UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Clinical and Experimental Sciences Academic Unit

Innate and adaptive natural killer cells in the liver and peripheral blood

Dr Theresa Hydes

MRC Clinical Research Fellow

Main Supervisor: Professor Salim Khakoo

Second Supervisor: Dr Tilman Sanchez-Elsner

Thesis for the Doctor of Philosophy

April 2017

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

Clinical and Experimental Sciences

INNATE AND ADAPTIVE NATURAL KILLER CELLS

IN THE LIVER AND PERIPHERAL BLOOD

Dr Theresa Hydes

Background and Aims: Natural Killer (NK) cells are innate-like lymphocytes, yet selected murine populations display antigen-specific 'memory' towards haptens and viruses. Liver-specific adhesion molecules CXCR6 and CD49a act as surface markers. Cytokines can also generate NK cells with memory-like behaviour. I aimed to identify CXCR6+ and CD49a+ NK cells in humans, define their phenotype and function and examine the role of cytokines in driving their differentiation and proliferation. Secondly, following the discovery of an association between reduced STAT4 expression and NK cell tolerance, I aimed to investigate if overactivation of the IL-12/STAT4 axis, linked to susceptibility of the autoimmune liver disease Primary Biliary Cholangitis (PBC), may generate hyperfunctional autologous NK cells in this condition, and whether NK cells with a liver-resident or memory-like phenotype play a role in mediating this.

Results: CD49a+ and CXCR6+ NK cells comprised 7.8% and 59.5% of intrahepatic NK cells (both <5% in the circulation). CD49a+ NK cells were present at high frequencies in only 10% of individuals. They harboured higher frequencies of KIR+ and NKG2C+ NK cells compared to their CD49a- counterparts, suggestive of clonal expansion and licensing and produced high quantities of Th1 cytokines. Conversely CXCR6+ NK cells were found universally and were immature ($CD56^{bright}CD16^{low}$) with upregulation of markers of tissue-residency and cell adhesion. Liver-resident CD49a+ NK cells underwent rapid expansion following culture with all activating cytokines, in contrast to CXCR6+ NK cells, however both phenotypes can be induced in the peripheral blood using IL-12 and IL-15. Cytokine-induced CD49a+CXCR6+ NK cells expressed markers of adaptive behaviour (NKG2C, IFNγ) and tissue-residency (CD69). Furthermore I present the first functional correlate of the PBC GWAS studies, identifying overactivation of the IL-12/STAT4 axis in NK cells in PBC. This is manifest by elevated resting levels of pSTAT4 and the generation of CXCR6+ and CD49a+ NK cells which produce high quantities of IFNγ in response to low doses of IL-12. Hyperfunctional NK cells may home to the biliary tree via their interaction with CXCL16 and potentially lyse autologous BECs.

Conclusion: Human hepatic CD49a+ NK cells display features consistent with adaptive behaviour, whereas CXCR6 is a marker of liver-residency. Cytokine stimulation generates hyperfunctional, potentially adaptive-like, CD49a+ NK cells in the blood and may induce their migration to the liver via CXCR6, linking peripheral activation of NK cells with liver-homing. These findings may have important implications for the development of locally acting immunotherapies for viral hepatitis and hepatocellular carcinoma. The identification of overactivation of the IL-12/STAT4 signalling cascade implicates NK cells in the pathogenesis of PBC and suggests a role for the newly discovered CXCR6+ and CD49a+ liver-resident NK cell subsets in autoimmunity. Inhibition of this pathway may provide a new therapeutic target for PBC.

Contents

List of tables

List of figures

List of accompanying materials

1. A comparison of CD49a+ and CXCR6+ Natural Killer cell populations in the human liver reveals distinct phenotypes suggestive of NK cell memory and migration. T.Hydes, M.AbuHilal, T.Armstrong, J.Primrose, A.Takhar, S. Khakoo. Poster presented at **International NK cell meeting 2016.**

2. Natural Killer cell hypersensitivity in Primary Biliary Cholangitis: a novel mechanism for autoimmunity. T.Hydes, J.Naftel, M.AbuHilal, T.Armstrong, J.Primrose, A.Takhar, S. Khakoo. Poster presented at **British Association for the Study of Liver Disease Annual Meeting 2016** (top-ranked poster).

3. Distinct Natural Killer cell populations in the human liver express markers of NK cell memory and migration. T.Hydes, M.AbuHilal, T.Armstrong, J.Primrose, A.Takhar, S. Khakoo. *J. Hepatology* 2016, vol. 64, S528. Poster presented at **International Liver Congress 2016**.

4. Natural Killer Cell Maturation markers in the human liver and expansion of an NKG2C+KIR+ population. T.Hydes, M.AbuHilal, T.Armstrong, J.Primrose, A.Takhar, S. Khakoo. *Lancet* 2015; 38, supplement 1, S45. Poster presented at **Academy of Medical Sciences Spring Meeting 2015**.

DECLARATION OF AUTHORSHIP

I, Theresa Hydes declare that the thesis entitled

INNATE AND ADAPTIVE NATURAL KILLER CELLS

IN THE LIVER AND PERIPHERAL BLOOD

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University;
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- Where I have consulted the published work of others, this is always clearly attributed;
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- Parts of this work have been published as shown on the list of accompanying materials

Signed: ……..

Date:……

Acknowledgements

I would like to thank my supervisor Professor Salim Khakoo who has played a hugely supportive role throughout this journey. Salim introduced me to the new world of natural killer cells and basic immunology, teaching me to ask questions and think like a scientist! He always had an open-door policy and has been a steady source of support, guidance and inspiration throughout the many challenges a PhD brings. He has worked hard to create opportunities for me to be successful in my field and encouraged me to become more confident as a scientist and I am extremely grateful.

I would also like to thank a number of other colleagues which have supported me. Firstly Dr Berenice Mbiribindi who not only provided excellent teaching in terms of laboratory techniques and analytical skills, but also helped me understand the importance of laboratory-based research and how despite things not always working first time, with determination and focus it is possible to generate something completely novel, which could be that first important step towards something big! You also always made me laugh and made my time in the lab so much fun! I would also like to thank Dr Annie Tocheva and Rocio Martinez-Nunez who both encouraged and inspired me every step of the way.

I would like to thanks my parents, Alan and June Hydes who could not be more supportive, thank you for always listening and encouraging me, always telling me I can do it! Also thank you to my amazing fiancée Jason, who I met at the beginning of this PhD in Southampton! A fellow medic and PhD student – thank you so much for making these last three years so happy!

I would like to acknowledge the Medical Research Council (MRC) who funded this work and the support of the National Institute for Health Research (NIHR) Clinical Research Network and the NIHR Wellcome Trust Research Facility at Southampton. I would like to thank Andrew Guy, the NIHR Clinical Trials Assistant for Hepatology at Southampton and the NIHR research and development nurses Abbie Morley and McDonald Mupudzi. I would like to acknowledge the support of Dr Angela Noll, Professor Lutz Walter and Dr Gabriela Salinas-Riester from German Primate Centre, who performed the RNA sequencing analysis of sorted hepatic NK cells presented in this thesis. I would also like to acknowledge the support of the FACS facility led by Richard Jewell and Carolann McGuire at the University of Southampton. Finally I would like to thank the hepatobiliary

surgeons, operating department practitioners and all the generous patients for kindly taking the time to help provide tissue for this study.

Definitions and Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
AMA	Antimitochondrial antibody
AMPK	Adenosine monophosphate-activated protein kinase
APC	Allophycocyanin
BAT	HLA-B associated transcript
BEC	Biliary epithelial cell
BSA	Bovine serum albumin
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CCRL	CC-chemokine receptor-like
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CHC	Chronic hepatitis C
CHS	Contact hypersensitivity
CMV	Cytomegalovirus
CRC	Colorectal carcinoma
CRTAM	Cytotoxic and regulatory T-cell molecule
CXCL	Chemokine ligand
CXCR	Chemokine receptor
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DNAM	DNAX accessory molecule
DNFB	2,4-Dinitrofluorobenzene
DTH	Delayed type hypersensitivity
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetraacetic Acid
EGTA	Ethylene glycol tetraacetic Acid
ELF	Enhanced liver fibrosis
ELISA	Enzyme-linked immuno sorbent assay
Eomes	Eomesodermin
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FBS	Fetal bovine serum
FcRγ	Fc receptor γ-chain
FITC	Fluorescein isothiocyanate
FMO	Fluorescence Minus One
GIST	Gastrointestinal stromal tumour
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Gene wide association study
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
hCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HEPES	Hydroxy ethyl piperazine ethane sulfonic acid
HIV	Human immunodeficiency virus

HLA	Human leukocyte Antigen
HLA-DR	Human leukocyte Antigen – antigen D related
HSECs	Hepatic sinusoidal endothelial cells
HSV	Herpes simplex virus
ICAM	Intracellular adhesion molecule
ID	Inhibitor of DNA binding
IFN	Interferon
IL	Interleukin
ILC	Innate-like lymphocytes
IP-10	Interferon gamma-induced protein 10
IQR	Interquartile range
ITGA	Integrin alpha
JAK	Janus kinase
KIR	Killer cell immunoglobulin like receptors
LIF	Leukaemia inhibitory factor
MACS	Magnetic activated cell sorting
mCMV	Murine cytomegalovirus
MEF	Myeloid elf-1 like factor
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MIC	MHC class I chain related molecule
MIP	Macrophage inflammatory protein
MNC	Mononuclear cells
MS	Multiple sclerosis

mRNA	Messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCAM	Neural cell adhesion molecule
NET	Neuroendocrine tumour
NFILS	Nuclear factor, interleukin 3 regulated
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIHR	National Institute for Health Research
NK	Natural killer
OXA	Oxazolone
PAMP	Pathogen associated molecular patterns
PATJ	PALS1-associated TJ protein
PBC	Primary biliary cholangitis
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDC	Pyruvate dehydrogenase complex
PE	Phycoerythrin
PMA	Phorbol 12- myristate 13-acetate
PMA/I	Phorbol 12- myristate 13-acetate and ionomycin
PRF	Perforin
PSC	Primary sclerosing cholangitis
RAG	Recombinase activating gene

RANTES	Regulated on activation, normal T expressed and secreted
REC	Research ethics committee
RNA	Ribonucleic acid
ROR	RAR-related orphan receptor
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute medium
S1PR1	Sphingosine-1-phosphate receptor 1
SDF-1	Stromal cell-derived factor 1
SH2B3	SH2B adaptor protein 3
SIV	Simian immunodeficiency virus
SR	Spontaneous resolution
STAT	Signal transducer and activator of transcription
SVR	Sustained virological response
SYK	Spleen tyrosine kinase
T-bet	T-box expressed in T cells
TGF	Transforming growth factor
Th	T helper
TIGIT	T-cell immunoreceptor with immunoglobulin and ITIM domains
TLR	Toll-like receptor
TNF	Tissue necrosis factor
TRAIL	Tumour necrosis factor–related apoptosis-inducing ligand
Tyk	Tyrosine kinase
UDCA	Ursodeoxycholic acid
ULBP	UL16 binding proteins

UV Ultra violet

VLA Very late antigen

VSV Vesicular stomatitis virus

1 Chapter 1: Introduction

1.1 Natural killer cells

NK cells are lymphocytes and are major components of the cellular innate immune response along with macrophages, dendritic cells, neutrophils, basophils and eosinophils. They are classically defined as $CD3^{-}CD56^{+}$ lymphocytes and make up 5-15% of the peripheral blood lymphocyte population. Functional NK cells are broadly divided into those which are $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$. $CD56^{bright}CD16^{-}$ NK cells highly express the high affinity IL-2 receptor and release large volumes of Th1 cytokines, whereas $CD56^{dim}CD16^{+}$ NK cells are more mature and responsible for natural and antibody-dependent cytotoxicity. $CD56^{bright}$ NK cells are thought to develop into $CD56^{dim}$ NK cells. Unlike T and B lymphocytes, NK cells do not require prior sensitization making them key effectors of first line immune defence where they have activity against both virally-infected and tumour-transformed cells. NK cells have several functions. They can initiate programmed cell death of target cells via the release of toxic lytic granules, hence the name 'natural killer' cell. Perforin creates pores in the target cell allowing granzymes to enter an induce death. NK cells also interact with the adaptive immune response by killing Immunoglobulin (Ig) G opsonised target cells through their interaction with the IgG Fc receptor CD16 (Antibody Dependent Cell-mediated Cytotoxicity, ADCC). Secondly they can secrete Th1 (interferon (IFN) γ, Tissue Necrosis Factor (TNF) α) and Th2 cytokines (IL-4, IL-13), which prime the adaptive immune response, in addition to mediating direct anti-viral effects in the case of IFNγ, halting the replication and spread of viruses through non-cytolytic mechanisms. NK cells also release a number of chemokines including CC-chemokine ligand (CCL) 3 (Macrophage Inflammatory Protein (MIP)-1α), CCL4 (MIP-1β) and CCL5 (RANTES) in addition to Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF). NK cells are among the earliest cells to respond to an insult and mount an inflammatory response. However as will be discussed later they also play a role in maintaining homeostasis and immune tolerance.

NK cell activation is required to trigger both direct cell killing and cytokine release, and this is dependent on the interaction of NK cell receptors and their ligands (**Table 1**) [1]. NK cell receptors can broadly be divided into the extremely polymorphic group of type 1 transmembrane glycoproteins, Killer cell Immunoglobulin-like Receptors (KIR), type 2 transmembrane glycoproteins, C-type lectin receptors, and natural cytotoxicity receptors. The former two have both activating and inhibitory loci whereas the cytotoxicity receptors are solely activating. NK cell activation can be achieved in multiple ways. Firstly NK cells can be activated via 'missing self' [2,3]. In health, all cells express

self Major Histocompatibility (MHC) receptors, however these can be downregulated in cells which become cancerous or become infected with a virus. When inhibitory KIR can no longer engage with paired MHC ligands the inhibitory signal is lost leading to NK cell activation. This process is dependent on NK cell licensing (**Fig 1-1**). In order to become an effector cell, NK cells need to have encountered a self-MHC ligand which can be recognised by the KIR types expressed by that cell (**Table 1**). For example if a patient is homozygous for Human Leukocyte Antigen (HLA) C2 and a NK cell expressing KIR2DL1 encounters this ligand for the first time, the NK cell will recognise HLA-C2 as a self MHC molecule and will not mount a response against the target cell, i.e. the NK cell is licensed and self-tolerant. Thereafter an encounter with a cell which fails to express HLA-C2, will result in NK cell activation and targeted cell killing. Unlicensed NK cells cannot exert this effect. Certain KIR-HLA interactions are stronger than others with clinically relevant consequences. The weaker binding affinity between KIR2DL3 and group 1 HLA-C alleles over KIR2DL2 results in significantly higher rates of both spontaneous and treatment induced resolution of Hepatitis C Virus (HCV) infection in this genotype, presumably as result of a weaker barrier to NK cell activation [4,5].

