THE EFFECT OF LIVER TRANSPLANTATION ON NATURAL KILLER CELL FUNCTION

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Thesis submitted for PhD degree

DECLARATION OF ORIGINALITY

I declare that the content of this thesis is the result of my own work carried out between 2010 and 2014. The laboratory work, analysis, illustrations and written work were all performed by myself except where otherwise specified in the text.

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ABSTRACT

Liver transplantation (LT) is a definitive treatment for end-stage liver disease and hepatocellular cancer. As donor-recipient HLA matching is not employed, there is potential for natural killer (NK) cell-mediated alloreactivity. In practise this is not observed, and to date the recipient NK response to LT has not been characterized. This thesis describes a detailed investigation into LT recipient NK cell phenotype, function and activity.

Lymphocytes were extracted from peripheral blood samples taken from LT recipients. Expression of activating receptors on NK cells was assessed using flow cytometry assays, and NK function was analysed with functional assays. Recipient NK cells exhibited a downregulated phenotype with reduced expression of activating receptors NKp30 and NKp46. There was associated hypofunctionality, with impaired NK cell cytotoxicity, degranulation and IFNγ production. These changes were found only within the differentiated $CD56^{dim}$ subset, and not in $CD56^{bright}$ cells, implying a maturation defect between the two stages of development. Similar findings were not observed in LT recipients infected with hepatitis C virus (HCV), indicating a counteracting activating effect of HCV.

To explore a mechanism for the downregulation observed a microarray experiment was performed, with subsequent qPCR analysis. In LT there was downregulation of STAT-4 with associated reduction in miR-155, a microRNA target of STAT-4 and a key regulator of NK differentiation. Functional assays demonstrated impairment of the IL-12/STAT-4 pathway in LT recipients. The altered NK phenotype and gene expression were not recapitulated *in vitro* with immunosuppressants. The influence of HLA mismatching on LT outcome remains controversial, and this was addressed by analysing donor HLA and recipient HLA and KIR typing. No associations between HLA and HLA/KIR mismatching, NK cell activation, and clinical outcomes were found.

Taken together, the data presented in this thesis indicate that LT induces recipient NK cell tolerance through altered peripheral maturation at a step prior to the acquisition of inhibitory receptors for HLA class I. Consistent with this, HLA-C matching does not influence NK alloreactivity in LT.

ACKNOWLEDGEMENTS

I am deeply grateful to Salim Khakoo for his endless support and supervision throughout this PhD. The encouragement, understanding and guidance I have received have made the experience a thoroughly enjoyable and rewarding one. I am also fortunate to have worked with some gifted colleagues in the laboratory whose advice and friendship have been much appreciated. In particular my thanks go to Marco Purbhoo, Kip Cheent, Lemonica Koumbi, Sorcha Cassidy and Mina-Olga Aletrari who have all helped me along the way and with whom I have made good friends. The many helpful discussions we have had in my hours of need were invaluable.

I thank my collaborators Dr Michael Heneghan at Kings College Hospital, and Dr Graeme Alexander at Addenbrookes Hospital for facilitating my access to the patients and databases that have been essential to this work. I am also grateful to Dr Trowsdale and Dr Traherene at Cambridge University, and Dr Ann-Margaret Little at Gartnavel Hospital in Glasgow for their help and expertise with the patient genetic typing.

I extend my sincere thanks to the patients for their generosity in donating their time and blood samples in order for me to carry out this study. None of the work presented here would have been possible without their kindness.

I am extremely grateful to the Wellcome Trust for affording me the opportunity to carry out this work, and for the excellent support offered through the structure of the fellowship programs.

This thesis is dedicated to my wife and our daughters. Clare has been loving and supportive throughout, and there is no doubt I would not have completed this without her. Sara and Anya are the joy of my life and put everything in context. Thank you.

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LIST OF ABBREVIATIONS

^{51}Cr	^{51}Chromium
6MP	6-Mercaptopurine
ACR	Acute Cellular Rejection
ADCC	Antibody-Dependent Cell Cytotoxicity
AICL	Activation-Induced C-Type Lectin
AIH	Autoimmune Hepatitis
ALD	Alcohol-Related Liver Disease
ALF	Acute Liver Failure
AML	Acute Myeloid Leukaemia
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
AST	Aspartate Transaminase
BAT3	B-Associated Transcript 3
BSA	Bovine Serum Albumin
CD	Crohn's Disease
cDNA	Complimentary Deoxyribonucleic Acid
CDR	Complimentary Determining Region
CMV	Cytomegalovirus
CO_2	Carbon Dioxide
C_T	Threshold Cycle
CTO	Celltracker™ Orange
CysA	Cyclosporine A
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNAM-1	DNAX Accessory Molecule-1
E:T	Effector:Target
ELISA	Enzyme-Linked Immmunosorbant Assay
ERK	Extracellular Signal-Regulated Kinase
FCS	Fetal Calf Serum

FDR	False Discovery Rate
FES	Feature Extraction Software
FK506	Tacrolimus
FLK2	Fetal Liver Kinase 2 Ligand
GDC	Genomic DNA Control
GM-CSF	Granulocyte Colony Stimulating Factor
GOI	Gene(s) Of Interest
GvHD	Graft Versus Host Disease
HA	Haemagglutinin
HBV	Hepatitis B Virus
HC	Healthy Control(s)
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
HS	Heparin Sulphate
HSC	Haematopoietic Stem Cell
HSCT	Haematopoietic Stem Cell Transplantation
ICAM	Intracellular Adhesion Molecule
IFNγ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
ILS	Institute Of Liver Studies
ILT	Immunoglobulin-like Transcripts
INR	International Normalised Ratio
IPA	Ingenuity Pathway Analysis
IPKB	Ingenuity Pathways Knowledge Base
ITAM	Immunoreceptor Tyrosine-Based Activation Motifs
ITIM	Immunoreceptor Tyrosine-Based Inhibitory Motif
JAK	Janus Kinase
KIR	Killer Immunoglobulin Ig-Like Receptor(s)
KLRG1	Killer Cell Lectin-Like Receptor G1
LAMP	Lysosomal-Associated Membrane Protein

LCMV	Murine Lymphocytic Choriomeningitis Virus
LFA-1	Lymphocyte Adhesion Molecule-1
LILR	Leukocyte Immunoglobulin-Like Receptor
LLT1	Lectin-like Transcript 1
LRC	Leucocyte Receptor Complex
LSEC	Liver Sinusoidal Epithelial Cell
LT	Liver Transplant
LT HCV	LT Recipients with HCV infection
LT non-HCV	LT Recipients without HCV infection
Mϕ	Macrophage
MAPK	Mitogen Activated Protein Kinase
MELD	Model Of End Stage Liver Disease
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIC	MHC Class I Chain-Related Protein
miR	Micro RNA
MLL5	Mixed Lineage Leukaemia-5
MMF	Mycophenylate Mofetil
NASH	Non-Alcoholic Steatohepatitis
NCAM	Neural Cell Adhesion Molecule
NCR	Natural Cytotoxicity Receptor
NK	Natural Killer
PBC	Primary Biliary Cirrhosis
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PILR	Paired Ig-Like Type 2 Receptor
PILRβ	Paired Ig-Like 2 Receptor β
PKCθ	Protein Kinase C-θ
PPC	Positive PCR Control
Pred	Prednisolone
PSC	Primary Sclerosing Cholangitis
PVR	Polio Virus Receptor

qRT-PCR	Quantitative Real Time Reverse Transcription Polymerase Chain Reaction
RIN	RNA Intergrity Number
RNA	Ribonucleic Acid
RTC	Reverse Transcription Control
SD	Standard Deviation
SHP	Src Homology-Containing Tyrosine Phosphatases
Sir	Sirolimus
SLT	Secondary Lymphoid Tissue
STAT	Signal Transducers And Activators Of Transcription
TC	T Cell
TCR	T Cell Receptor
TNFα	Tumour Necrosis Factor-α
UKELD	UK End Stage Liver Disease
ΔΔ CT	Delta-Delta C_T

CHAPTER 1: INTRODUCTION

1.1 Natural Killer Cells

1.1.1 Discovery and characterization

The innate arm of the immune system recruits humoral and cellular components with the ability to respond rapidly to cell stress and injury. These include a subset of lymphocytes that, unlike the T-cells and B-cells of adaptive immunity, do not require immune sensitization to carry out their functions. This distinguishing feature, which was first described in 1975, led to the term "natural killer" (NK) cells (Herberman, Nunn et al. 1975, Kiessling, Klein et al. 1975). Human NK cells are traditionally characterised by the absence of CD3 expression (therefore excluding T-cells) and expression of the surface marker CD56, an isoform of neural cell adhesion molecule (NCAM) (Ritz, Schmidt et al. 1988, Lanier, Testi et al. 1989). NK cells are present in non-human mammals, with orthologs in other vertebrates, and this conservation across species suggests a fundamental role in the host immunological response (Jansen, van de Haar et al. 2010). Because of their ancient lineage and lack of antigen specificity, NK cells were once thought primitive and functionally less sophisticated than their T- and B-cell cousins from the adaptive arm of the immune system. However, the last 3 decades have seen substantial advances in our understanding of NK cell biology, shedding light on the complexities of repertoire diversity, finely tuned systems controlling self-tolerance and activation, and key interactions between NK cells and adaptive immune cells. Indeed there is now accumulating evidence that NK cells exhibit properties of

adaptive immunity including longevity and immunological memory, analogous to features of T cell development (O'Leary, Goodarzi et al. 2006, Sun and Lanier 2011). However, several issues remain unresolved, particularly related to NK cell development and education, further underlining the intricate nature of these key effector cells.

1.1.2 Circulating natural killer cell subsets

NK cells can broadly be divided into two phenotypically and functionally distinct subsets, according to the density of CD56 expression (**figure 1.1**). Those expressing a high density of CD56 (termed $CD56^{bright}$) comprise approximately 10% of circulating NK cells, and up to 100% of NK cells found in secondary lymphoid tissues (SLTs). These $CD56^{bright}$ cells are abundant producers of cytokines, with limited ability to spontaneously lyse tumour targets (Cooper, Fehniger et al. 2001). Conversely NK cells with a low density of CD56 expression ($CD56^{dim}$ cells, comprising around 90% of circulating NK cells) are relatively weaker producers of cytokines upon stimulation, but exhibit potent cytotoxicity against target cells (Walzer, Jaeger et al. 2007). There is accumulating evidence that the two NK cell subsets represent different stages of NK cell maturation, such that $CD56^{bright}$ cells are thought to be precursors of $CD56^{dim}$ cells (Chan, Hong et al. 2007, Romagnani, Juelke et al. 2007, Takahashi, Kuranaga et al. 2007, Domaica, Fuertes et al. 2012).

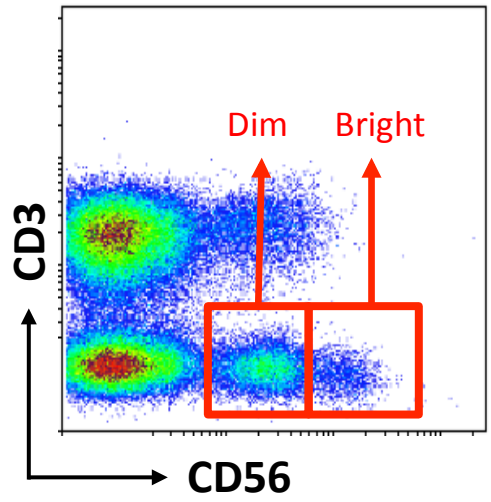


Figure 1.1 NK cell immunophenotype.
Representative flow cytometry plot showing NK cells and subsets when gating on peripheral blood lymphocytes. NK cells are characterized as CD3⁻ and CD56⁺. Within the NK cell population 2 distinct subsets can clearly be distinguished: CD56^{dim} (left-hand red box) and CD56^{bright} (right-hand red box).

NK cells can be further subdivided on the basis of CD16 (FcγRIIIA) expression so that overall five NK populations have been defined (Poli, Michel et al. 2009): 1) CD56^{dim}CD16^{-} 2) CD56^{dim}CD16^{bright} 3) CD56^{-}CD16^{bright} 4) CD56^{bright}CD16^{-} and 5) CD56^{bright}CD16^{dim}. Population 2 comprises the majority of CD56^{dim} NK cells which exhibit high effector functionality, whilst the roles of populations 1 and 3 are unclear.

1.1.3 Multifunctional immune cells

NK cells perform a pivotal role in the immune response, including direct killing of target cells, and production of important cytokine and chemokine factors that have a broader effect, influencing T-cell and antigen presenting cell (APC) responses (**figure 1.2**).

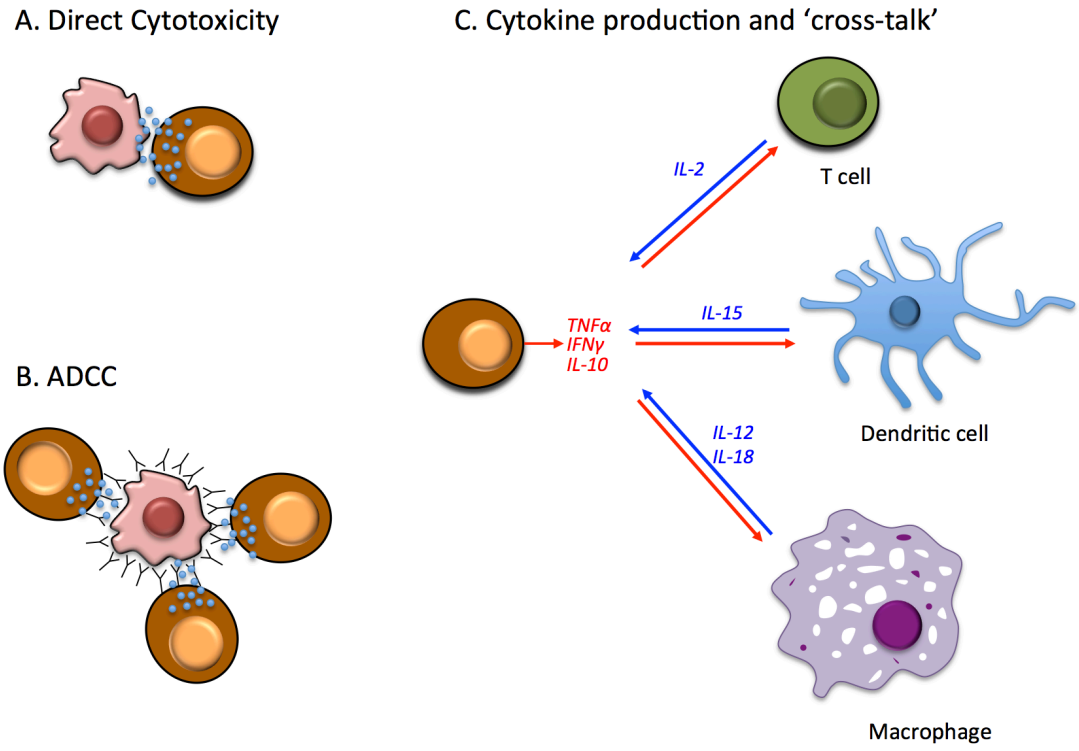


Figure 1.2 Functions of NK cells.

A *Once activated, NK cells can kill target cells directly through exocytosis of preformed granules containing perforin and granzyme.* ***B*** *NK cells express the Fc receptor and therefore also have the ability to perform antibody-directed cell cytotoxicity.* ***C*** *Additionally NK cells are producers of important cytokines and chemokines, influencing the T-cell response and APC recruitment and activation, including DC maturation. In turn, factors secreted by these cells promote NK cell activation, proliferation and maturation.*

Activated NK cells are potent killers with a similar mechanism of cytotoxicity to $CD8^+$ cytotoxic T-cells. Upon engagement with target cells, NK cells form an immune synapse, which is stabalised by the interactions of integrins (eg. LFA-1) with ligands on target cells (eg. ICAM-1) (Barber and Long 2003, Bryceson, March et al. 2005). This is followed by polarization of secretory lysosomes containing granzymes, perforin and Fas ligand (Trapani and Smyth 2002), and finally degranulation (exocytosis of the secretory granules). Degranulation is calcium (Ca^{2+})-dependent as evidenced by studies demonstrating defective NK cell degranulation in phospholipase C-γ-deficient mice, which have inhibited

intracellular Ca^{2+} mobilization (Caraux, Kim et al. 2006). Killing by NK cells is also achieved indirectly through antibody-dependent cell cytotoxicity (ADCC). The majority of $CD56^{dim}$ NK cells express CD16, which binds to the Fc portion of IgG antibodies and therefore antibody coated target cells trigger CD16-mediated NK activation and degranulation (Bryceson, March et al. 2005).

NK cells are not only cytolytic effector cells, but are also potent producers of cytokines, particularly interferon-γ (IFN-γ). Through IFN-γ, NK cells direct a Th1 immune response, activate and promote dendritic cell (DC) maturation, and upregulate major histocompatibility complex (MHC) class I expression (Moretta, Marcenaro et al. 2005, Vivier, Raulet et al. 2011). It is therefore logical that the cytokine-producing $CD56^{bright}$ subset predominates in SLTs, where NK cells are in close proximity to DCs and T-cells. Many other proinflammatory factors are secreted by NK cells including tumour necrosis factor-α (TNF-α), granulocyte colony stimulating factor (GM-CSF), interleukin (IL)-3, IL-8, CCL2, CCL3, CCL4, CCL5 and CXCL1 (Walzer, Dalod et al. 2005). NK cells can also play an immunoregulatory role through secretion of IL-10 and it is thought that direct killing of target cells may regulate T-cell responses by decreasing antigenic load (Vivier, Raulet et al. 2011). Furthermore, the influence of NK cells on virus specific T-cell responses has been investigated in murine lymphocytic choriomeningitis virus (LCMV) infection (a model for human hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infections) and in human chronic hepatitis B virus (HBV) infection (Waggoner, Cornberg et al. 2012, Peppa, Gill et al. 2013). These studies provide accumulating evidence that NK

cells can negatively regulate antiviral immunity by acting as rheostats for virus specific T-cell responses.

In addition to production of factors influencing other components of both innate and adaptive immunity, NK cells in turn rely on stimulation or priming by other cellular constituents of the immune response. For example, IL-12, IL-15 and IL-18 produced by macrophages and dendritic cells, are required for NK cell activation, proliferation and survival (Lucas, Schachterle et al. 2007, Guia, Cognet et al. 2008, Mortier, Advincula et al. 2009). Thus an important feature of NK cell functionality is "cross-talk" with other players in the innate and adaptive immune response.

1.1.4 NK cell receptors

NK cells do not exhibit the exquisite antigen specificity that dominates T and B cell function. Instead, NK cell activation is controlled by signals transduced by surface activating and inhibitory receptors. The balance of these signals determines whether an NK cell remains in an inhibitory state, or becomes activated. In health, the inhibitory signals dominate so that NK cells are tolerant toward self. This is achieved because the majority of NK inhibitory receptors bind to MHC class I ligands, which are expressed by all cells. As shall be explained in Section 1.1.9, the NK cell maturation process ensures that the majority of functional NK cells express an inhibitory receptor for self MHC class I. The importance of MHC and its role in alloreactivity is discussed in Section 1.2.

A useful way of classifying the many different activating and inhibitory NK receptors is according to MHC specificity, which is a key determinant of influence on NK cell development and function (**tables 1.1 and 1.2**)

1.1.4.1 MHC specific inhibitory receptors

In humans, two main families of inhibitory receptors engage MHC ligands, the inhibitory killer immunoglobulin (Ig)-like receptors, (KIR) and the C-type lectin like receptor CD94-NKG2A.

1. KIR: this polymorphic family comprises both activating and inhibitory members. KIR belong to the Ig-superfamily and nomenclature has been standardized based on structure (Andre, Biassoni et al. 2001), which consists of a type 1 transmembrane glycoprotein with two or three extra-cellular Ig-like domains (2D or 3D), and either a long (L) or short (S) cytoplasmic tail (**figure 1.3**). Thus KIR2DL* refers to inhibitory KIR with 2 extracellular Ig-like domains and a long cytoplasmic tail; receptors with differing amino acid sequences in key positions that effect ligand binding are suffixed with 1, 2, etc. (e.g. KIR2DL1, KIR2DL2, KIR2DL3).

Inhibitory receptors have long cytoplasmic tails that contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Receptor engagement with its ligand leads to ITIM phosphorylation and recruitment of Src homology-containing tyrosine phosphatases (SHP) with resultant downstream negative signaling. KIR2DL4 is an exception in that, although its cytoplasmic tail contains an ITIM, cross-linking of this receptor activates NK cells (Faure and Long 2002, Yusa,

Catina et al. 2002). This is achieved because KIR2DL4 associates with a membrane bound adaptor protein (FcεR1) containing activating motifs (see activating receptors below) (Kikuchi-Maki, Catina et al. 2005).

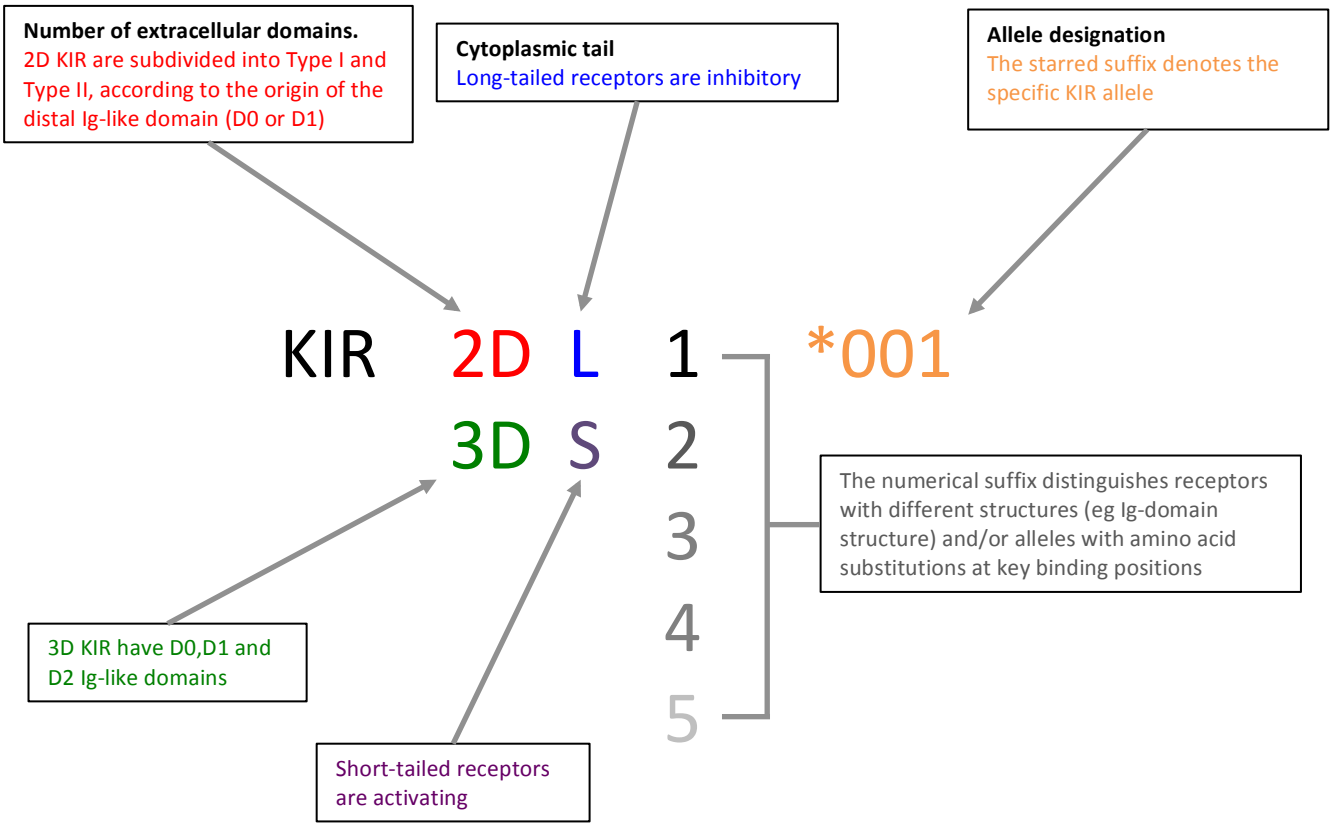


Figure 1.3 KIR nomenclature.

KIR are named according to structural features that are fundamental to their function.

The MHC class I ligands for inhibitory KIR have largely been defined (**table 1.1**), but KIR-ligand associations are by no means straightforward. Genetic and binding studies have demonstrated a high degree of polymorphism in both KIR and their ligands, which have significant effects on KIR-ligand interactions. Furthermore, it has been demonstrated that the peptide bound within the MHC groove influences the immune synapse and receptor signaling, uncovering a

further layer of complexity within the system (Malnati, Peruzzi et al. 1995, Fadda, Borhis et al. 2010). Whilst KIR are found in humans and other primates, they are not present in other mammals. Interestingly, in mice the Ly49 C-type lectin-like receptors (which are structurally very distinct from KIR) serve a very similar purpose to KIR. Like KIR, there are both activating and inhibitory Ly49 receptors, which exhibit substantial polymorphism and intracellular signaling pathways between KIR and Ly49 are similar. This indicates convergent evolution, supported by evidence demonstrating that activating KIR and Ly49 have evolved from their respective ancestral inhibitory receptors (Abi-Rached and Parham 2005).

Polymorphism within the KIR-HLA system creates variability in NK responses between individuals and populations, and this is of particular importance in KIR2DL1/2/3 interactions with HLA-C. KIR2DL2 and KIR2DL3 principally bind HLA-C molecules with an asparagine at residue 80 of the α-1 helix (group 1 alleles, HLA-C1), whereas KIR2DL1 binds HLA-C molecules that have a lysine at residue 80 (group 2 alleles, HLA-C2)[1]. Furthermore binding studies have demonstrated differences in the avidity of these interactions (Winter, Gumperz et al. 1998), so that, for example, the KIR2DL2-HLA-C1 interaction is stronger than KIR2DL3-HLA-C1. This has significant consequences, as KIR-HLA combinations have been associated with the host response to pathogens, influencing outcomes in diseases such as HIV, HCV and malaria. For example individuals homozygous for KIR2DL3-HLAC1 have a higher rate of resolution of

[1] Recent data has shown that some allotypes of KIR2DL2/3 can bind HLA-C2, but the significance of these interactions is unclear (Moesta and Parham, 2012).

acute hepatitis C virus infection compared to other KIR2DL2/3-HLA-C1/C2 genotype combinations (Khakoo, Thio et al. 2004). This is postulated to be because KIR2DL3-HLAC1 is the weakest inhibitory interaction, and so NK inhibition is most easily overcome in these individuals, allowing more efficient NK cell-mediated viral clearance (Parham 2004).

Table 1.1 NK inhibitory receptors and ligands.

Receptor	**Ligand**
MHC specific	
KIR2DL1	HLA-C2
KIR2DL2	HLA-C1/C2
KIR2DL3	HLA-C1/C2
KIR2DL5	?
KIR3DL1	HLA-Bw4
KIR3DL2	HLA-A3, -A11
CD94-NKG2A	HLA-E
LILR	Classical and non-classical MHC-I, UL18
Non-MHC specific	
2B4	CD48
KLRG1	Cadherins
CD161	LLT1

HLA – human leucocyte antigen; LILR – leukocyte immunoglobulin-like receptor; KLRG1 – killer cell lectin-like receptor G1; LLT1 – Lectin-like transcript 1

2. CD94-NKG2A: The other main MHC-specific inhibitory receptor is formed from a heterodimer between CD94 and NKG2A. CD94 is a 70kD disulfide bonded dimeric protein first described in 1990 (Aramburu, Balboa et al. 1990), that covalently associates with members of the C-type lectin-like receptor family NKG2* (Lazetic, Chang et al. 1996, Brooks, Posch et al. 1997). The NKG2* subunit determines whether the heterodimer transduces activating or inhibitory signals. Thus CD94-NKG2A is an inhibitory receptor because it utilizes the same cytoplasmic signaling apparatus as KIR (ie. via ITIMs and SHP) to transduce an inhibitory signal. Conversely because NKG2C and NKG2E contain activating motifs in their cytoplasmic tails, association with CD94 forms activating receptors (Lazetic, Chang et al. 1996). Human CD94-NKG2* bind to the non-classical MHC class I molecule HLA-E, which is ubiquitously expressed and relatively non-polymorphic (the ligand for CD94-NKG2* in mice is $Qa1^b$). Therefore in humans the CD94-NKG2A/HLA-E system is relatively conserved and, compared with KIR, does not contribute significantly to variability in NK cell responses.

Leukocyte immunoglobulin-like receptors (LILR, also known as immunoglobulin-like transcripts, ILT) are inhibitory receptors that bind classical and non-classical MHC class I. However their role in NK cell regulation is unclear as inhibitory KIR and NKG2A transmit much stronger inhibitory signaling (Navarro, Llano et al. 1999). LILR have been shown to bind UL18, an MHC class I mimic encoded by cytomegalovirus (CMV), which can actually increase NK cell induced cytolysis (Leong, Chapman et al. 1998).

1.1.4.2 Non-MHC specific inhibitory receptors

The MHC-specific inhibitory receptors described above are important in NK cell development, 'education' and self-tolerance. Some non–MHC specific inhibitory receptors have also been described. The 2B4 receptor is present on approximately 50% of NK cells and has dual activating and inhibitory roles depending upon interactions with adaptor proteins (Garni-Wagner, Purohit et al. 1993, McNerney, Lee et al. 2005). The ligand for 2B4 is CD48, and in mice two isoforms exist with different cytoplasmic domains that signal either activation or inhibition (Schatzle, Sheu et al. 1999). In humans 2B4 has been shown to be involved in the immune response to CD48-expressing melanoma cells, but its role in other pathology is not known (Vaidya, Stepp et al. 2005).

Killer cell lectin-like receptor G1 (KLRG1) is another inhibitory receptor that binds cadherins which are expressed on healthy cells, and therefore may play a role in inhibiting NK activity against healthy tissue (Colonna 2006). CD161 is a C-type lectin-like receptor that binds lectin-like transcript 1 (LLT1) which is expressed on activated plasmacytoid cells, DCs and activated B cells (Rosen, Cao et al. 2008). CD161/LLT1 interaction has been shown to inhibit NK cytotoxicity and IFNγ production, and may be important in the immune response to pathogens (Germain, Meier et al. 2011).

1.1.4.3 MHC specific activating receptors

Activating receptors are key to NK cell functionality. In contrast to the inhibitory receptors, most NK activating receptors transduce signals by association with membrane bound adaptor molecules, including DAP10 which facilitates PI3-

kinase activation and DAP12, CD3ζ and FcεRIγ which contain cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) (Joyce and Sun 2011). Ligation of the ITAMs leads to recruitment and activation of tyrosine kinases Syk and ZAP70 and results in downstream events including elevation of intracellular Ca^{2+} levels, and activation of mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK). Ultimately this signal cascade leads to initiation of NK cell-mediated cytotoxicity and production of cytokines including IFNγ (Lanier 2003).

Like the inhibitory receptors, activating receptors may be categorized according to MHC specificity, although the ligands are less well defined than for the inhibitory NK receptors (**table 1.2**). In contrast to the inhibitory NK receptors, the MHC specific activating receptors play a less prominent role in NK function.

Activating KIR possess short cytoplasmic tails and, due to the homology with inhibitory KIR, are thought to bind MHC class I molecules. However, few studies have been able to demonstrate this binding, and the ligands for several activating KIR remain elusive (see **table 1.2**). Direct binding of KIR2DS1 to HLA-C2 has been demonstrated (Biassoni, Pessino et al. 1997, Stewart, Laugier-Anfossi et al. 2005), but the strength of this interaction is weak compared with KIR2DL1:HLA-C2. More recently KIR2DS2 has been shown to recognize HLA-A11 (Liu, Xiao et al. 2014), but the significance of the activating KIR:HLA interactions is uncertain, and the function of activating KIR is unresolved.

NKG2C and NKG2E form heterodimers with CD94, and like CD94-NKG2A, recognize HLA-E. Recent studies have focused particularly on NKG2C expansion as a marker of a 'memory' NK cell phenotype in viral infections and may be indicative of a terminally differentiated or more mature/antigen experienced phenotype (Guma, Budt et al. 2006, Bjorkstrom, Lindgren et al. 2011, Malmberg, Beziat et al. 2012).

1.1.4.4 Non-MHC specific activating receptors

The natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 are highly influential in NK activation as demonstrated by cross-linking and blocking studies (Sivori, Vitale et al. 1997, Pende, Parolini et al. 1999). These receptors play a pivotal role in the NK response to tumour transformation and viral infection (Moretta, Bottino et al. 2001). NCRs belong to the Ig superfamily, and although viral haemagglutinins have been identified as ligands, cellular ligands for the NCRs have not been fully elucidated. Molecules that have been reported to interact with NKp30 include a CMV tegument protein pp65, leucocyte antigen-B-associated transcript 3 (BAT3) and heparin sulphate (Arnon, Achdout et al. 2005, Pogge von Strandmann, Simhadri et al. 2007, Hecht, Rosental et al. 2009), but the physiological significance of these is controversial.

More recently, the crystal structure of NKp30 has been determined, and a functional binding site for B7-H6 (a B7 family homolog that is expressed on several tumour cell lines and primary cancer cells) has been ascertained (Joyce, Tran et al. 2011); thus to-date B7-H6 is the most robust NKp30 ligand identified.

Table 1.2 Activating receptors and their ligands.

Receptor	Ligand
MHC specific	
KIR2DL4	HLA-G
KIR2DS1	HLA-C2
KIR2DS2	HLA-A11 ?HLA-C1
KIR2DS3	?
KIR2DS4	?
KIR2DS5	?
KIR3DS1	HLA-Bw4
CD94-NKG2C	HLA-E
CD94-NKG2E	HLA-E
Non-MHC specific	
NKG2D	MIC-A/B, ULBP1-4
NKp30	?Viral HA, B7-H6, BAT3, pp65, HS
NKp44	MLL5, ?Viral HA, flavivirus E-protein, HS
NKp46	?Viral HA
NKp80	AICL
2B4	CD48
DNAM-1	PVR, CD122
PILR	CD99

HA: haemagglutinin; HS: heparin sulphate; MLL5: mixed lineage leukaemia-5; AICL: activation-induced C-type lectin; DNAM-1: DNAX accessory molecule-1; PVR: polio virus receptor; PILR: paired Ig-like type 2 receptor

NKp30 and NKp46 are expressed on all resting and activated NK cells, but NKp44 is only significantly expressed on some NK cells upon stimulation with IL-2 (Fuchs, Cella et al. 2005). A ligand for NKp44 has recently been characterized

as an unusual isoform of the mixed lineage leukaemia-5 (MLL5) protein (Baychelier, Sennepin et al. 2013). Deletion of any one NCR reduces NK cell killing ability against tumour targets, highlighting their importance in NK cell effector function (Pegram, Andrews et al. 2011). Furthermore, it has been demonstrated that NKp30 has a central role in NK-dendritic cell interactions, which lead to DC apoptosis and maturation (Vitale, Della Chiesa et al. 2005).

NKG2D is an activating receptor expressed on both mouse and human NK cells. This receptor is related to the NKG2A/C family and is expressed as a homodimer, rather than forming a heterodimer with CD94 (Bauer, Groh et al. 1999). In humans several ligands for NKG2D have been characterized including MHC class I chain-related proteins A and B (MICA and MICB), and ULBP1, 2, 3 and 4. The expression of these proteins is stress-induced and therefore upregulated by damage or malignant transformation (Cosman, Mullberg et al. 2001, Steinle, Li et al. 2001). MIC gene expression is also reported to be related to heat shock response pathways, which are activated in tumours. Mouse data demonstrates that NKG2D deletion is associated with rapid lymphoma progression (Guerra, Tan et al. 2008). Therefore NKG2D plays an important role in tumour surveillance.

DNAM-1 is a co-stimulatory activating receptor expressed on around 50% of NK cells. It is an Ig-superfamily member and ligands include CD155 and CD112, which have been shown to be upregulated in some tumours (eg multiple myeloma) (Bottino, Castriconi et al. 2003, Soriani, Zingoni et al. 2009). DNAM-1 has also been shown to be involved in lysis of tumour cells not expressing other

NK activating receptor ligands, thus providing alternative mechanisms for NK activation. It is thought DNAM-1 plays an important role in stabalising the immune synapse between NK cells and their targets, as it associates with lymphocyte adhesion molecule-1 (LFA-1) and binds intracellular adhesion molecule (ICAM) (Shibuya, Campbell et al. 1996, Shibuya, Lanier et al. 1999). Other co-stimulatory receptors include NKR-P1 which can contain ITIMs and can also associate with ITAM containing adaptors and therefore may have a dual role depending on the situation (Arase, Arase et al. 1997, Carlyle, Martin et al. 1999) and paired Ig-like 2 receptor (PILβ), an activating receptor recognizing carbohydrate chains, thus potentially broadening NK cell recognition of target cells (Wang, Shiratori et al. 2008).

1.1.5 Diversity of receptor repertoire

Not all NK cells are the same. MHC specific receptor genes are expressed stochastically, and a key feature of NK cells is the variability in receptor expression between clones. Within an individual each NK cell clone expresses a unique repertoire of activating and inhibitory receptors, with cognate ligands as described above (Raulet, Vance et al. 2001). This therefore leads to differences in activation threshold and reactivity between NK cell clones. Additionally, genetic polymorphism (particularly in KIR) results in a further layer of diversity in NK receptor repertoire between individuals, and between populations (Shilling, Guethlein et al. 2002, Yawata, Yawata et al. 2008).

1.1.6 NK cell development

NK cells arise from haematopoietic stem cells (HSCs) and it is thought that early NK development occurs in the bone marrow. This is supported by murine studies demonstrating abnormal NK cell development and function when the bone marrow was ablated or disrupted (Seaman, Blackman et al. 1978, Kumar, Ben-Ezra et al. 1979). Although the thymus is not required for NK cell development (normal NK cell development is observed in athymic mice (Su, Ishikawa et al. 1993)), NK cell populations expressing CD127 and GATA-3 have been reported in the thymus (Vosshenrich, Garcia-Ojeda et al. 2006). NK cell precursors can also be found in lymph nodes and the liver, but it is unclear whether these populations represent distinct NK cell lineages or are derived from circulating immature bone marrow cells (Sun and Lanier 2011).

Acquisition of NK specific markers characterizes NK cell differentiation from precursor cells, and several stages of NK cell development have been described (Colucci, Caligiuri et al. 2003). The common lymphoid progenitor has been identified as the precursor cell that gives rise to NK cells as well as T- and B- cells (Kondo, Weissman et al. 1997). $CD34^+$ HSCs require contact with stromal cells to generate NK cells, whilst the more differentiated $CD7^+CD34^+$ HSCs can give rise to NK cells under the influence of IL-2 alone (Miller, Alley et al. 1994). Later studies have shown that cytokines such as c-KIT ligand, fetal liver kinase 2 (FLK2) ligand and IL-7 can replace the stromal cell requirements (Mrozek, Anderson et al. 1996, Williams, Moore et al. 1997). Furthermore, *in vivo* studies have demonstrated that IL-15 is crucial for the development of NK cells from early HSCs (Lodolce, Boone et al. 1998, Kennedy, Glaccum et al. 2000).

In mice the earliest NK precursor is a non-stromal bone marrow cell expressing CD122 (β chain shared by the IL-2 and IL-15 receptors) (Di Santo 2006), which is not present on human $CD34^+$ cells. In humans the co-expression of CD34 and CD45RA has been shown to characterize pre-NK cells, although $CD34^+CD45RA^+$ cells also give rise to non-NK lineage cells including B cells, T cells and DCs (Blom and Spits 2006). *In vitro* data suggest that these early precursors lose expression of CD34, and develop into NK intermediates expressing CD161. These cells lack the ability to lyse NK–sensitive cell lines, but when incubated with Daudi cells in IL-2 and IL-12 can differentiate into fully functional $CD56^+$ NK cells (Bennett, Zatsepina et al. 1996). The transition from $CD34^-CD161^+CD56^-$ intermediates to $CD3^-CD56^+$ NK cells is not well understood, but the high proportion of $CD34^{dim}CD45RA^+$ HPCs found in SLTs is suggestive that these sites are important for NK cell development. Furthermore, SLTs are enriched with $CD56^{bright}$ NK cells, which are generally understood to be precursors of $CD56^{dim}$ cells. This is supported by the following observations: (i) *in vitro* culture of $CD34^+$ cells with IL-2 or IL-15 generates $CD56^{bright}$ cells (Lotzova, Savary et al. 1993, Mrozek, Anderson et al. 1996); (ii) after $CD34^+$ stem cell transplantation, circulating donor derived $CD56^{bright}$ cells appear before $CD56^{dim}$ NK cells and the appearance of $CD56^{dim}$ cells coincides with a relative decrease in $CD56^{bright}$ cells (Shilling, McQueen et al. 2003, Vitale, Chiossone et al. 2004). Freud and Caligiuri (2006) have described 4 functionally distinct $CD56^{bright}$ NK cell developmental stages using CD34, CD117 and CD94 markers with cells transitioning from stage 1 $CD34^+CD117^-CD94^-$ NK cell progenitors, to stage 2 $CD34^+CD117^+CD94^-$ pre NK cells, followed by stage 3 $CD34^-CD117^+CD94^-$ immature NK cells and finally

$CD34^{-}CD117^{+/-}CD94^{+}$ $CD56^{bright}$ cells. The characteristics of each developmental stage (including capacities for cytokine production, cytotoxicity, lineage differentiation potential, and gene expression profiles) are consistent with previous predicted characteristics of NK intermediates. As immature NK cells develop into stage 4 $CD56^{bright}$ cells, they acquire CD94-NKG2A conferring MHC-I dependent inhibition, as well as activating receptors NKp46 and NKG2D. Additionally, this stage of development sees the acquisition of intracellular perforin and the capacity to produce IFNγ.

The progression of $CD56^{bright}$ cells into functionally mature $CD56^{dim}$ cells is associated with the acquisition of CD16 and KIR and partial loss of CD94 (Cooper, Fehniger et al. 2001). Recent data has elucidated CD57 as a marker for NK cell terminal differentiation. CD57 is expressed more frequently on $CD56^{dim}$ cells that lack NKG2A and express multiple KIRs, and cells expressing CD57 show no evidence of recent proliferation (Bjorkstrom, Riese et al. 2010, Lopez-Verges, Milush et al. 2010).

1.1.7 Natural killer cell education

1.1.7.1 Missing self

Normal NK cell function is dependent on the ability of NK cells to differentiate between healthy cells and target cells (eg non-self, transformed or infected cells). This is achieved through the surface expression of inhibitory receptors for self MHC class I molecules, which maintain NK cells in an inhibitory state when the receptors and ligands interact. This is essential for self-tolerance, as healthy cells

express activating ligands for NK cells, so that the lack of inhibitory ligands alone is enough to activate NK cells. This was demonstrated in studies of bone marrow transplantation where donor cells lacking an MHC class I allele of the recipient were frequently rejected by NK cells (Ohlen, Kling et al. 1989).

As described above, the two main families of inhibitory receptors are the KIR (which bind HLA-A, B and C) and CD94-NKG2A (which bind HLA-E). HLA class I genes are encoded within the HLA cluster on chromosome 6 separate to KIR genes located within the leucocyte receptor complex (LRC) on chromosome 19 (Yokoyama and Plougastel 2003). Therefore inheritance of KIRs and HLA is unlinked, so that genetic mechanisms cannot ensure that NK cells within an individual express cognate inhibitory receptors for self-HLA ligands. Yet nearly all circulating NK cells do express inhibitory receptors for self, and those that do not are hypofunctional (Fernandez, Treiner et al. 2005). The term 'education' has been coined to describe the broad processes during NK cell development resulting in self-tolerance; 'licensing' is a term used to describe the acquisition of function, which in NK cells is thought to be an MHC class I dependent process (Elliott and Yokoyama 2011). The mechanisms underlying these processes have not yet been fully characterized, but several credible models have been proposed, supported by experimental evidence.

1.1.7.2 Models for the generation of self-tolerance

The *'at least one'* model proposes that during development the initially random repertoire of NK receptor expression is modified so that all mature NK cells express one or more inhibitory receptors for a self MHC class I molecule

(Valiante, Uhrberg et al. 1997). This could involve either a selection process where only NK cells expressing inhibitory receptors for self MHC class I are allowed to expand, or deletion of cells lacking inhibitory receptors for self MHC class I. The fact that 10-20% of circulating NK cells do not express inhibitory receptors for self MHC class I seems to contradict this model. However, as has been mentioned, these cells are hyporesponsive, and may actually reflect immature cells, or those that have undergone altered development as a result of a developmental selection mechanism. Interestingly, functionality of these cells can be restored by cytokine stimulation with IL-2 (Fernandez, Treiner et al. 2005, Kim, Poursine-Laurent et al. 2005). Mouse studies have demonstrated that MHC class I specific receptor expression is acquired sequentially and that the average number of inhibitory receptors expressed by NK cells increases in hosts that lack MHC class I molecules (Roth, Carlyle et al. 2000). This suggests that an 'auditioning' process may take place during NK cell development whereby NK cells continue to express additional inhibitory receptors until a self-recognising one is expressed. However the existence of NK cells without inhibitory receptors does imply that this mechanism has a limited role in NK development.

