development NK cells are educated and selected and their receptors demonstrate some antigen specificity. In addition, NK cells can undergo clonal expansion during infection and generate long-lived memory cells (Sun et al., 2011 and Vivier et al., 2011).

There is now evidence that NK cells can adapt both phenotypically and functionally as a result of stimulation by viruses, cytokines and hapten antigens. This phenomenon has been termed NK cell memory but may also reflect functional NK cell maturation (Min-Oo et al., 2013). There are several routes by which NK cell function may be enhanced after re-exposure to a pathogen. Pathogen specific antibodies bound to antigens on the surface of the infected cell may initiate antibody dependent cellular cytotoxicity (ADCC) following recognition by the Fc receptor CD16 expressed on NK cells (Mandelboim et al., 1999). In addition, expansion of subsets of NK cells which express activating receptors which are able to bind pathogen ligands may increase the number of responsive cells. Alternatively, primary infection may induce some NK cells to differentiate into a more highly responsive state (Arase et al., 2002).

Specific NK cell subsets have been found to be expanded in individuals with cytomegalovirus (HCMV) infection. A subset of $NKG2C^{+}CD57^{+}$ NK cells has been found to be expanded in CMV^{+} individuals. NKG2C is an activating receptor while CD57 is a marker of terminal differentiation/maturation. The subset may therefore represent a group of long-lived and highly functional NK cells which play an important role in the immune response to HCMV and are capable of mediating an increased response to subsequent secondary infection. Such findings are in keeping with what has been reported in mice where long-lived NK cells showed an enhanced response to re-challenge with MCMV infection (Sun et al., 2009, Sun et al., 2011, Nabekura et al., 2014).

NK cells have classically been considered short-lived effector cells with a half life of approximately 12 days (Jamieson et al., 2004). However, more recent studies suggest that NK cells may become long-lived after ligation of activating receptors or stimulation with inflammatory cytokines (Sun et al., 2009, Sun et al., 2011). In particular, the cytokine IL-15 has been reported to play a central role in NK cell homeostatic proliferation and longevity (Lodolce et al., 1998, Sun et al., 2010). NK cells may therefore play a key role in vaccine induced immunity to infectious pathogens. Increasing evidence suggests that analysis of NK cell phenotype and function following vaccine adminstration may have an important role in the development of successful vaccine strategies.

1.9. Hepatitis C Virus (HCV)

HCV is a single stranded positive RNA virus of the Flavivirus family which infects over 170 million people worldwide. The vast majority of cases of HCV infection results in chronic infection with associated liver disease, including hepatic fibrosis, cirrhosis and increased risk of hepatocellular carcinoma (HCC). Liver failure due to HCV infection is also the leading indication for liver transplantation. Despite significant research efforts, there is currently no effective vaccine available for prophylactic prevention of HCV infection. Seven major HCV genotypes and more than 100 subtypes have been identified. The different HCV genotypes have been found to have distinct geographical distribution with different mechanisms of disease pathogenesis and varying levels of responsiveness to treatment (Rehermann B., 2009).

The HCV genome is approximately 9.6kb in length and encodes a single open reading frame for translation of a single HCV polyprotein. This polyprotein is then cleaved into at least 10 viral proteins which include both structural and non-structural proteins (Figure 1.2.). The structural proteins are cleaved from the HCV polyprotein by virus derived proteases in the first step of polyprotein processing. Non-structural proteins are then subsequently cleaved from the polyprotein by virus derived proteases. There are three HCV structural proteins: the core protein (which makes up the viral nucleocapsid) and two envelope proteins, E1 and E2, which are highly glycosylated proteins involved in entry into a host cell. There are seven non-structural proteins p7 (Signal Peptide), NS2 (Transmembrane protein), NS3 (HCV protease), NS4a (cofactor for protease), NS4b, NS5a and NS5b (an RNA dependant RNA polymerase). The structural proteins are located at the 5′ (N terminus) end of the ORF, followed by the signal peptide p7 and the non-structural (NS) proteins (Figure 1.2.) (Poordad and Dietrich, 2012).

Unlike other members of the Flavivirus family, HCV has narrow host specificity and is only transmitted by blood to blood contact between humans. HCV also displays significant tissue tropism and viral infection is largely confined to the hepatocytes of the liver. Much research has been carried out into the mechanism used by HCV to enter host cell during infection. The receptors CD81 and the scavenger receptor class B type I (SR-BI) have been identified as key HCV receptors, required for viral entry in a host cell. Glycosaminoglycans and low-density lipoprotein receptors such as apolipoprotein E are required for initial HCV attachment to cells, allowing HCV envelope proteins to interact

with CD81 and SR-B1. In addition, two tight junction proteins, claudin-1 (CLDN1) and occuldin have been identified as co-receptors for the later step of HCV entry. After viron entry into hepatocytes, the internal ribosomal entry site (IRES) sequence, located at the 5' end of the untranslated region of the viral RNA genome, is recognised by ribosomes in the host endoplasmic reticulum (ER) to mediate viral polyprotein translation (Yang and Zhu, 2015).

The HCV non-structural proteins make up the RNA replication machinery within ER derived structures known as the membranous web. Lipid droplets (LD) located at replication sites in the ER membranous web play an important role in viral RNA synthesis. HCV particle assembly involves the E1-E2 glycoprotein complex, recruitment of the LD associated core protein to package viral RNA as well as several viral and host factors. HCV then uses the lipoprotein production pathway to assemble further viral particles and to release them from infected cells (Yang and Zhu, 2015).

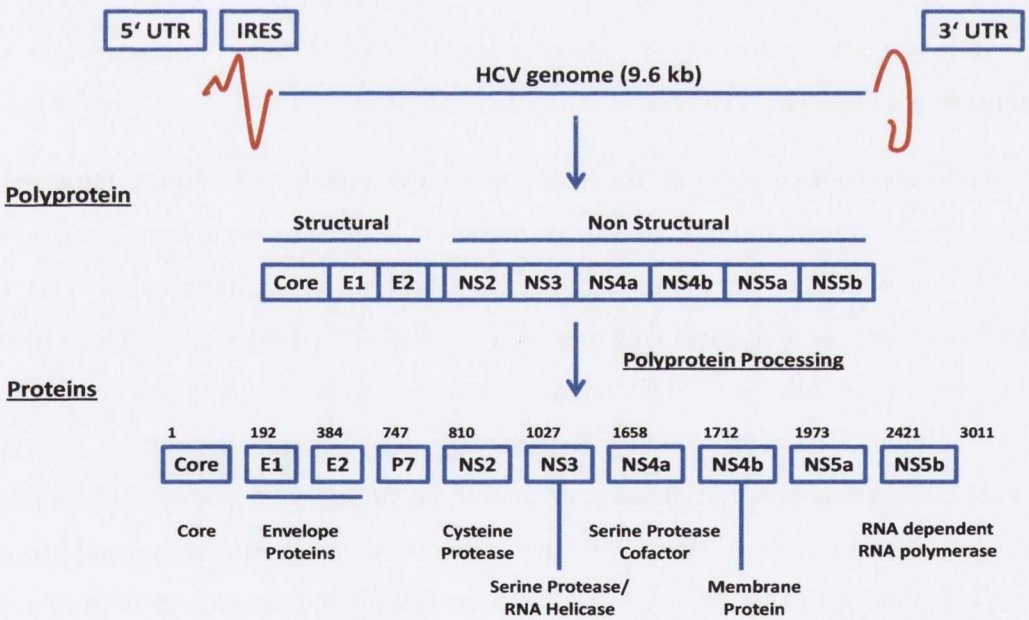


Figure 1.2. HCV genome structure and polyprotein processing. The 9.6.kb RNA genome encodes a single open reading frame for translation of a 3010 amino acid polyprotein. Both viral and host proteases cleave the HCV polyprotein into at least 10 viral proteins (structural and non-structural). Known functions for each viral protein are described (adapted from Poordad and Dietrich, 2012).

1.10. Natural history of HCV infection

Approximately 70%-80% of HCV infected individuals will develop chronic infection which can lead to serious liver disease, including liver fibrosis and scarring (cirrhosis) and hepatocellular carcinoma (HCC). The remaining 20% of individuals infected with HCV will spontaneously clear the virus. Clearance of virus in the acute phase of infection is largely asymptomatic and remains largely undetected in most cases. Identification of individuals who have resolved infection spontaneously can therefore prove difficult. (Hoofnagle J., 2002, Lee et al., 2014).

The majority of new cases of HCV infection are people who inject drugs and there is also a growing incidence of sexually transmitted infection among HIV positive men who have sex with men. The issue of HCV re-infection therefore remains a significant issue. Other populations at risk of HCV infection include patients who received blood transfusions, blood products or anti-D immunoglobulin before 1990 (before routine testing of blood products for HCV), patients with tattoos or body piercings, health care workers and dialysis patients. Estimates indicate that there are 3-4 million newly infected with HCV every year. Approximately 350,000 people die from HCV related disease each year (Westbrook and Dusheiko, 2014).

HCV replicates continuously in the liver and progressively establishes intra-hepatic infection (Westbrook and Dusheiko, 2014). Initial HCV infection is characterised by flu-like symptoms which are common to many acute infections. A smaller number of patients develop more specific symptoms which are characteristic of viral hepatitis. These include jaundice, dark urine, anorexia and abdominal discomfort. Approximately one third of HCV infected individuals develop jaundice. Symptomatic HCV infection has been associated with viral clearance without progression to chronic infection. HCV genotype and mode of acquisition are also factors in determining the course of HCV infection (Chen and Morgan, 2006). Detectable levels of HCV RNA have been identified after just 60 days of initial detection of viremia, however HCV RNA can remain undetectable in some individuals for up to 1 year post infection. In those who resolve HCV infection, the amount of time required for viral clearance has been found to vary significantly among individuals. Resolvers of infection have been reported to clear HCV RNA between 94 and 620 days after initial infection (Westbrook and Dusheiko, 2014).

Chronic HCV infection is the leading cause of end-stage liver disease, hepatocellular carcinoma and liver related death in the Western world. Chronic HCV infection is defined as six months of persistently detectable HCV RNA within the blood. In most cases chronic infection progresses slowly and is characterised by persistent inflammation of the liver. Up to 20% of chronic HCV patients develop liver cirrhosis within 30 years of HCV infection however the reported rates of progression vary significantly between HCV cohorts. A significant number of people only become aware of their HCV infection when they present with end-stage liver disease. Cirrhosis can remain asymptomatic for many years in some individuals while in other it is found to progress quickly, leading to HCC and hepatic decompensation. Once cirrhosis has developed, there is a reported 1-5% annual risk of HCC and a 3-6% annual risk of hepatic decompensation. Following decompensation of the liver, there is a 15-20% risk of death in the following year (Westbrook and Dusheiko, 2014).

Fibrosis progression is highly variable and several factors have been associated with increased risk of developing HCV related liver fibrosis and cirrhosis. These include age at infection, male gender, alcohol consumption, obesity, insulin resistance, type 2 diabetes, co-infection with hepatitis B or HIV, immunosuppressive therapy and genetic factors. Due to the large number of variables which influence disease progression it is difficult to predict prognosis and the effectiveness of treatment at various stages and patterns of disease. Further understanding of the natural history of HCV and its long-term consequences will enable more informed decisions regarding monitoring the need and urgency for treatment and the risks and benefits of treatment (Westbrook and Dusheiko, 2014).

Chronic infection with HCV can also result in several extra-hepatic manifestations including sicca syndrome, lichen planus, type 2 diabetes and non-Hodgkin's lymphoma. Fifteen to thirty-five percent of chronic HCV patients will develop clinical symptoms such as mixed essential cryoglobulinemia, systemic vasculitis, peripheral neuropathy, Raynaud's phenomenon and membranoproliferative glomerulonephritis. The risk of developing a non-Hodgkin's B cell driven lymphoma is also increased in these patientes, this is likely due to chronic B cell stimulation by the HCV. HCV is also reported to influence several metabolic pathways with patients more likely to develop insulin resistance, type 2 diabetes and increased rates of vascular disease (Westbrook and Dusheiko, 2014).

1.11. Recent advances in HCV treatment

Until recently the standard of care for treatment of HCV infection involved a protracted course of a pegylated IFN-α therapy, often administered in combination with the nucleoside inhibitor ribavirin. This treatment can result in significant side-effects and the effectiveness of this treatment has been found to vary significantly depending on HCV genotype (Preciado et al., 2014). Newly developed Direct Acting Anti-virals (DAA) which target specific viral proteins have been approved for HCV treatment since 2011. These IFN free therapies are extremely effective and have dramatically improved treatment responses and side-effects for patients. However, these drugs are prohibitively expensive for many, particularly in the developing world (Preciado et al., 2014). Due to these cost issues, US state Mediaid programmes have restricted DAA therapy to those with advanced liver fibrosis (Armstrong, 2014). Decisions regarding whether to treat immediately with less costly IFN treatment will therefore become an increasingly significant issue for patients and clinicians in the future. Delaying treatment until IFN-free options become available is not without risk of developing increasingly severe symptoms of HCV infection including fatigue and abdominal pain as well as progressive liver disease and HCC (Pho et al., 2015).

There are three major classes of DAAs which have been developed to date: NS3/4a protease inhibitors, NS5b polymerase inhibitors and NS5a inhibitors. The NS3/4a protease inhibitors were the first DAA to be developed and act by inhibiting the protease which is responsible for polyprotein cleavage, thereby blocking viral replication. First generation protease inhibitors include teleprevir and boceprevir which were approved for use in 2011. This was followed by simeprevir which was approved in late 2013 and has an improved safety profile. However all three protease inhibitors are only effective against HCV genotypes 1 and 4. Resistance to these drugs is also a significant issue. The NS5B polymerase inhibitors developed to date include nucleotide/nucleoside analog inhibitors which get incorporated into viral nucleic acid during replication, blocking the replication process. One such drug, sofosbovir, was approved for use in 2013. NS5a inhibitors also inhibit viral replication and assembly; however the exact function of the NS5a protein is unknown. Several NS5a inhibitors are currently in clinical trial and are expected to be approved in the near future (Dhingra et al., 2014).

The effectiveness of HCV treatment is measured by sustained virological response (SVR). SVR is defined as the absence of HCV RNA after cessation of antiviral therapy. SVR has

historically been tested at 24 weeks after stopping therapy but evaluation of SVR at 12 weeks post therapy is now commonplace in HCV clinical trials. Treatment with pegylated IFN-α and ribavirin has been found to have a SVR rate of just 40% in patients infected with HCV genotype 1 and 75% in those infected with genotype 2 or 3. IFN based therapy is also associated with significant side effects including fatigue, flu-like symptoms and depression. Following the approval of teleprevir and boceprevir, triple therapy of protease inhibitor + pegylated IFN-α + ribavirin was found to increase the SVR rate to 75% in genotype 1 patients. Unfortunately however the use of this treatment regimen has been limited due to a high number of adverse events which has resulted in a significant number of patients ending therapy early (Poordad et al., 2011).

Twelve weeks of sofosbovir based triple therapy is now the treatment of choice for those infected with HCV genotype 1 who are eligible to receive IFN treatment. An SVR rate of 89% in treatment naïve (genotype 1 infected) patients has been reported following this triple therapy treatment regimen. Data collected to date appear to suggest that DAA are most effective in those infected with HCV genotype 2. Sofosbovir + ribavirin therapy is the current standard of care for these patients (97% achieved SVR after 12 weeks of treatment). HCV genotype 3 has now become the most difficult to treat. Treatment with sofosbovir + ribavirin for 24 weeks is currently recommend for these patients however SVR rates are remain low in comparison with other HCV genotypes, particularly in patients who have failed to respond to previous IFN based therapy (60% achieved SVR after 24 weeks of treatment). Ongoing clinical trials aim to develop more effective DAA treatments for use in patients infected with this genotype (Dhingra et al., 2014).

1.12. Cellular immune response to HCV infection

Typically, HCV remains asymptomatic and undiagnosed in humans until alanine transaminase (ALT) levels rise, usually 8-12 weeks after initial infection. At this time, HCV specific antibodies and T cells also become detectable (Rehermann, 2009). T cells have been reported to have both protective and pathological roles in HCV infection. T cell IFN-γ and cytotoxicity responses appear to be an important component of a successful immune response to HCV. Early induction of IFN-γ production by T cells of the liver appears to be a key component of successful resolution of acute HCV infection (Rehermann, 2009, Rehermann, 2013). IFN-γ production results in inhibition of HCV

replication without killing the infected cell. T cell expression of perforin and successful induction of hepatocyte apoptosis has also been associated with resolution of infection in chimpanzee models of HCV infection (Watanabe et al., 2010).

Self-limited infection is characterised by broadly directed and sustained T cell responses which target a wide range of HCV epitopes. These early T cells responses have been found to decline quickly in those who subsequently develop chronic infection. HCV specific $CD4^+$ T cells in particular, are essential for the generation of a successful HCV specific immune response. Robust proliferation of HCV-specific $CD4^+$ T cells and IFN-γ production at the time of ALT elevation/clinical presentation has been associated with viral clearance. Correspondingly, initial $CD4^+$ T cell responses are found to be absent or weak in those who subsequently develop chronic infection (Dustin et al., 2014). Depletion of $CD4^+$ T cells *in vivo*, from HCV- recovered chimpanzees negated the protective effect of $CD8^+$ T cell mediated immunity upon re-challenge. This indicates that $CD4^+$ T cells are required for generation and maintenance of protective $CD8^+$ T cells in HCV infection (Grakoui et al., 2003). Importantly however, the presence of robust T cell responses after spontaneous clearance of HCV virus does not always confer protection against subsequent re-infection (Bukh et al., 2008, Schulze Zur Wiesch et al., 2007).

Adaptive immune responses against HCV are induced only weeks after infection even in cases of self-limiting infection. This delay may be due to viral strategies that reduce the impact of the innate immune response, limiting induction of IFN and other cytokines within the liver. Adaptive immunity appears to partially control HCV infection in those with chronic infection. HCV patients who are immuno-compromised or immuno-suppressed are found to develop more severe liver disease (Seeff, 2009). However, these immune responses can be misdirected and result in pathogenesis, liver damage in acute infection is attributed to T cell rather than virus mediated damage. In chronic infection, much of the inflammatory T cell response is not HCV specific (Klenermann and Thimme, 2012).

Chronic infections are characterised by reduced expression of the IL-7Rα chain CD127 on HCV-specific $CD4^+$ T cells. Individuals with chronic HCV infection have a higher proportion of $CD4^+$ T cells expressing Tim3 in comparison with those who clear infection. HCV specific $CD4^+$ T cells express higher levels of the inhibitory co-receptor PD-1. This attenuation in the $CD4^+$ T cell response contributes to a high level of $CD8^+$ T cell exhaustion in chronically infected patients due to a lack of T cell help. A lack of cytokine

survival signals (IL-2, IL-7, IL-15 and IL-21) also contributes to $CD8^+$ T cell exhaustion. This $CD8^+$ T cell exhaustion is characterised by expression of the inhibitory co-receptors such as PD-1, Tim3, Cytotoxic T Lymphocyte Associated protein (CTLA-4) and reduced expression of CD127 (Dustin et al., 2014).

1.13. The impact of host immune responses on HCV viral evolution

HCV replicates to high viral titre loads using an error prone RNA polymerase resulting in the generation of a group of related but genetically distinct viral variants or quasispecies. This genetic diversity and the high rate of genetic evolution presents challenges in vaccine design (Von Hahn et al., 2007). Selection of particular viral variants can provide escape from both humoral and cellular adaptive immune responses. In this way HCV has been shown to escape immune pressure from CTL and neutralising antibody in acute infection. However, the virus must balance the benefits of immune escape with any potential effect on viral fitness and replication (Timm et al., 2004, Dowd et al., 2009, Ray et al., 2005)

Functional $CD4^+$ and $CD8^+$ T cells, as well as NK cells, are thought to be the main components involved in the cellular immune response to HCV infection. Previous studies have investigated the relationship between MHC class I alleles and viral outcome in the Irish Anti-D cohort of HCV infected patients. These investigations have identified the HLA-A*03, HLA-B*27 and HLA-Cw01 alleles as significant determinants of viral clearance (McKiernan et al., 2004). Unlike other protective alleles, HLA-A*03 is a prevalent allele present in apporoximately 20% of the Caucasian population. HLA-A*03 is recognised by $CD8^+$ cytotoxic T cells (CTL) as well as NK cells. A novel T cell epitope (NS3 1080-1088), which is restricted by HLA-A*03 and is commonly targeted by women in the Irish Anti-D cohort has been identified by Fitzmaurice et al., 2011. $CD8^+$ T cell responses targeted against this epitope have been identified in 60% of patients, three decades after infection. $CD8^+$ T cell immune pressure has been found to result in selection of a common escape mutation within the epitope, resulting in a loss of viral fitness. A second prevalent mutation in this epitope is required to restore viral fitness. The requirement for two mutations could potentially explain the protective effect of HLA-A*03 (Fitzmaurice et al., 2011).

Antigen-specific recognition of virally infected or malignant cells is generally mediated by cytotoxic $CD8^+$ T-cells and is dependent on presentation of those viral or tumour antigens on MHC class I molecules. This has led to many pathogens evolving mechanisms to interfere with MHC expression in an attempt to evade T cell recognition. This manipulation of the host by viruses can range from interfering with antigen processing to direct blocking of HLA class I (Tortorella at al. 2000). It is therefore likely that NK cells are vital to the host's counteraction of many of the evasive mechanisms employed by viruses.

1.14. NK cells and the immune response to HCV infection

NK cells are part of the first line of defence against viral invasion following HCV infection, recruiting virus specific T cells and inducing the anti-viral response in the liver through rapid induction of cytotoxicity and release of cytokines. It has previously been reported that NK cells, co-cultured with the HCV replicon-containing hepatic cells, release soluble factors such as IFN-γ which suppress HCV RNA expression. Supernatants collected from primary cell cultures isolated from healthy donors as well as NK cell lines (NK-92 and YTs) have been shown to inhibit HCV replicon expression at both RNA and protein level (Li et al., 2004).

IFN-γ is a crucial anti-viral cytokine during early stages of infection which regulates gene transcription to aid viral clearance (Platanias and Fish, 1999). $CD56^{bright}$ NK cells from chronic HCV patients have been shown to be increased in number and to produce increased IFN-γ when compared HCV resolvers (Golden-Mason et al., 2008). The role of IFN-γ in NK cell mediated anti-HCV activity is further supported by the observation that antibodies to IFN-γ or IFN-γ receptors abolished the anti-HCV activity of NK cell supernatants (Li et al., 2004).

Abnormal NK cell function and abnormal receptor expression have been reported in HCV infection. Cell contact with HCV infected cells has been found to reduce NK cell functional capacity (Yoon et al., 2011). In addition, HCV infected cells have been shown to downregulate NKp30 and impair *ex vivo* NK cell functions (Holder et al., 2013). Freshly isolated blood and liver derived NK cells from chronic HCV patients have been reported to have significantly lower expression of NKp30 and NKp46 compared with healthy controls.

This study also showed that individuals with chronic HCV infection have higher frequencies of NK cells expressing NKG2A (Nattermann et al., 2006). In contrast, $NKG2A^+$ NK cells have also been shown to be associated with a positive response to HCV treatment (Harrison et al., 2010).

It has been shown that several genes encoding NK cell KIR receptors directly influence spontaneous as well as treatment induced resolution of HCV infection. Several studies have identified further genetic factors involved associated with outcome of HCV infection (Golden-Mason et al., 2011, Knapp et al., 2010, Suppiah et al., 2011 and Khakoo et al., 2004). Khakoo et al. (2004) has suggested that inhibitory NK cell interactions are likely to be important in determining antiviral immunity and that diminished inhibitory responses confer protection against HCV. This study identified genes encoding for KIR2DL3 and its ligand HLA-C1 to be directly influence resolution of HCV infection. This effect was in Caucasians and African Americans with low doses HCV but not in those with high dose of exposure (Khakoo et al., 2004). In addition, the expression of KIR3DL1 has been found to be decreased in HCV infection suggesting that this receptor may also be important in the prevention of development of chronic disease following HCV infection (Oliviero, 2009).

Further studies, carried out in the Gardiner lab have identified further NK cell genes to be involved in predicting risk of chronic HCV infection in the Irish Anti-D cohort (Dring et al., 2011). *KIR2DS3* gene frequency was found to be increased in chronically infected patients in this cohort. Although not reaching statistical significance, *KIR2DL5* was also more frequent in chronically infected patients. *KIR2DS3* was also found to predispose to chronic HCV infection in the presence of the *HLA-C2*. HLA-C2 is a KIR binding epitope, recognised by some KIR receptors though not KIR2DS3. Interestingly, a single centromeric KIR haplotype containing *KIR2DS3* and *KIR2DL5* was significantly increased in patients with chronic infection compared with resolved patients ($p=0.002$). As both of these genes are associated with a B type haplotype it is suggested that certain B type haplotypes may be increased in frequency in patients with chronic infection. This study also identified a SNP, adjacent to the *IL28B* gene as significantly associated with chronic HCV infection in the Irish Anti-D cohort. Two risk factors for development of chronic infection, *KIR2DS3* and the *IL28B-T* allele were found to synergise to increase the risk of developing chronic HCV infection (Dring et al., 2011).

This genetic polymorphism near the *IFNL3(IL28B)* gene which is associated with HCV infection outcome, in cohorts of both European and American descent (Dring et al., 2011,

Tillman et al., 2010, Ge et al., 2009). The functional effects of these IFNs are unknown and further research is required to fully establish the role of IFNs in HCV infection.

The factors which define a successful and protective immune response to HCV have not yet been fully elucidated. Further study to fully identify the host factors which determine the effectiveness of an individual's immune response to both HCV infection and vaccination will be required in order to develop improved therapies and vaccines directed against HCV. The study presented here attempts to address this gap in current knowledge. In particular, the role of NK cells in a successful immune response to HCV remains unclear. Given previous published evidence, we hypothesised that the NK cell immune response plays a significant role in determining clinical outcome following exposure to HCV. In order to address this hypothesis, several NK cell phenotyping, genotyping and functional studies were carried out to further examine the role of NK cells and specific NK cell receptors in recognition and outcome following HCV infection and anti-HCV immunisation. KIR gene frequency in those who developed chronic HCV infection and those who cleared infection were compared. The study described here also investigated a possible role of NK cells in the immune response induced following administration of a candidate HCV vaccine capable of inducing robust and HCV specific T cell responses.

1.15. Aims

The aims of the research detailed in this thesis were:

- To investigate the potential contribution of NK cell responses, mediated by the NK cell receptor KIR3DL2, in conferring the protective effect associated with the HLA class I allele *HLA-A*03* in the Irish Anti-D cohort of women infected with HCV.

- Investigate the effects of HCV peptide variation and receptor polymorphism on the level of KIR3DL2 binding to peptide tetramers complexed to its HLA-A*03 ligand.

- Investigate whether innate immune genes, including KIR receptor genes and their HLA ligands are associated with outcome in HCV viral infection.

- Investigate possible induction of NK cell activation and priming following administration of a candidate HCV vaccine.

Chapter 2

Materials and Methods

2.1. Study cohorts

2.1.1. Healthy Normal Donors

Human peripheral blood mononuclear cells (PBMC) were isolated from normal healthy donors, in the Trinity Biomedical Sciences Institute (T.B.S.I.) after informed written consent for donation of blood was provided. A 21 gauge butterfly needle was injected into the median cubital vein and peripheral blood was collected into tubes coated with the anticoagulant Lithium Heparin. All whole blood was processed in a Class II biological safety cabinet in a designated tissue culture suite. Ethical approval for collection of, and research on, venous blood was obtained by Dr. Clair Gardiner, School of Biochemistry and Immunology, Trinity College Dublin.

2.1.2. Paediatric cohort of post renal transplant patients

Fourteen paediatric patients who received a renal transplant at Children's University Hospital, Temple Street were included in this study. All patients had previously tested positive for at least one of two known ligands for the NK cell receptor KIR3DL2 i.e. HLA-A*03 or HLA-A*011. All patients had tested positive for previous or current infection with EBV. This study cohort was comprised of 10 patients who resolved EBV infection and 4 with chronic high viral load titres of EBV. This cohort included 5 female and 9 male paediatric patients with an age range of 6 – 16 years of age at the time this study was carried out. The age at the time of renal transplant surgery ranged from 1- 12 years of age. The time since renal transplant surgery ranged from 2-9 years. 3 patients received a transplant organ from a CMV positive donor. Further background information and clinical history for these patients is detailed in Appendix Table 1. Peripheral blood from this cohort was collected in Lithium-Heparin coated blood tubes in the Children's University Hospital, Temple Street, Dublin according to a protocol set out by hospital. PBMC were isolated from the 14 patients in this cohort. All whole blood was processed in a Class II biological safety cabinet in a designated tissue culture suite (T.B.S.I.). Ethical approval for collection of, and research on, venous blood from this cohort was obtained from the Ethics Committee, in the Children's University Hospital, Temple Street.

2.1.3. German Anti-D cohort of HCV infected women

DNA samples from 425 women from the larger German Anti-D cohort of HCV infected women were received from Dr. Thomas Berg and Dr. Janett Fischer (University of Leipzig, Germany) for use in this study. All women in this cohort were iatrogenically infected with HCV between 1978-1979 when 14 batches of anti-D immune globulin, contaminated with HCV genotype 1b from a single erythrocyte donor, was administered throughout East Germany for prophylaxis of rhesus isoimmunisation in pregnancy. These women have been prospectively followed in regional centres and a significant amount of background and clinical information has been documented on these patients. This study cohort included 302 women who became chronically infected with HCV and 102 who naturally resolved the infection. Additional background and clinical information on this cohort, including treatment outcome and current diagnosis, is detailed in Appendix Table 2. Results of HLA-A and HLA-B typing, previously carried out in these patients is also detailed in Appendix Table 3. Ethical approval for collection of samples from, and research on, this cohort of HCV infected patients was obtained from the University of Leipzig, Germany.

2.1.4. Healthy participants in a HCV vaccine trial (Oxford, U.K.)

PBMC from healthy participants in a phase 1 HCV vaccine trial, being led by the University of Oxford, were received from Eleanor Barnes, University of Oxford, U.K. for use in this study. The vaccine vectors being tested expressed NS proteins from HCV genotype 1b. Two of the vaccines being tested were based on rare serotypes of Adenovirus: Human Adenovirus 6 (Ad6) and Chimpanzee Adenovirus 3 (ChAd3). A third vaccine which was tested as part of this study was MVA vector based. Human PBMC samples from two cohorts of participants taking part in this study were examined as part of this study. The two cohorts received differing vaccine regimens. One cohort (HCV 001 cohort) received prime vaccination with either Ad6 or ChAd3 vector. The second cohort (HCV 003) received prime vaccination with ChAd3 vector which was followed 8 weeks later by boost vaccination with an MVA vaccine vector. The candidate HCV vaccines involved in this study were developed by Okairos. Vaccines were administered intramuscularly and all volunteers gave written informed consent prior to participation. These studies were conducted according to the principles of the Declaration of Helsinki and in accordance with good clinical practice. This clinical trial was ethically approved and registered with

the University of Oxford as clinical trial EudraCT N. 2007-004259-12 and with the ClincialTrial.gov database (ID: NCT01070407).

2.2. Cell culture

2.2.1. Growth and Maintenance of KIR3DL2 and LILRB1 expressing cell lines

All cell lines were maintained in RPMI-1640 (Roswell Park Memorial Institute) medium supplemented with 10% (v/v) FCS, 100U/ml Penicillin and 100ug/ml streptomycin. RPMI-1640 cell media, Penicillin, Streptomycin and Phosphate Buffered Saline (PBS) were purchased from Gibco (supplied by Invitrogen). Fetal Calf Serum (FCS) was purchased from Analabs.

Baf/3 Parental and Baf/3 LILRB1 transfectants for use in tetramer binding assays, as negative and positive controls respectively, were a gift from Veronique Braud, Institut de Pharacologie Moléculaire et Cellulaire, Université Nice, France.

Baf/3 cell lines (KIR3DL2 and LILRB1 transfectants) were supplemented with murine IL-3 (10ng/ml) and Blasticidin (15µg/ml) as a selective agent. Recombinant Murine IL-3 was purchased from ebioscience. Blasticidin S. Hydrochloride was purchased from Sigma. Cells were maintained in a humidified environment at 37°C and at 5% CO_2.

2.2.2. Cryopreservation of cells

For cryopreservation, cells were resuspended in freezing media (90% FCS, 10% Dimethyl Sulfoxide (DMSO) (Fisher Scientific) at a concentration of approximately 8 million/ml. This solution was aliquoted at 1ml/cryotube. Cryotubes were transferred to -80°C overnight and moved to liquid nitrogen for long-term storage.

For resuscitation, vials were removed from liquid nitrogen storage. Cells were thawed by pipetting small volumes of complete medium (heated to 37°C) into the cryovial and placing the cells in a 50ml volume of complete medium using a T75 tissue culture flask.

2.2.3. Isolation of Peripheral Blood Mononuclear Cells (PBMC) from whole blood

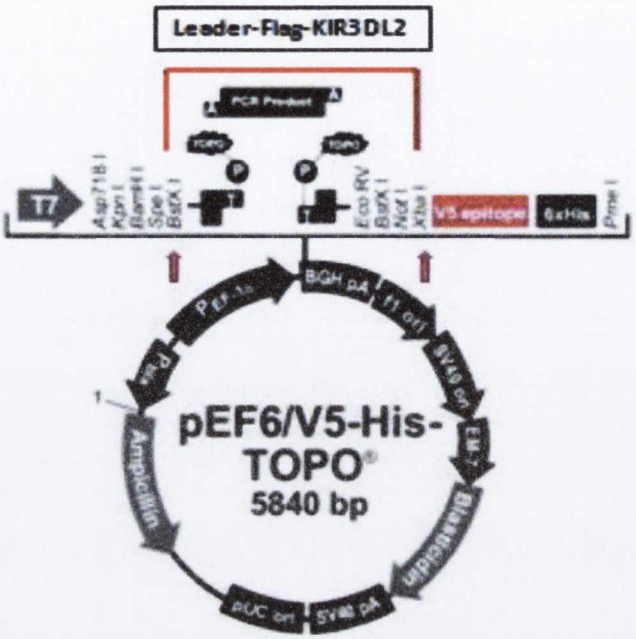
Human peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Lymphoprep Density Gradient Medium purchased from Biosciences. In brief, whole blood was diluted 1 in 2 in PBS followed by layering blood gently onto Lymphoprep, taking care to keep a sharp interface. Tubes were then centrifuged at 1200 rpm for 30 minutes, with no brake applied. The cloudy layer of lymphocytes was removed carefully; lymphocytes were washed with PBS before being resuspended in 50ml Red Blood Cell Lysis Buffer (155mM NH_4Cl, 10mM KCO_3 and 0.1mM EDTA). At both stages tubes were centrifuged at 1400 rpm for 10 minutes and the supernatant discarded. Finally, cells were washed in complete RPMI media for cell culture or FACS staining buffer (1% FCS in PBS).

2.3. Generation of stable highly expressing KIR3DL2 FLAG-Tagged Transfectants

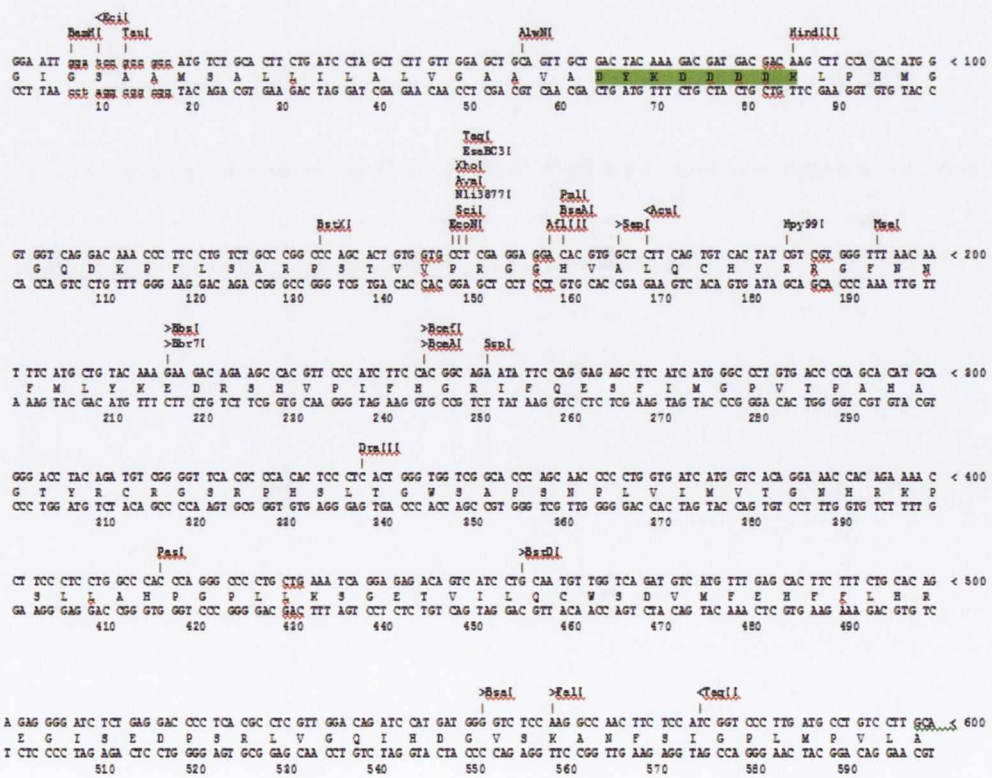
Stable Baf/3 transfectants expressing various KIR3DL2 alleles were produced using a Lonza Nucleofector kit V for transfection of DNA from five FLAG-Tagged plasmids containing inserts specific to 5 distinct alleles of the KIR3DL2 receptor (KIR3DL2*001, KIR3DL2*002 KIR3DL2*003 KIR3DL2*007 and KIR3DL2*009) were received from Geraldine O'Connor (University of Melbourne). These inserts had been incorporated into pEF6/V-His-TOPO vector after restriction digestion with BstXI and Xba1. This plasmid also contained Ampicillin and Blasticidin as bacterial and mammalian resistance markers respectively (Figure 2.1. (a)). A plasmid map of the pEF6/V5-HisTOPO vector illustrating the position of the Leader-FLAG-KIR3D2 insert and the antibiotic resistance genes present on the plasmid is detailed in Figure 2.1. (a). A detailed restriction map indicating sites of restriction enzyme digestion is also shown (Figure 2.1. (b)).

As part of this research study, plasmid DNA was fully sequenced before transfection into Baf/3 cells and sequencing results are detailed in Figure 3.2. Expression of NK receptors was monitored by flow cytometry using anti-KIR3DL2 (DX31) and anti-LILRB1 (VMP55) for cell surface staining. All cell lines were sorted using a Mo-Flo sorter to obtain cell stocks with high frequency of KIR3DL2 expression.

(a)


Leader-Flag-KIR3DL2
PCR Product
TOPO
T7
V5 epitope
6xHis
pEF6/V5-His-
TOPO®
5840 bp
BGH pA
f1 ori
SV40 ori
EM-7
Blasticidin
SV40 pA
pUC ori
Ampicillin
pEF-1α
Ori

(b)


BamHI
TaqI
AlwNI
HindIII
TaqI
EsaBC3I
XhoI
AvaI
Nli3877I
ScaI
EcoNI
PaeI
BsaAI
AflIII
SapI
AccI
Hpy99I
MseI
BstXI
BbrPI
BbvI
BsmFI
BsmAI
SapI
DraIII
PasI
BstXI
BsaI
BsaI
TaqII

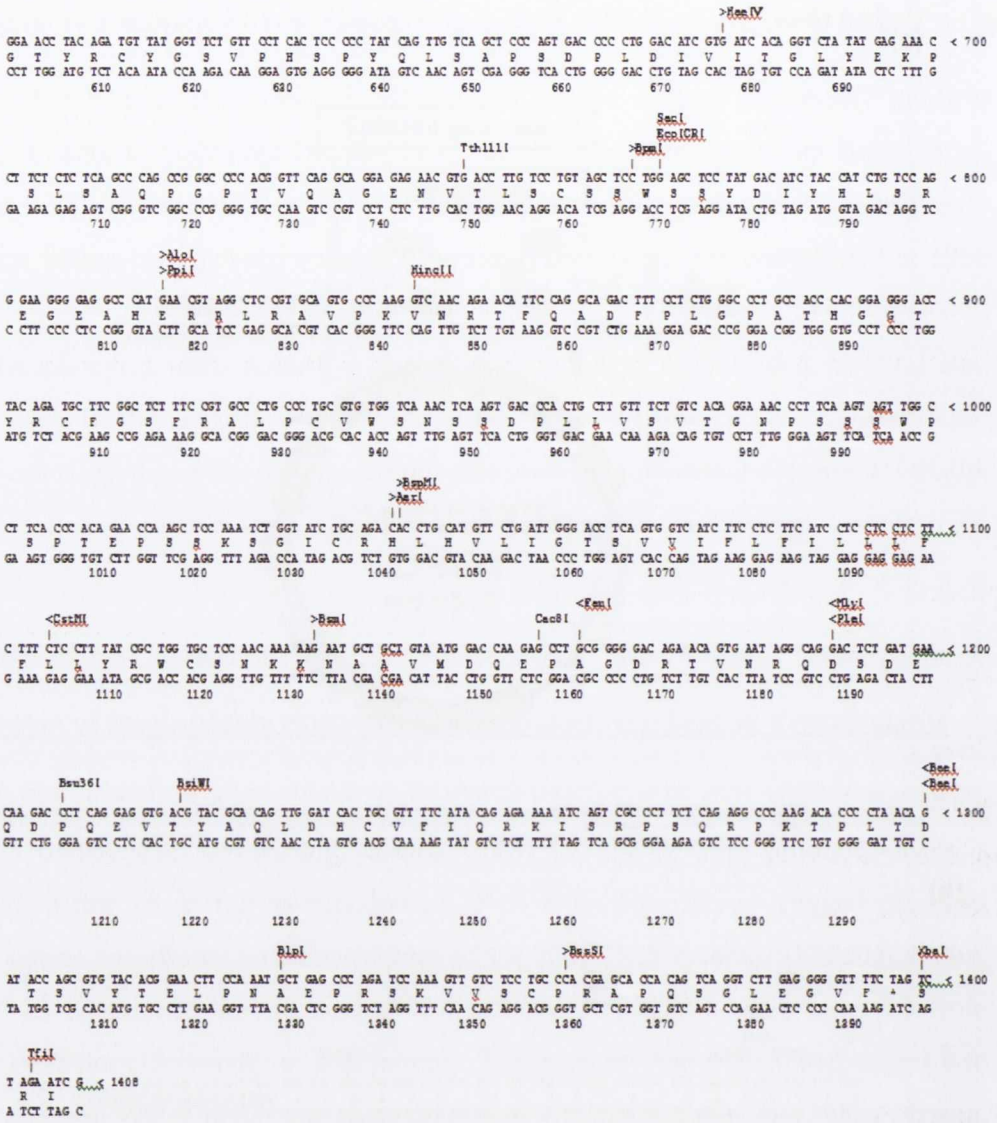


Figure 2.1. Plasmid mapping of pEF6/V5-HisTOPO plasmids. The location of the Leader-FLAG-KIR3DL2 insert and antibiotic resistance genes are illustrated in Figure 2.1.(a). A restriction map, detailing the sites of restriction enzyme digestion with the FLAG tag sequence highlighted in green (Figure 2.1.(b)).