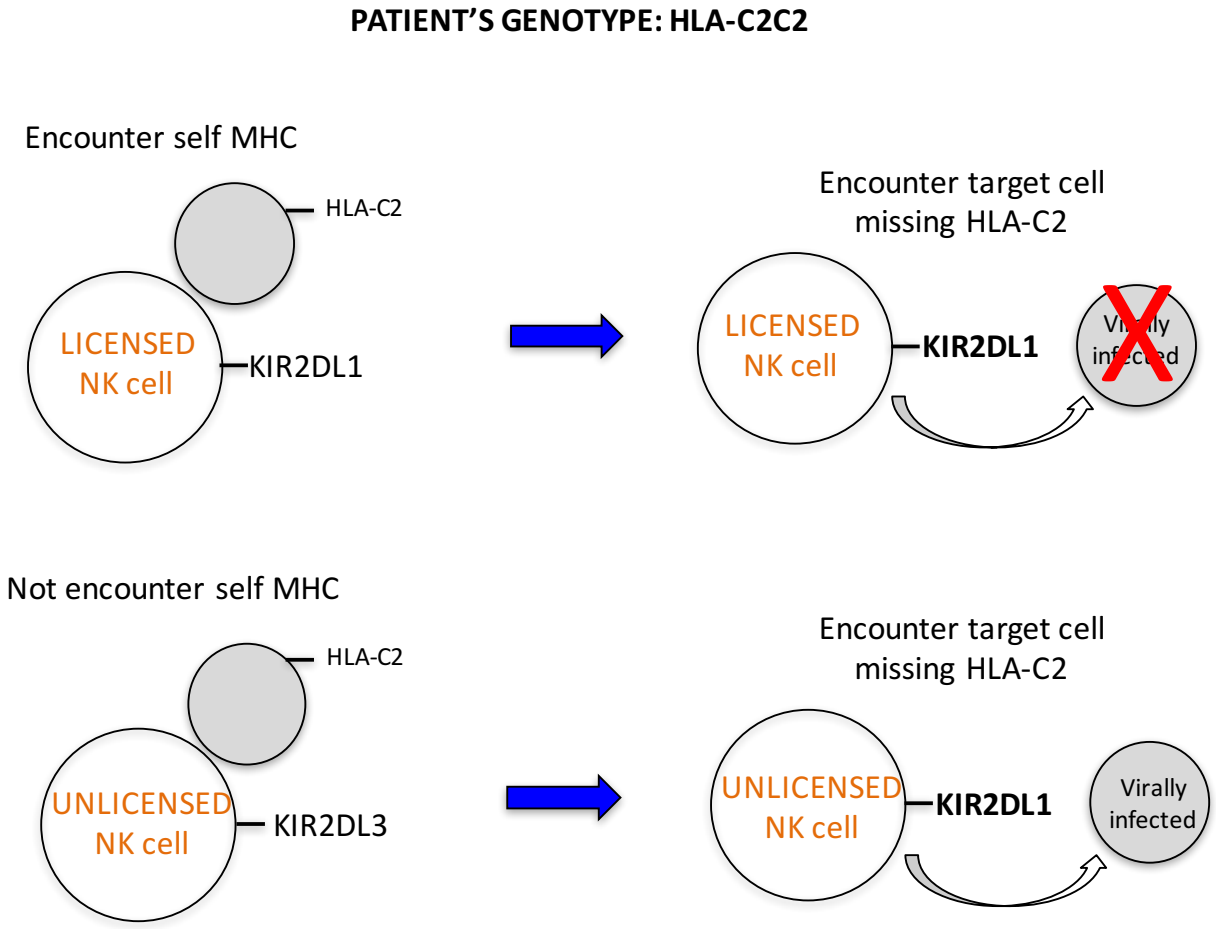
NK cells may also require the interaction of multiple activating receptors to become fully functional, 'induced-self recognition'. When cell signals from activating receptors exceed those of the inhibitory receptors, NK cells are activated**.** Finally an emerging pathway to activation involves interactions between peptide motifs presented by MHC molecules and inhibitory KIR receptors [6]. Changes at an amino acid level have been found to antagonise the inhibitory effect of known KIR-self MHC interactions resulting in NK cell activation. This is known as the 'altered self hypothesis' and demonstrates that NK cells expressing KIR can exhibit broad antigen specificity. Interestingly it has recently been shown that KIR2DL3-positive NK cells respond more efficiently to changes in peptide repertoire than those which are KIR2DL2-positive [7].

NK cells, and particularly KIR, display enormous diversity which can impact NK cell function. Diversity is seen in terms of KIR haplotype resulting in population diversity, at ligand level in terms of HLA expression, and at an allelic level at the KIR locus. The latter can lead to different individuals having different numbers of activating and inhibitory KIR. According to the 'rheostat model' this may be important for NK cell activation, as it proposes that NK cell reactivity is tuned by the number of self-MHC class I inhibitory receptors expressed, i.e. the greater number of self-MHC class I inhibitory receptors, the stronger the effector function and greater the frequency of engagement [8]. During education the NK cell balances its activation threshold according to the strength of the inhibitory input to allow NK cells maturation to be optimally determined by the inhibitory signals [9].

Table 1 NK cell receptors and their known ligands

Activating receptors		Inhibitory receptors	
Receptor	**Ligand**	**Receptor**	**Ligand**
KIR		**KIR**	
KIR 2DS1	HLA-C2	KIR 2DL1	HLA-C2
KIR 2DS2	HLA-C1	KIR 2DL2	HLA-C1
KIR 2DS3	-	KIR 2DL3	HLA-C1
KIR 2DS4	HLA-Cw4	KIR 2DL5 A/B	-
KIR 2DS5	-	KIR 3DL1	HLA-Bw4 + some HLA-B
KIR 3DS1	HLA-Bw4 + some HLA-B	KIR 3DL2	HLA-A3, A11
KIR 2DL4 (CD158d)	HLA-G	KIR 3DL3	-
C-type lectin receptors		**C-type lectin receptors**	
NKG2C/E:CD94	HLA-E	NKG2A:CD94	HLA-E
NKG2D	ULBP & MIC-A/B	Siglec-7	
Nkp80		LIR-1	
Natural cytotoxicity receptors			
NKp30	BAT3, B7H6, hCMV-pp65		
NKp44	Viral haemagglutinins		
NKp46	Viral haemagglutinins, neuroaminidase, heparin		
Co-receptors			
DNAM-1			
2B4			
Others			
CD16 (FCγ-RIIIA)			
CD161			

Figure 1-1 NK Cell Licensing



1.2 Natural killer cells in the liver

1.2.1 Natural killer cell phenotype and function in the liver

The liver is a fascinating organ in which to study NK cells. NK cells comprise approximately 30-60% of the lymphocyte population in the healthy liver [10–14] and preferentially reside in the sinusoids attached to hepatic sinusoidal endothelial cells (HSECs) [15]. It is well established that the liver is a mediator of immune tolerance and this is largely driven by NK cells. This is necessary as approximately 2000 litres of blood pass through the portal vein per day, exposing NK cells to large volumes of antigens through fenestrations in the endothelium. Furthermore HSECs can act as antigen-presenting cells (APC) themselves. Many reports have revealed different anatomical distributions of various NK cell subsets, described as being tissue-resident, including the cervix, uterus, brain, lung, intestine, placenta and secondary lymphoid tissue [16,17]. Tissue-resident NK cells may develop in situ from tissue-resident stem cells or innate-like lymphocyte (ILC) precursors [18,19]. There is also evidence however that they may traffic to certain organs, including the liver, through their interaction with chemokines via CC-chemokine receptor (CCR) 1/2/3/5/7, Chemokine Receptor (CXCR) 2/3/6 and CX_3CR-1, and adopt a tissue-resident phenotype in situ in response to local environmental stimuli [15,20,21]. While the liver is well established as a site of NK cell maturation in the foetus [22], only small percentages of NK cell precursors can be found in adulthood at this site [23].

Phenotype

NK cells have distinct phenotypic and functional features even in healthy livers. The liver is rich in more immature $CD56^{bright}$CD16- NK cells [10,11,15,24,25] and NK cells with this phenotype reside predominantly in the hepatic sinusoids [15]. Interestingly the phenotype of liver-resident $CD56^{bright}$ NK cells differs significantly to $CD56^{bright}$ NK cells isolated from the peripheral blood [15] . Hudspeth et al recently used nanostring technology to identify 50 differentially expressed genes between $CD56^{bright}$ NK cells isolated from the two tissues and confirmed their findings at a protein level. While liver-resident $CD56^{bright}$ NK cells showed reduced expression of the homing receptors, CCR7 and l-selectin in addition to adhesion molecules CD49e (ITGA5), CD11c (ITGAX) and DNAX Accessory Molecule (DNAM)-1, they highly expressed chemokine receptors CXCR6 and CCR5 [15]. These surface markers have been demonstrated to play an important role in the homing of $CD56^{bright}$ NK cells towards the liver in response to CCL3+ kupffer cells, CCL5+ NK and T-cells and Chemokine Ligand (CXCL) 16+ endothelial cells [15]. Liver-resident $CD56^{bright}$ NK cells were also positive for CD161 and CD69, a marker of tissue-residency known to inhibit sphingosine-1-phosphate receptor

1 (S1PR1) which promotes movement of lymphocytes out of tissue [15,25–27]. Conversely the transcriptional profiles of liver-resident and peripheral blood CD56^{dim} NK cells were nearly identical suggesting there may be free movement of these cells between the two compartments [15]. This may be mediated through their high expression of CCR3, CXCR2 and CX_3CR1 [15]. Hepatic CD56^{bright} NK cells have also been shown by other groups to express higher levels of the natural cytotoxicity receptors NKG2D, NKp44, NKp46, which recognise viral and tumour associated antigens, and stress ligands; in addition to CD94, compared to peripheral CD56^{bright} NK cells. In contrast differences were minimal between the CD56^{dim} subsets [11,25]. Studies differ in their reporting of NKG2A expression between compartments [11,25].

Function

Some variation exists between reports of the function of liver-resident NK cells. This may be a result of differing stimulation conditions or the presence of background liver disease. Expression of the death-ligand, Tumour necrosis factor–Related Apoptosis-Inducing Ligand (TRAIL), has been reported to be negligible at rest in the healthy liver [15,28], unlike in mice, however its expression can be induced on hepatic NK cells using IL-2 [28]. This does not occur on peripheral blood NK cells, where IFNα is required to generate the same effect. Furthermore there is evidence that liver-resident NK cells contain high levels of perforin and granzymes and are more cytotoxic towards HepG2 (hepatocellular cell line) and K562 (MHC-1 deficient cell line) targets than their peripheral blood counterparts [11,14,24,25,28]. In sharp contrast to the peripheral blood, liver-resident CD56^{bright} NK cells also display enhanced cytotoxicity [11]. Stegmann et al showed however that liver-resident CXCR6+ NK cells can degranulate in response to PMA/ionomycin but fail to release high levels of perforin and granzyme B, whereas CXCR6- liver-resident NK cells produce these proteins, but do not degranulate [29]. In health IFNγ production by liver-resident NK cells has been reported to be comparable to that of peripheral NK cells following stimulation with IL-12/IL-15 by Moroso et al [25] and IL-2/IL-12 by Hudspeth et al [15]. However Harmon et al showed IFNγ production by liver-resident CD56^{bright} NK cells to be significantly reduced compared to CD56^{bright} NK cells isolated from the peripheral blood in response to IL-2 [11].

Transcriptional profile

Interestingly NK cells isolated from the human liver have a unique transcriptional profile. Approximately 50% are T-bet^{low}Eomes^{high}, and the remainder are T-bet^{high}Eomes^{low} [29]. This contrasts to the blood where the majority are T-bet^{high}Eomes^{low} (CD56^{dim} NK cells), and a small

percentage are T-bet^{high}Eomes^{high} (CD56^{bright} NK cells) [29,30]. Nearly all T-bet^{low}Eomes^{high} liver-resident NK cells are CXCR6+, and all T-bet^{high}Eomes^{low} liver-resident NK cells are CXCR6-. This supports the premise that CXCR6+ CD56^{bright} NK cells remain in the liver, particularly the sinusoids due to the presence of CXCL16 [31,32], while CD56^{dim} NK cells move freely between the liver and the blood. Cuff et al recently examined NK cells isolated from HLA-mismatched donor and recipient livers and elegantly demonstrated that Eomes^{high} (CXCR6+ CD56^{bright}) NK cells cannot exit the liver and are long-lived, surviving for over 10 years [33]. This subset of NK cells were shown to be replenished via (1) the movement of peripheral blood Eomes^{high} NK cells into the liver, presumably through the expression CXCL16 and CCL5; and (2) the upregulation of Eomes on Eomes^{low} NK cells [33]. Therefore liver-resident NK cells in humans are CXCR6+ CD56^{bright} and are dependent on Eomes, but not T-bet.

Memory-like NK cells

Finally, the liver may also be a site of residence, and, or priming of a group of NK cells recently shown to display adaptive- or memory-like features. Liver-resident NK cells expressing the cell surface markers CD49a(DX5-) and CXCR6 have been shown to be capable of displaying antigen-specific memory against viruses and haptens in mice [19,34]. NK cells expressing these surface markers have since been identified in the human liver, but not the peripheral blood [11,15,29,35].

In summary the liver is an organ rich in NK cells. Approximately half are non-terminally differentiated tolerant CD56^{bright}CXCR6+ NK cells, which home to and reside in the liver. Local environmental signals including cytokines and antigens however are likely to shape the liver as site of NK cell differentiation, home to dynamic population shifts and the emergence of subsets with discrete functions, potentially including those with memory-like features.

1.2.2 The unique hepatic cytokine microenvironment

While the liver is rich in both innate and adaptive immune cells, the innate immune system dominates with large numbers of NK cells, non-classical Natural Killer T-cells (CD56+ CD3- lymphocytes), γδ T-cells, mucosal-associated invariant T-cells, and antigen-presenting Kupffer and dendritic cells [10,14,25,36,37]. These liver-resident immune cells are exposed to vast quantities of non-self antigens from food and bacteria as a result of seventy percent of the hepatic blood flow being supplied by the portal vein, draining the small intestine, colon and spleen. Liver-resident immune

cells therefore need to display a degree of tolerance during immunosurveillance and the liver is described as been in an 'immuno-privileged state'. This is well illustrated by the fact that liver transplant donors and recipients do not need to be HLA-matched.

During health the hepatic cytokine microenvironment is dominated by high levels of Transforming Growth Factor (TGF)-β and IL-10 released from kupffer cells and HSECs, in addition to IL-13 from T-helper cells. This can lead to enrichment of NK cells expressing inhibitory receptors, including NKG2A and a dampening of IFNγ production [38]. NK cells can also cross-talk with other cells within the immune system, for example by priming dendritic cells through the release of TGF-β and IL-10 to generate the expansion of regulatory T-cells [39] and inhibiting stellate cell activation [40]. Furthermore NK cells can eliminate over-stimulated macrophages, which in turn can control recruitment and activation of NK cells [41]. Interactions between NK cells and hepatocytes themselves are also important for maintaining homeostasis. Despite their low levels of of MHC-I expression [42,43], autologous hepatocytes are not killed by hepatic NK cells during health. Even during inflammation where intrahepatic IFNα and IFNγ levels are raised, leading to upregulation of TRAIL on NK cells and Th1 cytokine release, hepatocytes are protected through their ability to increase their expression of self HLA molecules in this state, which are recognised by inhibitory NK cell receptors [28,44]. Furthermore hepatocytes have been found to express the non-classical MHC molecule HLA-E. This binds the inhibitory receptor NKG2A, which is strongly expressed by hepatic NK cells and induces their production of TGF-β and IL-10 [45]. Therefore the whole immune network in the liver is optimised towards tolerance.

Central to my hypothesis is the effect of the unique hepatic cytokine environment on modulating NK cell phenotype and function. Lassen elegantly demonstrated this principal following the discovery in mice that NKG2A+ Ly49- (MHC binding receptor in mice) were hyporesponsive to stimulation with IL-12 and IL-18 in terms of IFNγ release, although cytolytic potential was preserved [38]. Adoptive transfer of splenic Ly5.1+ NK cells into Ly5.2+ mice showed NK cells migrate to liver and adopt the same phenotype and tolerant function as their resident counterparts, suggesting the liver cytokine environment induced this change. This appeared to be driven by IL-10 as blockade of this receptor resulted in a reduction in the frequency of hyporesponsive NKG2A+Ly49- cells [38]. Furthermore Harmon et al recently isolated peripheral blood NK cells from healthy donors and cultured them in liver-conditioned medium (LCM) for 24 hours [11]. Following stimulation with K562 target cells and IL-2, IFNγ production was reduced in both $CD56^{bright}$ and $CD56^{dim}$ NK cells compared to NK cells which were not exposed to the LCM, although CD107a expression was not altered. Taken together

this data suggests that the hepatic cytokine environment suppresses NK cell pro-inflammatory cytokine release.

Further evidence demonstrating the influence of the hepatic environment on NK cell function comes from liver transplant studies. Patients undergoing liver transplantation are not HLA matched, yet chronic rejection rates are significantly lower compared to other organ transplantations and in some cases immunosuppressive therapy can be withdrawn, providing further evidence of a tolerogenic immune environment in the liver. Work in the Khakoo laboratory has defined an unusual sub-population of $CD56^{dim}CD16$- NK cells in the peripheral blood of liver transplant recipients not found in healthy controls, suggesting that one step in NK cell maturation occurs in the liver [46]. Furthermore despite HLA mismatching, NK cells in the peripheral blood of recipients expressed lower levels of activating receptors and were tolerant in terms of both cytotoxicity and cytokine release. This hypofunctional phenotype occurred independent of the influence of immunosuppressants (**Fig 1-2**).

Whole genome microarray analysis followed by quantitative polymerase chain reaction (PCR) demonstrated an approximate ten-fold downregulation in Signal Transducer and Activator of Transcription (STAT) 4 mRNA in peripheral NK cells from individuals following liver transplantation compared to healthy controls ($p<0.0005$). This data has led me to hypothesize that migration of NK cells through the liver can alter their phenotype and function, with STAT4 downregulation leading to tolerant behaviour.