Others have proposed that non-MHC class I specific inhibitory receptors such as KLRG1 and 2B4 might explain how cells lacking MHC-class I specific inhibitory receptors achieve tolerance. However it has not been shown that their expression is increased in cells lacking MHC class I specific inhibitory receptors. In one study investigating NK cells from patients with transporter associated with antigen processing (TAP) deficiency (who do not express normal levels of MHC), increased NK levels of carcinoembryonic antigen–related cell adhesion

molecule 1 (CEACAM-1) were shown to inhibit NK mediated cytotoxicity (Markel, Mussaffi et al. 2004). Conversely, a more recent analysis of NK cells from TAP deficient individuals reported a significant effect of MHC class I on KIR repertoire, NK maturation and function (Sleiman, Brons et al. 2014). Furthermore, cells lacking MHC class I specific inhibitory receptors are hyporesponsive to target cells lacking ligands to any inhibitory receptor (Kim, Poursine-Laurent et al. 2005). These data suggest that that non-MHC class I specific receptors are unlikely to play a major role in NK hyporesponsiveness.

It has also been suggested that tolerance in NK cells lacking inhibitory receptors specific for self MHC class I is achieved through downregulated expression of the activating receptors. However, the activating receptors studied to date, including NKG2D, NKR-P1C and Ly49D, have been shown to be expressed at normal levels in the hyporesponsive cells (Fernandez, Treiner et al. 2005, Kim, Poursine-Laurent et al. 2005). However, the role of other activating receptors, particularly the NCRs, has not been studied in this context.

The '*arming'* and *'disarming'* models provide plausible explanations for the generation of self-tolerance. In the arming model, the presence of self MHC class I specific receptors allows precursor NK cells to become functionally mature (armed), whilst those lacking the self MHC class I specific receptors remain in an immature hyporesponsive state (similar to the 'at least one' model) (Wu and Raulet 1997, Raulet 2004). The disarming model dictates that NK cells lacking self specific MHC class I receptors are induced into a state of hyporesponsiveness by chronic stimulating encounters with self cells (equivalent to anergy in T cells)

(Raulet and Vance 2006). These two models crucially differ in the stage of maturation at which the tolerance is induced: this happens at an earlier stage in the arming model whereas in the disarming model, tolerance is induced in functionally mature cells. The arming model is supported by Kim et al (2005) who reported that Ly49-derived signals resulted in the development of highly responsive NK cells; these signals were separate to the normal inhibitory role of the Ly49 receptors, and the authors also showed that the ITIM in the Ly49 tail, but not normal SHP1 activity, was required for functional maturation of NK cells. These data suggest that the ITIMs play an important role in NK cell maturation, separate from the transduction of inhibitory signaling. These data however are not corroborated by models using transgenic mice with SHP-1 deficient NK cells (Lowin-Kropf, Kunz et al. 2000, Raulet and Vance 2006).

The disarming model predicts that multiple encounters between NK cells lacking self MHC class I specific receptors and host cells result in chronic stimulation (presumably through activating receptor-ligand interactions) and induce hyporeactivity. Experiments using a chimeric mouse model support the disarming hypothesis, as tolerance to MHC class I deficient bone marrow was observed in host NK cells regardless of the expression of MHC class I on the NK cells (Wu and Raulet 1997).

Whether through arming or disarming the cell type responsible for NK cell education remains unknown, although it is thought likely to occur within the bone marrow micro-environment. However, licensing is not restricted to the bone marrow and can occur in the periphery, as demonstrated in adoptive

transfer models of MHC class I deficient splenic NK cells into WT hosts (Elliott, Wahle et al. 2010, Joncker, Shifrin et al. 2010).

1.2 Transplantation

1.2.1 Overview

Transplantation refers to the engraftment of cells from one individual (donor) to another (recipient). Human transplantation was first recorded in 500 BC when Chinese doctor Pien Ch'iao reputedly swapped the hearts of warriors Gong Hu and Qi Ying, and several other accounts of crude limb, organ and blood transplantation (transfusion) have been documented throughout history (http://www.wellcomecollection.org/whats-on/events/in-or-out/transplant-timeline.aspx , Linden 2009). These were almost always unsuccessful due to immunological rejection. In the modern era, Peter Medawar's work in the 1940s in understanding the immunological processes involved in tissue rejection developed the field of transplantation biology (Medawar 1956). Since then, our understanding of transplantation has rapidly evolved, such that now it is routinely employed with relatively success in treating many varied medical conditions.

1.2.2 Immunological response to transplantation

The immune system has evolved exquisite and highly effective mechanisms to recognize and eliminate non-self tissue, and managing graft tolerance remains one of the great challenges of organ transplantation. Immunological rejection can occur in several forms and the strategies employed to reduce the risks of these occurring include the use of immunosuppressive agents and genetic donor recipient matching.

Recipient tolerance toward grafts depends upon the degree of genetic disparity between the donor and recipient. For example, xenografts (transplantation between different species) are poorly tolerated and often result in rapid immunological rejection, while autografts (transplantation of cells/organs within an individual) are not rejected at all as they do not express foreign antigens.

Allografts (grafts between members of the same species) are the most common form of transplantation in humans and tolerance varies not only according to genetic matching, but also depending on the type of graft. For example, the eye and placenta are considered immunologically privileged sites as they can be transplanted without eliciting an immune response. This is probably an evolutionary adaptation as inflammation in these sites would lead to catastrophic consequences. On the other hand hematopoietic stem cell transplantation requires a high degree of genetic matching to avoid a graft vs. host effect where the donor immune cells attack host tissues. In solid organ transplantation the liver is considered relatively tolerogenic (compared to the kidney, for example) and therefore genetic matching can be less stringent without adversely influencing outcomes in liver transplantation.

Rejection of transplanted tissue can take place through humoral and cell mediated mechanisms. The molecules that elicit immune responses of genetically disparate tissues are known as histocompatibility antigens. Whilst there are many loci encoding these antigens, the loci responsible for the most robust immunological reactions are encoded within the major histocompatibility complex (MHC). Within humans, the MHC is termed the human leukocyte antigen

(HLA) system and is encoded on the short arm of chromosome 6. HLA alleles are inherited as haplotypes, so that an individual carries one haplotype from each parent, and expresses both sets of alleles on the cell surface.

MHC class I molecules are expressed on all nucleated cells. Class II MHC molecules are only expressed on cells within the immune system which function as professional APCs such as dendritic cells, macrophages and B cells. The function of the MHC molecules is to present antigenic peptides on the cell surface, and this is reflected in their 3-dimensional structure. Class I molecules comprise 3 alpha domains and a β_2 microglobulin domain folded to form a peptide-binding groove. Class II molecules comprise an α and β chain forming the structure around the peptide groove. The two classes of MHC serve distinct purposes: class I MHC predominantly present peptides from within the cell, whilst class II MHC are responsible for presenting extracellular antigens. The ligands for class I and class II MHC/peptide complexes are largely present on lymphocytes, namely T cells and natural killer cells.

T cells are classically considered to be the main effectors in the adaptive immune response and whilst a detailed account of T cell development and function will not be discussed here, a brief overview of T cell antigen recognition and generation of self-tolerance follows.

T cells are defined by the T cell receptor (TCR), which recognizes MHC/peptide complexes. The TCR is a highly evolved molecule comprising two chains, the constituents of which determine the form of the receptor: either a αβ

heterodimer or a γδ heterodimer. The majority of peripheral T cells express the αβ receptor, whilst γδ cells make up higher proportions in certain tissues (eg gut intraepithelial lymphocytes). All the chains have a characteristic structure with an Ig-like variable (V) region at the N terminus and an Ig-like constant I region second domain. During T cell development rearrangement of hypervariable complimentary determining regions (CDRs) in the genes encoding the V segments of the α and β chains generates the extensive diversity in TCRs, so that a vast range of MHC-peptide complexes can be recognized. Tolerance to self is achieved centrally through clonal deletion of self-recognising receptors during development in the thymus or bone marrow, and peripherally through chronic stimulation resulting in hyporesponsiveness (anergy). Importantly, it is extremely difficult to induce tolerance in mature T cells, so that lifelong immunosuppression is required following transplantation (see Section 1.3)

1.2.3 Hybrid resistance

The missing self model of NK cell function (Ljunggren and Karre 1990) has intriguing consequences in the setting of transplantation, and the phenomenon of 'hybrid resistance' observed in mouse models highlights these (Kiessling, Hochman et al. 1977, Klein, Klein et al. 1978). Here two inbred strains of MHC class I homozygous mice are crossed, giving rise to MHC class I heterozygous offspring. When bone marrow from either parent is transplanted into the offspring, it is rejected (**figure 1.4**).

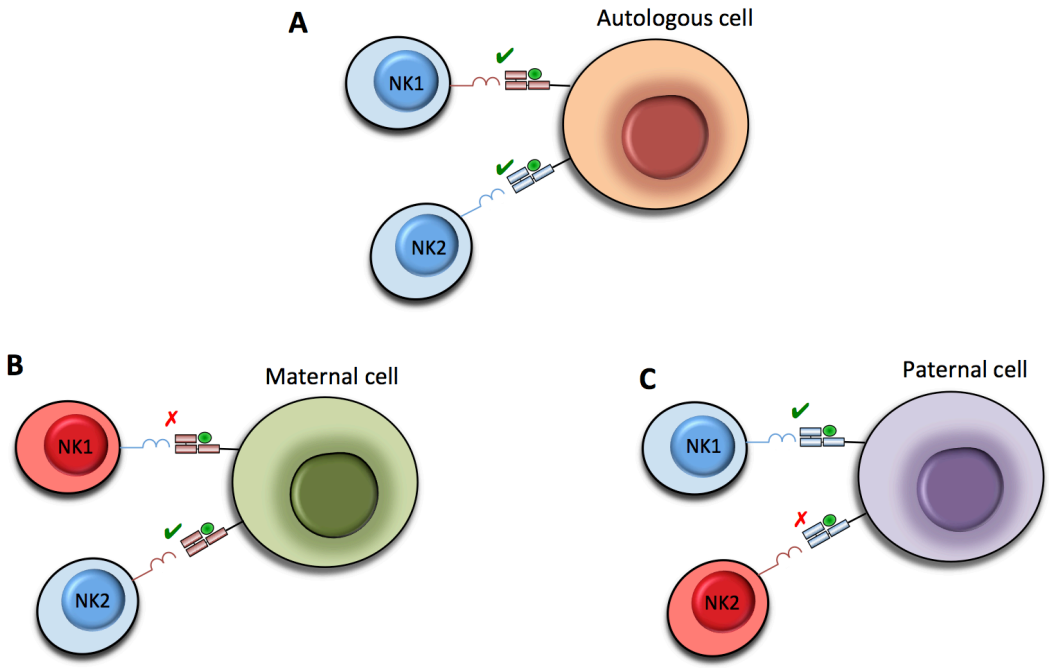


Figure 1.4 Hybrid resistance.

Parents homozygous for MHC I are crossed to create heterozygous F1 offspring. ***A*** *Autologous cells express MHC I of both parents, and thus are recognised as self by all NK clones expressing inhibitory receptors for either parental MHC.* ***B*** *Maternal cells only express maternal MHC I, and therefore are only recognised as self by F1 NK cell clones expressing inhibitory receptors for maternal MHC 1 (NK2). NK clones expressing only inhibitory receptors for paternal MHC I (NK1) do not receive an inhibitory signal, and reject the maternal cells.* ***C*** *a similar situation occurs when paternal cells are transplanted as they are rejected by NK2 cells which only recognise maternal MHC I.*

This cannot occur through T cell mediated mechanisms, as the offspring will express all MHC class I antigens of both parents, and therefore T cells reactive to these will have been deleted during development as described above. Rather the rejection occurs because of stochastic clonal expression of NK receptors. For example, NK cell clones expressing inhibitory receptors only for paternal MHC class I will not recognize maternal bone marrow cells as self, and therefore become activated upon interaction with these cells. Similarly, NK cell clones with inhibitory receptors for only maternal MHC class I will reject paternal bone

marrow. This has important implications in clinical practice in that alloreactivity is frequently observed in HSC transplantation (HSCT). Therefore a high resolution HLA matching strategy is employed in order to optimise outcomes in HSCT (Furst, Muller et al. 2013). Hybrid resistance is a key concept in NK response to transplantation and its relevance to liver transplantation and this thesis is expanded upon in Section 1.4.

1.3 Liver Transplantation

The process of liver transplantation is complex, expensive and, crucially, limited by the number of available donor organs. Therefore all possible strategies aimed at maximizing allograft utility should be employed. Multiple factors must be considered to optimise the transplant outcome. These may broadly be categorized into four groups:

Recipient. Comprehensive evaluation of the potential recipient is necessary to ensure that a liver transplant is appropriate, and once this has been established, that the patient will be able to undergo the rigours of a major operation (Leise, Kim et al. 2011, Martinez-Palli and Cardenas 2011). Within this process, treatments aimed at optimising the patient's physical state are employed, and transplantation should only proceed if the patient is deemed medically fit enough at the time an appropriate organ becomes available

Donor. The state of the donor graft is clearly a key factor in determining the success of liver transplantation. The ideal graft would come from an individual with no medical co-morbidities, and would be surgically dissected ('retrieved'), whilst the donor's circulation is intact (donation after brain death, DBD donor). This minimizes the warm ischaemia time, a critical period during which irreversible cellular damage can occur in the graft (Kootstra, Daemen et al. 1995, Jimenez-Galanes, Meneu-Diaz et al. 2009). The limited availability of donors and high demand for grafts means that most often these ideal conditions are not met. Increasingly 'marginal' grafts are utilized such as those with evidence of liver disease (eg steatosis), or those resected after the heart has stopped beating

(donation after circulatory death, DCD) (Callaghan, Charman et al. 2013). Our ability to modify these factors will remain limited until donation rates improve. Providing the best liver for engraftment also involves meticulous surgical resection such that the liver and hilar structures (vessels, bile duct) are preserved as intact as possible. Finally the length of time the between retrieval and engraftment (cold ischemia time) must be minimized.

Peri-operative care. Much of the improvements we have seen in liver transplantation outcomes over the last 4 decades have resulted from advances in the technical management around the operation. As well as optimising the recipient's health and donor liver as described above, perioperative monitoring and management of issues such as circulatory dysfunction, coagulopathy and sepsis are vital in improving outcomes (Nandhakumar, McCluskey et al. 2012).

Immunological tolerance. It is of paramount importance to ensure the recipient immune system tolerates the allograft to avoid rejection. The main strategies employed to maximize immunological tolerance are genetic matching to avoid alloreactivity and the use of immunosuppressant agents, which dampen the immune response to alloantigens. The extreme shortage of donor organs and severity of illness of potential recipients means that it is not practical to employ exact genetic matching strategies. As T cells are considered the main effectors of alloreactivity, the mainstay of immunosuppressive agents are directed at reducing T cell activation and proliferation. These agents are not thought to directly affect NK cell function (see Section 1.4) and therefore, theoretically, NK

cells could play a major role in organ rejection. This is the central theme in this thesis and is explored in greater detail in this and following chapters.

1.3.1 Indications, selection for listing and survival

The main indications for adult liver transplantation are acute liver failure, end stage chronic liver disease and hepatocellular carcinoma (HCC), whilst rare indications include liver based metabolic conditions causing systemic disease (O'Leary, Lepe et al. 2008). Whatever the aetiology of liver disease, the process involved in deciding whether an individual is a candidate for a liver transplant is complicated. The principles of utilitarianism and egalitarianism necessarily underpin the philosophy of transplant selection, where the precious limited resource of donor grafts must be employed to maximize their benefit. Central to the evaluation process is determining the severity of liver disease. Several scoring systems have been developed to address this, with the aim of reliably predicting prognosis without transplantation so that potential recipients can be categorized according to score. The Model of End Stage Liver Disease (MELD) score in the USA and UK End Stage Liver Disease (UKELD) score in the UK are employed for this purpose, as they have been proven to reliably predict mortality. Once an individual is determined to have disease severe enough to consider liver transplantation, extensive physical and psychosocial evaluation is carried out, in order to assess whether transplantation is feasible in the potential recipient.

Many factors influence post transplant survival, not least the physical characteristics of the individual recipient. Nonetheless, despite being a major

operation, liver transplantation has become a highly successful procedure, with post transplant survival rates currently 90% at 1 year and 70% at 5 years. This reflects extraordinary progress in all aspects of liver transplantation, from patient selection, pre-operative optimisation, surgical technique, perioperative care and management of immunosuppression. However, there is still a small but significant rate of acute and chronic rejection, and outcomes in transplantation for chronic hepatitis C are significantly poorer than most other indications. It is these challenges that research efforts must focus on, and provide the background to the studies in this thesis.

1.4 NK cells in liver transplantation

As NK cell tolerance of self involves inhibitory interactions with self HLA class I molecules, and liver allografts are not HLA matched to the recipient, there is a theoretical opportunity for NK cell alloreactivity in HLA mismatched transplantation. However, few studies have explored the effects of liver transplantation on recipient NK cells and the influence of the genetics of NK receptors/HLA ligands in NK allospecific reactivity is unresolved. In order to appreciate the theoretical potential of natural killer cell alloreactivity, a detailed understanding of the subtleties of inhibitory NK receptor/HLA ligand recognition is required.

As described in Section 1.1, NK cell receptors are expressed in a clonal and variegated manner so that within an individual, clones of NK cells express different repertoires of receptors. For example, some clones will express KIR2DL1 (recognizing HLA-C2) but not KIR2DL2/3 (principally recognizing HLA-C1), and vice versa. HLA and KIR genes are encoded on different chromosomes and are therefore not linked, so that an individual may not express the inhibitory HLA ligand for all the KIR encoded within his genotype. Therefore NK clones expressing KIR2DL2 (and not KIR2DL1) would be self reactive in an individual homozygous for HLA-C2. This does not occur, and it has been shown that NK cells that do not express inhibitory receptors to self ligands are hyporesponsive (Fernandez, Treiner et al. 2005). As discussed earlier, it is thought that NK cells undergo an educational process during maturation that allows full maturation of only those clones expressing inhibitory receptors recognizing self.

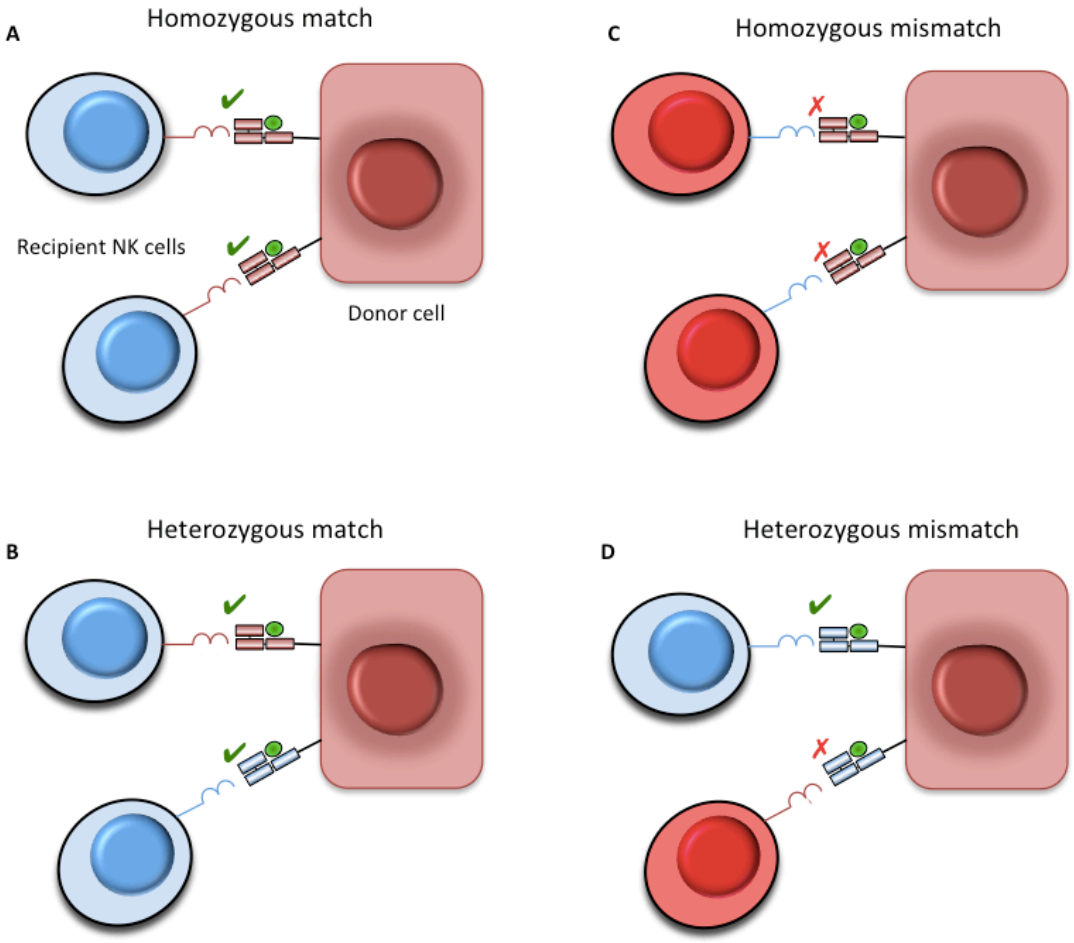


Figure 1.5 Schematic showing how HLA mismatch can induce NK cell activation.
Receptor colours represent cognate KIR/HLA combinations (eg blue KIR on NK cell recognises blue HLA ligand on donor cell). NK cells colours represent putative inhibition (blue) or activation (red). ***A, B*** *in matched LT match, the donor cells express HLA recognised by recipient inhibitory KIRs, and therefore an inhibitory signal is transduced (✔).* ***C, D*** *in mismatched LT, recipient NK cell clones express KIRs that do not recognise donor HLA ligands, and these clones may lose their inhibitory status (✗), becoming activated.*

The introduction of an allograft will theoretically challenge the recipient NK recognition system depending on degree of HLA matching and I will focus on the HLA-C1/C2-KIR2DL1/2/3 system to illustrate this.

In HLA-C matched transplantation, recipient NK cells are educated on recipient HLA-C alleles, which are also present on the donor cells. Therefore the donor cells are able to form inhibitory KIR-HLA-C interactions, and there is no NK alloreactivity. This will occur when the donor is either homozygous (**figure 1.5A**) or heterozygous (**figure 1.5B**) for the HLA-C alleles present in the recipient. In HLA-C mismatched transplantation, the donor liver expresses HLA-C alleles absent in the recipient. Therefore recipient NK cells educated on a recipient MHC background do not express inhibitory receptors for donor HLA-C and are released from inhibition. For example, in transplantation of HLA-C1C1 donor to HLA-C2C2 recipient, mature recipient NK cells educated on the C2 background will only express KIR2DL1 (and not KIR2DL2/3). These NK cells will not see an inhibitory ligand in the donor (which only expresses HLA-C1) and would theoretically become activated (**figure 1.5C**). Similarly in transplantation of HLA-C1C1 donor to HLA-C1C2, clones of NK cells in the recipient educated on the HLA-C2 background express only KIR2DL1 (and not KIR2DL2/3); these clones will not recognize the HLA-C1 homozygous donor and theoretically become alloreactive (**figure 1.5D**). **Table 1.3** illustrates the donor:recipient pairings that will result in match or mismatch with regard to HLA-C alleles.

This model predicts NK alloreactivity in transplantation according to HLA matching. However previous studies exploring NK reactivity and the influence of HLA on outcomes after liver transplantation are conflicting with disparate findings.

		Donor		
		C1C1	C1C2	C2C2
Recipient	C1C1	Match	MM for 158a	MM for 158b
	C1C2	Match	Match	Match
	C2C2	MM for 158a	MM for 158b	Match

Table 1.3 Donor:recipient HLA-C matching.

HLA-C genotype pairings resulting in match or mismatch are shown. In mismatch (MM) the NK cell clones predicted to be alloreactive are noted (eg MM for 158a implies NK cells expressing CD158a are alloreactive).

1.4.1 Genetic studies in transplantation

The effects of HLA mismatching between donor and recipient have been studied most extensively in HSCT. In this setting, mismatching can cause alloreactivity in two ways: 1) recipient NK cells can reject donor cells that do not express ligands for recipient NK inhibitory receptors (akin to hybrid resistance); 2) donor NK cells can lyse recipient cells that do not express ligands to donor inhibitory NK receptors (graft versus host disease, GvHD). Indeed in bone marrow transplantation NK alloreactivity in KIR epitope mismatch has been well described (Ciccone, Viale et al. 1988, Ciccone, Pende et al. 1992, Colonna, Brooks et al. 1993, Ruggeri, Capanni et al. 2002). Because of the high potential for NK and T cell driven alloreactivity in HSCT, high resolution HLA matching is employed. However, over the last 15 years, benefits of HLA mismatch have been demonstrated. In acute myeloid leukaemia (AML) KIR-ligand mismatch in haploidentical allogeneic BMT has been associated with increased survival, decreased relapse, and reduced GvHD (Ruggeri, Capanni et al. 2002, Ruggeri, Mancusi et al. 2007). It is thought that the major mechanism for these

advantages is a graft versus leukaemia effect, where donor NK cells lyse tumour target cells. These observations have led to NK cell based immunotherapy as an emerging research field in treatment of haematological malignancies (Miller, Soignier et al. 2005, Rubnitz, Inaba et al. 2010, Curti, Ruggeri et al. 2011).

In solid organ transplantation only a few studies have analyzed the effects of HLA-KIR mismatching, and these have been performed in liver and kidney transplantation. In renal transplantation, one study reported a beneficial effect of donor HLA-C2 homozygosity, and associations between stable renal function and high numbers of inhibitory receptors in the recipient genotype, matches for KIR2DL2/DS2, and mismatch for KIR2DL3 (Kunert, Seiler et al. 2007). Other influences of HLA and KIR on outcomes reported in kidney transplantation include: beneficial effect of recipient HLA-C2 on long-term graft survival (Hanvesakul, Kubal et al. 2011); a reduction in graft survival in KIR-ligand mismatch (van Bergen, Thompson et al. 2011); and an association between KIR2DS4 and graft rejection (Nowak, Magott-Procelewska et al. 2012). In contrast other studies have demonstrated no effect of KIR or HLA genotype on outcomes in kidney transplantation (Tran, Mytilineos et al. 2005, Tran, Unterrainer et al. 2013).

Early studies in liver transplantation have variably reported a negative effect of HLA matching on graft survival (Donaldson, Underhill et al. 1993, Rasmussen, Davies et al. 1995, Muro, Marin et al. 1999) or a benefit of HLA matching on reducing acute rejection and improving graft survival (Nikaein, Backman et al. 1995, Moya-Quiles, Muro et al. 2003).

Bishara *et al* (2005) reported HLA-C matching was associated with fewer rejection episodes in the first year post transplantation, whilst HLA-Bw mismatching had no effect on outcome. In an analysis of 446 liver transplant recipients Moya-Quiles *et al* (2007) observed a significant association between lower recipient HLA-Cw*07 (an HLA-C1 allele) frequency and acute rejection. The HLA-C2/KIR2DL1 interaction transduces a stronger NK inhibitory signal than HLA-C1/KIR2DL2/3 (Winter, Gumperz et al. 1998), and it has been postulated that donor HLA-C2 might therefore protect the graft from NK alloreactivity. This question was addressed in two retrospective studies but with inconsistent findings. In a cohort of 416 transplant recipients Hanvesakul *et al* (2008) reported the presence of HLA-C2 in the donor was protective in terms of development of chronic rejection, cirrhosis and graft loss in a dose related manner. This was not corroborated in a subsequent study of 913 genotyped liver donors, which found no effect of HLA-C2 on either graft or patient survival (Tran, Middleton et al. 2009). More recently, the effect of recipient KIR and donor HLA-C genotypes have been explored, again with disparate findings. Whilst Moroso *et al* (2011) found no effect of KIR/ligand mismatching on acute rejection, graft or patient survival, Legaz *et al* (2013) have recently reported a beneficial effect on short-term survival of recipient KIR2DL3- donor HLA-C1 matching.

In liver transplantation, there is a paucity of data assessing the effects of HLA and KIR genotypes and matching on NK cell activation or function. In a study of 31 liver transplant recipients, Oertel *et al* (2001) found increased cytotoxicity in recipient NK cells in the first 3 weeks post transplant in cases where there was

predicted NK cell alloreactivity (according to the model described earlier in this section).

Thus immunogenetic studies have not provided sufficient evidence to generate a consensus view of the effect of HLA matching on clinical outcomes in liver transplantation, and there is little data on the effects of matching on NK cell function.

1.4.2 Studies of NK phenotyping and function after transplantation

Most study designs into NK cell alloreactivity predicted by the missing self model, have focused on clinical outcomes according to HLA mismatching. In order to determine that NK cells are the effectors of alloreactivity or indeed tolerance, it is important to examine the effect of transplantation on the activation and functionality of NK cells. An early study of NK cell reconstitution after HSCT demonstrated increased numbers of $CD56^{dim}$ NK cells in the first few months post transplant, and reduced cytotoxicity of the $CD56^{bright}$ subset, which could be restored with IL-2 stimulation (Jacobs, Stoll et al. 1992). A subsequent study reported that early after HSCT $CD3^{-}CD56^{+}$ NK cells initially lacked CD16, and that all NK cells initially expressed CD94/NKG2A, acquiring KIR at late stages post transplant; whilst donor NK cells retained cytotoxicity against MHC class I deficient cell lines, they did not lyse recipient target cells, even in the setting of graft versus host disease (GvHD) (Vitale, Pitto et al. 2000). In another study of NK cells after bone marrow transplantation, NK cells from 5 of 24 recipients exhibited an immature phenotype at engraftment, lacking KIR, CD16

and NKG2D, and expressing low levels of NKp30, NKp46, 2B4 and NKG2A. These cells were hypofunctional, but after 30 days the phenotype and functionality had recovered (Shilling, McQueen et al. 2003). These studies give an insight into *donor* NK cell development and maturation in the setting of HSCT, but solid organ transplantation represents a significantly different context, as developing and mature *recipient* NK cells encounter donor cells in peripheral tissue.

NK cells were conventionally thought not to participate in solid organ transplant rejection. This was based on observations that NK cell depletion in rat recipients of liver, cardiac or skin allografts did not alter rejection outcomes (Markus, Selvaggi et al. 1993); conversely, in *RAG*$^{-/-}$ mice (which therefore lack T and B cells, but have normal NK cells), allografts survived indefinitely (Bingaman, Ha et al. 2000). However further experimental data in animals has challenged this view. For example NK cells have been shown to infiltrate rat liver allografts and produce potent chemokine and cytokine mediators of acute rejection (Kondo, Morita et al. 2000, Obara, Nagasaki et al. 2005). In CD28 knockout mice (in which there is blockade of the T-cell co-stimulatory signal), NK cell depletion prolonged cardiac allograft survival, thus implicating NK cells in rejection (Maier, Tertilt et al. 2001).

In human solid organ transplantation few studies have looked into NK cell phenotyping. In acute antibody mediated rejection of human renal transplants, NK cells have been found within the allograft (Hidalgo, Sis et al. 2010). In a study of NK cells in bronchoalveolar lavages in lung transplant recipients, NK cells from patients experiencing acute rejection episodes were found to be more

activated, expressing higher levels of NKp44, CD107a, HLA-DR and CD25 (Meehan, Sullivan et al. 2010). Another lung transplant study in paediatric recipients reported an expansion of $CD56^{bright}$ NK cells, with preserved functionality (Wiesmayr, Webber et al. 2012). The liver is of particular interest in NK cell alloreactivity because NK cells are enriched within the intrahepatic lymphocyte population (Gao, Radaeva et al. 2009). A study of 36 transplant recipients reported increased recipient NK cytotoxicity but no association with acute rejection was demonstrated (Oertel, Kohlhaw et al. 2001). A more recent study investigating recipient NK cell phenotyping after LT found a decrease in NK cell number, and increased NKp30 at one week post LT but this did not persist at 12 months (Pham, Piard-Ruster et al. 2012). In the specific setting of recurrent hepatitis C post LT, Varchetta et al noted a decrease in the proportion of NK cells at 7 days, and an increase in CD94/NKG2C expression which was associated with hepatitis C recurrence (Varchetta, Oliviero et al. 2009). Whilst these studies have provided some insight into the recipient NK response to allograft, detailed characterization of NK phenotyping and function after transplantation has not been performed.

1.5 Immunosuppressive therapy

Current standard immunosuppressive therapy for LT targets T-cells and adaptive immunity. In the initial phase corticosteroids are utilised, and calcineurin inhibitors form the mainstay of maintenance therapy (Knechtle 2011). The effect of these medications on NK cell activation is controversial and this is reflected in the literature. *In vitro* and *in vivo* immunosuppression assays have demonstrated conflicting effects across a number of heterogeneous studies as shown in **Table 1.4** (Gui, Ho et al. 1982, Wasik, Gorski et al. 1991, Alamartine, Sabido et al. 1994, Van Ierssel, Van der Sluys Veer et al. 1995, Vacher-Coponat, Brunet et al. 2006, Wang, Grzywacz et al. 2007, Thum, Bhaskaran et al. 2008, Kim, Kim et al. 2010, Morteau, Blundell et al. 2010, Meehan, Mifsud et al. 2013, Neudoerfl, Mueller et al. 2013). This gap in our current understanding will be further addressed in this thesis, with detailed phenotyping, functional and gene expression assays.

Table 1.4 Studies investigating the effect of immunosuppressive drugs on NK cells.

Year	Author/Journal	Drugs investigated	Methodology	Findings
1982	Gui et al *Infect and Immunity*	CysA	Murine, *in vitro* ^{51}Cr release assay	↓ NK activity
1984	Nair et al *J Immunol*	Pred	*In vitro*, ^{51}Cr release assay, ADCC	↓ NK activity
1991	Wasik et al *Transplantation*	FK506, CysA	*In vitro,* ADCC assay	No effect on activity
1994	Alamartine et al *Nephrol Dial Trans*	CysA, FK506, Pred, 6MP	*In vitro,* specific killing assay	Cys A/FK506 – **no** effect Pred/6MP - ↓ NK activity
1995	van Ierssel et al *APT*	Pred, Budesonide	CD patients, *in vivo,* ^{51}Cr release assay	↓ NK activity at Wk 2/4 but ↑ at wk 10
2006	Vacher-Coponat et al *Transplantation*	FK506, MMF, CysA, Aza	Renal transplant *in vivo* Europium release assay	↑ NK activity with FK506/MMF at 1 year
2007	Wang et al *Blood*	CysA	*In vitro*, FACS, Western blotting, Ca imaging, ^{51}Cr	No effect on cytotoxicity. Changes in receptors
2008	Wai et al *Transplantation*	Rapamycin, FK506, CysA	Rat, *in vivo /vitro,* IFN γ release/ killing assay	Rapamycin ↓ NK activity, but not FK506/CysA
2008	Thum et al. *Am J Reprod Med*	Pred	Recurrent miscarriage pts, *in vivo*, killing assay	↓ NK activity
2010	Morteau et al *PLoS*	CysA, FK506	Renal transplant pts *in vitro/in vivo,* LAMP/IFN γ	↓ NK activity
2010	Kim et al *J Leuc Biol*	FK506	HSCT *in vivo* and *in vitro*, ^{51}Cr, LAMP, killing assays	↓ NK activity
2013	Meehan et al *PLoS One*	MMF, CysA, Pred	*In vitro* CD107a, IFN γ, CFSE; *in vivo* lunge transplant recipients	NK activity dependent on stimulation used
2013	Neudoerfl et al *Front Immunol*	CysA, FK506, MMF, Sir	*In vitro* CD107a, IFN γ Elispot, proliferation assays	Altered NK subsets Effects varied with cytokines used

CysA, Cyclosporine A; FK506, Tacrolimus; Pred, Prednisolone; 6MP, 6-Mercaptopurine; MMF, Mycophenylate Mofetil; Sir, Sirolimus; ^{51}Cr, ^{51}Chromium; CD, Crohn's disease

1.6 Summary, hypotheses and aims of thesis

Natural killer cells are potent effector immune cells with the ability to distinguish between self and non-self, through a complex process of education and maturation that is not fully understood. Liver transplantation offers a unique opportunity to study NK cells as HLA matching is not performed, rendering allografts theoretically susceptible to NK alloreactivity. Despite this, outcomes for liver transplantation are good, with graft survival of 90% after 12 months. Therefore the hypothesis I wish to test is that LT leads to recipient NK cell hypofunctionality, with downregulation of activation. I aim to achieve this through detailed analysis of LT recipient NK cells combined with clinical data collection.

Aim 1: To investigate the effects of liver transplantation on recipient NK cell phenotype, function and gene expression and assess this in the context of:

i) ***genetics (ie. HLA-KIR)***

ii) ***therapeutics (ie. immunosuppression)***

Aim 2: To investigate the whether any recipient NK cell changes found have clinically relevant correlates in terms of graft and patient outcome.

CHAPTER 2: MATERIALS AND METHODS

2.1 Subject selection and sample collection

Peripheral venous blood was taken from study participants in EDTA blood bottles. Between 10-20 ml of blood was taken and either processed immediately or stored at 4^{o}C until processing. The maximum time between venesection and blood processing was 16 hours. All participants were given a patient information sheet (appendix 1), and gave informed consent for participation in the study (appendix 2).

2.1.1 Cross-Sectional Study

Individuals that had undergone liver transplantation were recruited from outpatient clinics at the Institute of Liver Studies (ILS) at Kings College London, and from St Mary's Hospital, Imperial College Academic Health Science Centre, London. Inclusion criteria were: aged over 18 and capacity to give informed consent. Exclusion criteria were: less than 4 weeks post-transplant and refusal to consent. A total of 109 patients were recruited. Patient characteristics are shown in **table 2.1**.

2.1.2 Longitudinal Study

Patients were recruited during their admission for work-up for liver transplantation at the ILS. Inclusion criteria were: age greater than 18. Exclusion criteria were: unable to give informed consent. After informed consent, blood was collected from each patient pre-transplant. If the patients went on to receive

a liver transplant, blood samples were taken in the 12 months post transplant. Twenty-eight patients were recruited from transplant work-up, 21 were listed for liver transplantation and 13 received an allograft during the follow-up period. Of these post transplant samples were available from 7 patients.

	Transplants		Healthy Controls		p-value
Total, n	109		31		
Male, n (%)	65	(59.6)	18	(58.1)	NS
Age, years (SD)	55	(11.7)	41	(13.4)	<0.0001
Aetiology, n (%)					
HCV	25	(22.9)			
ALD	17	(15.6)			
ALF	11	(10.1)			
PBC	11	(10.1)			
PSC	11	(10.1)			
Cryptogenic/NASH	7	(6.4)			
Subacute	5	(4.6)			
AIH	5	(4.6)			
Redo Transplant	3	(2.8)			
Other	14	(15.6)			
Time since T/P, days (range)	2285	(73-9597)			
Immunosuppression, n (%)	(data missing from 2 patients)				
Tacrolimus	89	(83.2)			
Sirolimus	3	(2.8)			
Cyclosporine	9	(8.4)			
Corticosteroid	43	(40.2)			
Mycophenylate	29	(27.1)			
Unknown	2				
Post transplant Biopsy Available	57	(53.3)			
Lab Data (range, SD)					
AST, IU/L	34	(13-119, 21)			
INR	1.07	(0.85-2.71, 0.32)			
Albumin, g/L	40.8	(16-47, 4.8)			
Bilirubin, µmol/L	10.8	(3-50, 7.6)			
Platelets, $x10^9$/L	234	(73-1042, 130)			
Tacrolimus level, ng/ml	4.7	(0.5-14.9, 2.4)			

Table 2.1 Demographic and clinical data for patients recruited into cross-sectional study.
ALD: alcohol-related liver disease; ALF: acute liver failure; PBC: primary biliary cirrhosis; PSC: primary sclerosing cholangitis; NASH: non-alcoholic steatohepatitis; AIH: autoimmune hepatitis; AST: aspartate transaminase; INR: international normalised ratio.

2.1.3 Healthy controls

After informed consent blood samples were obtained from individuals that had no history of transplantation or immunosuppression. For all of the assays described, each experiment comprised a healthy control sample alongside the liver transplant samples, in order to control for assay-to-assay variation.

2.2 Peripheral Blood Mononuclear Cell (PBMC) preparation

The whole blood samples were processed within 16 hours of venesection. The blood was centrifuged at 350g for 5 minutes and the plasma separated. The PBMCs were harvested via Ficoll-Paque™ (Amersham Biosciences, U.K.) density centrifugation, resuspended in 10% dimethyl sulfoxide (DMSO) in fetal calf serum (FCS, Lonza) and stored at -190^{o}C in liquid nitrogen. When required for assays, the cells were thawed, rapidly transferred and washed in RPMI 1640 medium (Biowhittaker, U.K.) and resuspended in RPMI 1640 supplemented with 10% FCS, 100 IU/ml penicillin-streptomycin and 2mM L-glutamine (Biowhittaker, U.K.), henceforth referred to as R10 culture medium.

2.3 Cell lines

2.3.1 K562

The K562 cell line is a human erythroleukaemic cell line derived from a patient with chronic myeloid leukaemia. These cells do not express surface MHC class I, and are therefore readily killed by NK cells (Koeffler and Golde 1980) .

2.3.2 P815

The P815 cell line is a mouse mastocytoma cell line that is resistant to NK cell killing, and expresses the Fcγ receptor. This can therefore be used as a target for redirected cytotoxicity after coating with murine antibodies to specific human receptors (Vadas, Nicola et al. 1983).

2.4 Cell culture

K562 cells were cultured and maintained in R10 culture medium at 37°C under 5% CO2. These were maintained in logarithmic phase at neutral pH by diluting the cultures at a 1 in 10 dilution every 72 hours. P815 cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Biowhittaker, U.K.) supplemented with 10% FCS, 100 IU/ml penicillin-streptomycin and 2mM L-glutamine. P815 cells were maintained in logarithmic phase at neutral pH by diluting cultures at a 1 in 10 dilution every 72 hours.

Target	Clone	Species	Isotype	Vendor	Cat. No	Fluorophore	Dilution
CD3	UCHT1	Mouse	IgG1	BioLegend	300428	PerCP	1:100
CD3	OKT3	Mouse	IgG1	eBioscience	57-0037-73	Pacific Blue	1:200
CD56	MEM188	Mouse	IgG2a	BioLegend	304604	FITC	1:10
CD56	HCD56	Mouse	IgG1	BioLegend	318318	PE	1:20
CD56	HCD56	Mouse	IgG1	BioLegend	318317	PECy7	1:100
CD158a,h	EB6B	Mouse	IgG1	Beckman Coulter	A09778	PE	1:10
CD158b1/2j	GL183	Mouse	IgG1	Beckman Coulter	IM2278	PE	1:10
CD337	AF29-4D12	Mouse	IgG1	Miltenyi Biotec	130-092-484	APC	1:10
CD335	9E2	Mouse	IgG1	Miltenyi Biotec	130-092-609	APC	1:10
CD314	BAT221	Mouse	IgG1	Miltenyi Biotec	130-092-673	APC	1:10
NKG2C	134591	Mouse	IgG1	R&D Systems	FAB138P	PE	1:50
CD16	3G8	Mouse	IgG1	BioLegend	302018	APC Cy7	1:20
CD57	HCD57	Mouse	IgG1	BioLegend	322216	Pacific Blue	1:100
CD107a	H4A3	Mouse	IgG1	eBioscience	51-1079-42	AF 647	1:200
TNFα	MAb11	Mouse	IgG1	BioLegend	502906	PE	1:50
IFNγ	B27	Mouse	IgG1	BD Pharmingen	554701	FITC	1:100
pSTAT4	38/p-Stat4	Mouse	IgG2b	BD Biosciences	558137	AF 647	1:5

Table 2.2 Antibodies used in flow cytometry assays.

2.5 NK cell phenotyping assays

Table 2.2 summarises the antibodies used in the flow cytometry experiments describe in this thesis.

2.5.1 Activation panel

PBMCs were suspended at a concentration of 1.5 x 10^6 cells/ml and 200ul aliquoted into a 96 well plate (3 x 10^5 cells/well). The cells were then incubated with a blocking buffer (10% human serum (Cambrex, U.K.) in FACS buffer (phosphate buffered saline (PBS, Invitrogen, UK) with 1% bovine serum albumin (BSA, Sigma, Poole, UK) and 0.1 % sodium azide)) on ice for 20 minutes, washed and stained with Aqua fixable live/dead stain (Invitrogen, U.K.) and then stained with fluorochrome-conjugated mouse antihuman IgG monoclonal antibodies as follows: PerCP-CD3 (BioLegend, U.K.) and FITC-CD56 (BioLegend, U.K.); half were stained with PE-CD158a,h (Beckman Coulter, U.K.), staining KIR2DL1, and half with PE-CD158b (Beckman Coulter, U.K.), staining KIR2DL2/3; cells from each half were stained with either APC-CD337 (NKp30) (Miltentyi Biotec, U.K.), APC-CD335 (NKp46) (Miltentyi Biotec, U.K.), or APC-CD314 (NKG2D) (Miltentyi Biotec, U.K.). Control wells were stained with appropriate IgG isotype controls for each fluorochrome. After staining the cells were washed twice with PBS and fixed in 1% paraformaldehyde (PFA), then read by flow cytometry.