2.4. Flow Cytometry

2.4.1. Flow Cytometry Materials

Anti-human LILRB1 FITC (Clone 292305) was purchased from R&D Systems.

Anti-human KIR3DL2 (unconjugated) was a gift from Dr. Daniela Pende (Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy).

IgM isotype control antibody was purchased from Biolegend.

PE conjugated secondary antibody (Goat $F(ab')_2$ Fragment Anti-Mouse IgM (μ)-PE) was purchased from Beckman-Coulter.

Customised HCV peptides were purchased and synthesised by Schafer-N (Denmark).

Fluorescently labelled (PE and BV-421 labelled) HLA-A*03 bound peptide tetramer were synthesised by our collaborators in the Laboratory of Experimental Immunology, Institute for Medicinal Microbiology and Immunology, Panum Institute, University of Copenhagen, Denmark.

Anti-CD3 BV605 (Clone OKT3) and anti-Ki67 PE (Clone Ki-67) were purchased from Biolegend

Anti-CD56 PE-Cy5 (Clone B159), anti-CD57 FITC (Clone NK-1), anti-Granzyme B AF700 (Clone GB11), anti-IL-2 PE-CF594 (Clone 5344.111), anti-CD25 V450 (Clone M-A251), anti-IFN-γ PE-Cy7 (Clone B27), and anti-CD107a APC (Clone H4A3) were purchased from BD.

Anti-CD8 APC-eFluor-780 (Clone RPA-T8) was purchased from ebioscience

Anti-Mouse Ig, κ/Negative Control (FBS) Compensation Particles Set was purchased from BD

Live/Dead Fixable Aqua Dead Cell Stain Kit was purchased from Biosciences

2.4.2. Fluorescence Activated Cell Sorting (FACS) of KIR3DL2 transfectants

Highly purified KIR3DL2 expressing Baf/3 cell lines were generated by labelling with anti-KIR3DL2 followed by PE labeled secondary antibody. KIR3DL2 positive cells were isolated and sorted on a DAKO MoFlo cell sorter in the centralised cell sorting facility in T.B.S.I. to result in populations of >95% purity.

2.4.3. Tetramer Binding Assay

HCV peptides for tetramer generation were identified in collaboration with Karen Fitzmaurice (University of Oxford, U.K.). Peptides of interest were purchased from Schafer-N. HLA-peptide tetrameric complexes of HLA-A*03 complexed with EBV or HCV peptides and their common variants were synthesised by our collaborators: Dr. Soren and Anette Buus, University of Copenhagen, Denmark.

5 x 10^5 Baf/3 transfectant cells were spun down and resuspended in 8µl conjugated tetramer and incubated for 25 minutes at 37°C. This was followed by additional surface staining for KIR3DL2 using unconjugated Q66 antibody in order to specifically analyse the receptor positive ($KIR3DL2^+$) population for tetramer binding. Cells were washed twice in staining buffer before being incubated in a 1:5 dilution of Q66 (KIR3DL2) antibody in PBS. Isotype control wells were also prepared by incubating cells in a 1:5 dilution of IgM isotype control antibody in PBS. Cells were incubated at 4° C for 20 mins, protected from light before being washed twice in staining buffer. Cells were then stained with a PE conjugated secondary goat antibody against the heavy chain portion of mouse IgM. A 1:200 dilution of this antibody in PBS was used. Again, cells were incubated at 4°C for 20 minutes, protected from light before being washed twice in staining buffer. After washing, cells were acquired using a BD FACS Canto II and analysed using Flow-Jo software.

2.4.4. Cell surface staining of freshly isolated PBMC from pediatric renal transplant patients

Cells were resuspended at 5x106 cells/ml PBS and aliquoted in 200ul in a round bottomed 96 well plate. Cells were washed twice in staining buffer before being incubated in a 1:5

dilution of Q66 (KIR3DL2) antibody in PBS. Isotype control wells were also prepared by incubating cells in a 1:5 dilution of IgM isotype control antibody in PBS. Cells were incubated at 4°C for 20 minutes, protected from light before being washed twice in staining buffer. Cells were then stained with a PE conjugated secondary goat antibody against the heavy chain portion of mouse IgM. A 1:200 dilution of this antibody in PBS was used. Again, cells were incubated at 4°C for 20 minutes, protected from light before being washed twice in staining buffer. Cells were then resuspended in 50ul Mouse Fc Block for 20 minutes. Following this, Extracellular Antibodies (antiCD3, antiCD56, anti-CD8, anti-NKG2C anti-CD94 and anti-CD57) were added to the cells at a concentration deemed optimal by titration. Cells were then incubated for 20 minutes at 4°C protected from light. Cells were then washed twice with staining buffer and acquired using a BD LSRFortessa.

2.4.5. CD107a cytotoxicity assay and Intracellular Staining of frozen PBMC from HCV vaccine trial

Cryopreserved PBMC from each subject in the longitudinal series were batch processed to reduce the coefficient of variation attributable to antibody staining and cytometric evaluation. Cryopreserved specimens were thawed, washed and resuspended in RPMI medium supplemented with 10% Fetal Calf Serum before incubation at 37 °C for 1 hour.

For T0 analysis, cells were plated at a concentration of $0.5x10^6$ cells/200µl of RPMI medium in a 96 well plate. $0.1x10^6$ 721.221 target cells ($2x10^5$/ml concentration) and anti-CD107a antibody were added to the cells for 5 hours with addition of BD Golgiplug and BD Golgistop after one hour. 721.211 target cells were received from Peter Parham, the protein transport inhibitors, Golgiplug and Golgistop were purchased from BD Biosciences. At the end of this incubation, Live/Dead aqua stain was added to the cells and cells were incubated at room temperature for 20 minutes. From this point samples were protected from prolonged exposure to light. Cells were then centrifuged at 1400rpm for 3 minutes and supernatant was removed. After washing with staining buffer (1% FCS in PBS), cells were resuspended in 50µl blocking buffer (10% human AB serum, 1% FCS in PBS) and incubated at 4°C for 5 minutes. An additional 50µl of staining buffer containing extracellular antibodies (anti-CD56, anti-CD3, anti-CD8, anti-CD25 and anti-CD57 or their respective isotype controls) were then added to the cells and incubated at 4°C for 20

minutes. The cells were again washed twice in staining buffer and then resuspended in 100µl of BD Cytofix/Cytoperm and incubated at 4°C for 20 minutes. The cells were washed twice in BD Perm/wash and resuspended in 100µl Perm/wash containing intracellular antibodies (anti-IFN-γ, anti-IL-2, anti-Granzyme B and anti-Ki67 or their respective isotype controls) and incubated at 4°C for 20 minutes. These cells were washed twice in staining buffer and finally resuspended in 300µl staining buffer.

In addition to the T0 analysis, identical extracellular and intracellular flow cytometry analysis was carried out following incubation of PBMC (for 18 hours, 3 Days or 5 Days) with rhIL-12 + rhIL-18 cytokine combination (12.5pg/ml rhIL-12 + 10ng/ml rhIL-18 or 5ng/ml rhIL-12 + 50ng/ml rhIL-18). Recombinant human IL-12 was purchased from Miltenyi Biotech. Recombinant human IL-18 was purchased from R&D systems.

Samples were analysed using a BD LSRFortessa.

2.4.6. Statistical Analysis

One or Two way analysis of variance (ANOVA), followed by Bonferroni's Multiple Comparison Test was used to test for statistically significant differences between groups using GraphPad Prism 5 software. Error bars represent standard error of the mean (SEM).

2.5. Genotyping of KIR and KIR epitopes

2.5.1. PCR Materials

Sequence specific primers and Internal Control Primers were purchased from Sigma. PCR reagents (Buffer, MgCl2 and Taq polymerase) were purchased from Sigma. Deoxynucleotide triphosphates (dNTPs) were purchased from Biosciences. Double distilled water (ddH_20) was purchased from Sigma. PCR was performed using a PTC200 (Bio-RAD-MJ Research) or a Px2 (Thermo Hybrid). Agarose was purchased from Fisher. 50X TAE buffer was purchased from Sigma. Nancy-520 for gel visualization was purchased from Sigma. Benchtop 1Kb and 100bp DNA ladder were purchased from Promega. Gels were viewed on a UVP GelDoc-It Imaging System (UVP Analytik Jena).

2.5.2. Polymerase Chain Reaction (PCR) Protocol

Patient samples were genotyped for the HLA-C1 and HLA-C2 epitopes and the KIR genes: KIR2DL2, KIR2DL3 and KIR2DS3. Genotyping was carried out using sequence specific primers. Primer stocks were reconstituted at a concentration of 100μM using ddH_20. Working stocks for each set of primers were generated by diluting the primer stocks in ddH_20. These working stocks were at 5X the final concentration used for the PCR so that a subsequent 1:5 dilution performed upon addition to the PCR reaction resulted in the desired concentration. Control primers were included in each reaction to ensure that the quality and quantity of DNA used was sufficient for amplification to occur. The reagents used in the PCR mixes are outlined in Table 2.1. Master-mixes, enough for the number of reactions being performed and containing all the components except the DNA were prepared. Individual DNA samples were pipetted into PCR tubes and 9μl of the Mastermix was added to each sample. Also included in each experiment was a 'No Template Control' (NTC) reaction, where ddH_20 replaced the DNA in the PCR reactions, as well as positive and negative control reactions where DNA of known genotype was tested. Following amplification, PCR products were run on gels consisting of 2% w/v agarose in TAE Buffer (0.04M Tris-Acetate, 0.001M EDTA) to which Nancy-520 had been added. Samples were electrophoresed at 100V for 30 minutes to TAE Buffer and viewed on a UVP Imaging system.

2.5.3. KIR Genotyping

Genotyping was carried out for *KIR2DL2, KIR2DL3*, and *KIR2DS3* using sequence specific primer (PCR-SSP) based on those previously described (Vilches et al. 2007). The reagents used in all PCR reactions are outlined in Table 2.1. KIR genotyping was carried out using sequence specific primers (Table 2.2.). Internal Control primers were included in each reaction to ensure that the quality and quantity of DNA used was sufficient for amplification to occur. These primers amplified non-polymorphic regions of the *HLA-DRA* gene (Table 2.3.). The PCR conditions are outlined below (Table 2.4.). As part of this research study, the PCR protocol for KIR genotyping was validated using DNA of known KIR gene content, supplied by the University of California at Los Angeles as part of the International KIR Exchange (http://www.hla.ucla.edu/cellDNA/Cell/summaryKir.htm).

2.5.4. HLA-C Genotyping

Patients were genotyped for both *HLA-C1* and *HLA-C2* using PCR-SSP methods based on those previously described (Tajik et al., 2010). The reagents used in all PCR reactions are outlined in Table 2.1. HLA genotyping was carried out for *HLA-C1* and *HLA-C2* using sequence specific primers (Table 2.2.). Internal control primers were included in each reaction which amplified non-polymorphic sequences of the Growth Hormone gene (Table 2.3.). The PCR conditions are outlined below (Table 2.5.). As part of this research study, the PCR protocol for HLA genotyping was validated using DNA of known gene content, which was isolated from HLA-typed cell lines. KT12 ($C1^+C2^+$), KT17 ($C1^+C2^+$), BM9 ($C1^-C2^+$), AMAI ($C1^-C2^+$). HLA- C*03:03 DNA ($C1^+C2^-$) was received as a gift from Richard Hagan (Irish Blood Transfusion Service (IBTS)).

Table 2.1. KIR/HLA genotyping PCR reaction mix

Reagent	Volume/reaction (µl)
ddH_20	2.15
5X Green GoTaq Flexi Buffer	2
$MgCl_2$ (25mM)	0.6
dNTP (10mM)	0.2
KIR/HLA Primer	2
Control Primer	2
DNA	1
GoTaq DNA Polymerase	0.05

A total volume of 10µl/reaction was used. A Mastermix comprising of ddH_20, 5X Buffer, $MgCl_2$, dNTP, Primers (including Internal Control primers) and GoTaq was added to 1µl sample DNA.

Table 2.2. Sequence Specific Primers for the identification of KIR and HLA genes

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Conc. each primer/reaction (µm)	Product Size (bp)
KIR2DL2	aaaccttctctctcagccca	gccctgcagagaacctaca	5	142
KIR2DL3	agaccctcaggaggtga	caggagacaactttggatca	5	156
KIR2DS3	cttgtcctgcagctcct	gcatctgtaggttcctcct	2.5	158
HLA-C1	gaggtgcccgcccggcga	cgcgcaggttccgcaggc	1.6	332
HLA-C2	gaggtgcccgcccggcga	cgcgcagtttccgcaggt	1.6	332

Working stocks for each pair of primers were generated by diluting the primer stocks in ddH_20. These working stocks were 5X the final concentration for each primer as outlined above.

Table 2.3. Internal Control for KIR and HLA genotyping

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Conc. each primer/reaction (µm)	Product Size (bp)
HLA-DRA (KIR)	gaggtaactgtgctcacgaacagc	ggtccataccccagtgcttgagaag	0.2	283
IC-2 (HLA)	gccttcccaaccattccctta	gtccatgtccttcctgaagca	1.6	1070

Working solutions for both internal controls were made by performing a 1:100 dilution of 100µM stocks with ddH_20. These working stocks were 5X the final concentration for each primer as outlined above. Internal Control Primer 1 was used in all KIR genotyping reactions. Internal Control Primer (IC)- 2 was used for HLA genotyping.

Table 2.4. PCR cycling conditions for amplification of KIR genes

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	95	2 minutes	1
Denaturation	94	10 seconds	} 10
Annealing	65	40 seconds	
Denaturation	94	20 seconds	} 20
Annealing	61	20 seconds	
Extension	72	30 seconds	
Final extension	72	7 minutes	1
Hold	10	-	

The various steps involved in the thermal cycling conditions of the PCR are illustrated. Temperature and duration of each step was also described. The number of cycles for each step is also included. The various steps involved in the thermal cycling conditions of the PCR are illustrated. Temperature and duration of each step was also described. The number of cycles for each step is also included.

Table 2.5. PCR cycling conditions for amplification of HLA genes

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	94	2 minutes	1
Denaturation	94	10 seconds	10
Annealing	65	60 seconds	
Denaturation	94	10 seconds	20
Annealing	61	50 seconds	
Extension	72	30 seconds	
Hold	10	-	

Various steps involved in the thermal cycling conditions of the PCR are illustrated. Temperature and duration of each step was also described. The number of cycles for each step is also included.

2.5.5. Statistical Analysis

Carrier frequency differences between populations were tested for significance by direct counting. Chi Squared test (χ^2) for statistically significant differences was used to calculate variance between populations and was carried out as implemented by StatCalc software EPI-INFO 3.5.1.

Chapter 3

Differential binding of HLA-A*03 restricted peptides to KIR3DL2

3.1. Introduction

3.1.1. The importance of peptide epitopes in the T cell immune response to pathogen

As previously described (Section 1.1), T cells of the adaptive immune system recognise protein antigens which have been broken down into peptide fragments and bound to membrane-bound glycoprotein receptors. In humans, these receptors are encoded by genes belonging to the HLA complex. The HLA gene complex is highly polymorphic with individuals expressing an average of 10 allotypes of HLA. In order to be presented to the immune system, individual peptides must specifically bind to a particular allotype of HLA. This process is known as HLA restriction (Parham, 2014).

T cell peptide epitopes are normally between 8-11 amino acids in length but epitopes up to 16 amino acids in length have been identified. The structural basis of peptide binding to MHC class I molecules has been extensively studied in mice and humans. The N and C termini of bound peptide interacts with invariant residues at either end of the HLA peptide binding groove. Although binding of peptide to an HLA molecule is dependent on peptide sequence, several key interactions between anchor residues and the HLA peptide binding motif are required for successful complexing of peptide to the HLA molecule. These anchor residues are found to be relatively conserved among peptides which bind the same HLA allotype. Specificity of peptide binding to HLA is dependent on key amino acid side chains on these anchor residues interacting with polymorphic residues in a series of pockets present in the peptide binding groove of the HLA molecule (Parham, 2014, McDonald, 2010).

The majority of peptides which are sampled by the immune system are derived from self-proteins and do not induce any significant immune response. Recognition of peptide by the TCR is therefore highly specific, with a T cell being capable of recognising a single peptide antigen. In order to be detected by the T cell immune response, peptide must tightly associate with the surface of the HLA molecule and T lymphocytes must make direct contacts with both the peptide and the surrounding surface of the HLA molecule through their TCR (Parham, 2014).

The majority of potentially antigenic peptides are removed at various stages of antigen processing and presentation and are never presented at the cell surface for recognition by the immune system. This peptide selectivity is partly derived from peptide-binding

requirements of the MHC HLA peptide binding groove: the majority of pathogen derived peptides are not successfully complexed to an HLA molecule. For HLA class I presentation, specifity requirements also apply during proteosome cleavage and transport into the ER. For MHC class II presentation, endocytic proteases and the B cell receptor are likely to contribute to peptide selectivity. The combined selectivity means that every protein is represented by an average of just less than one peptide on a given MHC isoform. This results in responding immune effector T cells being capable of recognising only a small number of foreign peptide epitopes derived from an infecting pathogen. The benefit of this host strategy is that it allows for efficient sampling of potentially antigenic proteins and allows for presentation of a sample of every protein synthesised in the cell. It is suggested that this strategy derives from the need for an immune response which is reliable as well as self-tolerant. High peptide selectivity and HLA restriction contribute to low foreign presentation diversity (Van den Berg, 2003).

An obvious disadvantage of this host strategy of low presentation diversity is that pathogens may remain completely undetected by the immune system. These immunogenic epitopes are critical for rapid elimination of infected cells and the generation of a pool of memory cells which will provide a more rapid immune response in the event of subsequent re-infection with the same pathogen. However, such epitopes are also a common target of viral immune escape mutations, which are induced by selection pressure and allow the virus to avoid detection by the T immune response (Van den Berg, 2003, Akram et al., 2012). NK cells of the innate immune system are also capable of recognising peptide complexed with HLA class I, providing an effective alternative mechanism for recognition of intracellular pathogens such as viruses (Parham, 2014).

3.1.2. The importance of peptide in the NK cell immune response

Like the TCR, KIR receptors on NK cells have also been shown to bind peptide: MHC class I complexes with distinct thermodynamic and kinetic properties (Maenaka et al., 1999). Peptide specificity has been demonstrated for all inhibitory KIR tested to date as well as some activating KIR. Peptide specificity was originally shown for KIR3DL1 and its HLA-B*27 ligand and then for KIR2DL1, KIR2DL2, KIR2DL3 and KIR3DL2 (Cassidy et al., 2014). KIR3DL2*001 was originally shown to bind specifically to HLA-A*03 and HLA-A*011 but not HLA-A*01, HLA-A*02 or HLA-A*24 (Wagtmann et al., 1995, Doring et al., 1996 and Pende et al., 1996). KIR3DL2 was subsequently found to

bind to HLA-A*03 and HLA-A*011 in a peptide specific manner. KIR3DL2*001 binding to tetramerised peptide complexes of either of these two HLA molecules was found to be highly dependent on the sequence of complexed viral peptide (Hansasuta et al., 2004).

Determination of the co-crystal structure of the KIR2DL2 and KIR3DL1 interaction with HLA class I ligand has previously shown that specific residues of KIR directly contact P7 and P8 residues of bound peptide (Boyington et al., 2000, Vivian et al., 2011). As has been seen with other KIR:HLA class I interactions, positions 7 and 8 of complexed peptide have been found to be of particular importance for promotion of HLA binding to KIR3DL2 (Hansasuta et al., 2004). KIR receptors therefore demonstrate relatively broad peptide specificity in comparison with the fine specificity of the TCR, with NK cell receptors recognising specific peptide motifs rather than individual peptides.

Both KIR3DL1 and KIR3DL2 bind HLA-B27 free heavy chain forms. Interaction of HLA-B27 heterotrimers with KIR3DL1 and KIR3DL2 has been shown to be dependent on the sequence of complexed peptide. Engagement of these receptors was found to inhibit NK cell IFN-γ production in a peptide dependent manner (Kollnberger et al., 2007). Interestingly, binding of KIR3DL1 to its HLA-Bw4 KIR binding motif has also been found to be dependent on KIR3DL1 polymorphism (Thananchai et al., 2007, O'Connor et al., 2007, O'Connor et al., 2014), though this has yet to be shown in the context of KIR3DL2.

Despite both binding to HLA-C1 ligand, KIR2DL2 and KIR2DL3 have been shown to confer different levels of protection against infectious diseases including HCV (Khakoo et al., 2004, Knapp et al., 2010). KIR2DL2 is a higher affinity receptor for HLA-C1 in comparison with KIR2DL3 and it has been suggested different that KIR2DL3$^+$ NK cell have a lower activation threshold and are more easily activated then KIR2DL2$^+$ NK cells. This may play a role in the protective effect of KIR genes in infectious diseases although NK cell functional experiments have shown no difference in inhibition of KIR2DL2$^+$ and KIR2DL3$^+$ NK cells by HLA-C1 (Moesta et al., 2008, Winter et al., 1998).

More recent studies involving these receptors suggest that functional differences in NK cell responsiveness may be related to the peptide presented by MHC class I. It is suggested that differences in the response to changes in peptide content of MHC class I may be one mechanism contributing to the protective effect of different KIR genes in infectious diseases such as HCV. Examination of NK cell functional responses in both KIR2DL2 and KIR2DL3 homozygous donors have found that individuals with different genotypes have different responses to changes in the peptide being presented by MHC class I. KIR2DL3$^+$

NK cells were found to be more sensitive to changes in the peptide content in comparison with KIR2DL2^{+} NK cells, with the result that, changes in peptide repertoire leading to more robust activation of KIR2DL3^{+} NK cells. There is therefore, increasing evidence of peptide specificity in inhibitory KIR interactions (Cassidy et al., 2015).

3.1.3. Peptide antagonism as a mechanism for NK cell activation

NK cell function is determined by a balance of signals from both activating and inhibitory KIR. The blocking of a receptors inhibitory signal can therefore lead to increased NK cell activation. It has been shown that peptides which bind to MHC class I but not KIR can act as altered peptide ligands for the inhibitory KIR2DL2/KIR2DL3, resulting in a corresponding reduction in KIR mediated NK cell inhibition. It is suggested that these peptides act as antagonistic peptides which block inhibitory KIR signalling, resulting in increased NK cell activation (Fadda et al. 2010). This process has been compared to TCR antagonism where antagonistic peptides block activation of CTL by cognate peptide (Fadda et al., 2010).

Inhibition of NK function by inhibitory KIR was also found to be highly sensitive to the presence of antagonist peptides which do not induce NK cell recognition. Three types of peptides have been identified: those which induce strong KIR mediated NK cell inhibition, those which induce low level inhibition and antagonistic peptides which block and are unable to induce NK cell inhibition. The relative quantities of each of these types of peptide as well as the nature of the peptides produced by infection or tumorigensis is therefore likely to affect the level of NK activation *in vivo* (Fadda et al., 2010).

Peptides inducing weak KIR binding were found to antagonise NK cell inhibition resulting in NK cell activation. This antagonistic effect was found to be dependent on the relative concentration of peptides inducing strong or weak KIR binding. Such antagonistic peptides have been shown to induce aggregation of KIR at the interface of the NK and target cells without induction of KIR mediated NK cell inhibition However, only high affinity peptides were capable of inducing inhibition of signalling which requires the formation of tight inhibitory microclusters suggesting that peptide antagonism of NK cells results from disruption of inhibitory synapse formation (Fadda et al., 2010, Borhis et al., 2013).

It is suggested that this sensitivity may allow NK cells to recognise and respond to small changes in the peptide: HLA class I complex presented at the cell surface during viral infection. (Fadda et al., 2010). KIR expressing NK cells have been shown to respond more readily to changes in peptide than to changes in HLA class I expression. This provides NK cells with an additional mechanism for viral recognition in cases where down-regulation of HLA class I molecules is not used as a mechanism for immune escape.

*3.1.4. Key CD8$^+$ T cell epitopes, restricted by HLA-A*03, are commonly targeted in the immune response to HCV*

As previously mentioned (Section 1.7.), the induction of escape mutations is a key mechanism used by virus in order to evade detection by the T cell adaptive immune response. In particular, HCV encodes an error-prone RNA polymerase enzyme which contributes to a high rate of viral mutations, making HCV particularly effective at evading immune recognition (Preciado et al., 2009).

Gene association studies have identified particular HLA alleles, including HLA-A*03 as being significantly associated with viral clearance of HCV infection (McKiernan et al., 2004). Unlike other protective alleles which have been identified, HLA-A*03 is a prevalent allele, present in 20% of Caucasian populations (www.allelefrequencies.net). A subsequent study identified novel T cell HCV epitopes (NS3 (1080-1088) and NS5B (2510-2518)) restricted by HLA-A*03 which are commonly targeted in the immune response to HCV and may contribute to the protective effect of this HLA allele (Fitzmaurice et al., 2011).

This study identified a mutation at position 9 in the NS3 epitope (K1088R) was present in 75% (9/12) of HLA-A*03$^+$ HCV patients compared with just 3% (1/33) in those who are HLA-A*03 negative. A second mutation in this epitope (T1087A) was present in 50% (6/12) of HLA-A*03$^+$ individuals compared with just 6% (2/33) in HLA-A*03$^-$ patients. The presence of both mutations was also detected in a significantly higher number of HLA-A*03$^+$ patients (6/12 (50%) compared with 1/33 (3%) in HLA-A*03$^-$ individuals). A mutation in the NS5B epitope (K22518R) was also observed in a significant number (8/15 (53%)) of HLA-A*03$^+$ patients compared with HLA-A*03- patients (2/45 (4%)). Using peptide specific cell lines, 6/10 patients with resolved infection were found to mount CD8$^+$

T cell responses to the NS3 (1080-1088) epitope by tetramer analysis (Fitzmaurice et al., 2011).

These results provide evidence that these HLA-A*03 restricted peptide epitopes are important targets of the T cell response *in vivo* following HCV infection. Targeting of such epitopes is likely to contribute to the protective effect of HLA-A*03 in HCV infection. One of the key immune evasion strategies of HCV is the development of escape mutations in key immuno-dominant epitopes which are highly recognisable by the immune system. Escape mutations may therefore provide information about the nature of the T cell response in infection cohorts (Fitzmaurice et al. 2011). In this cohort, the HLA-A*03 restricted NS3 (1080-1088) epitope was found to be targeted by $CD8^+$ T cell responses in those who resolved infection. Immune pressure imposed by $CD8^+$ T cells appears to drive the selection of an escape mutation at the key anchor site within this epitope (K1088R).

HCV replicon studies showed reduced replication efficiency in K1088R mutants in comparison with wild type virus. Normal replication efficiency was found to be restored in mutants with an additional second common mutation (T1087A), suggesting this second mutation effectively compensates for loss in viral fitness. It is suggested that the requirement for two mutations in this epitope in order to balance fitness and escape provides an advantage to the host which may contribute to the protective effect of HLA-A*03 alleles in HCV infection (Fitzmaurice et al., 2011).

HLA-A*03 molecules are also recognised by the NK cell receptor KIR3DL2. In undertaking this study we therefore hypothesised that NK cells and T cells may display differential recognition of HCV peptides presented on a HLA-A*03 background.

*3.1.5. HLA-A*03 restricted peptides are also recognised by the KIR3DL2 receptor on NK cells*

In addition to presenting peptide to TCR, HLA-A*03 is also a ligand for the NK cell receptor KIR3DL2. *KIR3DL2* is a framework gene within the KIR locus and is highly polymorphic with 84 reported variants of which 62 encode for potential proteins (Shaw and Kollnberger, 2012). The relative expression levels of these different alleles remain unknown. The contribution of allelic variation in *KIR3DL2* on the recognition of HLA class I is also unknown. The *KIR3DL2* gene contains multiple regulatory elements which

are not found in other KIR genes and are likely to result in differential transcriptional regulation (Shaw and Kollnberger, 2012).

A mean of 24.3% of NK cells in healthy individuals express KIR3DL2. These proportions are similar to what has been previously reported for other KIR. KIR3DL2 is expressed as a protein with a signal peptide (encoded for by exons 1-2 of the *KIR3DL2* gene), three immunoglobulin-like domains (exons 3-5) as well as stem (exon 6), transmembrane (exon 7) and cytoplasmic regions (exons 8-9). The immunoglobulin-like domains (D0, D1, D2) are 96, 102 and 98 amino acids in length respectively. The stem and transmembrane region of the KIR3DL2 protein are 24 and 20 amino acids in length respectively. The cytoplasmic tail of this protein is longer than that of other KIR3D proteins (96 amino acids) and includes two ITIM. The D0 immunoglobulin domain of KIR3DL2 is unique in comparison with other KIR3D proteins suggesting a role for this region in ligand binding (Shaw and Kollnberger, 2012). Several KIR3DL2 ligands have been identified, including HLA-A*03, HLA-A*011, HLA-B*27 and CpG-oligodeoxynucleotide (ODN) (Shaw and Kollnberger, 2012).

Ligation of the KIR3DL2 receptor on NK cells has been found to inhibit IFN-γ and cytotoxic function. The effects of receptor ligation on T cell function are less clear. A significant number of peripheral blood $CD4^+$ and $CD8^+$ T cells express KIR3DL2 relative to other KIR (mean of 5% and 9% KIR3DL2 expression respectively). Expression of this receptor is found also to be enriched in memory T cells (Chan et al., 2005). Increased expression of KIR3DL2 on activated $CD4^+$ T cells and NK cells has been reported in spondyloarthritis (Chan et al., 2005 and Bowness et al., 2011). KIR3DL2 expression is also increased on circulating and T cell lymphoma cells in patients with Sezary's Syndrome (Mussette et al., 2003, Poszepczynxka-Guigne et al., 2003). Both disorders are associated with bacterial infection and it has been suggested that exposure to bacteria may promote T cell expression of KIR3DL2 (Shaw and Kollnberger, 2012). $KIR3DL2^+$ $CD4^+$ T cells have been found exhibit many phenotypic characteristics of Th17 cells and are significant producers of IL-17 which may be suggestive of a role for KIR3DL2 in the differentiation of Th17 cells. (Shaw and Kollnberger, 2012).

3.1.6. Peptide: MHC tetramer binding assays

The development of fluorescently labelled peptide tetramer MHC: peptide complexes has allowed for flow cytometry based visualisation, quantification and characterisation of antigen specific T cells from a wide variety of biological samples. Peptide: MHC tetramer are now widely used for determining the frequency of viral specific T cells during viral infection and after vaccination. Tetramers can be combined with functional assays to provide detailed information to the antigen specific T cell response to viral infection. This technique is particularly useful in examining the number and function of antigen specific $CD8^+$ cytotoxic T cells which directly lyse their target cells following recognition by its TCR. Effective T cell staining requires the multimerisation of peptide: MHC complexes to allow for simultaneous binding of a large number of peptide: MHC tetramers to multiple TCR on the surface of the T cell. Although initially limited to MHC class I molecules, tetramer technology has been expanded to include MHC class II molecules, recognised by $CD4^+$ cells. This technology is now commonly used for measurement of immune responses in a large number of species including humans, mice, chimps, chickens and swine (Sims et al., 2010).

The peptide: MHC class I complex is composed of an invariant light chain (β_2 microglobulin), a polymorphic heavy chain with and a peptide of between 8-10 amino acids in length. MHC molecules that do not associate with peptides are unstable and dissociate. MHC class I tetramers consist of four peptide: MHC glycoproteins bound to a fluorescently labelled strepavidin molecule. The heavy and light chains of the MHC molecules are normally independently expressed in *Escherichia Coli* bacteria. The heavy chain is then refolded with β_2 microglobulin and the peptide of interest. Each MHC molecule is then biotinylated using the BirA enzyme. An MHC tetramer is formed following addition of a streptavidin molecule which binds four MHC-biotin complexes. This streptavidin molecule is fluorescently labelled, to allow for flow cytometric analysis of tetramer positive cells (Sims et al. 2010).

Monoclonal antibody staining can be carried out in combination with tetramer staining for surface and intracellular staining of cell populations. To date, the primary application of MHC tetramers has been to track antigen specific T cell populations using conventional flow cytometry. This has been very successful so far, although issues with standardisation of laboratory techniques in order to provide reliable, comparable results between laboratories still remain. Such tetramer assays can also be used to carry out tetramer guided

epitope mapping for identification of novel immune-dominant peptides from a pool of tetramers loaded with overlapping peptides derived from a larger protein antigen. PBMC stimulated with the antigen of interest are stained with different tetramer pools, with each pool containing 5-10 peptides. Peptides from pools which stain positively are then loaded on to MHC tetramer as individual peptides in order to identify specific peptides from an antigen (Sims et al., 2010).

*As HLA-A*03 has been associated with resolution of HCV in the Anti-D cohort, and is also recognised by KIR3DL2, we reasoned that part of this protective effect could be through interaction with NK cells. As recognition of HLA-A*03 by KIR3DL2 has been shown to be peptide specific, we investigated the impact of different HCV specific peptides, bound to HLA-A*03, on KIR3DL2 recognition. In addition, as KIR3DL2 is polymorphic, we investigated the possible impact of this on recognition of HLA-A*03.*

*We hypothesised that variation of HCV peptide presented by HLA-A*03 would affect binding to the KIR3DL2 receptor. In addition, polymorphism in KIR3DL2 could also influence recognition of HLA-A*03 ligand.*

3.1.7. Aims

- Investigate whether NK cells contribute to the immune response to HCV peptides through engagement of the KIR3DL2 receptor.
- Investigate peptide specificity of KIR3DL2 interactions with HCV specific peptides restricted by HLA-A*03.
- Investigate the effect of allelic variation at the KIR locus on recognition of HCV peptides restricted by HLA-A*03.

3.2. Results

3.2.1. KIR3DL2 allelic variation in the Southern Irish population

In order to investigate recognition of HLA-A*03 restricted HCV peptides by the KIR3DL2 receptor, previously published data from the Gardiner lab (Guinan et al., 2010), detailing the results of KIR3DL2 allelic typing in the Southern Irish population (n=105) was analysed and tabulated (Table 3.1.). Table 3.1. details KIR3DL2 allelic frequency in the Southern Irish population in order of decreasing prevalence. Like all KIR, the gene encoding the KIR3DL2 receptor displays significant allelic polymorphism resulting in high KIR diversity in the Irish population. *KIR3DL2*001, KIR3DL2*007, KIR3DL2*002, KIR3DL2*009 and KIR3DL2*003* were identified as the five most prevalent KIR3DL2 alleles in the Southern Irish population with a population frequency of greater than 8.1% (Table 3.1.).

Table 3.1. Frequency of allelic variants of the *KIR3DL2* genes in the Southern Irish population

KIR3DL2 allelic frequency (n=105)	
KIR3DL2 allele	**Frequency**
*KIR3DL2*001*	0.262
*KIR3DL2*007*	0.229
*KIR3DL2*002*	0.171
*KIR3DL2*009*	0.086
*KIR3DL2*003*	0.081
*KIR3DL2*005*	0.048
*KIR3DL2*010*	0.048
*KIR3DL2*011*	0.048
*KIR3DL2*004*	0.010
*KIR3DL2*008*	0.010
*KIR3DL2*006*	0.010

105 healthy normal donors from Southern Ireland were typed to the allele level and frequencies are shown (adapted from Guinan et al., 2010).

These allelic variants result from common nucleotide variations in the KIR3DL2 gene. Some of these nucleotide changes result in amino acid changes in the cytoplasmic and Immunoglobulin-like domains of the KIR3DL2 protein. Therefore, sequencing data detailing the nucleotide (Figure 3.1.(a)) and amino acid changes (Figure 3.1.(b)) present in each of the 5 most prevalent KIR3DL2 alleles was analysed (Figure 3.1.). Sequence data for these common KIR3DL2 allelic variants (available from the Immuno Polymorphism Database of the European Bioinformatics Institute (EBI) (www.ebi.ac.uk/ipd)), was aligned, collated together and tabulated (Figure 3.1.).

(a)

Nucleotide	12	132	216	303	321	322	324	337	438	456	474	893	1190
KIR3DL2*00101	G	C	C	T	G	G	A	C	A	C	G	G	C
KIR3DL2*00102										T			
KIR3DL2*00103	T												
KIR3DL2*00104					A								
KIR3DL2*00201	T										T		
KIR3DL2*00202											T		
KiR3DL2*00301								G					
KIR3DL2*00302				C				G					
KIR3DL2*00701	T					A	G						T
KIR3DL2*00702	T		T			A	G						T
KIR3DL2*00901										T		A	
KIR3DL2*00902	T									T		A	
KIR3DL2*00903	T	T								T		A	

Signal Peptide	Ig Domain I	Ig Domain II	Ig Domain III	Cytoplasmic

(b)

A.A.	108	113	158	298	397
KIR3DL2*001	A	L	E	R	T
KIR3DL2*002			D		
KIR3DL2*003		V			
KIR3DL2*007	T				M
KIR3DL2*009				H	

Ig Domain I	Ig Domain II	Ig Domain III	Cytoplasmic

Figure 3.1. Strategy for generation of KIR3DL2 allelic variants. Illustration of the (a) Nucleotide changes and (b) amino acid changes in each distinct region of the KIR3DL2 receptor present in all allelic variants of KIR3DL2*001, KIR3DL2*002, KIR3DL2*003, KIR3DL2*007 and KIR3DL2*009. Nucleotide and amino acid positions are detailed in the top row and nucleotide positions highlighted in red result in amino acid changes in the KIR3DL2 protein.

3.2.2. Sequence validation of KIR3DL2 plasmids

Before transfection into mammalian cell lines, the DNA sequence of all five plasmids was verified by sequence analysis carried out by Eurofins MWG Operon. Plasmid DNA sequence was verified by two separate sequencing reactions using specifically designed sequencing primers (Table 2.4. in Materials and Methods). An initial sequencing reaction sequenced the majority of the KIR3DL2 gene, giving reliable sequence data from nucleotide position 340 to 1327). A second sequencing reaction sequenced a smaller proportion of the KIR3DL2 gene, from nucleotide position 61 to 348. A contiguous sequence for each of the five plasmids was constructed from the results of both these sequencing reactions. Sequence alignment, comparing sequencing results with the consensus sequence for KIR3DL2*001 was then carried out to confirm the presence of the KIR3DL2 insert (Figure 3.2.). As expected, some segments of unreliable sequence were identified at the beginning and end of the sequence alignment. The expected nucleotide variation in KIR3DL2 allele (as described in Figure 3.1. above) was identified following analysis of these sequence alignments with nucleotide changes at positions 322, 324, 337 (Ig Domain I), 474 (Ig Domain II), 893 (Ig Domain III) and 1190 (Cytoplasmic region). From this sequencing data we confirmed that each of the five plasmids contain the correct KIR3DL2 insert, corresponding to each of the allelic variants of the KIR3DL2 receptor, and were suitable for generation of KIR3DL2 expressing allelic transfectants.

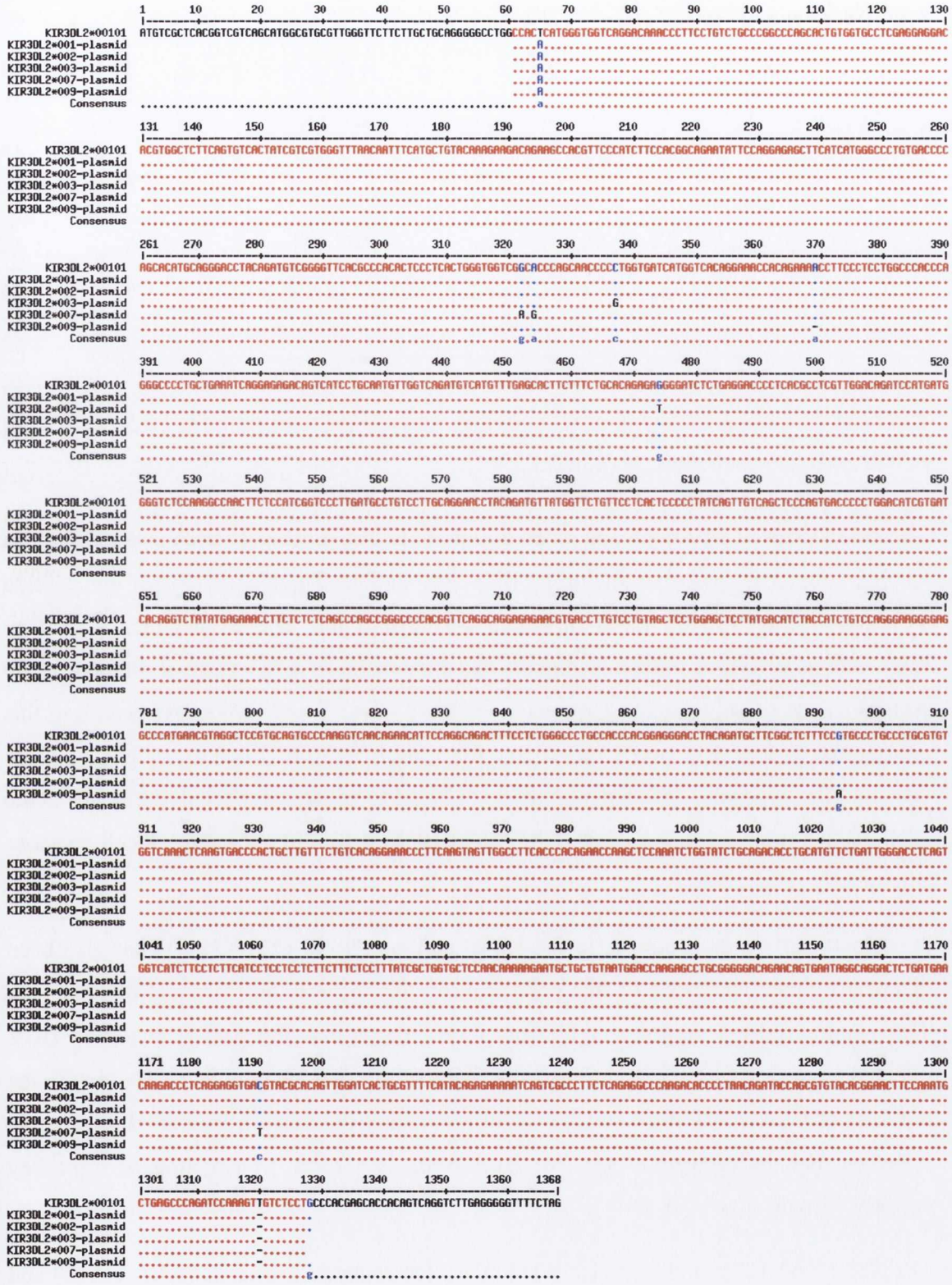


Figure 3.2. Sequence validation of KIR3DL2 plasmids. Results of sequence alignments of five plasmids containing allelic variants of the KIR3DL2 gene (KIR3DL2*001, KIR3DL2*002, KIR3DL2*003, KIR3DL2*007 and KIR3DL2*009) are shown. A contiguous sequence, constructed for each plasmid and combining results of the two sequencing reactions, is shown. Plasmid sequence was compared to the

consensus sequence of the KIR3DL2*001 allele (including the 63bp signal peptide). Nucleotide positions where sequencing results differ from the consensus sequence are highlighted in blue.