Figure 1-2 Changes in NK cell phenotype and function following liver transplantation

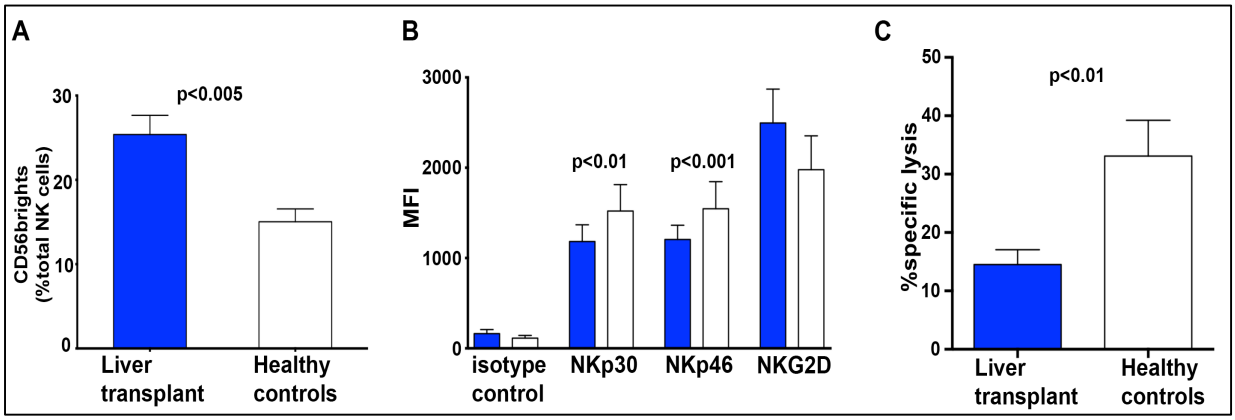


Fig 1-2 Changes in NK cell phenotype (n=84) and function (n=45) following liver transplantation: $CD56^{bright}$ NK cells (A), activating receptor expression (B) and cytotoxic function (C) were determined in liver transplant recipients (blue bars) and healthy controls (white bars) – *GUT* 2015, Khaleel Jamil [46]

Finally the liver is known to be rich in chemokines, including CCL3, CCL5 and CXCL16 released from both kupffer cells and HESCs, which play important roles in the homing of NK cells towards the liver. There is evidence that chemokines may be redistributed and upregulated during disease, for example CXCL12 is upregulated in hepatitis B and C leading to the recruitment of CXCR4+ liver-infiltrating lymphocytes [47].

Importantly the presence of hepatitis or cancer leads to a change in the intrahepatic cell milieu and switch to a more inflammatory cytokine environment dominated by IL-1, IL-2, IL-6, IL-12, IL-15, IL-18, IFNα, IFNγ and TNFα/β [48,49]. These signals may direct NK cell differentiation down discrete pathways, which could include the generation of a subset which has a strong Th1 cytokine profile and is highly cytotoxic. I hypothesise that the generation of 'hyperfunctional' NK cells may occur through upregulation of the transcription factor, pSTAT4 (**Fig 1-7**).

1.2.3 Cross talk between NK cells and other cells in the liver

NK cells cross talk with other immune and non-immune cells resident in the liver. As discussed later on they can both inhibit stellate cell activation via the release of IFNγ and kill activated stellate cells through TRAIL and FasL [50–52]. In terms of their interaction with innate immune cells, NK cells are activated by toll-like receptor (TLR) ligand-activated kupffer cells (liver-resident macrophages) via cell-to-cell contact [53]. In return NK cell derived-IFNγ can activate macrophages and increase the capacity of macrophages to kill phagocytosed bacteria. During liver injury, for example poly I:C/D galactosamine-induced fulminant hepatitis, Kupffer cell-derived IL-12 and IL-18 can enhance their cross talk with NK cells contributing to liver injury [54]. NK cells can also stimulate kupffer cell-dependent IL-6 production and it this way the two play a protective role against cholestatic injury [55]. Dendritic cells can induce NK cell activation, promoting the release of IFNγ and TNFα, and enhancing both NK cell cytotoxicity and proliferation through the release of proinflammatory cytokines (IL-12, IFNα). In HBV, dendritic cell-activated NK cells can lead to hepatocyte death via Fas/FasL interactions [56]. In turn IFNγ and TNFα released by NK cells stimulate dendritic cell maturation and migration towards inflamed tissue. NKT cells can also enhance NK cell cytotoxicity, specifically towards hepatocytes, through their release of IL-4 and IFNγ [57].

Finally NK cells can interact with adaptive immune cells. T-regulatory cells (in addition to kupffer cells and HSECs) can suppress NK cell activation via the release of TGFβ and IL-10, ameliorating neonatal bile duct injury [58] and inhibiting the anti-fibrotic functions of NK cells [59]. Furthermore

NK cells can help direct Th1 T-cell differentiation through their secretion of IFNγ and can kill activated CD8+ T-cells and NKT cells [60–62]. In this way NK cells can help orchestrate the immune response system by acting as potential regulators of other immune cells.

1.3 Natural killer cells in liver disease

Natural Killer cells can display an altered phenotype and function in liver disease and can significantly influence clinical outcomes. They are protective against viral infections, tumour growth and hepatic fibrosis, but can be detrimental by enhancing hepatocellular damage and inhibiting liver regeneration. A key issue which this projects aims to address in how these diseases are able to escape the NK cell response despite the rich NK cell population in the liver and their frequent exposure to antigens via HESC fenestrations, and whether loss of immune tolerance may lead to autoimmune disease. Building on our understanding that NK cells can undergo functional differentiation within the liver, I propose intrahepatic NK cells can be modulated by cytokines and antigens present in liver disease influencing clinical outcomes.

1.3.1 Hepatitis C

Hepatitis C is a common viral infection of the liver. It was identified in 1989 as the agent responsible for non-A non-B hepatitis [63]. It belongs to the Hepacivirus genus of the Flaviviridae family and is a small enveloped positive stranded RNA virus [63]. An estimated 216,000 people in the UK and 150 million people worldwide are infected [64]. Approximately 80% of exposed individuals fail to spontaneously clear the virus resulting in cirrhosis, liver failure and cancer [65]. A large number of novel direct-acting antivirals have emerged over the last 5 years and replaced pegylated interferon and ribavirin as the main stay of treatment. While sustained virological response (SVR) rates (absence of HCV RNA 6 months post treatment cessation) are generally over 95%, some difficult-to-treat groups remain, particularly patients who are infected with HCV genotype (G) 3, are cirrhotic and have failed previous treatment. Furthermore, these medications are unaffordable in some countries and there is currently no vaccine against HCV viral infection. Understanding the mechanisms that lead to viral clearance can impact vaccine design and lead to novel immunologic therapies. In addition to IFN-λ3/4 [66–68], polymorphisms within KIR and their HLA alleles are extremely influential in determining HCV related outcomes (spontaneous and treatment induced resolution) and can provide a discrete pathway to clearance [69] (**Table 2**).

Table 2 KIR and HLA polymorphism in determining HCV resolution

Spontaneous Resolution (SR)				Sustained Virological Response (SVR)			
Protective		**Susceptible**		**Protective**		**Susceptible**	
Genotype	*Population*	*Genotype*	*Population*	*Genotype*	*Population*	*Genotype*	*Population*
KIR2DL3L3 : HLA-C1C1	Caucasian, African American (n=1037), Puerto-Rican American (n=160) [4,70]	KIR2DL1: HLA-C2C2	Caucasian, African American (n=1037) [4]	KIR2DL3 : HLA-C1	UK caucasian (n=208) & Spainish caucasian (n=186) [5,71]	KIR2DL2 : HLA-C1	Spanish caucasian (n=186) [71]
KIR3DS1 : HLA-Bw4^{801}	Caucasian, African Americans (n=1037) [4]	KIR2DS3 : HLA-C2C2	Irish HCV cohort (n=543) [72]	KIR3DS1 : HLA-Bw4^{801} in HIV co-infected	Spanish caucasian, HCV G1 (n=60) [73]	KIR2DS2 : HLA-C1	Spansih caucasian (n=186) [71]
KIR2DL3 : HLA-DRB1*1201	Puerto-Rican Americans (n=160) [70]			KIR3DL1 : HLA-Bw4^{801} (dual & triple therapy)	Japanese (n=115, n=200) [74,75]	Centromeric motif BB	Spanish caucasian, HCV G1 (n=811) [76]
				KIR2DL2/S2 (dual therapy post liver transplant)	Caucasian (n=44) [77]		
				Centromeric motif AA	Spanish caucasian, HCV G1 (n=811) [76]		

Our knowledge regarding the phenotype and function of NK cells in chronic hepatitis C (CHC) has grown over the last 10 years although variability exists between studies. Ideally a large multicentre study is required which can stratify for both viral (HCV genotype) and clinical factors (presence of cirrhosis, HIV or Hepatitis B (HBV) co-infection) using the same methodology throughout to examine gene expression. A summary of publications comparing NK cells in the blood and liver in CHC compared to control groups is presented in **Tables 3 and 4**. A strong point of consensus exists for a 'cytotoxic, poor cytokine release' phenotype with polarisation towards Th2 cytokine production within NK cells isolated from the peripheral blood, a feature which persists in the presence of IFNα (**Table 3**) [78,79]. In common with the tolerant NK cells found in recipients post liver transplant, this phenotype is also associated with reduced STAT4 phosphorylation relative to STAT1 [80,81]. A particularly hypofunctional cohort of terminally differentiated CD56-CD16+ NK cells have also been found to be expanded in the blood of both HCV [82,83] and Human Immunodeficiency Virus (HIV) [84] infected patients, displaying not only a cytokine profile skewed towards MIP-1β production, but

also reduced cytotoxicity. Numbers can however revert back to those found in healthy controls with the introduction of IFNα therapy, a further example of the role of cytokines on NK cell phenotype.

NK cells isolated from the liver from HCV-infected individuals display altered phenotype and function compared to controls (**Table 4**). Enrichment of NK cells in the liver is generally lost [85,86]. Hepatic NK cells display increased natural cytotoxicity receptors, NKp44 [87] and NKp46 [79,86–88] but also increased expression of the inhibitory receptor NKG2A [87] and reduced NKp30 [86] which may help promote chronicity. As in health, hepatic NK cells are less mature than their blood counterparts with reduced CD16 [86] and KIR [85,87]. Function appears to be improved in terms of both IFNγ release [86] and cytotoxicity [79,86,87] in the liver compared to the blood. Such phenotypic and functional changes can influence clinical outcomes. For example, a greater percentage of hepatic NK cells is associated with response to IFN therapy [89], a high proportion of NKp46+ NK cells predicts treatment failure despite being associated with NK cell activation and increased NK cell cytotoxicity [86] and lower pre-treatment level of the hypofunctional CD56-CD16+ NK cell cohort has been linked to improved SVR rates [79]. Liver-resident NK cells from HCV-infected patients have been shown to express similar levels of chemokine receptors, CCR2, CCR5, CCR6, CXCR3, CXCR4, CXCR6 and CX_3CR1, to healthy controls [90].

Table 3 A comparison of NK cell phenotype and function in the peripheral blood in chronic hepatitis C compared to healthy controls

NK cell phenotype			
	Increase	**No change**	**Decrease**
% NK cells in lymphocyte population		Corado 1997 [91] Kawarbayashi 2000 [92] Natterman 2006 [93] Yamagiwa 2008 [89] Sene 2010 [94]	Corado 1997 [91] Meier 2005 [95] Morishima 2006 [96] Golden-Mason 2008 [97] Oliviero 2009 [98] Bonorino 2009 [85] Dessouki 2010 [99] Pembroke 2013 [86]
$CD56^{bright:dim}$ ratio	Meier 2005 [95] Morishima 2006 [96] Bonorino 2009 [85] Bozzano 2011 [100]	Pembroke 2013 [86]	
NKp30	De Maria 2007 [78]	Dessouki 2010 [99] Pembroke 2013 [86]	Natterman 2006 [93] Bozzano 2011 [100] Yoon 2011 [101]
NKp44	Ahlenstiel 2010 [79]		De Maria 2007 [78]
NKp46	DeMaria 2007 [78] Ahlenstiel 2010 [79] Kramer 2012 [88]	Dessouki 2010 [99]	Natterman 2006 [93] Pembroke 2013 [86]
NKG2A	Natterman 2006 [93]	Bonorino 2009 [85]	
NKG2C	Ahlenstiel 2010 [79]		
NKG2D	Oliviero 2009 [98]	Natterman 2006 [93] Sene 2010 [94] Pembroke 2013 [86]	Dessouki 2010 [99]
NK cell function			
	Increase	**No change**	**Decrease**
Cytotoxicity	Ahlenstiel 2010 [79] Dessouki 2010 [99] Oliviero 2009 [98] Morishima 2006 [96]		Natterman 2006 [93] Meier 2005 [95] Corado 1997 [91] Pembroke 2013 [86]
Th1 cytokine release		De Maria 2007 [78]	Ahlenstiel 2010 [79] Dessouki 2010 [99] Oliviero 2009 [98]

Table 4 A comparison of NK cell phenotype and function in the liver in chronic hepatitis C compared to healthy controls or chronic hepatitis B

NK cell phenotype			
	Increase	**No change**	**Decrease**
% NK cells in lymphocyte population		Meier 2005 [95] Boisvert 2003 [90]	Bonorino 2009 [85] Kawarbayashi 2000 [92] Sene 2010 [94] Yamagiwa 2008 [89] Kramer 2012 [88] Varchetta 2012 [87] Meier 2005 [95] Pembroke 2014 [86]
$CD56^{bright:dim}$ ratio	Meier 2005 [95]		Bonorino 2009 [85]
NKp30, NKp44		Natterman 2006 [93]	
NKp46	Kramer 2012 [88] Ahlenstiel 2010 [79] Varchetta 2012 [87]	Natterman 2006 [93]	
NKG2A	Bonorino 2009 [85]		
NKG2D	Sene 2010 [94] Oliviero 2009 [98] Varchetta 2012 [87]		
KIR			Bonorino 2009 [85]
NK cell function			
	Increase	**No change**	**Decrease**
Cytotoxicity	Bonorino 2009 [85] Pembroke 2013 [86] Ahlenstiel 2010 [79] Varchetta 2012 [87]		Kawarbayashi 2000 [92] Varchetta 2012 [87]
Cytokines	Pembroke 2013 [86]		Kawarbayashi 2000 [92]

1.3.2 Hepatitis B

Hepatitis B virus infects 400 million individuals worldwide. Clinical outcomes are dictated by the hepatic immune response which may lead to viral clearance, but at the expense of inflammation and hepatocyte death. NK cell function has therefore been described as a 'double-edged' sword in HBV [102]. Peripheral and intrahepatic NK cells have been shown to be activated and express high levels of the death ligand TRAIL, which contributes towards hepatocellular damage and inflammation [103], in addition to performing an immunoregulatory role by killing HBV-specific T-cells [61,62]. This cytolytic phenotype may be associated with the high levels of IL-8, IL-12, IL-15, IL-18 and IFNα [61,103]. On the other hand activated NK cells may promote viral clearance, inhibit stellate cells reducing progression to fibrosis [104] and protect against hepatocellular carcinoma (HCC) [105]. In acute and chronic hepatitis B however, NK cells have also been found to have impaired IFNγ production associated with high levels of IL-10 [106,107].

Few large studies have examined the role of KIR polymorphisms in this setting. A protective association between KIR2DL3 : HLAC1 homozygosity has been demonstrated, with KIR2DL1 : HLAC2 being disadvantageous [108]. A higher frequency of KIR haplotype A and lower frequency of haplotype B has also been observed in HBV infected patients compared to controls [108,109].

1.3.3 Hepatocellular Carcinoma

Hepatocellular carcinoma is the 9th most common cause of cancer death in the UK however this figure is predicted to rise nearly 60% between 2014 and 2035 [110]. Numerous groups have studied NK cell frequency in HCC and generally reported reduced absolute numbers of NK cells in the periphery and tumour-infiltrating areas of the liver, driven by a decrease in the CD56^{dim} subset, although numbers are higher in non-tumour infiltrating regions [111–113]. The reduction of CD56^{dim} NK cells is generally associated with more advanced HCC and correlates with poor survival [114]. Furthermore CD56^{dim} NK cells have been found be less cytotoxic and release lower levels of IFNγ in tumour infiltrating regions of patients with HCC [113,115], and it has been suggested that NK cell exhaustion may contribute towards more advanced HCC including multilobular disease and portal vein invasion [113,115–117].