2.5.2 Differentiation panel

PBMCs were prepared and plated as described above. After blocking the PBMCs were incubated with the following antibodies: PerCP-CD3 (Biolegend, U.K.), PECy7-CD56 (Biolegend, U.K.), PE-NKG2C (R&D systems, U.K.), APC Cy7-CD16 (Biolegend, U.K.), Pacific Blue-CD57 (Biolegend, U.K.). Control wells were stained with IgG-1 isotype controls for each fluorochrome, and after washing, the cells were fixed in 1% PFA and analysed by flow cytometry.

2.6 NK cell functional assays

2.6.1 Cytotoxicity assay

In order to avoid the expense, radiation exposure and limitations of the traditional ^{51}Chromium based cytotoxicity assay, a more novel flow-cytometry-based method for measuring NK cell specific cytotoxicity was employed. PBMCs (effector cells) were resuspended in R10 culture medium at a concentration of 1.5 x 10^5 cells/ml and stimulated with 1ng/ml recombinant human interleukin (IL)-15 (R&D Systems, U.K.). The cells were incubated overnight at 37^{o}C in 5% carbon dioxide (CO_2) in a 96-well U-bottom plate with a volume of 200ul/well.

The following day K562 (target cells) were resuspended in R10 culture medium and incubated for 1 hour at 37^{o}C in 5% CO_2 with the cell marker CellTracker™ Orange CMTMR (Invitrogen, U.K.). This is a dye that passes freely through cell membranes. Once inside the cell, the reagent reacts with thiol groups and is transformed into a cell impermeant fluorescent dye. The cells were washed twice

in PBS and resuspended in R10 culture medium at a concentration of 6 x 10^5 cells/ml. 50ul of cell suspension (3 x 10^4 cells) were transferred to each assay well of a 96 well U bottom plate. The IL-15 containing media was then removed from the stimulated PBMCs and these were resuspended in 50ul of fresh R10 medium. The PBMCs were stimulated by incubation with the K562 cells at an effector:target (E:T) ratio of 10:1 for 3 hours at 37°C in 5% CO_2, with no effector used as a negative control. The optimal E:T ratio was determined by titration of E:T from 1:1 to 40:1. After 4 hours the cells were transferred to a V-bottomed plate and the media removed. The cells were then incubated with Live/Dead fixable Aqua dead cell stain (Invitrogen, U.K.) in the dark on ice for 30 minutes, washed twice in PBS and resuspended in 1% PFA. Analysis by flow cytometry allowed calculation of specific cytotoxicity as follows:

% Dead K562 in test wells - % Dead K562 in control well.

2.6.2 CD107a

PBMCs were prepared and stimulated overnight with IL-15 as described for the cytotoxicity assay. The following day the IL-15 containing media was removed from the PBMCs, which were resuspended in 50ul of fresh R10 containing anti AF647-CD107a antibody (eBioscience, U.K.). K562 target cells were suspended in R10 medium at a concentration of 1.2 x 10^6 cells/ml and 50ul added to the PBMCs resulting in an E:T ratio of 5:1. This optimal ratio was determined by titration of E:T from 1:1 to 40:1. The cells were incubated at 37°C in 5% CO_2 for 1 hour, after which monensin (Golgi Stop, BD Biosciences, U.K.) was added to the

mixture at a final concentration of 3ug/ml. Monensin prevents the acidification of endocytic vesicles, blocking protein transport. Therefore this prevents degradation of reinternalised CD107a, and allows accumulation of intracellular cytokines in the Golgi apparatus, enhancing visualisation of these proteins for flow cytometry. After a further 3 hours incubation at 37°C in 5% CO_2 the cells were washed and incubated with blocking buffer for 20 minutes. The PBMCs were then stained with monoclonal antibodies to identify the NK cell population as follows: PerCP-CD3 (BioLegend, U.K.) and FITC-CD56 (BioLegend, U.K.). The cells were washed twice in wash buffer, fixed in 1% PFA and subsequently analysed by flow cytometry. For each experiment, spontaneous degranulation (CD107a expression) was determined for PBMCs in the absence of target cells.

2.6.3 Intracellular cytokine assay

PBMCs were prepared and stimulated the night before the assay as described above. On the day of the assay the PBMCs were incubated at 37°C in 5% CO_2 with target K562 cells at an E:T of 5:1 (optimal ratio titrated as described). After 1 hour Golgistop was added, and after a further 3 hours, the PBMCs were washed, incubated in blocking buffer, washed, and surface staining was performed with monoclonal antibodies: PerCP-CD3 (BioLegend, U.K.) and FITC-CD56 (BioLegend, U.K.). The cells were then permeabilised by incubation with Cytofix/Cytoperm reagent (BD Biosciences, U.K.), containing formaldehyde for fixation and saponin for cell permeabilisation. Permeabilisation was maintained by using Perm/Wash (BD Biosciences, U.K.) containing saponin for all subsequent washing and staining steps. Intracellular cytokine staining was then

performed by incubating the PBMCs for 30 minutes in the dark at 4°C with monoclonal antibodies as follows: FITC-IFNγ (BD Pharmingen, U.K.) and PE-TNFα (Biolegend, U.K.). Cells were subsequently washed in PBS and analysed by flow cytometry.

2.7 Immunosuppression assays

In order to assess the effect of commonly used immunosuppressant drugs on NK cell activation, *in vitro* immunosuppression assays were performed. PBMCs from healthy donors were incubated at 37°C in 5% CO_2 with varying concentrations of Prednisolone (Pred, Sigma, U.K.), Cyclosporine A (CysA, Sigma, U.K.) or Tacrolimus (FK506, Sigma, U.K.). PBMCs incubated with R10 culture medium alone were used as a control. Phenotyping and functional (cytotoxicity and CD107a) assays were performed as described above at baseline and at 16 and 96 hours of incubation. Comparisons were made between the drug assays and control (R10 medium alone).

2.8 DNA preparation

DNA was extracted from whole blood using the QIAmp blood kit (Qiagen, U.K.) according to the manufacturer's instructions. Samples were lysed using proteinase K and the lysate was loaded onto the DNeasy spin column. During centrifugation, DNA was selectively bound to the silica membrane as contaminants passed through. Remaining contaminants and enzyme inhibitors

were removed in two wash steps and DNA was then eluted in water. DNA purity and quantification was assessed by spectrophotometry.

2.9 HLA genotyping

Recipient DNA was sent for HLA genotyping to Dr Ann-Margaret Little's Histocompatibility & Immunogenetics laboratory in Gartnavel Hospital, Glasgow. The methodology utilized the Lifecodes HLA sequence-specific oligonucleotides (SSO) Typing kit (Gen-Probe, U.S.A), according to the manufacturer's protocol.

Donor HLA genotyping was obtained from records kindly provided by NHS Blood and Transplant (www.nhsbt.nhs.uk)

2.10 KIR genotyping

KIR copy number typing was performed by Dr James Traherne at Professor John Trowsdale's laboratory in Cambridge using a recently described high-throughput technology called qKAT (Jiang, Johnson et al. 2012).

2.11 NK cell purification

NK cell purification was performed with the Invitrogen Dynabeads Untouched Human NK cells kit. PBMCs were thawed and incubated with an antibody mixture which bound to all the non-NK cells. The cells were then incubated with Dynabeads, which bound to all the antibody bound cells. These cells were

separated by placing the mix in a magnet and aspirating the supernatant containing purified NK cells. The purity of the NK cells was assessed by flow cytometry, staining with monoclonal antibodies as follows: PerCP-CD3 (BioLegend, U.K.) and FITC-CD56 (BioLegend, U.K.).

2.12 Ribonucleic acid (RNA) preparation

Phenol-chloroform extraction of RNA from purified NK cells was performed using TRIzol reagent (Invitrogen, U.K.). The standard protocol was modified to obtain maximum yield of RNA from low numbers of cells. The NK cells were lysed and homogenized in TRIzol (10^6 cells/ml) by vortexing and incubation at room temperature for 10 minutes. Chloroform was then added (0.2 ml per 1ml of TRIzol) and the mixture shaken vigorously by hand for 15 seconds. This was incubated on ice for 5 minutes and then centrifuged at 4^{o}C for 15 minutes. The upper aqueous layer was aspirated and added to 500ul isopropranol/ml TRIzol used. This was incubated at -20^{o}C for 1 hour, followed by 15 minutes centrifugation at 4^{o}C, and the supernatant was discarded. 75% ethanol (500ul/ml TRIzol used) was added to the pellet, and this was incubated at -80^{o}C for 1-24 hours. The mixture was centrifuged 4^{o}C for 15 minutes and the supernatant discarded. The pellet was left for 10-15 minutes to allow the excess ethanol to evaporate and then resuspended in 20ul of DNase- and RNase-free water. DNase treatment was performed with the Turbo DNase kit (Ambion, U.K.) according to manufacturer's instructions. The RNA was then quantified by spectrophotometry and aliquots that were not immediately used were stored at -80 ^{o}C.

2.13 Microarray

NK cells were purified from freshly processed PBMCs and after counting, the cells were lysed and homogenized in 750ul TRIzol. The lysed cells were then sent on dry ice to Miltenyi Biotec in Germany for RNA extraction and microarray analysis. RNA was extracted using a standard TRIzol protocol and quality check performed via the Agilent 2100 Bioanalyzer platform (Agilent Technologies, U.S.A.). 20ng of each total RNA sample was used for the linear amplification of the RNA. Cy3-labelled cRNA was produced using the Agilent Low Input Quick Amp Labelling Kit (Agilent Technologies, U.S.A.) following the manufacturer's protocol. Hybridization was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit (Agilent Technologies, U.S.A.). Briefly, 600ng Cy3-labelled fragmented cRNA in hybridization buffer was hybridized overnight at 65^{o}C to Agilent Whole Genome Oligo Microarrays 8x60K using Agilent's recommended hybridization chamber and oven. The microarrays were washed once with a wash buffer for 1 minute at room temperature followed by a second wash with preheated wash buffer for 1 minute. A last washing step was performed with acetonitrile. Fluorescence signals of the hybridized Agilent Microarrays were detected using Agilent's Microarray Scanner System (Agilent Technologies, U.S.A.).

With the aid of a statistician, the Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files. The software determines feature intensities (including background subtraction), rejects outliers and calculates statistical confidences. For determination of differential gene expression FES derived output data files were further analysed using the

Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware, U.S.A.). The output data of the FES included gene lists with signal intensities normalised by dividing intensity values by their median. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2132.

Pathway and functional analyses were performed using Ingenuity Pathway Analysis 6.0 (IPA; Ingenuity® Systems, www.ingenuity.com). An Excel data set containing gene identifiers and corresponding expression values was uploaded into the IPA programme, and each gene identifier was mapped using the Ingenuity Pathways Knowledge Base (IPKB). The IPKB analyses identify the biological functions as well as the pathways from the IPA library that are most significant to the data set. Fisher's exact test was used to calculate a *p*-value determining the probability that each biological function and/or canonical pathway assigned to this data set was not due to chance alone.

2.14 Quantitative real time reverse transcription polymerase chain reaction (qRT-PCR)

In order to validate the microarray findings, candidate genes were chosen for gene expression analysis in a further cohort of samples. RNA was extracted from purified NK cells as previously described. The expression of 11 candidate genes was analysed using a custom array plate with wells pre-loaded with primers (RT^2 profiler PCR array, SABiosciences, U.K.). 200ng of RNA was used as a template to make complimentary deoxyribonucleic acid (cDNA) with the RT^2

First Strand Kit (SABiosciences, U.K.) according to the manufacturer's instructions. The cDNA was stored at -20^{o}C until required for the PCR assay.

For the real-time PCR assay, the cDNA was thawed and added to RT^{2} SYBR Green Mastermix and RNase-free water according to the manufacturer's instructions; 25ul of this PCR components mix was loaded into each well of the array plate. As well as the genes of interest (GOI), expression of two housekeeping genes was assayed for comparative quantitation. Furthermore the array plate contained three control wells per sample: a) positive PCR control (PPC) to test the efficiency of the PCR using a pre-dispensed artificial DNA sequence and the primer set that detects it; b) genomic DNA control (GDC) containing a primer set detecting non-transcribed genomic DNA; c) reverse transcription control (RTC) to test the efficiency of the cDNA synthesis reaction, containing a primer set detecting template synthesized from a built-in external RNA control.

The array plate was placed into the StepOne cycler (Applied Biosystems, U.K.) using absolute quantitation settings. After amplification the threshold cycle (C_T) for each well was calculated and expression of each GOI was calculated using the comparative C_T (delta-delta C_T, $\Delta\Delta C_T$) method. Dissociation (melting) curve analysis was performed to verify the PCR specificity, ensuring one PCR product per well. Quantitative RT PCR was also performed in triplicate on NK cells from 3 healthy donors incubated with either medium alone, or immunosuppressive drugs at mid therapeutic concentrations: either Prednisolone 20ng/ml, Tacrolimus 5ng/ml, or Cyclosporine A 275ng/ml. Gene expression was analysed between NK cells incubated with the drugs and control (medium alone).

MicroRNA-155 (miR-155) quantitative PCR was performed with the miScript PCR system (Qiagen, U.K.) according to the manufacturer's instructions as follows. Stored RNA extracted by the Trizol method described was thawed, and 200ng was added to the reverse transcription mastermix (containing HiSpec buffer, nucleics mix, reverse transcriptase mix, and RNase-free water) in a final volume of 20 µl. This was mixed gently and incubated for 60 minutes at 37°C, then 5 minutes at 95°C to inactivate the reverse transcription mix. The cDNA was then diluted in RNase-free water and 2ng was used for the PCR reaction. A 25 µl reaction mix was prepared comprising 2x Quantitect SYBR Green PCR master mix, 10x miScript universal Primer, 10x miScript Primer Assay (miR-155), template cDNA, and RNase-free water at room temperature (15–25°C). Human RNU6B (RNU6-2) miScript primers were used for normalization controls, and a no template control was used to exclude non-specific amplification. Reaction mixes were prepared in triplicate in a 96 well plate, which was sealed and centrifuged at 1000g for 1 minute. The plate was then placed into the StepOne cycler (Applied Biosystems, U.K.) using the recommended cycling conditions. After amplification the C_T for each well was calculated and expression of miR-155 was calculated using the $\Delta\Delta C_T$ method.

2.15 Intracellular pSTAT assay

As STAT4 expression was significantly downregulated in liver transplantation (see chapter 4), an assay evaluating phosphorylated intracellular STAT4 expression was performed. The Phosflow kit was used for this (BD Biosciences U.K.) according to the manufacturer's protocol. PBMCs were resuspended in R10

culture medium and stimulated with IL-12 at a concentration of 10ng/ml. After 60 minutes surface staining was performed with Pacific Blue-CD3 (eBiosciences, U.K.) and PECy7-CD56 (eBiosciences, U.K.). The cells were then fixed by incubation with pre-warmed Cytofix buffer (BD Biosciences U.K.) at 37^{o}C for 10-12 minutes. This was followed by incubation in Perm III buffer (1ml/10^{6} cells) on ice for 30 minutes. After washing, Phosflow antibody (AF647-anti STAT4 (pY693), BD Biosciences) was added and the mixture was incubated on ice in the dark for 60 minutes. The cells were washed, resuspended in PBS and analysed by flow cytometry. The assay was also performed using PBMCs from 3 healthy donors incubated for 72 hours with the immunosuppressive medications as previously described.

2.16 Clinical data

Patient information was recorded from clinical notes and database records. Data collected included: age, sex, date of transplant, time since transplant, aetiology of liver disease, immunosuppressive therapy, date of post-transplant biopsy (if done), biopsy details (including rejection and fibrosis score), episodes of acute cellular rejection (ACR) and history of antiviral treatment (if HCV positive). Laboratory data collected included serum alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TB), albumin, internationalised normalised ratio (INR), platelet count, serum concentration of immunosuppressive drugs (Tacrolimus, Cyclosporine, Mycophenylate) and CMV infection status.

2.17 Statistical analysis

Statistical data analysis was performed using GraphPad Prism version 6.0b software (GraphPad Software Inc., U.S.A). Descriptive statistics are shown as mean± standard error of the mean (SEM) as appropriate. Comparisons between groups were made using the χ^2 test or Fischer's exact test for qualitative data. Comparisons between the means were made using the unpaired *t*-test or one-way analysis of variance (ANOVA) as appropriate. All tests were two-tailed and a probability value (p-value) of less than 0.05 was considered significant. Multivariate analysis and logistic regression modelling were used to examine associations where multiple factors were involved. A p-value of less than 0.05 was considered significant.

Statistical analyses of the microarray gene expression data was performed with the statistical analysis software environment R together with packages available as part of the Bioconductor project (http://www.bioconductor.org). Data generated from the Agilent Feature Extraction software for each sample was imported into R. Raw data was quality assessed using scatter plot matrices, boxplots and principal components analysis. Replicate probes were mean summarised and quantile normalised using the pre-process Core R package. The limma R package (Smyth 2004) was used to compute empirical Bayes moderated *t*-statistics to identify differentially expressed genes between groups. A corrected p-value cut-off of less than 0.05 was used to determine significant differential expression.

CHAPTER 3: THE EFFECT OF LIVER TRANSPLANTATION ON NK CELL PHENOTYPE AND FUNCTION

In health, NK cell inhibitory signaling is dominant such that NK cells are held in constitutive inhibition, and therefore do not attack self cells. Release from inhibition can occur through loss of inhibitory and/or increase in activating signals so that the balance swings towards activation, and the NK cell becomes activated. NK inhibitory receptors and their ligands have been well described (see Chapter 1), but much less is known about the activating receptors. An important group of these receptors are the NCRs NKp30 and NKp46. Cross-linking studies have shown the NCRs to play a key role in NK cell activation, and conversely, blocking inhibits NK cell cytotoxicity (Sivori, Vitale et al. 1997, Pende, Parolini et al. 1999). Additionally the activating C-type lectin receptor NKG2D binds MICA and MIC B. The density of the activation receptors on the cell surface correlates with activation status and it has been shown that tumour targets are resistant to NK cells expressing reduced levels of activating receptors (Pessino, Sivori et al. 1998, Pende, Parolini et al. 1999, Sivori, Pende et al. 1999, Sivori, Parolini et al. 2000).

Several processes are associated with reduced NCR expression including malignant transformation (Garcia-Iglesias, Del Toro-Arreola et al. 2009, Pietra, Manzini et al. 2012), HIV infection (De Maria, Fogli et al. 2003) and drugs (Fiegler, Textor et al. 2013). Upregulation of NKp30 and NKp46 have been

demonstrated in chronic viral infections including HCV (Bozzano, Picciotto et al. 2011) and autoimmune disease (Rusakiewicz, Nocturne et al. 2013).

In Chapter 1 the potential for NK cell alloreactivity in LT was discussed in the context of HLA mismatch. Recipient NK cells educated on recipient HLA class I may not express inhibitory receptors for donor HLA (HLA-KIR mismatch), and theoretically should become activated, attacking donor tissue. However, as LT is generally well tolerated, my hypothesis is that in LT recipient NK cells become 'tolerised' by the donor graft. To test this hypothesis, detailed phenotyping and functional analysis of NK cells in LT recipients is presented in this chapter.

RESULTS

3.1 Expression of activating receptors NKp30, NKp46 and NKG2D

In order to determine the activating phenotype of recipient NK cells in liver transplant recipients, the surface expression of the NK activating receptors NKp30, NKp46 and NKG2D was determined.

3.1.1 Expression of activating receptors on total NK cells

Phenotyping of NK cells from 54 liver transplant (LT) recipients (36 non-HCV and 18 HCV) and 31 healthy controls was performed by flow cytometry. A five-fluorochrome panel was designed allowing measurement of activating receptor expression in total NK cells, $CD56^{bright}$ and $CD56^{dim}$ subpopulations, and KIR2DL1 (CD158a) and KIR2DL2/3 (CD158b) subsets. **Figure 3.1** shows the gating strategy employed in this phenotyping assay.

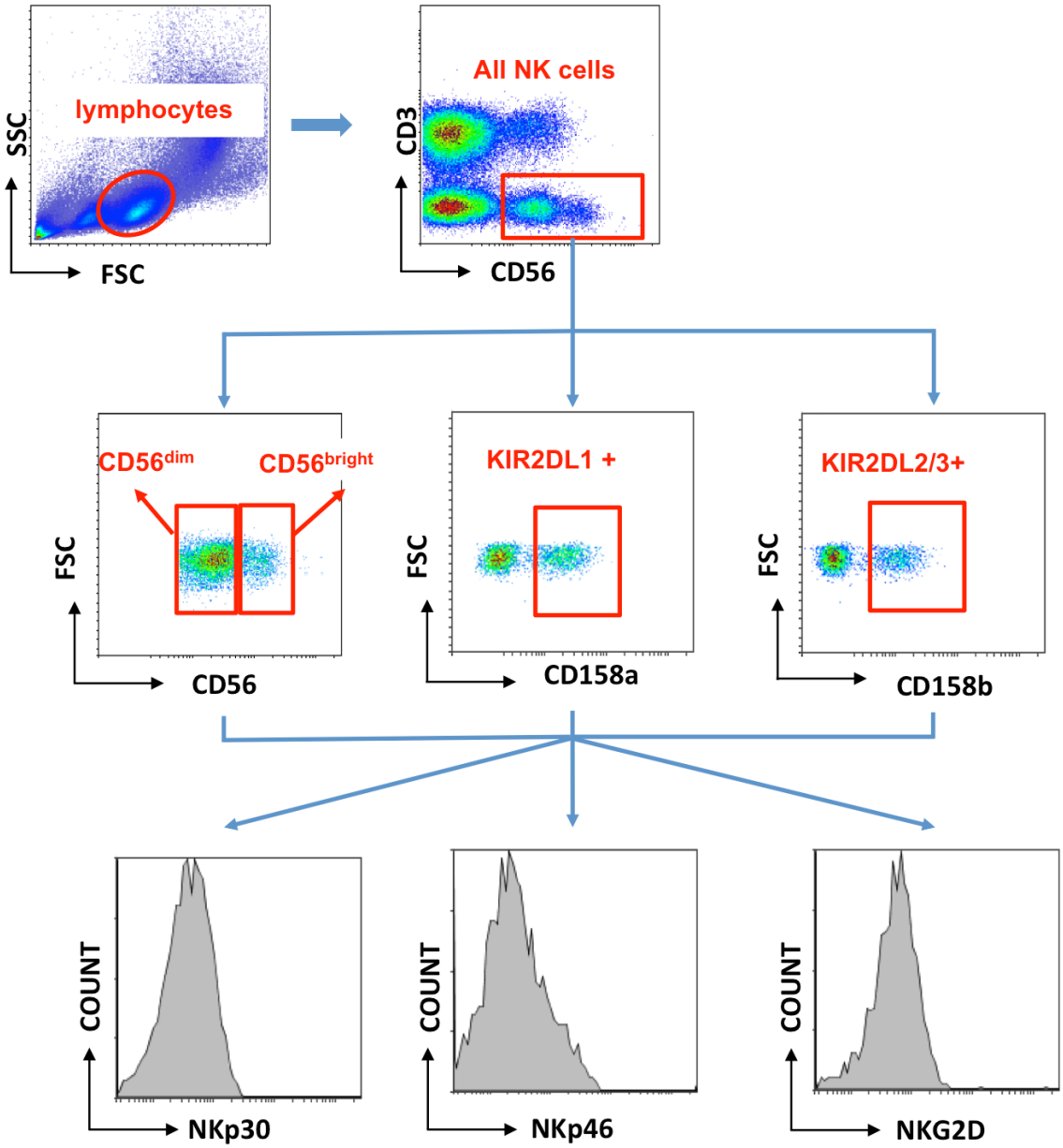


Figure 3.1 Gating strategies for NK cell activation phenotyping.

NK cells were identified as lymphocytes that were CD56+/CD3-. Total NK cells, and CD56^{bright}, CD56^{dim}, CD158a+ and CD158b+ subsets were then analysed for expression of the activating receptors NKp30, NKp46 and NKG2D

There was no difference in the mean fluorescence intensity (MFI) of any of the activating receptors between all LT recipients and healthy controls (**figure 3.2**).

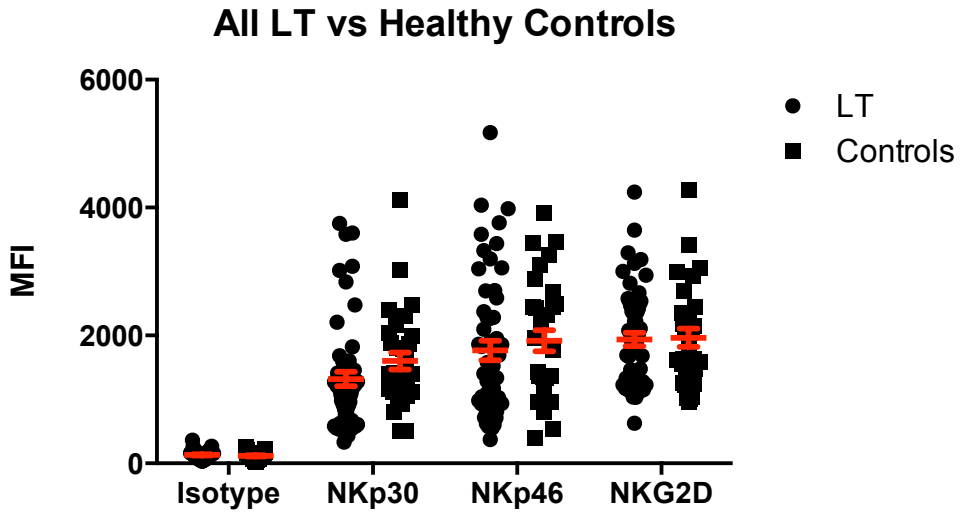


Figure 3.2 Surface expression of activating receptors on NK cells in LT recipients. *Comparison of activating receptor expression was made between LT recipients and healthy control subjects.*

HCV infection is associated with NK cell activation, and therefore the LT recipients with HCV infection (LT HCV) were analysed separately from LT recipients without HCV infection (LT non-HCV). This demonstrated highly significant down regulation of the natural cytotoxicity receptor NKp30 in LT non-HCV compared with both healthy controls and LT HCV ($p < 0.0001$ for both, **figure 3.3**). Furthermore, there was reduced NKp46 expression in LT non-HCV compared with healthy controls ($p=0.05$) and LT HCV ($p=0.006$). There were no differences in NKG2D expression between any of the groups and no differences were found between LT HCV and healthy controls.

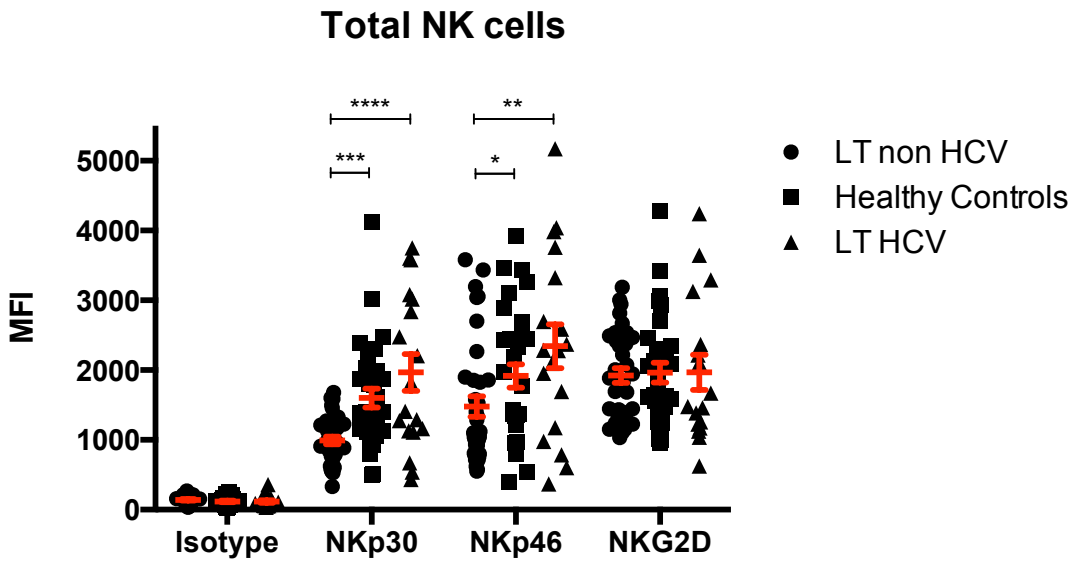


Figure 3.3 Activating receptor expression in HCV and non-HCV LT.

*Comparisons between HCV and non-HCV LT subsets and healthy controls are shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.1.2 Expression of activating receptors on NK subsets

NK cells undergo several stages of development and function varies accordingly. $CD56^{bright}$ cells are immature and are precursors of $CD56^{dim}$ NK cells. Furthermore, only fully functional 'licensed' NK cells express KIR. Therefore the expression of the activating receptors was examined in $CD56^{bright}$, $CD56^{dim}$ and $KIR2DL^{+}$ subsets.

Within KIR^{+} NK cells there was highly significant downregulation of both NKp30 and NKp46 in LT non-HCV compared with healthy controls ($p<0.0001$ and $p=0.00015$ respectively), and LT HCV ($p<0.0001$ for NKp30 and $p= 0.0028$ for NKp46) as shown in **figure 3.4.** The expression of NKp30 was significantly higher in LT HCV compared with HC ($p=0.013$). In order to assess activating expression in KIR^{-} NK cells, PBMCs were stained for KIR2DL1 and KIR2DL2/3,

and analysis performed in NK cells negative for both stains (LT non-HCV n=14, LT HCV n=8, HC n=12). In contrast to KIR^{+} cells, no differences in NCR expression was observed on KIR^{-} cells between the three groups, although there was increased NKG2D in LT non-HCV vs. controls (**figure 3.4**).

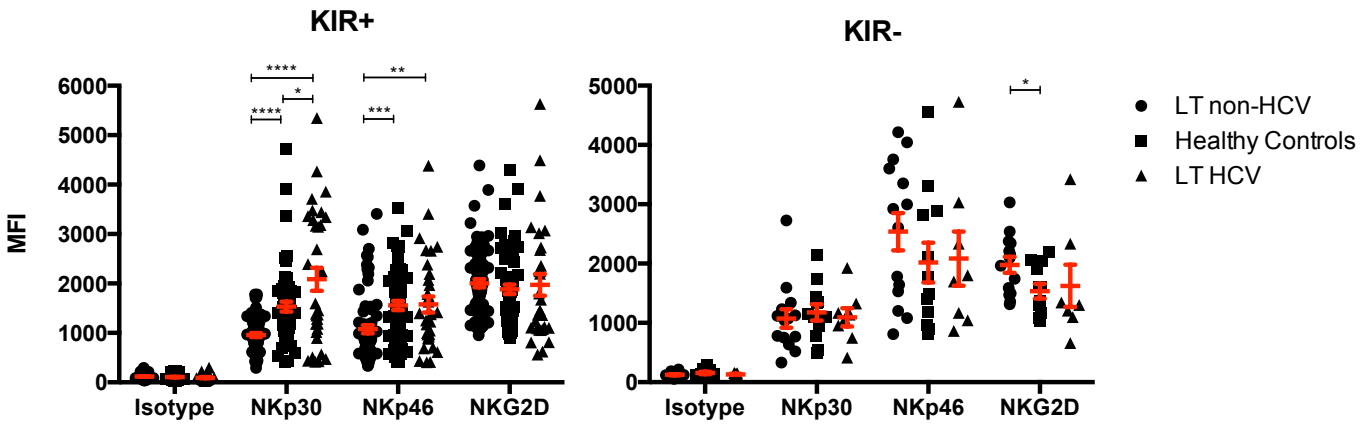


Figure 3.4 Activating receptor expression in KIR expressing NK cells.

*Analysis of KIR^{+} and KIR^{-} NK subsets are shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

Compared with healthy controls, in both LT groups there was a significant increase in the proportion of CD56^{bright} NK cells relative to total NK cells (p=0.012 and 0.0001 respectively, **figure 3.5A**). In LT non-HCV, reduction in NKp30 expression was present only in the CD56^{dim} population and not in the CD56^{bright} population as shown in **figure 3.5B,C.** Furthermore, the reduction in NKp46 expression was less marked in CD56^{bright} NK cells in comparison with CD56^{dim} NK cells (fold change in MFI vs healthy controls was -1.3 in CD56^{bright} vs -1.6 in CD56^{dim}). Once again, no reduction in any activating receptor expression was observed in NK cell subsets in HCV LT recipients compared with healthy controls.

Thus liver transplantation has a differential effect on NK subsets, with the most marked down regulation of natural cytotoxicity receptors observed in the mature $CD56^{dim}$ subsets.

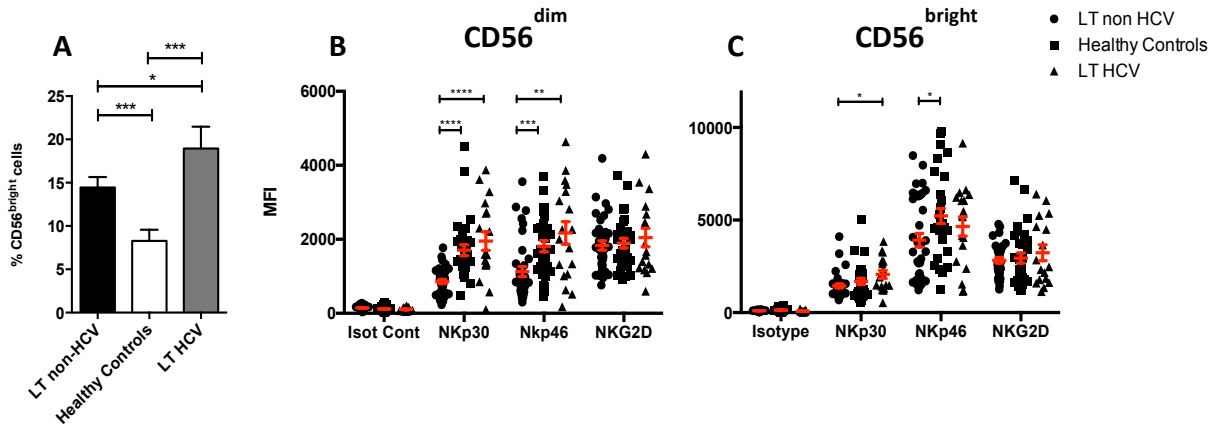


Figure 3.5 LT effects on $CD56^{bright}$ and $CD56^{dim}$ NK subsets.

***A** Relative percentage of $CD56^{bright}$ NK cells. **B, C** Activating receptor expression in $CD56^{dim}$ and $CD56^{brights}$ NK subsets. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.1.3 Age and expression of natural cytotoxicity receptors

The LT recipient cohort was significantly older than the healthy controls within this study (55 years vs 41 years, p<0.0001, see **table 2.1**). Studies in mice and humans have reported that ageing is associated with NK cell functional decline, but this has largely been observed when comparing the young with the very old (Rukavina, Laskarin et al. 1998, Solana and Mariani 2000, Mocchegiani and Malavolta 2004, Chiu, Martin et al. 2013). Therefore the age difference between LT recipients and healthy controls is unlikely to be of significance and indeed, there was no association between age and NCR expression within the entire cohort (**figure 3.6**).

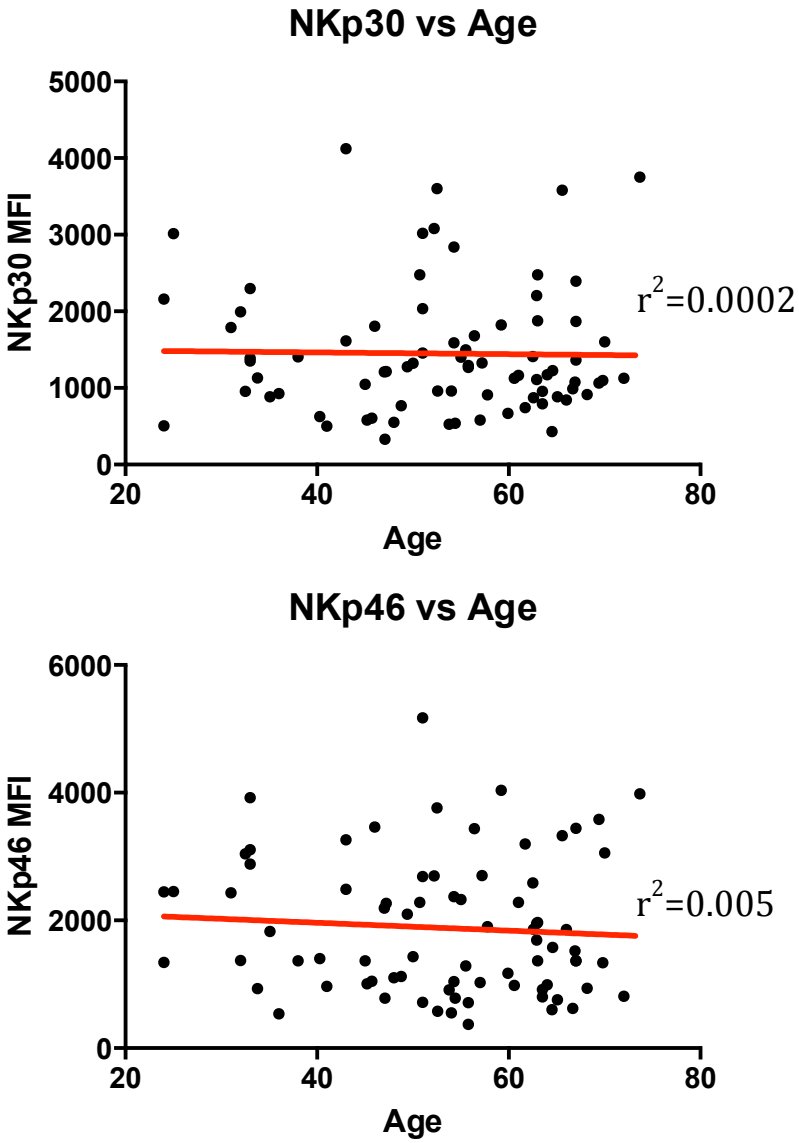


Figure 3.6 Age and NCR expression.

3.2 Effect of liver transplantation on NK cell function

The phenotyping assays demonstrated significant downregulation of activating receptors on liver transplant recipient NK cells. This effect was largely seen in the mature $CD56^{dim}$ subset and less so in the $CD56^{bright}$ population, which was expanded in LT recipients. Furthermore, the changes observed were not present in LT recipients with HCV infection.

To assess NK activation further, NK cell function was investigated. NK cell effector functions include cytokine production, cross-talk with dendritic cells and direct and indirect cell lysis. Therefore readouts of NK cell function include NK cell cytotoxicity, degranulation, and cytokine production. Experimental assays were designed to compare these functions in LT recipient NK cells with healthy controls and these are described below.

3.2.1 Cytotoxicity assay

Once released from inhibition, natural killer cells can directly lyse target cells through direct contact and exocytosis of perforin/granzymes containing granules (Shresta, Pham et al. 1998). In order to assess NK cell specific cytotoxicity I set up a flow cytometry based assay as described in the methods. Target MHC class I deficient K562 cells were labeled with a fluorescent marker before co-culture with effector cells for 4 hours. After incubation, the cells were stained with a live-dead stain, so that the proportion of target cells that had been killed was a measure of cytotoxicity (Kim, Donnenberg et al. 2007).

PBMCs from healthy donors were used to perform assay optimisation experiments. These were stimulated overnight with IL-15. The following day, K562 cells were incubated for 1 hour with a CellTracker™ Orange (CTO) dye. The K562 cells were then incubated with stimulated PBMCs for 4 hours followed by staining with a live-dead stain. The flow cytometry gating strategy used to assess specific cytotoxicity is shown in **figure 3.7**.

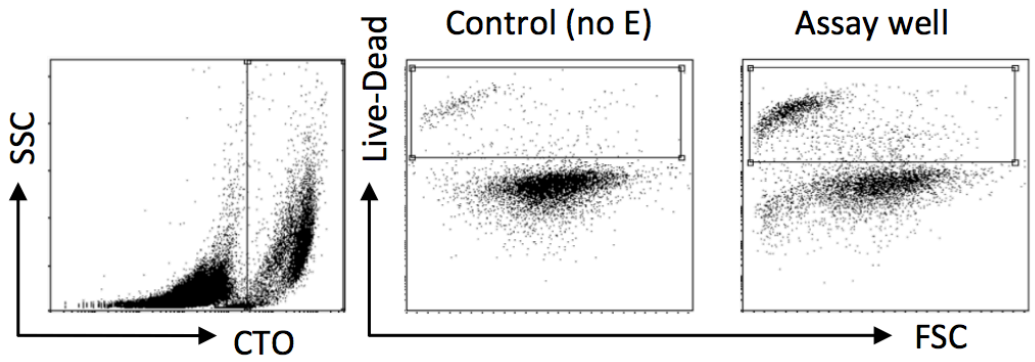


Figure 3.7 Specific cytotoxicity assay for NK cells.

The assay was performed as described in Materials and Methods and analyses performed by flow cytometry. K562 cells were identified as CTO positive and % death in these cells quantified as % live-dead stain positive. Specific cytotoxicity was calculated by subtracting % death in the control well (no effector cells) from % death in the assay well.

The target cells were identified as CTO positive, and a live-dead stain was used to quantify the proportion of dead cells. Specific cytotoxicity was calculated by subtracting the % target cell death with no effector cells from the % target cell death in assay wells as described in the Materials and Methods. To determine the optimal ratio for the assay the K562 cells (T) were incubated with PBMCs (E) at varying E:T ratios (**figure 3.8)**. Cytotoxicity increased sharply as the E:T ratio increased with a plateau at an E:T of 10:1 and therefore this ratio was used for all further cytotoxicity experiments.

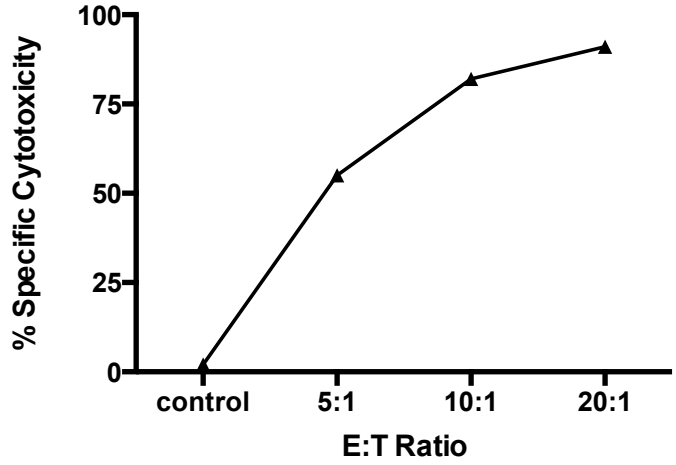


Figure 3.8 Determination of optimal E:T ratio for cytotoxicity assay.

The cytotoxicity assay was performed as described in the Materials and methods. PBMCs (E) were incubated with K562 cells (T) at varying E:T ratios to determine the optimal E:T ratio for cytotoxicity. Specific cytotoxicity was calculated relative to control which contained no effector cells.

The experimental assays demonstrated significantly reduced specific cytotoxicity by NK cells from non-HCV LT recipients compared to healthy control subjects (p=0.0004) as shown in **figure 3.9**.

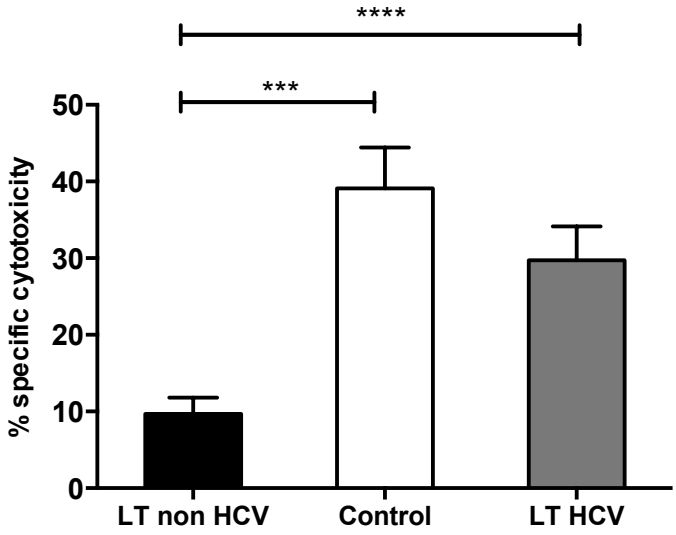


Figure 3.9 Specific cytotoxicity in LT recipient NK cells vs controls.

****p<0.001, ****p<0.0001.*

Moreover, correlating with the activating receptor data, this reduced cytotoxicity was not observed in HCV LT. Specific killing in HCV LT was significantly higher than in non-HCV LT ($p<0.0001$).

3.2.2 CD107a degranulation assay

A commonly used measure of NK cell activation is the CD107a (lysosomal-associated membrane protein-1, LAMP-1) assay. NK cells contain cytolytic granules within their cytoplasm that are released at the immunological synapse when NK cells are activated by target cell contact. The granules are lined by a membrane containing the CD107a protein, which is a glycoprotein making up approximately half of the proteins within the lysosomal membrane (Fukuda 1991). As NK cells degranulate, CD107a becomes externalized and this can be detected by antibody binding and thus is suitable for staining by flow cytometry (Alter, Malenfant et al. 2004).