*3.2.3. Identification of HCV peptide variants for HLA-A*03 tetramer generation.*

In order to investigate immuno-dominant HCV epitopes restricted by the protective HLA-A*03 and their interaction with their ligand, KIR3DL2, a panel of HLA-A*03 tetramers complexed with peptides derived from HCV was generated. The panel of HCV peptides was designed in collaboration with Dr. Karen Fitzmaurice (Peter Medawar Building for Pathogen Research, University of Oxford) and included peptides from the NS3, NS5B, E1, NS4, NS4B and Core proteins (Table 3.2.).

The NS3 (1080-1088) peptide, included in this study, has previously been characterised as an 'HLA footprint' associated with the HLA-A*03 allele. Three variations of this peptide were also included in the panel of tetramers. Substitutions in these peptides have been identified as having significantly higher frequency in HLA-A*03 positive women of the Anti-D cohort of HCV infected women. Cytotoxic $CD8^+$ T cell responses specific to this epitope were observed in 60% of patients from the Irish Anti-D cohort, more than three decades after exposure. Selected mutants were found to impact on recognition *in vitro*. One such mutant, included in our panel, NS3 (1080-1088) K9R, did not induce T cell response (Fitzmaurice et al., 2011).

A second HLA-A*03 footprint, in the NS5B epitope (NS5B (2510-2518)) has also been identified. Three variations of this peptide were also included in the panel of tetramers. This peptide did not induce a significant T cell response in the Anti-D cohort of HCV infected women. However, the strong association of this epitope (and its variants) with the presence of HLA-A*03 in the Anti-D cohort is evidence of selection pressure. It is possible therefore that the protective effect of HLA-A*03 is a result of targeting of these key epitopes (Fitzmaurice et al. 2011).

The NS3 (1267-1275) and NS4 (1858-1867) peptide epitopes, restricted by HLA-A*03 and HLA-A*11, also included in our panel of tetramers, have been previously reported as important targets in the CTL response to HCV (Sreenarasimhaiah et al., 2003, Bonilla e al., 2006, Alexander et al., 1997, Chang et al., 1999, Scognamiglio et al., 1999, Accapezzato et al., 2004). The remaining HCV peptides included in this panel have all been predicted to be restricted by HLA-A*03. The online prediction tool NetMHCpan (available at

www.cbs.dtu.dk/services/NetMHCpan) was used to check predicted binding to HLA-A*03. These comprised of NS3 (1639-1647), NS5B (2588-2596), NS3 (1390-1399), NS4B (1950-1958), and NS5B (2756-2764). Two additional EBV peptide tetramers were also synthesised for use as positive and negative controls for KIR binding (Table 3.2.). The EBV/EBNA3A peptide (RLRAEAQVK) has previously been shown to bind at high frequency to KIR3DL2. The presence of a V8R mutation in this peptide was found to abrogate KIR binding (Hansasuta et al. 2004).

Customised HCV peptides were purchased and synthesised by Schafer-N (Denmark). Fluorescently labelled (PE and BV-421 labelled) HLA-A*03 bound peptide tetramer were synthesised from these peptides by our collaborators in the Laboratory of Experimental Immunology, Institute for Medicinal Microbiology and Immunology, Panum Institute, University of Copenhagen, Denmark.

Table 3.2. Identification of HCV peptides for HLA-A*03 tetramer generation

HCV Protein	Position	Peptide Sequence
NS3	1080-1088	TVYHGAGTK
		TVYHGAGAK
		TVYHGAGAR
NS5B	2510-2518	KLTPPHSAK
		KLTPPHSAR
		SLTPPHSAK
NS3	1639-1647	VTLTHPITK
NS3	1267-1275	LGFGAYMSK
NS3	1227-1236	HLHAPTGSGK
NS4	1858-1867	GVAGALVAFK
NS3	1390-1399	HLIFCHSRKK
NS4B	1950-1958	SLTITQLLK
NS5B	2756-2764	RVFTEAMTR
NS5B	2588-2596	RVCEKMALY
Core	43-51	RLGVRATRK
EBV/EBNA3A (Positive Control)	603-611	RLRAEAQVK
EBV/EBNA3A (Negative Control)		RLRAEAQRK

15 HCV peptide tetramers were identified, synthesised and complexed with HLA-A*03. These included 3 variants of both the NS3 peptide (position 1080-1088) and the NS5b peptide (position 2510-2518). Two EBV/EBNA3A peptide tetramers were also generated for use a positive and negative control for KIR binding. Peptide sequence and location within the protein are also detailed.

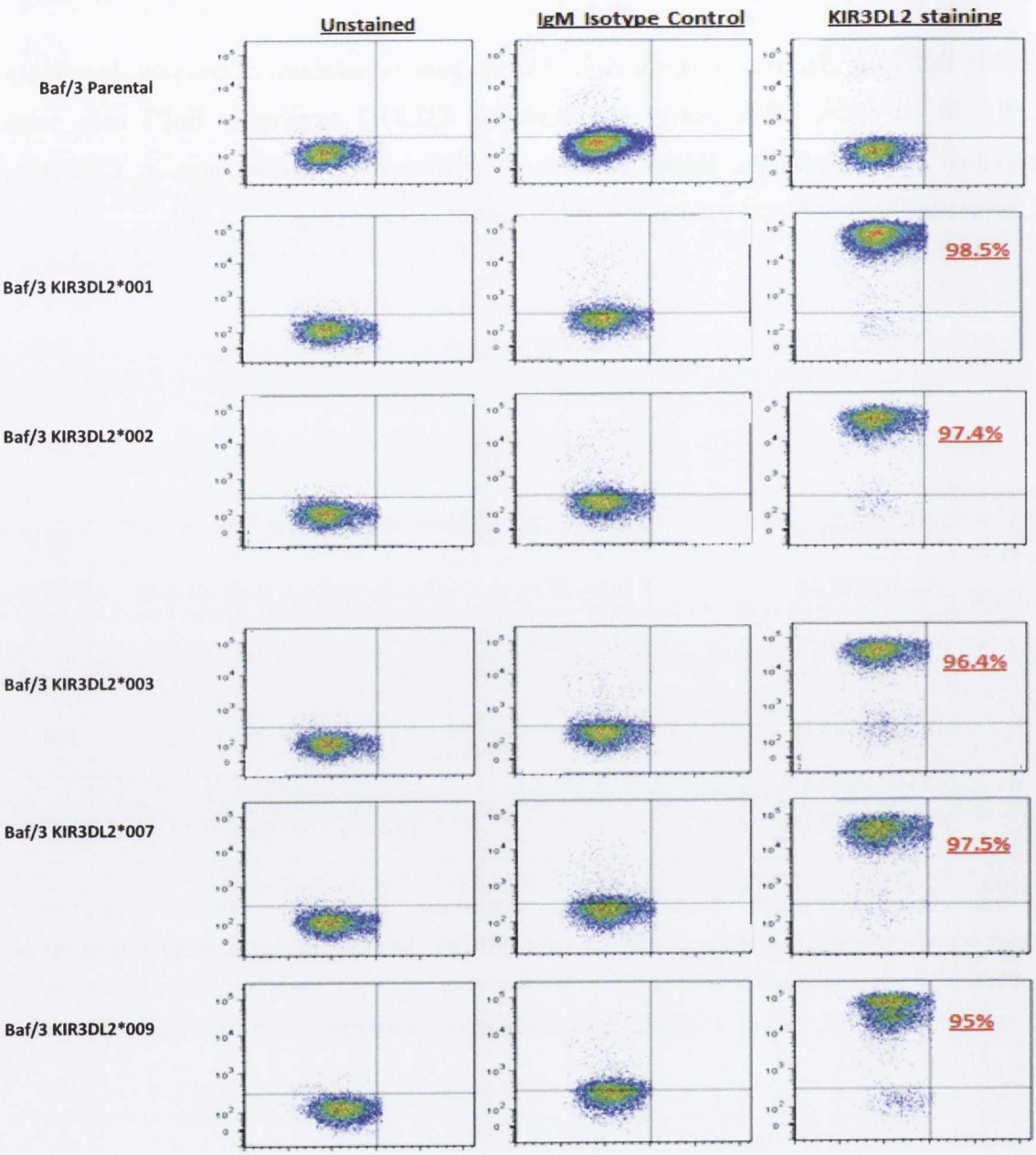
3.2.4. Generation of highly expressing Baf/3 KIR3DL2 transfectants

The previously validated plasmid constructs, containing inserts encoding for KIR3DL2 alleles common in the Irish population, were used to generate stable transfectants expressing the KIR3DL2 receptor. These plasmids also contained a FLAG epitope as an N-terminus fusion protein. The small size of the FLAG protein makes it unlikely to obscure other epitopes or alter function, secretion or transport of the fusion protein. FLAG–tagged KIR3DL2 transfectants were generated using Baf/3 Parental Cells (Mouse B cell line). Baf/3 cell lines produced consisted of the five most prevalent allelic variants:

KIR3DL2*001, KIR3DL2*002, KIR3DL2*003, KIR3DL2*007 and KIR3DL2*009 as detailed in Table 3.1.

Stable Baf/3 transfectants were sorted to provide pure populations of receptor expressing cells. In all cases, >95% purity was achieved. LILRB1 expressing Baf/3 cells were received from Veronique Braud (Institut de Pharacologie Moléculaire et Cellulaire, Université Nice) and were used as a positive control cell line (Figure 3.3.(a)).

(a)


Unstained
IgM Isotype Control
KIR3DL2 staining
Baf/3 Parental
Baf/3 KIR3DL2*001
98.5%
Baf/3 KIR3DL2*002
97.4%
Baf/3 KIR3DL2*003
96.4%
Baf/3 KIR3DL2*007
97.5%
Baf/3 KIR3DL2*009
95%

(b)

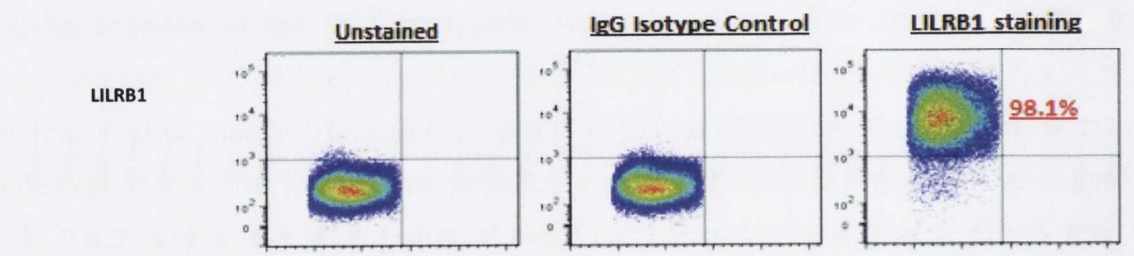

Unstained
IgG Isotype Control
LILRB1 staining
LILRB1
98.1%

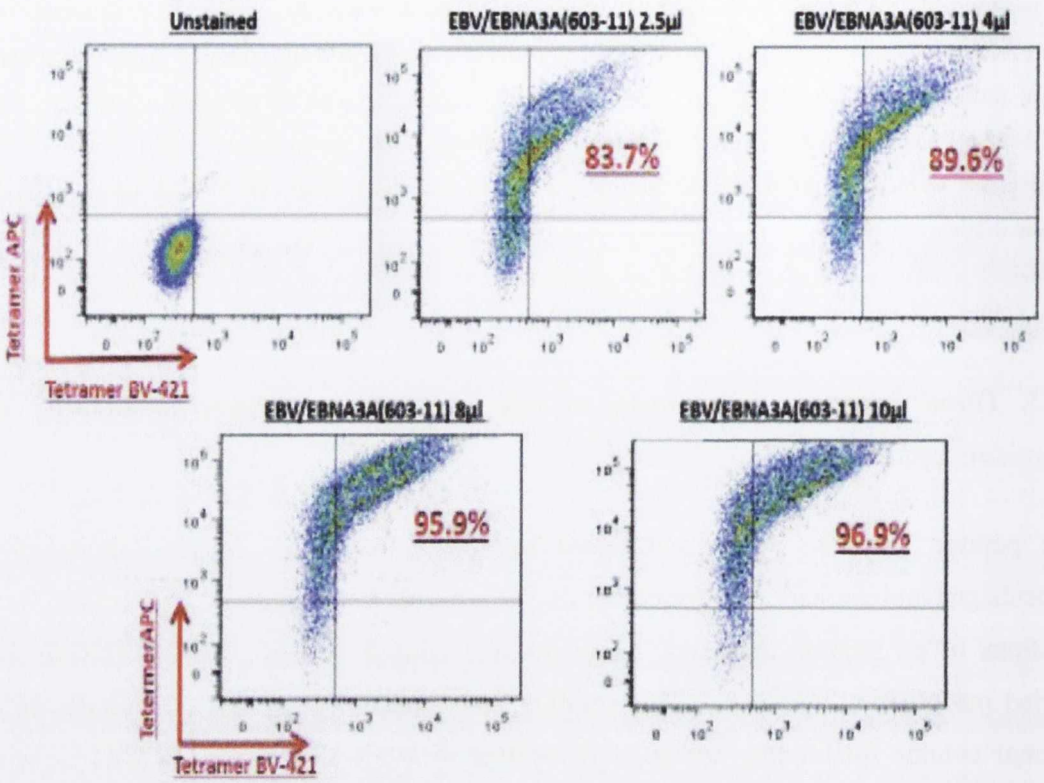
Figure 3.3. Generation of Baf/3 transfectants with high percentage expression of KIR3DL2. Using molecular cloning techniques followed by transfection into Baf/3 cell line, stable transfectants expressing the five most prevalent *KIR3DL2* alleles in the Southern Irish population were generated. All transfectants were then FACS sorted to generate pure populations as shown by flow cytometry analysis using unconjugated mouse antibody to human KIR3DL2 (DX31) followed by incubation with PE conjugated secondary Goat-Anti Mouse antibody (Figure 3.3(a)). For comparison, staining of Baf/3 parental cells is also shown. LILRB1 transfectants were also purified by cell sorting for use as positive control cell line for tetramer binding (Figure 3.3(b)).

3.2.5. Titration of peptide tetramers to determine optimal binding to LILRB1 and expressing cell lines

The peptide tetramers were synthesised by our collaborators in the University of Copenhagen and we had no information on the tetramer concentrations. Therefore, initial titrations of all peptide tetramers, using positive control cell line Baf/3 LILRB1 were carried out by flow cytometry. This resulted in establishment of 8µl of tetramer as the optimal volume for binding assays as it resulted in >95% binding to LILRB1 receptor. Figure 3.4.(a) illustrates representative binding with positive control tetramer EBV/EBNA3A (603-611) which is summarised in histogram format in Figure 3.4.(b). Titration of all HLA-A*03 tetramers showed similar high levels of binding to LILRB1 using this volume of tetramer (data not shown).

One approach used to reduce background signal in tetramer assays is to use two separate flourophores for each tetramer and to stain transfectants with a combination of both. Initially double staining with both APC and BV-421 fluorescently labelled tetramer was performed to reduce background staining. Only cells which stained double positive when analysed by flow cytometry were taken to be tetramer positive. However, our analysis revealed the skewed nature of dot plots when carrying out the assay with double staining, using both APC and BV-421 labelled tetramer. The skewed appearance of dot plots was consistent over several separate experiments, including all peptide tetramers and made it difficult to accurately determine the proportion of LILRB1 expressing cells bound to peptide: MHC tetramers.

(a)



(b)

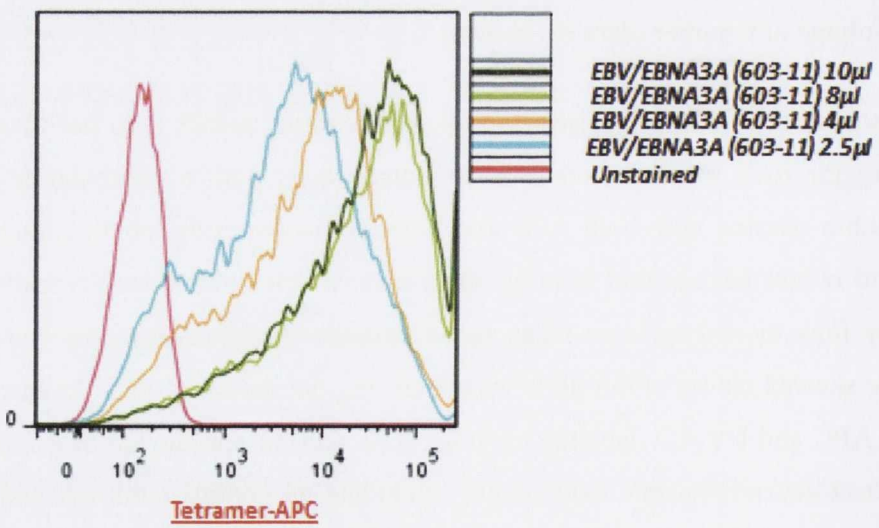


Figure 3.4. Determination of optimal peptide tetramer volume for assay. Titration of EBV/EBNA3A (603-611) peptide tetramer with Baf/3 LILRB1. Peptide assay was carried out using 2.5µl, 4µl, 8µl, 10µl volumes of both APC and BV421 conjugates for each peptide tetramer as indicated in figure 3.4(a). Figure 3.4(b) illustrates the same data information in the format of a histogram measuring APC levels.

3.2.6. Single Stained analysis of tetramer binding using positive control (LILRB1) cell line identifies that a higher proportion of KIR3DL2 expressing Baf/3 transfectants bind to APC labelled tetramer

The skewed nature of the dot plots from this initial data suggested that BV-421 labelled tetramer bound cell less frequently in comparison with the APC labelled tetramer. Single stained analysis of both APC and BV412 labelled conjugates of positive control EBV peptide tetramer to Baf/3 LILRB1 cells confirmed this (Figure 3.5.). Only approximately 30% of BV421 conjugated tetramer bound to positive control cell line, in comparison with 85% of the APC labelled tetramer. This was found for the whole panel of tetramers and was not unique to the EBV/EBNA3A peptide. As a result, we proceeded using only the APC conjugated tetramers.

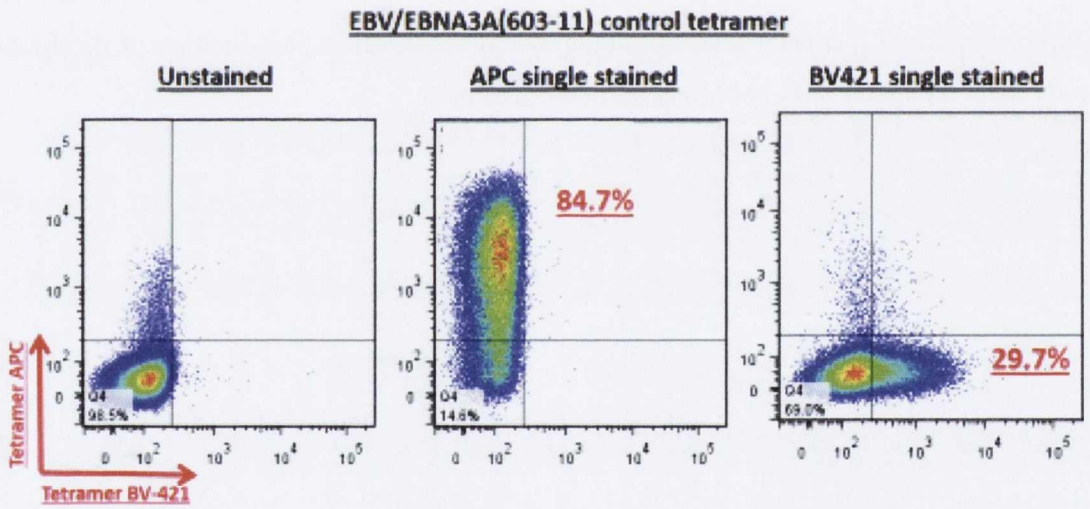
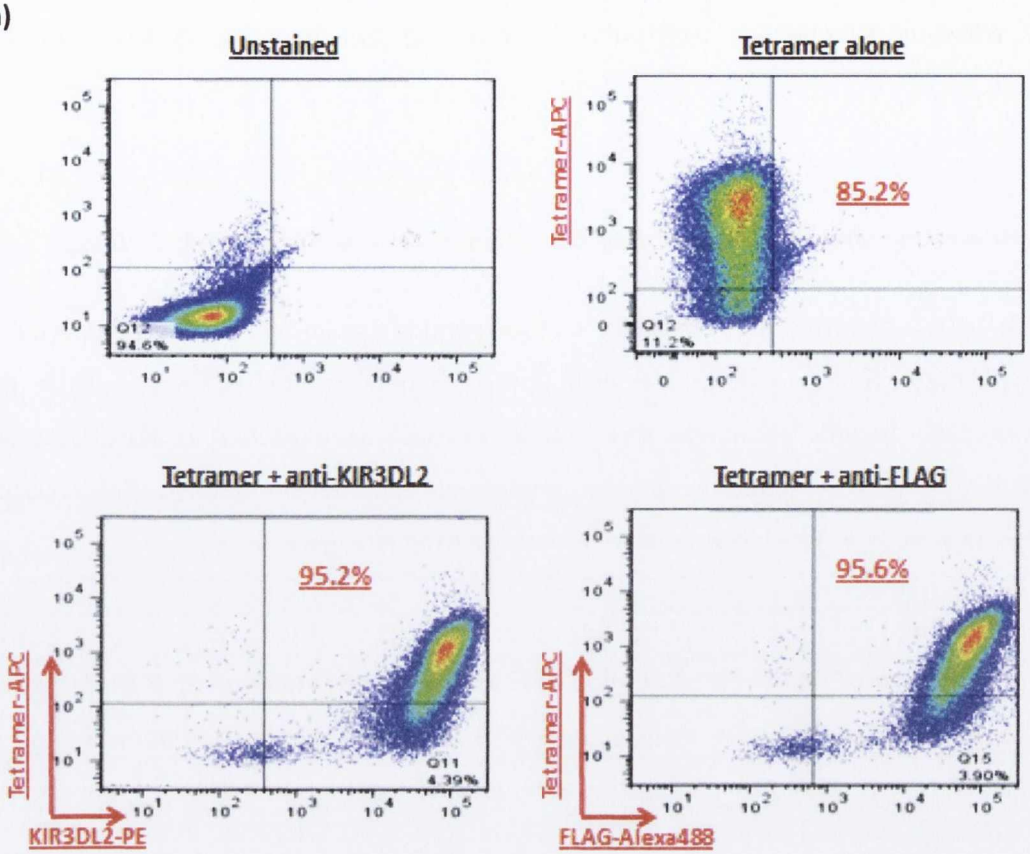


Figure 3.5. Differential binding of BV421 conjugated tetramer to Baf/3 LILB1 transfectants when compared to APC conjugated tetramer. Peptide assay carried out with separate, single stained analysis of both APC and BV421 labelled positive control tetramer (EBV/EBNA3A (603-611)), measuring the level of binding to Baf/3 LILRB1positive control transfectants.

3.2.7. Binding of either KIR3DL2 or FLAG does not interfere with tetramer binding to KIR3DL2

As we planned to identify KIR3DL2 positive cells by flow cytometry, we wanted to investigate whether antibody binding to either KIR3DL2 or FLAG epitope interfered with tetramer binding. To investigate this, KIR3DL2 transfectants were stained with antibody after incubation with tetramer to allow for optimal tetramer binding. Binding levels were compared with that of tetramer alone using highly expressing (>95% positive) Baf/3 KIR3DL2 transfectant cells. Binding of KIR3DL2 (DX31) antibody was found not to interfere with binding of positive control EBV/EBNA3A (603-11) peptide tetramer to KIR3DL2*001 transfectants (Figure 3.6. (a)). Binding of FLAG (M2) antibody was also found not to interfere with tetramer binding (Figure 3.6.(b)). Greater than 85% binding to positive control tetramer was seen with additional inclusion of antibodies in comparison with 77.8% binding seen without antibody incubation. The absence of inhibition allowed for further alteration of the protocol to include KIR3DL2 antibody (DX31), enabling gating of KIR3DL2 expressing cell population. It is also clear that ligation of the FLAG antibody to its ligand does not affect tetramer binding.

(a)



(b)

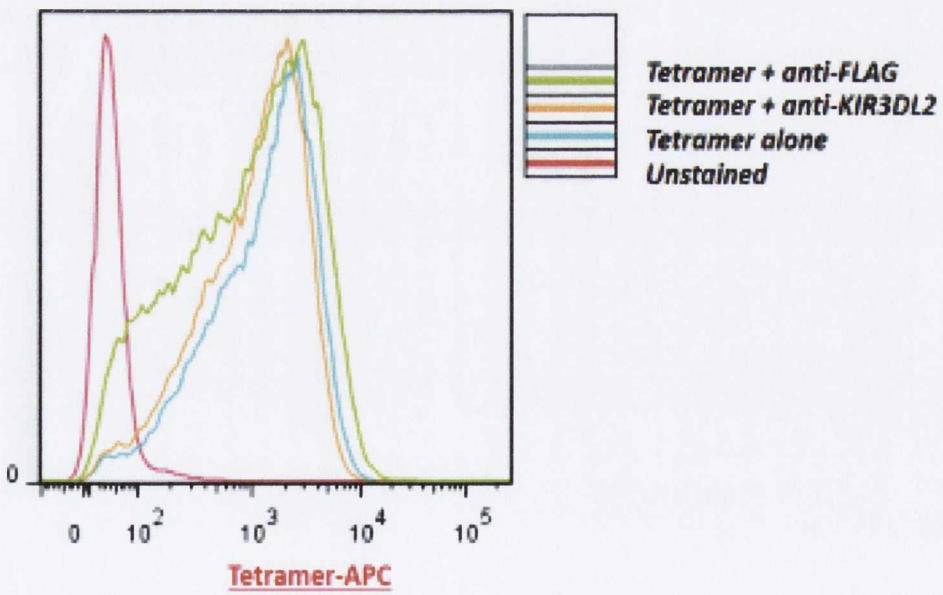


Figure 3.6. Binding of either KIR3DL2 or FLAG antibody does not block tetramer binding to KIR3DL2*001 transfectants. Tetramer binding assay was carried out to measure binding of EBV/EBNA3A (603-611) peptide tetramer to highly expressing Baf/3 KIR3DL2*001 transfectants. After incubation with tetramer, cells were stained with either anti-KIR3DL2 (DX31) or anti-FLAG antibody (Figure 3.6(a)). Figure

3.6(b) illustrates the same data information in the format of a histogram measuring APC levels. Data were compared with unstained samples as well as tetramer only control samples.

3.2.8. Absence of significant binding of HCV peptide tetramer to KIR3DL2 alleles

The EBV/EBNA3A (603-611) positive control peptide has previously been reported to bind with high affinity to KIR3DL2*001. Using our new updated strategy, positive and negative EBV peptide tetramers were found to bind as expected to KIR3DL2*001 transfectants. A V8R mutation in this peptide is reported to abolish KIR binding (Hansasuta et al. 2004) and again did so in our experiment (Figure 3.7.).

The experimental plan was to test the full panel of HLA-A*03 tetramers complexed with HCV peptides. To begin, levels of binding of NS3 (1080-1088) tetramer to KIR3DL2*001 was measured. While positive and negative EBV/EBNA3A control tetramers bound as expected, there was no significant binding seen with HCV NS3 (1080-1088) (Figure 3.7.).

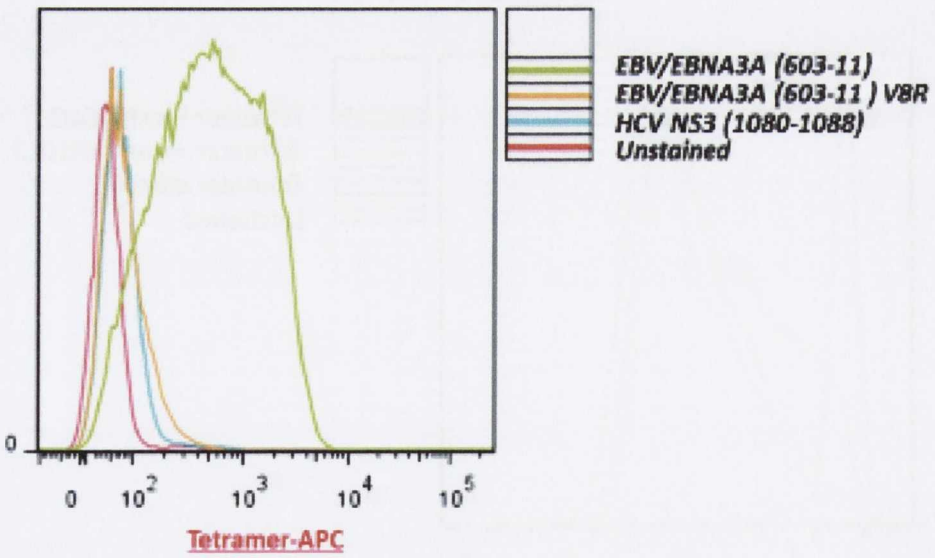


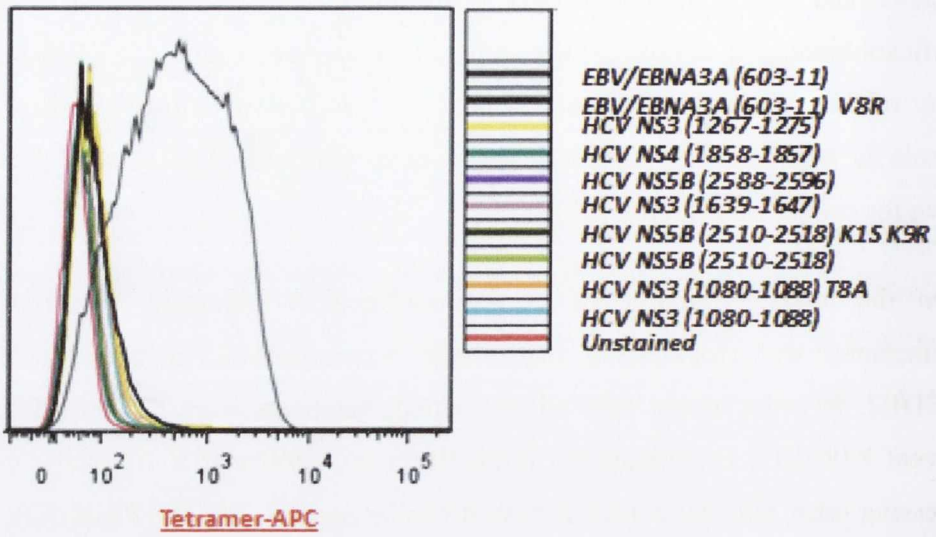
Figure 3.7. Positive and negative control EBV peptide tetramers behave as expected however no significant binding to HCV peptide NS3 (1080-1088) was detected. Histogram illustration of binding levels of HLA-A*03 tetramers complexed with NS3 (1080-1088) peptide to Baf/3 KIR3DL2*001 transfectants. Binding levels of EBV/EBNA3A (603-611) and EBV/EBNA3A V8R (603-611) peptide tetramer are also shown as positive and negative controls.

This binding assay was then expanded to include the other HCV peptide tetramers from our panel and results are summarised in histogram format in Figure 3.6. There was no significant binding of any of the HLA-A*03:HCV peptide tetramers to KIR3DL2*001. For clarity of data presented, the panel of peptides was divided into two groups. Unstained controls as well as positive and negative control EBV/EBNA3A (603-611) peptides are shown for comparison (Figure 3.8.).

Given the negative binding of all HLA-A*03:HCV tetramers with KIR3DL2*001 transfectants, we hypothesised that allelic polymorphism might allow binding to KIR3DL2. In order to test this, all our peptide tetramers were tested with each of the different KIR3DL2 expressing cell lines. However, additional Baf/3 allelic transfectants expressing other KIR3DL2 alleles showed similar negative results (Table 3.2). In fact, no tetramers bound apart from the positive EBV/EBNA3A peptide although some variability of binding was identified across experimental repetitions. This resulted in discounting of several significant outliers (noted as n=3 in Table 3.3.).

Somewhat unexpectedly however, initial results indicated that our positive control EBV/EBNA3A peptide tetramer bound KIR3DL2 transfectants with varying levels of specificity. KIR3DL2*001 expressing cells bound with the highest frequency of 87.9% while KIR3DL2*007 bound with the lowest frequency with an average of only 50.1% (Table 3.3. and Figure 3.9.). These results suggest that the level of binding of peptide:MHC tetramer to the KIR3DL2 receptor is dependent on allelic polymorphism. However, further analysis is required in order to confirm this.

(a)



(b)

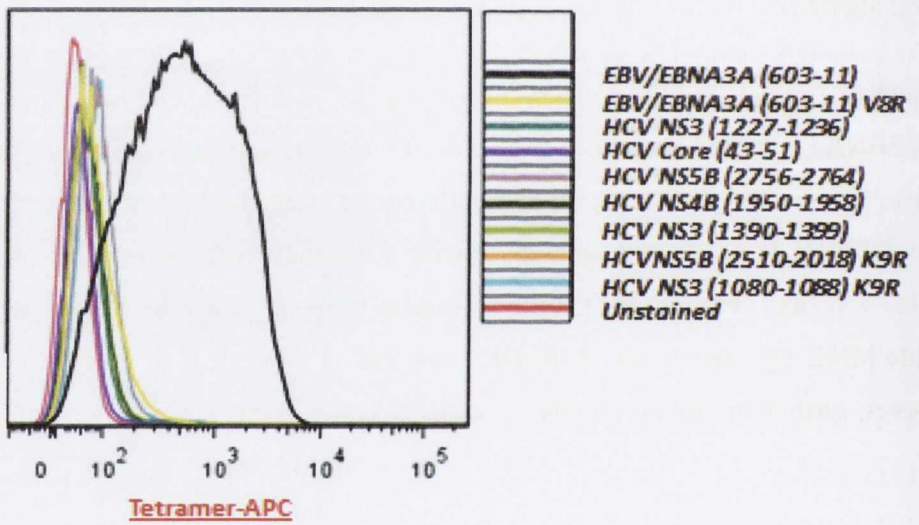


Figure 3.8. Representative histograms of Baf/3 KIR3DL2*001: HCV peptide tetramer binding assays. Figures 3.8(a) and 3.8(b) illustrate the binding pattern of 15 HCV peptide tetramer to Baf/3 KIR3DL2*001 transfectants. For clarity, the tetramer panel was divided into two separate groups of 8 and 7 HCV peptides. Representative data for positive and negative control EBV peptide tetramer are also included for both groups.

Table 3.3. Summary data from Baf/3 KIR3DL2: HCV peptide tetramer binding assays

HCV Peptide	Baf/3 transfectants (% tetramer positive)				
	KIR3DL2 *001	KIR3DL2 *002	KIR3DL2 *003	KIR3DL2 *007	KIR3DL2 *009
NS3 (1080-88)	6.6±0.7	6.6±3.4	9±7.7	5.1±1.6	9.3±7.2
NS3 (1080-88) T8A	8.7±0.9	7.4±3.1	7.6±4.0	5.5±1.6	8.6±5.2
NS3 (1080-88) K9R	15.6±9.1*	10±3.3 *	10±9.1	7.4±1.6	8.3±3.6
NS3 (1267-75)	8.7±2.6	10±3.7	7.9±4.1	8±4.4	10.5±4.6
NS3 (1227-36)	3.8±0.8	3.7±0.5	3.4±0.7	3.5±1.2	4.3±1.1
NS3 (1390-99)	7.8±3.12 *	12.8±9.9	8.9±7.4	6.8±3.4	11.4±7.5
NS3 (1639-47)	5.2±2.4 *	5.3±1.2	5.1±2.5	4.8±1.3	5.5±1.9
NS4 (1858-67)	4.7±1.3	5.4±1.3	5.5±2.6	4.9±1.1	5.7±2.9
NS4B (1950-58)	5.4±1.3	4.8±1.2	3.9±0.9	5.3±1.5	5.6±1.9
NS5B (2510-18)	5±1.3	5.5±1.8	5.2±2.7	4.9±1.6	5.3±1.9
NS5B (2510-18) K1S	8±7.4	4.72±1.7	4.7±2.4	4.4±1.3	5.4±1.8
NS5B (2510-18) K9R	6.8±2.2	6.7±2.6	5.1±1.4	4.4±1.4	8.3±6.2
NS5B (2756-64)	8.4±4.3	7.3±3.9	5.6±1.3	7.2±6.2	7.6±3.3
NS5B (2588-96)	8.9±2.5	7.3±1.7	6.5±2.7	5.7±2.6	8.5±2.7
Core Protein (43-51)	3.6±1.6	3.8±1.0	3.2±0.8	3.3±1.2	3.8±1.7
Control Peptides					
EBV/EBNA3A (603-11) (n=2)	87.9±1.0	85.6±11.7	76.1±11.5	50.1±13.2	66.9±4.9
EBV/EBNA3A (603-11) V8R (n=2)	9.7±8.2	8.7±6.6	4.6±4.1	3.9±2.0	8.27±4.6

Mean frequency of binding of 15 HLA-A*03:HCV peptide tetramers to Baf/3 transfectants expressing 5 common allotypes of the *KIR3DL2* gene are shown. Values given are averages of n=4 except where stated. Standard deviation is also shown. For comparison, corresponding values for positive and negative control EBV peptide tetramer are also included.

*Values shown are averages of n=3 experiments

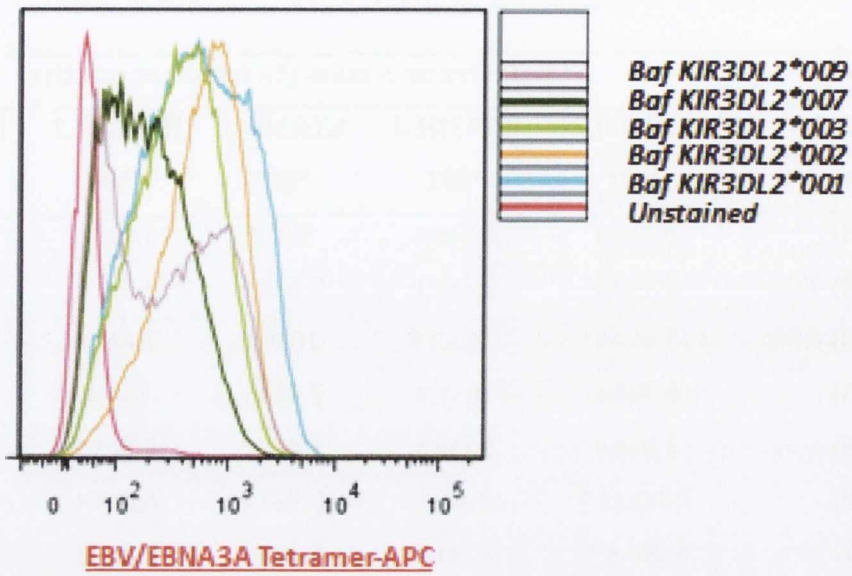


Figure 3.9. Positive control EBV/EBNA3A peptide binds to KIR3DL2 alleles with varying levels of specificity. Representative histogram of EBV/EBNA3A peptide tetramer binding assays with 5 Baf/3 KIR3DL2 allelic variants.

3.2.9. Differential binding of two HCV peptide tetramers to LILRB1 due to low folding efficiency

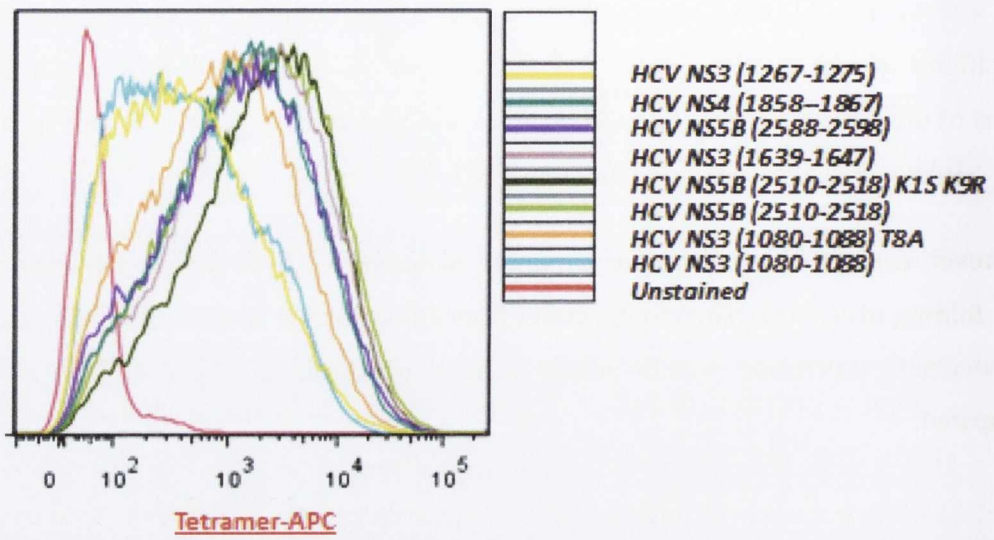
As part of our control study the full panel of HLA-A*03 complexed peptides were also tested using Baf/3 LILRB1 transfectants. Unexpectedly, initial binding assays with Baf/3 LILRB1 transfectants showed differential binding of HCV tetramers to the receptor. HCV peptide NS3 (1267-1275) showed lower levels of binding in comparison with the majority of tetramers which showed high levels of tetramer binding. NS3 (1080-1088) T8A, NS3 (1390-1399) and NS5B (2756-2764) appeared to show an intermediate binding in comparison with high and low binding tetramer groups (n=2). NS3 (1080-1088) also showed lower levels of binding but this was not reproducible (Figure 3.10.).