Crucially NK cell tumour surveillance is also impaired in the presence of pre-cancerous fibrosis and cirrhosis. This may be dependent on the expression of NK cell receptors, for example KIR3DS1 :

HLA-Bw4-80(I) is protective against HCC in patients with CHC, as is the activating receptor NKG2D [118,119]. Again the external cytokine environment may help shape the NK cell response as transcriptional profiling of NK cells treated with IFNα revealed upregulation of TRAIL promoting killing of HCV infected hepatoma cells [120]. The use of NK cell immunotherapy to treat HCC is therefore an extremely promising field with a number of early studies currently being undertaken using NK cell transfer, gene therapy, cytokine therapy and monoclonal antibodies to generate activated NK cells able to effectively kill hepatoma cells [121].

1.3.4 Hepatic fibrosis

Activated NK cells can protect against fibrosis via the release of IFNγ, which blocks stellate cell activation reducing extracellular matrix deposition [50]. Furthermore NK cells are able to induce apoptosis of hepatic stellate cells via TRAIL and FasL. This feature is associated with expression of NKp46 and NKG2D and is enhanced by IFNα [51,52]. Greater NK cell activation and cytotoxicity, particularly seen in NKp46^{high} cells, is associated with less hepatic fibrosis in patients with CHC [86,88,96]. The downside is that IFNγ, TRAIL and granzyme B released by activated NK cells can lead to hepatocyte death and increased periportal inflammation, limiting regeneration of the liver [86].

1.3.5 Alcohol and Non-Alcoholic Fatty Liver Disease

Alcohol-related liver disease and non-alcoholic steatohepatitis (NASH) are now the leading causes of cirrhosis in the UK and are growing in prevalence. Current management includes lifestyle changes and best supportive care following the development of cirrhosis, with no new therapies on the horizon. NK cell cytotoxicity is reduced in alcohol-related liver disease [122]. Consequences of this may include accelerated fibrosis and increased susceptibility to HCC. Similar findings have been reported in peripheral blood NK cells in obese patients in addition to reduced NK cell frequencies [123,124]. On the other hand, intrahepatic NK cells have been found to express enhanced markers of cytotoxicity in the livers of patients with NASH compared to non-alcoholic fatty liver disease (NAFLD) and healthy controls [125], which may help contribute towards hepatocyte death. This may reflect NK cell activation in the liver in the presence of inflammatory cytokine IL-12 and IL-18.

1.3.6 Autoimmune Hepatitis

Autoimmune liver disease is relatively rare compared to rheumatoid arthritis, Hashimoto's thyroiditis, coeliac disease, T1 diabetes mellitus and Graves disease. As described above this is probably a result of the fact that the rich immunological network in the liver is coordinated to maintain tolerance. Despite hepatic NK cells being activated and capable of cytolysis, they do not release high quantities of pro-inflammatory cytokines or kill autologous hepatocytes. Dysregulation of this state, resulting in activity against autoantigens can lead to autoimmune disease [126,127]. Few studies in humans have explored a role for NK cells in mediating autoimmune hepatitis and it is generally thought to be driven by autoreactive T-cells [128]. It is interesting to note however that the first study to identify hepatic NK cells ('pit cells') in the 1980s was in a patient with autoimmune hepatitis and showed them to be in contact with degenerating hepatocytes [129]. A recent murine study also reported that the activation of hepatic NK cells using polyI:C, a potent type 1 interferon inducer, led to an NK cell driven hepatitis with histology comparable to that of autoimmune hepatitis [130]. Blocking this cytokine pathway may therefore help modulate NK cell autoimmunity.

1.3.7 Primary Biliary Cholangitis

Primary Biliary Cholangitis (PBC) is chronic condition characterised by a progressive lymphocytic cholangitis. Immune-mediated non-suppurative destruction of the small intrahepatic biliary ducts leads to cholestasis and can advance to cirrhosis and liver failure in some individuals. Highly specific autoantibodies against the E2 components of the pyruvate dehydrogenase complex (PDC-E2) are found in 95% of individuals. PBC is estimated to affect approximately 1 in 5000-10,000 individuals in the developed world, and 1 in 1000 women over 40 [131]. Ursodeoxycholic acid (UDCA) is currently the only licensed treatment. It protects cholangiocytes and hepatocytes from the detrimental effect of excessive bile acids by decreasing plasma and biliary endogenous bile acid concentrations and stimulating hepatobiliary secretion. Therefore it addresses complications of cholestasis, but not the underlying cause. While UDCA use has been shown to lead to an improvement in liver biochemistry and inflammation seen at histology, there is no strong evidence it can prevent progression to cirrhosis and improve survival [132]. Moreover, approximately 40% of patients have been found to have an inadequate biochemical response [133].

Immunological studies in PBC have largely focused on anti-mitochondrial antibodies (AMA) produced by autoreactive B-cells, and activated CD4+ and CD8+ T-cells which bring about destruction of the small bile ducts [134]. Decreased number and function of T-regulatory cells and

IgM hyper-gammaglobinaemia is also seen. Interest has been redirected towards the innate immune response however following the publication of a number of GWAS studies (**Table 5)** [135–140]. These have identified 25 differentially expressed genes, in addition to those encoding HLA-II, in patients with PBC compared to healthy controls. Identified polymorphisms fall broadly into three biological pathways; TNFα, Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NFκB) and the IL-12/STAT4 pathway (IL-12A (encodes IL-12α (p35)), IL-12 receptor B2 subunit locus (encodes IL-12 receptor β2), STAT4/1, SH2B adaptor protein 3 (SH2B3), Tyk2), which has been the most consistently differentially expressed non-HLA loci across ethnicities. While IL-12A and IL-12RB polymorphisms were not associated with PBC susceptibility in Japanese cohorts, STAT4 remained a significant player [139,141]. Whether this latter pathway has a role in influencing PBC susceptibility through the activity of NK cells is unknown.

The phenotype and function of NK cells in PBC has not been well characterized, however within both the peripheral blood and liver there appears to be an increase in absolute numbers of NK cells and a bias towards a 'cytotoxic, poor cytokine release' profile [142]. The role of NK cells in PBC has been most extensively investigated by Shimoda et al using their expertise in isolating and culturing liver mononuclear cells (MNCs) and biliary epithelial cells (BECs) [143,144]. They demonstrate that hepatic NK cells from individuals with PBC have enhanced cytotoxicity against autologous biliary epithelial cells (BECs) compared to controls [145,146]. This aberrant activity only occurred however where NK cells were stimulated with TLR-4 ligands and in the presence of IFNα produced by TLR-3 ligand stimulated hepatic monocytes, demonstrating cross talk between the two immune cells [145]. Of note immunohistochemistry revealed an abundance of NK cells surrounding destroyed small bile ducts [145]. The group later demonstrated that NK cell function plays an important role in shaping the adaptive immune response in PBC [144]. Where the ratio of NK cells : BECs was high, NK cells lysed autologous BECs leading to the release of the PDC-E2 peptide intact within a microparticle, stimulating proliferation and cytokine release from CD4+ T-cells [144]. NK cells continued to facilitate BEC destruction where their ratio to BECs was lower through IFNγ release which led to the induction of HLA class II molecules on BECs which enabled CD4+ T-cells to become more cytotoxic towards them [144]. Finally it has also been suggested that NK cells induce BEC lysis through a TRAIL receptor 5, which is strongly expressed on cholangiocytes in PBC and is a mediator of cholestatic liver injury. The upregulation of TRAIL on hepatic NK cells by inflammatory cytokines may therefore trigger BEC destruction [147,148]

Intrahepatic chemokine receptors also appear to be influential in the pathogenesis of PBC. David Adams' group had previously shown that CXCL16 promotes lymphocyte adhesion to BECs in

inflamed human liver tissue. More recently they showed that CXCL16 is most strongly upregulated in PBC compared to other liver disease including alcoholic liver disease, with no change in serum levels [149]. CXCR6 expression was upregulated in cirrhosis compared to healthy livers on NK cells, but not CD56- or CD56+ T-cells, with the highest frequency of intrahepatic CXCR6+ NK cells found in the livers of individuals with PBC. Dynamic flow assays mimicking the sinusoid revealed that a small molecule inhibitor of CXCR6 could reduce migration of NK cells across the hepatic endothelium. This work identifies CXCR6+ liver-homing activated NK cells as potentially pathogenic in PBC. In summary PBC may represent a multi-lineage immune condition driven initially by innate immune activity which maintains auto-reactivity of the adaptive immune response.

Table 5 Summary of non-HLA susceptibility loci identified by GWAS studies in PBC

Study	Population	Associated gene	SNP
Hirschfield et al. *NEJM*. 2009. [135]	**North America & Canada** 536 cases 1536 controls	IL12A IL12RB2	rs6441286 rs3790567
Hirschfield et al. *Nature Genetics.* 2010. [136]	**Europe** 857 cases 3198 controls	IL12A IL12RB2 IRF5/TNPO3 ZPBP2 MMEL1	 rs10488631 rs11557467 rs3748816
Liu et al. *Nature Genetics*. 2010. [137]	**Italy** 453 cases 945 controls	IL12A IL2RB2 IRF5/TNPO3 ORMDL3/IKZF3 SPIB	 rs10488631 rs9303277 rs3745516
Mells et al. *Nature Genetics*. 2011. [138]	**UK** 1840 cases 5163 controls	IL12A IL12RB2 IRF5 ORMDL3 MMEL1 SPIB STAT4/1 DENND1B CD80 IL7R CXCR5 TNFRSF1A CLEC16A NFKB1 RAD51L1 MAP3K7IP1 ELMO1 IRF8	rs485499 rs17129789 rs12531711 rs7208487 rs10752747 rs3745516 rs10931468 rs12134279 rs2293370 rs860413 rs6421571 rs1800693 rs12924729 rs7665090 rs911263 rs968451 rs6974491 rs11117432
Nakamura et al. *American Journal of Human Genetics*. 2012. [139]	**Japan** 487 cases 476 controls	IL7R ORMDL3/IKZF3 CD80 STAT4 NFKB1 TNFSF15 POU2AF1	 rs4979462 rs4938534

Liu et al. *Nature Genetics.* 2012. [140]	**UK** 2861 cases 8514 controls	IL12RB2 STAT1, STAT4 IL12A SH2B3 Tyk2 DENND1B TMEM39A NFKB1 IRF5 DDX6 TNFRSF1A RAD51B SOCS1 IRF8 ORMDL3/IKZF3/ZPBP2 CRHR1 SYNGR1	rs72678531 rs3024921 rs7574865 rs2366643 rs62270414 rs668998 rs80014155 rs11065979 rs34536443 rs2488393 rs2293370 rs7665090 rs35188261 rs3807307 rs80065107 rs1800693 rs11064157 rs911263 rs1646019 rs12708715 rs80073729 rs11117433 rs8067378 rs17564829 rs2267407

1.3.8 Primary Sclerosing Cholangitis

Primary Sclerosing Cholangitis (PSC) is a chronic cholestatic condition characterised by destruction of the intra- and extra-hepatic biliary tree, leading to the formation of fibrous strictures and saccular dilatations. Eventually complete obstruction of the biliary ducts occurs leading to cirrhosis. It is an aggressive condition, affecting mainly men in their 20s and 30s and the majority of patients will require a liver transplant. The pathophysiology of PSC remains unknown, however it is classified as an autoimmune condition. Some patients have detectable autoantibodies, and there is an higher incidence of other autoimmune conditions [150,151]. While NK cell frequencies are increased in the peripheral blood and colonic mucosa, they are not enriched in the liver in PSC [13,152]. Furthermore NK cells in individuals with PSC are less cytotoxic [13]. Expression of TRAIL receptor 5 is more highly expressed on cholangiocytes in PSC however and may induce NK cell mediated cholangiocyte death via this mechanism instead [28]. Increased cholangiocyte expression of CXCL3 and CX_3CL1 has also been reported in PSC and may play a role in recruitment of peripheral blood NK cells expressing CXCR3 and CX_3CR1 to this site [153,154]. Lastly genetic variations in the MIC-A allele, affecting its binding affinity for NKG2D have been associated with disease susceptibility [155,156].

1.3.9 Summary

Liver disease is the only major cause of mortality increasing year on year thanks to a rise in alcohol consumption, obesity and viral hepatitis. Outside the field of HCV and HBV, therapies are strikingly limited. The alteration of NK cell function in all of these diseases, often closely linked to changes in the immune or metabolic environment in which they reside, highlights NK cells as an important target for further study. Manipulation of NK cell differentiation driven by microenvironmental changes induced by toxins, cancer and viruses may lead to the development of novel cell based therapies for a wide range of diseases.

1.4 Natural killer cell maturation

NK cells belong to the ILC family which develop from CD34+ haematopoietic stem cells in the bone marrow and foetal liver [157]. In vitro studies have revealed five stages in human NK cell development, although the transcription factor checkpoints for these are much less well defined compared to mice (**Fig 1-3** Freud, *Immunological Reviews*, 2006 [23]). KIR acquisition occurs between stages 4 and 5, also the transition from $CD56^{bright}$ to $CD56^{dim}$ [158]. The classical understanding of NK cell maturation is that they develop in the bone marrow and secondary lymphoid tissue prior to entering the circulation and may then enter and leave the liver unchanged.

While the bone marrow is certainly the predominant site of NK cell maturation, CD34+CD45+ and committed NK cell precursors have been identified in the adult human liver, albeit in frequencies of less than 1% of the lymphocyte population [21,159]. Furthermore CD34+ hepatic progenitors have been shown to be capable of giving rise to functional $CD56^{bright}$ NK cells in vitro [21]. Transplantation studies demonstrate that NK precursors appear to be recruited to the liver from the circulation and take on a liver-specific phenotype at maturation stage 3 [21]. This phenomenon was first demonstrated in 1993 following the identification of hepatic NK cells as 'low-density large granular lymphocytes' [160]. Adoptive transfer experiments demonstrated the migration of fluorescent-labelled high-density granular lymphocytes from the blood towards the liver and their transformation into the hepatic low-density fraction of the recipient rat, with morphological features consistent with hepatic resident cells.

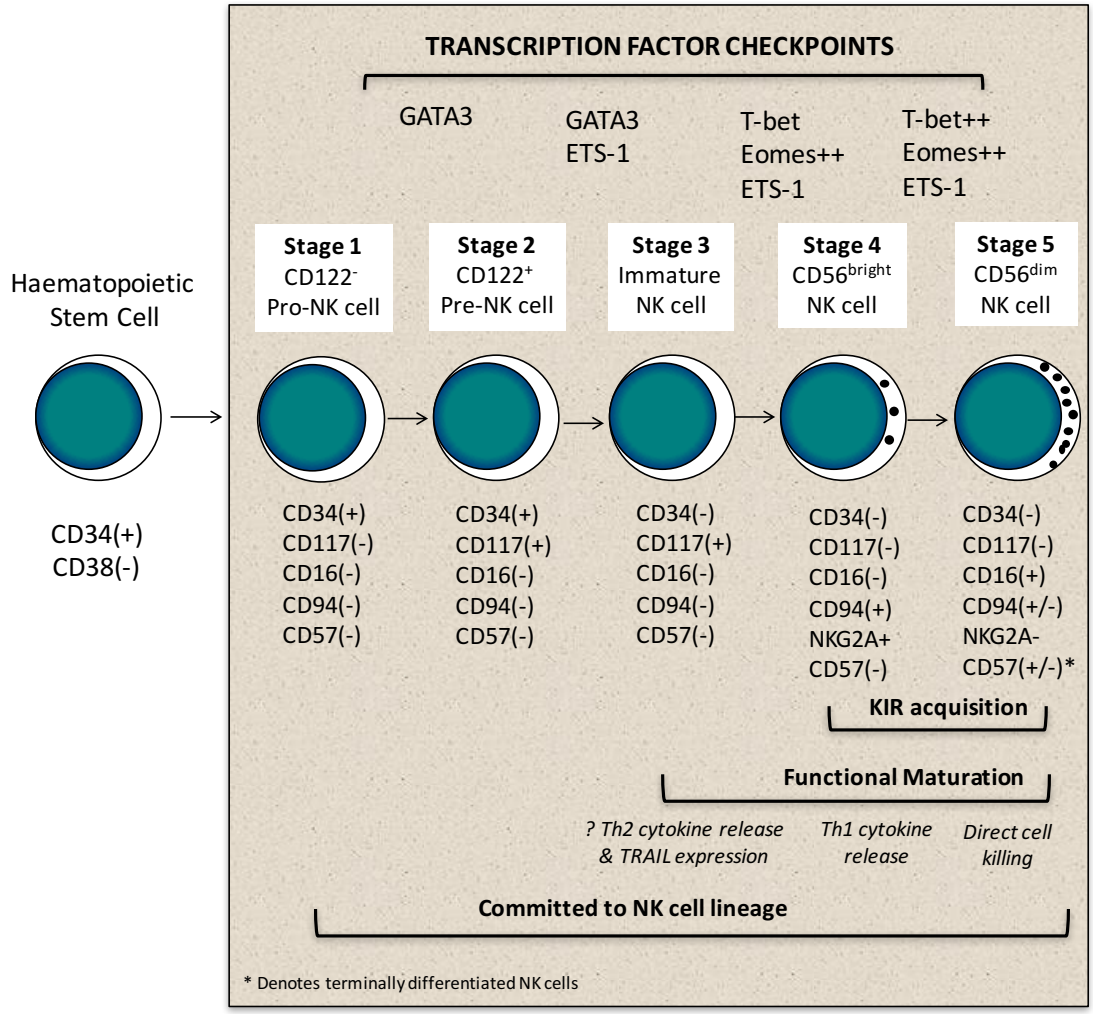
In mice NK cell maturation is dependent on transcription factors ID2, ID3, Ets-1, Nfil3 in addition to the T-box transcription factors, Eomes (encoded by EOMES) and T-bet (encoded by Tbx21) [161–164]. T-bet maintains the developmental stability of young NK cells and Eomes is essential for the sequential maturation of NK cells [163,165]. The intrahepatic microenvironment in mice is non-permissive for Eomes induction with only 50% of hepatic NK cells expressing this transcription factor, leading to the stability of $Eomes^{-}$ NK cells in the liver [19,163]. As described in the following section T-bet dependence in mice can drive hepatic NK cells down discrete pathways with novel phenotypes including adaptive-like feature [19,35].