Once again, the MHC class I deficient K562 cell line was used as a target to stimulate NK cells. For optimisation of the assay, NK cells from healthy donors were used. K562 cells were co-incubated with IL-15 stimulated NK cells and multiparameter staining performed. NK cells were identified as $CD56^{+}CD3^{-}$ and the frequency of degranulating NK cells was quantified by CD107a expression (**figure 3.10**).

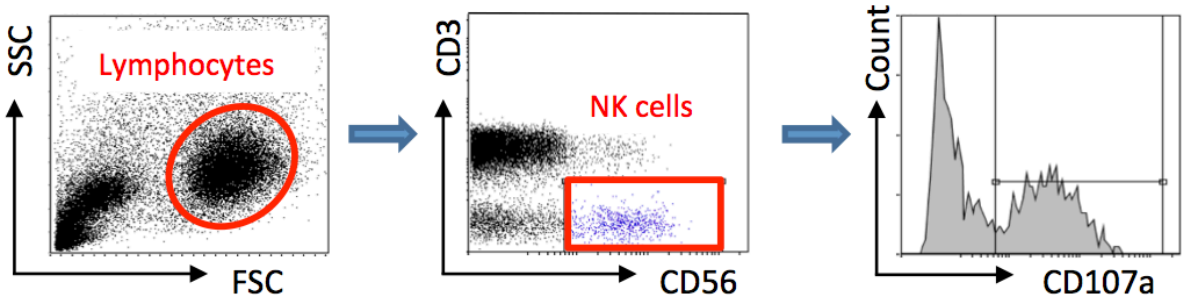


Figure 3.10 Gating strategy for CD107a (LAMP-1) assay.

NK cells were identified from the lymphocyte gate as CD3⁻CD56⁺. The frequency of CD107a expression within NK cells was measured.

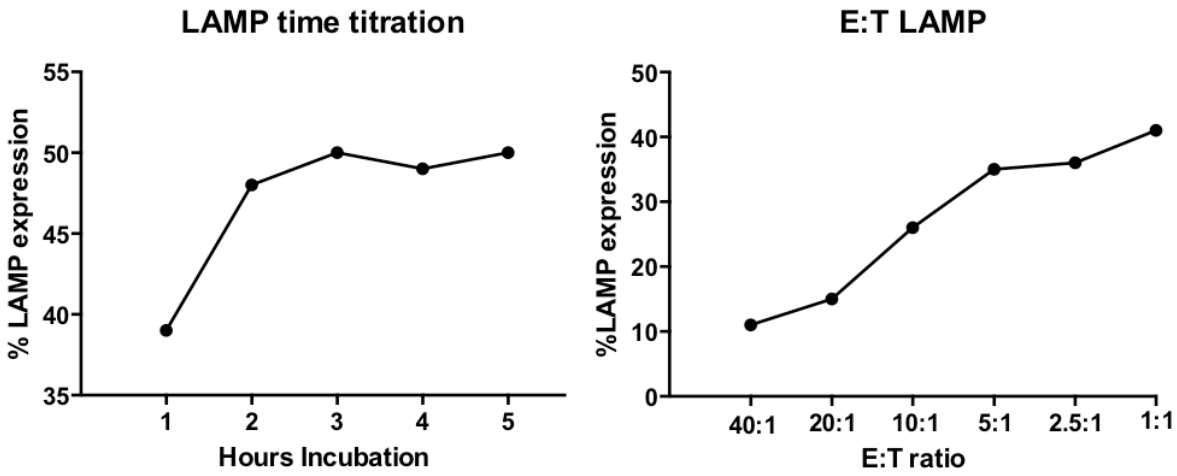


Figure 3.11 Optimisation of CD107a assay.

Determination of optimal incubation time and E:T ratio was performed by titrating the ratio of PBMCs:K562 cells and the length of co-incubation. CD107a expression in NK cells was quantified by flow cytometry as described.

To determine the optimal timing and E:T ratio for the assay, NK cells were incubated with K562 cells at E:T ratios from 40:1 to 1:1, and for between 1 and 5 hours **(figure 3.11**). The plateau for CD107a expression was between 3 and 5 hours, and for the E:T ratio, the plateau was at an E:T of 5:1. Therefore an E:T of 5:1 with an incubation time of 4 hours was used for all further CD107a assays.

A significant reduction in CD107a expression was observed in LT non-HCV recipients compared with healthy controls (p=0.035) and LT HCV recipients (p=0.036) as shown in **figure 3.12**. CD56^{bright} cells as well as CD56^{dim} NK cells express CD107a and as an increase in CD56^{bright} cells was found in LT (see Section 3.1), these NK subsets were analysed for CD107a expression. No differences were observed in CD107a expression in CD56^{bright} NK cells, but within the CD56^{dim} NK subset, CD107a expression was significantly reduced in non-HCV liver transplant recipients compared with healthy controls (p=0.025).

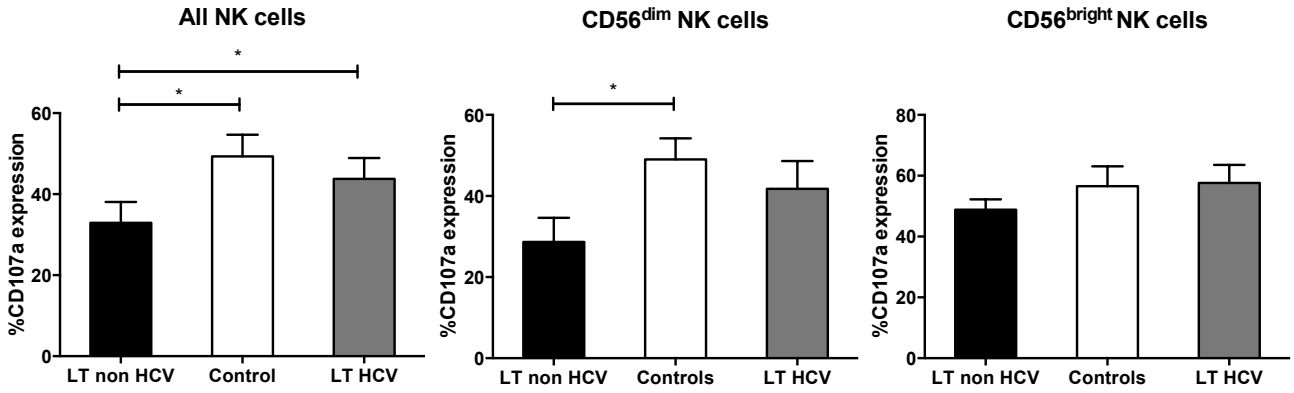


Figure 3.12 CD107a assay in LT recipients.

*Analyses of all NK cells, CD56^{dim} and CD56^{bright} subsets are shown. *p<0.05*

3.2.3 Intracellular cytokine assay

As well as cytolysis, cytokine production is a key function of NK cells. Early in the immune response NK cells release vast quantities of cytokines, particularly IFNγ and TNFα. These are important in the recruitment and activation of multiple other cells in both the innate and adaptive immune response (Moretta, Marcenaro et al. 2008, Fauriat, Long et al. 2010). As our data has demonstrated hypofunctionality of NK cell cytotoxicity in LT, cytokine production in stimulated NK cells was assessed with a flow cytometry based assay.

Whilst cytokine production in stimulated PBMCs can be measured by enzyme-linked immmunosorbant assay (ELISA) in supernatants, this is a non-specific method, which does not discriminate between the cell types producing the cytokines. In contrast, flow cytometry based methods can measure cytokine production in specific cell types, and thus an intracellular cytokine assay was employed to measure production of IFNγ and TNFα in NK cells. In order to determine the optimal conditions for the assay, healthy donor PBMCs were stimulated overnight with IL-15 and then co-cultured with target K562 cells at varying E:T ratios and incubation times. After the incubation, the PBMCs were stained for cell surface markers CD56 and CD3 to identify NK cells followed by permeabilisation. The cells were then stained with antibodies to intracellular cytokines and analysis was performed by flow cytometry. The time and E:T titrations are shown in **figure 3.13.** In order for both cytokines to be measured in the same assay wells, an E:T of 5:1, with a 4 hour incubation period was used for this assay.

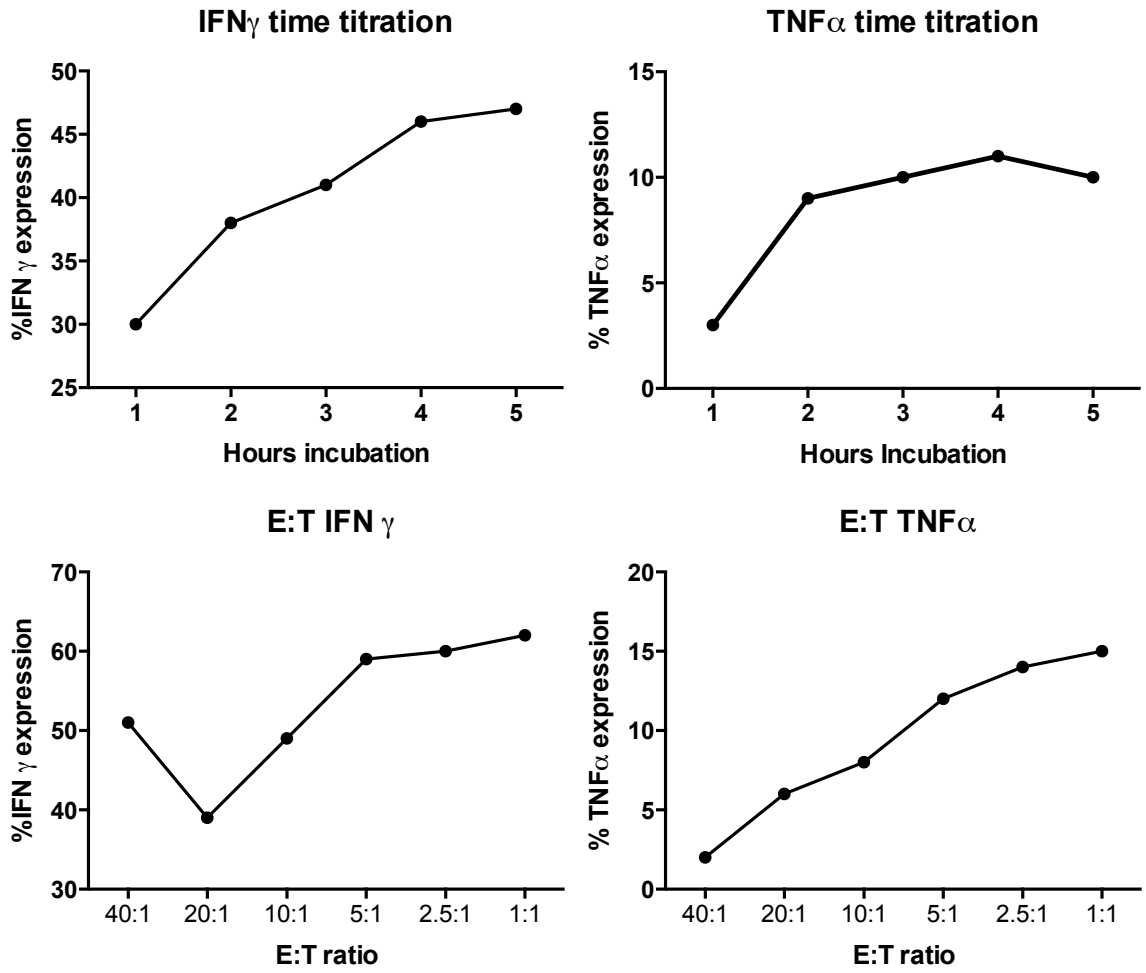


Figure 3.13 Optimisation of intracellular cytokine assay.

IL-15 stimulated PBMCs from healthy donors were co-incubated with K562 cells at varying E:T ratios and for varying incubation times as shown. The PBMCs were then surface stained for NK cells ($CD3^{-}CD56^{+}$) and permeabilised followed by intracellular cytokine staining. The optimal conditions allowing both IFNγ and TNFα quantification in the same assay were determined.

Following stimulation with K562 target cells, there was reduced intracellular expression of IFNγ in LT non-HCV recipient NK cells compared with healthy controls (p=0.020) and LT HCV recipients (p=0.001, **figure 3.14A**). The difference was also observed in the $CD56^{dim}$ NK cell subset (p=0.008 for both LT non-HCV vs. healthy control and LT non-HCV vs. LT HCV). In $CD56^{bright}$ NK cells IFNγ expression was significantly lower in LT non-HCV compared with LT HCV

(p=0.0001). No significant differences were observed in TNFα production between the 3 groups, although there was a trend towards reduced TNFα in LT non-HCV compared with controls in all NK cells (p=0.08) and in the $CD56^{dim}$ subset (p=0.06), **figure 3.14B**.

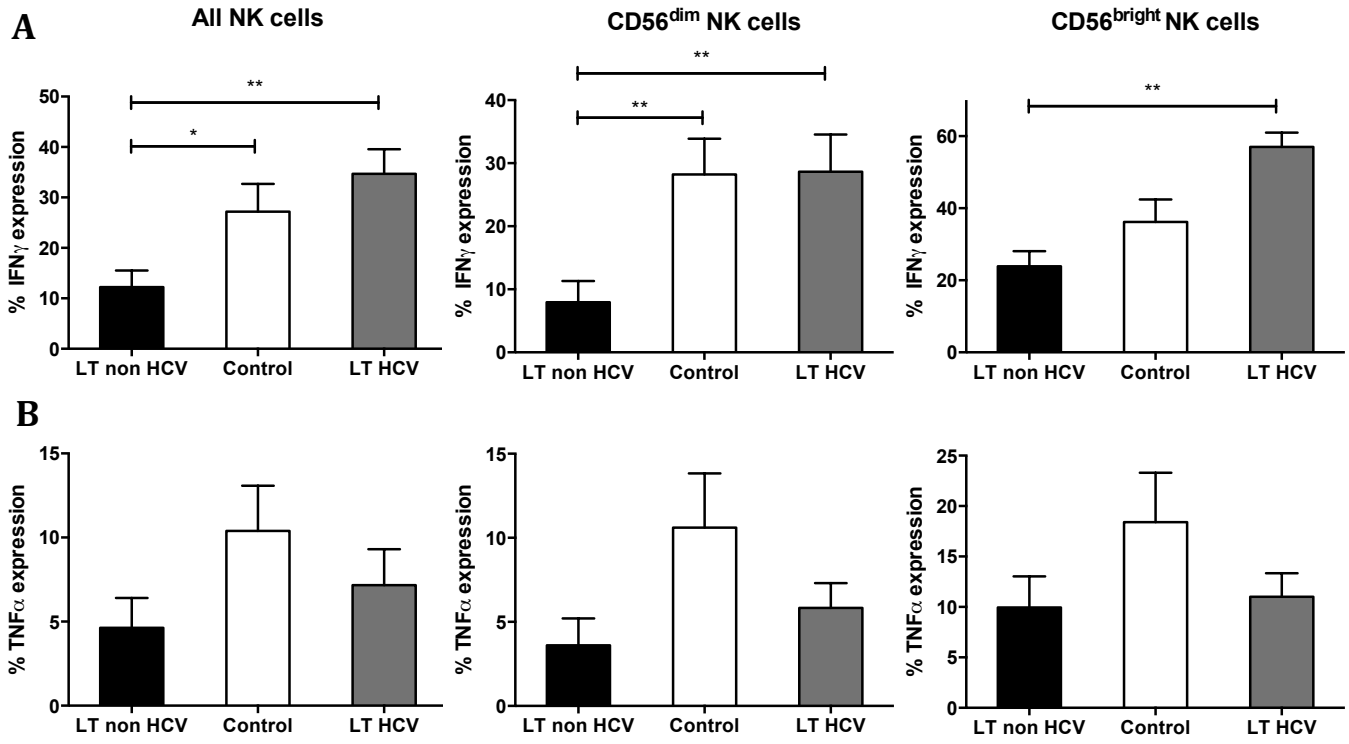


Figure 3.14 Intracellular cytokine expression in LT.

*Expression in all NK cells, as well as $CD56^{dim}$ and $CD56^{bright}$ subsets was determined using flow cytometry. **A** IFNγ expression **B** TNFα expression. *p<0.05, **p<0.01*

3.3 NK cell differentiation

The data thus far provides compelling evidence that in non-HCV liver transplantation recipient NK cells exhibit a hypofunctional phenotype. Interestingly, the differences in activation between LT recipients and healthy controls were observed in $CD56^{dim}$ but not in less mature $CD56^{bright}$ populations. This is suggestive of an altered NK differentiation and maturation process in LT recipients. The next stage of experiments was designed to establish whether markers of NK maturation correlated with these data.

CD16 (FCγRIII) is a transmembrane protein present on most $CD56^{dim}$ NK cells but expressed by very few peripheral $CD56^{bright}$ cells. It co-localises with CD3ζ and Fc-εR1-γ and binds to the Fc portion of IgG antibodies (Anegon, Cuturi et al. 1988). CD16 is acquired at advanced stages of NK cell differentiation, and the expression levels of CD16 correlates with the level of NK cell maturation (Moretta 2010). CD57 antigen is a surface marker previously associated with senescence in $CD8^+$ T cells, and more recently has been shown to be a marker of terminal differentiation in NK cells (Bjorkstrom, Riese et al. 2010, Lopez-Verges, Milush et al. 2010). There is increasing evidence that the activating receptor NKG2C is a marker of NK cell differentiation and adaptation particularly in response to viral infection (Lopez-Verges, Milush et al. 2011, Beziat, Liu et al. 2013).

Therefore CD16, CD57 and NKG2C are markers of mature NK cells. In order to determine whether the activation data was corroborated by maturation

phenotype, the surface expression of these was compared between LT recipients and healthy controls.

Flow cytometry assays were designed incooperating CD3, CD56, CD57, CD16, and NKG2C allowing analysis of the maturity markers in NK cells. Overall, there was no difference in expression of CD16 or CD57 between LT recipients (HCV and non-HCV) and healthy controls, but there was increased NKG2C in LT non-HCV compared to controls (p=0.019, **figure 3.15**). Within the LT cohort, there was a trend towards increased NKG2C positivity in CMV seropositive individuals (**figure 3.16**).

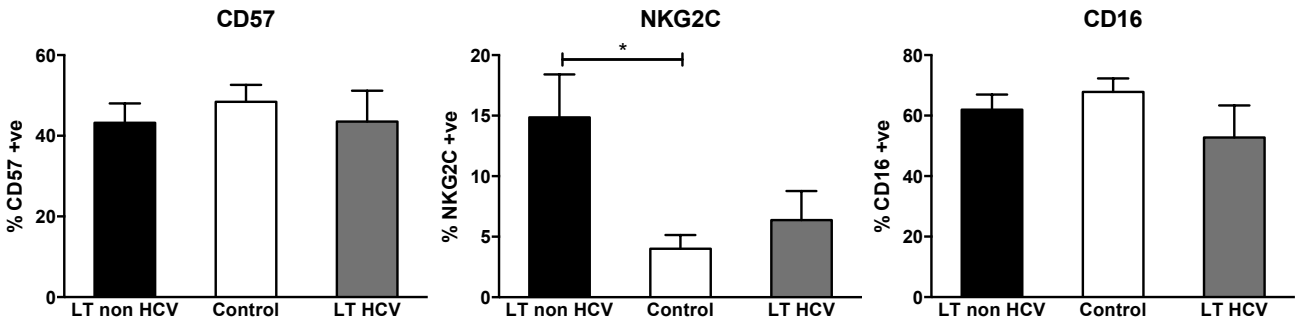


Figure 3.15 Markers of NK cell differentiation.

**p<0.05*

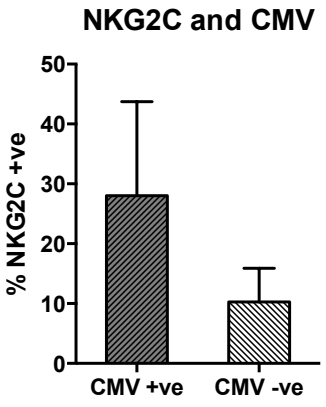


Figure 3.16 NKG2C and CMV.

3.4 Effect of immunosuppression on NK cells

The key experimental findings support a hypothesis that liver transplantation results in defective recipient natural killer cell maturation leading to a hypofunctional phenotype. Recipients of liver allografts necessarily take immunosuppressive medication, largely based around calcineurin inhibitors (Cyclosporine A and Tacrolimus). In the phenotyped cohort 47/54 (87%) LT recipients were on either Tacrolimus (43 patients) or Cyclosporine A (4 patients). These agents target the adaptive immune response, inhibiting T-cell activation and proliferation. The effect of immunosuppressive medication on natural killer cell activation is controversial. Several studies investigating this have reported conflicting findings (as discussed in Chapter 1), such that a consensus has not been reached. It is therefore important to address the fundamental question of whether the observed changes in recipient NK cells might be due to the immunosuppressive agents taken by the liver transplant recipients.

This was approached by performing a series of *in vitro* assays, incubating PBMCs from several healthy donors with commonly used immunosuppressants, followed by assessment of NK cell phenotype and function. The results of these experiments are described below.

PBMCs from healthy donors were incubated with increasing concentrations of the immunosuppressive medications or medium alone and phenotyping assays were performed at 16 and 72 hours. At the 16 hour time point, no dose effect

was observed on the expression of the activating receptors over the therapeutic ranges of any of the immunosuppressive agents (**figure 3.17A-C**). At the mid-therapeutic concentrations, there was a reduction in NKp30 ($p=0.05$) and NKG2D ($p<0.0001$) with Prednisolone **figure 3.17G**. At 72 hours there was a trend to downregulation of NKp30 and NKG2D with increasing concentrations of Prednisolone, but no other dose effects were observed (**figure 3.17D-F**). At mid-therapeutic concentrations, no significant effects of the immunosuppressive agents were observed on the expression of activating receptors (**figure 3.17H**).

The *in vitro* effects of immunosuppressants on NK cell function were also assessed. No effects were seen on cytotoxicity at either 16 or 72 hours. The only effect on CD107a expression was reduced expression with Cyclosporine A at 72 hours (**figure 3.18**). Thus the phenotypic and functional changes observed in liver transplantation were not recapitulated by the *in vitro* incubation assays.

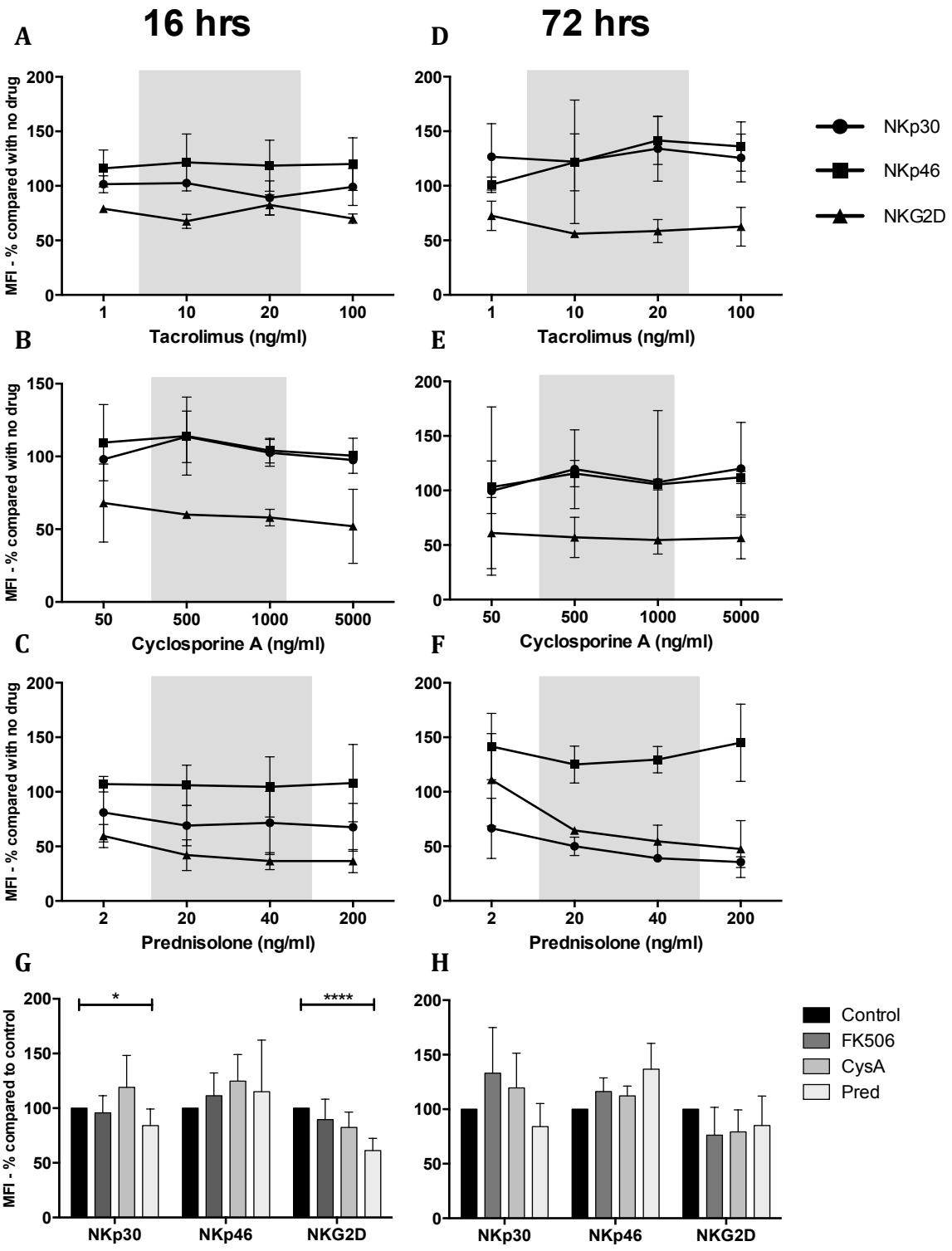


Figure 3.17 The effect of immunosuppressive drugs on NK cell phenotype in vitro.
Activating receptor expression at 16 hour ***(A-C)*** *and 72 hour* ***(D-F)*** *time points.* ***G, H*** *Summary of effect of immunosuppressive agents mid-therapeutic concentrations at 16 and 72 hours. *p<0.05, ****p<0.0001.*

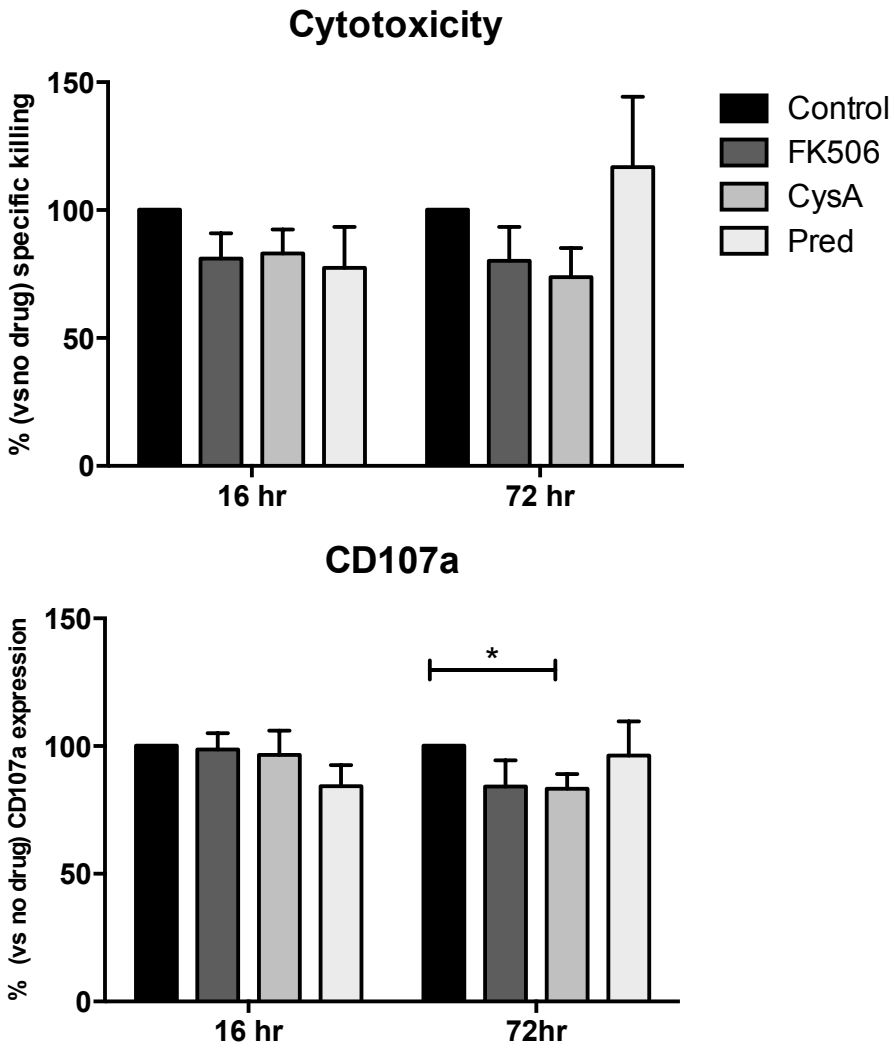


Figure 3.18 Effect of immunosuppressants on NK cell function.

*Results form cytotoxicity and degranulation assays are shown. *p<0.05*

3.5 Association of NK cell activation with clinical outcomes

Natural killer cell activation and functionality is clearly important in the host defense against infection and tumour transformation. Whilst NK alloreactivity against donor grafts is predicted in solid organ transplantation, it is unclear what contribution this makes to clinical outcomes such as rejection and graft survival. In order to investigate whether the reduced recipient NK cell activation found in our liver transplant recipient cohort was associated with clinically significant outcomes, clinical data was collected from case notes and databases. In particular, documented episodes of acute cellular rejection (ACR) were recorded, as well as the findings from any post-transplant biopsies taken including fibrosis score. NK cell activation was compared between individuals who had an episode of ACR and those that did not, and also between individuals who had developed advanced fibrosis at biopsy and those with early stage fibrosis (as defined in Chapter 2).

In order to control for the higher NCR expression observed in LT recipients infected with hepatitis C virus, these patients were excluded from the analysis of NCR expression vs ACR. Of the 36 LT non-HCV recipients 4 had documented episodes of ACR (11%). There was no difference in activating receptor expression between individuals that experienced an episode of ACR and those that did not (**figure 3.19**).

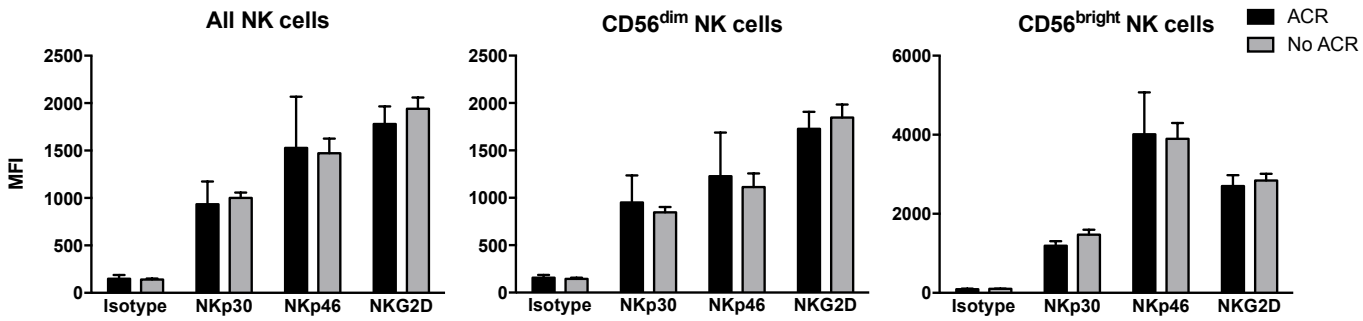


Figure 3.19 Activating receptor expression and acute cellular rejection.

Of the 54 LT recipients phenotyped, 47 (83%) had undergone a liver biopsy at follow-up. Only one LT non-HCV individual had advanced fibrosis at biopsy, therefore it was not possible to perform an analysis of NCR and development of advanced fibrosis in the LT non-HCV group. Within the LT HCV cohort, 17 individuals underwent a liver biopsy: 6 (35%) had evidence of advanced fibrosis and 11 (65%) had early stage fibrosis. There was significantly higher NKp46 expression in LT recipients with advanced fibrosis compared with those who had early stage fibrosis (**figure 3.20**). However the time to biopsy was significantly different between the two groups (830 days in F0-3 group vs 1659 days in F4-6 group, p=0.04), and this may well confound the results. No difference in NK cytotoxicity was observed between the two fibrosis groups (**figure 3.21**).

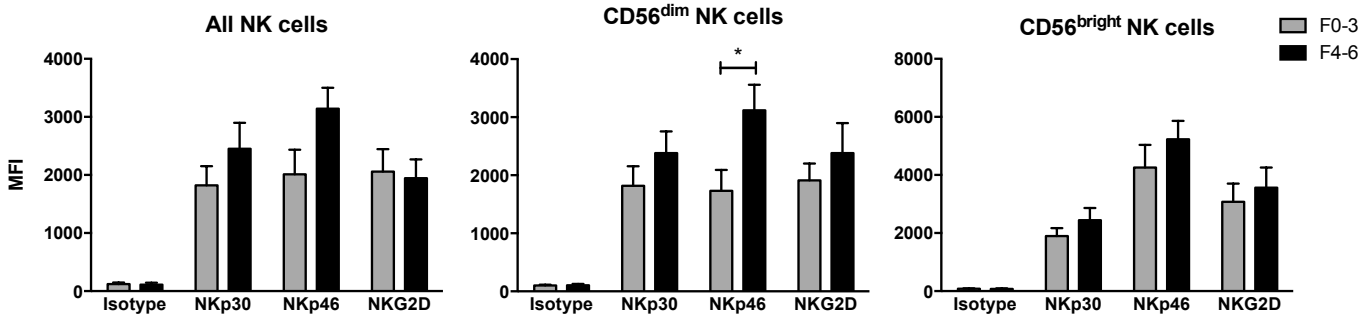


Figure 3.20 Activating receptor expression and fibrosis score.

**p<0.05*

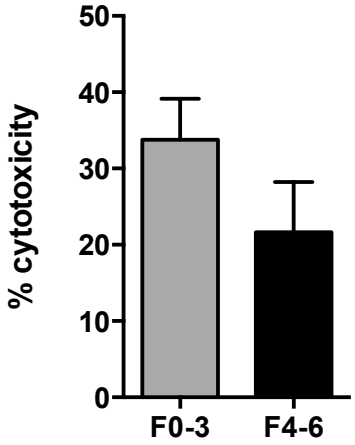


Figure 3.21 Cytotoxicity and fibrosis score.

3.6 Discussion

The phenotype and function of recipient natural killer cells after liver transplantation was assessed using flow cytometry based assays. Compared with healthy controls, I observed a significant downregulation in the expression of activating natural cytotoxicity receptors (NCRs) and, correlating with this, reduced functionality including cytotoxicity, degranulation and cytokine production.

These data challenge the conventional paradigm that recipient NK cells are not influenced by allografts. Previous animal studies have also provided evidence for NK cell involvement in transplantation. For example NK cells were found to infiltrate rodent cardiac allografts a few days after transplantation (Dresske, Zhu et al. 1997), and in mouse models, acute rejection of cardiac allografts has been associated with NK cells expressing NKG2D (Feng, Ke et al. 2009). Furthermore NK cells expressing the activation marker Ly49G2 have been shown to be present in murine cardiac allograft vasculopathy lesions, suggesting a role in chronic rejection (Russell, Chase et al. 2001, Uehara, Chase et al. 2005, Uehara, Chase et al. 2005)

However, the literature concerning NK cell activation in human solid organ transplantation is sparse. The expression of NKG2D ligands MICA/MICB on renal and pancreatic transplants has been associated with acute rejection (Hankey, Drachenberg et al. 2002, Suarez-Alvarez, Alonso-Arias et al. 2009, Suarez-Alvarez, Lopez-Vazquez et al. 2009). Another study demonstrated NK cells infiltrating the graft in acute rejection of renal transplants (Hidalgo, Sis et al.

2010). In liver transplantation, NK cells from recipients were shown to exhibit greater cytotoxicity in HLA mismatched LT compared with matched LT at day 21, but no association with acute rejection was observed (Oertel, Kohlhaw et al. 2001). Thus studies to date have provided very limited evidence of the NK response to allograft, but suggest that NK cells can be involved in acute rejection. My data set is the first to describe the long-term effects of transplantation on recipient NK cells, characterizing the peripheral NK cell phenotype in LT recipients. In demonstrating that NK cells do indeed exhibit downregulated activation, the results may explain why liver allografts are well tolerated.

One possible mechanism underlying this NK cell tolerance is a direct effect of interaction between circulating NK cells and the allograft; this could result in downregulated activation in mature NK cells, or altered maturation/development of immature cells. In order to address whether LT has similar effect on NK cells at different stages of development, phenotyping assays were designed to identify $CD56^{bright}$ and $CD56^{dim}$ subsets of NK cells, which differ in their stage of maturation. There was an increase in $CD56^{bright}$ cells (as a proportion of total NK cells) in LT recipients and the reduction of NCR expression was most marked in the $CD56^{dim}$ cells. $CD56^{bright}$ NK cells generally do not express KIR, and consistent with this, the NCR changes were most striking in KIR^+ NK cells whilst no downregulation in NCR expression was seen in KIR^- cells.

These data provide evidence of altered development of recipient NK cells in liver transplantation. In the LT recipient cohort, the higher relative abundance of peripheral $CD56^{bright}$ NK cells is suggestive of a change in maturation induced by

the allograft. The observed downregulation of activation in $CD56^{dim}$ NK cells, but not in the $CD56^{bright}$ subset suggests that the maturation defect occurs between the two stages of NK development, and that this results in hypofunctionality.

The *in vitro* immunosuppression assays were performed to investigate whether the findings in LT recipient NK cells could be a direct effect of immunosuppressive therapy. Nearly 90% of the cohort were taking calcineurin inhibitor based immunosuppression, and thus the assays were representative of the LT recipient cohort. Whilst there are limitations to the *in vitro* experiments, these data demonstrate that NK cell hypofunctionality is not a direct effect of immunosuppressive medication on mature NK cells. However, it is possible that the immunosuppressants have upstream effects on developing NK cells prior to full maturation. A previous study investigating the effects of Cyclosporine on NK cells reported influences on development, with increased $CD56^{+}CD16^{-}KIR^{-}$ cells, and increased IFNγ production (Wang, Grzywacz et al. 2007).

None of the reduced activation phenomena were observed in NK cells from liver transplant recipients with HCV infection. In fact, an increase in NCR expression was observed in HCV LT recipients compared with non-HCV. In the non-transplant setting, NK cells are thought to be chronically activated, and have been shown to express higher levels of activating receptors NKp30, NKp44, NKp46 and NKG2D (De Maria, Fogli et al. 2007, Ahlenstiel, Titerence et al. 2010, Harrison, Ettorre et al. 2010), with increased cytotoxicity (Duesberg, Schneiders et al. 2001, Golden-Mason, Madrigal-Estebas et al. 2008, Oliviero, Varchetta et al. 2009, Yoon, Shiina et al. 2009). As HCV recurrence is universal after liver

transplantation, this may similarly result in recipient NK cell activation, thus overcoming any transplant related hypofunctionality/tolerance.

Further work would assess the NK phenotype in the setting of other solid organ transplant (eg kidney transplantation), to investigate if our findings are specific to liver transplantation, or are more generalized phenomena. In a larger cohort, it may be possible to elucidate whether aetiology of liver disease (other than HCV vs non-HCV) has an effect on post transplant NK functionality. The mechanism through which NK cells activation and maturation becomes altered is the subject of investigation in the next chapter.

CHAPTER 4: GENE EXPRESSION ANALYSIS OF RECIPIENT NK CELLS AFTER LIVER TRANSPLANTATION

The data presented in the previous chapter provides evidence that recipient natural killer cells undergo abnormal maturation following liver transplantation with associated downregulation of activating receptors and hypofunctionality in response to cytokine stimulation. This was not observed in LT recipients infected with hepatitis C virus, and the *in vitro* assays demonstrated that the changes are not a direct effect of immunosuppressive agents. This led to the hypothesis that recipient NK cells become tolerised by the donor allograft, an effect abrogated by the presence of HCV infection.

The next aim of the study was investigation into the mechanisms through which the observed changes in recipient NK cells occur. As there seems to be a maturation defect, a plausible explanation is that immature NK cells undergo altered development after encountering the allograft. Perhaps hyporesponsiveness is a result of chronic stimulation and exhaustion.

Gene expression in recipient NK cells could provide a valuable insight into the pathways involved in NK tolerance. In particular, comparison of gene expression between NK cells from healthy controls, and those from LT recipients could provide insight into the mechanisms underlying tolerance. Furthermore comparison between HCV and non-HCV recipient NK cells may provide an answer as to how HCV infection counteracts the tolerising effect of LT.

Microarray is a powerful research tool, which offers a non-hypothesis driven approach by assessing changes in gene expression between groups. The advantage of this methodology is that the expression of tens of thousands of genes can be assessed. The Agilent Whole Genome Oligo Microarrays contain over 40,000 genes, and in this study the groups analysed were liver transplantation recipients without HCV infection (LT non-HCV), recipients with HCV infection (LT HCV) and healthy controls (HC). Clearly microarray provides useful information upon which further experiments can be based. However the gene expression data must be verified by further experiments using standard quantitative PCR techniques, and this is the approach taken in these experiments.

RESULTS

4.1 Microarray

The microarray experiments were carried out on the Affymetrix platform by Miltenyi Biotec and analysis of relative expression of each gene was determined using the Agilent Feature Extraction Software. The company provided the quality control and scatterplot data and images described below. With the assistance of a statistician, the data was examined and entered into pathways analysis software in order to assess pathways and biological functions that were significantly represented by differential gene expression.

4.1.1 Quality Control

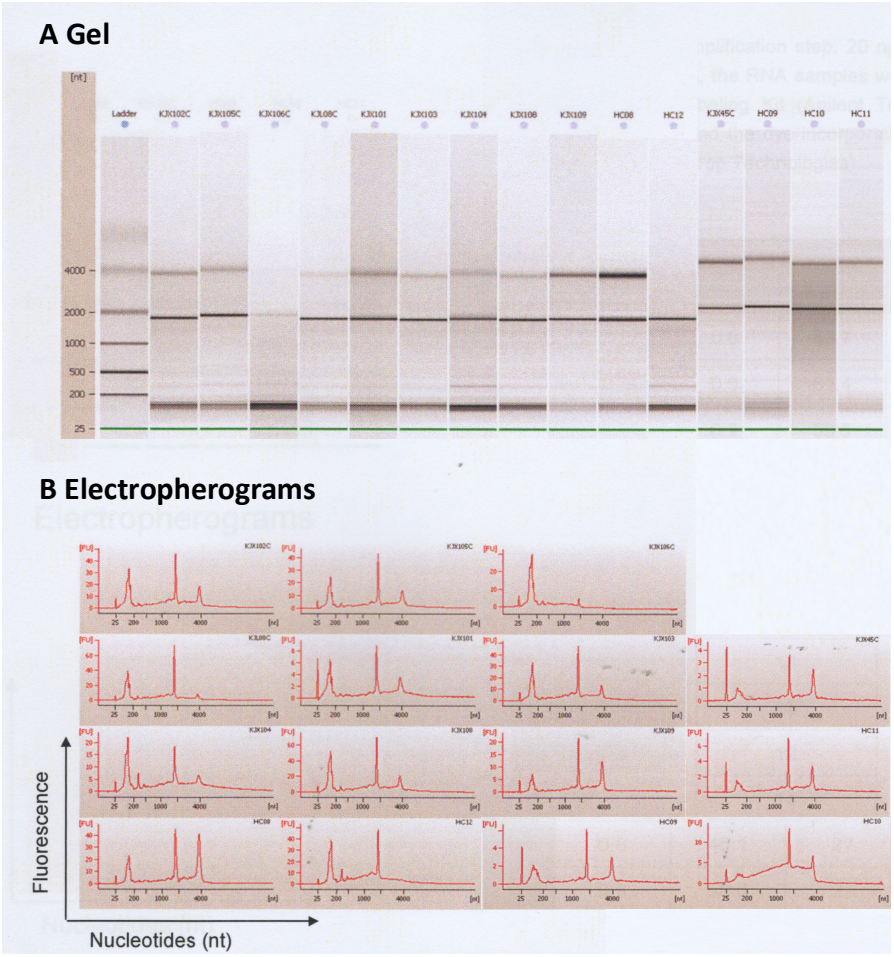


Figure 4.1 RNA quality control.

Gel image (A) and electropherogram (B) of total RNA samples. As a reference the molecular weight ladder (in nucleotides, nt) is shown in the first lane. The lowest migrating green band represents internal standard. The two prominent peaks within the electropherograms represent ribosomal RNA: left 18S, right 28S. Scaling of the y-axis is done automatically, relative to the strongest signal within a single run.

RNA was extracted from 15 LT recipients and the quality was assessed via the Agilent 2100 Bioanalyzer platform. The results are visualized in gel images and electropherograms (**Figure 4.1**). The software also allows generation of an RNA Integrity Number (RIN) to check integrity and overall quality of total RNA samples (Fleige and Pfaffl 2006). Originally, RNA was extracted from 15 samples, but 3 were excluded due to poor quality as determined by RIN.