Analysis of titration data showed not all tetramers displayed a similar intensity in maximum fluorescent peaks using the optimal tetramer volume (8µl). Four peptides displayed a downward shift in fluorescent peak (Average MFI of $1.14x10^4$ in comparison with overall average of $2.8x10^4$). These included three peptides which had displayed lower levels of binding (NS3 (1267-1275), NS3 (1390-1399) and NS5B (2756-2764)). However, this was not the case for NS3 (1080-1088) T8A which also displayed lower levels of binding.

Technical reports from the tetramer synthesis company Schafer-N reported that two of these peptides, NS3 (1267-1275) and NS3 (1390-1399), have lower folding efficiency (<50% and <80%). The LILRB1 receptor has previously been reported to have a broad binding specificity which is independent of peptide. From our results, while some variation in terms of differential binding of HCV peptides was seen, this is likely to be due to variation in the folding efficiency of individual tetramers.

However, none of the other peptide tetramers, including the EBV peptide tetramer, showed low folding efficiency. Therefore, results from EBV peptide binding assays, using Baf/3 transfectants expressing specific allelic variants of *KIR3DL2,* could still be accurately compared.

(a)



(b)

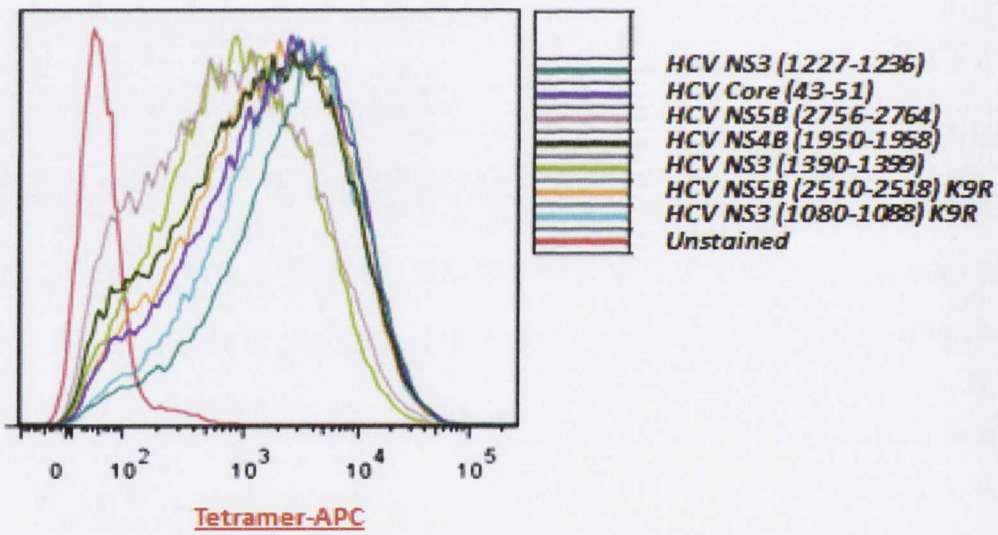


Figure 3.10. Baf/3 LILRB1 shows differential binding to HLA-A*03:HCV peptide tetramers. Representative histograms of Baf/3 LILRB1:HCV peptide tetramer binding assays. Figures 3.6(a) and 3.6(b) illustrate the binding pattern of 15 HCV peptide tetramer to Baf/3 KIR3DL2*001 transfectants. For clarity, the tetramer panel was divided into two separate groups of 8 and 7 HCV peptides. Representative data for positive and negative control EBV peptide tetramer are also included for both groups.

3.2.10. Decreased NK cell expression of CD57 in chronic high viral load carriers of EBV infection in a paediatric cohort of post renal transplant patients

As previously described, our tetramer assays demonstrated differential binding of an HLA-A*03 restricted EBV peptide (EBV/EBNA (603-611)) to allelic variants of the KIR3DL2 receptor. This suggested that KIR3DL2 may play a role in EBV infection outcome. Following on from this we investigated the possibility that NK cells could contribute to the immune response to EBV infection through KIR3DL2 recognition of viral peptide presented by its HLA ligand (HLA-A*03 or HLA-A*011).

EBV is a human gamma Herpesvirus which infects 95% of adults by the age of 30 (Cohen et al., 2000). EBV infection usually occurs during childhood and is transmitted through saliva (Gerber et al., 1972). EBV preferentially infects B and epithelial cells but infection is not exclusive to these cell types. Following initial infection, EBV establishes a latent infection within memory B cells allowing lifelong infection to be established (Hutt-Fletcher et al., 2007 and Rickinson et al., 2007). Childhood infections are usually asymptomatic or result in mild symptoms which are hard to distinguish from other mild viral infections. However, EBV infection in young adults can result in infectious mononucleosis, a normally benign self-limiting lymphoproliferative disease which occurs in 25%-70% of adolescents following primary infection (Candy et al., 2002).

In addition, EBV may result in significant complications following solid organ transplantation due to the use of immunosuppressive therapy to prevent organ rejection. In a significant number of post-transplant patients, primary EBV infection can result in chronic high viral load titres (C.H.L.) for months or years (Bingler et al., 2008). High EBV viral load is a risk factor for the development of EBV related post-transplant lymphoproliferative disease (PTLD). PTLD is prevalent in 10% of transplant recipients following transplant of EBV^+ donor organs into EBV^- individuals (Taylor et al., 2005).

In light of our previous finding from our tetramer binding assays, we undertook a pilot project in a subset of HLA-A*03/ HLA-A*011 positive patients who were previously exposed to EBV (n=14). In collaboration with Jaythoon Hassan (NVRL, UCD) and Sheila Boyle (CUH Temple Street) we received blood samples from a paediatric cohort of post renal transplant patients who have been previously HLA typed and tested for EBV viral load levels due to the risk of EBV related lympho-proliferative disease post transplantation (n=14, 5 female and 9 male). This cohort consisted of 10 patients who had resolved EBV

infection and 4 patients diagnosed as chronic high viral load carriers of EBV infection (C.H.L. group). PBMC were isolated from the whole blood of these individuals. This cohort ranged in age from 6-16 years and patients had undergone transplant surgery between 2 and 9 years previously. Age at the time of transplant surgery ranged from 1-12 years. Further background and clinical information in this cohort is described in Appendix Table 1.

This pilot study aimed to:

- *Identify any interaction of EBV: HLA-A*03 tetramer with KIR3DL2 expressing NK cells in patients that had been infected with EBV.*
- *Stratify patients to examine whether there was any correlation between EBV: HLA-A*03 tetramer binding and clinical outcome following EBV infection.*
- *Identify an NK cell phenotype associated with development of chronic EBV infection*

In order to carry out this first aim, binding of fluorescently labelled (EBV/EBNA3A(603-11):HLA-A*03) peptide tetramer to KIR3DL2$^+$ NK cells was measured in these patients. This tetramer was previously shown to differentially bind to Baf/3 transfectants expressing various allotypes of KIR3DL2 (Figure 3.7.). However no significant binding of tetramer to KIR3DL2$^+$ NK was identified in these patients (data not shown).

A key aim of this pilot study was to test the hypothesis that NK cells play a role in the immune response to EBV infection in HLA-A*03/HLA-A*011$^+$ individuals through engagement of the KIR3DL2 receptor. If this is true, it is possible that EBV infection may be associated with an expansion in the number of KIR3DL2 expressing NK cells. However, the overall percentage of NK cells was found to be statistically unchanged in resolvers of EBV infection compared with C.H.L. group (8.9±4.0% and 12.4±12.3% NK cells respectively). Similarly, average expression of the KIR3DL2 receptor was unchanged in resolvers of EBV infection compared with those with chronic high viral load titres. (21.6±12.8% and 19.2±9.4% KIR3DL2$^+$ NK cells respectively). These levels are broadly similar to what is seen in the normal Irish population where an average of 29% of NK cells have been reported to express KIR3DL2 (Dunphy, S., Unpublished).

In this patient cohort, 5 resolvers of EBV infection tested positive for CMV antibody before transplantation. None of the high viral load carriers tested CMV^+. Donor information for 4 of these CMV^+ patients revealed that n=2 received transplants from CMV^+ donors while the remaining 2 received a transplant which was CMV-. Interestingly, those CMV^+ patients who received a transplant from a CMV^+ donor had a higher frequency of KIR3DL2 expression in comparison with CMV^+ patients who received a transplant from a CMV^- donor (29.0% and 26.1% $KIR3DL2^+$ NK cells in those with CMV+ donor compared with 9.2% and 7.8% in those with CMV^- donors. Although the numbers in this pilot study are small, these results suggest that re-exposure to CMV through donor transplantation may result in an expansion in the $KIR3DL2^+$ NK cell subset.

As previously described (Section 1.6.), the proportion of $CD57^+$ and $NKG2C^+$ NK cells has been found to be expanded in individuals exposed to CMV infection (Section 1.9.). Recent reports suggest that this NK cell subset is important in the 'memory like' NK cell response to viral infection by which the NK cell immune response is modulated by previous exposure to viral infection (White et al., 2014). We hypothesised these NK cell subsets may be differentially expanded in resolvers and chronically infected patients following EBV infection. Therefore, in addition to KIR3DL2, we also compared NK cell expression of these receptors in this cohort of post renal transplant patients to test whether receptor expression was associated with EBV infection outcome (Table 3.3.). In addition, the expression of CD94 which forms a heterodimer with NKG2C was also measured in this cohort.

We found no difference in NK expression of NKG2C and CD94 when comparing CHL patients with resolvers of EBV infection (30.3±12.3% $NKG2C^+$ and 74.6±10.0% $CD94^+$ NK cells in chronically infected patients compared with 37.0±26.5% and 70.0±16.1% respectively in resolvers). However, NK expression of CD57 was found to be significantly decreased in chronically infected patients in comparison with those who resolved infection (44.6±14.7% and 65.7±14.2% $CD57^+$ NK cells respectively p=0.03 CI=2.6, 39.5). $CD8^+$ T cell expression of CD57 was also decreased in those who are chronically infected although this was not statistically significant (14.8±8.7% $CD57^+$ NK cells compared with 22.9±15.7% in resolvers of infection).

Table 3.4. Analysis of NK cell receptor expression in a paediatric cohort of HLA-A*03$^+$/HLA-A*011$^+$ post renal transplant patients infected with EBV

	Resolvers (n=10)	C.H.L. (n=4)	
Age	12±3.2	8±1.8	
% NK cells	8.9±4.0	12.4±12.3	
% KIR3DL2$^+$ NK cells	21.6±12.8	19.2±9.4	
% CD57$^+$ NK cells	65.7±14.2	44.6±14.7 *	p=0.03 CI=2.6, 39.5
% NKG2C$^+$ NK cells	37.0±26.5	30.3±12.3	
% CD94$^+$ NK cells	70.0±16.1	74.6±10.0	
% KIR3DL2$^+$ CD8 T cells	3.7±1.6	3.6±0.9	
% CD57$^+$ CD8$^+$ T cells	22.9±15.7	14.8±8.7	

Mean percentage of NK cell extracellular expression of the receptors KIR3DL2, CD57, NKG2C and CD94, as measured by flow cytometry is detailed. For comparison, CD8$^+$ T cell expression of KIR3DL2 and CD57 is also shown.

* Average NK CD57 expression was found to be significantly decreased in those with high viral load titre of EBV following statistical analysis using unpaired T test analysis

3.3. Discussion

*3.3.1. HCV peptide HLA-A*03 tetramer binding assays*

Our studies have centred on the role of NK cell receptors in the anti-HCV immune response. Previous studies have reported associations between several KIR alleles and initial sustained clearance response to HCV infection. The T cell immune response to pathogen infection is known to be peptide specific. NK cell immune responses have also been shown to be peptide dependent. There is previous evidence to suggest that peptide sequence affects the level of binding to the NK cell inhibitory KIR receptors (Kollnberger et al., 2007, Hansasuta et al., 2004). However, this study described here found no evidence of peptide dependent NK cell responses and it remains unclear what impact peptide variation has on the overall NK cell response to HCV.

Most of the HCV peptides in our panel of synthesised HLA-A*03 restricted tetramers represent epitopes which have been previously reported to be common targets for $CD8^+$ T cells in the immune response to HCV infection (Fitzmaurice et al. 2011). We therefore hypothesised that some of these peptide epitopes may also be important in the NK cell immune response to HCV which is mediated through the KIR3DL2 receptor. It is possible that changes in peptide epitopes that increase the affinity of certain MHC class I ligands for inhibitory and activating KIR may affect the activation of specific NK cell subsets and thus influence the nature of the subsequent immune response generated.

Somewhat unexpectedly, none of the HCV peptide tetramers generated demonstrated any significant binding to the NK cell receptor KIR3DL2, expressed with high frequency, on Baf/3 cells. Various common alleles of KIR3DL2 were also tested for levels of binding, yielding similar results. There is therefore no evidence to support a role for KIR3DL2 interactions in the protective effect of HLA-A*03 in HCV infection. However, this result does confirm that binding of KIR3DL2 to its ligand displays significant peptide specificity with KIR3DL2 expressing transfectants showing high levels of binding to EBV: HLA-A*03 tetramers but no significant binding to HCV peptide tetramers.

Several quality control procedures were carried out as part of this tetramer study. The plasmids used for generation of KIR3DL2 expressing transfectants which were received from our collaborator Geraldine O'Connor were tested by sequencing analysis for the presence of the KIR3DL2 insert before transfection into Baf/3 cells. Transfection of Baf/3 was carried out using a specialised kit for transfection of such cell lines (Amaza Cell Line Nucleofector Kit V, Lonza). The resultant cell lines were routinely tested to ensure high frequency of expression of the KIR3DL2 receptor. In addition, transfectants were incubated with fluorescently labelled KIR3DL2 antibody as part of our tetramer assay in order to accurately determine frequency of tetramer binding to $KIR3DL2^+$ cells.

The cell lines which were generated were not tested for the presence of mycoplasma, however transfectants were found to bind as expected to the positive and negative EBV peptide tetramer, showing no indication of contamination issues. Peptides for MHC tetramer generation were purchased from Schafer-N and these peptides were shipped to our collaborators at the University of Copenhagen. Folding efficacy of the peptides tetramers were confirmed in technical reports. We had no reliable mechanism to independently verify the peptide sequence of the panel of tetramers. Several of these peptides (NS3 (1080-1088), NS5B (2510-2518), NS3 (1267-1275) and NS4 (1858-1867)) have

previously been shown to induce T cells responses (Fitzmaurice et al., 2011, Sreenarasimhaiah et al., 2003, Bonilla et al., 2006, Alexander et al., 1997, Chang et al., 1999, Scognamiglio et al., 1999, Accapezzato et al., 2004). This could possibly be tested experimentally. Importantly however, the remainder of the HLA-A*03 restricted peptides included in our tetramer panel have not been previously tested or have been shown not to induce T cell responses. Future studies involving these peptides may involve investigations into the effect of KIR3DL2 polymorphism on the frequency and intensity of interactions with HCV peptides presented by HLA-A*011, an alternative KIR3DL2 ligand.

The LILRB1 receptor is known to bind to the β_2 microglobulin domain which is conserved among HLA class I molecules resulting in broad binding specificity. For this reason, Baf/3 LILRB1 transfectants were received from our collaborator Veronique Braud for use as a positive control for peptide binding. Similar to the KIR3DL2 expressing cell lines, LILRB1 transfectants were routinely tested to ensure high frequency of expression of the LILRB1 receptor. In our control study, the LILRB1 receptor was found to bind to the panel of peptide tetramers with varying levels of specificity. However, this is likely due to variation in peptide folding efficiency and is unlikely to be true in a biological setting.

In conclusion, the results described here provide no evidence that KIR3DL2 mediated NK cell responses are dependent on the sequence of bound peptide. This is in contrast with previous evidence that the level of NK cell inhibition, induced following ligation of inhibitory KIR with its MHC ligand, is peptide dependent (Cassidy et al. 2015, Hansauta et al. 2004).

3.3.2. EBV peptide binds to KIR3DL2 alleles with varying levels of specificity

Our results also found that allotypes of the KIR3DL2 receptor bind differentially to EBV/EBNA3A positive control peptide, presented on a HLA-A*03 background. The KIR3DL2*007 and KIR3DL2*009 allelic variants were found to bind EBV/EBNA3A at a lower frequency in comparison with the other three KIR3DL2 alleles tested, particularly *KIR3DL2*001* which bound at the highest average frequency. These results suggest that KIR3DL2 responses may be allotype dependent. In addition, differential binding of KIR3DL2 alleles to EBV peptide may be suggestive of a role for this receptor in the

immune response to EBV. These results raise the possibility that specific allotypes of KIR3DL2 may be associated with clinical outcome following EBV infection.

These results suggest that KIR3DL2 polymorphism may affect the NK cell response to EBV. Interestingly, binding of KIR3DL1 to its HLA-Bw4 KIR binding motif has also been found to be dependent on KIR3DL1 polymorphism. In addition, allelic polymorphism in KIR3DL1 has been shown to play a role in the peptide specificity of this receptor. (Thananchai et al., 2007, O'Connor et al. 2007, O'Connor et al., 2014). However, this role has not yet been confirmed in the context of KIR3DL2 polymorphism. Further work into generation of a second panel of HLA-A*03 peptide tetramers, derived from EBV immune epitopes, for use in further tetramer binding assays may therefore yield interesting results.

Like the KIR receptors, the LILRB1 gene is also polymorphic. With this in mind, further investigations may also be carried out into the effect of allelic variation as well as peptide specificity on levels of HCV peptide binding to this receptor. This may involve peptide binding assays, similar to those carried out with KIR3DL2, following generation of transfectants expressing allelic variants of the LILRB1.

The large degree of variability that is seen in receptor genes allows for an effective immune response to pathogens which are constantly evolving to evade the host immune response. This study has centred on the role of NK receptors in the immune response to HCV. It is now accepted that inhibitory KIR possess peptide specificity and that NK cell binding can be modulated by the presence of bound peptide. However, the effect of substitutions in the peptide sequence which is presented to the NK cell on the overall anti-viral response remains unclear (Cassidy et al., 2014).

Previous studies have identified that KIR3DL2 interaction with ligand is peptide specific. The results described here provide evidence that this interaction may also be dependent on KIR3DL2 allotype. These results also provide evidence that the NK cell receptor KIR3DL2 may play a role in the immune response to EBV infection.

3.3.3. Frequency of CD57$^+$ NK cells is decreased in chronic high viral load carriers of EBV infection in a paediatric cohort of renal transplant patients infected with EBV

Analysis of NK cell phenotype in this cohort of EBV infection provided no evidence for a role for the KIR3DL2:HLA-A*03/HLA-A*011 receptor:ligand interaction in the immune response to EBV infection. All patients, included in this study had tested positive for one or both ligands for the KIR3DL2 receptor however, those who resolvers of EBV showed no difference in NK cell KIR3DL2 expression in comparison with those who remained chronically infected. However, the numbers in this pilot study (n=10 resolvers and n=4 C.H.L.) are small and an expansion of this cohort, particularly in the C.H.L. group would be required in order to make definitive conclusions. In addition, although not possible for this pilot study, it would be ideal to include a healthy age-matched control group for comparison with EBV infection groups.

Bearing these limitations in mind, the data described here suggests that re-exposure of patients to CMV through transplantation of a CMV$^+$ donor organ may result in an expansion of the KIR3DL2$^+$ NK cell subset. As previously described, NK cell phenotype has been previously reported to be altered following CMV exposure, however, to our knowledge; no associations with KIR3DL2 expression have been identified to date. Expansion in this cohort may reveal statistically significant differences in KIR3DL2 expression following re-exposure to virus. Relevant clinical information on all 14 donors, including CMV status of donor is detailed in Table 1 of the appendix section.

In addition, resolution of EBV infection was found to be significantly associated with increased NK cell expression of CD57. This suggests that this subset of NK cells play a role in the successful response to EBV infection. CD57 is a marker of NK cell terminal differentiation. This subset may therefore represent long-lived, highly responsive and highly functional NK cells which remain in the circulation following primary EBV infection.

If this is the case, further analysis of this cohort may identify impaired NK functions in the C.H.L. group. Analysis of NK cell cytotoxicity and cytokine production in response to cytokine stimulation may therefore yield interesting results in this EBV infection cohort. Previous studies have identified a high level of CD8$^+$ T cell exhaustion in this cohort following transplantation irrespective of EBV viral load. This dampening of the CD8$^+$ T cell functional response may reflect an overall reduction in the responsiveness of immune

cells which extends to the NK cell subset of lymphocytes. However it is important to bear in mind that these patients have received immunosuppressive therapy post organ transplantation. This is an important factor contributing to the reduction in immune cell function seen in these patients.

Certain cancers and other chronic conditions have been associated with development of infectious mononucleosis following EBV infection. These include nasopharengeal carcinoma, Hodgkin's lymphoma and Burkitt's lymphoma (Parkin et al., 2006). Several HLA class I polymorphisms have been associated with the development of infectious mononucleosis and Hodgkin's lymphoma following primary EBV infection (McAulay et al., 2007, Niens et al., 2006 and Huang et al., 2012).

The *HLA-A*02* allele has been found to be associated with chronic high viral loads while the HLA-B*08 allele is significantly prevalent in individuals who resolved their high viral load (Moran et al., 2011). *HLA-A*26* and *HLA-B*38* have been associated with development of EBV^+ B cell origin post-transplant lymphoproliferative disease (PTLD) (Reshef et al., 2011). Other small studies have identified *HLA-DR7* as a risk factor for the development of symptomatic EBV infection in transplant patients (Hocker, 2013). However, the role of host genetics in predicting EBV infection outcome remains unclear. Ideally, a larger genetic study examining frequencies of KIR (and their HLA ligands) in an EBV infection cohort would be carried out to confirm any association between KIR3DL2 and EBV infection outcome.

Chapter 4

KIR2DS3 is not a risk factor for chronic infection in the East-German Anti-D cohort of HCV infection

4.1. Introduction

4.1.1. Disease association studies and the Anti-D cohorts of HCV infection

Most individuals exposed to HCV (50-85%) will go on to develop chronic infection with associated risks of liver cirrhosis and hepatocellular carcinoma (Westbrook and Dusheiko, 2014). Newly developed DAAs which are now becoming available have significantly improved patient outcomes but the problems of cost, treatment resistance and the lack of a prophylactic vaccine for prevention of HCV infection still remain. Identification of the factors involved in HCV disease development, therefore remains an area of significant research importance, and a large number of genetic studies have been carried out which further characterise the molecular basis for disease and the potential for development of new therapeutic approaches.

Exposure to HCV can lead to several clinical outcomes on the basis of presence of virus or virus antibody: HCV Ab^-, HCV RNA^- (seronegative, aviremic), HCV Ab^+, HCV RNA^- (spontaneous resolver) and HCV Ab^+, HCV RNA^+ (chronically infected). Those chronically infected can be further subdivided into those who clear HCV RNA following therapy (sustained virological responders) and those who do not (Knapp et al., 2010). In the past, genetic studies to identify factors which predispose to development of chronic HCV have been hampered by a lack of appropriate control populations of patients that have spontaneously cleared infection. Identification of such well-defined cohorts can prove difficult due to the mild clinical symptoms associated with infection (Cox et al., 2009).

A role for the adaptive immune response in HCV infection outcome has been well documented. A role for the innate immune system in the response to HCV is less well understood. The innate response to infection is characterised by detection of viral infection, activation of effector cells and the production of antiviral cytokines such as type 1 IFNs (Vilches and Parham, 2002). NK cells of the innate immune system have the ability to directly induce apoptosis of HCV infected hepatocytes while at the same time producing a range of antiviral cytokines. An increasing number of genetic and functional studies have provided evidence that NK cells are centrally involved in all stages of HCV infection. There is evidence that NK cell depletion or dysfunction may contribute to HCV persistence (Golden-Mason and Rosen, 2006 and Golden-Mason et al., 2008). Due to the considerable genetic diversity in the NK cell receptors that regulate NK cell function, several studies by

our research group and others have questioned whether the natural variation in NK cell receptor genetics may contribute to altered NK cell function with a resultant impact on HCV disease progression.

Gene association studies have been particularly useful in evaluating the contribution of innate immune genes to disease outcome. In many viral infection settings, the presence of many inherent variables makes it difficult to determine the specific key components involved in the protective immune response to viral infection. Factors such as ethnicity, gender, age, the presence of co-morbidity and co-infections and the duration of infection are known to affect disease outcome. The occurrence of two large single source outbreaks of HCV in both Ireland and the former East Germany has provided novel cohorts for investigation of the natural history of the virus and the factors affecting disease outcome (Kenny-Walsh et al., 1999, Weise et al., 2000). These outbreaks of iatrogenic infection occurred following accidental infection of Anti-D immunoglobulin directed against the Rhesus-D factor on red blood cells. Contaminated Anti-D Immunoglobulin was later administered to Rhesus negative mothers for prevention of Rhesus disease in current and subsequent pregnancies, resulting in iatrogenic infection (Kenny-Walsh et al., 1999, Weise et al., 2000).

The Anti-D cohorts of HCV infection have proven very informative in the study of HCV disease progression as they lack many of the confounding variables which often prove problematic when carrying out disease association studies. The patient population is extremely homogenous due to the nature of exposure to pathogen. All patients affected were female, fertile, and of a young age at time of infection. All were infected through the same route with a similar level of inoculum. In addition, all were infected with a single source of HCV of defined genotype (genotype 1b in the both Irish and German outbreaks) (Kenny-Walsh, 1999, Wiese et al., 2000).

Due to these factors, these two cohorts of women have been extensively studied and have been found to have a comparatively high rate of resolution (≈50%) (Kenny-Walsh et al., 1999 Wiese et al. 2000). This type of cohort allows for identification and study of spontaneous resolvers to identify the immuno-genetic factors that determine whether an individual will eliminate virus or develop chronic lifelong infection. Investigations into these cohorts have led to a greater understanding of viral pathogenesis, virus-host interactions and viral evasion strategies.

4.1.2. Irish cohort of female recipients of HCV (genotype 1b) contaminated Anti-D Immunoglobulin from a single infection source

The occurrence of the Irish outbreak of HCV infection in women who received contaminated Anti-D Immunoglobulin only came to light in the 1990s, although the majority of these women were found to have been infected from 1977-1979. Contamination with a single HCV genotype (genotype 1b) could be traced back to a single, acutely infected, plasma donor. Contaminated batches were found to contain between 9,600 and 200,000 viral genomes/vial (Kenny-Walsh et al., 1999).

In total, 795 women were confirmed as anti-HCV positive, 55% of these were found to be chronically infected (circulating HCV RNA viremia). In addition, 33 women, who developed jaundice within 6 months of receiving Anti-D immunoglobulin, were found to be anti-HCV negative (Kenny-Walsh, 1999). This cohort was found to have a lower rate of transmission and persistence of HCV in comparison to reported outcomes of disease progression in transfusion associated hepatitis (Seeff et al., 1992). Symptomatic infection and development of jaundice was significantly associated with a resolving course of infection (Barrett et al., 2001). 17 years after infection, only 55% of those who were initially described as chronically infected remained HCV RNA positive. 363 chronically infected women from this cohort underwent liver biopsy, 98% showed some signs of inflammation. In 93% of cases, inflammation was mild or moderate (Barrett et al., 2001).

Despite the restrictions associated with retrospective studies, valuable information, relating to progression of acute disease has been obtained from a clear and detailed review of past clinical events in this Irish cohort. Clinical re-evaluation of this cohort has identified a relatively benign course of HCV infection. Only a small proportion (2%) of those who became chronically infected (viral RNA positive) had developed cirrhosis 17 years after infection. In addition, sequential biopsies carried out two decades after initial infection, demonstrated signs of mild to moderate necro-inflammatory activity in 40% of chronically infected women in the Irish cohort. Comparatively higher levels of cirrhosis and related complications have been observed in other cohort studies involving post-transfusion outbreak of acute HCV (Kenny-Walsh et al., 1999). The clinical history of this patient cohort (as described in Kenny-Walsh et al., 1999 and Barrett et al., 2001) is summarised in Table 4.1.

4.1.3. German cohort of female recipients of HCV (genotype 1b) contaminated Anti-D Immunoglobulin from a single infection source

A second patient cohort was confirmed to be accidentally infected with HCV (genotype 1b) through contaminated anti-D blood products administered to Rhesus negative women post-partum in Germany in 1978/9. The source of the outbreak was identified in January 1979 and all the women involved were recalled for repeated screening of alanine transaminase (ALT) levels to test for possible liver damage. A total of approximately 3,000 women were involved, aged between 16 and 38 at the time of infection. The median age of infection was 24. In a sub-group of 1018 of these women, 10% showed no evidence of disease 6 months after Anti-D administration, as measured by ALT levels of less than 34mIU/ml. Forty-nine per cent of those who developed chronic infection, displayed a symptomatic course of infection with 22% displaying symptoms of jaundice (icteric infection) (Wiese et al., 2000).

A 20 year follow up analysis of this cohort was carried out, re-examining this sub-cohort (n=1018) of HCV infected women for symptoms and physical findings (Wiese et al., 2000). Ultrasound imaging of liver and spleen was carried out and levels of albumin, α-fetoprotein, anti-HCV antibodies and HCV RNA were also evaluated. After 20 years, 55% of diseased women remained HCV RNA positive (of these, 7% were found to be non-responders to therapy). Three per cent were classed as HCV carriers who displayed chronic viremia (HCV RNA^{+}) with normal ALT levels and no signs of liver disease. Liver cirrhosis was identified in just 0.4% of diseased patients. Liver biopsies obtained from 220 viremic women showed minimal or moderate grade hepatitis in the vast majority of cases.

This study concludes that this cohort of young women with no hepatic comorbidities may clear HCV infection in as many as 50% of cases or develop mild chronic HCV with a low risk of cirrhosis progression within 20 years (Wiese et al., 2000). Further re-evaluation of this cohort, carried out at 25 and 35 years after infection confirmed that long-term outcome in this otherwise healthy young female cohort is dependent on the initial (either natural or treatment induced) course of HCV infection (Wiese et al., 2014). These findings are similar to what has been reported in the Irish Anti-D cohort of HCV infection with both cohorts displaying similarly high levels of viral clearance, a good rate of response to IFN therapy and slow rates of fibrosis progression. The clinical history of this patient cohort (as described in Wiese et al., 2000) is summarised in Table 4.1.

Table 4.1. Clinical manifestations and natural history of HCV infection in the Irish and German Anti-D cohorts

	Irish Anti-D cohort	German Anti-D cohort
Source of infection	Plasma from 1 HCV (genotype 1b) infected donor was used to prepare 16 batches of Anti-D Immune globulin administered to women for prophylaxis of rhesus isoimmunisation in pregnancy (1977-1978)	Plasma from 1 HCV (genotype 1b) infected donor was used to prepare 14 batches of Anti-D Immune globulin administered to women for prophylaxis of rhesus isoimmunisation in pregnancy (1978-1979)
Identification of HCV outbreak	Routine regional screening of blood donors for HCV antibodies identified a high proportion of older, rhesus negative women among HCV^+ donors (1994)	A high proportion of cases of non-A non-B hepatitis in women receiving Anti-D immunoglobulin was reported (1979)
HCV Screening Programme	62,667 women who had received Anti-D Ig between 1970 and 1994 were referred for HCV testing (1994)	2,867 exposed women screened for abnormal ALT levels (1979)
Number of women with confirmed HCV infection	704 women were HCV Ab^+ showing evidence of past or current HCV infection 390 women were HCV RNA^+ (chronically infected) and offered clinical assessment and therapy	Study in 1018 of these women reported: 90% (n=917) had developed acute HCV with an increase in ALT levels 49% (n=497) developed symptomatic acute infection 22% (n=221) displayed an icteric course of infection.
Age at time of infection	28 (Mean)	24 (Median)
Long-term outcome following HCV infection	**1999:** 304 of 390 HCV RNA^+ women reported symptoms with fatigue being the most common (n=248) 47% (n=176) showed slightly elevated ALT levels Just 8% (n=31) showed significantly elevated levels of ALT Liver inflammation was identified in 98% of women (n=356) but this was mostly mild or moderate (41% (n=150) and 52% (n=190) respectively). 51% (n=180) displayed liver fibrosis but only 2% (n=7) had definite or probable cirrhosis	**2000:** 65% (n=599) of 917 with active or past infection reported constitutional symptoms, in particular, weakness and fatigue 85% (n=774) remained HCV Ab^+ while 55% (n=500) remained HCV RNA^+ Just 0.4% (n=4) displayed overt liver cirrhosis
Associations between the host immune response and HCV infection outcome	Icteric hepatitis was reported in 20.6% of those who cleared infection but only 3.4% of chronically infected women The HLA class II DRBI*01 allele was present in 28.8% of resolvers but only 8.7% of PCR positive women	Just 43% of those with an icteric course of acute infection developed long-term chronic infection in comparison with 60% of those with anicteric infection

Previously reported clinical history of both the Irish (Kenny-Walsh et al. 1999, Barrett et al., 2001) and German (Wiese et al. 2000) Anti-D cohorts is detailed.

4.1.4. KIR: HLA genes as predictors of HCV infection outcome

As previously mentioned (Section 1.5), the KIR family consists of highly polymorphic NK cell receptors, which play an important role in regulating NK cell function (Vilches and Parham, 2002). The KIR locus is rapidly evolving and displays significant population diversity, resulting in individuals possessing varying numbers of KIR genes (Vilches and Parham, 2002). In addition, a given KIR gene may be expressed on some, but not all NK cells within an individual. The ligands for KIR are encoded by the human leukocyte antigen (HLA) class I gene loci (chromosome 6) which are also highly polymorphic. This variety in receptor:ligand interaction results in great diversity in KIR regulation of NK cell function. HLA group 1 allotypes are defined by an asparagine residue at position 80 in the alpha helix while HLA group 2 molecules contain a lysine residue at this position (Colonna et al., 1993, Moretta et al., 1993, Winter and Long, 1997).

Several HLA haplotypes have been associated with HCV viral clearance in the Irish Anti-D cohort (McKiernan et al. 2004). The HLA class I genes, *HLA-A*03*, *HLA-B*27* and *HLA-Cw*01* have been reported to occur more frequently in those with HCV clearance compared with those who developed chronic infection. In contrast, the *HLA-B*08* gene was found to occur more often in those with chronic infection (McKiernan et al., 2004). Similar associations involving HLA class II alleles have also been identified with *HLA-DRB1*0101*, *HLA-DRB1*0401* and *HLA-DRB1*15* being linked to viral clearance while *HLA-DQB1*0201* has been associated with development of chronic infection (McKiernan et al. 2004, Fanning et al., 2000, Barrett et al., 1999).

KIR must be present in combination with their specific HLA ligands in order to mediate a biological effect. The genes encoding HLA ligands for the KIR receptors segregate independently of KIR genes. It is therefore possible for an individual to lack one or more cognate ligands for their KIR repertoire. This may influence the inhibition or activation potential of NK cells. As the interaction of KIR and HLA is epistatic, analysis of combined or compound genotypes is often required to see a particular impact or association.

Several studies have identified the importance of the KIR-HLA receptor interaction in controlling the NK cell anti-viral response to HCV infection. In addition, some KIR:HLA

compound genotypes have been reported to confer a protective effect against developing chronic hepatitis while others are found to be more prevalent in those who develop persistent infection. For example, the KIR2DL3: HLA-C1 interaction has been previously associated with HCV clearance while KIR2DL1: HLA-C2 has been associated with chronic infection (Khakoo et al., 2004, Knapp et al., 2010, Romero et al., 2008). *KIR2DL2* and *KIR2DL3* have been proven to segregate as alleles of the same locus (Khakoo et al., 2004). Genotypes which confer a lower inhibitory response and increased activating potential (such as the KIR2DL3: HLA-C1 interaction) are thought to be advantageous, resulting in a more effective response to pathogen. NK cells with more activating and less inhibitory receptor: ligand systems respond to viral infections more effectively than NK cells in individuals lacking these important systems (Moesta et al., 2008).

The frequency of *KIR2DS2* has also been reported to be decreased in a cohort of Korean patients with chronic HCV in comparison with uninfected healthy control populations (Sung et al., 2011). Recent studies in a Brazilian population reported the frequency of the *KIR2DL2*$^{+}$*HLA-C1*$^{+}$ compound receptor:ligand genotype was found to be increased in chronic HCV patients in comparison with healthy control donors. *KIR2DS2*, the *KIR2DS2*$^{+}$*HLA-C1*$^{+}$ compound genotype and *KIR2DS3* were also found to be associated with chronic infection independently of *KIR2DL2* (Vasconcelos et al., 2013).

In addition, previous studies from our research group, on the Irish Anti-D cohort, have reported *KIR2DS3* frequency to be increased in chronic HCV infection (0.314 in comparison with 0.194 in those who resolved infection p=0.002). Further genotype analysis, incorporating HLA class I ligand frequencies, showed that *KIR2DS3* was only significantly associated with development of chronic HCV infection in the presence of HLA-C2. Additional haplotype reconstruction analysis resulted in the identification of a specific centromeric KIR haplotype, containing *KIR2DS3*, which predisposes to HCV chronic infection in this cohort. A haplotype containing *KIR3DL3*, *KIR2DS2*, *KIR2DL2*, *KIR2DL5*, *KIR2DS3* and *KIR2DL1* was found to be significantly more prevalent in chronic HCV patients in comparison with those who resolved infection (0.1535 versus 0.0909, p=0.002).

4.1.5. IFNλ3 gene as a predictor of HCV infection outcome

This same study published by our research group also reported that *KIR2DS3* acts in synergy with the presence of an *IFNλ3* (*IL-28B*) *–T* allele to increase the risk of development of chronic HCV infection in the Irish Anti-D cohort (Dring et al., 2011). This polymorphism (rs12979860) near the IFNλ3 gene has also been previously associated with resolution of HCV infection in other European cohorts, including the German Anti-D cohort (Thomas et al., 2009, Tillman et al., 2010). When the frequency of C and T alleles were compared in the Irish cohort, the T allele was found to be significantly increased in those who developed chronic infection in comparison with spontaneous resolvers of infection (0.395 and 0.134 respectively). In addition, both the CT and TT genotypes were significantly increased in the chronically infected group compared with those who resolved infection, indicating that the *IFNλ3-T* allele has a dominant effect. IFNλ3 genotypes were found to be in Hardy-Weinberg equilibrium for spontaneous resolvers but not in chronically infected group, further indicating influence of the T allele. *KIR2DS3* was found to be significantly associated with chronic infection irrespective of the IFNλ3 allele. The presence of both risk factors was found to result in a synergising effect, resulting in significantly increased risk of chronic infection compared with the presence of either marker alone (Dring et al., 2011).

As previously mentioned (Section 4.1.4.), the HLA class I genes *HLA-A*03*, *HLA-B*27*, *HLA-C*01* genes have been associated with spontaneous clearance of HCV infection in the Irish Anti-D cohort (McKiernan et al., 2004). Further studies in this cohort revealed that the *HLA-A*03* gene is significantly associated with HCV clearance only in the presence of a favourable *IFNL3* (C/C) genotype. The protective effect of the *HLA-A*03* gene was not found to be statistically significant in those with a non C/C genotype. Contrastingly, the *HLA-B*27* and *HLA-C*01* genes are significantly increased in resolvers of HCV infection in the non-C/C group only. The HLA class II alleles *HLA-DQB1*0201* and *HLA-DRB1**0301 were found to significantly predispose to development of chronic infection irrespective of *IFNλ3* genotype. Similarly, DQB1*0501 and DRB1*0101 were associated with resolution of infection, irrespective of *IFNλ3* genotype (Fitzmaurice et al., 2014).

This SNP has also been previously associated with resolution of HCV infection in the German Anti-D cohort (Tillman et al., 2010). In the German cohort, 64% of those with C/C homozygous genotype for this polymorphism had cleared viral infection. This compares with 24% and 6% in women with a C/T and T/T genotype respectively. C/C

positive patients were also more frequently affected by symptoms of jaundice (32.7% in comparison with 16.7% in non C/C patients). In patients with a C/T or T/T (non C/C) genotype, those who had developed jaundice were significantly more likely to demonstrate spontaneous clearance (42.9% in comparison with 13.7% in those who did not develop jaundice). Interestingly, this association between development of jaundice and clearance of infection was not seen in those with a C/C genotype with no significant difference in spontaneous clearance rates in those who developed jaundice in comparison to those who did not (56.3% versus 60.6%). These results suggest that the association between development of jaundice and viral clearance is dependent of *IFNλ3* genotype (Tillman et al., 2010). A subsequent study by this research group reported that the predictive value of this *IFNλ3* polymorphism is limited in patients with a mutation in the *CCR5* gene (CCR5Δ32). Favourable response rates were associated with the *IFNλ3*genotype only in CCR5 wild-type homozygous women while HCV clearance in CCRΔ32 carriers remained poor even in patients with the IFNλ*3* C/C genotype. This suggests that both mutations are independent genetic determinants of HCV infection outcome.

4.1.6. Innate immune genes as predictors of treatment response in chronic HCV patients

Several KIR:HLA genes have been associated with treatment response in Japanese patients being treated for chronic HCV infection (Nozawa et al. 2013, Sung et al., 2011). In the Japanese cohort, the centromeric A/A KIR haplotype and *HLA-Bw4* were both significantly associated with a sustained virological response (SVR) to pegylated IFN-α + ribavirin therapy. In contrast, *KIR2DL2* or *KIR2DS2* was associated with non-response to therapy in these patients. In addition, a second polymorphism in the *IFNL3* gene (rs8099917) was found to be associated with response to treatment in this cohort. The *IFNL3* TT genotype in combination with *KIR3DL1*:*HLA-Bw4* was found to be predictive of good response to therapy. However, the *IFNL3* TT genotype in addition to the presence of *KIR2DL2*$^{+}$*HLA-C1*$^{+}$ compound genotype was related to non-response (Nozama et al., 2013).