In humans, the opposite occurs, with hepatic NK cells being predominantly $Eomes^{high}T\text{-}bet^{low}$ (CXCR6+), and circulatory NK cells $Tbet^{high}Eomes^{low}$ in the case of $CD56^{dim}$ NK cells, and $Eomes^{high}Tbet^{high}$ for $CD56^{bright}$ NK cells [11,30,33,166]. The intrahepatic NK cell population in

humans is likely to be composed of a small frequency of CD34+ stem cells and NK cell precursors, approximately 50% $CD56^{bright}Eomes^{high}T\text{-}bet^{low}$ liver-resident NK cells (Eomes dependent), and approximately 50% conventional $CD56^{dim}Tbet^{high}Eomes^{low}$ NK cells which matured in the bone marrow [11,29,33]. Liver-resident $CD56^{bright}Eomes^{high}T\text{-}bet^{low}$ NK cells have been shown to be replenished from the circulation, and through the upregulation of Eomes on $Eomes^{low}$ NK cells suggesting these subsets are part of the same lineage [33]. It is also possible that liver-resident CD34+ haematopoietic stem cells may give rise to $CD56^{bright}Eomes^{high}T\text{-}bet^{low}$ NK cells. However CXCR6+CD69+ NK cells have recently been identified in human lymphoid tissue (bone marrow, spleen, lymph nodes), with comparable phenotype and function to liver-resident $CD56^{bright}Eomes^{high}T\text{-}bet^{low}$ (CXCR6+) NK cells, and were not found to undergo early hyperexpansion in the bone marrow following haematopoietic stem cell transplantation, unlike conventional NK cells [167].

Finally the $Eomes^{high}T\text{-}bet^{low}$ transcriptional profile of the dominant liver-resident NK cell population ($CD56^{bright}$CXCR6+), raises the possibility that this may be a subset of ILC-1 lymphocytes. Cuff et al compared whole transcriptome data from NK cells and ILC-1 subsets isolated from the tonsil and liver-resident $Eomes^{high}$ NK cells and reported no significant overlap [33]. Therefore liver-resident CD3-CD56+$Eomes^{high}T\text{-}bet^{low}$ lymphocytes are thought to be bona fide NK cells.

Figure 1-3 Stages of development of a human NK cell



Adapted from Freud, *Immunological Reviews*, 2006 [23]

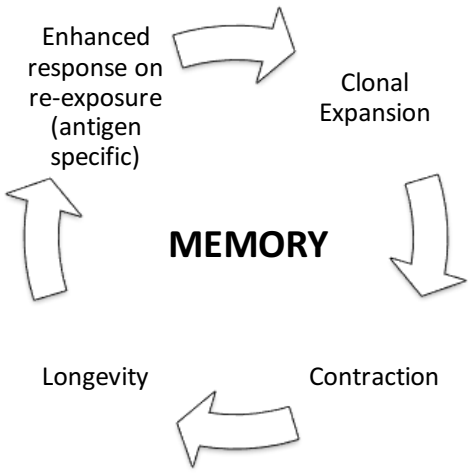
1.5 Memory and memory-like natural killer cells

1.5.1 Introduction

The innate immune system is ancient, with fundamental features being conserved from plants to mammals highlighting its necessity for survival [168]. One such feature is antigen recognition receptors, which are encoded in the germline DNA and recognise fixed pathogen associated molecular patterns (PAMPs) with no requirement for gene rearrangement. The result is a highly effective but short-lived activated cell. In addition there is a non-clonal distribution of cells with all cells of a certain class been identical. Our traditional understanding of NK cells as members of the innate immune system has been challenged however following the discovery of NK cells with memory-like features in mice, with some early work in humans. NK cells appear to be able to express a novel type of 'memory' in response to not only antigens, but also cytokines, in some instances demonstrating robust antigen specificity and in others responding to a wide range of stimuli [169].

Attributes which have led researchers to identify certain NK cells as adaptive are based on our understanding of T-cell memory, i.e. the ability of specific subsets to undergo clonal expansion and subsequent contraction, longevity and an augmented functional response on re-exposure to the original stimuli (**Fig 1-4**). Current nomenclature refers to NK cells as possessing 'memory' where an enhanced functional response is antigen specific and 'memory-like' where NK cells show other features consistent with memory but without antigen specificity. This is an exciting field as NK cells do not express recombinase activating genes (RAG) required to re-arrange gene segments in T and B lymphocytes to create an infinite number of receptors leading to antigen specificity. The mechanism by which NK cells develop memory-like features is unknown. The majority of this work has been performed in mouse models and our knowledge of memory-like NK cells in humans is limited. Despite the vast range of methodology and sensitizing agents used to demonstrate NK cell memory, an intriguing phenomenon common to nearly all studies is the residence of these populations in the liver leading to the intriguing question of whether NK cells gain memory in the antigen-rich liver and is their development induced or supported by the hepatic cytokine environment. A summary of key papers published in the field of NK cell memory are presented in **Tables 6, 7 and 8** and **Figure 1-5**.

Figure 1-4 Immunological definition of 'memory'



1.5.2 Antigen-specific memory NK cells in mice and macaques

Hapten-induced contact hypersensitivity

Some of the earliest work in this field came in 2006 when O'Leary demonstrated that RAG deficient mice, but not mice also deficient in NK cells, could mediate hapten induced contact hypersensitivity (CHS) [170]. This is a type III delayed-type hypersensitivity (DTH) reaction requiring a learnt response specific to the original sensitizing hapten thought to be mediated by adaptive lymphocytes only. Haptens are reactive chemicals that modify self-proteins causing them to be recognised as foreign antigens. The group were able to show that the T and B cell independent CHS reaction to DNFB and OXA was specific to the original sensitizing hapten, and the hapten could be recalled for up to four months. To support the hypothesis that the CHS reaction was mediated by NK cells, an adoptive transfer study was performed demonstrating that NK cells were recruited to the site of CHS in naïve mice following a hapten challenge only if from sensitised donors. NK cells with hapten-specific memory features possessed the MHC receptor, Ly49C-I, suggesting that CHS memory NK cells are licensed, and were isolated only from donor livers.

In 2010 Paust suggested that the chemokine receptor and adhesion molecule, CXCR6 may be a surface marker of NK cells with memory-like features [34]. CXCR6 interacts with CXCL16 expressed on HSECs, hepatocytes and particularly cholangiocytes [32]. Engagement with CXCL16 is essential to ensure the presence of CXCR6+ NK cells within the liver [15]. Interestingly CXCL16 is upregulated by IFNγ and hepatic CD4+, CD8+ and CD56+ T-cells express high levels of CXCR6 in

inflammatory liver disease [32]. With prior knowledge that memory-like NK with activity against haptens may reside in the liver and that CXCR6 is expressed on the majority of hepatic NK cells in rats [171], CXCR6 was selected as a target.

CXCR6+ NK cells were generally restricted to the liver in mice, with populations of <5% in the circulation and a small percentage in the spleen [34]. Adoptive transfer of sensitized CXCR6+/- NK cells revealed only primed CXCR6+ subsets could elicit a CHS reaction towards OXA and DNFB, and only those subsets from the liver. Introduction of a CXCR6 neutralizing antibody into sensitized mice 12 hours prior to hapten re-challenge blocked this response. In vitro cytotoxicity assays demonstrated that hapten specific cell killing was limited to CXCR6+ hepatic NK cells, importantly demonstrating that hapten-sensitised NK cells can mediate killing of target cells that have sufficient expression of MHC-1 and inhibitory signals can be overridden through this mechanism. Interestingly the group later described hepatic NK cell mediated CHS to be dependent on IL-12, IFNα and IFNγ, perhaps through their ability to drive activation and proliferation of this subset [172]. This work provides evidence that an inflammatory cytokine environment is required to support the effector function of memory NK cells. IFNγ production by memory NK cells themselves however was found to be regulated by the interaction of CXCR6 and CXCL16 [172].

In 2013 Peng et al sought to identify other surface markers of NK cells capable of mediating hapten-induced memory [19]. They phenotyped NK cells from a variety of organs in mice and identified that unique to the liver, only 50% of CD161+ NK cells expressed CD49b (DX5) (Very Late Antigen (VLA) α2 chain). Selecting this as a negative marker, the group demonstrated that hepatic CD49b- NK cells could confer CHS by sensitising RAG-1/-1 mice with OXA and transferring CD49b+/- NK cells to wild type recipients. Immunised mice were re-challenged a month later and only recipients of hepatic CD49b- cells mounted a CHS reaction. The group identified the liver as the site of hapten priming by demonstrating migration of labelled haptens exclusively to this organ. Syngeneic bone marrow transplant work and adoptive transfer studies using bone marrow and liver MNCs demonstrated that CD49b- NK cells originated from hepatic stem cells and were dependent on T-bet rather than Eomes for development. Furthermore CD49b- NK cells which trafficked to the liver remained in situ and in a steady state, but were capable of migrating to local sites of inflammation. Surprisingly CD49b- NK were non-terminally differentiated with low expression of Ly49 in addition to low CD107a and IFNγ. Gene microarray analysis also revealed upregulation of CD49a (integrin α1 or VLA α1 chain) in this group. CD49a heterodimerises with β1 integrin (CD29) to form the α1β1 integrin duplex and is a receptor for collagen and laminin [173]. Through this interaction, CD49a plays a role in cell adhesion, leukocyte migration and T cell proliferation and may maintain CD49a+

NK cells in the liver. Recipients of sensitised $CD49a^{+}CD49b^{-}$ NK cells displayed a vigorous CHS response following hapten re-challenge supporting this phenotype as a robust maker of NK cells capable of hapten mediated CHS. Whether this subset has the potential to developed memory towards viral antigens is unknown.

NK cell memory against viral antigens

In 2009 Sun used a murine cytomegalovirus (mCMV) model to demonstrate the ability of Ly49H+ NK cells to exert adaptive behaviour [174]. Following mCMV exposure, Ly49H+ NK cells underwent expansion up to 1000-fold, contracted, then persisted for 70 days in lymphoid and non-lymphoid tissue including the liver. On re-exposure Ly49H+ NK cells showed more rapid degranulation and enhanced cytokine production. Adoptive transfer into naive mice followed by re-challenge demonstrated phenotypic and functional stability in addition to antigen-specificity, with later studies showing that mCMV memory NK cells fail to show enhanced function or proliferation following infection with influenzae or listeria [175]. It is important to consider however that these novel features are a result of the known interaction between the pathogen specific NK receptor, Ly49H, and mCMV encoded MHC-1 homolog, m157. Ly49H+ NK cells are not activated by mutant mCMV lacking m157 [175]. The group went onto provide mechanistic insights into their findings, demonstrating that memory features were lost in IL-12 and STAT4 deficient mice [176] and were also dependent on the co-stimulatory molecule DNAM-1 [177]. The group later described the importance of mitophagy in maintaining the memory-like NK cell pool [178]. During proliferation NK cells accumulate polarised dysfunctional mitochondria leading to a build up of reactive oxygen species (ROS). During contraction a protective mitophagy pathway returned ROS levels to baseline and reduced the mitochondrial mass promoting long-term survival of remaining effector cells. Activation of adenosine monophosphate-activated protein kinase (AMPK) or inhibition of mechanistic target of rapamycin (mTOR) during the contraction-to-memory phase also led to increased autophagic activity and enhanced memory NK numbers through an Atg3-dependent mechanism [178].

In addition to exploring the role of hepatic CXCR6+ NK cells in mediating hapten specific CHS, Paust et al also addressed the potential of these cells to develop a DTH reaction towards viruses [34]. Adoptive transfer of splenic and hepatic NK cells from RAG deficient mice exposed to virus-like particles from influenza or irradiated Vesicular Stomatitis Virus (VSV) revealed hepatic NK cells could mediate a vigorous DTH response when re-challenged with the original antigen. Findings were replicated with HIV-1 despite mice being HIV resistant and not under evolutionary pressure from this disease. RAG deficient mice were also protected from median lethal doses of VSV

following immunization with irradiated VSV. Mice sensitized to influenza and VSV could no longer mount a DTH response however following anti-CXCR6 administration. The vaccination potential of UV irradiated VSV was also lost in this setting highlighting a potential mechanistic role for CXCR6 in mediating NK memory.

Similar adoptive transfer methods were employed by a separate group shortly after, demonstrating robust antigen-specific NK cell memory against the vaccinia virus for which no cognate germline-encoded receptor has been identified [179]. This was mediated by hepatic Thy1(+) NK cells leading to viral clearance and protection against lethal doses of vaccinia using Thy1(+) NK cells primed with live or attenuated virus [179].

In 2015, the first evidence of antigen-specific NK cell memory in primates was published [180]. Splenic and hepatic NK cells from rhesus macaques infected with S-HIV$_{SF162P3}$ and simian immunodeficiency virus (SIV)$_{mac251}$ demonstrated significant activity against Gag antigen pulsed dendritic cells, but not towards those which were untreated or treated with another peptide [180]. Remarkably NK cells isolated from macaques 5 years post vaccination using adenovirus vectors expressing SIV$_{mac239}$ and HIV-1 Env could display robust antigen-specific killing of dendritic cells pulsed with matched, but not mismatched peptides [180]. These results demonstrate that antigen specific memory following both infection and vaccination, is capable of being preserved without ongoing antigen stimulation [180]. In common with murine studies only a marginal response was seen in the case of NK cells isolated from the peripheral blood suggesting NK cells with antigen memory are tissue-resident, although perhaps not liver specific as suggested in previous studies [180].

Identification of CD49a+ and CXCR6+ NK cells in the human liver

Since starting this project, both CD49a+DX5- [35] and CXCR6+ [11,15,29,33] NK cells have been identified in the human liver. Both were largely absent from the peripheral blood. A summary of the main findings from each paper are presented in **Table 8**. The Karolinska Institute identified CD49a+ NK cells in 12 out of 29 individuals undergoing resection for cancer, or from donor livers not used for transplantation. The highest frequency reported was 12.7% of the total hepatic NK cell population. Their transcriptional profile was T-bet+Eomes-, as seen in mice [19]; more closely resembling conventional CD16+ NK cells, than the liver-resident CD16-Eomes^{high}T-bet^{low} subset described above [11,33]. Intrahepatic CD49a+ NK cells displayed phenotypic and functional

features consistent with potential adaptive-like properties. Specifically, they showed upregulation of activating receptors NKp30, NKp46 and NKGD along with the adhesion molecule DNAM-1, and downregulation of inhibitory NKG2A. The majority of CD49a+ NK cells were positive for NKG2C and KIR. Furthermore, high resolution KIR phenotyping revealed that KIR expression is not only oligoclonal, with a single dominating inhibitory KIR profile in some cases, but is also biased towards self-HLA [35]. These findings suggest CD49a+ NK cells are activated and may represent a subset of licensed NK cells which have undergone clonal expansion. CD49a+ NK cells were also found to produce high quantities of IFNγ, TNFα and GM-CSF, and demonstrated enhance proliferative capabilities in culture. In addition, they maintain a stable phenotype. Interestingly despite upregulation of NKG2C, expansion was not enhanced by the presence of HLA-E transfected targets. CD49a+ NK cells were not able to produce high levels of perforin or granzyme A however and degranulated poorly in response to stimulation with PMA and inonomycin. It is therefore not clear if these lymphocytes would be able to lyse target cells presenting antigens against which they had developed sensitivity. Finally, as described below, clonal expansion of NKG2C+ NK cells has been observed in the peripheral blood of people following infections with certain viruses, in particular hCMV (**Table 7**). No correlation was observed between the frequency of CD49a+ NK cells and hCMV serological status however, excluding hCMV as a trigger for the development of this unique subset. Overall the group concluded that CD49a+ NK cells are the likely human counterpart of murine CD49a+ NK cells with adaptive-like features.