Principal Components Analysis

This is used to cluster the microarrays by groups according to the most differentially expressed genes by order of significance.

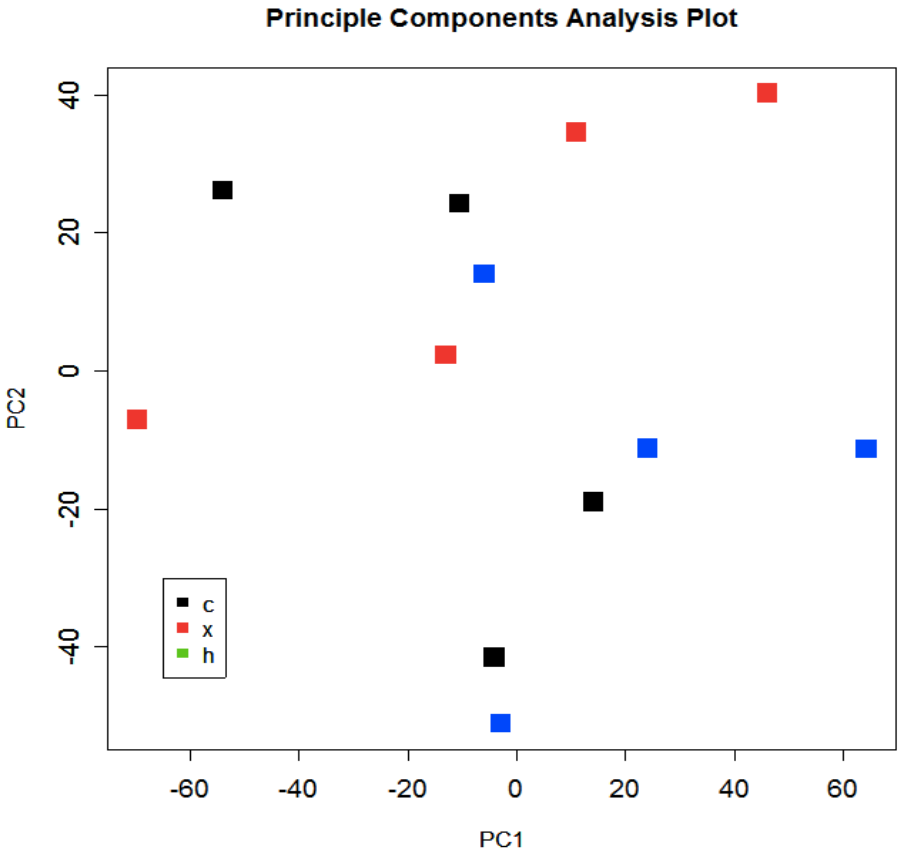


Figure 4.2 Principal Components Analysis.

There was no clustering of the three groups (c=LT HCV, x=LT non-HCV, h= healthy control) in the principal component analysis.

The results of this analysis are demonstrated in **figure 4.2**. There was no clustering of the biological groups (ie. the neither the healthy controls, LT non-HCV nor LT HCV patients clustered) and this suggests that there was no consistent global change in gene expression between the groups.

The data was further analysed using Rosetta Biosoftware, which compares two single intensity profiles in a ratio experiment. The groups were each compared in this way and the scatter plot matrix shown in **figure 4.3** is an example of the output result (comparing healthy controls with LT HCV).

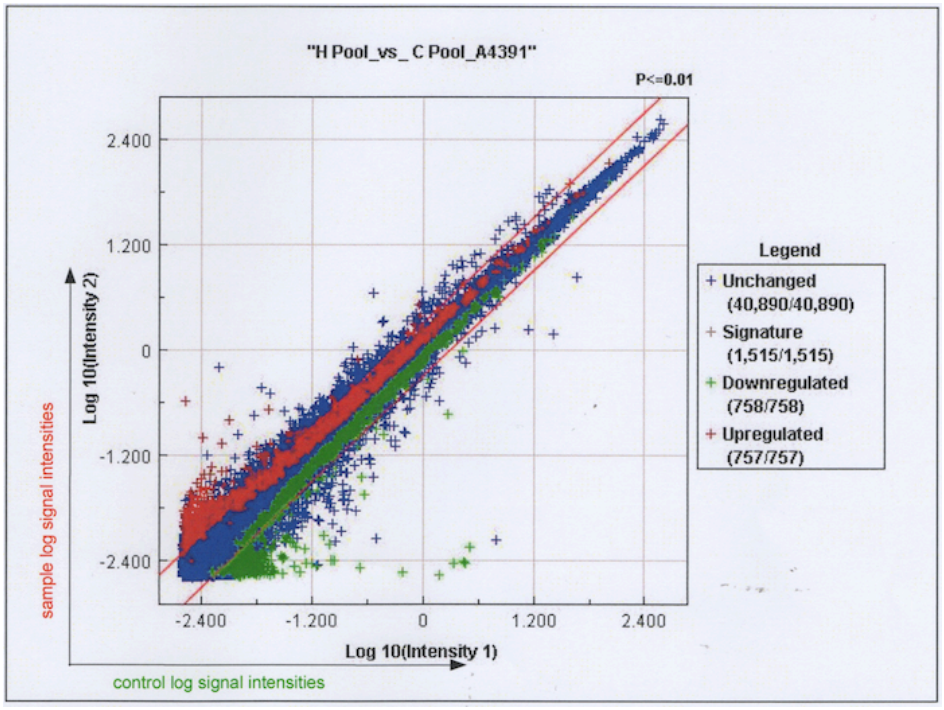


Figure 4.3 Scatter plot of signal intensities of all spots.

As an example the data of one array experiment is shown (healthy controls vs LT HCV). The signal intensities of each feature represented by a dot are shown in double logarithmic scale. x-axis: control-log signal intensity; y-axis: sample-log signal intensity. Red diagonal lines define the areas of 2-fold differential signal intensities. Blue cross: unchanged genes. Red cross: significantly upregulated genes (p-value <0.01). Green cross: significantly downregulated genes (p-value <0.01).

The boxplot in **figure 4.4** compares the pre-normalised probe intensity levels between the samples in this microarray experiment. The samples were all comparable with similar medians for the expression of all genes, indicating no single sample had significantly different intensity levels. This implies that there was no problem with any single array in terms of comparability.

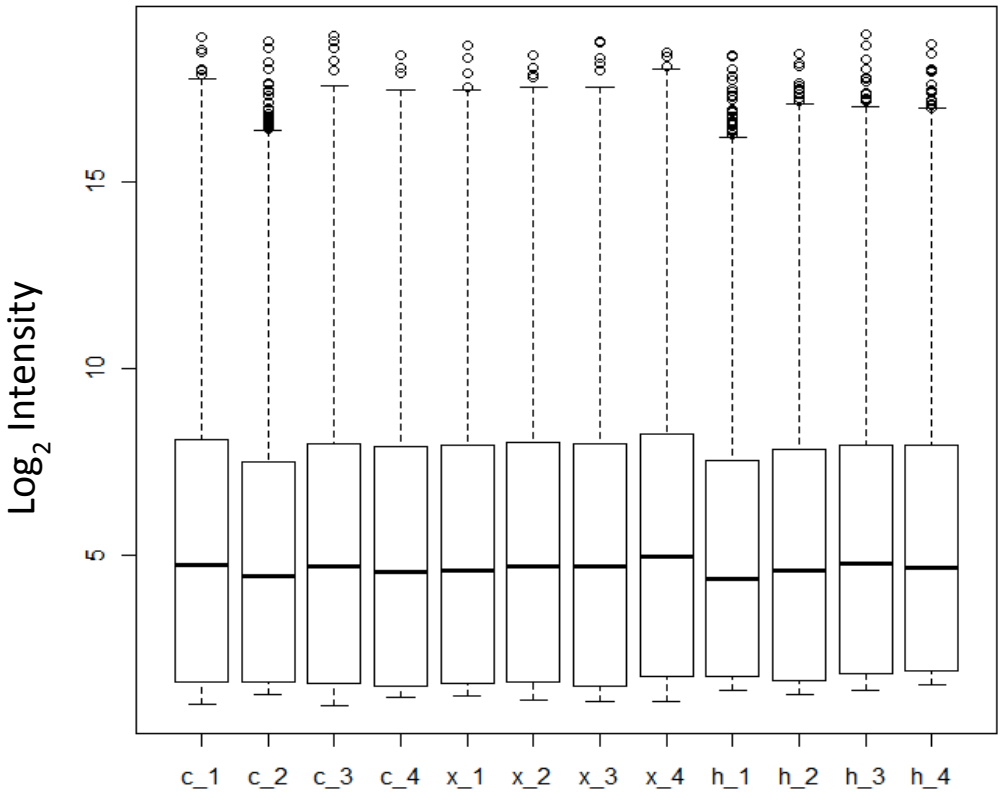


Figure 4.4 Boxplot of pre-normalised expression data.

This boxplot demonstrates that the samples in this microarray experiment all had similar probe signal intensity, with similar medians and distributions. Either end of the box represents the upper and lower quartile, and the line in the middle of the box represents the median. Horizontal lines, connected to the whiskers represent the limits of values not considered to be outliers. Outliers (circles) are values lying more that 1.5 x interquartile range. This quality control tool indicates that no single sample had significantly different expression values from the other, and thus supports the comparability of the samples in this experiment. c = LT HCV, x = LT non-HCV, h = healthy control.

4.1.2 Differentially expressed genes in LT

Although many hundreds of genes demonstrated significantly differential expression between the 3 groups, those genes that were expressed at very low level in all 3 groups (normalized $\log_2$ expression value <6.65) were excluded from further analysis. From the remaining dataset, and using a *p*-value cutoff of 0.05, 858 genes were differentially expressed between the three groups. **Figure 4.5** is a Venn diagram illustrating the number of genes differentially expressed between the groups.

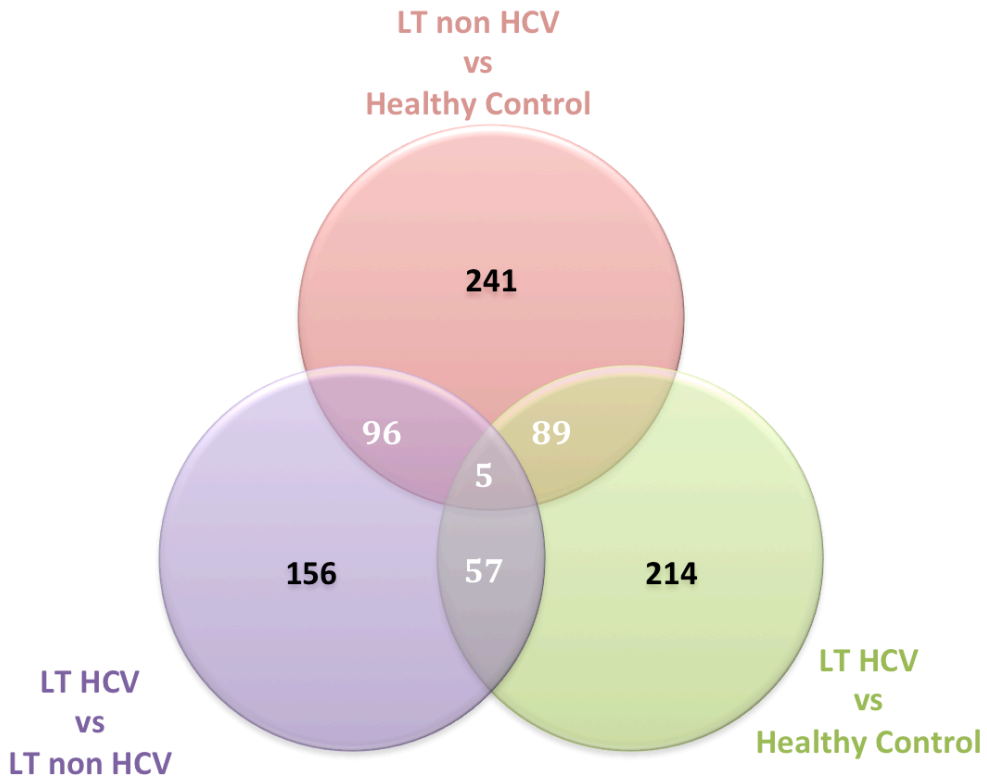


Figure 4.5 Gene expression in recipient NK cells after LT.

Venn diagram showing distribution of 858 differentially expressed genes between the three groups on microarray. Figures in black indicate the number of genes differentially expressed between 2 groups only, and figures in white (within the intersections) indicate the numbers of genes differentially expressed in common between two or three sets of groups.

Appendix 3 shows the full gene lists for differentially expressed genes. As there are multiple analyses performed in microarray, a false discovery rate (FDR) correction was performed. With such correction, there was no significant differential expression of any gene between the 3 groups. The likely explanation for this is the low number of samples used in this experiment.

4.1.3 Pathway analysis

The gene lists and corresponding expression values derived from the microarray analysis were input into the Ingenuity® Pathway Analysis Software which uses the Ingenuity® Pathways Knowledge Base (IPKB) to identify biological functions as well as pathways that are most significantly effected by the genes. The results of these analyses generate biological pathway diagrams as shown in **figures 4.6 - 4.8.**

The most significantly affected canonical pathways were IL-4 signaling ($p=4.47x10^{-5}$), PKCθ signaling ($p=2.38x10^{-4}$) and JAK/STAT signaling ($p=2.46x10^{-4}$).

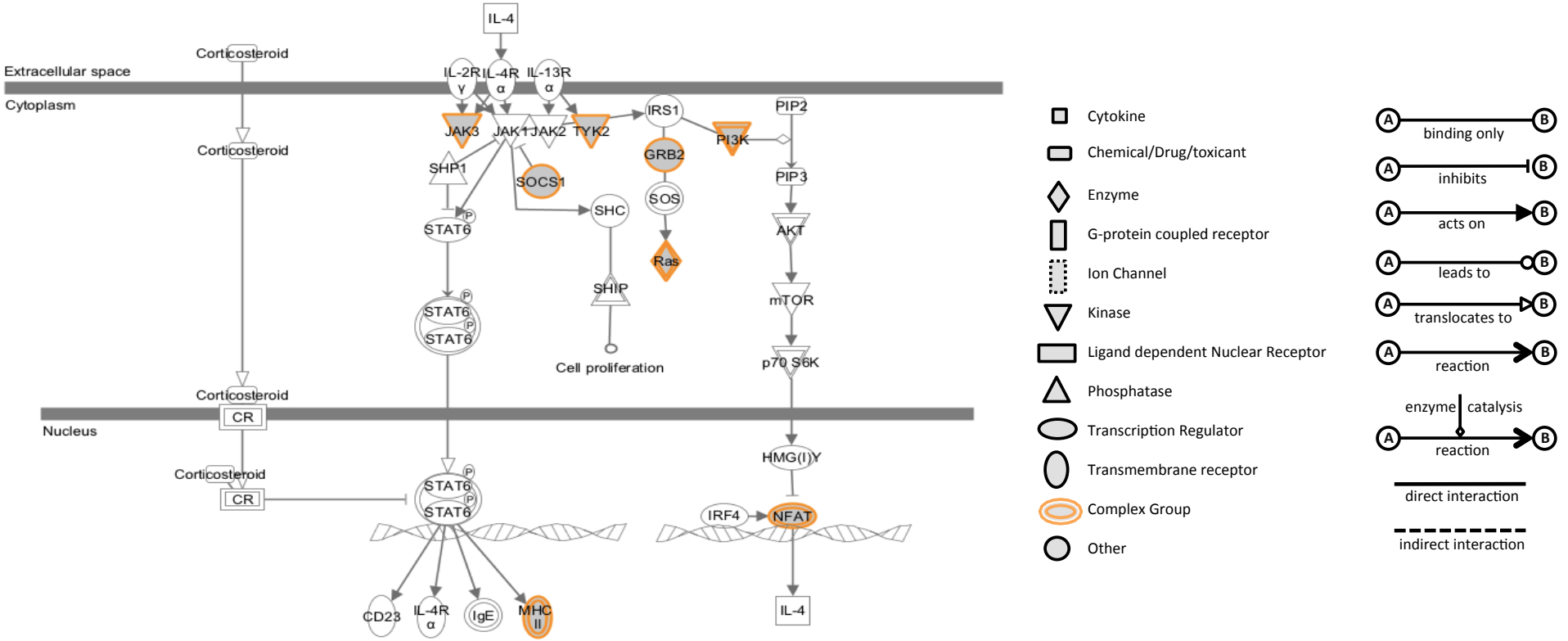


Figure 4.6 IL-4 signaling affected by gene expression differences between groups.

The IPA analysis demonstrates several genes involved in IL-4 signaling were significantly differentially expressed between the groups. These included JAK3, SOCS1, PI3K and NFAT.

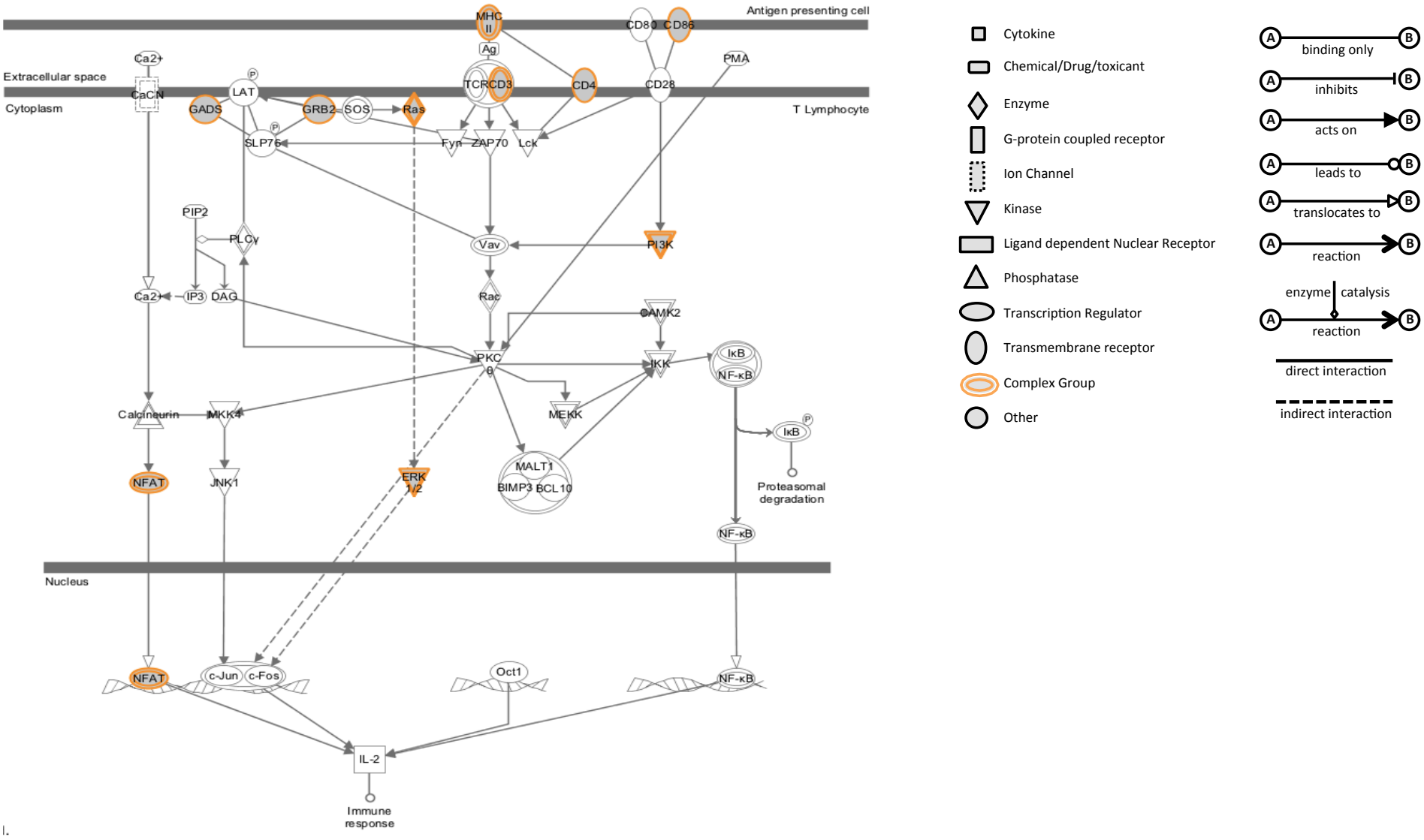


Figure 4.7 PKCθ signaling affected by gene expression changes between groups.

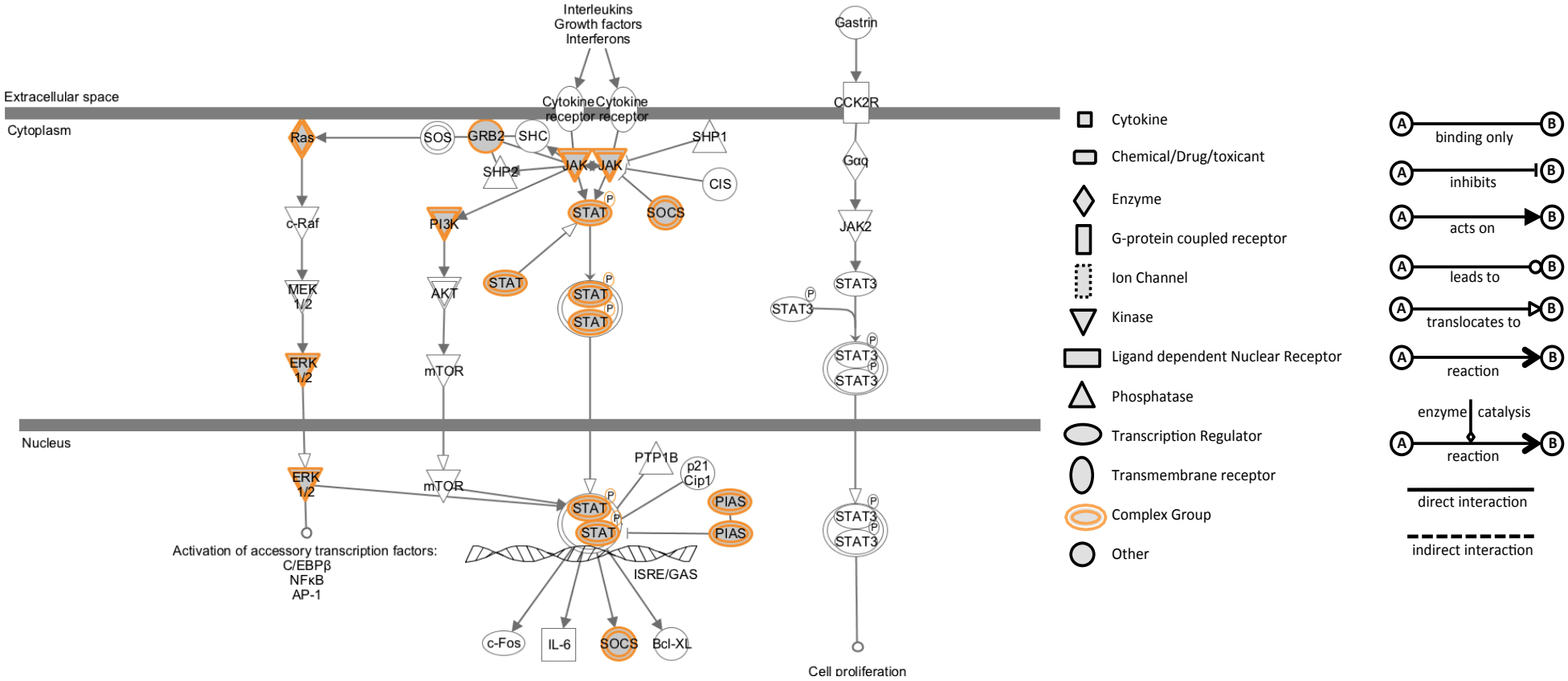


Figure 4.8 JAK/STAT signaling pathways.

Expression of genes crucial to JAK/STAT signaling was significantly different between the 3 groups. In particular STAT4 and JAK3 were differentially expressed, and these are important in the differentiation of NK cells.

4.1.2 Systems analysis

The IPA software also analyses associated network functions most significantly affected by differential expression, and the highest scoring were:

1. DNA replication, recombination and repair, post translational modification , cell cycle
2. Drug metabolism, cell cycle, cell morphology
3. Antigen presentation, haematologival system development and function, inflammatory response.

Biological functions most affected between the study groups were:

1. Haematological disease ($p=2.58x10^{-4}$ to $3.16x10^{-2}$)
2. Immunological disease ($p=2.58x10^{-4}$ to $3.16x10^{-2}$)
3. Inflammatory response ($p=1.33x10^{-3}$ to $3.16x10^{-2}$)

The most significantly affected molecular and cellular functions were:

1. Cellular development ($p=1.57x10^{-6}$ to $3.16x10^{-2}$)
2. Cellular growth and proliferation ($p=1.57x10^{-6}$ to $3.16x10^{-2}$)
3. Cellular function and maintenance ($p=4.38x10^{-5}$ to $3.16x10^{-2}$)

4.2 Quantitative RT-PCR

Microarray is a powerful tool for investigating gene profiling, interrogating the expression of thousands of genes on one experiment. However the very nature of this technique means that vast numbers of comparisons between groups are made and can result in false positives; this is accounted for by FDR correction. In order to achieve statistical significance, many more microarrays would have to be performed; this would have proved prohibitively expensive and was certainly beyond the budget of this project. However, the utility of the data provided is in the generation of possible candidate genes to explore further. Further investigation of these genes of interest (GOI) can then be performed by measuring expression in more samples with qPCR. This then enables determination of whether differential gene expression is indeed significant.

Based on the pre-FDR significant *p*-values, the gene lists were studied and candidate genes were selected for further analysis. We used our previous data to generate our hypothesis that NK cells develop tolerance through decreased activation related to altered development to select candidate genes. Thus genes that were likely to effect NK cell maturation or activation, or involved in the response to HCV infection were of interest. **Table 4.1** shows the genes chosen for further analysis by qPCR and the functions of the genes. Eight genes were selected from the microarray data because of significant differential expression between groups. *IFI44L* and *KIR2DS3* were also selected as, although not reaching statistical significance, there were high fold difference expression values (3.09 and 6.99 respectively) in LT HCV vs LT non-HCV. One further gene (*IKZF2*) was selected because of recent publications suggesting an important role in NKp46 expression (Narni-Mancinelli, Jaeger et al. 2012).

Gene	Differentially expressed	*p* value	Function
STAT4	HC > LT non-HCV HC > LT HCV	0.047 0.071	Type 1 IFNs signal through STAT-4. Important in activation and IFNγ secretion in response to IL-12.
JAK3	LT non-HCV > HC	0.040	IL-2,4,7 signalling. Downstream effects include transcription, maturation.
IKZF2	LT non-HCV > LT HCV HC > LT HCV	N/A	Also known as *helios.* Zinc finger transcription factor. Regulation of lymphocyte development
IL29	LT-HCV > HC LT HCV > LT non-HCV	0.012 0.027	Also known as *IFNλ1*. Antiviral activity
SMAD7	HC > LT HCV HC> LT non-HCV	0.045 0.012	Blocks TGFβ (which blocks lymphocyte activation)
SOCS1	LT HCV > HC	0.017	JAK-STAT downstream – regulates signalling (-ve feedback).
SOX13	HC > LT HCV HC > LT non-HCV	0.008 <0.001	Transcription factor – cell development
IFI44L	LT HCV > LT non-HCV HC > LT non-HCV	N/A	Exhibits antiviral activity against HCV
TNFRSF18	LT HCV > HC LT non-HCV > HC	0.014 0.028	Also known as *GITR.* Possibly inhibits NK cell cytotoxicity.
ZNF683	LT HCV > HC LT non-HCV > HC	0.007 0.022	Transcription factor.
KIR2DS3	LT HCV > LT non-HCV LT HCV > HC	N/A	Activating KIR

Table 4.1 Names and functions of genes of interest.

The genes of interest were selected from the microarray dataset. The selection was based on knowledge from the published literature; candidate genes were those likely to be involved in NK cell activation, maturation or development, or in the immune response to HCV infection.

The method of extracting RNA for the qPCR experiments required significant adaptation for optimisation. This was largely due to the problem of low starting number of cells (a few as $1x10^5$ NK cells after purification from PBMCs). Column based RNA extraction tended to yield very limited levels of RNA, as much of the RNA was lost on the columns. Thus the TRIzol phenol-chloroform extraction method was employed and optimised to maximize RNA yield. Modifications found to have a significant effect on yield were incubation at -80^{o}C for 1 hour after the isopropanol precipitation step, and using in-solution DNase (rather than a column based method).

The qPCR reactions were performed on an array plate pre-loaded with primers for the 11 genes of interest as well as 2 housekeeping genes (SMA and GAPDH) (SABSciences, U.K). There were also 3 control wells per sample: a) positive PCR control (PPC); b) genomic DNA control (GDC); c) reverse transcription control (RTC) and the purpose of these is described in the **Methods** chapter.

4.2.1 STAT4 expression reduced in LT

Quantitative RT-PCR was performed on samples from 13 healthy controls, 12 LT HCV patients and 17 LT non-HCV patients. The experiments were validated using the controls described and the housekeeping genes were used to determine relative gene expression between groups $\Delta\Delta C_T$ method. **Figure 4.9** is a heatmap representation of the qPCR data for all the genes of interest quantified. The results are summarised in **table 4.2**, which includes the fold changes and *p*-values for the genes where the differential gene expression was either significant ($p<0.05$) or nearly significant ($p=0.05$-0.07).

The most consistent difference between LT and healthy controls was downregulation of *STAT4* gene expression. This occurred in both LT groups compared with healthy controls (p=0.0001, -10.73 fold change, and p=0.01, -3.78 fold change in LT non-HCV and LT HCV respectively). Compared with controls, in LT non-HCV there was also upregulation of *ZNF683* (*Hobit*, a repressor of IFNγ expression (van Gisbergen, Kragten et al. 2012), p=0.06, 2.03 fold change) and downregulation of *KIR2DS3* (p=0.05, -2.14 fold change).

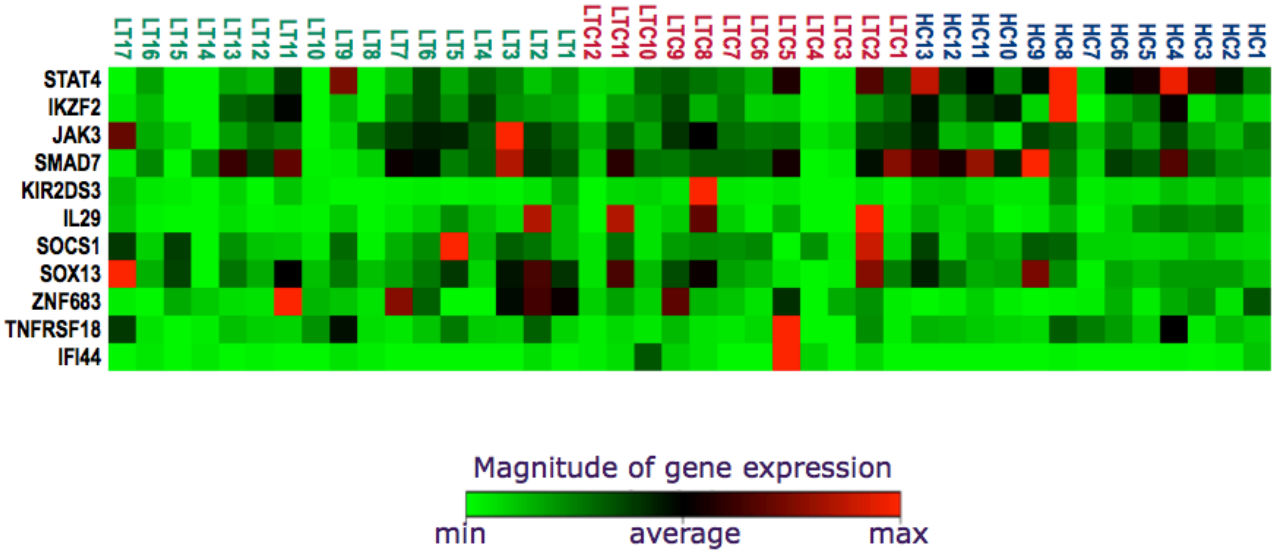


Figure 4.9 Heat map representation of gene expression between the 3 groups.
All the candidate genes selected for qPCR are represented. The black and red colouration reflects higher gene expression; this can be seen in STAT4 expression, where the healthy controls (HC) are mostly red or black, reflecting upregulation compare with the two LT groups (LT= LT non-HCV, LTC= LT HCV).

The only candidate gene differentially expressed with near significance between LT HCV and LT non-HCV was *IF144L* (an interferon induced protein, p=0.07, 3.14 fold upregulation in HCV, consistent with the activation of interferon stimulated

genes (ISGs) as found in chronic HCV infection). When comparing all LT (ie. HCV and non-HCV) with controls, downregulation of *STAT4* (p=0.0001, -6.97 fold change) and *IKZF2* (*Helios*, a transcription factor controlling NKp46 expression (Narni-Mancinelli, Jaeger et al. 2012)) (p=0.06, -2.26 fold change) and upregulation of *ZNF683* (p=0.07, 2.10 fold change) were found.

	All LT vs HC		LT non-HCV vs HC		LT HCV vs HC		LT HCV vs LT non-HCV	
Symbol	**Fold Change**	**p value**	**Fold Change**	**p value**	**Fold Change**	**p value**	**Fold Change**	**p value**
STAT4	-6.97	**0.0001**	-10.73	**0.0004**	-3.78	**0.01**	2.84	ns
IKZF2	-2.26	0.06	-2.88	ns	-1.61	ns	1.79	ns
ZNF683	2.10	0.07	2.03	0.06	2.20	ns	1.08	ns
IF144L	1.99	ns	1.14	ns	4.38	ns	3.85	0.07
KIR2DS3	-2.47	ns	-2.14	0.05	-3.01	ns	0.71	ns

Table 4.2 qRT PCR analysis for the candidate genes identified by microarray analysis.

Expression of the genes of interest was quantified in 13 healthy controls (HC), 17 LT non-HCV and 12 LT HCV patients. Using housekeeping genes, the $\Delta\Delta C_T$ method was employed to determine relative expression. The fold-change refers to expression in the first group compared to the second in the header column. Negative fold change reflects downregulation. Only the genes where there was significant (p<0.05, in bold) or near significant (p=0.05-0.07) difference in expression in at least one analysis are shown. The most consistent and significant finding was down regulation of STAT4 in LT.

4.2.2 Immunosuppression and gene expression

The effects of immunosuppressive medication on expression of the candidate genes in NK cells were assessed by qRT PCR on NK cells from 3 healthy donors incubated with Tacrolimus, Cyclosporine A, Prednisolone, or medium alone. None of the gene expression changes observed in LT were replicated in these *in vitro* assays (**Table 4.3**) and in general, aside from KIR2DS3, differences observed *in vitro* were in the opposite direction to those observed *in vivo*.

	Fold Regulation compared with no drug		
Symbol	**Tacrolimus**	**Cyclosporin A**	**Prednisolone**
STAT4	8.0463	-1.0952	10.3711
IKZF2	2.5727	6.1457	5.6047
ZNF683	-3.5245	-4.9738	-28.0862
IF144L	-5.5939	-36.9022	-15.4974
KIR2DS3	-17.0462	-25.5979	-17.6655

Table 4.3 Effects of immunosuppressants on gene expression.

Levels of candidate gene expression were quantified by qPCR in healthy NK cells after incubation with immunosuppressant drugs and relative expression compare to incubation in medium alone is shown

4.2.3 Reduced IL-12 induced STAT4 phosphorylation in LT

STAT4 is transcriptional activator that is phosphorylated following stimulation of lymphocytes by IL-12. In order to determine if the low mRNA levels observed had functional consequences, STAT4 phosphorylation in response to IL-12 stimulation was measured. There was significantly reduced STAT4

phosphorylation in LT recipients (n=8) compared to healthy controls (n=8) (p<0.001) (**figure 4.10**).

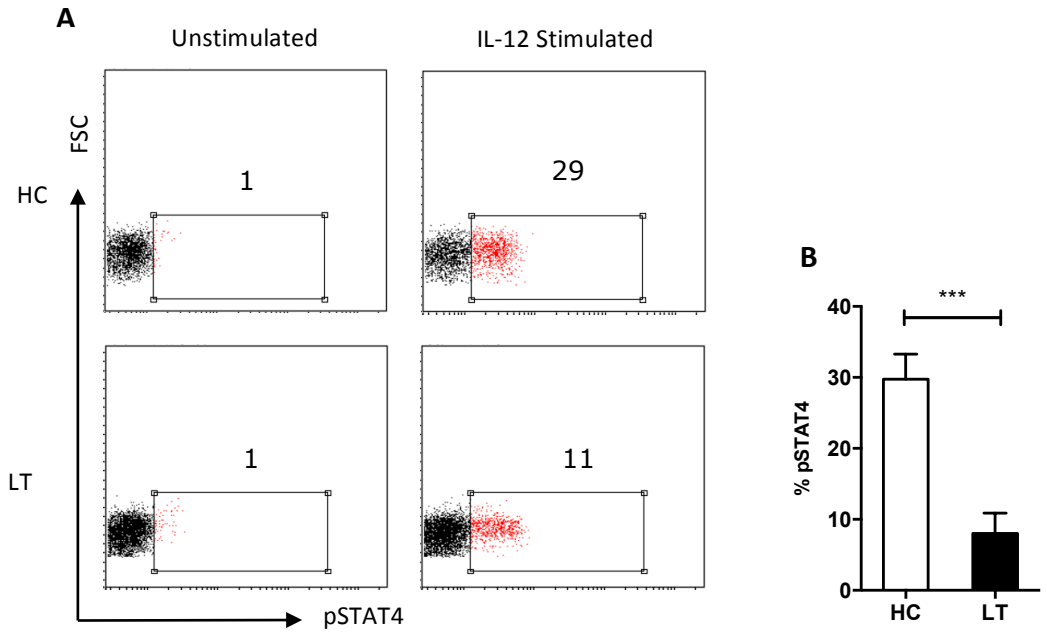


Figure 4.10 STAT4 phosphorylation after IL-12 stimulation.

*Intracellular pSTAT4 was measured in NK cells using a FACS based intracellular staining method. A representative FACS plots demonstrating little pSTAT4 expression in unstimulated NK cells; after IL-12 stimulation, there is increased pSTAT4, with a smaller increase seen in LT versus HC. The results of experiments in 8 LT recipients and 8 healthy donors are summarized in B. ***p<0.001.*

In order to exclude this effect as being directly due to immunosuppressive medication the effects of Tacrolimus, Cyclosporine A and Prednisolone on IL-12 induced STAT4 phosphorylation were analysed. No change was observed in STAT4 phosphorylation following incubation with any of these agents (**figure 4.11**). Thus the tolerant NK cell phenotype is associated with defective STAT4 signaling, and this was not related to the direct effect of immunosuppression on mature NK cells.

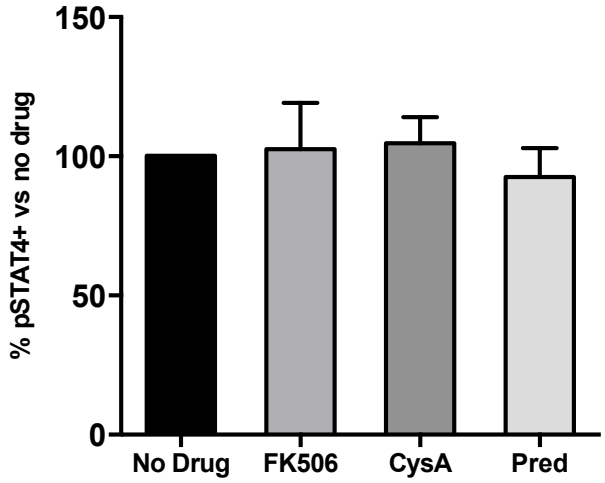


Figure 4.11 Effect of immunosuppression on IL-12 induced STAT4 phosphorylation.

PBMCs from healthy donors incubated with the immunosuppressive agents demonstrated no difference in pSTAT4 levels compare with medium alone. N=3. FK506= Tacrolimus, CysA= Cyclosporine A, Pred = Prednisolone.

4.2.4 MicroRNA-155 expression in LT

STAT4 has many important downstream cellular effects, and downregulation of STAT4 expression and activation may have broad functional consequences. To investigate if the effects of STAT4 down modulation had significant effects on NK cells *in vivo*, we studied the expression of microRNA-155 (miR-155), a key regulator of NK cell differentiation and activation, that is a target of STAT4 (Trotta, Chen et al. 2012, Zawislak, Beaulieu et al. 2013). Quantitative RT-PCR performed on isolated NK cells from post-LT patients and healthy controls demonstrated a 4.9 fold down-regulation of miR-155 in LT (p=0.04, **figure 4.12**), which is consistent with the observed defect in STAT4 signaling. This suggests that effects of STAT4 downregulation have an on-going effect on NK cells in post transplant patients.

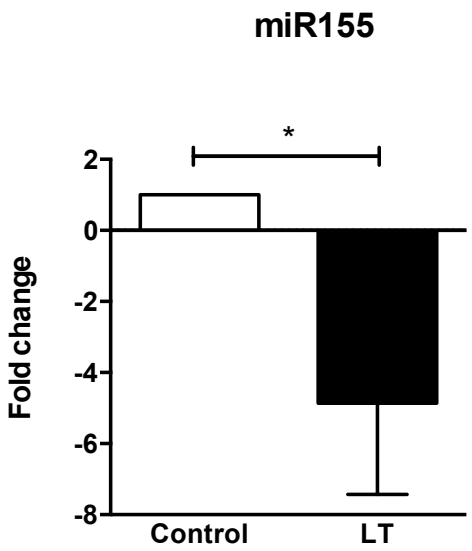


Figure 4.12 miR-155 expression in LT.

*There was a 4.9-fold downregulation of expression of miR-155 in LT recipients compared with healthy controls. Experiments were performed on samples taken from 7 LT patients and 7 healthy controls. * p<0.05.*

4.3 Discussion

In this chapter gene expression profiling was utilized to explore mechanisms underlying the observed differences in NK cell phenotype and function in liver transplant recipients. The initial approach employed was DNA microarray, which has the advantage of assessing the expression of tens of thousands of genes in one experiment. This technique allows for a global comparison of gene expression between groups in biologically distinct conditions, and therefore provides hypothesis-generating data that may help explain the differences between the groups. As such, hitherto unthought-of mechanisms can be elucidated, and then explored in further experimental analysis.

Interestingly, when all the differentially expressed genes were analysed through the pathways analysis software, the biological networks and functions most affected were related to the immunological and inflammatory response, cellular development, growth and proliferation. This is consistent with the previous observation that NK cells in LT exhibit a hypofunctional phenotype and that this is related to altered maturation and differentiation. The pathway analysis demonstrated that a number of genes involved in key immunological signaling pathways were differentially expressed between the groups.

Protein Kinase C-θ (PKCθ) is known to be a key regulator of signal transduction in activated T cells (Isakov and Altman 2012), but has also been shown to be important in NK cell activating signal transduction, and deficiency in PKCθ has been shown to result in defects in NK transcriptional activation and IFNγ

secretion (Tassi, Cella et al. 2008). Therefore alterations in the PKCθ pathway may be one mechanism through which the altered phenotype in recipient NK cells arises.

The pathway analysis also found Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling to be significantly altered between the study groups. The canonical JAK/STAT pathways are the principal signaling mechanism for many cytokines and growth factors, which are crucial to cell proliferation, differentiation, apoptosis and migration (Rawlings, Rosler et al. 2004). Therefore alterations in JAK/STAT signaling would have significant consequences for NK cell biology from activation, to differentiation and maturation. The confirmation by qRT-PCR of downregulation in *STAT4* expression provides more compelling evidence that JAK/STAT signaling is indeed involved in the alteration in recipient NK cell function after LT.

The *STAT4* gene is located on chromosome 2q32 and encodes the STAT4 protein. This is an important transcription factor whose expression is restricted to myeloid cells, thymus and testis (Yamamoto, Quelle et al. 1994), and is known to be required in the development of Th1 cells from naïve CD4+ T cells (Kaplan 2005). STAT4 is expressed at very low levels in resting cells, but T cell production of STAT4 can be stimulated by phytohaemagglutinin (PHA) (Bacon, Petricoin et al. 1995). STAT4 is activated by binding of IL-12 to its receptor (IL-12R); this results in dimerization of the IL-12R, phosphorylation of JAK2, which in turn phosphorylates STAT4. Phospho-STAT4 (pSTAT4) homodimerizes and translocates into the nucleus where it has multiple effects on gene transcription,

with resultant promotion of key activating cytokines including IFNγ and TNFα (Wurster, Tanaka et al. 2000). STAT4 has been shown to be important in NK cell activation, and PBMC production of IFNγ is impaired after HSCT as a consequence of STAT4 deficiency, with significantly reduced *STAT4* gene expression (Robertson, Chang et al. 2005). In the present study, not only was *STAT4* gene expression downregulated, but STAT4 phosphorylation in response to IL-12 was also reduced. Our data therefore suggests that the tolerant NK cell phenotype observed in LT recipients may be associated with defective STAT4 signaling and is consistent with the observed reduction in NK cell production of IFNγ as described in **Chapter 3**.