In addition, the *KIR2DS5* allele was also found to be associated with SVR in the previously mentioned study of Brazilian patients with chronic HCV which was carried out by Vasconcelos et al. (2013). In a Spanish cohort of HCV infection, the *KIR2DL2*001* allele was found to be more frequency in non-SVR patients while the *KIR2DL3*001* allele was associated with sustained response to therapy. The associated *KIR2DL3*001*$^{+}$*HLA-C1*$^{+}$

genotype was also significantly associated with SVR. Similar to what was seen in the Japanese cohort, the centromeric A/A genotype was more frequent in patients with an SVR. In contrast, the centromeric B/B genotype was associated with non-response to treatment (Vidal-Castineira et al., 2014).

Thus, there is increasing evidence that HLA genes, as well as the innate immune genes *IFNλ3* and KIR play a role in determining viral outcomes in terms of both initial response to infection and long-term treatment response. However, the interaction of these genes with each other is not well understood. Recent studies have attempted to address this, reporting that *IFNλ3*, HLA-C and KIR allelic variants additively predict response to pegylated IFN-α + ribavirin therapy following chronic HCV infection in a cross-sectional European Study. They suggest that these findings may be indicative of a role for NK cell activation (partially mediated by IFNL3) in treatment induced HCV clearance (Suppiah et al., 2011). A recent study in the Irish Anti-D cohort reported that HLA Class I and Class II genes are significantly associated with viral outcomes, even when impact of IFNL3 and KIR2DS3 genes are taken into account. They also identified a clear additive effect between the different genes indicating likely independent and separate but cumulative immune events (Fitzmaurice et al., 2014).

KIR and HLA genes have not been previously investigated in the German Anti-D cohort of HCV infection. The study outlined in this chapter examined the hypothesis that several prevalent KIR:HLA associations, specifically KIR2DS3:HLA-C2 and KIR2DL2/3:HLA-C1, are significant factors in predicting prognosis in the German Anti-D cohort of HCV infected women, as has been previously reported by our research group and others in other cohorts of HCV infection.

We undertook to investigate the contribution of several KIR and HLA class I genes to resolution of infection in the German Anti-D cohort and determine whether these genes or their associated compound KIR:HLA genotypes contributed to outcome of HCV infection.

4.1.7. Aims

- Investigate associations between innate immune genes and HCV resolution in the German Anti-D cohort.

- Examine whether KIR and HLA genes contribute to the course or outcome of infection as has been seen in other cohorts of HCV infection.

4.2. Results

4.2.1. HLA typing and treatment response in the East German Anti-D cohort of HCV infection

DNA from 425 women from an East German cohort of women, infected with HCV through contaminated Anti-D immune globulin was kindly provided by Dr. Thomas Berg and Dr. Janett Fischer (University of Leipzig, Germany). All women in this cohort were accidently infected with HCV between 1978-1979 when 14 batches of anti-D immune globulin, contaminated with HCV genotype 1b from a single erythrocyte donor, was administered throughout East Germany for prophylaxis of rhesus isoimmunisation in pregnancy. These women have been prospectively followed in regional centres and a significant amount of background and clinical information has been documented on these patients. This study cohort included 302 patients diagnosed as chronically infected (PCR RNA^{+}) and 108 who had resolved infection without treatment (PCR RNA^{-}). Further information on this cohort, relating to HCV viral load, response to treatment, current diagnosis and the presence of liver cirrhosis was also documented. Detailed clinical information of the patients included in this study is provided in Appendix Table 2. This cohort has also been previously typed for various HLA genes: *HLA-A1* (377 individuals), *HLA-A2* (376 individuals), *HLA-B1* (377 individuals) and *HLA-B2* (373 individuals). Detailed clinical information relating to the patients included in this study is provided in Appendix Table 2. Results from HLA typing of this cohort are detailed in Appendix Table 3.

4.2.2. No difference in KIR gene frequency in the East German Anti-D cohort of HCV infection in comparison with a healthy control population

In order to investigate the contribution of KIR genes to the development of HCV infection, patients were genotyped for the following KIR genes: *KIR2DS3, KIR2DL3* and *KIR2DL2.* The frequencies of individual KIR genes in an East-German cohort of women, infected

with HCV via administration of contaminated Anti-D immunoglobulin, were compared to those in a healthy control group (Momot et al., 2004). KIR frequency data was also compared to corresponding data from the Irish Anti-D cohort previously published from our research group (Dring et al., 2011) as well as an healthy Irish control group (D. Middleton – www.allelefrequencies.net) (Table 4.2.).

Carrier frequency distributions, determining the proportions of individuals testing positive for each of the 3 KIR genes tested were broadly similar to what had been previously reported in the Irish Anti-D cohort and in healthy control populations from both regions. *KIR2DL2* frequency ranged between 0.503 and 0.510 and *KIR2DL3* frequency ranged between 0.896 and 0.93 across the four separate cohorts (Table 4.2.) (Dring et al., 2011, Momot et al., 2004). *KIR2DS3* frequency in the German Anti-D cohort was found to be 0.282, in comparison with 0.23 in a healthy control population (Table 4.2.).

Table 4.2. Phenotypic frequencies of several KIR genes in the East German Anti-D cohort of HCV infected women

	German		Irish	
	Anti-D (n=425)	**Healthy (n=100)**	**Anti-D (n=543)**	**Healthy (n=200)**
KIR2DS3	0.282 (120)	0.230 (23)	0.256 (139)	0.280
KIR2DL3	0.896 (381)	0.910 (91)	0.899 (488)	0.930
KIR2DL2	0.504 (214)	0.510 (51)	0.503 (273)	0.510

The frequencies of *KIR2DL2, KIR2DS3* and *KIR2DL3* are shown. For comparison, corresponding frequencies from Irish Anti-D cohort (Dring et al., 2011) and healthy populations from Germany and Northern Ireland (Momot et al., 2004, D. Middelton (unpublished) respectively) are also shown. N numbers detailing the number of individuals in each study are shown. N numbers detailing the number of individuals in each group is shown in parentheses.

4.2.3. No difference in HLA-C gene frequency in the East-German Anti-D cohort of HCV infection in comparison with a healthy control population

KIR and HLA genes are inherited on different chromosomes and both receptor and ligand are required for a functional interaction to occur (Parham et al., 2005). *HLA-C1* alleles

encode ligands for the KIR2DL3 receptor (including its common allotype *KIR2DL2*). *HLA-C2* alleles encode ligands for the KIR2DL1 receptor. In order to investigate the contribution of KIR ligands (HLA) to the development of HCV infection, carrier frequency distributions, determining the proportion of individuals positive for each of the HLA-C KIR binding motifs, were calculated (Table 4.3.).

Although no significant differences in HLA gene frequency were identified in the German Anti-D cohort, *HLA-C2* carrier frequency did show a trend towards increased frequency in this cohort in comparison with healthy controls (0.649 compared with 0.567 p=0.071). Although not reaching statistical significance, this low P value may be suggestive of a real difference in average frequency of *HLA-C2* between healthy and diseased populations as has been reported in other HCV cohorts (Table 4.3(a)). It is likely that there are insufficient numbers in this study population to reliably test our hypothesis. An increase in sample numbers in our study population may therefore yield statistically significant genotypic differences.

Corresponding allelic frequencies of both *HLA-C1* and *HLA-C2*, determining the proportion of a particular allele of a gene among all alleles, were also calculated (Table 4.2(b)). Evaluation of allelic frequencies showed similar non-significant trends with a marginal increase in *HLA-C2* frequency the German Anti-D cohort in comparison with healthy control populations (Table 4.3(b)).

Table 4.3. HLA-C typing in the East German Anti-D cohort of HCV infected women

(a)

Phenotypic Frequency

	German		Irish
	Anti-D (n=425)	**Healthy (n=150)**	**Anti-D (n=543)**
HLA-C1	0.828 (352)	0.820 (123)	0.865 (424)
HLA-C2	0.649 (276)	0.567 (85)	0.575 (73)

(b)

Allelic Frequency

	German		Irish	
	Anti-D (2n=850)	**Healthy (2n=300)**	**Anti-D (2n=1086)**	**Healthy (n=270)**
HLA-C1	0.590	0.627	0.639	0.680
HLA-C2	0.410	0.373	0.361	0.320

Phenotypic (Table 4.3(a)) and allelic (Table 4.3(b)) frequencies of HLA-C1 and HLA-C2 are shown. For comparison, corresponding frequencies from Irish Anti-D cohort (Dring et al., 2011) and healthy population from Germany and Southern Ireland (Schonberg et al., 2011 and Guinan et al., 2010 respectively) are also shown. N numbers illustrating the number of individuals in each study are shown in parentheses. The number of individuals in each group is shown in parentheses.

4.2.4. KIR2DS3:HLA-C2 is not a risk factor for development of chronic infection in the East German cohort of HCV infected women

We also tested whether *KIR2DS3* was associated with predisposition to chronic HCV infection as has been previously reported in the Irish Anti-D cohort (Dring et al., 2011). No such significant associations were identified in the German Anti-D cohort, indeed, *KIR2DS3* carrier frequency was found to be marginally decreased in chronically infected patients from the German cohort when compared with those who spontaneously resolved

infection, (0.333 in spontaneous resolvers compared with 0.262 in those with chronic infection) (Table 4.4(a)). Importantly however, this result was not statistically significant by Chi Squared analysis.

As previously mentioned, the *KIR2DS3* gene has previously been reported to predispose to development of chronic HCV infection in the presence of *HLA-C2* in the Irish Anti-D cohort. We therefore examined whether the frequency of *HLA-C2* was increased in those who developed chronic infection. However, analysis of *HLA-C2* frequency in the German Anti-D cohort showed no significant association with chronic infection (Table 4.4(b)).

We also tested whether carrier frequency of *KIR2DL3*, and its allelic variants *KIR2DL2*, was associated with resolution of HCV infection as has been previously reported (Khakoo et al., 2004). The frequency of *KIR2DL3* was not significantly increased in those who resolved HCV infection (0.907 compared with 0.890 in chronically infected patients) (Table 4.4(a)). Similarly, the frequency of its HLA ligand: HLA-C2 was not associated with HCV infection outcome (0.870 in those who resolved infection compared with 0.815 in chronically infected patients (Table 4.4(b)). The frequency of the *KIR2DL2*, an allotype of *KIR2DL3,* was statistically unchanged (0.526 in chronically infected patients compared with 0.463 in resolvers of infection) (Table 4.4(a)).

Table 4.4. Comparison of KIR/HLA distribution in the East German Anti-D cohort of HCV infected women

(a)

	German Anti-D		Irish Anti-D	
	Chronic HCV (302)	Resolvers (108)	Chronic HCV (296)	Resolvers (247)
KIR2DS3	0.262 (79)	0.333 (36)	0.314	0.194
KIR2DL3	0.890 (269)	0.907 (98)	0.898	0.928
KIR2DL2	0.526 (159)	0.463 (50)	0.527	0.486

(b)

	German Anti-D		Irish Anti-D	
	Chronic HCV (302)	Resolvers (108)	Chronic HCV (268/258)	Resolvers (220/215)
HLA-C1	0.815 (246)	0.870 (94)	0.843 (226)	0.890 (196)
HLA-C2	0.672 (203)	0.602 (65)	0.597 (154)	0.549 (118)

Phenotypic frequencies of KIR2DL2, KIR2DS3 and KIR2DL3 (Table 4.4(a)) and HLA-C1 and HLA-C2 (Table 4.4(b)) are shown. For comparison, corresponding frequencies from Irish Anti-D cohort (Dring et al., 2011) are also shown. N numbers detailing the total number of individuals in each study are shown in parentheses. The number of individuals in each group is shown in parentheses.

The *KIR2DS3:HLA-C2* compound genotype has also been previously associated with development of chronic infection in the Irish cohort. In light of these results and previous findings (Dring et al., 2011, Khakoo et al., 2004, Knapp et al. 2010), we examined the frequency distribution of *KIR2DS3* and *KIR2DL3* in the presence of either one or two copies of the *HLA-C2* or *HLA-C1* allele. In this way, we went on to test whether *KIR2DL3* was associated with resolution of HCV infection on a HLA-C1 background as has

previously been reported. Similar analysis was carried out for the KIR2DS3: HLA-C2 interaction which has been previously associated with an increased risk of developing chronic HCV in the Irish Anti-D cohort (Table 4.5.).

Distribution of *KIR2DS3* in the presence of either one or two copies of *HLA-C2* was found to be similar to what has been previously reported (Figure 4.5(a)). The frequency of *KIR2DL3* in the presence of at least one copy of its ligand *HLA-C1* was found to be non-significantly increased in chronic patients in comparison with resolvers (p=0.147). The presence of this KIR and two copies of *HLA-C1* was found to be non-significantly decreased in chronic patients (p=0.112). These patterns are similar to what has been previously reported in other HCV infected cohorts (Khakoo et al., 2004) (Figure 4.5(b)).

Table 4.5. Comparison of distribution of two compound genotypes associated with HCV clinical outcome *KIR2DS3:HLA-C2* and *KIR2DL3:HLA-C1* in the German Anti-D cohort

(a)

	Chronic HCV (302)	Resolvers (108)	X^2 (p value)	O.R.
KIR2DS3	0.262 (79)	0.333 (36)	2.03 (0.154)	0.71
HLA-C2	0.672 (203)	0.602 (65)	1.74 (0.187)	1.36
KIR2DS3$^+$C2$^+$	0.172 (52)	0.204 (22)	0.53 (0.465)	0.81
KIR2DS3$^+$C2/C2	0.046 (14)	0.037 (4)	0.16 (0.685)	1.26

(b)

	Chronic HCV (302)	Resolvers (108)	X^2 (p value)	O.R.
KIR2DL3	0.890 (269)	0.907 (98)	0.05 (0.815)	0.92
HLA-C1	0.815 (246)	0.870 (94)	1.75 (0.186)	0.65
KIR2DL3$^+$C1$^+$	0.715 (216)	0.707 (85)	2.10 (0.147)	0.68
KIR2DL3$^+$C1/C1	0.288 (87)	0.370 (40)	2.52 (0.112)	0.69

	Chronic HCV (681-685)	Resolvers (348-352)	O.R.
Khakoo et al., 2004 (n=1037)			
HLA-C1	0.798 (547)	0.855 (301)	-
KIR2DL3$^+$HLA-C1/C1	0.267 (182)	0.339 (119)	1.41 (0.02)

Table 4.5(a) details the distribution of two variations of *KIR2DS3:HLA-C2* compound genotypes. Table 4.5(b) details the distribution of two variations of *KIR2DL3:HLA-C1* compound genotypes. Frequencies of individual KIR and HLA genes are also shown. For comparison, corresponding distributions for a separate cohort of HCV infection originating from U.S.A. and U.K. is also shown (Khakoo et al., 2004). N numbers detailing the total number of individuals in each study are shown. The number of individuals in each group is shown in parentheses. X^2 values and Odds ratios are also given.

4.3. Discussion

The genetic diversity inherent in NK cells genes makes their genes likely candidates for involvement in disease development. There is significant evidence that genetic factors play a significant role in HCV disease progression (Dring et al., 2011). Cohorts such as the German and Irish Anti-D cohorts of HCV infected women are extremely useful in gene association studies such as that described in this chapter. This type of cohort allows for direct comparison of patients who resolve infection naturally and those who develop chronic HCV infection and severe disease.

As mentioned previously, findings from our research group have previously identified the NK cell receptor gene *KIR2DS3* as a risk factor associated with lower rates of spontaneous clearance and lower levels of responsiveness to treatment in the Irish Anti-D cohort of HCV infected women. A synergising effect of this gene in the presence of the *IFNL3-T* allele has also been reported to result in a substantial increase in risk of chronic infection (Dring et al., 2011). In contrast, the KIR2DL3/HLA-C1 receptor ligand interaction has been associated with increased levels of viral clearance following HCV infection (Knapp et al., 2010, Khakoo et al., 2004). The study described in this chapter examined whether similar associations between KIR:HLA and disease progression exist in the German Anti-D cohort of HCV infection.

Several quality control measures were put in place in order to ensure the validity of the results from this genotyping study. KIR and HLA genotyping of patient samples was carried out using sequence specific primers (PCR-SSP) which had been previously validated and published (Vilches et al., 2007, Tajik et al., 2010). Before the analysis of patient samples, the PCR protocol for both KIR and HLA genotyping was validated using DNA of known gene content. In addition, internal control primers were included in each reaction as part of this protocol in order to ensure that the quality and quantity of DNA used was sufficient for amplification to occur. Following analysis of the patient cohort, a random selection of 10 out of 425 samples were re-tested and showed no difference in *KIR2DS3* genotype.

4.3.1. KIR2DS3 and HLA-C2 gene frequencies in the German Anti-D cohort

Although trends for particular KIR and HLA associations were observed in this current study, no definitive associations were identified. KIR and HLA genotyping results from the German Anti-D cohort revealed gene frequencies which were broadly in line with what has been previously reported in both healthy and HCV infection cohorts (Dring, 2011 and Khakoo, 2004). *KIR2DS3* was found to have a gene frequency of 0.282 in the German Anti-D cohort in comparison with 0.23 in healthy German counterparts. These gene frequencies are similar to what has been previously identified in the Irish population which showed a *KIR2DS3* frequency of 0.256 in the Irish Anti-D cohort in comparison with 0.28 in a healthy Irish population. Of note however, comparison of healthy and patient groups from Irish and German cohorts showed contradictory patterns in *KIR2DS3* frequency. Frequency of *HLA-C2* was found to be statistically unchanged in this Anti-D cohort in comparison with healthy control populations (0.410 compared with 0.320 in a healthy German cohort).

In the Irish Anti-D cohort, *KIR2DS3* has been previously reported to be significantly increased in chronic HCV patients. When analysed in terms of HLA class I ligands, this KIR was found to predispose to chronic HCV infection only in the presence of *HLA-C2* genetic background. Such associations were not identified in this study of the German Anti-D cohort. Indeed, this study of the German Anti-D cohort identified a marginal decrease in frequency of *KIR2DS3* in those who developed chronic infection compared to resolvers (0.262 in comparison with 0.333 in those who resolved infection). Importantly however, this trend was not statistically significant.

Multiple KIR genes with different HLA specificities are inherited on different haplotypes and HLA genotype can have a significant impact on NK cell responses. Further investigations into KIR haplotype structure and frequency in the German population may account for the lack of an association between *KIR2DS3* frequency and development of chronic infection in this Anti-D study population. It is possible that *KIR2DS3* and *HLA-C2* genes are acting as members of a larger haplotype of genes that are inherited together in linkage disequilibrium (Dring et al., 2011). Thus, it is likely that it is a haplotype rather than individual genes that are contributing to predisposition towards development of chronic viral infection. It is likely that *KIR2DS3* is one marker of this haplotype in the Irish Anti-D cohort. Other genes associated with such haplotypes may influence the course of HCV infection and further KIR genotyping of the German Anti-D cohort may therefore

reveal significant associations with HCV infection outcome. The results shown here support the theory that a combination of other receptors work together with members of the KIR receptor family to influence the contribution of KIR genes to HCV development and progression. It is probable that these receptors work in combination to provide the balance of signals to result in either activation or inhibition of NK cell function.

KIR2DS3 has previously been implicated as a risk factor in other viral infections (Zhi-Ming et al., 2007 and Wauquier, et al., 2010). It has also been identified as a risk factor for graft-vs-host disease after stem cell transplant for haematological malignancies (McQueen et al., 2007). However, evidence suggests that it is not a functional receptor. The *KIR2DS3* gene has been lost from some populations and the KIR2DS3 protein has been shown to have little or no cell surface expression in transfection (VandenBussche et al., 2009). No significant avidity for HLA-C1, HLA-C2 or any HLA class I epitope has been identified and its ligand remains unknown (Moesta et al., 2010).

Previous work from our research group identified that the association between *KIR2DS3* and development of chronic HCV infection was only seen on a *HLA-C2* genetic background (Dring et al., 2011).

HLA-C2 is not a ligand for *KIR2DS3.* Our results suggest that the receptor interacting with HLA-C2 is likely to be encoded by a gene in linkage disequilibrium with *KIR2DS3* within the NK complex or the wider genomic region of the leukocyte receptor complex. It is likely that it is this gene, rather than *KIR2DS3* itself, which is influencing HCV disease development through interaction with HLA-C2. This may explain why *KIR2DS3* gene was not associated with chronic HCV infection in this study cohort. Indeed, this possibility was originally raised by our research group following analysis of KIR:HLA genotyping results in the Irish Anti-D cohort (Dring et al., 2011). This previous study identified a specific centromeric haplotype block that was associated with development of chronic infection following exposure to HCV. In addition to *KIR2DS3,* this haplotype contained both *KIR2DL1* and *KIR2DL2* which encode receptors which bind to HLA-C2, making them candidate genes for explaining the increased risk of chronic infection associated with *KIR2DS3* and *HLA-C2* (Dring et al., 2011).

Another important factor of note when comparing data from the Irish and German Anti-D of HCV infection is that the subgroup from the German Anti-D cohort examined in this study included a comparatively lower number of resolving patients in comparison with the Irish Anti-D cohort which have been previously studied by our research group (n=108

versus n=247 in the Irish Anti-D cohort). This may also explain the lack of significant differences when the study population is sub-divided based on clinical outcome following initial exposure to virus. Ideally an equal number of chronically infected and HCV resolvers would be examined. Analysis of further patient samples from patients who resolved infection may reveal significant associations between KIR genotype and HCV infection outcome.

4.3.2. KIR2DL3 and HLA-C1 gene frequencies in the German Anti-D cohort

As previously mentioned, the KIR2DL3:HLA-C1 interaction is a relatively weak inhibitory interaction. We hypothesised that this interaction would be protective in HCV infection because it would be more easily overridden by activating signal than a stronger inhibitory interaction such as KIR2DL2:HLA-C1 or KIR2DL1:HLA-C2. The protective effect of the *KIR2DL3/HLA-C1* compound genotype has been previously reported in relatively heterogenous HCV patient cohorts, infected through blood transfusion or intravenous drug use (Khakoo et al., 2004 and Knapp et al., 2010). Similar, although non-significant, trends have been previously reported in the Irish Anti-D cohort by our research group. This finding is consistent with HIV disease models in which activating interactions are advantageous for viral clearance (Martin et al., 2002). It has been suggested that the evolutionary conservation of the MHC-C1 motif across primate species is due to the protective effect conferred by the *KIR2DL3:HLA-C1* compound genotype, resulting in a selective advantage of this genotype in combating viral infection (Khakoo et al., 2004). Our study aimed to determine whether this genotype was associated with resolution of HCV infection in the German Anti-D cohort.

This genetic study of the German Anti-D cohort provides no evidence of a statistically significant association between *KIR2DL3* or *HLA-C1* and resolution of HCV infection. Gene frequencies were found to be broadly similar to those previously reported in both healthy and diseased populations (Dring et al, 2011 and Khakoo et al., 2004). Although no statistically significant associations were identified, patterns identified in the German Anti-D cohort are similar to what has been previously reported in the Irish Anti-D and other HCV infection cohorts.

Although not significant by Chi squared analysis, these results may support the previous findings of a protective effect of *KIR2DL3:HLA-C1* interaction following HCV infection,

due to its relatively weaker inhibitory potential in comparison with *KIR2DL2*. More extensive investigations into a larger cohort population may reveal these non-significant trends to be indicative of a real long-term difference in frequency of these innate immune genes. However, it is also important to note that the association of the KIR2DL3:HLA-C1 receptor ligand interaction with spontaneous clearance was only observed in those exposed to low infectious doses of HCV through intravenous drug use or needle stick injury. A significant protective effect was not seen in those with high dose exposure (through transfusion of contaminated blood or concentrated blood products), where the innate immune system may be expected to be overcome by high viral load (Khakoo et al., 2004). A further study by Knapp et al. (2010) that the advantageous effect of this KIR:HLA interaction was strongest in those who acquired HCV from intravenous drug use, suggesting that concentration of inoculum is important. This may explain the lack of significant associations between *KIR2DL3/HLA-C1* genotype and viral clearance in this study of the German Anti-D cohort (and previous studies in the Irish Anti-D cohort), although similar trends were identified.

A combination of factors have been implicated in determining disease outcome in HCV infection. Identification of the factors involved in the persistence of viral infection could lead to development of specific prognostic tests and more effectively managed treatment for patients. Much of the early work in this area has focused on the role of the adaptive immune system; however recent studies carried out by our research group provide evidence for a role for innate immune genes in regulating HCV disease progression (Dring et al., 2011). Future study on this cohort may include genotyping for a wider range of KIR and HLA genes, including allele typing for *KIR2DS3* as well as *KIR2DL1* and *KIR2DL2*. Genotyping of Bw4 and Bw6 ligand motifs present on HLA-B and some HLA-A alleles may also reveal interesting results. Genotyping of the *IFNL3* polymorphism has previously been carried out in the German Anti-D cohort (Tillman et al., 2010). It may be possible to obtain *IFNL3* genotyping data for the donors used in our study in order to carry out similar KIR:HLA genotyping analysis on sub-groups of this cohort stratified according to *IFNL3* genotype. It may also be useful to determine whether the KIR2DL3:HLA-C1 is significantly associated with sustained response to pegylated IFN-α + ribavirin therapy as has been previously reported in other cohorts (Knapp et al., 2010). Given the recent significant advances in HCV treatment, it will also be important to examine whether KIR genotype can be used as a predictor of patient response to newly developed DAA therapies.

Chapter 5

NK cell functional responses following administration of Adenoviral prime and MVA boost candidate HCV vaccines

5.1. Introduction

5.1.1. Development of vaccines for prevention of infectious diseases

The introduction of large scale vaccination programmes has had a major impact on public health worldwide, resulting in control of many infectious diseases, improved quality of life and increased life expectancy. Some of those more successful vaccines to date include those developed against Smallpox, Polio, Hepatitis A, Hepatitis B and Measles, Mumps and Rubella. Although currently available vaccines are extremely effective, continued efforts to develop new and improved vaccines will required in order to ensure the continued success of vaccination programmes into the future. Successful vaccines must be capable of eliciting a robust but non-pathogenic immune response which provides long-term protection against infection. Such long-term immunity requires the induction of the adaptive immune response and immunological memory. This involves both humoral (antibody) and cellular immune responses with the induction of virus specific memory T cells (Slifka, 2014).

There are several types of vaccines which have been developed to date. These include live attenuated vaccines, inactivated vaccines, subunit vaccines, DNA vaccines, toxoid vaccines and recombinant vector vaccines. Live attenuated vaccines contain a version of the living microbe which has been altered and is no longer virulent. In contrast, inactivated vaccines are produced by killing of the disease causing microbe by chemical or heat treatment. Subunit vaccines contain only the most antigenic peptides which are capable of stimulating a robust immune response. Vaccines containing bacterial toxins which have been inactivated by treatment with formalin are capable of inducing robust immune response resulting in successful immunisation against infection. DNA vaccines are taken up by some cells of the recipient and induce production of immunogenic antigens which stimulate a protective immune response. Similarly, recombinant vector vaccines use an attenuated virus or bacterium as a carrier vector to expose the cells of the recipient to microbial DNA. Viral vectors work by inserting DNA from a potentially harmful microbe into recipient cells, mimicking a natural infection and inducing an immune response. Microbial DNA can also be inserted into an attenuated bacterial vector causing the bacteria to display immunogenic antigens and inducing an immune response (www.vaccines.gov/more_info/types).

The duration of vaccine induced immunity will vary depending on the vaccine and the course of natural pathogen infection. Not all antibody responses are equally long-lived and there are antigen-specific characteristics which determine the magnitude and durability of the antibody response to each particular antigen (Slifka et al., 2014). The durability of vaccine induced T cell memory responses and their role in vaccine-induced protection against disease remains unclear. Studies have shown that antiviral T cell memory can be maintained for decades after vaccination (Hammeralund et al., 2003, Crotty et al., 2003). It is generally accepted that most vaccines will require the induction of a combination of both humoral and T cell immune responses in order to be effective. However, several studies suggest that viral specific T cells are not required for protection against infection (Edgehill-Smith et al., 2005, Monath et al., 2002, Guirakhoo et al., 2006). In addition, a reported candidate HIV vaccine failed to protect against infection despite a robust induction of virus specific T cell responses following vaccine administration (Buchbinder et al., 2008).

The vast majority of vaccines require one or more boost vaccinations following initial prime vaccination regardless of whether they are non-replicating vaccines of live attenuated vaccines. In many cases, boost vaccination is required for long-term protection even in the case where natural infection results in life-long immunity. This requirement for a boost vaccination is likely due to issues with vaccine safety which require reduced pathogenicity. This will improve the safety profile of the vaccine but may also result in a reduction in antigenic load and the subsequent antiviral immune response which is induced. In most cases, initial prime vaccination will provide short-term protection against infection. Boost vaccination are designed to further increase the levels of immunity but the longevity of this protective effect varies depending on the vaccine type and the disease involved (Slifka et a., 2014).

To date, most vaccine trials have focused on the ability of candidate vaccines to induce an adaptive immune response which has the benefits of being pathogen specific and also resulting in the production of long-lived memory response. There is however increasing evidence that NK cells of the innate immune system are capable of undergoing alterations in both receptor expression and effector function following natural exposure to pathogen or vaccination. Despite the recent advances in HCV treatment in the last 2-3 years, the development of a prophylactic vaccine for prevention of HCV infection has become an area of significant research importance. The study described in this chapter examined

whether a candidate HCV vaccine was capable of activating NK cell innate responses which may contribute to successful protection against infection.

5.1.2. NK cells are phenotypically and functionally altered following exposure to potentially pathogenic antigens

A small number of clinical studies carried out to date suggest that NK cells contribute to the effector phase of vaccine induced immune responses, secreting cytokines and directly killing infected cells. The proportion of responding NK cells is found to vary between individuals and by vaccine, suggesting that functionally discrete subsets of NK cells with different activation requirements may be involved (White et al., 2014).

The concept of 'memory-like' NK cells which display some of the characteristics of adaptive immunity has been of the subject of much research in recent years. In mouse CMV infection, NK cells have been reported to proliferate and persist at high frequencies for several months and showed increased cytokine producing and degranulation functions following reactivation (Cooper et al., 2009, O'Leary et al., 2006 and Sun et al. 2009). Human CMV infection has been reported to result in an expansion of an $NKG2C^+$ NK cell subset in individuals who are seropositive (Guma et al, 2004). More recent studies have associated development of acute CMV infection with the expansion of long-lived 'memory-like' NK cells marked by the expression of CD94/NKG2C dimers and CD57 (Lopez-Verges et al., 2011, and Muntasell et al., 2013).

EBV coinfection in children has been found to boost CMV induced NK cell differentiation. Co-infected children were found to have higher proportions of $NKG2C^+$ NK cells in the peripheral blood in comparison with CMV^+EBV^- children. This $NKG2C^+$ NK subset were found to be highly responsive with significant degranulation and IFN-γ production following target cell stimulation. *Ex vivo* NK degranulation response to target cell stimulation was found to be increased in CMV^+ children and EBV coinfection was also found to be associated with high levels of plasma IL-15. In vitro EBV infection of PBMC from EBV^-CMV^+ children was found to result in increased proportions of $NKG2C^+$ NK cells (Saghafian-Hedengren et al., 2013).

This $NKG2C^+CD57^+$ NK cell subset has also been found to be expanded in CMV^+ individuals co-infected with other viral infections such as Hantavirus, Chikungunya virus

and Hepatitis B and C (Bjorkstrom et al., 2011, Petitdemange et al., 2011 and Beziat et al., 2012). Taken together, these studies suggest that exposure to CMV results in a long-term alteration in distribution of effector cells, which in turn modulates subsequent immune responses to viral infections.

A Puumala Hantavirus outbreak allowed for a prospective study of clinical samples, following NK cell responses for 16 months following initial presentation with symptoms, to be carried out (Bjorkstrom et al., 2011). In this study, NK cell numbers were found to rapidly expand and persist at elevated levels for up to 60 days after hantavirus infection, extending far beyond the period of a normal innate immune response (Bjorkstrom et al., 2011). Plasma levels of IL-15 were also found to be elevated up to 60 days after disease onset in the majority of patients suggesting that this cytokine is required for NK cell proliferation and persistence. Furthermore, the proliferating NK cells were found to express high levels of Bcl-2 compared with non-proliferating NK cells, suggesting that Bcl-2 up-regulation may prevent early apoptosis of proliferating NK cells contributing to the accumulation of this cell population (Bjorkstrom et al., 2011).

In addition, a high proportion of the expanding population of NK cells were found to express the activating receptor NKG2C and were functional (i.e. expressed a licensing inhibitory KIR receptor and were capable of responding to target cell stimulation) (Bjorkstrom et al., 2011). These observations support the newly developing line of thought that human NK cells may naturally possess features not traditionally associated with the innate immune response (Sun and Lanier, 2009). However it remains unclear whether these features allow for adaptation of the NK cell repertoire to future infection with the same or similar pathogens.

Further studies, undertaken around the same time, have attempted to address this question in the context of vaccination. Horowitz et al. (2010) has reported that NK cells can act as effector cells of the acquired immune responses after vaccination. This study reported prolonged NK cell cytokine production and degranulation responses *in vitro* after re-stimulation of PBMCs with inactivated rabies virus in vaccinated but not unvaccinated individuals. NK cells also showed up-regulation of the activating receptor CD69 and increased perforin secretion in response to inactivated rabies virus after rabies vaccination. Importantly, NK cells were found to make up over 70% of total IFN-γ secreting and degranulating cells in the first 12-18 hours after re-challenge with inactivated virus with

NK cells undergoing extensive proliferation during post-vaccination recall response (Horowitz et al., 2010).

This recall 'memory' response was seen to be dependent on IL-2 production by $CD4^+$ T cells. IL-12 and IL-18 cytokine production by other accessory cells were also reported to synergise with IL-2 to activate NK cells after vaccination (Horowitz et al., 2010). Interestingly, pre-vaccination NK cells were found to respond to stimulation with inactivated rabies virus when cultured with post-vaccination T cells (Horowitz et al., 2010). This study concludes that pre and post vaccination NK cell effector responses are augmented in an antigen specific manner by vaccination. These results also show that NK cells are major contributors to the post vaccination response particularly in initial stages after re-challenge with virus. The authors also suggest that IL-2 mediated NK cell activation may now be considered as an indicator of vaccine efficacy. Measurement of IL-2 production by $CD4^+$ T cells, NK cell IFN-γ production and NK cytotoxicity may therefore be used to evaluate levels of vaccine induced immunity in order to determine vaccine efficacy (Horowitz et al., 2010).

Interestingly, more recent reports from this research group, looking at individuals vaccinated against Diphteria/Tetanus infection, suggest that the $CD56^{dim}CD57^-$ subset of NK cells may make the most significant contribution to the NK cell recall response (White et al., 2014). This study reported that a higher proportion of this subset expressed CD25 (IL-2R-α) and produced IFN-γ in comparison with $CD57^+$ NK cells. These findings may have implications for the ability of NK cells to contribute to the effector response following vaccination, and for vaccine effectiveness in older people who have increased levels of NK cell CD57 expression (White et al., 2014). A recent study from this research group, which examined NK cell responses to pertussis and Influenza vaccines, suggests that HCMV serostatus influences the NK cell contribution to vaccine induced adaptive immunity and may therefore have an impact on efficacy of established and newly developed vaccines (Nielson et al., 2015).

A similar study previously investigated NK cell phenotype and function following vaccination against influenza virus. This study reported increased number of IFN-γ producing NK cells in response to vaccination despite the overall lymphocyte frequency remaining stable. Interestingly, the frequency of IFN-γ producing NK cells was most substantially increased following stimulation with inactivated whole influenza virus. NK cells were found to make up a significant proportion of IFN-γ producing lymphocytes

following stimulation of PBMC but only after vaccine administration (Long et al., 2008). A subsequent study reported an increase in NK number in the peripheral blood following influenza vaccination. This increase was most pronounced in the $CD56^{bright}$ subset of NK cells. In addition, the number of $CD56^{bright}CD25^{+}$ NK cells was found to increase following vaccine administration (Jost et al., 2011).

Another study showed that exposure of previously naïve individuals to a single malaria infection resulted in robust and long-lived NK cell IFN-γ responses to subsequent stimulation with *Plasmodium falciparum* infected red blood cells (PfRBC). NK cell responses to PfRBC following exposure to malaria showed a classical recall pattern with IFN-γ responses remaining high four months post-infection. However, the relative contribution of NK cells to the IFN-γ response was found to reduce with time following exposure. In addition, NK cells IFN-γ response to PfRBC was found to be dependent on T cell help. T cells but not NK cells were found to be required for IFN-γ response to PfRBC (McCall et al., 2010).

5.1.3. HCV vaccine development

Current HCV therapies are, for many patients, ineffective, expensive, lengthy and associated with significant toxicity. New Direct Acting Antivirals (DAA) agents for HCV treatment have been available since 2011, but at high cost (> €25,000 per patient) (McHutchison et al., 2009). In addition, the issue of viral re-infection remains a problem.

Co-infection with HIV and HCV is also a major clinical problem with HCV being the most common cause of death in patients co-infected with HIV on combination antiretroviral therapy (cART). Co-infection with both viruses is common due to similar transmission routes and is associated with increased risk of chronic HCV infection, progression to liver fibrosis, end stage liver disease and death (Klenermann and Kim, 2007). The level of successful viral clearance following treatment for chronic HCV infection is lower in HIV co-infected individuals and ranges from 20-60% success depending on genotype (Torriani et al., 2004). Current medical guidelines recommend that co-infected patients start cART treatment at higher $CD4^{+}$ T cell counts than mono-infected HIV patients, increasing the costs involved for both therapy regimes as well as the potential side-effects.

There is therefore a significant clinical need for an effective vaccine for prevention of HCV infection. Despite significant research efforts, previous HCV vaccine trials have failed to identify a safe and effective vaccine against HCV infection. Several studies have suggested that broad $CD4^+$ and $CD8^+$ T cell responses which target multiple antigens are associated with virologic control of HCV. These responses should also be sustained and functional, for example, resulting in increased amounts of IFN-γ production or cell proliferation. It has therefore been suggested that induction of a robust T cell response through vaccination may provide for effective control of acute HCV infection (Thimme et al., 2001, Lechner et al., 2000). This has been tested using chimpanzee challenge models using a target immunogen, delivered in recombinant, replication deficient human adenovirus constructs. The target immunogen was comprised of the NS3-NS5B region from HCV genotype 1b which contains many $CD4^+$ and $CD8^+$ epitopes (Folgori et al., 2006). In vaccinated animals, strong T cell responses (both $CD4^+$ and $CD8^+$) were induced. A robust response to challenge was associated with viral clearance in 4 out of 5 animals, suggesting that vaccination protocols which induce T cell responses may protect against persistent infection by limiting early viral replication upon challenge (Folgori et al., 2006).

5.1.4. Clinical trial of Adenoviral based HCV vaccines in healthy volunteers (HCV 001 cohort)

Following on from this, two adenoviral vectors based on rare serotypes were tested in healthy human volunteers for robust induction of T cell responses (Barnes et al., 2012). Adenoviral based vectors have been shown to shown to be more effective at priming of immune responses in comparison with other approaches such as Modified Vaccinia Ankara (MVA). However, the high prevalence of Adenoviral infection in man may result in vaccine efficacy being impaired by high levels of neutralizing antibodies. Thus, vectors based on rare serotypes of Adenovirus were employed: human Adenovirus 6 (Ad6) (prevalence of approximately 22%) and the serologically distinct chimp Adenovirus 3 (ChAd3) (seroprevalance of 12%). These vaccines were tested for safety and efficacy when used alone or in combination as a prime boost vaccine regimen (Barnes et al., 2012).

These vaccines were found to have good safety profiles and were associated with sustained $CD8^+$ and $CD4^+$ T cell priming responses which targeted a broad range of antigenic regions. T cell responses were also measured following stimulation with HCV peptide

pools corresponding to the viral gene products reproduced in the vaccine insert (Non-structural NS3-NS5B region). The Adenoviral vectors were found to be potent inducers of priming responses which exceeded 1,000 SFC/million PBMC and these responses were readily detected up to 1 year post vaccination by ELISpot. Multimer staining revealed detectable and functional responses after 1 year indicative of a long-term memory population. Importantly, an increased size of response was associated with an increase in response breadth, improving the likelihood of peptide recognition of the infecting strain and limiting viral escape *in vi*vo (Barnes et al., 2012).

Alteration of these vectors as prime and boost vaccines suggested that cross-strain recognition is possible. Vaccine induced immune responses were increased following heterologous boost vaccination; however, these boost responses were lower in magnitude in comparison with that observed following prime vaccination (Barnes et al., 2012). It is unclear whether this would lead to a decline in protection against challenge and alternative vaccine approaches may therefore be required. It has also been suggested that neutralizing antibodies raised against the heterologous vector may limit host exposure to antigen (Rauch et al., 2009).

5.1.5. Clinical trial of MVA based HCV vaccine in healthy volunteers (HCV 003 cohort)

Vaccine boost responses may potentially be enhanced by using alternative vector combinations. The adenoviral vectors used in this study (described in Barnes et al., 2012), belong to the same genetic subgroup which may result in problems with serologic cross-reactivity. As an alternative, a second clinical trial is currently being led by the same research group, in order to assess the safety of a new candidate MVA vaccine containing the same vaccine insert construct. The first aim of this clinical trial was to confirm the safety of the MVA vaccine in healthy individuals when administered alone or in combination with the ChAd3 priming vaccine (HCV003 cohort). These vectors have also been shown to be capable of inducing robust and HCV specific T cell responses in these individuals (Swadling et al., 2014).

This clinical trial aims to develop simple, affordable and effective vaccine strategies that can be given alone or in combination to prevent HCV, HIV and co-infection based on novel and viral vectors for in vivo delivery of antigens. Given positive results so far, this trial will assess the safety and immunogenicity of adenovirus prime/MVA boost HCV

vaccines in a key target group of people infected with HIV on cART. Given positive results, it is hoped that this would be followed by efficacy studies to assess the ability of the vaccine to protect against HCV infection in HIV-infected patients (Barnes et al., 2012). This trial aims to optimise treatment, reduce mortality and improve the quality of life of patients with HIV through better prevention of co-infection with HCV. It is also hoped that this programme will contribute to the development of further innovative strategies to prevent HCV and HIV co-infection, enhance basic knowledge for the prevention of co-infection and inform the design of future phase II/III clinical trials (Barnes et al., 2012).

The factors which determine vaccine efficacy and result in prevention of HCV infection remain to be fully elucidated. To study this, NK cell functional analysis was carried out on cryopreserved PBMC samples from the HCV001 and HCV003 cohorts of participants in the HCV vaccine trial currently being led by our collaborators in the University of Oxford. We were also provided with HCV peptide pools (consisting of NS3 and NS4 peptides) derived from the vaccine insert. From the results of previous studies evaluating NK cell function following administration of various vaccines, we hypothesised that Adenoviral based HCV vaccination would result in increased NK cell activation in vivo. We also hypothesised that vaccinated individuals would show robust NK cell functional responses to in vitro cytokine stimulation and HCV peptide stimulation.