A number of groups have recently described CXCR6+ NK cells in the human liver (**Table 8**) [11,29,33,181]. There is now consensus that this chemokine receptor is a marker of liver-resident NK cells. As described above approximately half of NK cell found in the human liver are $CD56^{bright}$CD16-$Eomes^{high}$T-bet^{low}CXCR6+. NK cells with this phenotype highly express other markers of adhesion and tissue-residency. Furthermore they have been shown to remain in the liver for over ten years, rather than re-circulating. Unlike CD49a+ NK cells, CXCR6+ NK cells do not highly express KIR or produce high quantities of pro-inflammatory cytokines, although there is a lack of consensus regarding their ability to degranulate and produce cytolytic proteins (**Table 8**). Therefore in people, CXCR6 (expressed on $Eomes^{high}$T-bet^{low} NK cells) appears to be a surface marker of immunotolerant liver-resident NK cells present in significant frequencies throughout the population, and not a marker of NK cell memory.

1.5.3 Cytokine-Induced memory-like NK cells

Comparative studies in mice and humans have demonstrated that cytokines can lead to increased clonal NK cell proliferation and longevity in addition to significantly enhanced function on secondary exposure to further cytokines [182,183]. Cooper et al exposed splenic NK cells from RAG deficient mice to IL-12, IL-18 and low dose IL-15 prior to Carboxyfluorescein Succinimidyl Ester (CFSE) labelling and transfer into a naive recipient [182]. Donor derived NK cells demonstrated enhanced proliferation and longevity, but had a similar cytokine profile to naïve host NK cells. On re-stimulation three weeks later with IL-12 and IL-18, pre-activated NK cells from were able to mount an amplified Th1 cytokine response compared to non-pre-activated controls. Their enhanced capacity to release IFNγ persisted for up to three weeks. Interestingly the group were able to identify daughter generations of pre-activated NK cells and demonstrate that enhanced functional properties could be passed on to NK cell progeny and were not a result transcriptional changes induced by proliferation.

These findings were later reproduced in humans [183]. Purified human NK cells were exposed to IL-12, IL-15 and IL-18 followed by prolonged culture in low dose IL-15. After 7,14 and 21 days cells were harvested and re-stimulated with IL-12 and IL-15 or K562 target cells. NK cells re-stimulation with cytokines and target cells displayed enhanced IFNγ release and proliferative capacity. In common with cytokine pre-activated NK cells in mice, degranulation was not enhanced and again adaptive features could be passed on to their progeny. Adaptive features were associated with expression of NKG2C in common with viral-driven NK cell expansion, in addition to CD94, CD69, NKp46 and NKG2A, but not KIR or CD57. Attempts to establish the mechanism driving this phenotype revealed no alteration in IL-12 or IL-18 receptor expression, in addition to no difference in STAT4 phosphorylation. Furthermore IFNγ mRNA was not more abundant in NK cells following re-stimulation in the same individual suggesting a post-transcriptional or translational mechanism behind the observed enhanced cytokine release, i.e. this phenotype may be a result of changes at an epigenetic level. Indeed epigenetic modifications may be responsible for the maintenance of memory-like responses in NK cells.

A more comprehensive analysis of triggers required to induce this functional phenotype revealed that no one cytokine was essential, but a combination of cytokines was required, for example IL-15 and IL-18, IL-15 and IL-12 in addition to IL-12 and IL-18 would be sufficient. The same group later identified that pre-activation of NK cells with IL-12, IL-15 and IL-18 leads to upregulation of the high affinity IL-2 Rαβγ, resulting in IL-2 hyper-responsiveness [184]. Culture with picomolar concentrations of IL-2 subsequently led to enhanced proliferation, cytotoxicity and IFNγ release.

The discovery of cytokine-induced memory-like NK cells has resulted in a huge leap forward for NK cell immunotherapy due to their ability to address a number of factors which previously hampered work in this field. Specifically, cytokine-induced memory-like NK cells demonstrate enhanced anti-tumour activity in vivo, provide sustained NK cell effector function, and expand in response to cytokines providing sufficient effector cell numbers. Most significantly Romee et al recently demonstrated that allogenic cytokine-induced memory-like NK cells can proliferate, expand and exhibit anti-leukaemic function after adoptive transfer into patients with relapsed or refractory acute myeloid leukaemia [185]. This formed the first in-human phase 1 trial of adoptively transferred cytokine-induced memory-like NK cells.

1.5.4 Expansion of NKG2C+ NK cells in the peripheral blood post viral infection

Much less is known about memory-like NK cells in humans and work in this field has taken a different path to murine studies, perhaps a result of different investigative techniques (**Table 7**). The expansion of long-lived NKG2C+ NK cell populations in the peripheral blood of patients post viral infection including CMV, HBV, HCV, HIV, Chikungunya and Hantavirus is a common feature of all early studies [79,98,186–190]. The phenotype of this expanded population has been remarkably consistent with high expression of markers of terminal differentiation CD16, CD57 and KIR [188–190]. Interestingly expanded NKG2C+ NK subsets found following infection with CMV and hantavirus demonstrate oligoclonal KIR expansion with skewing of the KIR repertoire towards self-specific KIR in the case of CMV [189,190]. This supports the premise that these cells have undergone clonal expansion and are predominantly licensed. In common with their $CD56^{dim}$ phenotype, NKG2C+ cells demonstrate high levels of perforin and display vigorous ADCC [190]. While this subset are generally hyperfunctional, activation can be triggered using numerous stimuli including cytokines or HLA-E transfected targets [189,190]. Furthermore evidence suggests that NK cells can be primed by CMV are ready for efficient expansion on encountering a second virus [187,189]. Therefore unlike liver-resident memory NK cells found in mice, expanded NKG2C+ NK cells do not appear to demonstrate antigen specificity. Full characterisation of the phenotype and function, including any adaptive features of NKG2C+ NK cells in the human liver is therefore worthwhile.

NKG2C is an activating receptor, forming a lectin-like heterodimer with CD94 to bind the non-classical HLA-E ligand in humans [191]. A small number of studies have proposed a mechanistic relationship between NKG2C and the reconfiguration of the NK cell population post viral infection.

Deletion of the KLRC2 gene which encodes NKG2C leads to increased susceptibility to HIV, higher viral titres and rapid disease progression [192]. Furthemore upregulation of HLA-E occurs in HIV [193], hCMV [194] and hantavirus [189] perhaps leading to the consistent finding of NKG2C+ NK cell expansion between viruses. A further group suggest NKG2C copy number dictates surface levels of NKG2C in hCMV seropositive patients independent of HLA-E dimorphism, and that NKG2C engagement between NK cells from NKG2C+/+ and hemizygous individuals prompt differences in calcium influx, proliferation and degranulation [195]. There is currently no strong evidence however to support a functional role for NKG2C in driving the proliferation and longevity of certain NK subsets following viral infection and this receptor may act as a surrogate marker only. Béziat et al recently demonstrated that 'HLA-specific KIR expanded NKG2C-' NK cells adopted the same functional phenotype, clonal expansion and stability post CMV as their NKG2C+ counterparts [190]. Interestingly the altered KIR repertoire remained stable for years, unlike for example the contraction seen following Chikungunya virus [188]. Furthermore in vitro generation of these cells following stimulation with HLA-E transfected 221 cells and IL-15 revealed a less mature CD57- population [190]. This work suggests the stability of this phenotype may be the result of stem-cell renewal.

A novel subset of NK cells seen to undergo expansion in hCMV seropositive patients was recently discovered by the Kim group and is characterised by the absence of FcRγ (g- NK cells) [196,197]. These cells were strongly NKG2C and KIR positive, suggesting the latter markers may simply represent a pre-activated state. The key characteristic of g- NK cells is their vigorous antibody-dependent functional response towards hCMV infected cells, a feature shared with both NKG2C+/- populations [197]. The group recently extended this work, identifying g-NK cells to be deficient in multiple transcription factors including SYK, suggesting that epigenetic modifications of the SYK promoter may be responsible for inducing a stably maintained pool of antigen experienced NK cells, continuously re-shaped following repeat exposure to a related antigen in the presence of autologous immunoglobulins [198]. Their reliance on viral specific antibodies and signalling via CD16 crosslinking appears to be a feature shared with NKG2C+ NK cells, with multiple studies failing to show enhanced activity of NKG2C+ NK cells towards hCMV infected targets [197,199], unless in the presence of hCMV specific antibodies [200]. In common with NKG2C+ NK cells, g- cells display broad antiviral activity, demonstrating enhanced functional capabilities against herpes simplex virus (HSV)-1 infected target cells [197] and undergoing expansion following infection with influenza [198].

CMV status can therefore be a significant confounder in studies which use human NK cells, leading to clonal expansion of an activated, mature NKG2C+ population with high expression of self-HLA

KIR molecules. hCMV is a beta-herpes virus with a reported prevalence of 55-100%. It is a lifelong infection and therefore positive serological status is more common with increasing age. While it can cause severe disease manifestations in neonates and patients who are immunocompromised, individuals are generally asymptomatic and therefore examination of previous exposure using CMV immunoglobulins or CMV PCR is required. Stratification of patients according to CMV status is therefore highly relevant to all studies examining NK cell phenotype and function.

1.5.5 Summary

Three distinct groups of memory and memory-like NK cells have been described: (1) murine hepatic resident NK cells demonstrating antigen specificity towards haptens and viruses, (2) clonally expanded NKG2C+/KIR+ NK cells found in the peripheral blood of humans post viral infection and (3) cytokine-primed NK cells which can demonstrate longevity, enhanced proliferation and amplified IFNγ release on re-stimulation (**Fig 1-5**). At the time this project was commenced, the existence of CD49a+ and CXCR6+ NK cells in the human liver was unknown and it is extremely exciting to consider whether they may demonstrate antigen-specific memory and how they are influenced by cytokines, particularly the 'adaptive-phenotype inducing cocktail' of IL-12, IL-15, IL-18 and IL-2. Adaptive capabilities of hepatic NKG2C+ NK cells have also not been fully explored in the human liver. Furthermore, cytokine-induced memory-like NK cells provide an interesting layer of complexity when analysing the influence of the hepatic cytokine environment on the induction of hyperfunctional NK cells, and an exploration of whether cytokines can induce liver-resident NK cells to display adaptive qualities would be worthwhile. NK cell 'memory' can therefore be targeted in some instances and diverse in others, opening up wide therapeutic possibilities including priming memory-like NK cells to respond to virus or cancer specific peptides, but also using therapeutically available cytokines to boost NK cell function on repeated stimulation.

Table 6 Summary of studies demonstrating memory-like features of NK cells in mice

MURINE STUDIES						
Surface marker	**Study Summary**	**Stimulus**	**Memory-like features**	**Hepatic involvement**	**Receptor expression**	**Ref.**
Viral Antigens						
Ly49H+ NK cells	Adoptive transfer of Ly49H+ NK cells sensitised to mCMV into Ly49H+ deficient mice. Memory features blocked in IL-12 receptor or STAT4 deficient mice.	mCMV (m157-Ly49H interaction)	Enhanced capacity to undergo expansion following mCMV. Longevity (> 2 months). Enhanced degranulation & IFNγ release on restimulation specific to mCMV. Stable phenotype on adoptive transfer	Ly49H+ NK cells underwent 1000 fold expansion in liver (100 fold in spleen). Ly49H+ NK cells persist in the liver, peripheral blood & lymph nodes.	Mature: ↑ KLRG1, CD43, Ly6C ↓ CD27	Sun 2009 [174] Sun 2012 [176]
CXCR6+NK cells	Adoptive transfer of splenic & hepatic NK cells from RAG-/- mice exposed to virus-like particles & lethal doses of VSV & influenza. Blockade of protective effects using anti-CXCR6.	Virus-like particles from influenza, VSV, HIV-1	Sensitised CXCR6+ NK cells can mediate a DTH response to virus-like particles in an antigen specific manner & provide an effective vaccination from median lethal doses of VSV	Only hepatic NK cells could mediate a vigorous DTH response when re-challenged with virus-like particles	Not defined	Paust 2010 [201]
Haptens						
Ly49C-I+ NK cells	RAG-/- mice can mediate CHS. Adoptive transfer of NK cells from sensitized donors were actively recruited to the site of CHS in naïve mice following hapten challenge	OXA, DNFB	Longevity Hapten specificity	Only NK cells isolated from the donor liver of sensitized mice could induce CHS	Mature: Ly49C-I+	O'Leary 2006 [170]
CXCR6+ NK cells	Adoptive transfer of sensitized CXCR6+/- NK cells followed by hapten challenge & inhibition of CXR6 using neutralizing antibody. In vitro cytotoxicity assays.	OXA, DNFB	Hapten-specific CHS in vivo & hapten-specific cell killing in vitro	Hapten-specific CHS restricted to hepatic CXCR6+ NK cells	Not defined	Paust 2010 [201]
CD49a+ CD49b- NK cells	Adoptive transfer of sensitized CD49b- NK cells followed by hapten re-challenge. Hapten labelling. Syngeneic bone marrow transplantation & adoptive transfer studies. Gene microarray. Phenotyping & functional assays.	OXA	CHS response CD49a+CD49b- NK cells remain in a steady state in the liver	Only CD49a+ CD49b- hepatic NK cells could confer CHS to haptens. The liver was identified as the site of hapten priming. CD49b- NK cells originated from hepatic stem cells.	↓ Ly49 ↑ NKG2C ↓ CD107a ↓ IFNγ	Peng 2013 [19]
Cytokines						
	Splenic NK cells from RAG-/- mice were stimulated with IL-12, IL-15, IL-18 prior to transfer into a naive recipient.	IL-12, IL-15, IL-18	Cytokine exposed NK cells from recipient mice displayed greater IFNγ release on re-stimulation & enhanced proliferation. Memory-like features were transferred to NK cell progeny.	Nil known	Phenotypically similar to naïve NK cells	Cooper 2009 [182]

Table 7 Summary of studies demonstrating memory-like features of NK cells in humans