MicroRNA-155 has been shown to be an important target of STAT4, with consensus STAT4 binding motifs present in the promotor of the *bic* gene that encodes miR-155. In mice miR-155 is associated with accelerated NK cell maturation, and deletion of this miRNA has been shown to result in defects in NK cell maintenance and homeostasis (Zawislak, Beaulieu et al. 2013). In the LT cohort a 4.9-fold downregulation of miR-155 compared with healthy controls provides further evidence of downstream consequences of defective STAT4 signaling. It is plausible that this deficit in miR-155 may cause a similar maturation defect in humans as is observed in mice. In **chapter 3**, NK cells from LT recipients expressed increased NKG2C, a marker that has been shown to be associated with terminal differentiation of NK cells and a 'memory' phenotype (Lopez-Verges, Milush et al. 2010, Moretta 2010, Lopez-Verges, Milush et al. 2011). Thus the data presented in this study suggests that, as is seen in mice, miR-155 may also be associated with NK maturation in humans, with decreased

levels found in LT associated with altered differentiation and ultimately hypofunctionality.

The downregulation in *STAT4* expression was present in LT regardless of HCV status, suggesting that this is a phenomenon related to transplantation itself. However, as described in **Chapter 3**, NK cells in HCV LT recipients have preserved functionality. Interestingly, in the analysis comparing LT HCV with LT non-HCV increased expression of *IFI44L* was observed. As this is an interferon-induced protein, known to be activated in HCV infection, these data support our hypothesis that HCV infection *counteracts* (rather than *prevents*) the tolerance induced by LT.

The effects of immunosuppressive agents on NK gene expression have not previously been reported. Tacrolimus inhibits calcineurin phosphatase, and in T cells has been shown to result in inhibition of calcium-dependent events including IL-2 gene transcription, nitric oxide synthase activation, cell degranulation, and apoptosis (Thomson, Bonham et al. 1995). Tacrolimus may also enhance expression of the transforming growth factor beta-1. Thus it might be expected that gene expression involving these pathways would be affected in NK cells from LT recipients receiving Tacrolimus. Whilst none of the selected candidate genes are directly involved in these pathways, the analysis of the microarray data set suggested that IL-4 signalling is significantly affected in LT. In murine NK cells IL-4 and IL-2 synergistically enhance IFNγ production (Bream, Curiel et al. 2003), thus it is possible that the observed effects on IL-4 signaling in our LT recipient cohort is a consequence of immunosuppression.

Since I found that the CD56^{bright} population is expanded after LT, some of the gene expression differences might be related to this. Previous studies analysing gene expression in CD56^{bright} and CD56^{dim} NK subsets and have reported differential gene expression profiles between the two populations which reflect their functional diversity (Hanna, Bechtel et al. 2004, Wendt, Wilk et al. 2006, Wilk, Kalippke et al. 2008). For example, in CD56^{dim} NK cells mRNA expression of KIR genes, CD16, and granzyme were much greater than in CD56^{bright} NK cells. However no study has reported differences in *STAT* gene expression, or of IL-12 or IL-4 between the NK subsets. Therefore the differences in *STAT4* gene expression and signaling found in our LT cohort are not likely to be related to the observed changes in NK subsets. Instead, we propose that interactions between the allograft and recipient NK cells lead to the observed alterations in STAT4 signaling, contributing to the reduced activation and resulting NK tolerance.

The microarray dataset clearly has its limitations, most obviously being the small sample size. This was addressed by performing qPCR on a few genes of interest selected from the microarray in samples from a further cohort. However, if time and budget allowed, it would be interesting to perform microarray on many more samples. This would enhance the statistical power of the results, and without doubt provide many more genes and pathways to explore further. I chose to focus on a small set of genes for further analysis by qPCR, and explored *STAT4* in detail. There were many other genes that were differentially expressed, and pathways (such as PKCθ signaling) that could equally have been investigated. Future

studies could explore the different gene sets and pathways in detail, shedding further light on the mechanisms underlying recipient NK tolerance in liver transplantation.

An interesting study to take this further would be to analyse gene expression in a different transplant cohort as a comparator with our LT recipients. For example, one could quantify NK gene expression in renal transplant recipients. Is *STAT4* expression similarly affected in this group? If so this would suggest that the tolerance mechanism is a general transplant phenomenon; however if NK *STAT4* expression is not affected, this would support a hypothesis that induction of NK tolerance is specific to liver allografts. This would be consistent with the clinical observation that liver allografts are in general better tolerated than other solid organ transplants.

CHAPTER 5:THE EFFECT OF KIR-HLA GENOTYPE ON LIVER TRANSPLANTATION

As NK cell tolerance of self involves inhibitory interactions with self HLA class I molecules, and liver allografts are not HLA matched to the recipient, there is a theoretical opportunity for NK cell alloreactivity in HLA mismatched transplantation. As described in the Chapter 1, NK cell receptors are expressed in a clonal and variegated manner so that within an individual, clones of NK cells express different repertoires of receptors. For example, some clones will express KIR2DL1 (HLA-C2 specific) but not KIR2DL2/3 (HLA-C1 specific but with some cross reactivity with HLA-C2), and vice versa. HLA and KIR genes are encoded on different chromosomes and are therefore not linked, so that an individual may not express the inhibitory HLA ligand for all the KIR encoded within his genotype. Therefore NK clones expressing KIR2DL2 (and not KIR2DL1) would be self reactive in an individual homozygous for HLA-C2. This does not occur, and it has been shown that NK cells that do not express inhibitory receptors to self ligands are hyporesponsive (Fernandez, Treiner et al. 2005). The mechanisms for this are uncertain, but it is thought that NK cells undergo an educational process during maturation that allows full maturation of only those clones expressing inhibitory receptors recognizing self. The various theories for this are discussed in Chapter 1.

Immunogenetic studies have not provided sufficient evidence to generate a consensus view of the effect of HLA matching on clinical outcomes of liver

transplantation, and there is little data on the effects of matching on NK cell function. The phenotyping and functional data described in Chapter 3 has clearly demonstrated that NK cells are hypofunctional after LT. The results are consistent with an effect of LT on NK cell development and maturation that is independent of KIR expression. The altered phenotype was observed largely on $CD56^{dim}$ KIR^{+} subsets, suggesting the effect on NK cells occurs at an immature stage of development, prior to acquisition of KIR. It follows therefore that KIR-HLA mismatching is unlikely to cause predicted NK allospecific reactivity, or influence LT outcomes. In order to explore this further, immunogenetic analysis was performed in the recruited cohort of liver transplant recipients, focusing on associations between HLA alleles, HLA matching, HLA-KIR combinations, KIR haplotype and NK cell activation. Any effects of HLA/KIR on clinical outcomes were also examined.

RESULTS

5.1 Genotype and NK cell activation

5.1.1 Recipient KIR genotype

DNA was extracted from whole blood from all study recruits using the Qiagen DNeasy kit and quantified by spectrophotometry. Aliquots were sent to the KIR genotyping laboratory in Cambridge for full KIR allelic typing including copy number (James Traherne, Trowsdale laboratory, Cambridge). Of the 109 LT recipients recruited, KIR typing was possible in 106 (97%). **Figure 5.1** shows the distribution of the KIR alleles amongst the LT recipients and the copy number of the KIR alleles present within individuals is depicted **figure 5.2.**

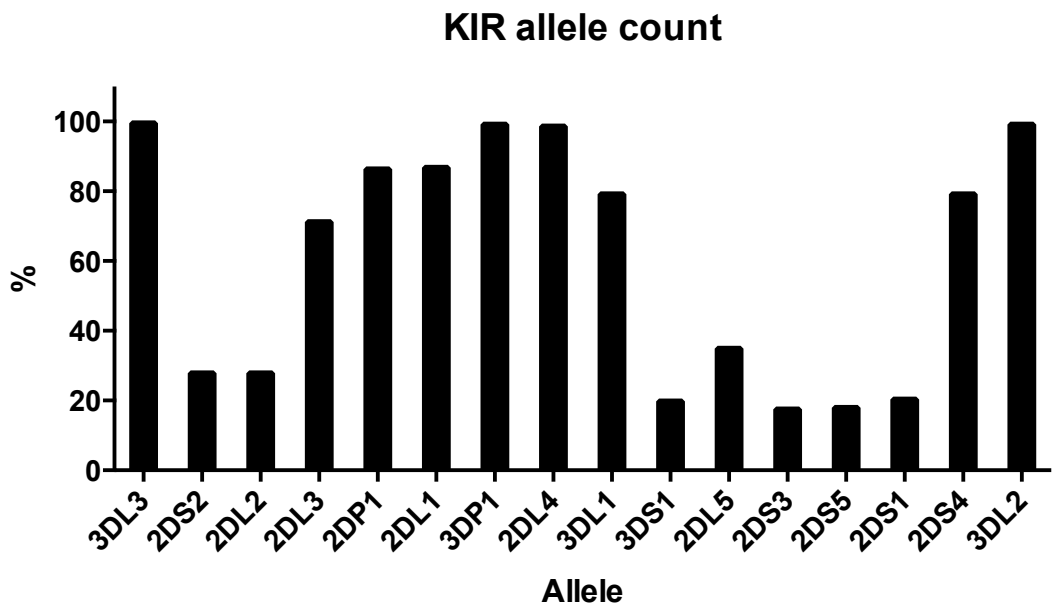


Figure 5.1 The distribution of KIR alleles amongst LT patients.

The frequency of all 16 current known KIR genes (including the 2 pseudogenes KIR2DP1 and KIR3DP1) is shown.

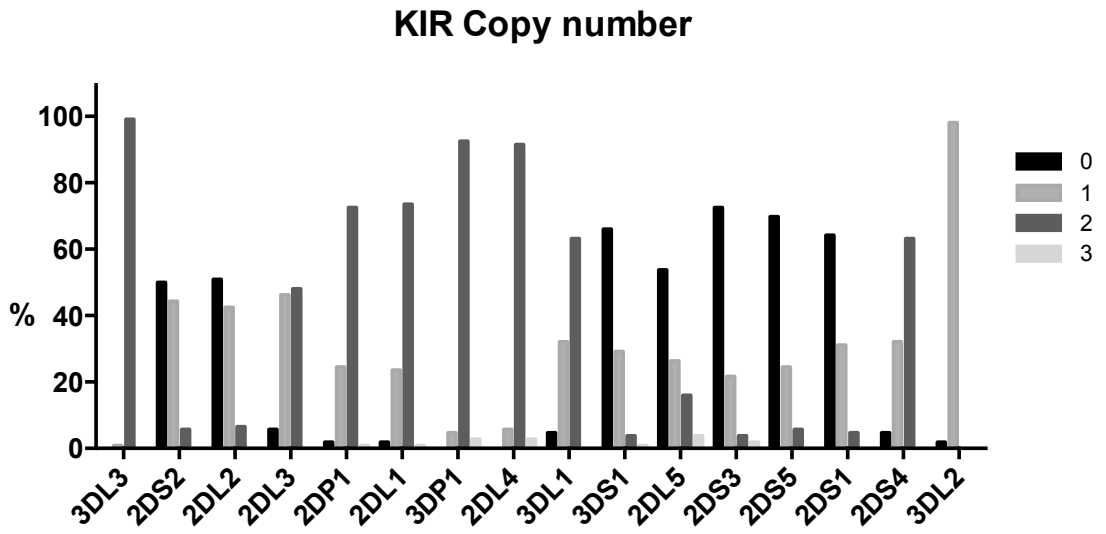


Figure 5.2 KIR copy number.

The frequency of individuals carrying either 0, 1, 2 or 3 copies of each KIR allele is shown

In addition to the diversity in KIR gene content between individuals, KIR alleles exhibit substantial polymorphism (Middleton and Gonzelez 2010), resulting in a great variation in KIR genotype within and between populations. However, it has proved useful to categorise KIR genotypes into two broad haplotype groups termed A and B depending upon KIR gene content. Haplotype B contains one or more of the following genes KIR2DL2, KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1 (KIR Nomenclature Committee, http://www.ebi.ac.uk/ipd/kir/nomenclature.html).

Group A haplotypes contain none of these genes, and consequently group B haplotypes contain more activating KIR genes. This distinction has biological significance as associations have been observed between haplotype and diseases including TB, viral infections and relapse of leukaemia following stem cell

transplantation (Kulkarni, Martin et al. 2008, Cooley, Trachtenberg et al. 2009, Symons, Leffell et al. 2010, Braun, Larcombe et al. 2013). In the present study 42 individuals (40%) were homozygous for group A haplotypes (ie. AA, termed Haplotype A in the following analyses) and 64 individuals (60%) were either homozygous or heterozygous for group B haplotypes (termed Haplotype B in the analyses). In the liver transplantation cohort there was no association between KIR haplotypes and expression of activating receptors or cytotoxicity (**Figure 5.3**).

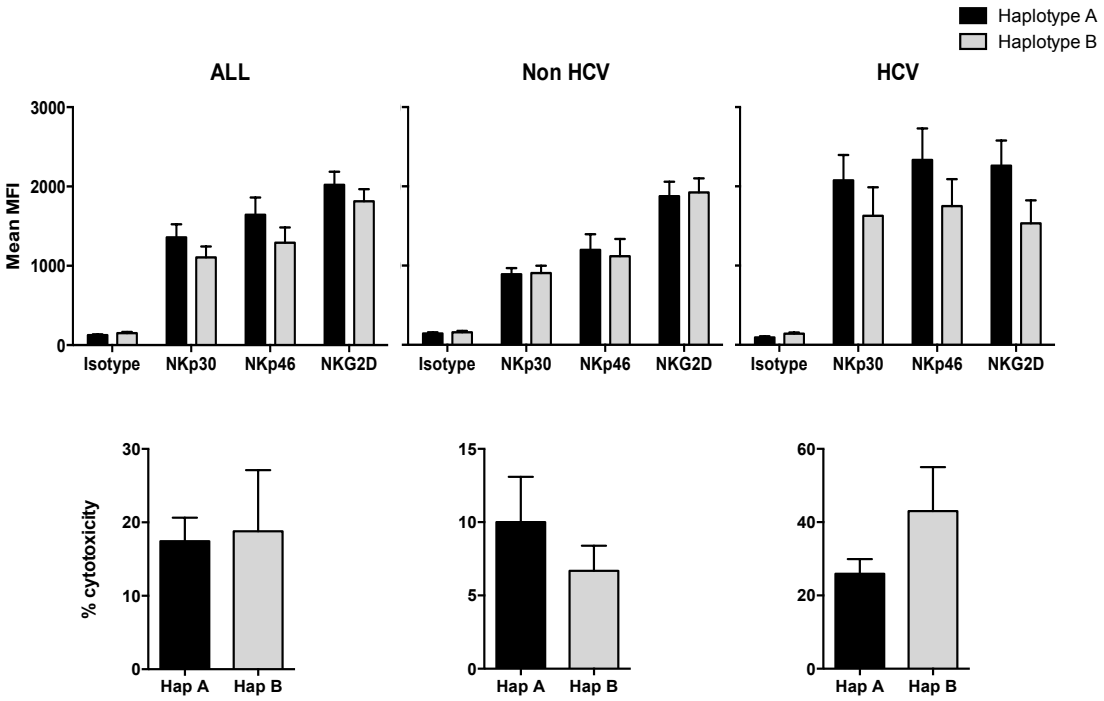


Figure 5.3 KIR haplotype and NK cell activation.

Association between KIR haplotype and the expression of the activating receptors was analysed (haplotype A: All n=33, non-HCV n=20, HCV n=13; haplotype B: All n=18, non-HCV n=13, HCV n=5). Similarly NK cytotoxicity was assessed according to haplotype (A: All n=15, non-HCV n=8, HCV n=7; B: All n=6, non-HCV n=4, HCV n=2).

5.1.2 Donor and recipient HLA genotype

HLA-B and -C interactions with KIR dominate NK inhibitory receptor signaling (Young, Rust et al. 1998, Yawata, Yawata et al. 2006) and therefore genes encoding these epitopes were analysed. Donor HLA typing data was obtained from the UK blood and transplant (UKBT) database, with 2 digit allelic typing information available. Recipient HLA typing was performed using DNA extracted from recipient PBMCs in collaboration with the Histocompatibility and Immunogenetics laboratory at Gartnavel General Hospital, Glasgow (Helen McFarlane, Ann-Margaret Little, Glasgow).

Locus	***Motif***	***Position 77***	***Position 80***	***Alleles***
HLA-B	Bw4	N (asparagine)	I (isoleucine)	B*51, B*52, B*53, B*57, B*58,
HLA-B	Bw4	N (asparagine)	T (threonine)	B*13, B*27, B*37, B*44
HLA-B	Bw4	D (aspartic acid)	T (threonine)	
HLA-B	Bw4	S (serine)	T (threonine)	
HLA-B	Bw6	G (glycine)	N (asparagine)	B*7, B*8, B*14, B*18, B22, B*35, B*39, B*45, B*55, B*56, B*62, B*64
HLA-B	Bw6	S (serine)	N (asparagine)	
HLA-C	C1		N (asparagine)	Cw1, Cw7, Cw8, Cw9, Cw10, Cw12, Cw13, Cw14, Cw16
HLA-C	C2		K (lysine)	Cw2, Cw4, Cw5, Cw6, Cw15, Cw17

Table 5.1 HLA-B and -C allele classification according to KIR binding epitopes.

HLA alleles were categorised into groups according to the amino acid sequence encoded within the KIR binding epitope of the HLA molecules, which determines KIR binding specificity. Group 1 HLA-C alleles encode asparagine at position 80, whilst group 2 HLA-C alleles encode lysine at position 80 (Mandelboim, Reyburn et al. 1996). HLA-B alleles are categorized according to the residues encoded at

positions 77 and 80 as shown in **table 5.1** (Gumperz, Barber et al. 1997, Kulkarni, Martin et al. 2008).

In Chapter 3, a hyporesponsive phenotype in LT recipient NK cells was observed, with reduced expression of the natural cytotoxicity receptors NKp30 and NKp46, only in the absence of Hepatitis C infection. The most marked effect was seen in the $CD56^{dim}$ (mature) subset of NK cells. In order to assess whether the presence of any of the HLA-B or -C alleles in either the donor or recipient was associated with NK cell activation, the expression of the NCRs in $CD56^{dim}$ NK cells was analysed in subgroups categorised according to the presence of each allele, as shown in **figure 5.4**. There was no significant association between the presence of any of the HLA-B or -C alleles and expression of the activating receptors NKp30 and NKp46, although in LT non-HCV, there was a trend towards increased NKp46 expression when HLA-Bw4 was present in the recipient.

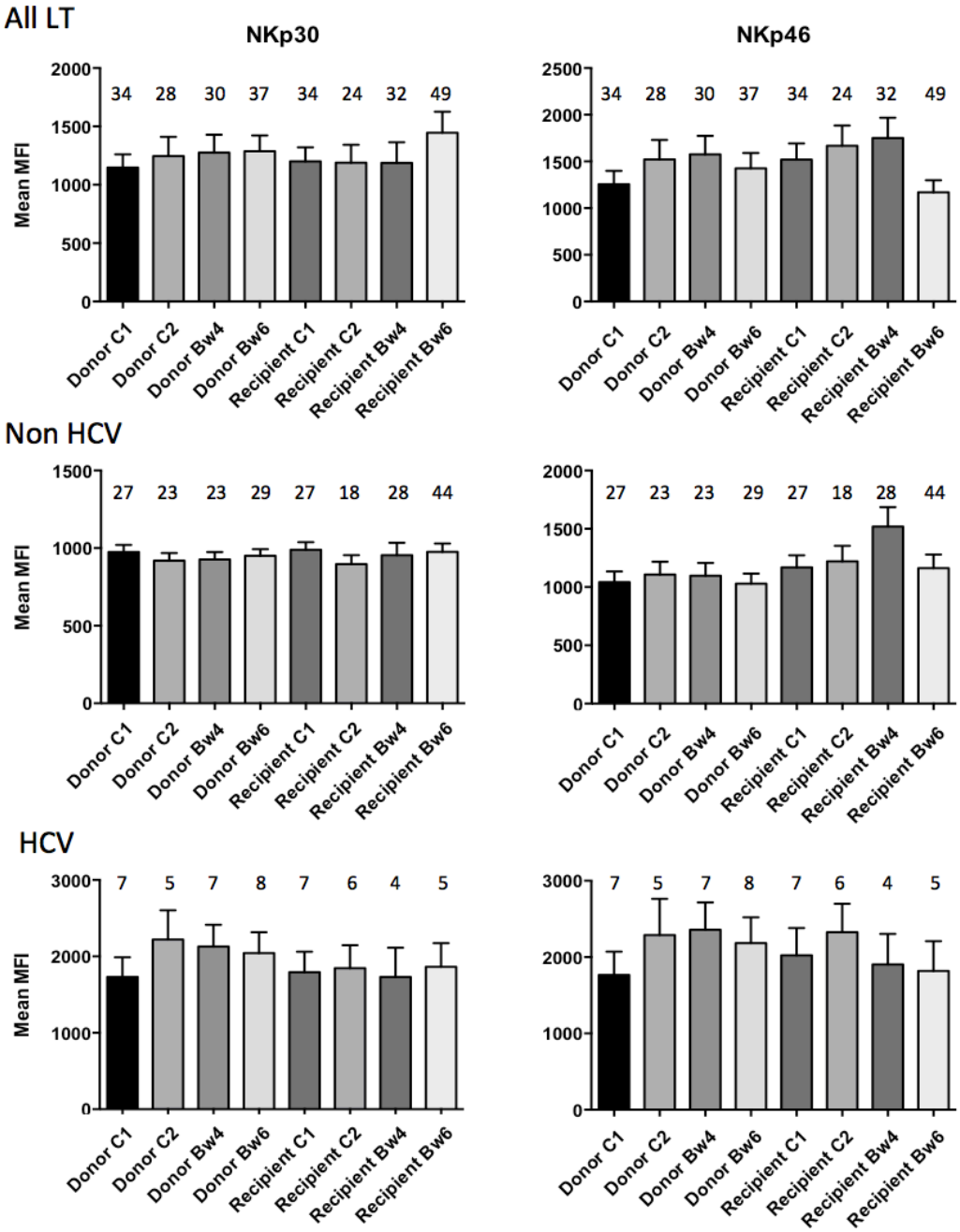


Figure 5.4 HLA alleles and NCR expression.

Analysis of association between the presence of donor and recipient HLA-Bw4/Bw6 alleles and HLA-C1/C2 alleles and expression of the natural cytotoxicity receptors is demonstrated. The numbers in each group are indicated above each bar.

5.1.3 KIR2DS3

The microarray and qPCR gene expression experiments described in Chapter 4 suggested a role for the activating receptor KIR2DS3 in LT HCV. There was higher gene expression of KIR2DS3 in LT HCV compared with both LT non-HCV and healthy controls. Therefore I sought to assess whether the presence of the KIR2DS3 allele was associated with NK activation. There was no statistical difference in the presence of KIR2DS3 between LT non-HCV and LT HCV (26% vs. 32% respectively, p=0.61). However, within the LT cohort, the presence of the KIR2DS3 allele was associated with higher expression of NCRs (**figure 5.5**). In all patients there was higher NKp46 expression in the presence of KIR2DS3 (p=0.004), and within the LT HCV subgroup increased expression of both NKp30 (p=0.04) and NKp46 (p=0.02) were associated with KIR2DS3.

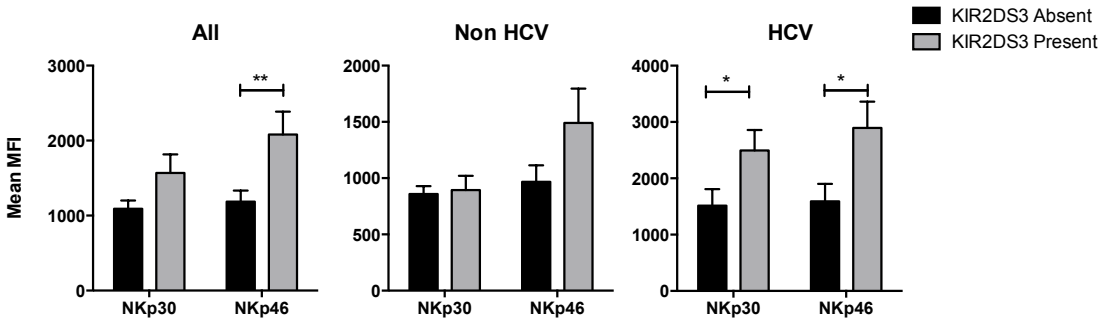


Figure 5.5 NK activation and KIR2DS3.

*Comparison of NCR expression in the presence and absence of the KIR2DS3 allele was performed. KIR2DS3 absent/present: All n=32/19; LT non-HCV n=22/11; LT HCV n= 10/8. *p<0.05; **p<0.01.*

5.1.4 HLA matching

The key question of whether HLA mismatching influences NK alloreactivity was addressed by comparison of NK activation between HLA-B and -C matched and mismatched LT recipients. The analysis of NK activating receptor expression in $CD56^{dim}$ NK cells is summarised in **figure 5.6**. There was no difference in expression of NKp30, NKp46 or NKG2D in either HLA-C of HLA-B mismatched LT compared to matched LT. Consistent with these phenotyping data, no differences in cytotoxicity were found between HLA matched and mismatched groups (**figure 5.7**).

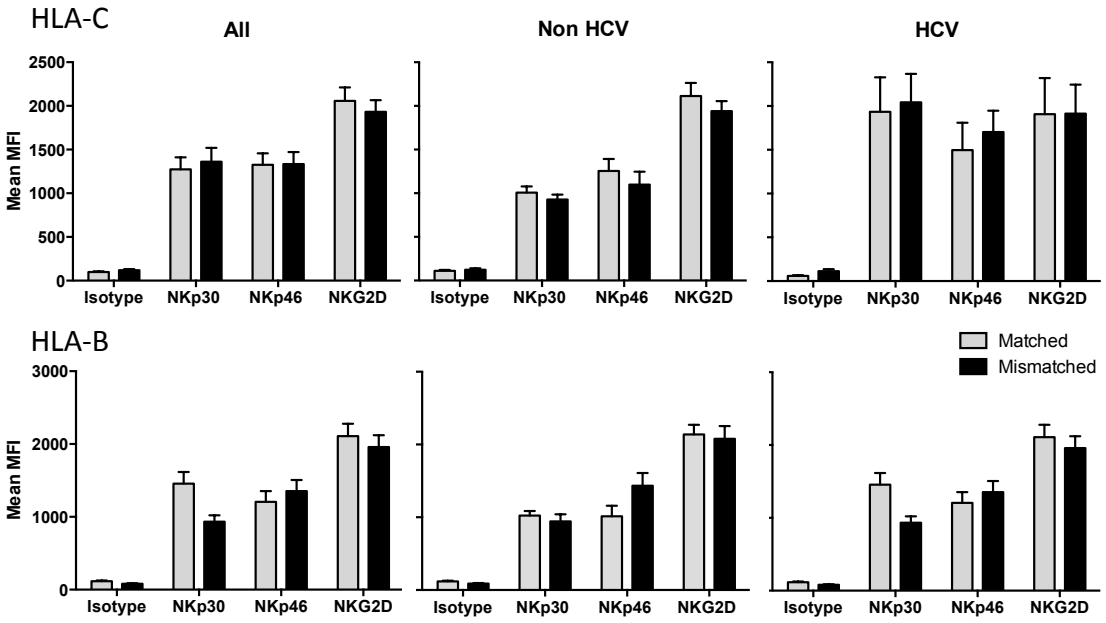


Figure 5.6 HLA-B and -C matching and NK activation.

Association between HLA-B and -C matching and recipient NK activating receptor expression was analysed. HLA-C: All n=41, non-HCV n=27, HCV n=14; HLA-B: All n=34, non-HCV n=24, HCV n=10.

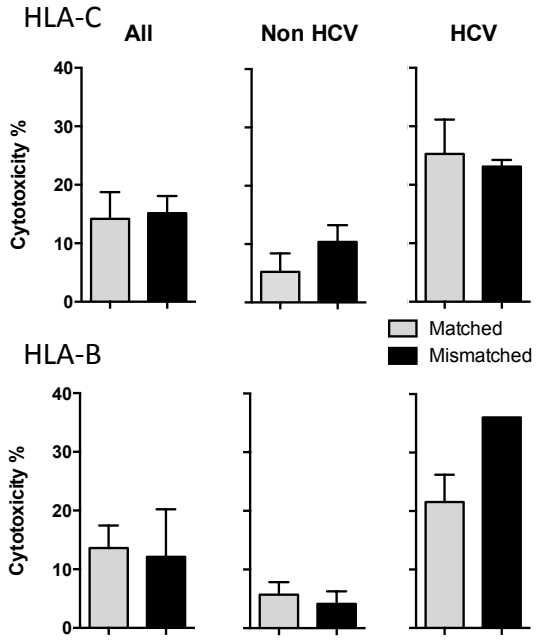


Figure 5.7 HLA-B and -C matching and NK cytotoxicity. *NK cytotoxicity according to HLA-B and -C matching is shown. HLA-C: All n=17, non-HCV n=10, HCV n=7; HLA-B: All n=12, non-HCV n=7, HCV n=5.*

The influence of matching was explored in more detail through analysis of specific subtypes of HLA-B and -C matching. For example, HLA-C matching in terms of NK allospecificity includes both donor HLA-C1C1 to recipient HLA-C1C1and donor HLA-C1C2 to recipient HLA-C1C1 (see **table 1.3** in Chapter 1). The expression of the NCRs in all the possible matching and mismatching HLA-B and -C combinations are shown in **figures 5.8 and 5.9**. There was no significant association between types of donor:recipient HLA-C matching and NKp30 expression in the whole LT cohort, and this remained the case in the LT non-HCV subgroup. In LT HCV there was increased NKp30 in C1C2:C1C2 (matched) LT compared with C1C1:C1C1 (matched), C1C2:C1C1 (matched), C1C2:C2C2 (matched) and C1C1:C1C2 (mismatched) LT. In addition there was increased NKp30 in C2C2:C1C2 (mismatched) LT compared with C1C2:C1C1 (matched) and C1C2:C2C2 (matched) LT. In the whole cohort, there was increased NKp30 expression in Bw4Bw6:Bw4Bw6 (matched) LT compared with Bw4Bw6: Bw6Bw6 (matched), Bw4Bw4:Bw4Bw6 (mismatched) and Bw6Bw6:Bw4Bw6

(mismatched) LT). In the HCV LT subgroup NKp30 expression was higher in Bw4Bw6:Bw4Bw6 (matched) LT compared with Bw6Bw6:Bw4Bw4 LT (mismatched). In LT non-HCV no donor:recipient HLA-C combinations were associated with NKp46 expression. Nkp46 expression was significantly higher in Bw4Bw6:Bw4Bw6 (matched) LT compared with Bw4Bw6:Bw6Bw6 (matched LT in all patients and this remained the case in both the HCV and non-HCV subgroups. NKp46 was also higher in Bw4Bw6:Bw4Bw6 (matched) LT compared with Bw6Bw6:Bw6Bw6 (matched) LT in the whole cohort and non-HCV subgroup. Finally, in the whole cohort, Bw4Bw6:Bw4Bw6 (matched) LT was associated with higher NKp46 expression than Bw6Bw6:Bw4Bw6 (mismatched) LT.

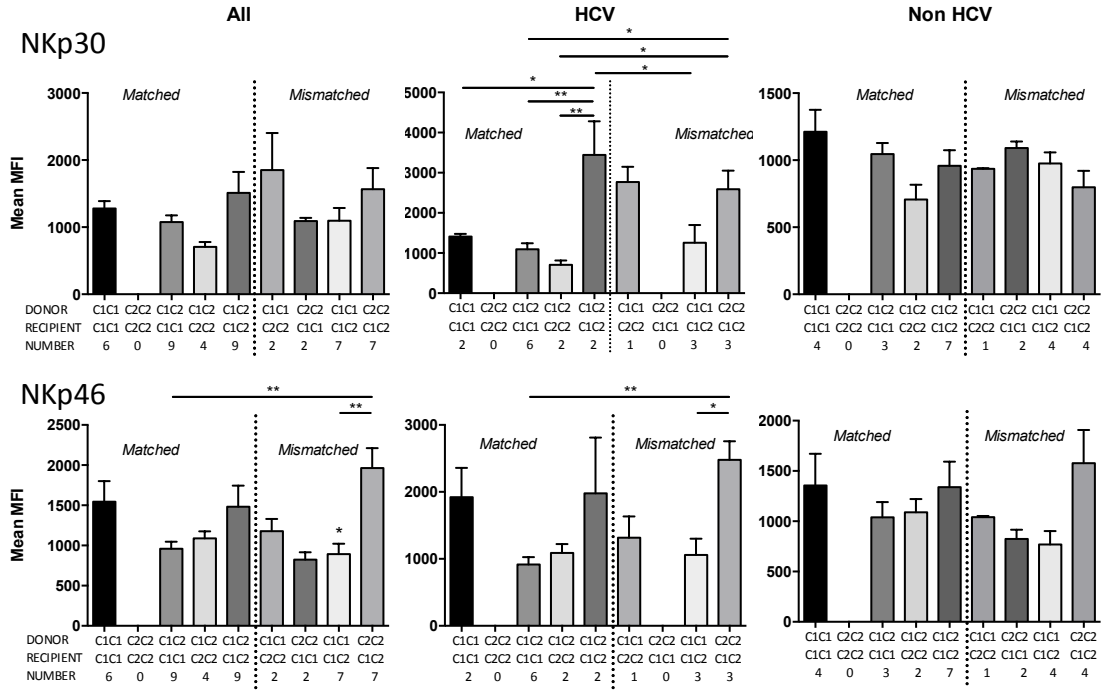


Figure 5.8 HLA-C matching subtypes and NCR expression.

Comparisons were made between each donor/recipient HLA-C pairing subtype. One-way ANOVA was used to determine if there was a significant difference between the means, with Tukey's correction for multiple comparisons. The donor and recipient genotypes and

*the number of patients in each subgroup are indicated below each graph. *p<0.05; **p<0.01*

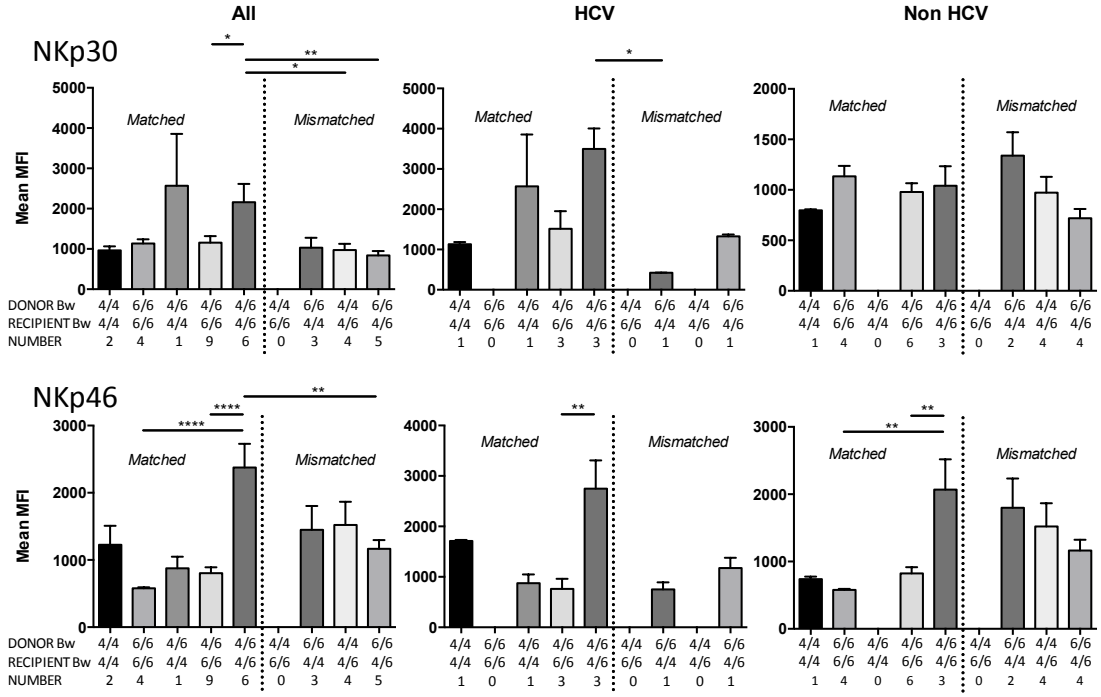


Figure 5.9 HLA-B matching subtypes and NCR expression.

*Comparisons were made between each donor/recipient HLA-B pairing subtype. One-way ANOVA was used to determine if there was a significant difference between the means, with Tukey's correction for multiple comparisons. The donor and recipient genotypes and the number of patients in each subgroup are indicated below each graph. *p<0.05; **p<0.01; ****p<0.0001*

As NK cell receptors are expressed stochastically, different clones of NK cells within an individual recipient may be predicted to be alloreactive, determined by KIR expression and degree of HLA matching. This is explained in the introduction and an example is illustrated in **figure 1.5D**. The specificity of KIR2DL1 for group 2 HLA-C epitopes and KIR2DL2/3 for group 1 HLA-C epitopes provides us with an opportunity to assess whether predicted NK alloreactivity does indeed occur within specific NK cell clones expressing inhibitory KIR with no HLA-C ligand present in the donor. This was achieved through evaluation of expression of activating receptors in KIR2DL1 (CD158a) and KIR2DL2/3 (CD158b) NK cell

subsets. If the observed hyporesponsive phenotype found in my cohort of liver transplant recipients was due to an effect on potentially alloreactive NK cells, analysis of the NK subsets would be expected to demonstrate differential expression of activating receptors determined by KIR expression and type of HLA-C mismatch. For example, in C1 mismatched LT (where HLA-C1 is present in the recipient and not the donor), recipient CD158b+ NK cells would be predicted to be alloreactive; therefore the tolerising effect of the allograft would be observed in these cells, generating the hyporesponsive phenotype. The results of this analysis are summarised in **figure 5.10**. There were no differences in expression of the activating receptors between KIR2DL1 and KIR2DL2/3 subsets in either HLA-C1 mismatched or HLA-C2 mismatched LT, and this remained the case when HCV and non-HCV subgroups were analysed.

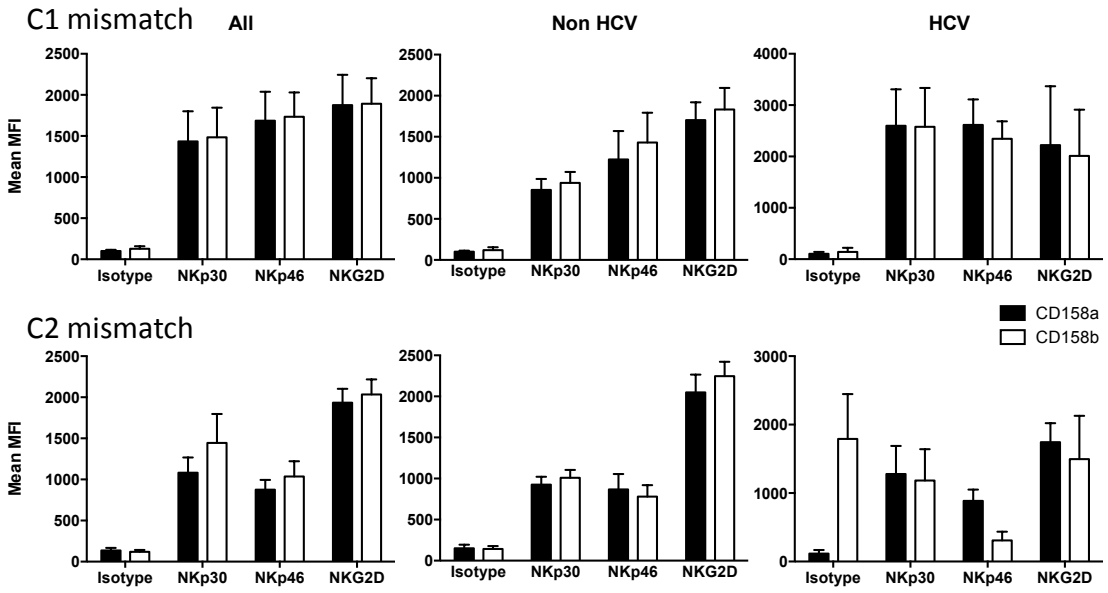


Figure 5.10 HLA-C mismatching and activating phenotype in KIR subsets.

Activating receptor expression in NK cells according to expression of inhibitory KIR with no ligand present in the donor. C1 mismatch: All n=9, non-HCV n=6, HCV n=3; C2 mismatch: All n=9, non-HCV n=5, HCV n=4.

5.1.5 HLA-C/KIR matching

Inhibitory signaling in the HLA-C/KIR2D- system is determined by the strength of the signal transduced by the KIR-ligand interaction. Binding and functional studies have established that the binding of KIR2DL1 with HLA-C2 is the strongest inhibitory combination, followed by KIR2DL2/HLA-C1, with KIR2DL3/HLA-C1 conferring the weakest inhibition (Winter, Gumperz et al. 1998, Moesta, Norman et al. 2008). It has been hypothesized that these differences in inhibitory signaling result in variable susceptibility to disease. Khakoo *et al* (2004) found KIR2DL3/HLA-C1 was associated with greater resolution of acute Hepatitis C infection was compared with KIR2DL2/HLA-C1 and KIR2DL1/HLA-C2. The authors postulated that the weaker inhibitory signaling conferred by KIR2DL3/HLA-C1 is more easily overcome, resulting in stronger NK antiviral responses. These data were independently corroborated in a subsequent study (Romero, Azocar et al. 2008). Furthermore in pregnancy, the combination of maternal KIR2DL1 and fetal HLA-C2 has been associated with recurrent miscarriage, growth retardation and pre-eclampsia (Hiby, Walker et al. 2004). This is thought to be because the stronger inhibitory combination renders uterine NK cells hypofunctional, providing susceptibility to the disorders.

I sought to investigate whether the variability of inhibitory signaling conferred by the HLA-C/KIR2D- combinations had significant influence on NK activating receptor expression within our study cohort of LT recipients. As all individuals carry KIR2DL1, the analysis focused on KIR2DL2 and KIR2DL3 alleles in combination with donor HLA-C1C1 homozygous and HLA-C1C2 heterozygous genotypes. Activating receptor expression was compared between each possible

HLA-C1/KIR2DL2/3 combination as shown in **figure 5.11**. There was no association between the genotype combinations and natural cytotoxicity receptor expression.

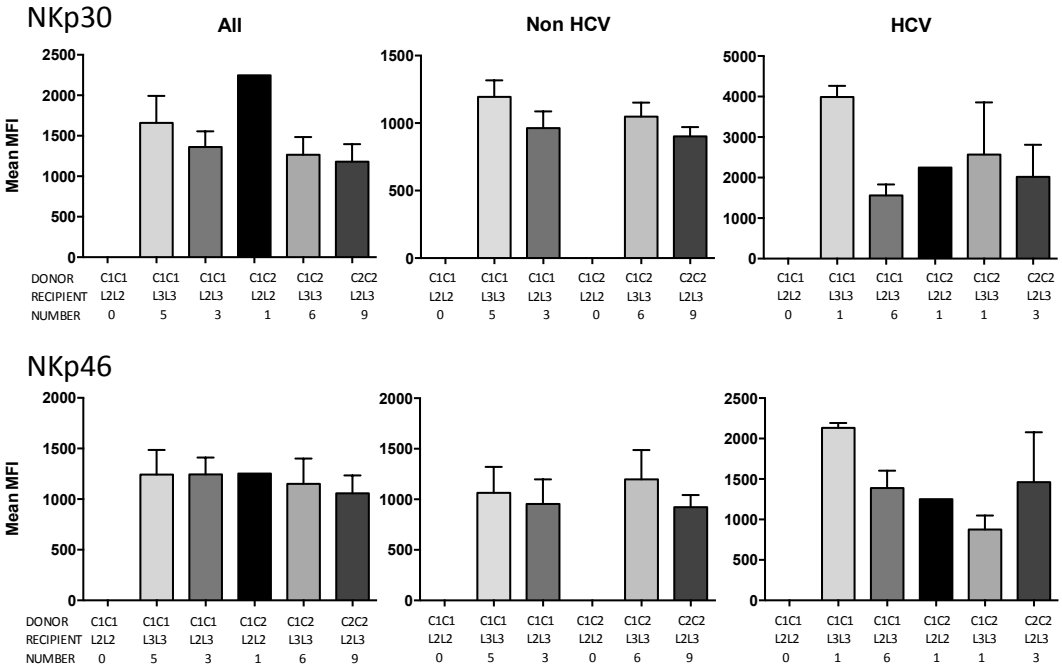


Figure 5.11 HLA-C/KIR combinations and NCR expression.

Comparisons were made between each donor/recipient HLA/KIR pairing subtype. One-way ANOVA was used to determine if there was a significant difference between the means, with Tukey's correction for multiple comparisons. The donor and recipient genotypes and the number of patients in each subgroup are indicated below each graph.