5.1.6. Aims

- Investigate NK cell *in vivo* activation following vaccine administration in the HCV 001 and HCV 003 cohorts taking part in a HCV vaccine trial.
- Investigate priming of NK cell functions in these cohorts following vaccine administration.
- Investigate NK cell functional 'recall response' following restimulation of vaccinated individuals with HCV peptide pools corresponding to the gene products present in the vaccine insert.

5.2. Results

5.2.1. No evidence of in vivo activation of NK cell function following administration of Adenoviral-Based prime vaccination against HCV (HCV001 cohort)

Participants in the HCV001 study had received prime vaccination with one of two Adenovirus vectors expressing NS proteins from HCV genotype 1b. These two vaccine vectors were constructed based rare serotypes of Human and Chimpanzee Adenovirus. These were Human Adenovirus 6 (Ad 6) with a seroprevalance of approximately 22% and Chimpanzee Adenovirus 3 (ChAd3) with a seroprevalance of 12% (Barnes et al., 2012). Different sub-groups had also received escalating doses of either vaccine. The specific vaccine regimens administered are detailed in Table 5.1. PBMC from 10 healthy participants in the HCV001 study cohort, taken at three different time-points (before, 2 and 4 weeks post prime vaccination), were analysed.

Table 5.1. HCV001 Vaccine Study Design

Group	Priming Vector	Dosage (vp)	Number
1	Human Ad6	$5x10^8$	2
2	Human Ad6	$5x10^9$	3
3	Human Ad6	$2.5x10^{10}$	2
4	Chimp Ad3	$5x10^9$	2
5	Chimp Ad3	$2.5x10^{10}$	1

Priming vector used and vaccine dose (number of viral particles) administered to 5 sub-groups of the HCV001 vaccine trial cohort are illustrated.

We initially investigated whether there was any evidence of *in vivo* NK cell activation post vaccination, measuring NK cell intracellular IFN-γ production in response to addition of target cells (Figure 5.1.(a)). Overall NK cell IFN-γ production remained unchanged post vaccination with a mean of 18.3±8.8% IFN-γ^+ NK cells pre-vaccination (Tw0) compared

with 16.6±5.3% at 2 weeks post-vaccination (Tw2) and 16.7±5.4% at 4 weeks post-vaccination (Tw4).

Overall, average percentage IFN-γ^+ NK cells (n=10) remained unchanged in this cohort.

A second functional output, NK cell cytotoxicity, was also measured simultaneously in samples taken pre and post vaccination. Mean percentage CD107a$^+$ NK cells remained unchanged post vaccination with an average of 28.4±13% of NK cells expressing CD107a at Tw0 compared with 24.7±11.4% at Tw2 and 27.7±11% at Tw4.

Overall average percentage CD107a$^+$ NK cells (n=10) remained unchanged in the cohort. This study of NK cell cytotoxicity and cytokine production responses therefore provides no clear evidence of *in vivo* NK cell activation following administration of Adenoviral based vaccine.

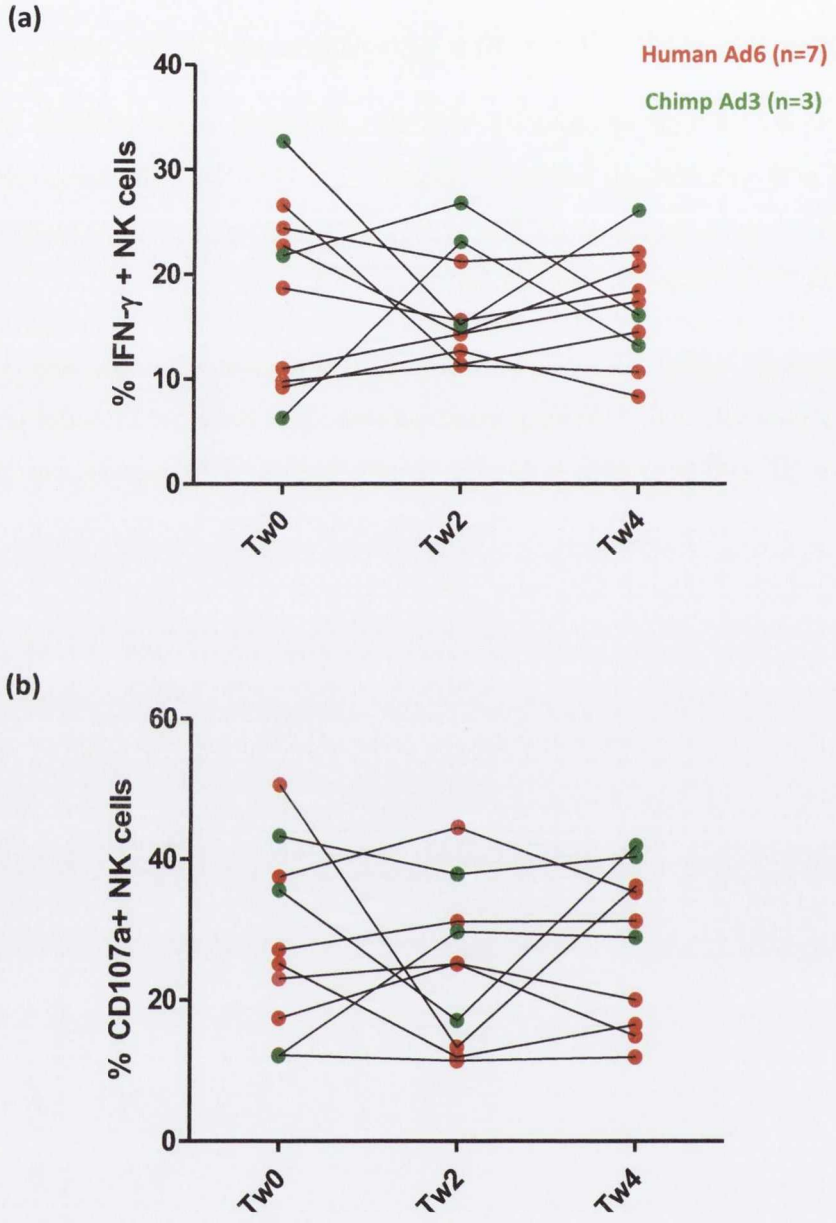


Figure 5.1. NK cell function is unchanged following administration of Adenovirus-Based HCV vaccine. $CD3^{-}CD56^{+}$ NK cells from 10 healthy normal vaccine trial participants were analysed by flow cytometry. Percentage NK cells staining positive for (a) intracellular IFN-γ production and (b) CD107a expression were measured in PBMC taken before vaccination (Tw0) as well as 2 and 4 weeks post vaccination (Tw2 and Tw4 respectively). Cells were incubated with target cells (721.221) for 5 hours before staining. Coloured dots represent distinct vaccine regimens administered to sub-groups of the HCV cohort, as outlined in Table 5.1. Repeated measures 1-way ANOVA analysis of the data, followed by Bonferroni Multiple Comparisons Test was carried out comparing different time-points.

5.2.2. PBMC showed normal cytokine responsiveness following resuscitation from cryopreservation, with up-regulation of the cytokine receptor CD25 (IL2-Rα)

In order to confirm normal cytokine responsiveness of PBMC after resuscitation from cryopreservation, NK upregulation of the IL-2 receptor α (CD25) was measured in these donors (n=10), following stimulation of PBMC with IL-12+IL-18 cytokine combination to induce NK cell activation. PBMC were stimulated with either Basal Dose (12.5pg/ml IL-12 + 10ng/ml IL-18) as survival factors or High Dose (5ng/ml IL-12 + 50ng/ml IL-18) cytokine combination. NK cell immune responses were analysed after 18 hour (Figure 5.2.(a)), 3 Day and 5 Day stimulation (data not shown). As expected, NK cell expression of the cytokine receptor CD25 was induced following High Dose cytokine stimulation, indicating normal cytokine responsiveness in these samples (Figure 5.2.(a)). Basal levels of CD25 expression were found to be increased in comparison with *ex vivo* (T0) analysis of PBMC (no cytokine stimulation) (Figure 5.2.(b)). Average NK cell expression of CD25 at Tw0 increased from 2.7±1.3% to 8.8±4.7% following incubation with Basal Dose cytokine for 18 hours. Expression levels were increased to 18.2±12.1% with High Dose stimulation. Importantly, NK expression of CD25 was unchanged following vaccine administration (Figure 5.2.(a) and (b)).

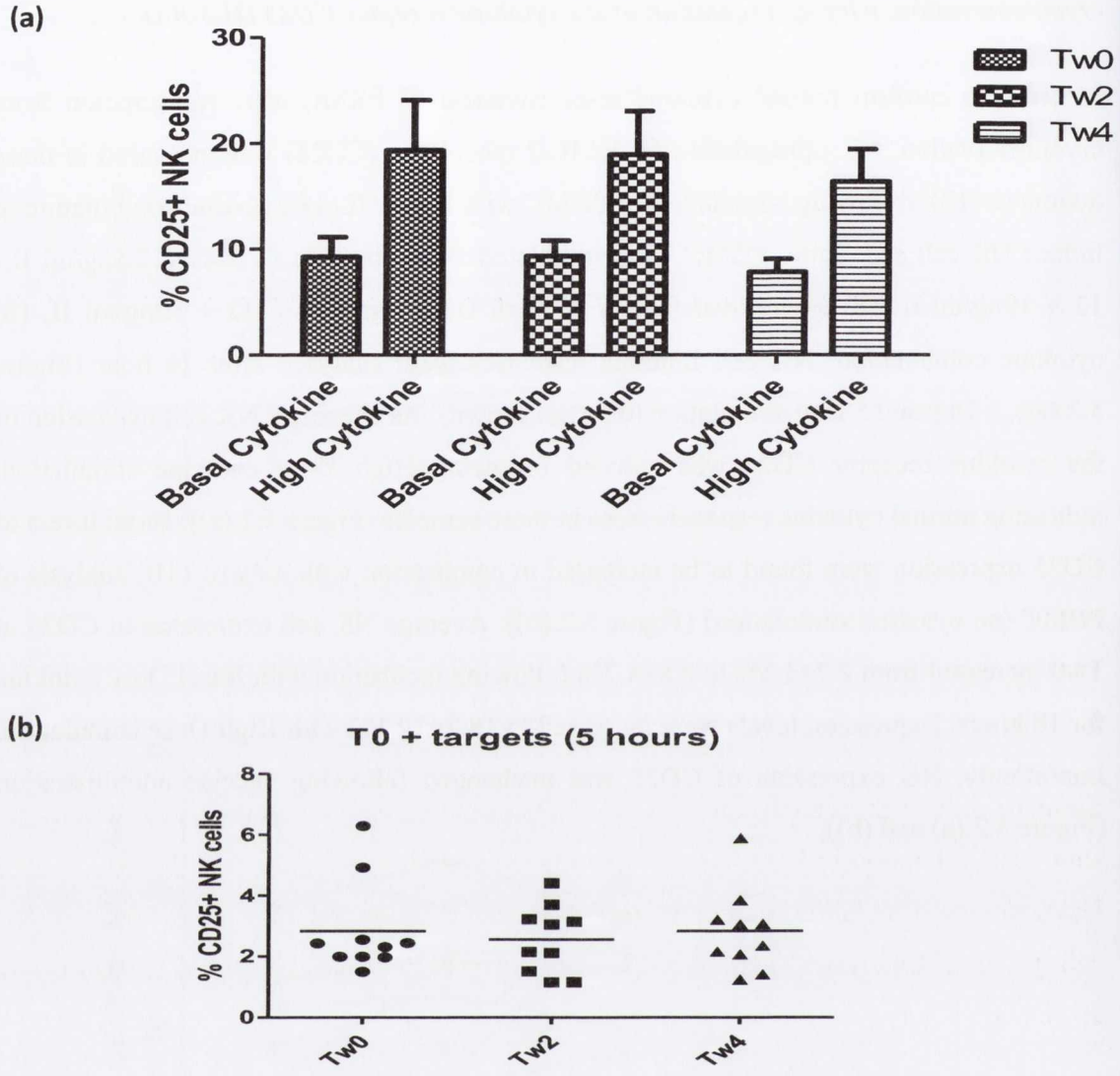


Figure 5.2. Induction of the CD25 receptor on NK cells following cytokine stimulation. Percentage $CD3^-$ $CD56^+$ NK cell expression of CD25 was measured in PBMC samples from 10 participants, taken before vaccination (Tw0) as well as 2 and 4 weeks post vaccination (Tw2 and Tw4 respectively). (a) PBMC were stimulated for 18 hours with either Basal Dose (12.5pg/ml IL-12 + 10ng/ml IL-18) or High Dose (5ng/ml IL-12 + 50ng/ml IL-18) cytokine combination. Percentage $CD25^+$ NK cells were analysed by flow cytometry. Repeated measures 2-way ANOVA analysis of the data, followed by Bonferroni Multiple Comparisons Test was carried out comparing pre and post vaccination levels at each cytokine concentration. (b) After resuscitation, PBMC were rested for 1 hour at 37°C before addition of target cells (721.221) for 5 hours. T0 analysis of percentage $CD25^+$ NK cells was carried out by flow cytometry. Repeated measures 1-way ANOVA analysis of the data, followed by Bonferroni Multiple Comparisons Test was carried out comparing different timepoints.

5.2.3. NK cell IFN-γ response to cytokine stimulation is attenuated at two weeks post vaccination with partial recovery by four weeks post prime vaccination in the HCV001 cohort of vaccine trial participants

In order to investigate whether there was any evidence of NK cell priming following vaccination, NK effector functional responses were also measured following treatment with IL-12+IL-18 cytokine combination for varying time-courses (Figure 5.3.(a)). Intracellular NK cell IFN-γ production in response to High Dose cytokine stimulation after 18 hour stimulation was found to be significantly attenuated at 2 weeks post vaccination compared to pre vaccination levels with a reduction from 31.0±17.9% NK cells producing IFN-γ at Tw0 to 22.5±16.5% at Tw2. This impairment in responsiveness to High Dose cytokine appeared to show a partial recovery at 4 weeks post vaccination although this was not statistically significant (27.9±16.4% IFN-γ^+ NK cells) (Figure 5.3.(b)). In contrast, responsiveness to Basal Dose cytokine was found to be broadly unchanged following vaccine administration (15.9±8.2% at Tw0 compared with 12.9±8.0% at Tw2) (Figure 5.3.(b)).

Similar results were seen following 3 and 5 Day stimulation with High Dose cytokine. No significant alteration in Basal Dose response post vaccination was seen following 3 and 5 Day cytokine stimulation (Figure 5.3.(a)). This data therefore provides no evidence of NK cell priming following Adenoviral vector vaccination, indeed there is a short-term attenuation in response following vaccination.

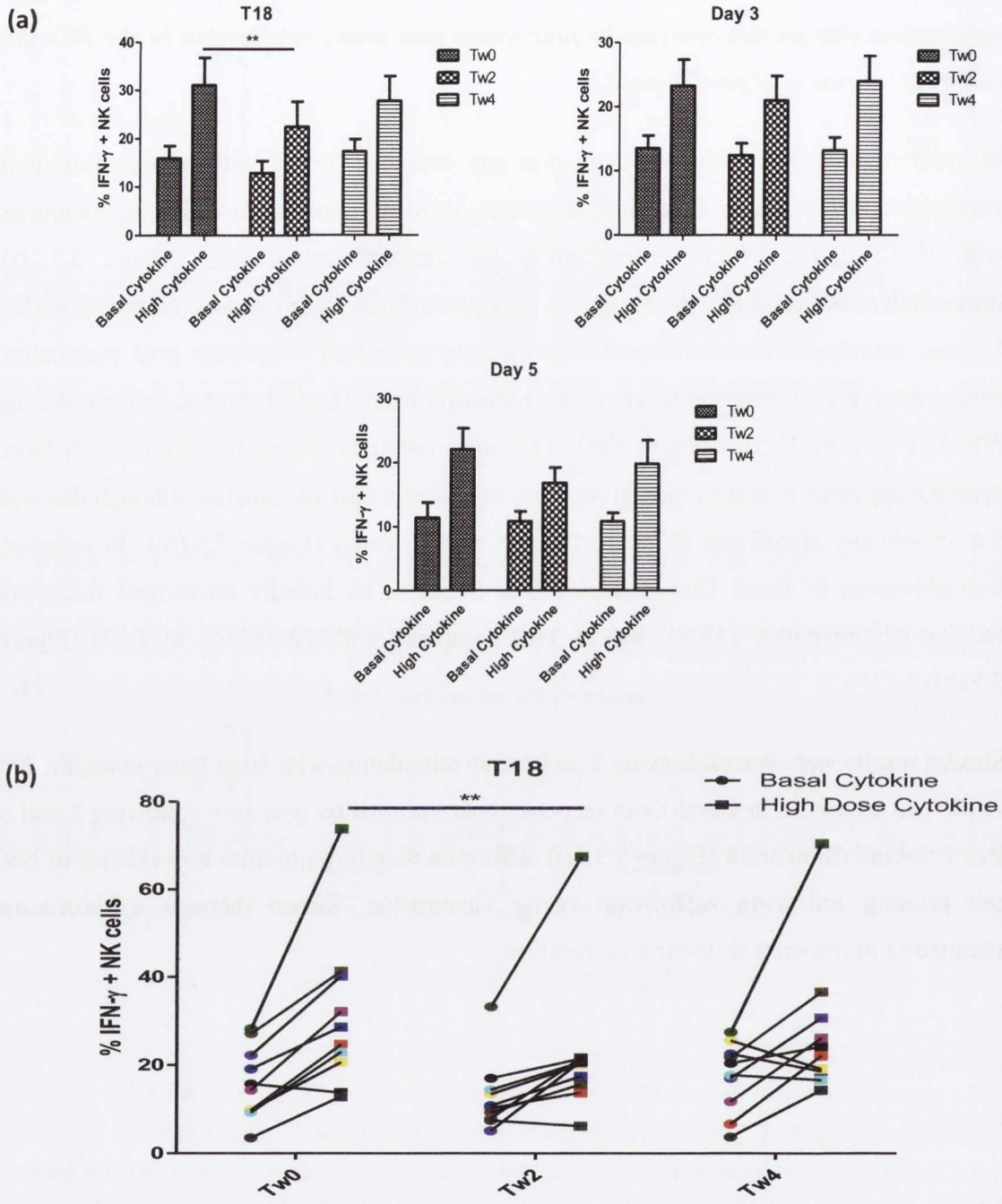


Figure 5.3. Short-term attenuation in cytokine induced IFN-γ produced by NK cells post vaccination. Percentage $CD3^{-}CD56^{+}$ NK cells producing IFN-γ were measured in PBMC samples, taken from 10 participants, before vaccination (Tw0) as well as 2 and 4 weeks post vaccination (Tw2 and Tw4 respectively). (a) PBMC were stimulated for 18 hours, 3 or 5 Days with Basal or High Dose IL-12 + IL-18 cytokine combination. Percentage positive NK cells were analysed by flow cytometry following intracellular staining. Repeated measures 2-way ANOVA analysis of the data followed by Bonferroni Multiple Comparisons Test was carried out comparing pre and post vaccination levels at each cytokine concentration. Individual donor data from 18 hour stimulation experiments are shown in (b).

5.2.4. NK cell cytotoxicity response to High Dose cytokine stimulation is unaffected following administration of priming vaccine to the HCV001 cohort

CD107a assays, carried out simultaneously in PBMC from these donors, showed no significant changes in NK cell cytotoxicity response to High Dose cytokine stimulation following vaccine administration (Figure 5.4.(a)). Mean CD107a expression following 18 hour stimulation was 39.2±11.5% at Tw0, this compared with 37.1±14.4% at Tw2 and 37.8±12.1% at Tw4 (Figure 5.4.(b)).

Somewhat surprisingly, NK cell cytotoxicity in response to 18 hour Basal Dose stimulation was found to be significantly boosted at Tw2 post vaccination when compared with pre-vaccination (Tw0) levels (Figure 5.4. (b)). Percentage $CD107a^+$ NK cells increased from 29.3±8.8% at Tw0 to 39.0±19.1% at Tw2. Levels were found to decrease to 34.0±10.9% at Tw4. This is in contrast with the trends in NK cell IFN-γ production, described above, which showed attenuation in cytokine responsiveness immediately after vaccination (Figure 5.3(b)). Examination of individual data from these donors following 18 hour stimulation showed that the statistically significant result seen in Basal Dose stimulated cells may be biased by data from just 2 out of 10 donors (labelled in orange and light blue) who showed large increases in NK cytotoxicity at Tw2 when compared to pre-vaccination levels (Figure 5.4.(b)). Re-analysis of this data without these two donors may therefore reveal significant changes in NK cell cytotoxicity responses following vaccination administration.

Following 5 Day High Dose cytokine stimulation, NK cell cytotoxicity is marginally (non-significantly) increased post vaccination when compared to pre-vaccination levels, with average levels of cytotoxicity increasing with time after vaccination (32.2±12.6% $CD107a^+$ NK cells at Tw0 compared with 36.6±15.5% at Tw2 and 42.1±14.9% at Tw4) (Figure 5.4(a)). However, this non-significant trend was not seen following 18 hour or 3 Day stimulation. Thus, it remains unclear from this data whether there is a real significant change in NK cell cytotoxicity following vaccine administration.

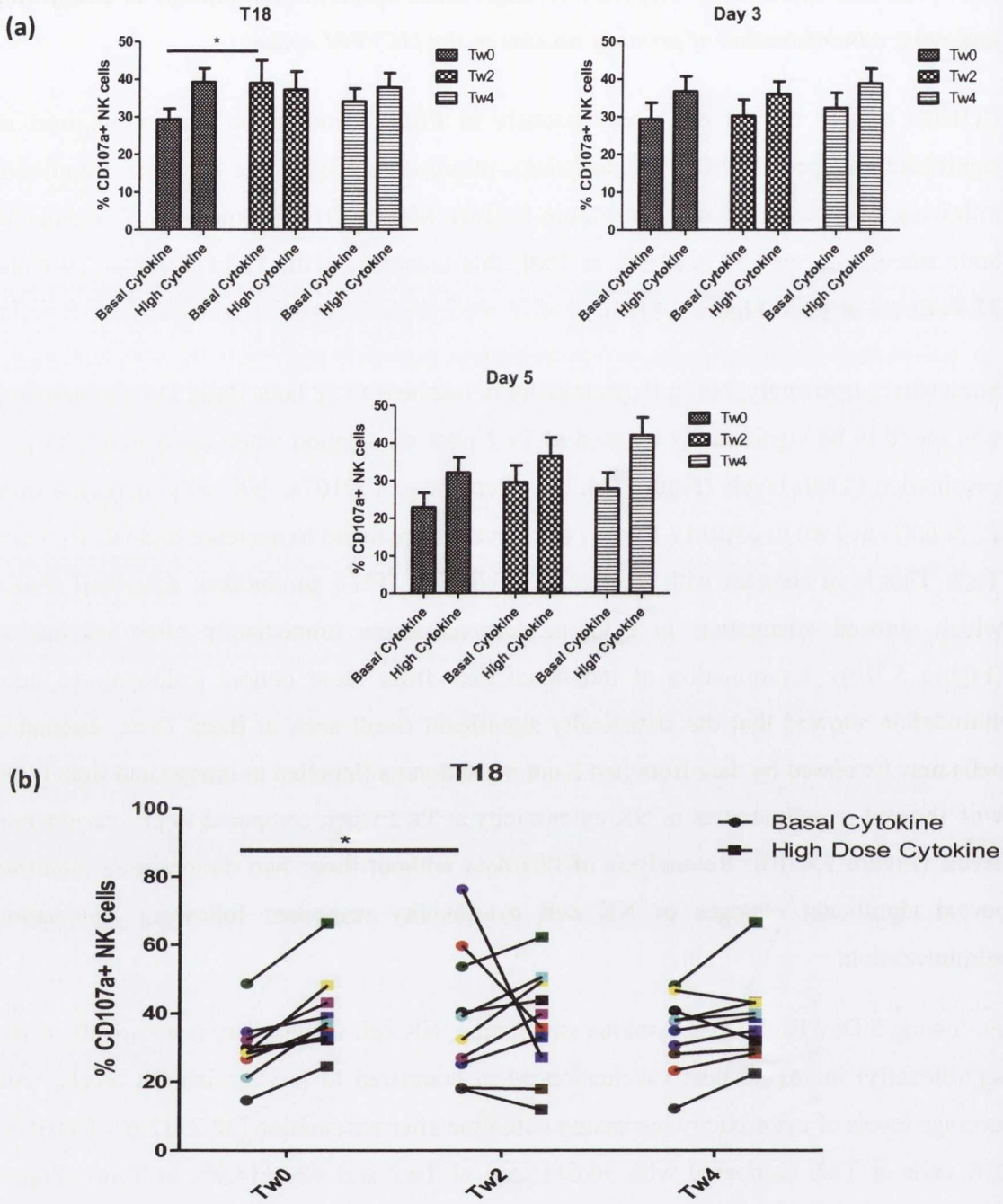


Figure 5.4. NK cell cytotoxicity is increased or unchanged post vaccination. Percentage $CD3^{-}CD56^{+}$ NK cells expressing CD107a were measured in PBMC samples, taken from 10 participants, before vaccination (Tw0) as well as 2 and 4 weeks post vaccination (Tw2 and Tw4 respectively). (a) PBMC were stimulated for 18 hours, 3 or 5 Days with Basal or High Dose IL-12 + IL-18 cytokine combination. Percentage positive NK cells were analysed by flow cytometry following intracellular staining. Repeated measures 2-way ANOVA analysis of the data followed by Bonferroni Multiple Comparisons Test was carried out comparing pre and post vaccination levels at each cytokine concentration. Individual donor data from the 18 hour stimulation experiments are shown in (b).

5.2.5. No evidence of a robust NK cell functional response in the HCV001 cohort following stimulation with HCV peptide pools

In addition to investigating NK cell priming following cytokine stimulation, we also looked for evidence of a 'recall' NK cell functional response post vaccination in 9 of these vaccine trial participants. In order to do this, PBMC taken before and after vaccination, were stimulated with HCV peptide pools corresponding to the viral gene products reproduced in the vaccine insert. PBMC were stimulated with Basal Dose cytokine combination + peptide pools (1μg/ml/peptide) derived from the HCV genotype 1b NS3/NS4 protein. As these peptide pools are dissolved in DMSO, responses were compared to cells stimulated with Basal Dose in combination with DMSO, added as a vehicle control (Figure 5.5.). NK cell responses were measured after 18 hour stimulation. Longer peptide stimulations (3 Day and 5 Day cytokine stimulation) which were also carried out were found to result in low cell viability (< 50% viability), likely due to the presence of DMSO (data not shown).

NK cell IFN-γ responses remained unchanged following peptide stimulation compared to vehicle control samples, however graphing of the data shows a consistent decrease in NK cell IFN-γ production in peptide stimulated PBMC (Figure 5.5.(a)). This decrease was most pronounced at Tw4 with mean percentage IFN-γ^+ NK cells decreased from 24.1±9.4% in vehicle control samples to 15.2±9.4% in peptide treated samples. In addition, IFN-γ responses in the vehicle control samples (Basal Dose cytokine + DMSO) were increased in comparison with samples stimulated with Basal Dose cytokine alone. In contrast, both basal dose treated and peptide stimulated samples demonstrated similar levels of NK cell IFN-γ production (See Figure 5.3.). Importantly however, NK cell IFN-γ responses remained largely unaffected by peptide stimulation following vaccination (Figure 5.5.(a)).

Mean CD107a expression was found to be largely unchanged following peptide stimulation in comparison with vehicle control samples, though small but consistent decreases in peptide treated samples were reported. Similar to the patterns identified in NK cell IFN-γ production, these differences were most pronounced at Tw4 with 35.3±12.4% CD107a$^+$ NK cells in vehicle control samples compared with 23.7±7.8% in peptide treated samples (Figure 5.5.(b)). Importantly, NK cell cytotoxicity responses remained largely unaffected by vaccine administration. This data therefore provides no evidence of an NK cell 'recall' response in individuals vaccinated against HCV following re-stimulation with HCV peptide pools corresponding to the gene products engineered into the vaccine insert.

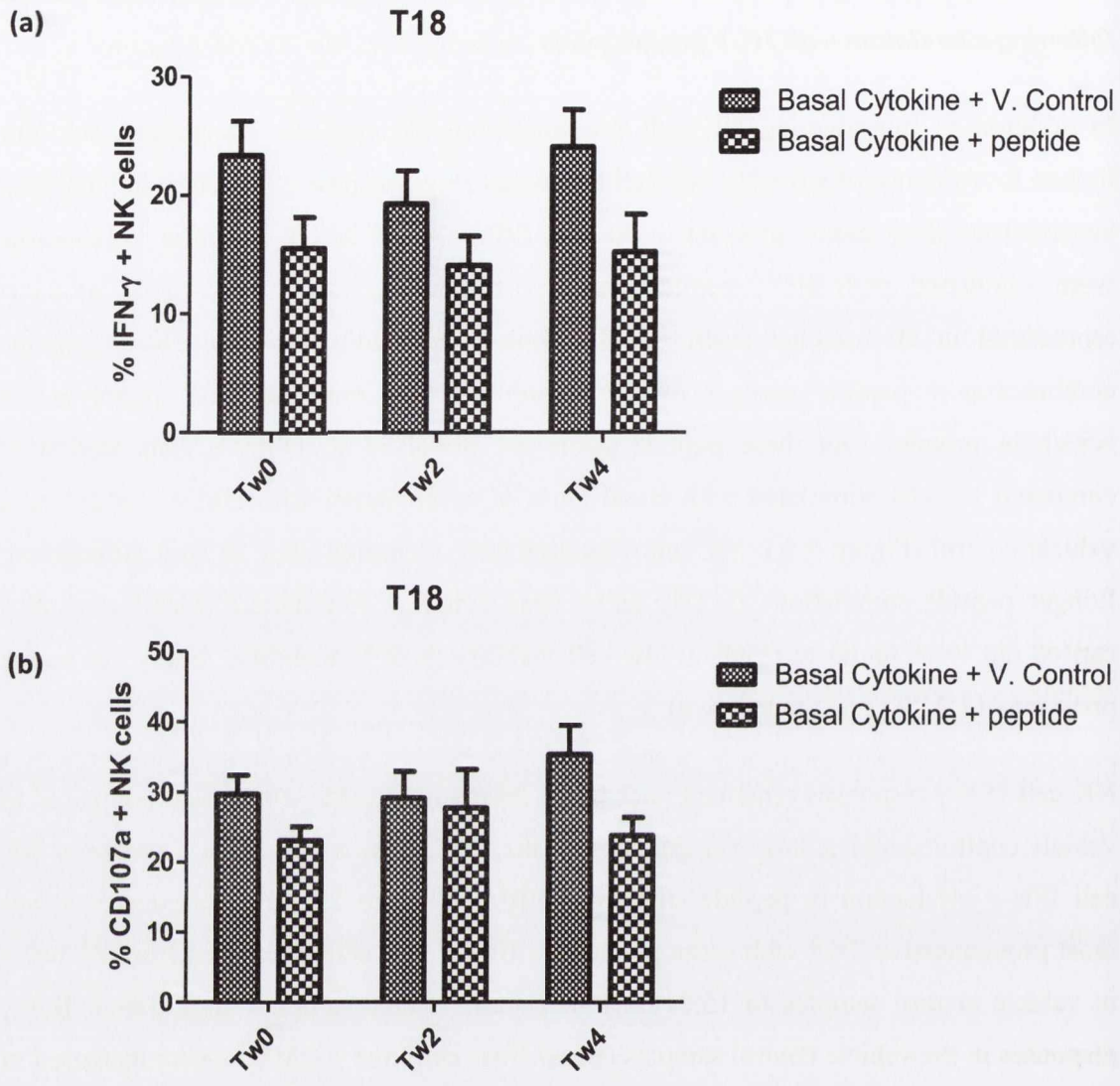


Figure 5.5. No change in NK cell functional responses following stimulation with HCV peptide pools. Percentage NK cells staining positive for (a) intracellular IFN-γ and (b) CD107a expression were measured in samples PBMC samples, from 9 participants, taken before vaccination (Tw0) as well as 2 and 4 weeks post vaccination (Tw2 and Tw4 respectively). PBMC were stimulated for 18 hours with Basal Dose IL-12 + IL-18 cytokine combination + DMSO (Vehicle Control) or HCV peptide pool (1μg/ml each peptide). Percentage positive NK cells were analysed by flow cytometry following intracellular staining. Repeated measures 2-way ANOVA analysis of the data followed by Bonferroni Multiple Comparisons Test was carried out comparing vehicle control and peptide stimulated groups at each timepoint.

5.2.6. No significant in vivo activation of NK cell function following administration of Chimp Adenoviral Prime/MVA boost vaccination regimen to the HCV003 cohort

Following on from this data, additional PBMC samples from a second study cohort (HCV003) of healthy participants taking part in this HCV vaccine study were tested. These participants had received prime vaccination with previously described Chimp Adenovirus vaccine (ChAd3) ($2.5x10^{10}$ v.p.); this was followed 8 weeks later by MVA based boost vaccination ($2x10^{8}$ v.p.). PBMC from 5 participants in the HCV003 study, taken before, as well as 2 and 4 weeks after prime vaccination were tested. In addition, samples taken before, and 1 and 4 weeks post boost vaccination were also analysed (Tw8, Tw9, Tw12).

Analysis, similar to that carried out in the initial HCV001 cohort from this vaccine study, was performed in these PBMC in order to investigate possible differential NK cell activation and priming following administration of a second vaccine regimen, which included MVA boost vaccination.

In order to determine whether there was any evidence of *in vivo* NK cell activation post vaccination, further flow cytometry based NK cell functional assays were carried out, measuring intracellular IFN-γ in NK cells following addition of target cells after resuscitation from cryopreservation. Similar to what was seen in the HCV001 cohort, there was no significant alteration in NK cell IFN-γ production following vaccine administration.

The mean percentage of IFN-γ^+ NK cells was increased from 12.5±1.5% pre-vaccination to 14.2±3.04% at Tw2. These levels were found to decrease again at Tw4 (12.9±1.8%) (Figure 5.6.(a)). Levels of NK cell IFN-γ production were further decreased at Tw8 (10.6±2.6%). However, these levels increased to 14.0±6.1% at 1 week post boost vaccination (Tw9). Interestingly, levels remained elevated compared to pre-boosting levels after a further 3 weeks (13.4±4.4% at Tw12) reported (Figure 5.6.(a)).

A second functional output, NK cell cytotoxicity, was simultaneously measured in samples taken pre and post vaccination. Similar to what was seen with NK cell IFN-γ production, cytotoxicity responses remained unchanged following vaccine administration. Percentage NK cells expressing the cytotoxicity marker CD107a increased marginally from 20.9±4.4% CD107a$^+$ NK cells at Tw0 (pre-vaccination) to 24.9±4.8% at Tw2 post vaccination. These levels remained similar at all post vaccination time-points (24.0±6.5%, 22.9±7.9%, 24.3±8.2% 23.9±8.7% CD107a$^+$ NK cells at Tw4, Tw8, Tw9 and Tw12 respectively).

Both measures of NK cell function showed similar non-significant trends in the HCV cohort. In addition to CD107a assay, levels of intracellular NK cell Granzyme B staining was also analysed in tandem with CD107a assay as second output/measure of cytotoxicity, however no significant alteration in percentage expression or Mean Fluorescence Intensity (M.F.I.) was reported post vaccine administration (data not shown).

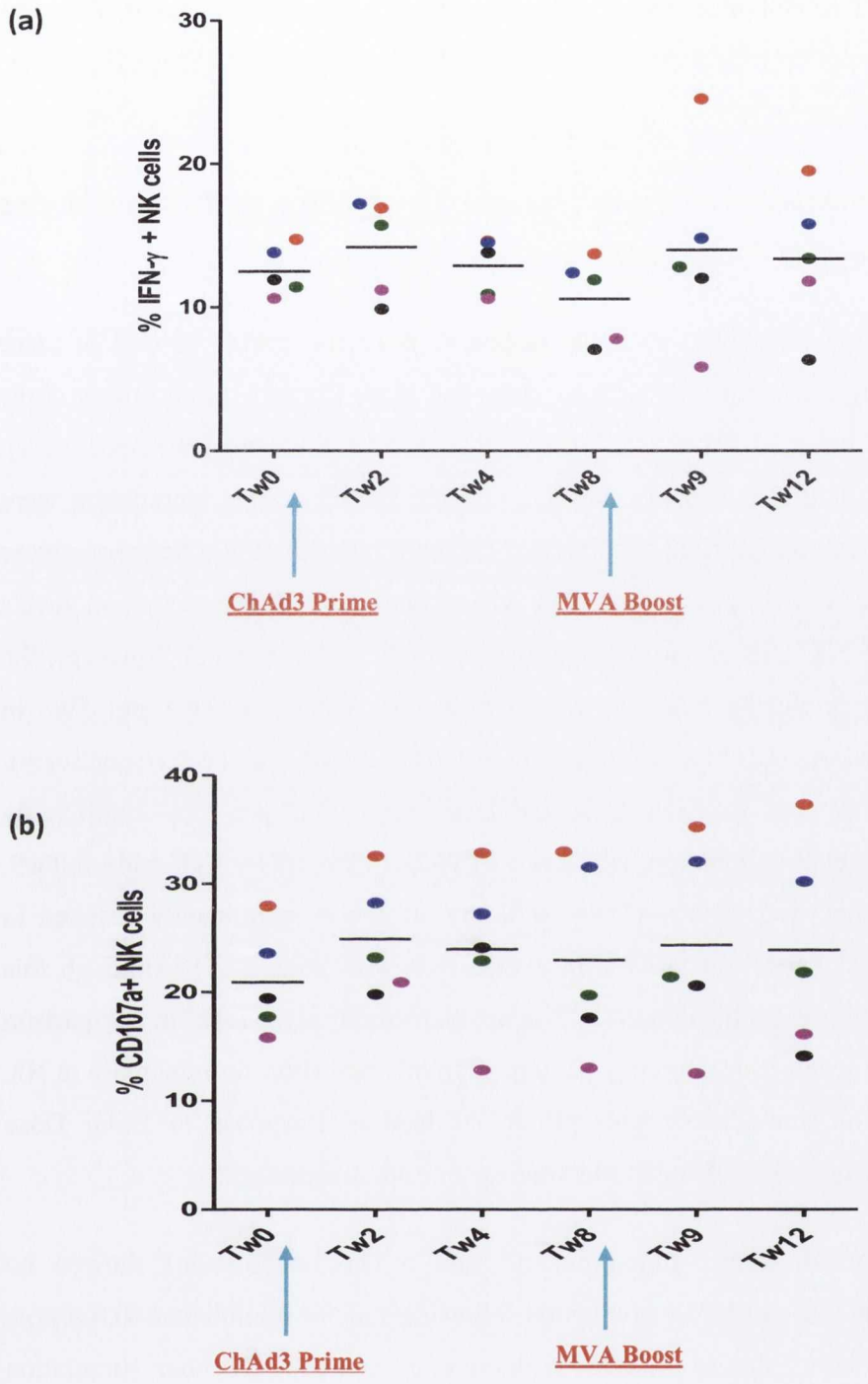


Figure 5.6. NK cell function is unchanged following administration of Adenovirus Prime/MVA Boost-Based HCV vaccine regimen. Percentage NK cells staining positive for (a) intracellular IFN-γ and (b) CD107a expression were measured in PBMC samples taken from 5 participants before prime vaccination (Tw0) as well as 2 and 4 weeks post vaccination (Tw2 and Tw4 respectively) and before boost vaccination (Tw8) and 1 and 4 weeks post boost vaccination (Tw9 and Tw12 respectively). Cells were incubated with

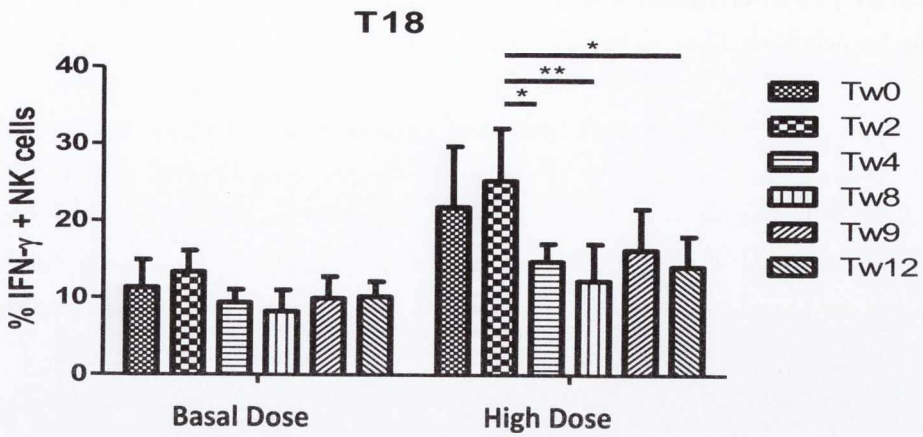
target cells (721.221) for 5 hours before staining. Repeated measures 1-way ANOVA analysis of the data, followed by Bonferroni Multiple Comparisons Test was carried out comparing different timepoints.