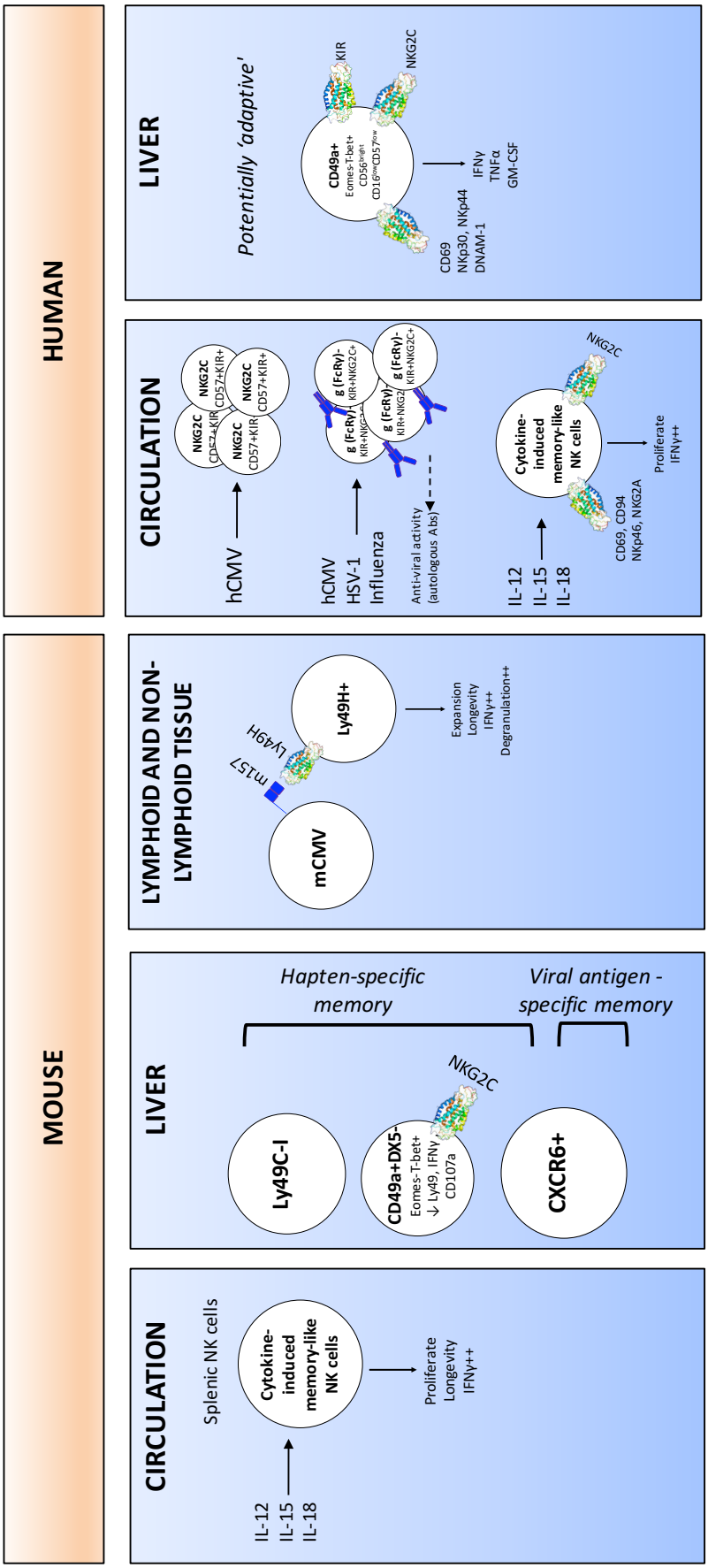
HUMAN STUDIES				
Surface marker	**Stimulus**	**Memory-like features**	**Receptor expression**	**Ref.**
Viral Antigens				
NKG2C+ NK cells	hCMV	CMV infection, but not EBV/HSV, associated with ↑ NKG2C+ NK cells in adults / children / post congenital infection. NKG2C gene copy number may influence NKG2C+ NK cell frequency. NKG2C+ NK cells expand in transplant patients experiencing hCMV reactivation & persist long term, even after clearance of active infection	↓ NKp30, NKp46 ↑ KIR, CD85j, CD57	Guma 2004 [186] Monsiváis-Urenda 2010 [202] Noyola 2012 [203] Muntasell 2013 [195] Lopez-Vergès 2011 [204] Foley 2012 [205]
NKG2C+ NK cells	HIV (? primed by hCMV)	Expansion NKG2C+ NK cells in HIV-1+ patients. Conflicting findings re: whether this disappears on consideration CMV serological status. ↑ NKG2C:NKG2A+ ratio in hCMV patients who develop HIV-1 Deletion KLRC2 gene (encodes NKG2C) → ↑ risk contracting HIV, ↑ viral titres & rapid disease progression	Not defined	Guma 2006 [187] Mela 2007 [206] Brunetta 2010 [207] Thomas 2012 [192]
NKG2C+ NK cells	HBV (? primed by hCMV)	Expansion NKG2C+ NK cells in peripheral blood in HBV. Expansion NKG2C+ NK cells in peripheral blood of patients with HBV who were hCMV positive.	Not defined	Oliviero 2009 [98] Béziat, 2012 [208]
NKG2C+ NK cells	Chikungunya virus	Transient clonal expansion NKG2C+KIR+ NK cells after acute infection with Chikungunya virus followed by contraction	CD57+ KIR+	Petitdemange 2011 [188]
NKG2C+ NK cells	HCV (? primed by hCMV)	Expansion of NKG2C+ NK cells in peripheral blood in HCV. Expansion of NKG2C+ NK cells in peripheral blood of patients with HCV who were hCMV positive. Expanded cells show enhanced cytotoxicity & IFNγ/TNFα release on stimulation with Ig coated / HLA-E expressing cells (dampened by KIR engagement)	Clonal expansion of inhibitory KIR specific for self HLA-I	Ahlenstiel 2010 [79] Béziat 2012 [208]
NKG2C+ NK cells	Hantavirus (? primed by CMV)	4x ↑ in NKG2C+ NK cells post infection - persist for > 2 months. NKG2C^{+} NK cells uniformly express a single inhibitory HLA-C binding KIR supporting recent clonal expansion. NKG2C+ cells exposed to hantavirus showed ↑ cytotoxicity & Th1 cytokine release on stimulation with HLA-E+ target. ↓ numbers of NKG2C+ NK cells in CMV IgG-ve patients with lack of expansion.	CD56^{dim} ↑ KIR, CD57 NKG2A negative	Björkstrom 2011 [189]
NKG2C+ NK cells	EBV	hCMV seropositive children co-infected with EBV had a higher % of NKG2C+ NK cells.		Saghafian -Hedengren 2013 [209]
KIR+ NK cells	hCMV	Analysis of alterations in the KIR repertoire in > 200 donors following viral infection revealed skewing of inhibitory KIR repertoire towards self-specific HLA-C1 post CMV infection (not EBV/HSV) suggesting clonal expansion of licensed cells. Both NKG2C+/- KIR+ NK cells showed poor IFNγ expression, but a strong ADCC response. Expanded subsets maintained stable cell numbers & phenotype even in absence of NKG2C. hCMV drives the expansion of CD56^{dim} KIR+NKG2A- NK cells, even in absence of NKG2C. The expanded subset expressed activating KIR that could trigger NK cytotoxicity & IFNγ release.	Strong expression activating KIRs (KIR2DS2/4, KIR3DS1) NKG2C++ CD56^{dim} NKG2A negative	Béziat 2013 [190] Della Chiesa 2014 [210]
FcRγ- NK cells (g-NK cells)	hCMV	g- NK cells are expanded in hCMV seropositive individuals & have greatly enhanced functional capacity compared to conventional NK cells against hCMV & HSV in the presence of virus specific Igs. Long term stability.	↑ KIR, NKG2C ↓ NKp30/46	Hwang 2012 [196] Zhang 2013 [197] Lee 2015 [198]
Cytokines				
NKG2A/C+ NK cells	IL-12, IL-15, IL-18 in any combination	Purified human NK cells were exposed to IL-12, IL-15 & IL-18 followed by culture in low dose IL-15. After 7,14, 21 days cells were harvested & re-stimulated with cytokines/K562 cells. NK cells re-stimulated with cytokines had enhanced IFNγ release & proliferative capacity & passed features onto daughter cells.	Adaptive features associated with NKG2A/C but not KIR/CD57	Romee, 2012 [183]

Table 8 Summary of studies identifying murine liver-resident memory-like NK cell equivalents in the human liver

Surface marker	Tissue	Phenotype	Function	Ref.
CD49a+ NK cells				
CD49a+ NK cells	Liver (12/29 individuals) Not in afferent (portal vein) or efferent (hepatic vein) blood Low frequencies in foetal livers	CD56^{bright}, CD16-, CD57- CD7+NKp30+NKp44+CD122^{low}NKG2A-NKG2D+CD69+DNAM-1+ > 80% KIR+ (oligoclonal expression pattern) Majority NKG2C+ T-bet+Eomes- RORγt, HELIOS, CD127 negative (not non-NK CD3-CD56+ ILC1)	High IFNγ, TNFα, GM-CSF (stimulation with PMA/I) Perforin^{low}, Granzyme A^{low}, Granzyme B^{high} Poor degranulation (stimulation with PMA/I) CD49a+ NK cells expand ~ 800-fold in vitro cultures over 3 weeks & maintain phenotype (irradiated PBMCs as feeder cells, IL-15 – no differences with HLA-E transfected or untransfected 721.221) CD49a- NK cells also expanded to a similar degree - upregulated CD49a, T-bet, perforin, CD16	Marquardt 2015 [35]
CXCR6+ NK cells				
CD56^{bright} CD16- (CXCR6+) NK cells	Liver: 50% CD56^{bright} (localise in hepatic sinusoids)	Liver-resident CD56^{bright} NK cells – CD69+, CCR5+, CXCR6+. Also CD161+, l-selectin-, CD49e-, CD11c- DNAM-1-, CCR3-, CXCR2-, CX_3CR1-, TRAIL-. Liver-resident CD56^{dim} 'conventional' NK cells – CD69-CXCR6-CCR5-, express selected integrins, selectins, CX_3CR1	Liver-resident CD56^{bright} NK cells released similar levels of IFNγ to peripheral CD56^{bright} NK cells following IL-2/IL-12 stimulation. Chemotaxis assays show liver-resident CD56^{bright} CCR5+CXCR6+ NK cells home towards CCL3, CCL5 (expressed by kupffer cells, NK cells & T-cells) & CXCL16	Hudspeth 2015 [15]
CXCR6+ NK cells	Liver: just < 60% CXCR6+ Same frequency in liver tissue & perfusion liquid (paired samples)	Same frequency of CXCR6+ NK cells in healthy controls & chronic hepatitis B CXCR6+ NK cells: CD56^{bright}CD16^{low}CD57-CD69+ Not enriched in HLA-DR+ NK cells **Hepatic NK cells:** (1) T-bet^{high}Eomes^{low} – CXCR6- (2) T-bet^{low}Eomes^{high} (5-88%, mean 48%) – CXCR6+, barely detectable in circulation Same for healthy controls & HBV livers / blood CXCR6, not CD49a, correlates with TF profile **Peripheral blood NK cells:** T-bet^{high}Eomes^{low} (even peripheral CXCR6+ NK cells)	**CXCR6+ vs CXCR6- NK cells**: Produce less IFNγ in response to IL-12/IL-18 (similar with PMA/I) Produce less TNFα/MIP-1β with PMA/I GM-CSF production similar Degranulate better (CD107a), but produce low quantities OF granzyme B & perforin ex vivo TRAIL high (only HBV infected livers examined)	Stegmann 2016 [29]
Eomes^{high}T-bet^{low} CD56^{bright} NK cells	Liver: Eomes^{high}T-bet^{low} (47.2% perfusate, 61.7% parenchyma, 1.8% peripheral blood) Eomes negative subset not seen	**Liver-resident Eomes^{high}T-bet^{low}** Nearly 90% CD56^{bright} **Liver-resident CD56^{bright} NK cells** 68.3% CXCR6+ (3.1% in the blood) 97.5% NKG2D+ (92.6% in the blood) 8.6% NKG2C+ (19.1% in the blood) 13.2% NKG2A+ (5.1% in the blood) 93.1% NKp46+ (87.7% in the blood) 8.9% NKp44+ (absent in the blood) **Liver-resident CD56^{dim} NK cells** 1.3% CXCR6+ (0.6% in the blood)	**Liver-resident CD56^{bright} NK cells** 60% granzyme B (>75% blood) 92% perforin (63.7% blood) 28.6% CD107a (9.3% blood) (Target K562 cells) 53.2% CD107a (Target K562 cells + IL-2) Same for CD56^{bright} Eomes^{high}T-bet^{low} / Eomes^{high}T-bet^{low} 21.4% IFNγ (68.6% blood) (IL-2 + Target K562 cells) Lower for CD56^{bright} Eomes^{high}T-bet^{low} vs. Eomes^{high}T-bet^{low} **Liver-resident CD56^{dim} NK cells** 24.1% IFNγ (32.3% blood)	Harmon 2016 [11]

Eomes^{high} NK cells	Liver	**Eomes^{high} (CXCR6+) vs Eomes^{low} (CXCR6-CD16+) NK cells (RNA-seq & protein)** Most altered canonical pathways associated with exiting the circulation & tissue retention High CXCR6 & CCR5, lower CX_3CR1 Higher CD69, lower S1PR1 Higher ITGA1 (CD49a) & ITGAE (CD103) at transcript, but not protein level Lower KIR, higher CD94 Examination NK cells from donor livers before & after HLA-mismatched liver transplant (LT), revealed only Eomes^{low} NK cells exit the liver Examination livers from patients who had previously received HLA-mismatched LT now undergoing 2nd LT & post-transplant biopsies revealed Eomes^{high} NK cells resided in liver for at least 13 years; Eomes^{low} NK cells could not be detected after 3 years. Examination of early post LT biopsies revealed Eomes^{low} NK cells only are rapidly recruited from the circulation. By day 8 new subsets of both Eomes^{low} & Eomes^{high} NK cells are seen. Culture liver-resident Eomes^{low} NK cells (IL-15, IL-15+TGF-β, IL-15+IL-12) → Eomes^{high}T-bet^{low} NK cells Culture peripheral blood Eomes^{low} NK cells (IL-15+TGF-β) → Eomes^{high}T-bet^{low} NK cells (↑ CCR5, CD49a, CD103; ↓ CX3CR1, S1PR1; no change CXCR6) Eomes-transduced NKL cells expressed higher levels CD49a & CD103, no change S1PR1	**Eomes^{high} vs Eomes^{low} NK cells** *K562 co-culture/IL-12/IL-12+IL-18/IL-1β+IL-23:* Eomes^{high} degranulate better, but have reduced cytotoxic capacity against K562 target cells Similar IFNγ, GM-CSF Lower TNFα Eomes^{high} NK cells Lower perforin & granzyme B, higher granzyme K Low production LIF & IL-22 by both cells	Cuff [33]

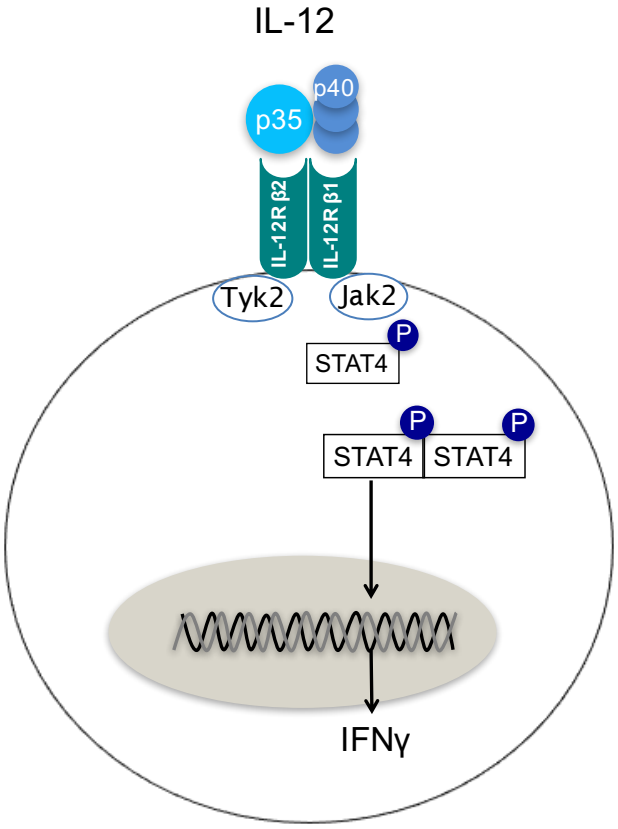
Figure 1-5 Summary of memory and memory-like NK cells identified in mice and humans



1.6 The role of STAT4 in NK cell differentiation and function

STAT4 is expressed in T-cells, activated blood monocytes, macrophages, dendritic cells and NK cells [211] . It is well known for its role in driving the differentiation of Th1 and Th17 T cells [90], monocyte activation and IFNγ production [91]. IL-12 is composed of two subunits, p35 (encoded by the IL-12A gene) and p45 (encoded by the IL-12B gene). Following IL-12 binding the two parts of the IL-12 receptor, IL-12Rβ1 and IL-12Rβ2, heterodimerise and activate janus kinase (JAK) 2 and Tyk2 receptor-associated JAK kinases. These tyrosine kinases phosphorylate STAT4 which homodimerises via its SH2 domain and translocates to the nucleus to activate gene transcription for cytokines including IFNγ and TNFα (**Fig 1-6**). Of the two IL-12 receptor subunits, IL-12RB1 is constitutively expressed, whereas IL-12RB2 is upregulated by IFNγ, therefore creating a positive feedback loop. In addition to the well-known IL-12-STAT4-IFNγ pathway, the STAT4 transcription factor also binds the IL-12 sensitive enhancer on PRF1, which in turn binds to myeloid ELF-1 like factor (MEF), which is essential for perforin production. In this way STAT4 may influence NK cytotoxicity although the exact mechanism is less well established.

Figure 1-6 IL-12 – STAT4 signalling pathway



While IL-12 is the most potent stimulus for STAT4 phosphorylation, Th1 cytokines (IL-2 and IFNα), in addition to IL-23 have also been shown to induce STAT4 activation [214,215]. IL-23p19 heterodimerises with IL-12p40 to form IL-23, a key component of the Th17 signalling pathway; IL-23 mediated induction of Th17 lymphocytes can however be inhibited by IFNγ.

Our understanding of the role of STAT4 in NK cells is supported by a knockout mouse model demonstrating reduced IFNγ production, decreased cytotoxicity and reduced IL-12 driven proliferation [92] (**Table 9**). Furthermore STAT4 deficiency blocks memory-like behaviour displayed by Ly49H+ NK cells against mCMV [176]. The IL-12R-STAT4 axis does not however appear to lead to the adaptive features displayed by cytokine pre-activated NK cells [183]. It would be interesting to explore any potential role of STAT4 in the induction of hepatic resident CD49a+ and CXCR6+ NK cells.