5.2 Association of HLA and KIR genotypes with clinical outcomes

Thus far the cellular assays have demonstrated that HLA and KIR genotypes have little bearing on NK cell activation. Few previous studies have investigated the influence of genetics and recipient NK cell function, but have focused on clinical outcomes such as rejection, or graft and patient survival. As the studied cohort was taken from allograft recipients attending liver outpatient clinics and donor genotyping was only available for the current graft, assessment of graft and patient survival were not applicable readouts of relevant clinical outcomes. Instead, episodes of acute cellular rejection, and degree of fibrosis at liver biopsy were considered more appropriate markers of clinical outcome. This information was obtained through patient clinical records and departmental databases. **Table 5.2** shows the number of patients in whom genotyping and biopsy data were available, as well as the number of patients diagnosed with at least one episode of acute cellular rejection (ACR). For the purposes of this analysis, an episode of ACR was attributed if there was a diagnosis of ACR made on the basis of clinical, biochemical and/or (but not necessarily) biopsy information.

	Number		
Recruited	109		
HLA-C			
Donor	97		
Recipient	98		
Both	87		
HLA-B			
Donor	102		
Recipient	79		
Both	72		
Recipient KIR	106		
Donor HLA-C and Recipient KIR	94		
	Non-HCV	*HCV*	
Episode of ACR	12	1	*p=0.29*
Fibrosis score 0-3	33	16	
Fibrosis Score 4-6	1	8	*p=0.0025*

Table 5.2 Genotyping and clinical outcome data.

The number of patients in whom donor and/or recipient HLA/KIR genotyping was available is shown, as well as the number with each of the analysed clinical outcomes. HCV was significantly associated with advanced fibrosis (but not ACR), and thus fibrosis analysis was performed only within the HCV subgroup.

Fibrosis stages were divided into early stage fibrosis (fibrosis score 0-3) or advanced fibrosis (fibrosis score 4-6) according to the Ishak scoring system (Ishak, Baptista et al. 1995). Of the total cohort of 109 liver transplant recipients, both donor and recipient HLA-C and HLA-B genotyping was available in 87 (80%) and 72 (66%) patients respectively. Both donor HLA-C and recipient KIR typing were available in 94 (86%) of cases. ACR occurred in 13 (12%) patients and a biopsy was available in 58 (53%). Of the patients with liver biopsy data, only 1 of 34 (3%) non-HCV recipients had advanced fibrosis, compared with 8 of 24 (33%) HCV recipients (p=0.0025). In contrast, ACR was not associated with HCV infection status (p=0.29), with only one HCV LT recipient experiencing ACR.

5.2.1 Acute Cellular Rejection

Recipients were grouped according to group A or group B KIR haplotypes as previously described and the incidence of ACR within each haplotype was compared. There were no differences in ACR between the two groups of KIR haplotypes (**figure 5.12**).

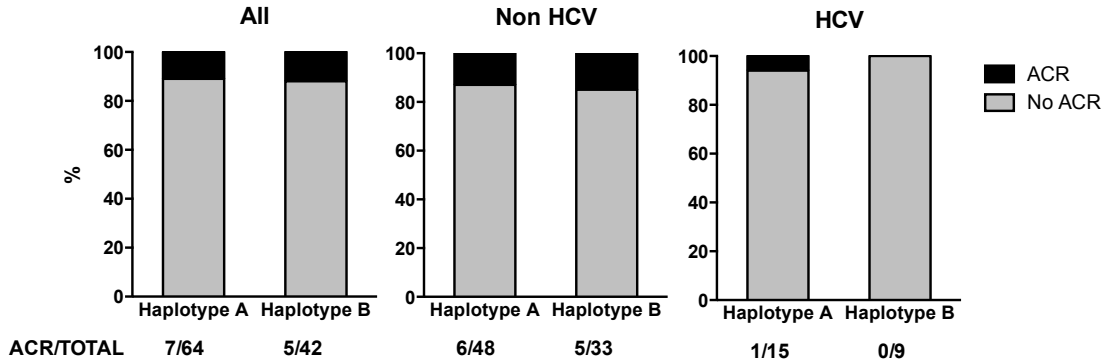


Figure 5.12 KIR haplotype and clinical outcomes.

Association between KIR haplotype and incidence of acute cellular rejection was analysed. The numbers in each group are indicated below the bars.

The presence of HLA-B and -C alleles within the donor and recipient were then compared with respect to incidence of ACR as shown in **figure 5.13**. ACR incidence was not significantly different between the groups.

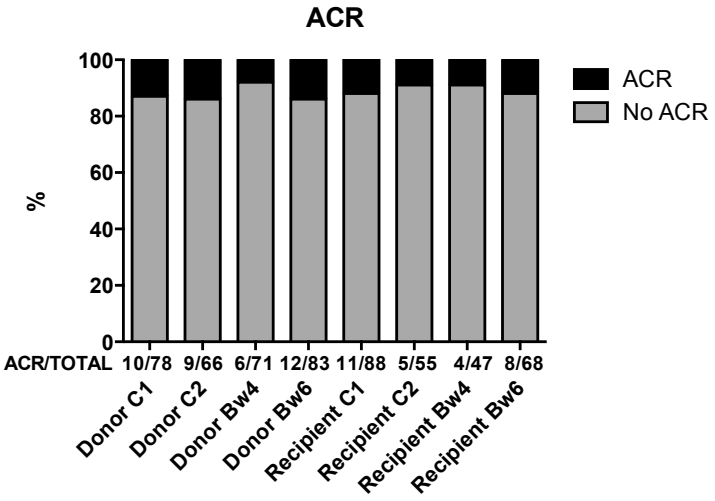


Figure 5.13 HLA alleles and ACR.

The rates of acute cellular rejection in the presence of HLA-C1/C2 and HLA-Bw4/Bw6 alleles in donor and recipient are shown. Numbers in each group are indicated below the bars.

Furthermore, there was no significant association between the incidence of ACR and HLA-B or -C matching, although there was a trend towards *less* ACR in both mismatched groups (**figure 5.14**).

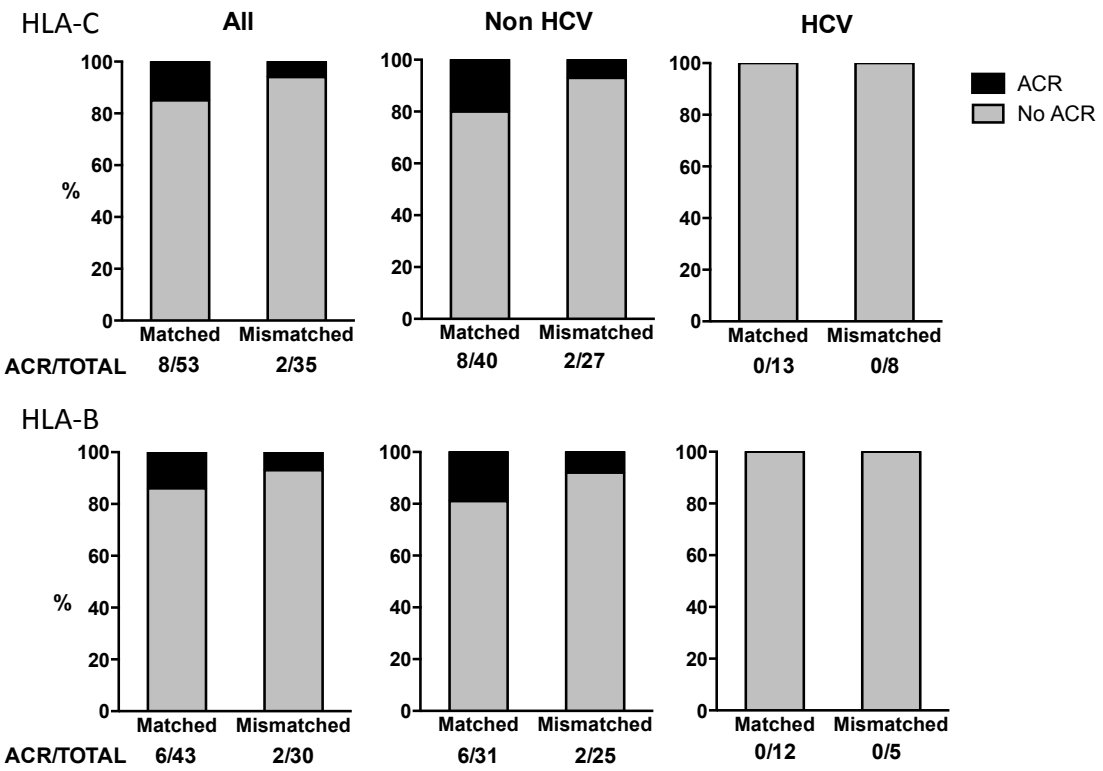


Figure 5.14 HLA matching and ACR.

Association between HLA-B and -C matching and episodes of ACR was assessed; numbers in each group are indicated below the bars.

This was explored in more detail by analysing whether specific subtypes of HLA-B or -C matching were associated with the incidence of ACR (**figure 5.15**). In this analysis, there was a significantly higher incidence of ACR in donor Bw6Bw6 to recipient Bw6Bw6 matching – ACR occurred in 3 of 6 transplants (50%) compared with an incidence of 7.5% in the other HLA-B matching subtypes (p=0.016, Fisher's exact test).

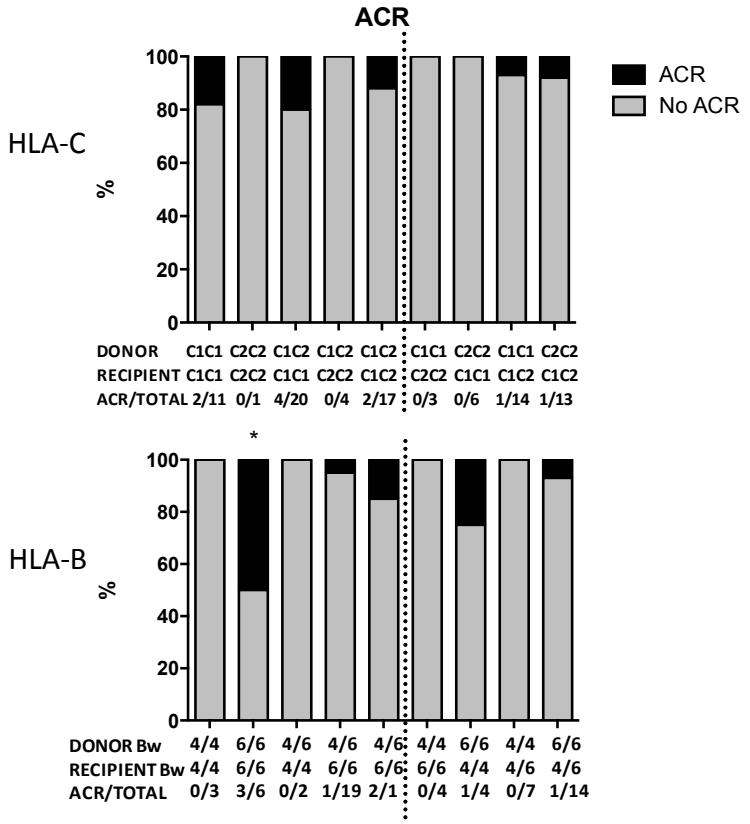


Figure 5.15 HLA matching subtypes and ACR.

*Association between subtypes of HLA-C matching and ACR was assessed. Numbers in each group indicated below the bars. *p<0.05 (Fisher's exact test)*

Analysis of HLA-C/KIR genotype combinations is shown in **figure 5.16.** There was no association between the various combinations and incidence of ACR.

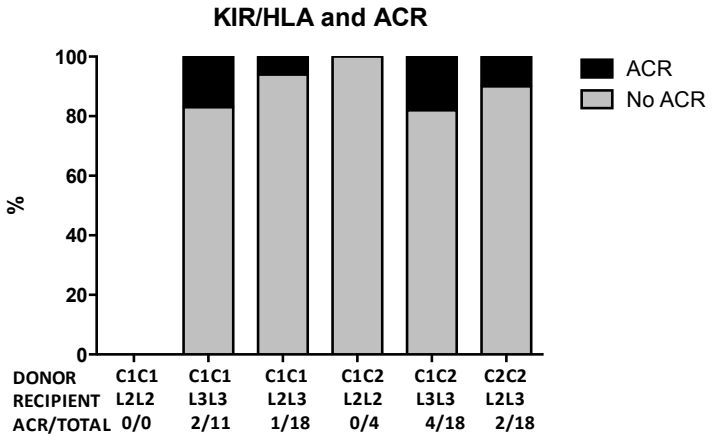


Figure 5.16 KIR/HLA combinations and ACR.

Analysis of association between ACR and HLA/KIR combinations is shown. The numbers in each group are indicated below the bars.

5.2.2 Fibrosis

Acute cellular rejection may be a short-term marker of alloreactivity in liver transplantation, as it occurs most commonly within three months of the operation. Practical longer-term assessment of allograft damage is a more challenging task as differentiating between chronic rejection, disease recurrence or *de novo* liver disease is often not possible on the basis of clinical signs and biochemical investigations. The gold standard for assessing the state of the graft is histological assessment of a liver biopsy. However, this is not routinely carried out post transplant, and only performed if clinically required. In the recruited cohort of patients, 34 non-HCV transplant recipients underwent a liver biopsy, but the majority of these were early post-transplant (median time to biopsy 11 months post transplantation) for assessment of acute cellular rejection. Only 1 of the LT non-HCV recipients had advanced fibrosis, and none had chronic rejection, and thus within this cohort it was not possible to assess the effect of genotype on long-term outcomes.

However the hepatitis C cohort did provide an opportunity to study the influence of genetics with longer-term outcomes, as liver biopsies are routinely carried out on a yearly basis. This is because hepatitis C recurrence is universal, and reinfection of the graft very often leads to accelerated liver fibrosis, with cirrhosis occurring in up to 30% of grafts at 5 years (Gane, Naoumov et al. 1996, Forman, Lewis et al. 2002). This can lead to graft failure and need for re-transplantation. For this reason, regular histological assessment of liver damage is carried out with liver biopsy. It is known that NK cells are important in the immune response to hepatitis C, and in the non-transplant setting, HLA-KIR

genotypes influence the outcome of acute infection (Khakoo, Thio et al. 2004, Romero, Azocar et al. 2008). In liver transplantation, the influence of HLA/KIR on recurrent hepatitis C was evaluated by de Arias et al (2009) in 151 LT recipients. The authors reported an association between HLA-C mismatch and histological recurrence of hepatitis, and that the presence of KIR2DL3 had an additive effect.

In the present recruited cohort of patients, 24 of the 25 individuals with HCV infection had a biopsy available with the median time to biopsy of 35 months post-transplant. Eight of the 24 (33%) had advanced fibrosis (**see table 5.2**), and analysis was performed to assess the association between genotypes and development of advanced fibrosis. Only one HCV patient lacked KIR2DL3, and therefore it was not possible to carry out an analysis of the effect of KIR2DL2/3. Advanced fibrosis was seen in a similar proportion of haplotype A and B individuals (**figure 5.17**). Furthermore, there was no significant association between the presence of any HLA-B or -C alleles and advanced fibrosis (**figure 5.18**).

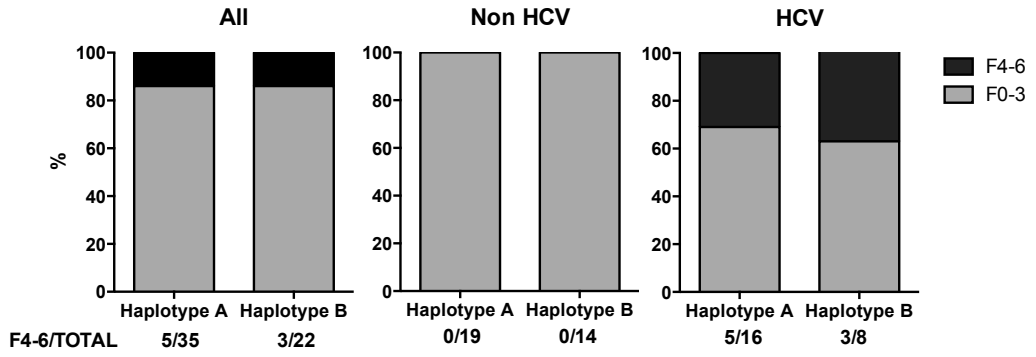


Figure 5.17 KIR Haplotype and fibrosis.

Association between KIR haplotype and advanced fibrosis in LT was assessed. Numbers in each group indicated below the bars.

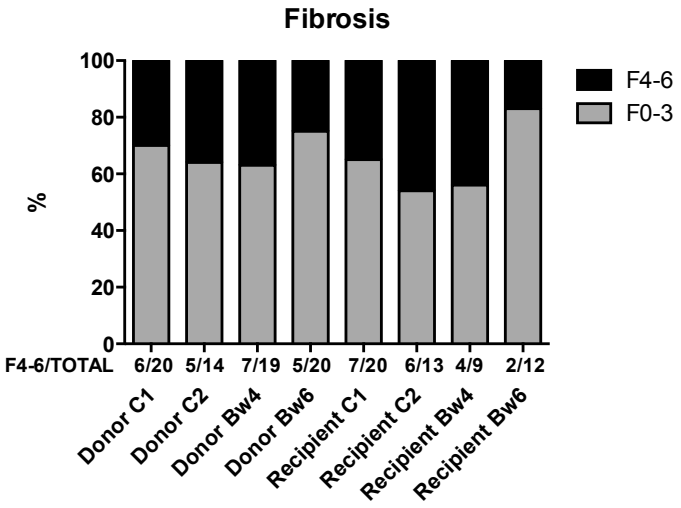


Figure 5.18 HLA alleles and fibrosis.

Association between the presence of donor and recipient HLA-C1/C2 and HLA-Bw4/Bw6 alleles and advanced fibrosis was analysed. Numbers in each group indicated below the bars.

As the KIR2DS3 allele was associated with NCR expression in HCV LT, association between this allele and development of advanced fibrosis was analysed within the HCV cohort (**figure 5.19**). There was a trend towards more advanced fibrosis in recipients with KIR2DS3 (50% F4-6) compared with those that did not carry the allele (25% F4-6), but this did not reach statistical significance (p=0.36).

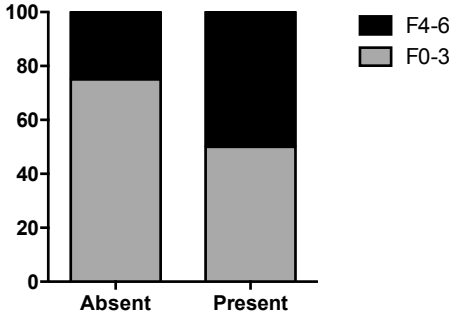


Figure 5.19 KIR2DS3 and fibrosis.

The rates of advanced fibrosis in the presence and absence of the KIR2DS3 allele was analysed.

The analysis of HLA-B and -C matching is shown in **figure 5.20**. No patient with HLA-B mismatch developed advanced fibrosis, compared with 5/12 (41%) HLA-

B matched patients, although this did not reach statistical significance (p=0.110, Fisher's exact test). No other associations were observed between matching (including subtypes) and advanced fibrosis in post LT HCV.

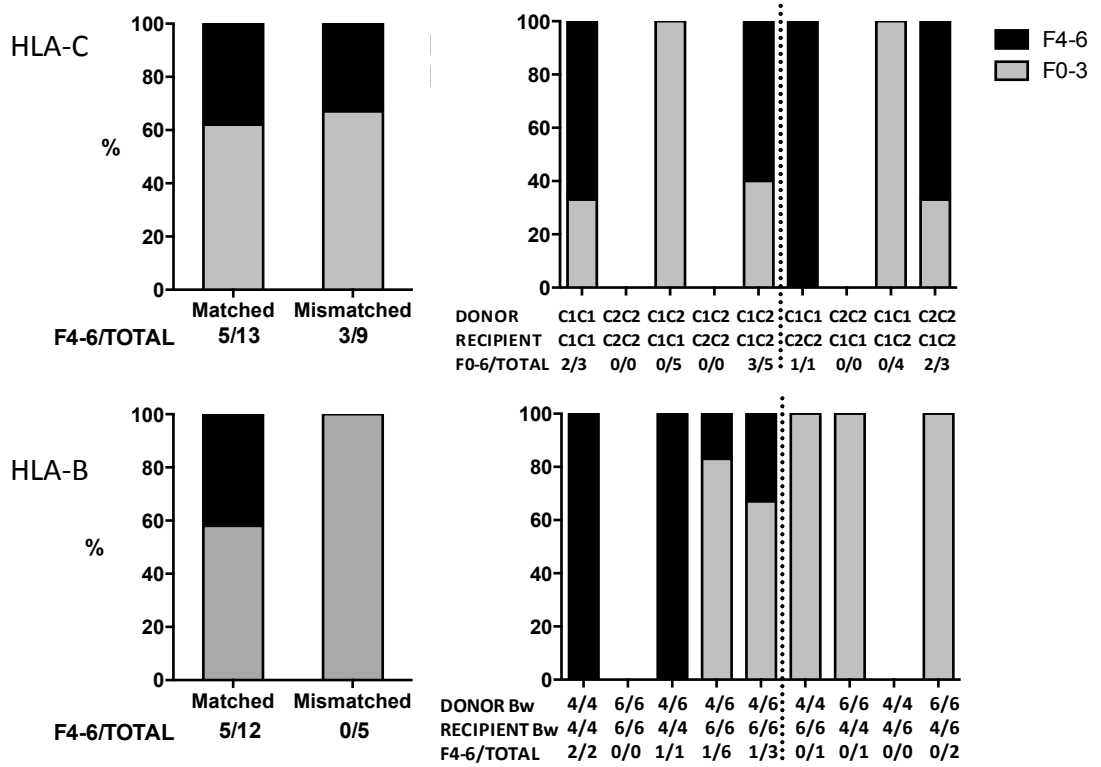


Figure 5.20 HLA matching and fibrosis.

Associations between HLA-B or -C matching and fibrosis stage at biopsy were analysed. The numbers in each group are indicated below the bars.

The analysis of HLA/KIR combinations and development of advanced fibrosis demonstrated no significant associations (**figure 5.21**). However, no patient with HLA-C1C1/KIR2DL3L3 matching developed advanced fibrosis, compared with 7/17 (41%) in the other HLA/KIR genotype combinations. This did not reach statistical significance.

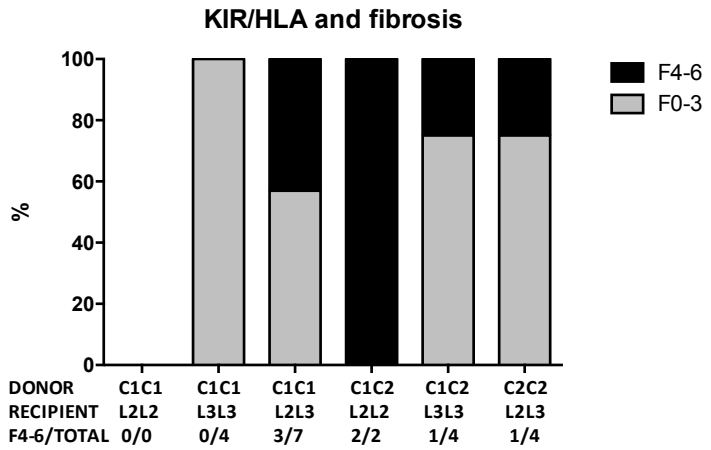


Figure 5.21 HLA/KIR matching and fibrosis.

Assessment of HLA/KIR combinations and development of advanced fibrosis was performed. The numbers in each group are indicated below the bars.

5.3 Discussion

The data presented in this chapter describe a detailed analysis of the influence of KIR and HLA genotyping on NK activation in liver transplantation. The associations between genotyping and clinical outcomes are also presented.

When assessing the effects of KIR genotype, individuals were divided into haplotypes A and B according to KIR gene content. As haplotype B contains more activating receptors, one might hypothesise that NK cells from haplotype B individuals would have a more activating phenotype. However, the functional assays did not demonstrate any effect of haplotype on activation. The function of the activating KIR is poorly understood and the lack of association between KIR haplotype and function implies that, in LT at least, the KIR do not influence recipient NK cell alloreactivity or tolerance. This is consistent with the phenotyping and functional data presented in Chapter 3, which demonstrated that the tolerised phenotype of NK cells was independent of inhibitory KIR expression.

More detailed analyses of NCR expression, NK function, and clinical outcomes according to donor HLA, recipient HLA, and recipient KIR typing were performed. Previous studies have explored a possible protective role in LT of the presence of HLA-C2 alleles in the donor, due to the relatively stronger inhibitory interaction between HLA-C2 and KIR2DL1 (Hanvesakul, Spencer et al. 2008, Tran, Middleton et al. 2009). I found no associations between NK cell phenotype or function and the presence of any HLA-C1, HLA-C2, HLA-Bw4, or HLA-Bw6

alleles in either donor or recipient. In addition the lack of association between KIR-HLA matching and NK phenotype is an important finding, further supporting the emerging theme that recipient NK cells do not become tolerised through KIR-ligand interactions.

The analysis of the KIR2DS3 allele provided interesting results. The trend towards increased carriage of the KIR2DS3 allele in HCV recipients is consistent with the gene expression data, and with previous studies reporting an association between KIR2DS3 and chronic HCV infection (Dring, Morrison et al. 2011, Fitzmaurice, Hurst et al. 2014). In our cohort, within KIR2DS3 carriers transplanted for HCV infection, there was higher NCR expression and a trend toward increased fibrosis. At present little is known about the functional role of KIR2DS3 especially since transfection experiments demonstrate that there is little or no surface expression of the protein (VandenBussche, Mulrooney et al. 2009). Furthermore the ligand for KIR2DS3 remains unknown. One possible explanation for our findings is that KIR2DS3 is in linkage disequilibrium with a gene (within the LRC) that has a functional role in the immune response to HCV.

The analysis of genetics and clinical outcome demonstrated similar findings, in that there was no association between HLA-B or –C matching and episodes of ACR or development of fibrosis. The lack of genetic influence on clinical outcomes supports the current clinical strategy of not matching for HLA in LT. That genetics do not seem to affect outcomes, even when specific KIR/HLA combinations are analysed, leads to the question of why predicted 'missing-self' allospecificity is not observed. Recipient NK cells lacking inhibitory KIR specific

for donor HLA must have over-riding mechanisms preventing activation. One possible explanation is that the presence of other non-HLA specific inhibitory receptors such as 2B4, LIR, and KLRG1 (described in section 1.1.4.4) prevents activation. Other KIR independent factors affecting recipient NK functionality might include the influence of cytokines and other cellular factors generated in the context of LT, and interactions with other immune cells that might regulate NK cell function.

In the **Introduction** chapter NK development and education was discussed in detail. The maturation and education processes leading to functional NK cells take place in stages across different sites, including secondary lymphoid tissues. The acquisition of surface KIR is a key stage in this process, and as our data shows that neither KIR genotype nor expression have an effect on the development of tolerance, I propose that the mechanism leading to tolerance occurs at a stage of NK cell development prior to, and independent of KIR expression. As such interactions between the liver allograft and developing NK cells resulting in downregulated activation are not due to direct interference to the licensing process.

The analysis presented in this chapter is limited by several complicating factors. The heterogeneity of underlying liver disease is one such factor, as this may affect NK cell activity. Separating HCV from non-HCV was an attempt to control for this, but I was unable to further sub–divide the recipient group into specific aetiologies because of low numbers. Viral infections other than HCV are known to influence the function of NK cells, which could mask the effects of HLA and KIR

genotypes. Additionally, dividing the analysis into specific donor-recipient genetic subgroups resulted in low numbers within each group, making statistical analysis somewhat challenging.

In summary, in liver transplant HLA and KIR genetics do not significantly influence recipient NK cell function, and importantly, do not influence short or long-term clinical outcomes. Thus, based on these data, HLA matching should not be carried out in LT.

CHAPTER 6: A LONGITUDINAL ANALYSIS OF LIVER TRANSPLANT RECIPIENT NK CELL ACTIVATION

The data presented in the previous chapters provide evidence of functional and maturation alterations in NK cells taken from a cohort of liver transplantation recipients with comparisons made with a cohort of healthy control individuals. This cross-sectional analysis demonstrates significant tolerance in LT recipient NK cells. But how do these recipient NK cell changes evolve with time after liver transplantation? To date there have been few studies assessing longitudinal changes in recipient NK cells after transplantation. In HSCT, previous studies have reported changes in frequency of NK subsets and impairment in reactivity to target cells in the early post transplant period (Jacobs, Stoll et al. 1992, Vitale, Pitto et al. 2000). The timing of acquisition of receptors and functionality by donor NK cells after HSCT has also been described (Shilling, McQueen et al. 2003). An early liver transplantation study reported increased NK cytotoxicity in HLA mismatched versus matched grafts in the first month post LT (Oertel, Kohlhaw et al. 2001); however no longitudinal changes were found. Subsequent studies have noted transient alterations in NK cell frequency and activating receptor expression early post LT that did not persist at 12months (Varchetta, Oliviero et al. 2009, Pham, Piard-Ruster et al. 2012).

Thus, as detailed characterization of longitudinal changes in NK activation has not previously been done, a longitudinal study was performed, with individuals recruited at assessment for liver transplant eligibility. In those individuals

transplanted, samples were taken at intervals post-LT and phenotyping assays were performed. Comparison of NK cell activation pre- and post-transplant was performed to assess the evolution of changes in the first year post LT.

RESULTS

Recruitment into the longitudinal study took place at the pre- liver transplant assessment stage. Patients with potentially transplantable liver conditions (eg end stage cirrhosis, hepatocellular carcinoma) were admitted for assessment and investigations to assess eligibility for transplant. 28 patients were recruited at pre-assessment at Kings College Hospital. Of these, 21 (75%) were listed for transplant, and during the study period, 13 underwent a liver transplant (46% of recruited cohort). Five of these patients were repatriated to local regions, and repeated endeavours to obtain blood samples from these patients at their local hospitals proved unsuccessful. One patient was diagnosed with HCC recurrence during the follow-up period and was excluded from the study. As a result the longitudinal study comprises an analysis of pre and post transplant NK cells from 7 patients (see **figure 6.1**)

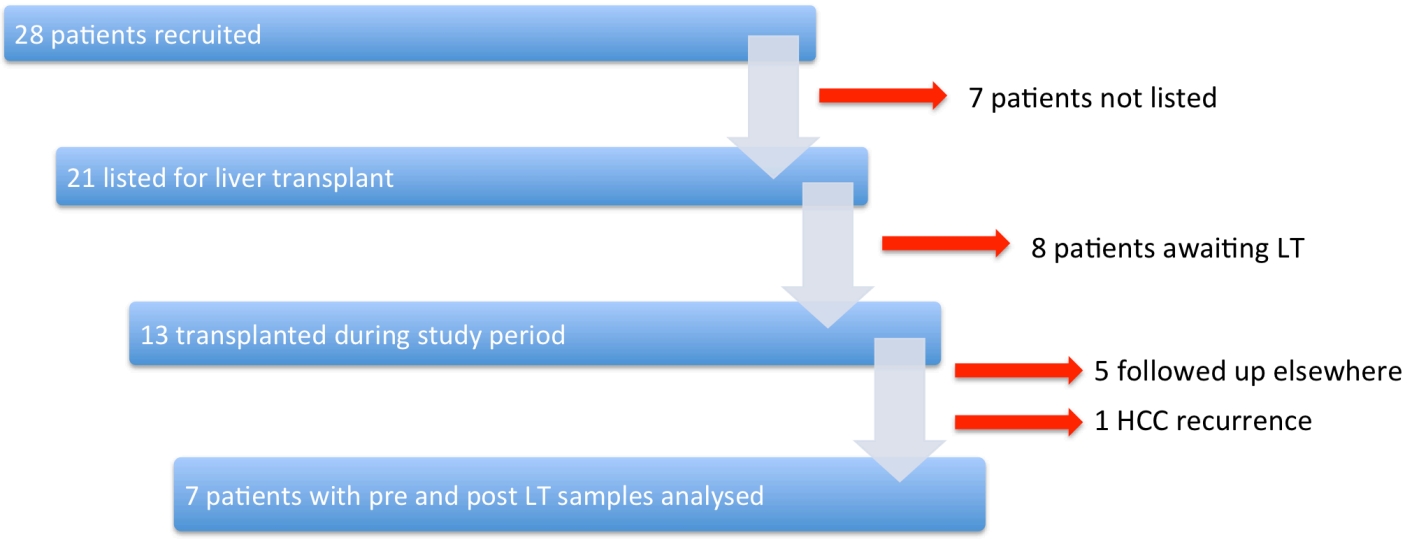


Figure 6.1 Schematic showing the recruitment into the longitudinal study.

Furthermore the timing of blood sampling proved problematic. It was anticipated that blood samples would be taken at specific intervals post transplant (eg at weeks 1, 4, 8, 16, 24, and 48), but a combination of factors ultimately made this unachievable. The two main factors were patients not attending scheduled appointments, and the fact that the appointments were at a different site (Kings College Hospital) from the academic department and laboratory where I was based (Imperial College London).

Table 6.1 shows the timing and number of sample obtained in this study. The number of post transplant samples collected ranged from 1-3 collected between week 7 and week 49 post-transplant.

Patient	**Timing of sample post liver transplant**		
#1	Week 7		
#2		Week 36	Week 49
#3			Week 45
#4	Week 8	Week18	Week 36
#5	Week 8		Week 34
#6	Week 9		Week 30
#7	Week 3		

Table 6.1 Timing of blood sampling in longitudinal cohort.

6.1 Changes in NK cell subsets

Figure 6.2 shows the changes in NK cell frequency and $CD56^{bright}$ subsets in each study participant. Where there was more than one post LT sample taken, the mean percentages are shown. There was no significant difference between pre- and post-LT in the percentage of NK cells as a proportion of total lymphocytes (**figure 6.2A**). However in 5 individuals the NK percentage remained fairly static, whereas in two there was a substantial increase. The proportion of $CD56^{bright}$ NK cells also did not change significantly from pre- to post LT, although in 5 of the 7 patients there was a reduction in NK percentage (**figure 6.2B**).

In a further analysis, the timing of the post LT samples was categorised to assess whether any trends could be detected longitudinally over the 12 months following LT (**figure 6.2C** and **6.2D**). Compared to pre-transplant levels, this demonstrated an increase in NK cell percentage up to week 12, with a reduction back to baseline from weeks 12 to 34, and levels remained stable up to week 49. The relative proportion of $CD56^{bright}$ NK cells followed a very similar pattern.

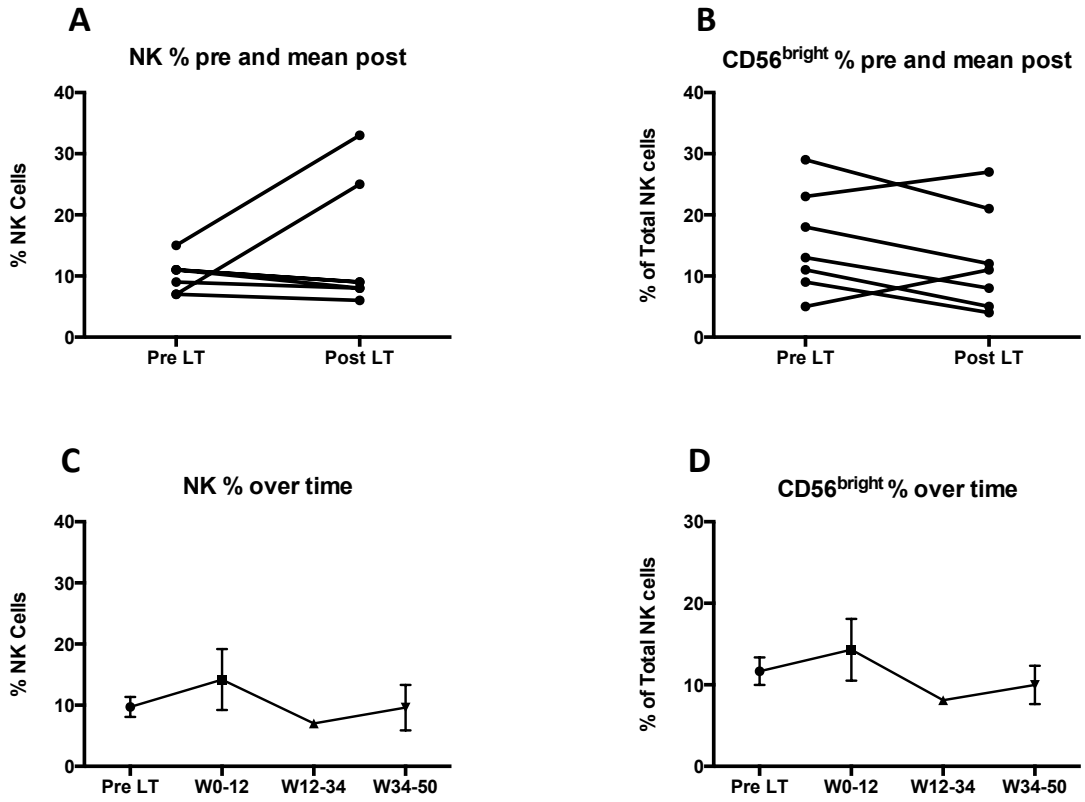


Figure 6.2 NK cell changes longitudinally post liver transplant.

Comparisons were made between pre- and post-LT samples. ***A*** *and* ***B*** *show the changes in individual study recruits; where there was more than one post LT sample, the mean % is displayed. The proportion of NK cells did not significantly change, but in 2 individuals, there was a substantial increase post LT. The relative proportion of the CD56^{bright} subset of NK cells reduced in 5 of the 7 patients, and modestly increased in the other 2.* ***C*** *and* ***D*** *demonstrate the pooled data at catergorised time periods post LT. Both NK cell and CD56^{bright} proportions followed a similar pattern of increasing in the first 12 weeks post LT, then falling back to baseline by week 24, and remaining stable up to week 49.*

6.2 Activating receptor expression

The expression of the NK cell activating receptors NKp30, NKp46 and NKG2D were assessed through FACS analysis as described in the cross-sectional study. The expression levels as determined by MFI were compared between pre- and post transplant samples. The results are depicted in **figure 6.3**.

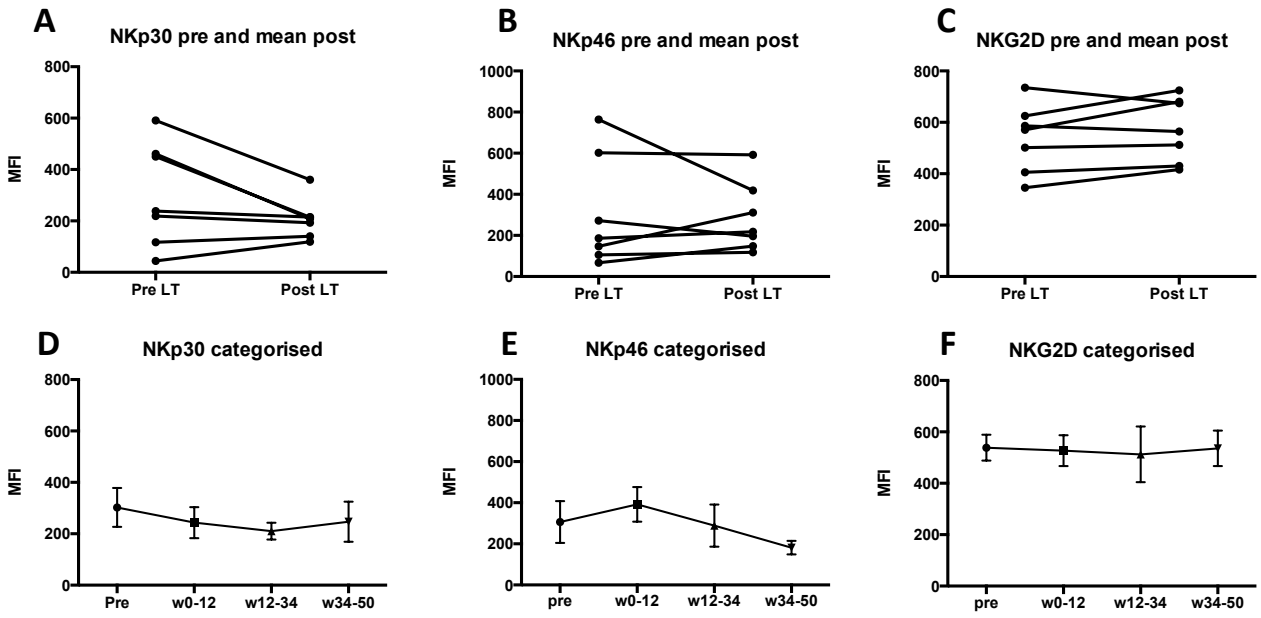


Figure 6.3 Longitudinal changes in activating receptor expression.
A-C *shows the individual patient data comparing pre-LT with mean post LT activating receptor expression. There were no consistent changes of any of the receptors analysed.* ***D-F****: pooled data categorizing the post LT samples into time periods. NKp30 expression declined to week 34, with levels recovering by week 50. NKp46 expression initially upregulated to week 12 but then declined to week 50, whereas NKG2D expression remained stable.*

Overall there was no consistent down- or up-regulation of receptor expression observed. The individual patient data is shown in **figure 6.3A-C** and the pooled data categorizing samples into time periods post LT are summarised in **figure 6.3D-F**. There was a slight reduction in NKp30 expression between pre-LT and

week 34 post-LT which returned to baseline between weeks 34 and 50. Conversely, NKp46 expression increased at weeks 0-12, and then steadily declined up to week 50 post-LT. The levels of NKG2D remained stable throughout the 12-month follow-up period.

6.3 Longitudinal changes in NKG2C and CD16 expression

As altered NK cell maturation was shown to be associated with a tolerant NK cell phenotype, with increased NK cell expression of NKG2C demonstrated in LT, the surface expression of maturation markers NKG2C and CD16 was also evaluated in the longitudinal cohort.

There was a wide variation in baseline CD16 expression pre-LT as seen in **figure 6.4A**. There was no significant change in the mean post LT expression of CD16 and this was also true of NKG2C expression (**figure 6.4B**). The changes in expression of these markers over the 12-month period are depicted in **figure 6.4C** and **6.4D**. There appeared to be a reduction in CD16 expression at weeks 12-34, but this did not persist at week 50. The levels of NKG2C were stable throughout the follow-up period, and thus the increase seen in the cross-sectional study was not recapitulated.

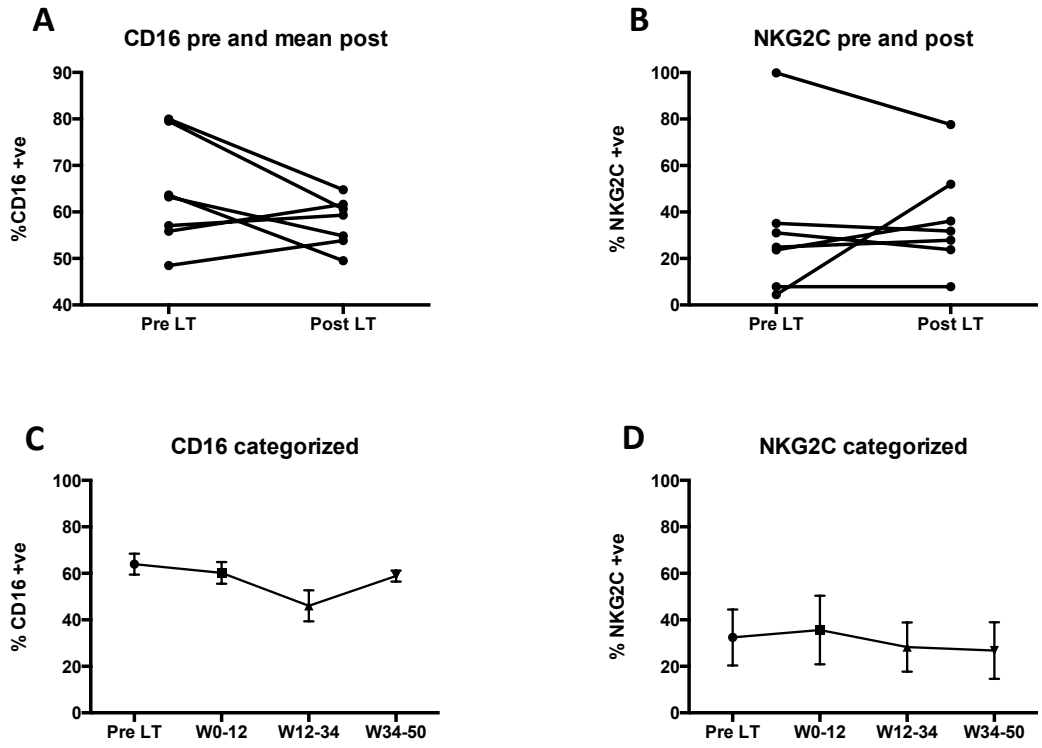


Figure 6.4 Longitudinal changes in CD16 and NKG2C.

A,B: *The changes from pre- to post-transplant expression of CD16 and NKG2C in the individual patients are shown. No significant trends were observed.* ***C,D:*** *pooled data with samples categorized into time periods post-LT. CD16 expression dipped at weeks 12-34 but subsequently recovered, whilst NKG2C expression did not changes throughout the period.*

6.4 Discussion

In this study an attempt an attempt was made to elucidate the evolution of NK cell changes towards the tolerant phenotype previously described. Somewhat surprisingly, the cross-sectional findings were not recapitulated, with no significant changes in NK activation, and no alterations in NK subsets.