5.2.7. No significant increases in cytokine induced NK cell IFN-γ production following prime/boost vaccination of the HCV003 cohort

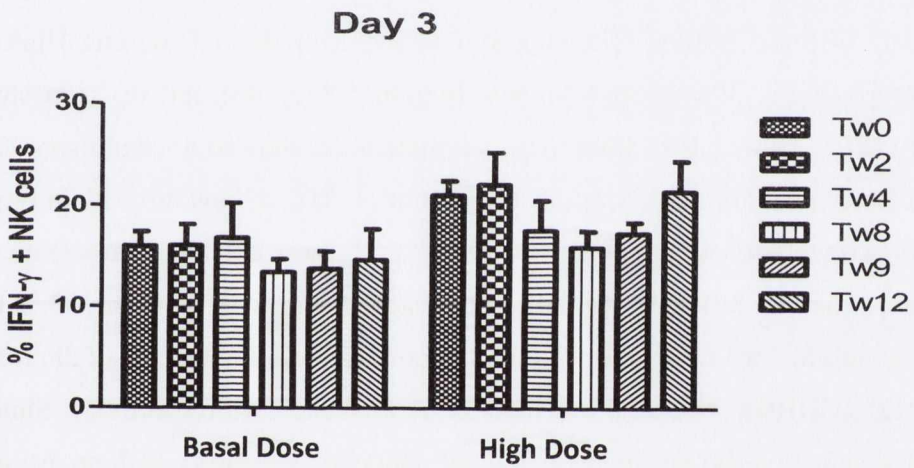
To test for NK cell priming, NK functional responses were also measured in 4 of these individuals following stimulation with Basal Dose and High Dose IL-12+IL-18 cytokine combination for 18 hours, 3 Days and 5 Days (Figure 5.7.(a),(b) and (c) respectively). Overall mean NK cell IFN-γ response to 18 hour High Dose cytokine stimulation were broadly unchanged following prime vaccination. Of note, NK cell IFN-γ responses were marginally increased at Tw2 post vaccination when compared to pre-vaccination levels (21.8±15.8% IFN-γ^+ NK cells at Tw0 compared with 25.3±13.6% at Tw2). However, the proportion of IFN-γ producing NK cells was significantly reduced at Tw4 and Tw8 in comparison with Tw2 levels (14.8±4.6% and 12.3±9.6% at Tw4 and Tw8 respectively). Cytokine induced response to High Dose cytokine stimulation was non-significantly increased following boost vaccination with levels of 16.2±10.7% IFN-γ^+ NK cells at Tw9. Again, the mean proportion of IFN-γ^+ NK cells at Tw12 was significantly reduced in comparison with Tw2 levels (14.1±7.9% in comparison with 25.3±13.6). Although this increase in NK cell IFN-γ production at Tw2 is not statistically significant in comparison with pre-vaccination levels, this data may be indicative of a real short term increase in NK cell IFN-γ production immediately post prime vaccination. Response to Basal Dose cytokine showed no significant change following vaccine administration.

Cytokine stimulation for longer time-points (3 and 5 Day stimulation) showed no significant changes in NK cell IFN-γ production following vaccine administration. Of note however, this data shows similar patterns to those seen following 18 hour stimulation (Figure 5.7.(b) and (c)).

(a)


T18
% IFN-γ + NK cells
40
30
20
10
0
*
**
*
Basal Dose
High Dose
Tw0
Tw2
Tw4
Tw8
Tw9
Tw12

(b)


Day 3
% IFN-γ + NK cells
30
20
10
0
Basal Dose
High Dose
Tw0
Tw2
Tw4
Tw8
Tw9
Tw12

(c)

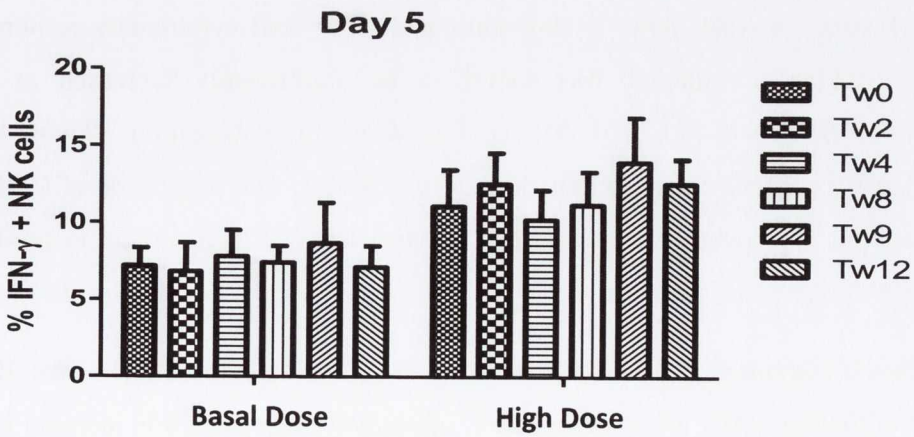

Day 5
% IFN-γ + NK cells
20
15
10
5
0
Basal Dose
High Dose
Tw0
Tw2
Tw4
Tw8
Tw9
Tw12

Figure 5.7. Short -term non-significant increase in cytokine induced IFN-γ production by NK cells following vaccine administration. Percentage NK cells producing IFN-γ were measured in 4 participants, before prime vaccination (Tw0) as well as 2 and 4 weeks post vaccination (Tw2 and Tw4 respectively) and before boost vaccination (Tw8) and 1 and 4 weeks post boost vaccination (Tw9 and Tw12 respectively). PBMC were stimulated for (a) 18 hours, (b) 3 Days or (c) 5 Days with Basal or High Dose IL-12 + IL-18 cytokine combination. Percentage positive NK cells were analysed by flow cytometry following intracellular staining. Repeated measures 2-way ANOVA analysis of the data followed by Bonferroni Multiple Comparisons Test was carried out comparing pre and post vaccination levels at each cytokine concentration.

5.2.8. NK cell cytotoxicity response to prolonged cytokine stimulation is significantly increased following boost vaccination of the HCV003 cohort

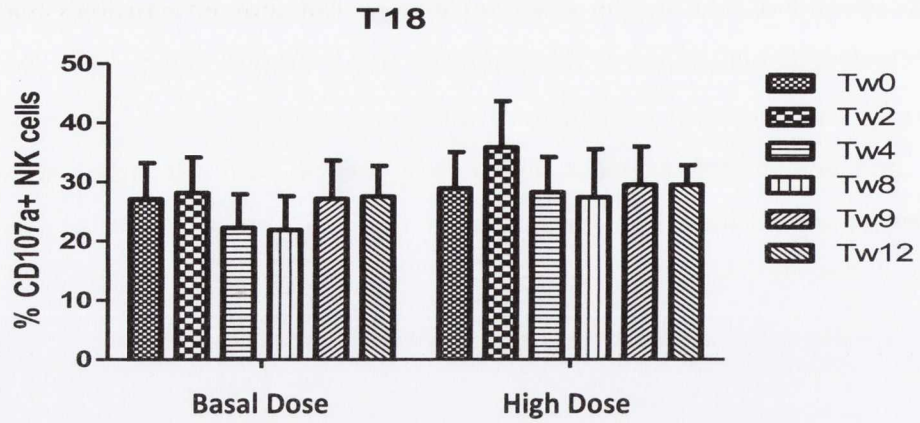
NK cell cytotoxicity, a second measure of NK cell effector function was measured simultaneously in these donors, following stimulation with Basal Dose and High Dose cytokine for 18 hours, 3 Days and 5 Days (Figure 5.8.(a), (b) and (c) respectively). Analysis of PMBC following 18 hour cytokine stimulation showed no significant change in NK cell cytotoxicity following vaccine administration. NK cell cytotoxicity in response to High Dose cytokine stimulation was found to be marginally (non-significantly) increased immediately following initial prime vaccination with a mean 28.9±12.2% $CD107a^+$ NK cells at Tw0 compared with 35.8±15.6% at Tw0. An average of 28.3±11.8% and 27.4±16.2% $CD107a^+$ NK cells was reported at Tw4 and Tw8 respectively. Similarly, MVA boost vaccination did not increase NK cell cytotoxic capability with an average of 29.5±12.8% and 29.5±6.6% $CD107a^+$ NK cells at Tw9 and Tw12 respectively.

Similar trends in NK cell cytotoxicity were identified after 3 Day cytokine stimulation, although average levels of CD107a expression were increased in comparison with 18 hour stimulation (Figure 5.8. (b)). After 5 Day stimulation, NK cell cytotoxicity response to High Dose cytokine stimulation was found to be significantly increased at Tw9 (41.3±11.7%) and Tw12 (40.7±10.5%) compared to pre-vaccination (Tw0) levels (28.9±7.8%) and Tw2 levels post vaccination (30.8±8.36 %). The proportion of $CD107a^+$ NK cells was also significantly increased at Tw9 and Tw12 in comparison in pre-boost vaccination (29.8±9.1% at Tw8).

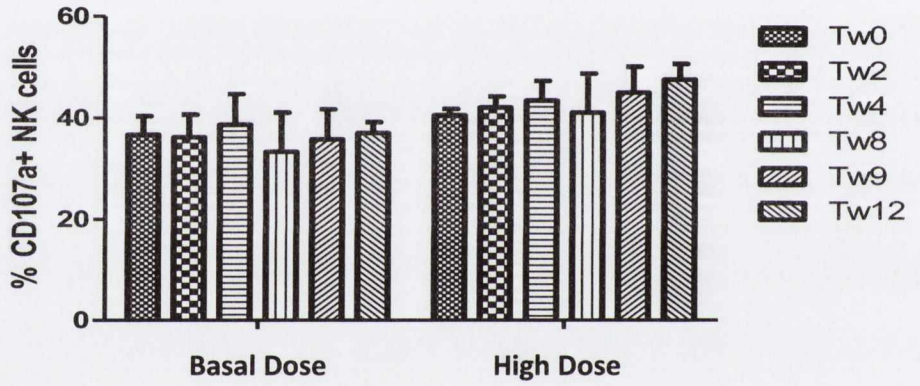
NK cell cytotoxic responses to prolonged exposure to activating cytokines (IL-12 + IL-18) are elevated following MVA boost vaccination. Importantly however, this increase in NK cell functional response is not seen following 18 hour or 3 Day cytokine stimulation which

may be indicative of a role for the adaptive immune response in activating NK cell responses following administration of this candidate HCV vaccine (Figure 5.8.). This NK cell cytotoxicity data, taken in combination with corresponding data of NK cell IFN-γ production, provides limited evidence for priming of NK cell functional responses following vaccine administration.

(a)


T18
% CD107a+ NK cells
50
40
30
20
10
0
Basal Dose
High Dose
Tw0
Tw2
Tw4
Tw8
Tw9
Tw12

(b)


Day 3
% CD107a+ NK cells
60
40
20
0
Basal Dose
High Dose
Tw0
Tw2
Tw4
Tw8
Tw9
Tw12

(c)

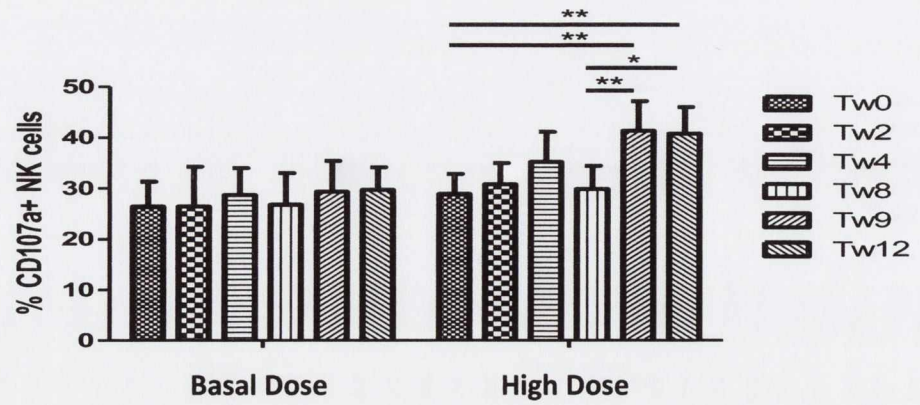

Day 5
**
**
*
**
% CD107a+ NK cells
50
40
30
20
10
0
Basal Dose
High Dose
Tw0
Tw2
Tw4
Tw8
Tw9
Tw12

Figure 5.8. NK cell cytotoxicity is increased post prime vaccination. Percentage NK cells expressing CD107a were measured in 5 participants, before prime vaccination (Tw0) as well as 2 and 4 weeks post prime (Tw2 and Tw4 respectively) and before boost vaccination (Tw8) and 1 and 4 weeks post boost vaccination (Tw9 and Tw12 respectively). PBMC were stimulated for (a) 18 hours, (b) 3 or (c) 5 Days with Basal or High Dose IL-12 + IL-18 cytokine combination. Percentage positive NK cells were analysed by flow cytometry following intracellular staining. Repeated measures 2-way ANOVA analysis of the data followed by Bonferroni Multiple Comparisons Test was carried out comparing pre and post vaccination levels at each cytokine concentration.

5.2.9. No significant NK cell functional responses, in the HCV003 cohort, following stimulation with HCV peptide pools

In addition to investigating NK cell priming we looked for evidence of a 'recall' response in NK cell function post vaccination of these HCV003 vaccine study participants. As previously described (Section 5.2.5.), PBMC taken before and after vaccination, were stimulated with HCV peptide pools corresponding to the viral gene products reproduced in the vaccine insert (HCV genotype 1b NS3/NS4 protein). NK cell IFN-γ production (Figure 5.9. (a)) and cytotoxicity (Figure 5.9. (b)) responses were measured after 18 hours stimulation. As before, responses were compared to cells stimulated with Basal Dose + DMSO, added as a vehicle control.

As had been seen in the initial cohort of vaccine study participants examined (HCV001), NK cell IFN-γ production was decreased in peptide stimulated samples in comparison with DMSO control stimulated cells. This difference was most pronounced at Tw2 with a mean of 10.4±1.2% IFN-γ^+ NK cells in peptide stimulated samples compared with 22.4±5.6% in control samples) (Figure 5.9.). However, these differences were not statistically significant. Overall production in IFN-γ in peptide stimulated samples remained very similar at all time-points and was unaffected by vaccination (Figure 5.9. (a)).

Measurement of NK cell cytotoxicity as measured by CD107a assay showed similarly low levels of NK cell function following stimulation with HCV peptide pools. This decrease was found to be statistically significant at Tw2 (11.1±2.9% CD107a$^+$ NK cells in peptide stimulated cells compared with 27.2±10.2% in control samples) and at Tw12 (9.9±2.8% in peptide stimulated cells compared with 25.2±7.2% in control samples). The proportion of CD107a$^+$ NK cells, following peptide stimulation, remained largely unchanged at all time-points and was unaffected by vaccination. Of note however, DMSO control stimulation

samples showed small, non-significant, increases in NK cell function in PBMC taken post vaccine administration. These trends were similar but at a higher level compared to what reported in samples stimulated with cytokine alone.

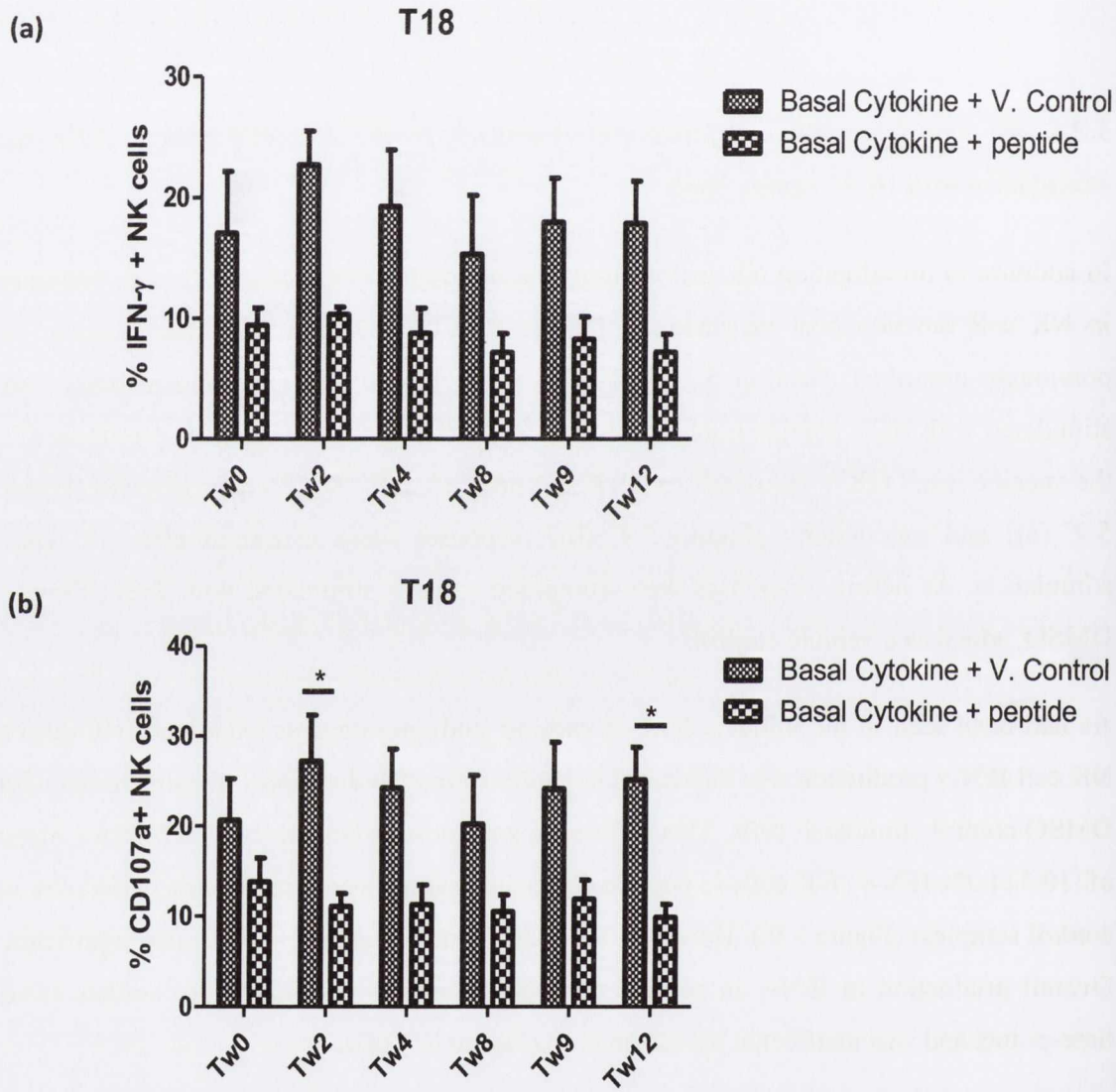


Figure 5.9. Non vaccine specific decrease in NK cell function following re-stimulation assays using HCV peptide pools. Percentage NK cells staining positive for (a) intracellular IFN-γ and (b) CD107a expression were measured in PBMC samples from 5 participants, taken before vaccination (Tw0) as well as 2 and 4 weeks post vaccination (Tw2 and Tw4 respectively). PBMC were stimulated for 18 hours with Basal Dose IL-12 + IL-18 cytokine combination + DMSO (Vehicle Control) or HCV peptide pool (1μg/ml each peptide). Percentage positive NK cells were analysed by flow cytometry following intracellular staining.

Repeated measures 2-way ANOVA analysis of the data followed by Bonferroni Multiple Comparisons Test was carried out comparing vehicle control and peptide stimulation groups at each timepoint.

5.3. Discussion

The study described here investigated NK cell functional responses following administration of a candidate HCV vaccine. The results of this study indicate that human (Ad6) and Chimp (ChAd3) Adenoviral based candidate HCV vaccines have varying effects on NK cell functional responses in healthy individuals. The results described here also provide some evidence that NK cell cytotoxicity response to prolonged *in vitro* cytokine stimulation is enhanced in individuals following vaccination with Chimp Adenoviral Prime/MVA boost vaccine regimen (Figure 5.8.). However we found no evidence of *ex vivo* activation of NK cells following vaccine administration.

PBMC samples used in this study were not freshly isolated prior to analysis but have been cryopreserved and stored in liquid nitrogen by our collaborators before transport to our research group for analysis of NK cell functional responses. Cryopreservation of these samples allowed for batch processing of PBMC samples and allowed for all samples relating to each individual participant to be analysed simultaneously in order to remove some of the variation associated with flow cytometry analysis. However, several technical issues have been associated with flow cytometry analysis samples following resuscitation from cryopreservation. These include a variable degree cell death in PBMC cultures after resuscitation from liquid nitrogen and a low proportion of $CD3^{-}CD56^{+}$ NK cells in these PBMC upon flow cytometry antibody staining (normal range of between 2% and 5% of PBMC). Several quality control measures were carried out before analysis of PBMC from vaccine trial participants in order to ensure validity of results from this vaccine study. These included carrying out antibody titrations as well as optimisation of cytokine stimulation protocols, flow cytometry staining protocols and gating strategies using cryopreserved PBMC samples from healthy normal volunteers, recruited from the School of Biochemistry and Immunology, TCD. These PBMC were isolated from fresh whole blood, cryopreserved and stored in liquid nitrogen before being resuscitated and cultured for use in these optimisation experiments. In addition, Live-Dead Aqua was included in the staining protocol for flow cytometry analysis in order to ensure that dead cells were excluded from our data analysis. This is particularly important following resuscitation of cell from liquid nitrogen.

5.3.1. No evidence of significant NK cell activation in vivo following administration of a candidate HCV vaccine

Ex vivo (T0) analysis of the HCV 001 cohort showed no significant evidence of an increase in NK cell functionality following administration of an Adenoviral based vaccine (Figure 5.1.(a)). These results are in contrast with previous reports of enhanced NK cell functional responses following vaccination against influenza and rabies virus. Seven out of ten individuals analysed from the HCV 001 cohort received Human Adenoviral based vector (Ad6) while the remaining 3 donors received ChAd3 vector.

The results in these 2 donors correlate somewhat with what was seen in the HCV003 cohort of volunteers from this HCV vaccine study who also received the ChAd3 prime vaccination. MVA boost vaccination 8 weeks after prime vaccination was seen to have a more pronounced effect on NK cell IFN-γ production when compared with cytotoxicity. Cytotoxicity responses were found to be more sensitive to initial prime vaccination. Although these trends are not statistically significant, analysis of additional patients from this cohort may reveal real significant changes in mean NK cell functional responses in terms of CD107a expression and IFN-γ production.

5.3.2. NK cell cytotoxicity response to long-term cytokine stimulation is increased post vaccination

Our studies, investigating NK cell response to short-term cytokine stimulation also contrast with previous reports of enhanced NK cell functional responses following vaccination (Horowitz et al., 2010, Long et al., 2008, Jost et al., 2011) NK cell IFN-γ production in the HCV001 cohort in response to 18 hour cytokine stimulation was found to be significantly decreased at 2 weeks post vaccine administration. This attenuation appears to be partially recovered after 4 weeks post vaccination. In contrast, NK cell cytotoxicity in response to cytokine remained unchanged following vaccine administration.

The results from the HCV 001 cohort may be correlated with recent reports suggesting a dichotomy or dissociation between NK cell degranulation and cytokine production functions during HCV infection. Recent studies have reported that NK cells in chronic HCV patients are polarized towards cytotoxicity while IFN-γ production is attenuated (Oliviero et al., 2009, Ahlenstiel et al., 2010, Pelletier et al., 2010). It has also been

previously reported that the level of NK cell degranulation is positively correlated with the magnitude of the HCV-specific T cell response whereas NK cell IFN-γ production is negatively correlated with HCV-specific T cell response (Pelletier et al., 2010). It has been suggested that this may be explained by the finding that although IFN-γ is important for induction of DC maturation, TNF-α, another cytokine also highly produced by NK cells, has been shown to be a more potent inducer of DC maturation (Vitale et al., 2005).

Responses to cytokine stimulation in the HCV003 cohort revealed similar results with no significant increase in NK cell IFN-γ production at 2 weeks post vaccination compared with pre-vaccination baseline levels. In contrast to this, IFN-γ production was found to be significantly reduced at Tw4, Tw8 and Tw12 in comparison with Tw2 levels. It is possible that this decrease may be indicative of a return to baseline levels following an initial increase immediately after prime vaccination. This attenuation in responses is not recovered following boost vaccination. Importantly however, these trends were not seen following longer cytokine stimulation (3 Days and 5 Days).

After 18 hour cytokine stimulation there was no change in NK cell cytotoxicity response following vaccine administration. Similar trends were seen after 3 Day stimulation. Interestingly however, NK cell cytotoxicity was found to be significantly increased only after boost vaccination. The percentage of $CD107a^+$ NK cells was significantly increased at 1 and 4 weeks post boost vaccination compared to pre-prime and pre-boost vaccination levels.

The seemingly contrasting results between the HCV001 and HCV003 cohorts would suggest that administration of the ChAd3 vector may result in more potent NK cell activation in comparison with human Ad6 vaccination. Previous studies by our collaborators, using these vectors found that the ChAd3 vector was more effective as a priming vector in comparison with its boosting effect. For this reason, this vector was chosen as the priming vector of choice for future clinical trials. Importantly however, both vectors have been previously been reported to induce sustained, broad, functional T cell responses (Barnes et al., 2012). Ideally however, further work, analysing additional volunteers from the HCV003 study group would be carried out to confirm these results in a larger cohort.

The MVA based vaccine studied in this chapter has previously been shown to boost T cell response following ChAd3 prime vaccination (Swadling et al., 2014). The data described in this this provides some evidence of enhanced NK cell cytotoxicity response to

prolonged cytokine stimulation in individuals following Ad6 Prime + MVA boost vaccination.

5.3.3. No robust NK cell responses following stimulation with HCV peptide pools

Re-stimulation assays using HCV peptide pools corresponding to the gene products present in the vaccine insert showed a trend of decreased NK cell cytotoxicity and IFN-γ production in comparison with vehicle control treated samples. This was unexpected as previous studies have shown robust induction of T cell function in vaccinated individuals following re-stimulation with these peptide pools. Previous studies have also shown robust induction of NK cell function in vaccinated individuals following antigen re-stimulation (Horowitz et al., 2010, Long et al., 2008). Importantly however, this type of study has not been looked at in the context of HCV infection and previous studies have used inactivated whole virus for re-stimulation assays in vaccinated individuals (Horowitz et al., 2010, Long et al., 2008). These factors may explain the lack of responses seen in our study.

The most obvious explanation for the lack of functional responses is peptide toxicity due to the presence of DMSO. However, vehicle control samples (containing DMSO + Basal Dose cytokine) showed increased levels of NK cell function in comparison with samples stimulated with Basal Dose cytokine only but also in comparison with peptide stimulated samples. This would suggest that the lack of responses is not due to the presence of DMSO.

Future work on these vaccine study participants may involve analysis of serum/plasma samples from donors in both the HCV001 and HCV003 cohort. ELISA analysis measuring cytokine production in such samples may support and provide further evidence of what has been described in PBMC samples by flow cytometry in this and previous studies (Barnes et al. 2012). Elispot data measuring T cell responses in the HCV001 cohort (Barnes et al., 2012), may be correlated with the NK cell responses seen in this study.

Chapter 6

General Discussion

One hundred and seventy million people are estimated to be chronically infected with hepatitis C virus. A significant proportion of those infected with HCV will develop serious liver disease such as cirrhosis and hepatocellular carcinoma and it is the leading cause for liver transplantation in the Western world. In addition, chronic HCV infection can lead to the development of extra-hepatic disease including type II mixed cryoglobulinemia (De Re et al., 2008). The current standard therapy is comprised of a lengthy and expensive and protracted course of interferon-alpha and ribavirin which are associated with significant side-effects and a relatively high rate of treatment failure (approximately 30% in genotype 1b infected patients).

Newly developed 'interferon free' therapies, including direct acting anti-virals such as protease inhibitors, are beginning to be rolled out and have greatly improved patient outcomes but at significant cost. This is a significant problem as a large number of HCV infections are found in developing countries where cost makes treatment inaccessible to many patients. The problems of treatment resistance and re-infection with virus also remain significant issues. There is, therefore, a need for design and development of a vaccine to prevent or improve development of disease symptoms. Despite significant research efforts, an effective vaccine against HCV infection has not yet been made available.

Some individuals will naturally clear HCV infection while the majority will develop chronic disease. The factors which determine the effectiveness of the response against HCV remain unclear. Previous evidence suggests that the innate immune response plays a significant role in determining HCV infection outcome. Further study to fully elucidate the factors which determine the effectiveness of an individual's immune response to both HCV infection and HCV vaccination will be required in order to develop improved therapies and vaccines directed against HCV. The study presented in this thesis has attempted to address this gap in current knowledge with the aim of characterising and identifying the innate immune factors which define a successful immune response to both HCV infection and HCV vaccination.

There is increasing evidence that the cellular innate immune response is a key determinant of viral infection outcome. NK cells are a central component of the innate immune system with important roles in the protection against cancer and viral infections such as HCV (Lanier, 2008). NK cells can directly kill virally infected cells through perforin dependent lysis and production of anti-viral cytokines such as IFN-γ, TNF-α and GM-CSF. The study

described here examined the role of NK cells in the immune response following exposure to HCV antigen in two important biological settings: following HCV infection and following administration of a candidate HCV vaccine.

NK cells are potent producers of IFN-γ as part of the initial immune response to intracellular pathogens such as HCV. In addition, they have the capability to modulate the subsequent adaptive immune response to viral infection, inducing a $CD4^+$ Th1 type response, which is effective against intracellular pathogens, resulting in $CD4^+$ T cell activation and IFN-γ production (Martin-Fontecha et al., 2004). $CD8^+$ cytotoxic T cells are another important component of the immune response to intracellular pathogen and share many similarities with NK cells in terms of effector function. When activated, both produce perforins and granzymes giving them the capability to lyse a virally infected or transformed cell. However, unlike NK cells, virus specific $CD8^+$ and $CD4^+$ T cells of the adaptive immune response remain in the circulation as long-lived effector memory cells long after viral clearance (Sun and Lanier, 2011).

In carrying out studies such as that described in this thesis, it is important to bear in mind the limitations associated with human studies and analysis of human samples. These include small sample size and heterogeneity within patient cohorts which can result in seemingly contradictory results. Characteristics such as age, gender, ethnicity, route of infection often vary significantly between individuals within a patient cohort. Small sample size is also a particular issue when carrying out studies in patient cohorts. Several factors were taken into account in the design of our human studies in order to reduce the potential impact of this inherent variability. The German Anti-D cohort analysed in this study are a relatively homogeneous patient cohort and are therefore very informative as they lack many of the confounding variables which are associated with other studies of HCV infection. All patients in this cohort are female, fertile and of similar age at the time of HCV infection. In addition, these women were infected by a single source of HCV (genotype 1b) and were infected through the same route, receiving a low level of inoculum. In addition, clinical outcomes for this cohort have been well described, allowing for direct comparison of spontaneous resolvers and those who develop chronic infection (Dring et al., 2011). In the context of our HCV vaccine study, the ability to carry out a prospective study, following a cohort of individuals over time is also beneficial. The design of this vaccine study also allowed for paired statistical analysis to be carried out for comparison of pre and post vaccination samples from the same individuals.

6.1. The role of NK cell KIR receptors in the immune response to HCV

It is now well established that a large range of both inhibitory and activating NK cell receptors regulate NK cell activity. The KIR are one of the largest and most diverse NK cell receptor families and many details regarding these receptors and their interaction with ligand remain to be elucidated. Research in this area to date has been hampered by the large degree of variation in KIR expression between populations. Polymorphism within KIR genes and the significant level of linkage disequilibrium between KIR genes has also made it difficult to confirm potential KIR gene associations with various diseases.

Certain KIR and HLA alleles have been previously associated with HCV clearance whilst others have been associated with chronic infection. KIR: HLA associations with HCV infection outcome have been identified in several (ethnically distinct) populations. In addition, several KIR genes have also been associated with treatment outcome following administration of pegylated interferon + ribavirin therapy. Whether similar KIR associations exist in individuals receiving newly developed DAA therapies remains to be established. This question will be the subject of increasing research interest into the future as these new treatments come into widespread use. KIR and HLA genes have also been associated with development of HCV related diseases such as HCC and HCV related lymphoproliferative disease.

The results of the tetramer binding assays described here provide no evidence to suggest that KIR3DL2 interactions with HLA-A*03 ligand play a significant role in the protective effect of this HLA allele in HCV infection. Interestingly however, additional peptide tetramer binding assays involving EBV/EBNA3A peptide, presented on the HLA-A*03 background, appear to suggest that the strength of the KIR3DL2 receptor: ligand interaction is dependent on receptor allotype. If true in a biological setting, it is possible that this could affect the subsequent immune response to viral infection, resulting in individual variation in levels of inhibition and activation of NK cells, dependent on KIR3DL2 allotype. This may have implications in terms of identifying further innate immune genes (and specific allelic variants of these genes) which may be predictive of outcome in EBV and other viral infections.

Another aspect of this study assessed the role of KIR genes in the immune response to HCV infection, examining the frequency of KIR genes in combination with their HLA ligands in a cohort of HCV infected patients. This study examined KIR genotype in the

German Anti-D cohort for the first time. Previous work from the Gardiner research group reported that the *KIR2DS3* gene is associated with chronic HCV infection in the Irish Anti-D cohort. *KIR2DS3* has also been associated with increased risk of pegylated IFN + ribavirin treatment failure in HIV/HCV co-infected patients (Keane et al., 2013). The genotyping study in the German Anti-D cohort which is described here examined the importance of KIR receptor genes in the clinical progression of viral infection, specifically HCV infection. Analysis of KIR and HLA frequency in those who developed chronic infection compared with those who spontaneously cleared the virus showed no statistically significant associations with HCV infection outcome in this cohort.

No statistically significant associations between *KIR2DS3*, *KIR2DL3* (or their HLA ligands) and HCV infection outcome were identified in this study despite previous findings in other cohorts of HCV infected patients. This suggests that, given the diversity of the KIR gene family, it may be insufficient to analyse simple compound genotypes without accounting for the other KIR genes present which contribute to the balance of signals which determine NK cell activity and functionality. In fact, it may be necessary to examine the whole NK cell receptor repertoire in order to determine the role of NK cells in the immune response to disease. Similarly, the significant allelic variation which is present within the KIR family may also have to be taken into account when carrying out disease association studies. Newly developed technologies such as next generation sequencing may facilitate this type of detailed analysis into the future. The role of KIR genes in HCV infection outcome should also be examined in additional cohorts and this should ideally incorporate a diverse range of ethnicities.

6.2. The role of NK cells in the immune response following administration of Adenoviral based HCV vaccine regimen

Several factors have delayed development of an effective HCV vaccine to date. There are seven HCV genotypes which are uniquely distinct with nucleotide sequences that differ by at least 70%. In addition, the virus is highly adept at escaping immune detection by antibodies and $CD8^+$ T cells of the adaptive immune system. The high mutation rate which is associated with HCV replication is the mechanism for this viral immune evasion. The primary function of any successful vaccine against HCV infection is the prevention of HCV infection in those who have not yet been exposed to the virus. However, another important benefit of a HCV vaccine could be to prevent re-infection in those who have

been cured of HCV following DAA treatment but who are at ongoing risk of re-exposure to the virus. It is not yet known whether DAA therapy will be as effective in clearing subsequent HCV infections in these patients.

There is significant evidence that spontaneous resolution of initial HCV infection is protective against development of chronic infection and disease following subsequent exposure to the virus. This suggests that the development of a HCV vaccine which is capable of preventing viral persistence may be of significant clinical importance. The factors which determine a protective immune response against HCV infection have yet to be elucidated but are likely to involve production of antibodies which are capable of ADCC as well as induction of a cellular immune response, including the production of memory T cells. However, the induction of a long-lasting, protective innate immune memory response is also likely to contribute to a protective cellular immune response to immunisation (Rydyznski et al., 2015).

To our knowledge, this study examined, for the first time, the effect of a candidate HCV vaccine on NK cell cytokine production and cytotoxic function. The candidate vaccine examined in this study has previously shown some promise and is capable of robustly inducing T cell adaptive immunity. IFN-γ production by NK cells following stimulation by pathogen is known to direct the subsequent adaptive immune response towards the induction of Th1 type responses. We therefore hypothesised that, through the induction of highly functional NK cells following vaccination, NK cells played an important role in the induction of the T cell adaptive immune responses to a HCV vaccine, culminating in the generation of memory T cells. Perhaps surprisingly, our *in vivo* analysis found no significant evidence of increased NK cell functional responses post vaccine administration, despite these vaccines being previously reported to induce robust $CD8^+$ and $CD4^+$ T cell responses in this cohort of vaccine recipients.

Results from the HCV 001 cohort suggest that the induction of T cell immune responses, previously reported in this cohort, arises independently of NK cell IFN-γ production. Interestingly however, although there is a decrease in NK cell IFN-γ production following vaccination of this cohort, NK cell cytotoxicity remains largely unaffected. These results are comparable with data from patients chronically infected with HCV which showed an attenuation in NK cell IFN-γ responses in comparison with healthy controls, with a polarisation towards NK cytotoxicity responses.

Interestingly, recent studies into LCMV infection in mice have suggested that NK cells can actively suppress the development of a protective but potentially pathogenic population of virus specific T cells in the early stages of infection (Waggoner et al., 2012). NK cell activation has been found to limit T cell immunity, enhancing immune pathology and promoting chronic infection (Lang et al., 2012). In addition, the depletion of NK cells has been found to prevent T cell exhaustion, resulting in the control of viral infection (Cook et al., 2013). It has been proposed that NK cells are capable of lysing both $CD4^+$ and $CD8^+$ T cells during initial infection when type 1 IFN is present and NK cells are cytolytically activated (Waggoner et al., 2014, Rydyznski et al., 2015). The generation of B cell immunity has also been found to be impaired by NK cells (Rydyznski et al., 2015).

Although this has been reported only in a mouse model of LCMV, activated NK cells may play a similar role in modulating the induction of the T cell mediated immune responses following administration of this candidate HCV vaccine. The lack of significant NK cell activation following vaccination of these participants may allow for the promotion of T cell vaccine responses by preventing NK cell induced T cell exhaustion. This may explain the differences in NK cell and T cell functional responses in these participants following administration of vaccines which have been previously shown to induce sustained and functional T cell responses.

From the results of this study it remains unclear whether NK cells are significant contributors to the activation of the adaptive immune response following administration of a HCV vaccine as has been previously reported in the context of influenza and rabies vaccination. Further study into the role of NK cells in the vaccine induced immune response is required and will likely provide further important information which will guide the development of future vaccines directed against HCV and other viral infections.

6.3. Future Perspectives

Following on from this research, there are several interesting findings which are worthy of further investigation. This study provides evidence that KIR3DL2 allotypes bind to EBV peptide with varying levels of specificity. Further peptide binding assays may be carried out to further investigate the influence of KIR3DL2 polymorphism on receptor binding to EBV peptide. This may reveal a novel role for NK cells in the anti-viral response to EBV infection. This would involve development and synthesis of a panel of EBV peptide

tetramers which are predicted to bind HLA-A*03. HLA-A*011 is another known ligand for KIR3DL2. Similar tetramer binding assays involving a group of HLA-A*011 restricted HCV and EBV peptides may also reveal differential binding to the KIR3DL2 receptor. Results from the post renal transplant cohort of EBV infection showed decreased NK cell expression of the CD57 maturation marker in patients with chronic high EBV viral load titres in comparison with those who resolved infection. Numbers in this study are small (n=10 resolvers and n=4 C.H.L.) and ideally would be expanded. This would also allow for further analysis of the CMV serology of the transplant donors which may also yield interesting results in terms of whether re-exposure to CMV infection from CMV^+ donor organs results in sustained increases in NK cell KIR3DL2 expression. Ideally however, a large genetic study would be carried out to confirm an association between KIR3DL2 (or its individual allotypes) with EBV infection outcome. However, acquisition of a sufficiently large number of samples, from a patient cohort with EBV infection may prove difficult and may not be feasible.

Further investigations into the genetic background of the German Anti-D cohort may reveal significant associations between particular genes and outcome/treatment prognosis following HCV infection. This may involve carrying out further genotyping studies investigating the frequencies of NK cell receptor genes, including additional KIR genes that are in linkage disequilibrium with *KIR2DS3*, as well as their HLA ligands. Previous work from our research group identified a centromeric KIR haplotype which was significantly associated with development of chronic HCV infection. In addition to *KIR2DS3* and *KIR2DL2*, this haplotype also includes the *KIR2DL1*, *KIR2DS2* and *KIR3DL3* genes (Dring et al., 2011) whose gene frequency is yet to be determined in the German Anti-D cohort. Perhaps significantly, the KIR2DL1 receptor is known to bind HLA-C2. These candidate genes may therefore contribute to increased risk of development of chronic HCV infection which has been previously associated with the *KIR2DS3* and *HLA-C2* genes. Experimental protocols for PCR-SSP allele typing of *KIR2DS3* have also been developed by our research group and this type of in-depth analysis in the German Anti-D cohort may also reveal interesting associations between individual *KIR2DS3* alleles and disease outcome/treatment prognosis in HCV infection. In addition, further research will be required in order to identify a ligand for the KIR2DS3 protein and confirm a functional role for this receptor, as these are key questions which remain to be answered.

Further analysis of NK cell functionality following administration of a candidate HCV vaccine may also provide interesting results. Results from the HCV 003 cohort, described

in this study, provide some evidence for increased NK cell cytotoxic function following administration of a candidate HCV vaccine. However, these results were only significant following *ex vivo* cytokine stimulation of PBMC. Analysis of additional donors from this cohort may reveal significant evidence of *in vivo* NK cell activation post vaccination. In addition, evaluation of cytokine levels in plasma samples, taken from these participants in the vaccine trial, may further support the results described in this and previous studies. In addition, previous Elispot data, which measured T cell responses in the HCV 001 cohort (Barnes et al., 2012), may be analysed in order to investigate possible correlations with the NK cell responses described in our study.

These candidate HCV vaccines are currently being tested on HIV^+ patients who are undergoing anti-retroviral therapy. The safety of these vaccines and their ability to induce robust immune responses in HIV^+ patients will be examined. Analysis of NK cell responses in these participants may yield interesting results which could contribute to the development of future clinical trials involving these or other candidate HCV vaccines. As previously described (Section 5.3.), several technical issues have been associated with analysis of frozen PBMC samples. These issues relate to a variable amount of cell death following resuscitation from cryopreservation, which can result in a low proportion of $CD3^-CD56^+$ NK cells. Future study in additional patient cohorts would allow for analysis of freshly isolated PBMC samples in order to minimise this issue.

The vaccine study described in this thesis examined NK cell functional responses at 2, 4, 8, 9 and 12 weeks post vaccine administration. As part of the innate immune system, NK cells are one of the first cell types to be activated in the immune response to foreign antigen. Future studies, examining NK cell function at earlier time-points (including 18 hours following administration of the candidate HCV vaccine) may therefore yield interesting results.

In conclusion, NK cells play an important role in the immune response to challenge with HCV antigen. Further study is required in order to gain a greater understanding of the mechanisms that control NK cell activation and inhibition and the role of NK cells in human diseases, particularly viral infection.

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Appendix

Appendix Figure 1. Background information and Clinical history of the Paediatric Cohort of Post-Renal Transplant patients.

Patient	HLA-A	EBV status pre-tx	Current EBV VL	EBV VL Detected	CMV status pre-tx	Group	DOB	DoTx	Donor CMV
1	A3/A24	N/A	Not detected	Not detected (5 yr)	Negative	REI	27/04/2000	20/07/2005	Negative
2	A1/A11	Positive	Detected	Detected (2 years)	Negative	CHL	18/08/2003	07/11/2012	Negative
3	A2/A3	Negative	Not detected	Not detected (1 yr)	Positive	REI	30/08/2006	30/05/2012	Negative
4	A3/A11	Negative	Not detected	Detected (2 years)	Negative	CHL	04/09/2007	15/02/2012	Negative
5	A3/A68	Negative	Not detected	Detected (5 years)	Negative	CHL	23/03/2007	07/11/2012	Negative
6	A2/A3	Positive	Not detected	Detected (5 years)	Negative	CHL	04/02/2005	24/09/2009	Negative
7	A3	Negative	Not detected	Not detected (1 yr)	Negative	REI	21/09/2000	06/03/2012	Negative
8	A1/11	Positive	Not detected	Not detected (2 Years)	Negative	REI	29/05/2004	22/05/2012	Negative
9	A2/A3	Positive	Not detected	Not detected (2 years)	Positive	REI	05/07/1999	04/12/2011	Positive
10	A1/A3	Positive	Not detected	Detected (1 year)	Positive	REI	26/01/2004	14/12/2007	Positive
11	A2/A3	Positive	Not detected	Not detected (3 Years)	Positive	REI	28/09/1998	13/03/2011	Negative
12	A1/A3	Positive	Not detected	Not detected (3 Years)	Negative	REI	13/05/1998	27/01/2010	Positive
13	A3/A34	Negative	Not detected	Not detected (3 Years)	Positive	REI	07/12/2005	29/05/2007	N/A
14	A3/A68	Negative	Not detected	Not detected (3 Years)	Negative	REI	17/12/2004	24/12/2011	Negative

Background information including date of birth (DOB), date of transplant (DoTx) and HLA-A type are detailed. EBV status both pre and post transplant is also provided. Patients are sub-divided into two groups based on EBV viral load: Chronic High Viral Load Carriers (CHL) and resolvers of EBV infection (REI). Pre-transplant CMV status of recipient and donor is also detailed.

Appendix Figure 2. Background information and Clinical history of the German Anti-D cohort of HCV infected patients.

Number	D.O.B.	Primary Outcome	Viral load	Therapy	Current Diagnosis	Cirrhosis
AD-007	16/05/1953	1	Pos	REL 08	NR	F3
AD-018	22/03/1956	0	Neg	0	spon res	
AD-034	21/08/1952	1	Neg	NR 95; SVR 99	SVR	
AD-036	04/12/1951	1	Neg	SVR 01	SVR	
AD-042	03/02/1955	1	Pos	0	chronic no treatment	
AD-045	16/08/1953	1	Neg	SVR 04	SVR	
AD-052	01/10/1950	1	Pos	0	chronic no treatment	
AD-053	23/07/1950	1	neg	SVR 07	SVR	
AD-055	13/06/1955	0	neg	0	spon res	
AD-059	13/05/1950	1	neg?	in therapy	SVR	
AD-063	23/06/1954	1	pos	0	chronic no treatment	
AD-065	29/12/1953	1	neg	SVR 93	SVR	
AD-069	18/02/1947	1	pos	0	chronic no treatment	
AD-077	18/03/1954	0	neg	0	spon res	

AD-088	19/05/1949	1	pos	0	chronic no treatment
AD-091	04/02/1952	0	neg	0	spon res
AD-093	05/07/1950	1	pos	NR 02, 05	NR
AD-096	11/01/1952	1	pos	0	chronic no treatment
AD-099	20/10/1952	0	neg	0	spon res
AD-110	08/05/1953	1	neg	SVR 97	SVR
AD-111	25/06/1955	1	pos	0	chronic no treatment
AD-113	08/07/1955	0	neg	0	spon res
AD-114	12/10/1955	0	neg	0	spon res
AD-115	21/04/1948	1	neg	SVR 98	SVR
AD-118	27/05/1956	1	pos	NR 95	NR
AD-123	24/02/1953	1	pos	0	chronic no treatment
AD-131	06/10/1946	1	pos	NR 00	NR
AD-137	09/03/1946	0	neg	0	spon res
AD-144	18/11/1949	0	neg	0	spon res
AD-145	20/05/1947	0	neg	0	spon res
AD-146	20/08/1948	1	neg?	NR	
AD-147	11/12/1950	0	neg	0	spon res

AD-149	27/07/1954	1	neg	SVR 92	SVR
AD-152	27/01/1945	2	neg	0	inoculated no symptoms
AD-155	14/02/1953	1	pos	NR 95	NR
AD-156	10/04/1955	1	pos	0	chronic no treatment
AD-159	29/08/1951	0	neg	0	spon res
AD-160	23/12/1950	0	neg	0	spon res
AD-163	30/08/1952	1	neg	SVR 93	SVR
AD-169	29/08/1954	1	pos	NR 02	NR
AD-172	15/06/1953	0	neg	0	spon res
AD-174	10/11/1952	2	neg	0	inoculated no symptoms
AD-175	07/11/1948	1	pos	in therapy 09	NR
AD-176		missed			
AD-177	31/10/1948	1	pos	0	chronic no treatment
AD-181	08/02/1951	1	neg	SVR 00	SVR
AD-183	28/03/1941	0	neg	0	spon res
AD-190	14/11/1953	0	neg	0	spon res
AD-191	26/09/1949	1	neg	SVR 05	SVR
AD-192	29/11/1957	1	pos	?	chronic no treatment

AD-193	04/04/1941	1	pos	treatment ended 00	NR
AD-194	07/10/1951	1	pos	96; NR	NR
AD-195	07/05/1952	1	neg	SVR	SVR
AD-196	14/10/1948	1	neg	REL 99; SVR 01	SVR
AD-197	21/07/1954	1	neg	SVR 06	SVR
AD-209	30/01/1953	0	neg	0	spon res
AD-218	25/06/1957	0	neg	0	spon res
AD-219	27/01/1955	1	pos	NR 02	NR
AD-233	08/02/1957	2	neg	0	inoculated no symptoms
AD-235	16/04/1950	1	pos	0	chronic no treatment
AD-236	10/02/1946	0	neg	0	spon res
AD-267	12/05/1943	1	pos	2001	NR
AD-275	16/11/1953	1	pos	?	chronic no treatment
AD-283	05/12/1950	2	neg	0	inoculated no symptoms
AD-284	13/09/1955	1	pos	2005	NR (2010 re-infection and treatment)
AD-285	09/07/1955	0	neg	0	spon res
AD-286	17/01/1953	1	pos	0	chronic no treatment
AD-287	13/10/1952	1	neg	SVR 00	SVR

AD-288	23/10/1952	1	pos	NR 96	NR	
AD-289	01/02/1950	0	neg	0	spon res	
AD-290	14/03/1937	0	neg	0	spon res	
AD-292	27/12/1947	2	neg	0	inoculated no symptoms	
AD-293	07/07/1951	0	neg	0	spon res	
AD-294	30/04/1955	1	pos	NR 01	CH	Ci
AD-296	01/09/1952	0	neg	0	spon res	
AD-297	11/04/1951	0	neg	0	spon res	
AD-298	09/04/1953	1	pos	0	chronic no treatment	
AD-299	16/05/1954	0	neg	0	spon res	
AD-300	11/01/1954	1	pos	NR 93	NR	
AD-301	24/10/1948	0	neg	0	spon res	
AD-302	01/09/1954	0	neg	0	spon res	
AD-303	25/02/1953	0	neg	0	spon res	
AD-304	29/06/1947	0	neg	0	spon res	
AD-305	20/06/1950	1	pos	0	chronic no treatment	
AD-307	08/10/1947	1	pos	0	chronic no treatment	
AD-308	02/03/1946	1	neg	SVR 96	SVR	

AD-309	22/11/1947	0	neg	0	spon res
AD-310	03/09/1951	0	neg	0	spon res
AD-311	21/01/1948	1	pos	0	chronic no treatment
AD-312	01/01/1952	1	pos	0	chronic no treatment
AD-313	15/06/1949	1	pos	0	chronic no treatment
AD-315	18/10/1948	0	neg	0	spon res
AD-316	05/11/1951	1	neg	SVR 97	SVR
AD-317	02/06/1955	0	neg	0	spon res
AD-318	29/03/1943	0	neg	0	spon res
AD-320	09/06/1953	0	neg	0	spon res
AD-321	13/06/1952	1	pos	0	chronic no treatment
AD-322	04/01/1948	0	neg	0	spon res
AD-323	03/04/1945	0	neg	0	spon res
AD-324	15/02/1946	1	neg	SVR 06	SVR
AD-325	17/01/1954	0	neg	0	spon res
AD-326	02/11/1949	missed			
AD-327	05/06/1950	1	pos	0	chronic no treatment
AD-328	20/03/1954	0	neg	0	spon res

AD-330	02/07/1951	1	pos	0	chronic no treatment	
AD-332	01/05/1949	1	pos	0	chronic no treatment	
AD-333	16/01/1946	1	pos	1999	NR	
AD-334	30/10/1951	missed				
AD-335	06/12/1953	1	neg	SVR 00	SVR	
AD-336	21/10/1952	1	pos	2004	NR	
AD-337	21/08/1951	1	pos	NR 05	CH	Ci
AD-338	25/07/1951	1	neg	SVR 06	SVR	
AD-339	29/01/1945	1	pos	Ther 09	NR	
AD-340	05/05/1905	1	neg	SVR 01	SVR	
AD-341	05/03/1952	1	pos	0	chronic no treatment	
AD-342	01/11/1953	1	pos	2002	NR	
AD-343	21/09/1949	1	pos	REL 09	NR	
AD-344	22/02/1953	1	z.Z. neg	Therapy since 10	?	
AD-347	08/09/1953	1	pos	2004	NR	
AD-348	16/01/1948	1	neg	SVR 03	SVR	
AD-349	21/06/1955	missed				
AD-350	17/02/1949	1	pos	0	chronic no treatment	

AD-351	10/01/1954	1	pos	ja	NR	
AD-352	05/08/1952	1	pos	2005	NR	
AD-353	21/03/1953	1	pos	NR 96	NR	
AD-354		0	neg	0	spon res	
AD-355	18/11/1952	1	neg	SVR 05	SVR	
AD-356	13/11/1953	1	pos	2006	NR	
AD-357	09/02/1954	1	pos	2007	NR	
AD-358	26/01/1954	1	pos	2008	CH	Ci
AD-359	09/06/1953	2	neg	0	inoculated no symptoms	
AD-360	03/04/1956	1	neg	SVR 06	SVR	
AD-361	02/11/1952	1	pos	0	chronic no treatment	
AD-362	20/01/1960	missed				
AD-363	29/04/1948	0	neg	0	spon res	
AD-364	31/10/1951	1	pos	0	chronic no treatment	
AD-365	17/05/1955	1	pos	0	chronic no treatment	
AD-366	13/06/1949	1	pos	NR 09	NR	
AD-368	06/03/1950	missed				
AD-369	13/05/1948	1	pos	NR	NR	

AD-370	08/04/1950	1	pos	0	chronic no treatment	
AD-371	26/12/1946	1	pos	REL 06	NR	
AD-372	12/01/1948	1	pos	NR 05	CH	CHe Ci
AD-373	10/12/1946	missed				
AD-374	11/05/1956	1	neg	SVR 98	SVR	
AD-376	23/09/1951	1	pos	0	chronic no treatment	
AD-378	01/08/1952	0	neg	0	spon res	
AD-379	15/12/1952	1	pos	0	chronic no treatment	
AD-380	03/01/1956	missed				
AD-381	06/02/1957	1	pos	0	chronic no treatment	
AD-382	07/07/1957	1	pos	NR 04	NR	
AD-383	04/03/1945	1	pos	0	chronic no treatment	
AD-384	29/01/1953	2	neg	0	inoculated no symptoms	
AD-385	05/07/1955	1	pos	0	chronic no treatment	
AD-386	30/08/1949	1	pos	NR 97	NR	
AD-387	24/05/1955	1	neg	SVR 05	SVR	
AD-389	19/03/1950	1	pos	0	chronic no treatment	Ci
AD-391	19/09/1955	1	bisher neg.	2009	SVR	

AD-392	31/08/1935	1	pos	NR 94	NR	
AD-394	29/04/1941	1	pos	NR 05	NR	
AD-396	21/05/1951	0	neg	0	spon res	
AD-397	09/05/1942	1	pos	0	chronic no treatment	
AD-398	11/03/1949	1	pos	NR 08	NR	
AD-401	01/08/1950	1	neg	SVR 09	SVR	
AD-402	16/04/1950	0	neg	0	spon res	
AD-403	29/03/1955	1	neg	SVR 06	SVR	
AD-404	25/10/1956	1	pos	REL 01	NR	
AD-405	06/03/1959	1	neg	SVR 08	SVR	
AD-408	15/07/1953	1	pos	0	chronic no treatment	
AD-409	17/12/1953	1	pos	0	chronic no treatment	
AD-410	21/08/1943	1	neg	SVR 07	SVR	
AD-412	17/03/1954	1	pos	0	chronic no treatment	
AD-413	10/08/1943	1	pos	0	chronic no treatment	
AD-414	02/09/1950	1	neg	SVR 99	SVR	
AD-415	29/02/1948	1	?	bis 04 keine	chronic no treatment	
AD-416	26/09/1945	1	pos	?	chronic no treatment	Ci

AD-417	23/11/1943	missed			
AD-419	03/03/1956	0	neg	0	spon res
AD-420	29/12/1947	1	pos	bis 04	NR
AD-421	07/11/1953	1	pos	0	chronic no treatment
AD-422	16/08/1955	1	Doku fehlt	REL 97	NR
AD-425	29/10/1956	1	neg	SVR 07	SVR
AD-427	11/03/1949	1	pos	0	chronic no treatment
AD-428	31/12/1955	1	pos	NR 05	NR
AD-429	29/09/1953	1	pos	0	chronic no treatment
AD-431	16/01/1942	1	pos	NR 99	NR
AD-432	07/11/1944	1	pos	REL	NR
AD-433	07/04/1955	missed			
AD-434	22/03/1959	missed			
AD-435	05/03/1961	missed			
AD-436	24/12/1949	0	neg	0	spon res
AD-437	30/08/1953	1	?	01 REL (Ther 09)	CHd
AD-438	20/02/1940	0	neg	0	spon res
AD-439	01/11/1949	missed			

AD-440	29/03/1961	missed				
E10-00002	19/03/1961	0	neg	0	spon res	
E10-00055	24/07/1959	1	pos	REL 95	NR	
E10-00056	11/03/1954	1	pos	NR 98	NR	
E10-00057	28/12/1951	1	pos	NR 07	NR	
E10-00058	13/10/1954	1	pos	REL 08	NR	
E10-00059	14/09/1951	1	neg	SVR 08	SVR	
E10-00061	03/10/1942	1	pos	NR 97	NR	
E10-00062	04/04/1956	1	pos	NR 95	NR	
E10-00063	11/06/1951	1	pos	NR 92	CH	Ci
E10-00064	04/06/1955	1	neg	SVR 95	SVR	
E10-00113	15/10/1954	1	pos	REL 05	NR	
E10-00124	27/11/1956	1	pos	Treatment ended 92	CH	Ci
E10-00143	15/11/1954	0	neg	0	spon res	
E10-00150	05/03/1959	1	neg	SVR 03	SVR	
E10-00151	04/05/1957	1	neg	SVR 97	SVR	
E10-00152	19/08/1944	1	pos	NR 04, 06	CH	Ci
E10-00153	08/07/1949	1	pos	NR 06	NR	

E10-00159	25/01/1953	1	LTx, Re-Therapie	z.Z. Therapie	LTx	Ci
E10-00243	03/07/1956	1	neg	SVR 00	SVR	
E10-00244	30/10/1959	0	neg	0	spon res	
E10-00245	22/04/1958	1	pos	NR 99, 06	NR	
E10-00246	26/02/1957	1	neg	SVR 97	SVR	
E10-00269	24/02/1959	1	pos	0	chronic no treatment	Ci
E10-00284	02/09/1956	0	neg	0	spon res	
E10-00302	16/03/1952	1	pos	NR 95	CH	Ci
E10-00320	12/06/1955	1	neg	SVR 08	SVR	
E10-00322	30/11/1950	1	neg	SVR 02	SVR	
E10-00323	23/03/1960	1	pos	0	chronic no treatment	
E10-00324	02/06/1955	0	neg	0	spon res	
E10-00325	01/04/1943	1	neg	SVR 01	SVR	
E10-00351	27/01/1957	1	pos	NR 96	NR	
E10-00397	24/09/1955	1	pos	0	chronic no treatment	
E10-00440	06/09/1956	1	neg	SVR 05	SVR	
E10-00441	01/08/1941	1	pos	NR 92, 06	NR	
E10-00455	14/11/1957	1	pos	REL 96; 09	NR	

E10-00459	20/12/1957	1	pos	0	chronic no treatment
E10-00460	03/10/1948	0	neg	0	spon res
E10-00493	30/06/1957	1	neg	SVR 05	SVR
E10-00502	12/11/1952	1	pos	0	chronic no treatment
E10-00523	25/08/1955	2 (NE)	neg	0	inoculated no symptoms
E10-00548	15/11/1951	0	neg	0	spon res
E10-00549	08/05/1948	1	pos	0	chronic no treatment
E10-00550	04/10/1960	1	pos	0	chronic no treatment
E10-00559	17/05/1952	0	neg	0	spon res
E10-00560	15/07/1959	1	pos	0	chronic no treatment
E10-00589	02/07/1955	1	pos	REL 97	NR
E10-00625	16/03/1950	1	pos	0	chronic no treatment
E10-00626	27/09/1957	1	pos	NR 97; 09 in THER	NR
E10-00662	22/09/1948	1	pos	REL 05	NR
E10-00680	09/10/1961	0	neg	0	spon res
E10-00691	21/12/1940	0	neg	0	spon res
E10-00692	10/12/1956	0	neg	0	spon res
E10-00693	18/08/1952	1	neg	SVR 01	SVR

E10-00703	10/05/1954	1	neg	SVR 97	SVR	
E10-00706	20/02/1953	0	neg	0	spon res	
E10-00707	17/10/1956	0	neg	0	spon res	
E10-00708	17/05/1953	1	pos	0	chronic no treatment	
E10-00709	12/04/1953	0	neg	0	spon res	
E10-00710	20/10/1956	1	neg	SVR 95	SVR	
E10-00720	23/07/1955	0	neg	0	spon res	F3
E10-00721	12/07/1942	1	neg	SVR 10	SVR	
E10-00722	25/04/1954	0	neg	0	spon res	
E10-00724	19/05/1959	1	neg	SVR 05	SVR	
E10-00728	26/03/1955	1	pos	0	chronic no treatment	
E10-00733	17/01/1948	1	pos	0	chronic no treatment	
E10-00735	12/12/1958	0	neg	0	spon res	
E10-00736	18/05/1956	2 (NE)	neg	0	inoculated no symptoms	
E10-00738	31/05/1954	1	pos	0	chronic no treatment	
E10-00739	15/10/1949	0	neg	0	spon res	
E10-00740	08/01/1956	0	neg	0	spon res	
E10-00753	22/09/1954	0	neg	0	spon res	

E10-00754	01/05/1959	0	neg	0	spon res	
E10-00762	03/05/1956	1	pos	0	chronic no treatment	
E10-00770	19/11/1957	1	pos	NR 06	CH	Ci
E10-00780	03/01/1953	1	pos	0	chronic no treatment	
E10-00786	12/08/1958	0	neg	0	spon res	
E10-00789	27/08/1952	1	pos	NR 96	NR	
E10-00798	29/09/1948	1	pos	REL 04	NR	
E10-00822	02/11/1951	1	neg	SVR 05?	SVR	
E10-00824	09/01/1951	0	neg	0	spon res	
E10-00825	23/03/1952	0	neg	0	spon res	
E10-00826	27/01/1955	1	neg	SVR 92	SVR	
E10-00836	19/07/1960	1	pos	REL 00; z.Z. in THER	NR	
E10-00858	30/12/1961	1	pos	NR 92	NR	
E10-00862	10/09/1954	1	pos	0	chronic no treatment	
E10-00865	30/08/1951	1	pos	NR 93	NR	
E10-00867	16/05/1939	0	neg	0	spon res	
E10-00875	27/12/1958	0	neg	0	spon res	
E10-00883	05/12/1954	0	neg	0	spon res	

E10-00900	23/04/1951	0	neg	0	spon res
E10-00901	10/05/1950	1	pos	0	chronic no treatment
E10-00903	08/02/1945	1	pos	NR 91; REL 04	NR
E10-00904	31/03/1960	0	neg	0	spon res
E10-00905	09/01/1954	0	neg	0	spon res
E10-00906	15/03/1944	1	neg	SVR 94	SVR
E10-00907	07/01/1960	1	pos	REL 06	NR
E10-00908	15/12/1956	1	pos	0	chronic no treatment
E10-00918	24/11/1957	1	neg	SVR 01	SVR
E10-00920	13/03/1956	1	pos	95	NR
E10-00921	03/10/1954	1	pos	0	chronic no treatment
E10-00930	30/04/1940	1	pos	NR 93	NR
E10-00931	14/03/1953	1	pos	0	chronic no treatment
E10-00934	30/05/1941	1	pos	0	chronic no treatment
E10-00935	11/11/1956	1	neg	SVR 06	SVR
E10-00954	11/12/1958	1	pos	NR 02	NR
E10-00963	31/08/1941	0	neg	0	spon res
E10-00964	25/09/1954	1	pos	NR 07	NR

E10-00965	19/12/1958	0	neg	0	spon res	
E10-00967	03/12/1957	1	pos	NR 02	NR	
E10-00968	06/12/1960	1	neg	SVR 00	SVR	
E10-00970	01/01/1940	0	neg	0	spon res	
E10-00978	25/05/1951	1	neg	SVR 03	SVR	
E10-00986	24/09/1949	1	pos	0	chronic no treatment	Ci
E10-00989	20/03/1947	1	neg	SVR 03	SVR	
E10-00990	12/07/1958	1	pos	0	chronic no treatment	
E10-00991	13/01/1952	1	neg	SVR 99	SVR	
E10-00995	20/03/1958	1	pos	0	chronic no treatment	
E10-00996	22/02/1955	1	pos	THER 10	NR	
E10-01002	22/07/1948	1	pos	0	chronic no treatment	Ci (2008 LTx)
E10-01004	17/12/1954	1	neg	SVR 09	SVR	
E10-01005	24/01/1952	1	neg	SVR 99	SVR	
E10-01016	15/01/1959	1	pos	NR 09	NR	
E10-01046	30/06/1955	1	pos	NR 07	NR	
E10-01064	21/07/1951	0	neg	0	spon res	
E10-01065	19/08/1960	0	neg	0	spon res	

E10-01066	20/06/1954	1	pos	0	chronic no treatment
E10-01067	29/04/1958	1	pos	REL 03	NR
E10-01074	24/03/1952	1	pos	REL 95	NR
E10-01076	23/08/1955	1	neg	SVR 03	SVR
E10-01108	14/11/1955	1	pos	REL 96	NR
E10-01111	03/11/1953	1	pos	0	chronic no treatment
E10-01112	04/01/1960	1	pos	REL 06	NR
E10-01113	10/08/1950	1	pos	0	chronic no treatment
E10-01114	23/03/1957	0	neg	0	spon res
E10-01128	19/05/1959	1	pos	REL 05	NR
E10-01143	22/01/1959	1	pos	NR 05	NR
E10-01146	08/07/1949	1	pos	NR 08	NR
E10-01154	26/09/1946	1	pos	0	chronic no treatment
E10-01164	03/11/1959	1	pos	0	chronic no treatment
E10-01167	02/04/1951	1	pos	NR 04, 06	NR
E10-01168	22/07/1948	1	pos	NR 02	NR
E10-01169	08/11/1951	1	neg	SVR 06	SVR
E10-01199	15/09/1958	1	neg	SVR 09	SVR

E10-01200	28/02/1956	1	pos	REL 96	NR	
E10-01221	03/11/1957	1	neg	SVR 04	SVR	
E10-01224	05/03/1944	2 (NE)	neg	0	inoculated no symptoms	
E10-01225	06/08/1953	0	neg	0	spon res	
E10-01230	24/01/1953	1	pos	0	chronic no treatment	
E10-01235	06/04/1949	1	pos	NR 03		
E10-01260	09/10/1953	1	pos	REL 06	NR	
E11-00021	20/08/1955	1	neg	SVR 01	SVR	
E11-00059	07/07/1953	1	neg	SVR 04	SVR	
E11-00070	25/03/1960	0	neg	0	spon res	
E11-00071	30/09/1953	1	pos	0	chronic no treatment	
E11-00085	11/09/1957	1	pos	NR 07; THER 11	CH	Ci
E11-00086	27/07/1954	1	pos	REL 09	NR	
E11-00089	21/02/1962	0	neg	0	spon res	
E11-00090	01/07/1959	1	pos	0	chronic no treatment	
E11-00151	18/09/1953	1	pos	0	chronic no treatment	
E11-00158	14/02/1957	1	pos	REL 02	NR	
E11-00195	21/02/1950	1	pos	0	chronic no treatment	

E11-00205	19/05/1950	1	pos	REL 07	NR
E11-00335	18/03/1958	1	neg	SVR 98	SVR
E11-00374	02/10/1959	1	pos	REL 05	NR
E11-00375	17/01/1946	1	pos	NR 99	NR
E11-00400	12/06/1957	1	pos	REL 07	NR
E11-00424	10/05/1959	1	pos	NR 05	NR
E11-00525	30/10/1960	1	neg	SVR 07	SVR
E11-00596	04/09/1950	1	pos	0	chronic no treatment
E11-00623	17/05/1952	1	pos	0	chronic no treatment
E11-00745	02/12/1956	1	neg	SVR 2010	SVR
E11-00810	15/04/1958	1	pos	REL 08	NR
E11-00839	28/12/1950	1	pos	0	chronic no treatment
E11-00840	02/12/1952	1	pos	NR 00; 09	NR
E11-00891	03/11/1958	1	pos	REL 08	NR
E11-00892	17/10/1953	1	neg	SVR 06	SVR
E11-00917	18/11/1959	1	neg	SVR 01	SVR
E11-00918	28/08/1953	1	neg	SVR 02	SVR
E11-00991	15/09/1954	1	neg	SVR 07	SVR

E11-01032	23/03/1953	0	neg	0	spon res	
E11-01054	05/10/1953	1	pos	REL 98	NR	
E11-01055	17/05/1959	0	neg	0	spon res	
E11-01056	17/07/1958	1	pos	REL 00	NR	
E11-01068	27/04/1960	1	neg	SVR 02	SVR	
E11-01069	09/09/1961	1	pos	0	chronic no treatment	F3
E11-01101	08/02/1953	1	pos	0	chronic no treatment	
E11-01108	29/04/1957	1	neg	SVR 92	SVR	
E11-01126	14/10/1960	1	neg	SVR 04	SVR	F3
E11-01169	09/02/1957	1	pos	0	chronic no treatment	Ci
E11-01178	26/12/1956	1	pos	NR 03	NR	
E11-01193	22/08/1947	1	neg	SVR 07	SVR	
E11-01231	16/08/1959	1	neg	SVR 04	SVR	
E11-01260	05/01/1960	1	?	?	chronic no treatment	
E11-01350	13/06/1946	1	pos	NR 95	NR	
E11-01358	11/09/1958	0	neg			
E11-01368	21/09/1951	1	pos	REL	CHd	
E11-01392	20/01/1954	1	neg	SVR	SVR	

E11-01431	19/03/1955	0	neg	0	spon res	
E11-01472	29/10/1958	0	neg	0	spon res	
E11-01489	17/07/1954	0				
E11-01508	26/10/1944	1	neg	SVR		
E11-01515	10/02/1955	0	neg	0	spon res	
E11-01554	22/04/1952	1	neg	Therapy	SVR	
E11-01639	10/02/1958	1	pos	0	chronic no treatment	
E11-01657	10/02/1955	0	neg			
E11-01707	01/03/1953	1	pos	0		
E11-01805	17/12/1954	1	neg	SVR	SVR	
E11-02022	12/12/1961	1	pos	0	chronic no treatment	
E11-02287	21/03/1959	0	neg			
E11-02395	25/05/1957	1	pos	NR	NR	
E11-02558	05/11/1962	1	pos	0	chronic no treatment	Ci
E11-02896	17/02/1957	1	pos			
E11-02897	13/01/1951	1	pos	0	chronic no treatment	
E11-02898	05/02/1951	1	pos	0	chronic no treatment	
E11-02899	22/07/1953		0	0	0	spon res

E11-02900	21/05/1954	1	neg	SVR 05	SVR	
E11-02902	21/04/1956	1	pos	0	chronic no treatment	
E11-02903	27/10/1954	1	pos	0	chronic no treatment	
E11-02905	30/08/1951	0	neg	0	spon res	
E11-02906	03/11/1950	1	pos	NR 05	CH	
E11-02907	21/10/1954	1	pos	0	chronic no treatment	
E11-02908	25/08/1952	0	neg	0	spon res	
E11-02909	16/04/1963	1	neg	SVR 08	SVR	
E11-02910	26/06/1959	1	pos	REL 07	CH	
E11-02911	27/12/1953	1	pos	0	chronic no treatment	
E11-02912	11/11/1956	1	pos	0	chronic no treatment	Ci
E11-02913	16/07/1955	1	pos	NR	CH	
E11-02914	07/06/1950	1	pos	NR 02	CH	
E11-02915	06/11/1956	1	neg	SVR 02	SVR	
E11-02916	27/11/1957	1				
E11-02917	07/11/1959	1	pos	0	chronic no treatment	Ci
E11-02918	25/04/1954	0	neg	0	spon res	

Background information, including date of birth and primary outcome of HCV infection, is detailed (Spontaneous clearance: 0 and 2, chronic infection: 1). Details of viral load and current diagnosis is also provided. Treatment outcome is also detailed where applicable (CH: chronic hepatitis, SVR: sustained virological response, NR: non-responder). The presence of liver cirrhosis is also detailed where applicable.

Appendix Figure 3. HLA typing of the German Anti-D cohort of HCV infected patients.

ID	HLA-A1	HLA-A2	HLA-B1	HLA-B2
AD-001	2	24	51	0
AD-002	2	24	51	57
AD-003	1	11	8	35
AD-004	2	24	41	44
AD-005	3	25	27	0
AD-006	3	24	7	51
AD-007	2	23	44	73
AD-008	2	3	7	35
AD-009	1	25	18	57
AD-010	24	68	44	57
AD-011	2	3	13	51
AD-012	11	24	15	40
AD-013				
AD-014	1	11	8	0
AD-015	68	0	44	0
AD-016	1	24	8	35
AD-017	1	0	8	18
AD-018	2	3	8	50
AD-019	1	33	8	14
AD-020	29	32	40	44
AD-021	2	0	18	44
AD-022	2	3	27	57
AD-023	3	68	35	44
AD-024	1	11	8	35
AD-025	3	0	7	0
AD-026	1	2	35	44
AD-027	1	2	15	51
AD-028	3	0	7	51
AD-029	2	24	8	27
AD-030	1	32	35	51
AD-031	2	11	35	51
AD-032	1	31	39	44
AD-033	2	24	13	40
AD-034	2	24	15	18
AD-035	24	32	27	35
AD-036	2	0	57	0
AD-037	2	24	7	18
AD-038	1	2	8	39
AD-039	25	32	18	57
AD-040	2	3	15	27
AD-042	2	0	8	44
AD-043	2	3	35	57
AD-044	2	3	7	44
AD-045	3	66	40	41
AD-046	24	0	27	40
AD-048	11	68	7	18
AD-049	2	3	27	51
AD-050	2	0	7	15
AD-051	2	3	18	40
AD-052	2	74	7	35
AD-053	1	2	8	35

AD-054	1	29	7	8
AD-055	2	29	18	44
AD-056	3	23	7	44
AD-057	2	29	38	44
AD-058	24	36	8	15
AD-059	2	24	44	51
AD-062	24	29	7	44
AD-063	1/2	2/36	40	57
AD-065	3	25	7	15
AD-066	30	31	13	51
AD-067				
AD-068	3	30	13	35
AD-069	26	68	40	44
AD-070	1	0	7	8
AD-071	2	32	44	55
AD-072	2	3	14	35
AD-074	1	30	14	0
AD-075	2	3	7	39
AD-076	1	2	35	44
AD-077	1	3	8	27
AD-079	2	29	41	44
AD-080	1	2	8	18
AD-081	1	11	8	35
AD-082	24	25	13	18
AD-083	1	3	40	51
AD-084	3	68	44	51
AD-085	26	33	39	58
AD-086	1/11	11/36	35	0
AD-087	1	11	8	52
AD-088	24	68	7	51
AD-089	2	30	13	55
AD-090	2	23	44	51
AD-091	2	3	27	35
AD-092	25	0	27	44
AD-093	11	26	7	38
AD-094	1	68	8	14
AD-095	1	2	15	57
AD-096	3	32	15	51
AD-097	3	31	7	44
AD-098	24	74	7	44
AD-099	2	33	14	58
AD-100	2	0	27	40
AD-101				
AD-102	2	0	15	44
AD-103	2	31	15	40
AD-104	2	24	38	39
AD-105	2	3	7	18
AD-106	2	32	7	44
AD-107	24	26	38	40
AD-108	1	3	15	49
AD-109	1	3	37	44
AD-110	1	2	7	35
AD-111	11	24	35	0
AD-112	23	32	40	49/50
AD-113	3	0	14	15
AD-114	2	0	15	18

AD-115	2	32	7	27
AD-116	3	31	7	51
AD-117	2	0	15	40
AD-118	1	26	8	38
AD-119	3	0	7	35
AD-120	2	29	13	44
AD-121	3	31	7	27
AD-122				
AD-123	2	24	44	51
AD-124	24	0	18	40
AD-125	1	3	8	35
AD-126	1	0	27	44
AD-127	11	33	44	58
AD-128	3	24	7	49
AD-129	1	3	8	27
AD-130	3	11	8	13
AD-131	2	26	13	44
AD-132				
AD-133	2	3	35	52
AD-134	2	0	7	15
AD-135	2	3	40	57
AD-136	3/25	32/66	18	0
AD-137	2	26	44/49	44/50
AD-138	2	29	44	0
AD-139				
AD-140	1	30	13	57
AD-141	1	3	8	35
AD-142	1	2	8	35
AD-143	31	0	18	40
AD-144	2	0	7	40
AD-145	1	2	44	51
AD-146	24	68	7	55
AD-147	1/2	2/36	57	0
AD-148	2	0	51	0
AD-149	3	11	35	0
AD-150	3	24	15	51
AD-151	2	3	15	18
AD-152	2	0	27	40
AD-153	3	24	7	35
AD-154	1	68	8	44
AD-155	3	0	7	0
AD-156	2	0	15	57
AD-157	3	11	18	51
AD-158	2	25	44	0
AD-159	2	31	40	44
AD-160	2	24	15	27
AD-161	29	31	18	44
AD-162	2	0	15	35
AD-163	3	26	40/55	40/56
AD-164	2	26	35	49
AD-165	1	25	8/51	8/78
AD-166	2	24	15	44
AD-167	24	0	40	44
AD-168	2	29	44	0
AD-169	2	24	35	40
AD-170	25/32	26/32	40	44

AD-171	2	3	7	44
AD-172	3	23	7	49
AD-173	1	26	8	44
AD-174	1	3	7	8
AD-175	2	23	44	0
AD-176	2	29	7	44
AD-177	3	26	27	38
AD-178	1	2	15	35
AD-179				
AD-180	2	24	38	44
AD-181				
AD-182				
AD-183	1	0	7	18
AD-184				
AD-185				
AD-186				
AD-187				
AD-188				
AD-189				
AD-190				
AD-191	1	1	8	35
AD-192	3	30	13	57
AD-193	3	68	51	55
AD-194	1	24	15	35
AD-195	1	24	15/62	35
AD-196	2	3	7	39
AD-197	1	2	15	40
AD-198	3	23	35	44
AD-199	24	32	18	44
AD-200	2	24	7	27
AD-201	1	2	8	51
AD-202	24	29	35	58
AD-203	32	68	40	44
AD-204	1	2	8	57
AD-205	1	3	7	8
AD-206	3	23	44	51
AD-207	03	0	07	15
AD-208	1	68	8	15
AD-209	1	24	49	51
AD-210	3	24	35	39
AD-211	3	23	35	44
AD-212	2	24	8	38
AD-213	2	0	7	15
AD-214	1	2	41	44
AD-215	3	24	51	55
AD-216	2	0	44	0
AD-217	1	26	8	45
AD-218	2	0	7	15
AD-219	1		8	
AD-219	3	29	27	44
AD-220	24	30	18	0
AD-221	2	3	15	51
AD-222	3	0	7	27
AD-223	3	32	35	40
AD-224	1	23	8	0
AD-225	1	32	8	40

AD-26	1	2	7	37
AD-27	3	25	7	18
AD-28	2	11	44	56
AD-29	11	68	14	35
AD-30	24	26	38	40
AD-31	2	11	40	52
AD-32	1	24	8	55
AD-33				
AD-34	2	24	15	50
AD-35	1	2	8	50
AD-36	3	32	27	40
AD-37	3	26	44	
AD-38	2	24	7	18
AD-39	1	1	8	35
AD-40	24	66	7	40
AD-41	2	11	44	
AD-42	2	0	13	40
AD-43	1	11	7	8
AD-44	2	0	51	40
AD-45	25	26	18	44
AD-46	31	74	40	55
AD-47	2	0	7	18
AD-48	2	29	44	
AD-49	11	30	7	13
AD-50	1	30	8	18
AD-51	1	31	37	57
AD-52	2	11	15	27
AD-53	24	32	7	35
AD-54	2	0	7	44
AD-55	1	29	8	15
AD-56	2	0	27	41
AD-57	3	0	7	35
AD-58	1	2	8	15
AD-59	1	26	35	55
AD-60	3	29	7	44
AD-61	24	29	44	0
AD-62	2	68	15	40
AD-63	2	26	7	55
AD-64	2	0	44	57
AD-65	24	68	40	44
AD-66	3	24	7	42
AD-67	3	24	14	15
AD-68	2	0	27	38
AD-69	2	0	40	44
AD-70	2	3	35	57
AD-71				
AD-72	2	26	40	57
AD-73	11	32	51	52
AD-74	1	3	7	42
AD-75	2	29	44	0
AD-76	30	31	40	51
AD-77	1	0	8	0
AD-78	2	3	8	39
AD-79	2	68	18	38
AD-80	2	3	7	35
AD-81	24	26	27	35

AD-282	1/2	2/36	8/38	8/39
AD-283	24	68	8	40
AD-284	24	30	40	51
AD-285				
AD-286	2	0	13	15
AD-287	2	24	35	0
AD-288	2	0	15	44
AD-289	1	23	8	35
AD-290	2	3	15	35
AD-291	1	0	15	55
AD-292	2	0	35	40
AD-293	31	68	40	0
AD-294	2	24	13	37
AD-295	1	0	18	57
AD-296	34	68	14	44
AD-297	2	24	15	44
AD-298	1	2	15	57
AD-299	2	0	15	51
AD-300	1	2	8	40
AD-301	3	68	7	14
AD-302	1	3	8	35
AD-303	1	31	8	35
AD-304	23	32	15	44
AD-305	24	68	13	49
AD-307	24	68	35	44
AD-308	2	24	15	35
AD-309				
AD-310	11	1	8	40
AD-311	1	3	8	18
AD-312	1	23	35	55
AD-313	1	0	8	35
AD-314	3	24	51	56
AD-315	3	30	7	27
AD-316	1	0	35	57
AD-317	2	0	18	41
AD-318	2	3	52	56
AD-319				
AD-320	1	33	13	52
AD-321	1	29	51	55
AD-322	2	0	27	44
AD-323	2	33	14	56
AD-324				
AD-325	1	24	35	53
AD-326	2	29	38	44
AD-327	1	3	8	15
AD-328	1	3	7	35
AD-329	2	0	15	44
AD-330	24	31	40	51
AD-331	3	3	14	15
AD-332	1	24	8	35
AD-333	1	2	8	35
AD-334	1	25	15(62)	57
AD-335	2	0	7	27
AD-336				
AD-337	2	30	13	44

AD-338	29	30	18	44
AD-339	2	31	51	0
AD-340	3	68	35	44
AD-341	2	0	7	40
AD-342	3	11	14	39
AD-343	2	2	27	57
AD-344	3	29	44	51
AD-345	24	0	35	0
AD-346				
AD-347	2	0	44	57
AD-348	3	68	35	44
AD-349	2	11	7	40
AD-350	2	31	7	0
AD-351	1	23	8	49
AD-352	2	24	7	40
AD-353	1	2	8	57
AD-354	1	11	15	0
AD-355	2	33	14	41
AD-356	1	26	38	51
AD-357	3	26	7	44
AD-358	1	2	7	57
AD-359	2	24	49	57
AD-360	24	66	13	57
AD-361	2	24	27	40
AD-362	2	24	7	40
AD-363	1	32	8	18
AD-364	1	24	8	0
AD-365	25	23	44	51
AD-366	1	2	37	40
AD-367				
AD-368	2	30	15	57
AD-369				
AD-370	3	11	13	35
AD-371	2	24	14	51
AD-372	2	30	13	44
AD-373	2	24	27	51
AD-374	3	26	38	44
AD-375	30	68	13	35
AD-376	3	0	7	35
AD-377	2	29	35	44
AD-378	3	24	35	44
AD-379				
AD-380				
AD-381	2	26	44	51
AD-382	1	2	35	40
AD-383	3	36	13	57
AD-384	1	2	8	50
AD-385	2	0	13	15
AD-386	3	29	15	44
AD-387	2	0	8	15
AD-388				
AD-389	2	0	15	44
AD-390				
AD-391	1	2	8	0
AD-392	23	24	44	51
AD-393	31	0	56	57

AD-394	26	30	38	52
AD-395	3	26	7	0
AD-396				
AD-397				
AD-398	1	0	35	39
AD-399	24	33	14	15
AD-400	2	0	7	18
AD-401	2	31	7	35
AD-402				
AD-403	2	11	35	51
AD-404	2	0	27	40
AD-405	1	2	14	50
AD-406	24	26	7	38
AD-407	2	0	44	0
AD-408	2	68	44	49
AD-409				
AD-410	2	66	41	44
AD-411	2	32	8	27
AD-412	2	68	13	14
AD-413	3	68	14	35
AD-414	26	31	18	27
AD-416	11	29	41	55
AD-417	2	11	7	44
AD-418				
AD-419				
AD-420				
AD-421				
AD-422				
AD-423	2	3	35	40
AD-426				
AD-427				
AD-428				
AD-429				
AD-430				
AD-431				

Results of HLA-A (A1/A2) and HLA-B (B1/B2) typing of German Anti-D cohort are shown.