One possible explanation for these disparate findings is that NK tolerance through hypofunctionality develops over a longer time period than the 12

months evaluated in this study. Perhaps repeated and prolonged interactions between recipient NK cells and the graft are required in order to promote the conditions necessary for NK cell tolerance. This would be consistent with an anergy type model of NK tolerance, whereby persistent NK stimulation leads to exhaustion and hypofunctionality. Two previous studies have evaluated NK cell changes longitudinally post LT. In a paediatric cohort increased expression of both NKp30 and NKG2D was observed in the first week post transplant, and this subsequently returned to baseline (Pham, Piard-Ruster et al. 2012). There was also a reduction in total circulating NK cells early post transplant but once again this did not persist. The second study evaluating NK changes in recurrent HCV infection after liver transplantation also reported a transient decrease in NK cells early post transplant, but no persistent changes (Varchetta, Oliviero et al. 2009). There was also a transient decrease in NKG2D and a slowly progressive increase in NKG2C positive NK cells, but no changes in NCR expression were reported. These studies are largely consistent with the data presented in this chapter, demonstrating little persistent measurable changes overall in NK activating phenotype in the first 12 months after liver transplant. The differences that are present could be attributed to the distinctive patient cohorts – ie. paediatric patients and HCV infection.

The major weaknesses in this longitudinal study relate to the lack of numbers and inconsistency in sample collection post transplant. Whilst a reasonable number of patients were initially recruited (28), the problems with drop-out were not anticipated. In particular, the follow-up of patients at geographically distant sites (Belfast and Plymouth) presented a genuine challenge. I established

collaborations with the local hepatologists in charge of these patients and obtained agreements for samples to be taken at clinic follow-up, but these never materialized. Of the patients that were included in the study, the logistical challenges of blood sampling at follow-up could have been better anticipated. Most pertinently, a local collaborator at Kings College Hospital able to take blood samples would have been ideal. This would have addressed the problems with erratic, non-scheduled clinic attendances, so that samples could be taken and processed at short notice. These problems notwithstanding, the present study adds data in an area where current knowledge is very limited, and provides evidence that NK cell tolerance does not appear to develop in the first year post transplant, and therefore is likely to be an evolving process over a longer term.

Further evaluation of longitudinal NK cell changes after liver transplantation is certainly warranted. Future studies should assess a larger cohort of patients, and extend the follow-up period beyond 12 months, perhaps up to 2 years. It would be interesting to assess NK functional readouts such as degranulation, cytotoxicity and cytokine production. Furthermore, characterization of the longitudinal NK response according to HCV recurrence and development of rejection could provide meaningful data that could help direct clinical studies aimed at treating these problematic conditions.

CHAPTER 7: DISCUSSION

Natural killer cells are key effectors in the immune response to cellular insults caused by viral infection and tumour transformation. The mechanisms through which NK cells discriminate between self and non-self are pivotal in their function as early players in the immune response. Importantly, NK cells do not depend on pre-sensitisation and recognition of specific alloantigens to become activated, as employed by T- and B-cells. Rather, in health, NK cells are kept in constitutive inhibition through dominant interactions between inhibitory surface receptors and MHC class-I ligands, which are present on healthy cells. The KIR comprise the major family of polymorphic inhibitory NK cell receptors recognizing HLA-A, -B and –C ligands. Several layers of complexity exist within the KIR-HLA system, the nuances of which have only recently been elucidated. For instance KIR and HLA class I are highly polymorphic and NK receptors are expressed in a stochastic fashion so that, even within an individual, there is great variability of receptor expression between clones. Furthermore strength of binding and inhibitory signal transduction between KIR and HLA vary depending on the specific KIR and HLA allelic subtypes. Thus there is extensive variation in NK cell repertoire, target cell specificity and activation threshold within an individual, between individuals and at the population level.

In order to ensure that NK cells are not auto-reactive, they undergo a process termed ‘education’, whereby only those cells expressing inhibitory receptors for self are allowed to mature and develop full functionality (termed ‘licensing’). It is

here that our understanding of NK cell biology is limited, as although several plausible mechanisms for NK education and licensing have been proposed, full characterization of the processes involved remains elusive.

Organ transplantation presents a particular challenge when it comes to NK cell reactivity. Recipient NK cells would be predicted to be alloreactive to donor grafts in any scenario where the donor expresses HLA that is not present in the recipient (HLA mismatch). In liver transplantation this situation is common, as HLA matching is not routinely employed. Yet in clinical practice NK-mediated rejection is not observed and outcomes are generally good. Little is known as to the functionality of recipient NK cells in LT or whether HLA mismatch causes activation. Additionally, conflicting data has caused controversy as to whether any donor or recipient HLA allotype confers a benefit in LT.

The role of NK cell reactivity after solid organ transplantation is currently unresolved. The conventional view that NK cells do not significantly contribute to alloreactivity was based on early NK depletion studies in animals. Since then, a number of animal models have shown that NK cells may well influence graft tolerance (Dresske, Zhu et al. 1997, Russell, Chase et al. 2001, Uehara, Chase et al. 2005, Uehara, Chase et al. 2005, Feng, Ke et al. 2009).

The phenotype and function of recipient natural killer cells after liver transplantation was assessed using flow cytometry based assays. I found several key differences in NK cells between LT recipients and healthy controls. In LT there was an increase in the relative abundance of immature $CD56^{bright}$ NK cells

compared to $CD56^{dim}$. I showed that the mature $CD56^{dim}$ subset has a downregulated activating phenotype, with reduced expression of the natural cytotoxicity receptors NKp30 and NKp46 but not downregulation of NKG2D. Furthermore, in KIR^{+} NK cells, which are conventionally considered to be 'licensed', the difference in receptor expression was most striking.

$CD56^{bright}$ and $CD56^{dim}$ dim NK cells form distinct subsets, differing in their stage of maturation, cytotoxicity, cytokine production and KIR expression. During NK cell development NK cells mature from $CD56^{bright}$ into $CD56^{dim}$ cells, and therefore the data suggests that in the presence of a liver allograft, NK cell maturation alters, with down regulation of activating receptors between the two stages of development.

NK cell function was assessed through response to incubation with K562 cells. This cell line stimulates activation of NK cells and is highly sensitive to NK cell killing as it lacks expression of MHC class I. In LT there was significantly reduced NK killing of K562 cells as measured by specific cytotoxicity. The other read-outs of NK function analysed were surface expression of CD107a (LAMP-1), and intracellular expression of the cytokines IFNγ and TNFα. Compared with healthy controls, in LT there was reduced expression of CD107a and IFNγ after incubation of NK cells with K562 cells.

These data provide evidence of altered development of recipient NK cells in liver transplantation. NK cell precursors arise from $CD34^{+}$ haematopoeitic progenitor cells and therefore early development occurs in the bone marrow. (Raulet 1999,

Colucci, Caligiuri et al. 2003, Yokoyama, Kim et al. 2004). However, the pathways and location of NK cell development and maturation have not been fully characterised. There is some evidence that committed NK cell precursors traffic from the bone marrow into secondary lymphoid tissue (SLT), such as lymph nodes where terminal differentiation occurs *in situ* (Di Santo 2006). The NK cells in SLT are nearly all $CD56^{bright}$, whilst the majority (~90%) of circulating peripheral blood NK cells are $CD56^{dim}$, and it is generally understood that $CD56^{bright}$ NK cells mature directly into $CD56^{dim}$ cells (Caligiuri 2008). The less mature $CD56^{bright}$ subset are potent producers of cytokines, but do not kill target cells efficiently, unlike their more differentiated $CD56^{dim}$ counterparts. In our LT recipient cohort, the higher relative abundance of peripheral $CD56^{bright}$ NK cells is suggestive of a change in maturation induced by allograft. An increase in $CD56^{bright}$ proportion has been observed in other conditions including immunodeficiency and chronic infection (Zimmer, Bausinger et al. 2007, Barcelos, Sathler-Avelar et al. 2008, Villa-Forte, de la Salle et al. 2008). In our study, the observed downregulation of activation in $CD56^{dim}$ NK cells, but not in the $CD56^{bright}$ subset suggests that LT induces a maturation defect between the two stages of NK development, and that this results in hypofunctionality. A similar observation was recently reported in NK cells from an individual with suspected immunodeficiency exhibiting a maturation defect leading to increased $CD56^{bright}$ NK cells, and $CD56^{dim}$ cells which could not acquire a terminally differentiated phenotype (Domaica, Fuertes et al. 2012).

The limited data from the longitudinal cohort suggests that the changes observed in recipient NK cells do not occur during the first 12 months after LT. This is

consistent with previous studies demonstrating modest changes in NK subsets and activating receptor expression very early on after LT but few persistent phenotypic changes at one year (Varchetta, Oliviero et al. 2009, Pham, Piard-Ruster et al. 2012). The disparate findings between the longitudinal and cross-sectional study (where median follow up was 55 months) implies that the development of tolerant NK tolerance is a slow, evolving process. It may be postulated that this requires multiple interactions between recipient NK cells and the liver allograft; the repeated interactions could result in the altered NK maturation, and associated hypofunctionality or even exhaustion, in a process analogous to the anergy that occurs in T cells.

The lifespan of NK cells is of particular importance when considering the recipient immune response to solid organ transplantation. Until relatively recently, the dogma has been that mature NK cells in the periphery were terminally differentiated and unable to proliferate. A mature NK cell was thought to die soon after performing its effector functions after activation, or if it did not become activated, it would be short-lived, dying with a half-life of around 2 weeks (Jamieson, Isnard et al. 2004). However more recent data in mice and humans has demonstrated that NK cells are able to become long-lived and last several months or even years after becoming activated (Cooper, Elliott et al. 2009, Sun, Beilke et al. 2011). Therefore it is likely that in the setting of liver transplantation, mature recipient NK cells, educated and licensed pre transplant, persist in the periphery for at least a few months. These NK cells maintain functionality and this could explain why the tolerant phenotype was not

observed in the longitudinal analysis, assessing NK activation in the first 12 months.

Several studies have demonstrated that donor immune cells within the engrafted liver, so-called passenger leucocytes, persist within the recipient (Schlitt, Kanehiro et al. 1993, Lukomska, Winnock et al. 1997). Circulating donor haematopoietic cells can be found in the recipient for up to 4 weeks post transplant it has been postulated that this donor chimerism explains why liver transplants are tolerogenic (Starzl, Demetris et al. 1993, Rao, Starzl et al. 1996, Verdonk, Haagsma et al. 2011). Given that the liver immune population is enriched with NK cells, comprising up to 40% of hepatic lymphocytes (Nakatani, Kaneda et al. 2004), NK chimerism potentially plays an important role in the recipient immune response. Moroso and colleagues (2010) assessed the phenotype and function of donor derived NK cells after LT. They found that a high proportion of hepatic NK cells were $CD56^{bright}$ which unusually exhibited strong cytotoxicity, and they proposed that these could exert alloreactivity against recipient immune cells entering the liver, thus protecting the allograft. They also noted that donor $CD56^{dim}$ NK cells, B cells, dendritic cells, macrophages and T cells mix with recirculating recipient NK cells for up to 2 weeks post transplant. This provides a potential mechanism for donor-recipient immune interactions to influence the recipient NK response in the early post transplant period. However, as the chimerism is not thought to persist beyond a month, it is not clear whether it has a significant influence in the longer term.

None of the reduced activation phenomena were observed in NK cells from liver

transplant recipients with HCV infection. I propose that this is related to the opposing effects of LT on NK cells (NCR down-regulation) and HCV infection (NCR up-regulation). In the non-transplant HCV setting, NK cells are thought to be chronically activated, and have been shown to express higher levels of activating receptors NKp30, NK44, NKp46 and NKG2D (De Maria, Fogli et al. 2007, Ahlenstiel, Titerence et al. 2010, Harrison, Ettorre et al. 2010) with increased cytotoxicity (Duesberg, Schneiders et al. 2001, Golden-Mason, Madrigal-Estebas et al. 2008, Oliviero, Varchetta et al. 2009, Yoon, Shiina et al. 2009). Furthermore, in a recent published study, intrahepatic NK cells in HCV infection were shown to retain functionality against target cells (Cosgrove, Berger et al. 2014). As HCV recurrence is universal after liver transplantation, this may similarly result in recipient NK cell activation. My data suggests that this does indeed occur, and overcomes transplant related hypofunctionality and tolerance.

As well as exhibiting a hypofunctional phenotype, I demonstrated that LT recipient NK cells have altered gene expression in pathways particularly involving activation and cell differentiation. Specifically I discovered that in LT recipients there are reduced levels of *STAT4* mRNA with associated reduction in STAT4 phosphorylation in response to IL-12 stimulation. I propose that these changes in the STAT4 signalling pathway are related to the NK hypofunctionality and altered maturation observed in LT recipients. The low levels of STAT4 observed are not related purely to an increase in the $CD56^{bright}$ subset as it has been shown that STAT4 levels are similar between the $CD56^{dim}$ and $CD56^{bright}$ subsets in humans (Yu, Mao et al. 2010). The gene expression data also

demonstrated a trend towards lower expression of *IKZF2,* also known as *helios,* in LT recipient NK cells. *Helios* encodes a member of the Ikaros transcription factor family, and in a murine model, *helios* downregulation was shown to regulate NK cell reactivity via NKp46 (Narni-Mancinelli, Jaeger et al. 2012). Interestingly the authors postulated that NKp46 engagement actually downregulates NK cell responsiveness and that silencing of *helios* restores NK activation, in an MHC-independent education process. Our data provides further evidence that *helios* may be involved in altered NK differentiation, with associated down-regulation of NKp46.

The development of NK cells between mice and humans is difficult to compare as mice do not have CD56 as an NK cell marker, and therefore it is difficult to draw exact parallels between the two species. However consistent with miR-155 being a STAT4 target in both species I observed downregulation of this microRNA in NK cells from LT patients. Interestingly in the mouse miR-155 is important for viral driven NK cell proliferation and NK "memory" responses (Zawislak, Beaulieu et al. 2013). In its absence there are more mature NK cells. As I found increased NKG2C expression in LT non-HCV, but not LT HCV, my data suggests that in humans, miR-155 may also be associated with NK cell maturation and their response to viral infection.

The effect of immunosuppressive drugs used in transplantation on NK cells is controversial. A study analysing phenotypic and functional changes in NK cells incubated with Cyclosporine *in vitro* demonstrated a reduction in

$CD56^{dim}CD16^{+}KIR^{+}$ NK cells but these cells retained their cytotoxicity, and expressed increased NKp30 (Wang, Grzywacz et al. 2007). Several other investigations into immunosuppressant effects of NK cells have reported conflicting results, and no clear consensus has been reached (Wasik, Gorski et al. 1991, Poggi and Zocchi 2005, Vacher-Coponat, Brunet et al. 2006, Wai, Fujiki et al. 2008, Kim, Kim et al. 2010, Morteau, Blundell et al. 2010, Moustaki, Argyropoulos et al. 2011). In order to determine whether any of the changes observed were the result of immunosuppressive drugs, I performed *in vitro* incubation assays of NK cells with commonly used immunosuppressants. Over 90% of the cohort was taking calcineurin inhibitor based immunosuppression, and thus the assays were representative of the recipient population. By performing the assays with drug concentrations spanning the therapeutic ranges, I was able to exclude a dose effect of the medications on the expression of activating receptors. Neither the phenotypic, functional or gene expression findings observed in recipient LT were recapitulated in these assays. Whilst there are limitations to these short-term incubation experiments, it is unlikely that the effects observed are related to the direct effects of immunosuppressive agents on mature NK cells, but more likely due to more complex effects on NK cell maturation during the $CD56^{bright}$ to $CD56^{dim}$ transition: the point at which NK cells acquire inhibitory receptors for HLA-A, -B and -C.

Consistent with this I did not observe any relationship with expression of inhibitory receptors, HLA-C and hypofunctionality. Thus it is unlikely that in LT tolerance of NK cells to the allograft is mediated by KIR:HLA-C interactions. As the phenotypic and functional changes were largely seen in $CD56^{dim}$ NK cells, but

were not restricted to NK cells expressing mismatched KIR, our data suggests that recipient NK cells are 'disarmed' in a KIR-ligand independent manner. The clinical data supports this hypothesis, as there was no association between HLA mismatch or indeed KIR-HLA combinations and the key measurable clinical outcomes of ACR and fibrosis. Furthermore, in terms of clinical outcome, there was no benefit of donor HLA-C2, and so our data supports the larger of the previous genetic studies, which found no impact of donor HLA-C allotype and graft survival (Tran, Middleton et al. 2009).

NK cells comprise a higher percentage of the lymphocyte population within the liver as compared to the periphery (Krueger, Lassen et al. 2011) and there is increasing evidence that a high proportion of these are phenotypically immature (Kim, Iizuka et al. 2002, Gordon, Chaix et al. 2012). Thus it is plausible that recipient NK cells become 'tolerised' by undergoing an altered maturation process during contact with the allograft. My findings support this hypothesis and suggest impairment of IL-12 signalling as a mechanism through which the NK cell hypofunctionality occurs (**figure 7.1**). Whilst these potential effects clearly require further investigation, my characterization of recipient NK cell hypofunctionality provides an insight in to the observed tolerability of liver allografts, a model for future studies into NK cell alloreactivity in solid organ transplantation, and suggests that HLA matching is unlikely to influence NK cell associated rejection.

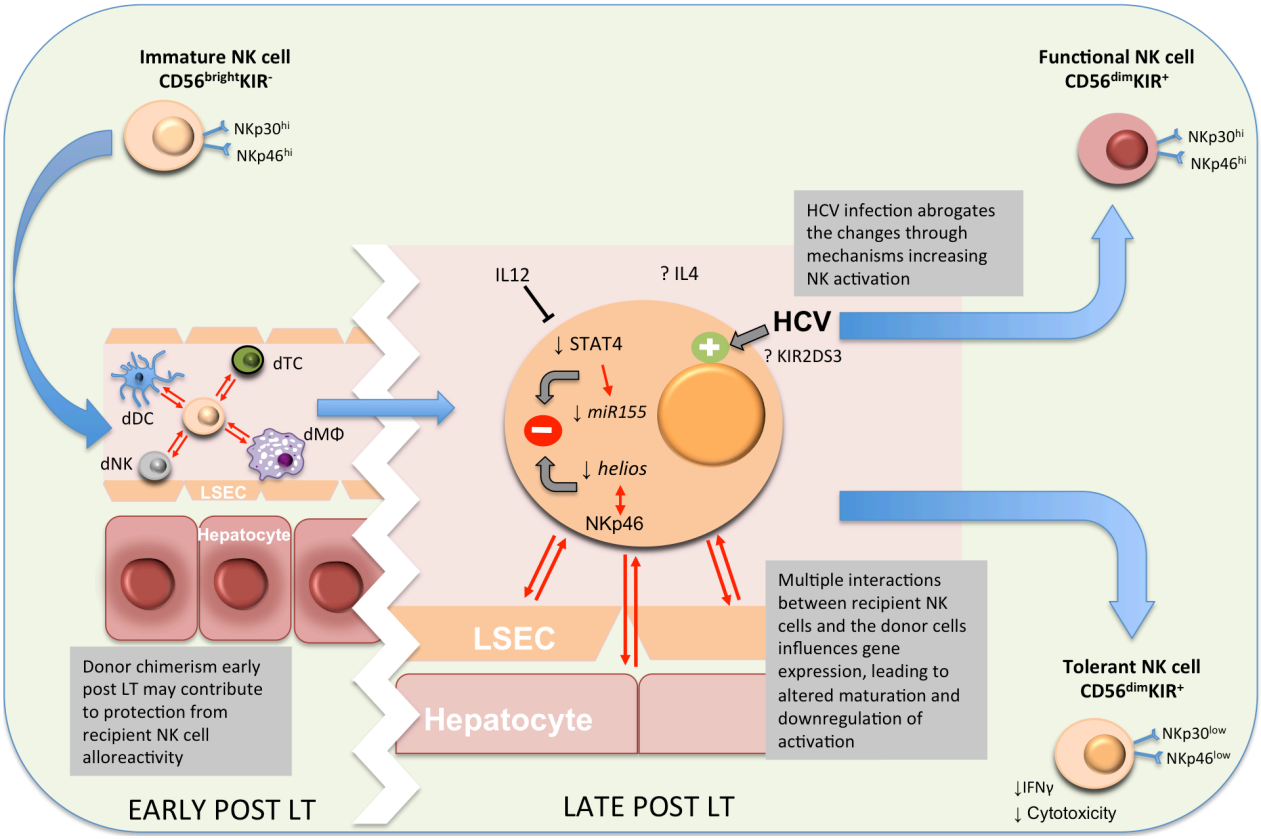


Figure 7.1 Mechanisms of recipient NK cell tolerance after liver transplantation.

Interactions between donor cells and the recipient NK cells shape the recipient NK cell response after engraftment, leading to tolerance. These phenomena are counteracted by the activating effects of HCV infection. dDC, donor dendritic cell; dTC, donor T cell; dNK donor NK cell; dMϕ, donor macrophage; LSEC, liver sinusoidal epithelial cell.

CHAPTER 8: CONCLUSION

In summary I have demonstrated that after liver transplantation there is a significant change within recipient NK cells, with an expansion of the $CD56^{bright}$ population, and downregulation of expression of the activating natural cytotoxicity receptors NKp30 and NKp46. This effect is most marked in the mature KIR^{+} $CD56^{dim}$ subset of NK cells, and there is associated hyporesponsiveness, with reduced cytotoxicity, degranulation and cytokine production. This implies a defect in NK maturation, and to investigate this further, I performed gene expression analysis using microarray and qRT PCR. I demonstrated that genes involved in activation and differentiation signalling pathways are differentially expressed in LT recipient NK cells, and that there is perturbation in the STAT4 pathway with associated downregulation of a miR-155, a key microRNA target of STAT4. MiR-155 is influential in NK maturation in mice, and our data supports a role for miR-155 in human NK cell development. Donor-recipient genetic analysis demonstrated no influence of HLA-KIR matching on NK activation or clinical outcomes, consistent with the hypothesis that NK tolerance in LT occurs independent of KIR-ligand interactions. Analysis of HCV infected LT recipients demonstrated that the NK function was preserved, yet *STAT4* expression was still downregulated. This suggests that HCV has a counteracting effect to LT on NK cells, leading to activation, which abrogates the tolerising effect of LT.

Important directions for future studies would be aimed at further characterization of the mechanisms underlying NK cell tolerance. In particular

investigation of interactions between NK cells and the donor graft through analysis of intrahepatic NK cells and hepatocytes could provide insights into how the maturation changes develop. It would also be interesting to further explore some of the other genes and signaling pathways (eg PKCθ) found in the microarray experiment. A more comprehensive longitudinal study, extending analysis beyond 12 months post transplant, would allow assessment of the evolution of NK cell changes that lead to tolerance. Finally, it would be important to explore the clinical utility of understanding NK tolerance in liver transplant. Given that our data suggests role for STAT4 for in development NK tolerance, it is possible that STAT4 could be a therapeutic target in liver transplantation. Lisofylline is a STAT4 inhibitor that has been shown to have anti-inflammatory effects, reducing expression of cytokines including IFNγ and TNFα through interruption of IL-12 signaling (Yang, Chen et al. 2003, Yang, Chen et al. 2004). Mouse models have demonstrated benefits of lisofylline in Type 1 (autoimmune) diabetes (Yang, Chen et al. 2006, Grant, Moran-Paul et al. 2013), and in light of the data presented in this thesis, it would be interesting to evaluate whether the anti STAT4 effects could be utilised in the setting of liver transplantation.

The ultimate aspiration of this and potential future studies is to exploit the knowledge of the mechanisms involved in NK tolerance to inform clinical research aimed at maximising graft outcomes in LT.

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Imperial College London

Imperial College Healthcare NHS Trust

CONSENT FORM

Title of Research Project: Assessing NK cell function and activity after Liver Transplantation
Principal Investigators: Professor Salim Khakoo
Study number:

Please initial boxes if you agree with each statement ↓

1. I confirm that a member of the research team has explained this project to me. I have had the opportunity to ask questions and these have been answered fully. ☐

2. I confirm that I read and understood the Patient Information Sheet dated / / *Version* for the study named above. ☐

3. I understand that my participation is entirely voluntary. If I decide **not** to participate my medical care will not be affected in any way. I also understand that I can withdraw from this study at any time and without giving any reason. ☐

4. I agree that authorised members of the research team, Imperial College Academic Health Sciences Centre or regulatory authorities may look at my medical notes. Any such access will be in strict confidence. ☐

5. I understand that donated samples may be used to research the genetic causes of serious diseases. ☐

6. I understand that donated samples will **not** be used for genetic tests that could adversely affect my insurance or employment. ☐

7. I understand that if the research produces results that are of direct clinical importance to me, or my family, I will be informed. I agree that my GP may also be informed. ☐

8. I understand that it may be necessary to send samples to other institutions for specialist tests. No identifying information or clinical information would ever be sent with such samples. ☐

9. I agree that any samples that I donate may be used in future research projects that comply with all relevant regulations and which have approval from an independent ethics committee. Under these circumstances, my samples would be retained indefinitely in a secure and properly regulated biobank for future use [OPTIONAL] ☐

10. I understand that any samples that I donate are a gift and that I shall receive no financial reward for participating in this study. ☐

11. I agree to participate in this study ☐

_____	_____	__/__/__
Signature of patient	Name of patient	Date
_____	_____	__/__/__
Signature of person taking consent	Name of person taking consent	Date
_____	_____	__/__/__
Signature of researcher	Name of researcher	Date

Appendix 2

Patient Information Sheet

Imperial College London

Imperial College Healthcare NHS Trust

Department of
Hepatology
10th Floor QEQM
Building
St Mary's Hospital
London, W2 1PG
Tel: 0207 886 6454

PATIENT INFORMATION SHEET

Title of Research Project: Assessing NK cell function after Liver Transplantation
Principal Investigators: Professor Salim Khakoo
Study number:

Dear Sir or Madam,

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.

This information sheet aims to explain the purpose of the study and what will happen to you if you take part. The later sections give you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for taking the time to read this information sheet.

Salim Khakoo
Professor of Hepatology
Department of Hepatology

Introduction
The departments of Hepatology at Kings College London and Imperial College London carry out research into many different diseases of the liver. These diseases include liver cancer, infections and inflammatory diseases.

Our work involves staff at Kings College Hospital, London, and St Mary's Hospital, London

What is the purpose of this study?
Liver diseases are an increasingly common problem in the UK and around the world. One of the treatments for severe liver disease is liver transplantation. In most patients liver transplantation is very successful, but rejection does sometimes occur. The reasons why some people suffer rejection whilst others do not are unclear.

We wish to analyse cells from the immune system of patients after liver transplantation to study whether there is a link between genetic make-up and rejection of the organ. This may help us decide whether specific 'matching' patients to livers improves outcomes after liver transplantation.

One of the biggest problems in liver transplantation is that in patients with hepatitis C, the new liver almost always becomes infected. This can lead to severe damage and may even result in liver failure.

As part of our study, we will analyse immune system cells in patients who have liver transplants for hepatitis C. We will compare patients who develop severe re-infection with those that do not, and assess whether there are genetic factors involved in determining outcomes.

In summary, *our aims are:*

- *To understand more about why certain patients suffer rejection after a liver transplant*
- *To assess why some patients with hepatitis C have a worse outcome than others.*

Why have I been chosen?
You have been chosen because you are being seen in a clinic or other department that is working with us. The doctor or nurse looking after you has suggested that you may be suitable to participate in this study. We believe that it would be useful to include your blood in our study for analysis, (or if you are having a biopsy, the liver tissue that will be removed as a standard part of your care).

In summary, *we are asking for volunteers from the following group:*

- *Patients who have undergone liver transplantation for chronic liver disease*

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive in any way.

What will happen if I take part? What do I have to do?
All patients who agree to participate will be asked to:

- Read and sign a consent form
- Provide a blood sample. This may be done at the same time as your routine blood tests in the clinic or arranged at another time. 20ml of blood will be taken (the equivalent of 4 teaspoonsful) with a needle from a vein.
- Allow us to collect some details from your medical records (in strict confidence)

- You may be asked to provide further blood samples at the same time as routine blood samples are taken as part of your standard care.

The consent form highlights some of the key points about participating in this study. You will be asked to initial a number of paragraphs and sign & date the form. You will be given a copy of this form.

The whole study will take up to three years to complete, but your involvement would be much more limited. Some patients will be asked for a blood sample just once, and others will be asked to provide samples at several time points. At most your total participation will be around 1 hour.

You will not have to attend any further extra appointments or undergo any other extra tests.

What if I am to undergo a biopsy.
If you are going to have a liver biopsy as part of your normal care, your doctor will have explained why and what this involves. You will **not** undergo a biopsy or operation as part of this study.

If you do undergo a biopsy we would look at the results of the standard tests performed as part of your routine care. No extra tissue will be taken, other than that which is required for your biopsy or operation. You will not have to undergo any extra procedure.

What will you do with the samples?
The samples will be studied in a variety of ways. We will analyse the proteins, DNA and other chemicals in them. We will compare the levels of these different chemicals in patients with different diseases and outcomes after liver transplantation.

Sometimes it is necessary to send samples to external laboratories – in the UK or abroad – for specialist tests. Only anonymised samples would be sent to an external lab, no personal details or clinical details about your case would be shared.

What are the possible side effects of taking part? What are the possible disadvantages and risks of taking part?
As you will not receive any extra treatment we do not foresee any potential side effects.

Some patients may develop a bruise after giving a blood sample. This will heal naturally with no treatment. Of course, you may be having a blood test anyway as part of your normal clinic assessment.

What are the possible benefits of taking part?
We do not anticipate any direct benefit to you. However, the information we get may help improve the future diagnosis or treatment of people with liver disease. You will not be paid for participating in this study.

What if something goes wrong?
It is highly unlikely that anything will go wrong during your participation in this study.

Imperial College London holds insurance policies which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault.

This does not affect your legal rights to seek compensation. If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator (Dr Khaleel Jamil – details below). The normal National Health Service complaint complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial AHSC Joint Research Office.

Will my taking part in this study be kept confidential?
All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you that leaves the hospital will have your name and address removed so that you cannot be recognised from it.

Our procedures for handling, processing, storage and destruction of their data are compliant with the Data Protection Act 1998.

It is unlikely that we will discover any information that is of direct importance to you or your family. If we do so, we will contact you and your GP to inform you of this. If you DO NOT wish us to contact you or your GP about any such information, please discuss this with the researcher. Your wishes will be recorded and observed.

What happens when the research study stops? What will happen to the results of the research study?
When the study stops we will analyse all of the results. The study will be written up and may be published in medical journals and presented at conferences. No patients will identifiable in any published results.

Our results will also be shared with the organisations and charities that support our work, including patient support groups. Again, no patients will be identifiable in any information that is shared with these groups.

If you wish to receive feedback on the study, you can contact us and we will send you a summary of the results and conclusions of the study. The contact details are listed below.

Any remaining samples will be kept securely, complying with rules set out in the Human Tissue Act. They may be used in future research projects that have received approval from an independent ethics committee. You would not be contacted again for further permission to use the samples in such future studies. Any samples kept beyond the end of this study may be held securely in a properly regulated biobank. The biobank will ensure that any samples are kept safely and are only used in ethically approved studies into human diseases. If you would rather that your samples were not used in this way, you may specify this on the consent form at Section 9.

Who is organising and funding the research?
This study is being organised by the Hepatology Department at Imperial College London, in co-operation with the following NHS Trusts:

- Imperial College Healthcare NHS Trust (St Mary's Hospital)
- Kings College Hospital NHS Trust

It is being funded by a the Wellcome Trust

Who has reviewed the study?
This study was given a favourable ethical opinion for conduct in the NHS by Surrey Research Ethics Committee (REC).

Contact for further information
Dr K Jamil (khaleel.jamil@nhs.net)

Department of Hepatology, 10th Floor QEQM Building, St Mary's Hospital, London, W2 1PG
Tel: +44 (0)207 886 6454

Thank you for reading this information and considering participation in this study.
You are asked to retain this Patient Information Sheet for your future reference

Appendix 3

Differentially Expressed Genes on Microarray

Common elements in "LT HCV vs LT non-HCV", "LT HCV vs HC" and "LT non-HCV vs HC":

XLOC_l2_013783
lincRNA:chr2:878560-878991_F
FAM57B
ENST00000523112
LOC389033

Common elements in "LT HCV vs LT non-HCV" and "LT HCV vs HC":

GSTM2
TMED3
lincRNA:chr1:119700127-119706377_R
BCORP1
lincRNA:chr6:43815372-43929922_R
RGPD5
KLHL29
HIST1H2BD
SIGLEC15
PTAR1
GLT25D2
TBKBP1
DVL3
YPEL4
LOH12CR1
lincRNA:chr9:23671778-23672397_F
ENST00000409590
lincRNA:chr16:87325199-87333574_F
A_33_P3327140
SSTR3
LYG1
PRSS53
PXN
lincRNA:chr6:155203224-155205268_F
STX4
HIST1H2BB
LOC440944
LONP1
CDK11B
TUBGCP6
lincRNA:chr8:37264155-37278889_F
CHKB
NDE1
SMPD3
A_33_P3423845
APBB1
AMY1C
SIGLECP3
ENST00000425471
IL29
ZCCHC17
SNORA74A
HEBP2
A_33_P3324904
AP3S2
LSS
HIST2H4B
lincRNA:chr10:67332969-67346819_R
LOC153577
PAXIP1
SLC26A11
ZNF226
GOLGA6L9
PDE4D
SLC27A3
SIL1

Common elements in "LT HCV vs LT non-HCV" and "LT non-HCV vs HC":

ACBD7
LGALS1
A_33_P3247077
THC2668129
CT47A11
ITM2A
KIF21B
A_33_P3345614
ENST00000366116
HIST1H2BO
FMNL1
ZNF713
ENST00000397064
lincRNA:chr6:86386083-86388435_R
CYP4F30P
LOC100128714
PNRC2
lincRNA:chr3:106619835-106630535_R
ESRRA
A_33_P3311646
A_33_P3229390
LOC100134663
BAP1
GBA2
A_33_P3244361
PANK4
LOC100287314
DPEP3
NADKD1
ENST00000366347
lincRNA:chr10:101192360-101200085_R
EFCAB4B
XLOC_l2_007876
lincRNA:chrX:102074319-102166119_R
COX6B2
ZMYND11
TRABD
KIAA1875
C1orf177
AK097143
GBP6
KLHL22
lincRNA:chr16:86511545-86542401_F
GPATCH1
FAM156B
LOC284379
A_33_P3258846
MAPK3
LOC100506380
AMN
NCOR1
C17orf59
LGALS3BP
NR1H2
DLEU1
THC2630254
PSMG2
PRRC2A
LUZP6
THC2566648
ATP2B4
SBNO1
GSDMD
LINC00092
WDR11
ASGR2
BC157839
lincRNA:chrX:102074319-102166119_F
ATP13A3
ENTPD1
AURKAPS1
TTC28
FBRSL1
RPS2P32
Q9N083
HIPK2
PPP1R14A
CR604878
lincRNA:chr1:94159362-94171362_F
ADAMTS7
AI198876
CDC34
NEK1
LOC100507645
ZSCAN2
IKZF1
HCFC2
SNCB
A_33_P3381292
RNFT1
ATP11B
A_33_P3406255
ENST00000447197
RNF168
SERPINB9
DDX39B

Common elements in "LT HCV vs HC" and "LT non-HCV vs HC":

TOP3A
lincRNA:chr18:74524062-74532337_R
lincRNA:chr6:167312435-167323185_R
A1BG-AS1
DYRK1B
AY358225
RPP14
IQGAP1
lincRNA:chr14:58744247-58760847_R
C20orf141
THC2780215
ITGB3BP
MPDU1
lincRNA:chr20:37075485-37076211_R
LOC100506035
ZBTB37
PAPL
HEBP1
IL18BP
ZNF683
LEPREL2
CYTH3
SOX13
FAM27A
C7orf55
lincRNA:chr19:24218385-24247285_F
ENST00000391681
lincRNA:chr16:88812349-88821799_F
PET112
lincRNA:chr13:48380599-48492024_R
MYOM2
WDR19
AIF1L
SYNE1
PBX4
LINC00339
LOC100133050
SPATA5L1
C2orf49
TNFRSF18
RELB
ACSF3
BEGAIN
LOC400099
GUSBP1
ENST00000357658
NIPSNAP3A
DUSP18
STYXL1
ENST00000522992
EIF2C1
ENST00000356801
PLOD3
CXorf49B
C9orf102
TCHP
CEP95
lincRNA:chr13:33868250-33874375_R
WDR41
lincRNA:chr7:104639816-104641977_R
MSH3
lincRNA:chr5:93440069-93451144_F
PPIP5K2
lincRNA:chr6:22056867-22194604_R
LOC100505854
GNAZ
AX721280
MST1
TRIT1
FCHO2
KRT81
C6orf64
lincRNA:chr7:105525814-105538089_F
ANKRD33
RPS24
SMC4
lincRNA:chr8:58126971-58142821_R
CHPT1
MDH1B
MED7
PAWR
lincRNA:chr14:61529972-61553247_R
LRR1
SPATA13
OXSR1
SMAD7
KRT86
INSL3
NUDT18

Elements only in "LT non-HCV vs HC":

FAM118A
CERS3
lincRNA:chr2:64434091-64478485_R
IFT88
UBE2M
CREB3L1
LCE1D
SEH1L
lincRNA:chr7:139115835-139124860_F
lincRNA:chr6:43988722-43993947_R
lincRNA:chr3:114043485-114052926_R
HNRNPA2B1
lincRNA:chr2:193943030-194203155_R
lincRNA:chr10:6623494-6637944_F
LOC645553
XLOC_005365
lincRNA:chr1:180915452-180922102_R
DCP2
SPRNP1
A_33_P3287388
lincRNA:chr8:143489971-143501023_F
TPT1
lincRNA:chrX:100696094-100725244_R
CCL24
lincRNA:chr11:130730240-130745290_F
UTRN
INPP1
PMS2P5
lincRNA:chr6:42166947-42172520_R
RBM20
SFTPA2
EIF2C3
MYOM1
PITX3
LNPEP
lincRNA:chr20:47715043-47723493_F
LY75
CNST
A_33_P3349597
ENST00000297020
FAM21C
lincRNA:chr2:216473880-216713730_R
RB1
lincRNA:chr6:44046036-44046378_R
NKIRAS2
A_33_P3240340
TCP11L1
CSDA
HLA-DQB1
lincRNA:chr19:57751638-57764913_F
ZNF274
MEX3D
PDSS2
NAA60
lincRNA:chr11:94380477-94405252_F
IP6K2
ARHGEF6
B3GALNT2
lincRNA:chr4:120400703-120402090_F
PCDHGA7
DND1
MT1F
HDGFRP2
TMEM9
ZBTB43
ARFRP1
KIR3DL2
A_33_P3362239
A_33_P3278303
C2CD4C
GFRA3
GPA33
lincRNA:chr8:123989344-123994919_F
TSTD1
AK130723
CRIP1
LOC100507637
FAM131C
lincRNA:chr3:194190336-194206820_R
AS3MT
lincRNA:chr5:95919519-95995094_R
A_33_P3233000
GABPB2
MAN2B1
UTP18
RAD23B
C16orf45
NFATC2
lincRNA:chr4:24814752-24827052_F
SEC23A
lincRNA:chr2:55388021-55396371_F
TAOK3

ATRN
ZNF138
KCNQ1
C16orf87
ORAI2
FAT3
lincRNA:chr12:54360060-54360161_F
CAB39L
ARAF2P
ALG13
ATF7IP
LOC399744
SKP2
PDXDC1
lincRNA:chr2:179375079-179386329_F
INTS8
ITGB7
RBM39
ADSL
STX2
WBSCR16
COQ10B
ASNS
A_33_P3391167
MCEE
GNB2
TRAIP
DIABLO
FGR
lincRNA:chr14:23982560-23989835_F
CD4
PER2
LRFN3
lincRNA:chr8:104254399-104295074_F
ZNF248
DRAM2
FKBP4
CK825926
ENST00000378340
TTC15
METTL16
FAM106CP
TOMM34
CAND1
A_24_P307065
lincRNA:chr18:59236195-59282370_F
lincRNA:chr11:2017748-2019065_F
LOC284373
A_33_P3273399
LOC648987
UCP2

CYGB
GNS
ATXN7L1
lincRNA:chr11:75122727-75130002_F
TPRA1
LGALS9C
GRB2
LEMD3
lincRNA:chr2:204620380-204666130_F
ZHX3
FUT7
TIMMDC1
lincRNA:chr6:81153106-81172770_R
MCMBP
MDM4
lincRNA:chrX:53707005-53710958_R
RNF19B
BSCL2
ADI1
APEX1
HPS4
AK091697
LTBP4
RBBP9
LAX1
lincRNA:chr1:201509727-201545677_F
ENST00000474213
LPP
MAN1A1
DDX31
lincRNA:chr1:173831852-173837251_R
LOC100130654
YKT6
A_33_P3326312
FAM76B
JAK3
KIAA0195
CTNS
ALPPL2
PIM2
C6orf204
KIAA0748
CDH22
lincRNA:chr6:155240958-155286533_R
EPB41L4A
SNX18

MIA3
SLC38A10
PMS2P4
C1orf112
C20orf72
SPINK7
lincRNA:chr17:38667099-38690799_F
ANKRD20A8P
lincRNA:chr7:130614967-130616965_R
PPA1
C1orf162
ENST00000390539
C1orf109
lincRNA:chr12:32571608-32576908_F
HLA-C
ZNF484
TRAPPC3
NOTCH2NL
lincRNA:chr21:30749379-30901454_R
A_33_P3231319
WDR24
FRYL
STAMBPL1
GINS2
ADAM10
lincRNA:chr20:56156369-56171644_R
RNPEP
MAD1L1
lincRNA:chr5:134465126-134478076_R
OGFOD1
ATG4D
lincRNA:chr7:63922340-63962340_F
UBAP1
CEACAM21
SLC20A2
MT3
DCAF5
RBM4
FLJ42200
GFM2
RPS6KC1
NBPF10
FBXO3
AK025047
lincRNA:chr2:42076869-42077718_F
STAT4
EIF4A1
CD86
BCAP29
DPYSL2
A_33_P3287314

Elements only in "LT HCV vs LT non-HCV":

RGS14
DCAF15
ANAPC7
SLC25A46
ADAMTS10
VPS39
ENST00000398067
HIST1H2BL
KRTAP3-3
NP105946
S100A5
A_24_P212565
MTMR9
MRGPRG
PRKACA
HIST1H2BK
LRRC37A2
MAST3
HIST1H2BG
AGPAT4
LOC100130152
TMEM87B
DOHH
HRAS
lincRNA:chr18:33016877-33028102_F
EGLN2
lincRNA:chr3:65012510-65037885_F
GAA
lincRNA:chr20:37049482-37049747_F
A_33_P3381762
TOX2
HIST2H2AC
SURF6
RNF123
ZRANB2
lincRNA:chr1:147706526-147727651_R
CHAT
MED25
UBE2Q1
ZNRF2
HIST1H3I
lincRNA:chr3:106526585-106630535_R
ZNF346
KLHL17
SNX1
HIST1H2BN
ARHGEF1
FNIP1
DA938875
SLC35B2
TYK2
CCDC142
ILF3
CLK2
IL11RA
PIK3CD
SH3GLB2
KBTBD13
PTGER2
CHI3L2
PDGFRB
MTMR1
FAM73B
C20orf94
AP2A1
RAB27B
CASP10
ZBTB24
TMEM141
lincRNA:chr12:2947389-2952675_R
SEPT7L
EEF1DP3
SRP68
UBTF
AXIN1
GLCCI1
HIST1H2AH
FAR1
C22orf43
lincRNA:chr5:52531518-52727218_R
Q8WY88
lincRNA:chr6:42725472-42759772_F
ARHGAP17
HIST1H1C
M6PR
PTRHD1
ANKRD12
OLA1
KATNAL1
KIAA1841
SIN3B
BRPF1
AK096443
SCARNA7

TSC22D4
SNORA70B
RNF40
TCF25
IQSEC1
EXOC7
TOR1AIP1
TBC1D3
ZNF862
lincRNA:chr1:207971227-208042127_F
RBM18
IRAK2
DGKD
lincRNA:chr5:33162475-33162948_R
AKAP8L
LOC148413
AFG3L1P
ASB2
SMU1
UNC119
HIST1H1E
PKNOX1
MAFK
CENPBD1
CEP350
FAM108A1
EML4
SIPA1
A_24_P280897
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RNU1-5
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USP3

Elements only in "LT HCV vs HC":

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KCNK12
SFI1
RUFY1
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SGSH
GRAP2
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SIGIRR
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UBE2Z
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KRT18
CD3G
TMX2
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SOCS1
ABCA2
DENND1C
ANGEL2
SCAI
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JPH3
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PCNXL2
ENO2
PSMG4
OTUD5
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VASP
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RAB11FIP3
POLR3C
AOAH
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SEMA4D
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PPOX
LPHN1
ABCA5
TRIM13
SDR39U1
DYNC1H1
CEACAM19
CCDC9
ANKAR
WDR13
RRM1
ZNF83
HSF2
RUNX1
PCSK7
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NUP85
IL18
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MAD2L1
ZRSR2
LRCH4

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MRPS34
HMBOX1
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PPP5C
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GGA1
EFHD2
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CREM
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PIAS3
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PSMA4
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MLH3
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APLP2
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ZNF585B
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CG030
VRK1
MFN1
ACADVL
HEXA
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KRT72
X81001
BZW2
FAH
RASSF7