Our preliminary data indicate disruption of the STAT4 axis in peripheral blood NK cells following liver transplantation, perhaps as a result of the Th2 and Th3 intrahepatic cytokine microenvironment in the donor liver, resulting in 'tolerant' NK cells in terms of both cytokine release and direct cell killing (**Fig 1-7**). In addition STAT4 phosphorylation is reduced in CHC resulting in the 'cytotoxic, poor cytokine release' phenotype commonly seen, although this is enhanced by IFNα, [35,36,93]. Furthermore, downregulation of STAT4 can predict more aggressive HCC tumour behaviour [218]. I therefore hypothesise that upregulation of the transcription factor, pSTAT4, may lead to the generation of 'hyperfunctional' NK cells with enhanced production of Th1 cytokine and direct cell killing (**Fig 1-7**).

STAT4 may also present an important therapeutic target for the autoimmune liver disease, PBC. Six GWAS studies have identified STAT4, along with IL-12 and the IL-12 receptor, as susceptibility loci for the development of PBC (**Table 5**) [135–140]. These findings are supported by a mouse model of autoimmune cholangitis, in which deletion of the gene coding for the IL-12p40 subunit led to disease suppression [219]. Therefore blocking components of the IL-12 signalling axis may provide novel treatment options for PBC. Upregulation of STAT4 could lead to dysregulated hyperfunctional autoreactive NK cells, breaching NK cell tolerance. This finding would provide the first functional correlate to the PBC GWAS studies. Whether NK cell autoreactivity is mediated through memory-like behaviour, with activity against PDC-E2, or induced by a cocktail of inflammatory cytokine in the liver, presents a completely novel path for investigation (**Fig 1-7**).

STAT4 polymorphisms have also been associated with susceptibility to Rheumatoid Arthritis and Systemic Lupus Erythematosus [220], and potentially Autoimmune Hepatitis [221]. Furthermore STAT4-deficient mice demonstrate resistance towards models of autoimmune disease [222]. IL-12 polymorphisms have also been linked to psoriasis, and variants within the IL-23 receptor are associated with susceptibility to both psoriasis and Crohn's disease [223–225]. Therefore further investigation of the role of the IL-12/STAT4 pathway on NK cell effector function may have implications for other autoimmune conditions.

The generation of hyperfunctional potentially 'memory-like' NK cells using cytokines, presents huge therapeutic possibilities in terms of vaccine development and the introduction of novel cell-based therapies for viral hepatitis and primary liver cancer. Furthermore, blocking the activity of NK cells with this phenotype, perhaps using the STAT4 axis, may help slow the progression of autoimmune disease, providing a completely innovative therapeutic role for memory-like NK cells **(Fig 1-7**).

Figure 1-7 Hypothesis: The influence of the hepatic cytokine environment on STAT4-driven NK cell differentiation

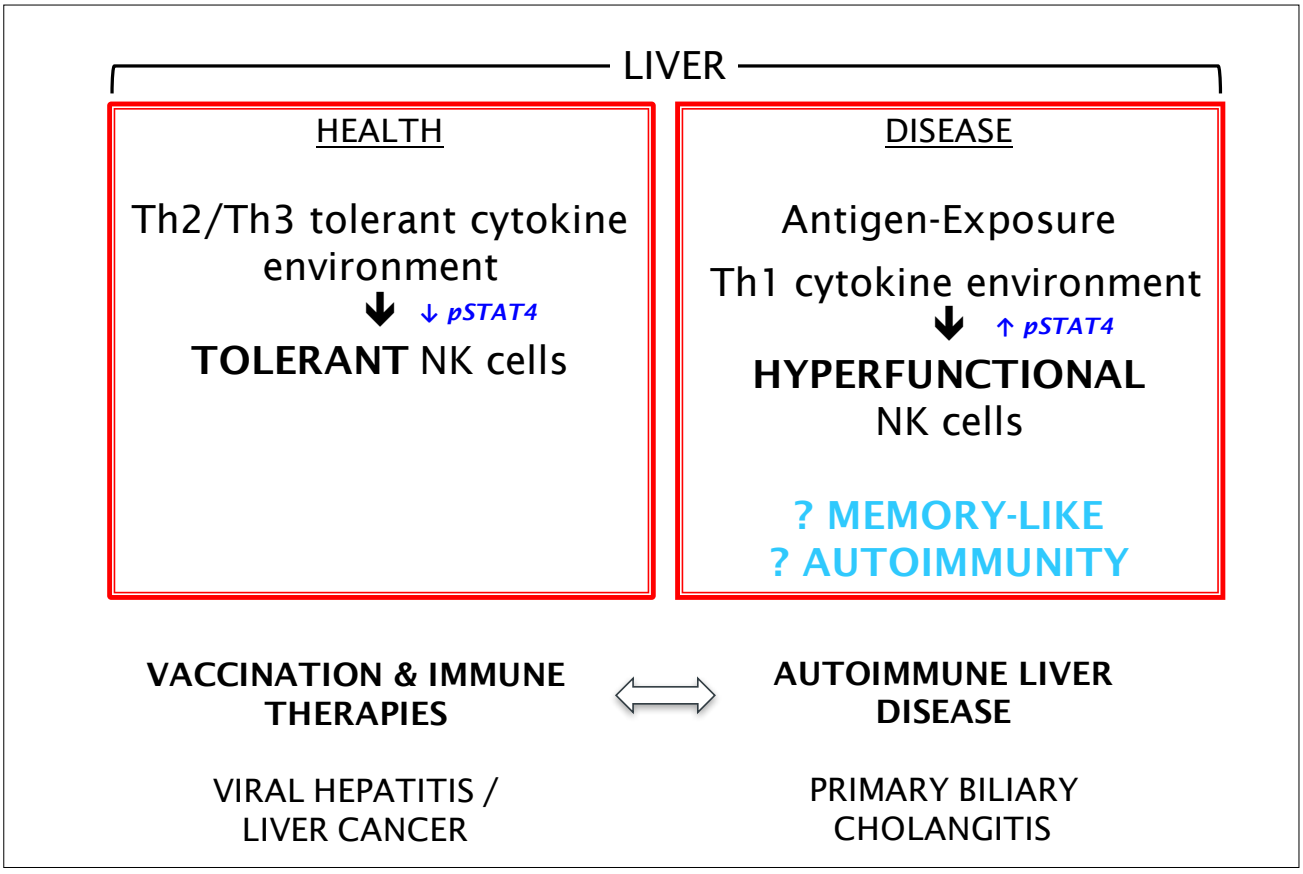


Table 9 A summary of key studies demonstrating the influence of the STAT4 axis on NK cell function

Findings	Role of STAT4	Reference
NK cell tolerance		
STAT4 knock out mouse	NK cells in STAT4 knock out mice exhibit reduced IFNγ release, decreased cytotoxicity against YAC-1 NK cell targets & reduced proliferation of pre-activated NK cells in response to IL-12	Thierfelder 1996 [92]
Tolerant NK cells found in the peripheral blood of patients who have undergone non-HLA-C matched liver transplantation	7x downregulation of STAT4 post liver transplant vs. healthy controls (10x downregulation in post liver transplant for non-HCV) 5x downregulation MicroRNA155, a target of STAT4 (role in NK cell differentiation & accelerated maturation)	Jamil 2016 [46]
NK cells in CHC adopt a 'cytotoxic, poor cytokine release' phenotype	Preferential phosphorylation of STAT1 over STAT4 in response to IFNα therapy in CHC & CMV Reduced STAT4 phosphorylation directly linked to 'cytotoxic, poor cytokine release' NK phenotype in CHC	Miyagi 2010 [81] Edlich 2012 [80] Sarasin-Filipowicz 2008 [93]
Downregulation of STAT4 in HCC	RT-PCR from tumour & non-tumour tissue revealed downregulation of STAT4 in tumour tissue Negative correlation between STAT4 expression & tumour TNM stage, hepatic vein invasion, size, differentiation & worse tumour-free survival	Wubetu 2014 [218]
NK cell memory		
Ly49H+ have memory against mCMV. On adoptive transfer sensitised Ly49H+ cells undergo expansion in liver, demonstrate longevity & amplified degranulation & IFNγ release on restimulation	NK cells from mice deficient in IL-12 receptor or STAT4 failed to expand post mCMV & mediate enhanced protection on re-exposure (STAT4-dependent IFNγ–independent mechanism)	Sun 2009 [174] Sun 2012 [176]
Cytokine pre-activated human NK cells display enhanced IFNγ release & proliferation on re-stimulation	There was n*o* significant upregulation of IL-12 receptor expression at an mRNA or protein level in this group & *no* increase in levels of phosphorylated STAT4	Romee 2012 [183]
NK cell autoimmunity		
GWAS studies to identify susceptibility loci in PBC	GWAS have shown an association between IL12A, IL12RB2, Tyk2, SH2B3 & STAT4 with disease susceptibility	Hirschfield 2009 [135] Hirschfield 2010 [136] Liu 2010 [137] Mells 2011 [138] Nakamura 2012 [139] Liu 2012 [140]

1.7 Natural killer cells and autoimmune disease

NK cells display altered distribution, phenotype and function in a number of autoimmune diseases (**Table 10**). While they have not been identified as been directly pathogenic, they may play a role in shaping the adaptive immune response. I am interested in whether overactivation of the IL-12/pSTAT4 axis may lead to hyperfunctional NK cells, and whether this NK cell subset may display autoreactivity. I am particularly interested in this model in the autoimmune liver disease, PBC given the recent identification of susceptibility polymorphisms within this pathway in multiple GWAS studies (**Table 5**). Observations regarding NK cells in autoimmune liver disease are discussed in section 1.4 'NK cells in liver disease', however a summary of the main regulatory and pro-inflammatory functions of NK cells in other autoimmune diseases is presented below as similar biological pathways are likely to be important.

NK cells are thought to display altered tolerance where activating signals outbalance inhibitory signals. This may occur through upregulation of activating receptor ligands on autologous cells through exposure to cytokines, antigenic peptides or a viral or bacterial infection [226,227]. NK cells may loose their tolerance and kill self-cells, for example oligodendrocytes in multiple sclerosis (MS). Abnormally activated NK cells can amplify the adaptive immune response, by inducing maturation of APCs, eliminating T-regulatory cells and secreting inflammatory cytokines (IL-17, IL-22, TNFα, IFNγ) which may maintain the necessary environmental milieu for autologous lymphocyte activity (**Table 10**). However NK cells can also help maintain homeostasis for example by secreting IFNγ which inhibits Th17 differentiation and killing activated T-cells and macrophages (**Table 10**).

Therefore the cytotoxic capacity and cytokine profile of NK cells, in addition to their expression of activating receptors, important for cell-to-cell interactions, play an active role in determining outcomes in autoimmune disease both directly and through interactions with the wider immune response. Furthermore a number of biological agents currently used to treat common autoimmune conditions are likely to work in some part through their influence on NK cell activity (**Table 10**). It will be interesting to see if hyperfunctional cytokine-induced memory-like NK cells, or adaptive liver-resident NK cells play any role in mediating autologous NK cell activity.

Table 10 Summary of the role of NK cells in autoimmune disease

Disease	NK cell phenotype and function	Biological Agents
Psoriasis	**Inflammatory** 5-8% lymphocytes in psoriatic lesions are NK cells – $CD56^{bright}$CXCR3+CCR5+ CD69+[228] Home towards CCL5 & CXCL10 [228] Secrete IFNγ → activate keratinocytes [228] Secrete IL-22 → proliferation keratinocytes & production IL-20 [229] Skewing NKG2A+ over NKG2C+ NK cells → lack of recognition leading to expansion autoreactive T-cells [230]	Ustekinumab (human IgG1κ, targets p40 subunit of IL-23 & IL-12 receptors) Secukinumab (IL-17A inhibitor) Adalimumab / Certolizumab / Ertanercept / Golimumab / Infliximab (TNFα inhibitors)
Rheumatoid Arthritis	**Inflammatory** Expansion of $CD56^{brigt}$ NK cells in synovial fluid [231]: Secrete IL-22 & TNFα → proliferation fibroblast-like synoviocytes [232] Secrete IFNγ → B-cell activation & dendritic cell maturation [231] Induce osteoclast differentiation from monocytes [233] **Regulatory** Secrete IFNγ → inhibits Th17 T-cell differentiation [231] Direct cell killing of activated T-cells and macrophages [234,235]	Anakinra (IL-1 receptor) Tocilizumab (IL-6 receptor) Rituximab (binds CD20) Adalimumab / Certolizumab / Ertanercept / Golimumab / Infliximab (TNFα inhibitors)
Inflammatory Bowel Disease	**Inflammatory** NK cells secreting IL-17 (along with ILC3s, Th17, NKT and γδ T-cells), IL-22 and IFNγ are enriched in inflamed mucosa [236] NK cells are stimulated to produce IL-17 following stimulation with IL-23 or on engagement of PAMPs [237]	Natalizumab (monoclonal antibody, binds α4β1 integrin) Vedolizumab (humanized monoclonal antibody, binds α4β7 integrin) Adalimumab / Certolizumab / Golimumab / Infliximab (TNFα inhibitors)
Multiple Sclerosis (Relapsing-Remitting)	Reduced cytotoxicity of NK cells in peripheral blood [238,239] **Inflammatory** NK cells are abundant in acute inflammatory MS lesions NK cells can kill autologous oligodendrocytes (produce myelin) [240] **Regulatory** Periods of relapse are associated with transient reduced number and reduced cytotoxicity of NK cells [239] *Interferon β / Daclizumab / Natalizumab* → expansion $CD56^{bright}$ NK cells (correlated with positive response) → inhibition of T-cell activation & T-cell killing via granzyme A & K, inhibit inflammation [241–243] *Rituximab / Alemtuzumab* → activity reliant on NK cell ADCC towards lymphocytes [244,245]	Natalizumab (monoclonal antibody, binds α4β1 integrin) Alemtuzumab (binds CD52) Daclizumab (binds CD25, subunit of IL-2 receptor) Interferon β

1.8 Hypothesis

I propose that pro-inflammatory cytokines may generate ‘hyperfunctional’ NK cells in the liver and peripheral blood with potential ‘memory-like’ features and that these may have important implications for liver disease, including autoimmune liver disease.

1.9 Thesis Aims

1. **Characterise liver-resident NK cells**

I will examine phenotypic differences between NK cells in the human liver and peripheral blood, with the aim of identifying liver-specific NK cell subpopulations and potential markers of NK cell ‘memory’.

2. **Investigate if markers of liver-residency can be induced on NK cells from the peripheral blood**

I aim to establish whether NK cells with potential liver-resident or memory-like phenotypes can be induced in the peripheral blood using cytokines.

3. **Investigate the role of peripheral blood NK cells in liver disease**

I aim to determine whether liver-homing and memory-like NK cells in the peripheral blood play a role in liver disease, and whether this is related to their response to cytokines.

2 Chapter 2: Materials and Methods

2.1 Isolation of mononuclear cells from liver tissue

Patients were recruited from University Hospital Southampton NHS Foundation Trust. Liver tissue was obtained from the margin of 73 adults undergoing resection for hepatic metastases or primary liver cancer. Liver tissue was weighed then infiltrated with chelating buffer (1x Phosphate Buffered Saline (PBS) (BioWhittaker, Belgium) 50ml, HEPES 28mg (Sigma, Poole, UK), EGTA 9.5mg (Sigma), adjusted to pH 7.4 and warmed at 37°C for 30 minutes prior to use) at 1ml/min and the perfusate collected. Cells were isolated from the liver parenchyma using collagenase digestion (Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies, UK) 50ml, TIV Collagenase 18mg (Collagenase from Clostridium histolyticum, Sigma), calcium chloride 90µl (Sigma) warmed at 37°C for 30 minutes prior to use) infused at 2ml/min, followed by mechanical disaggregation (the capsule was removed using scissors and tissue cut into 2mm pieces then disrupted using a tissue homogenizer), or mechanical disaggregation alone. Hepatic MNCs were isolated by layering 35ml of cell suspension from the perfusate and liver parenchyma onto 15ml Ficoll-Paque (GE Healthcare, Sweden) and centrifuged at 2000rpm for 30 minutes (brake / acceleration 0/0). Cells were washed three times in PBS and counted. Cells required for immediate analysis were handled accordingly, and the remainder were resuspended in foetal bovine serum (FBS) (Hyclone, ThermoScientific, Northumberland, UK) supplemented with 10% dimethyl sulfoxide (DMSO) (Sigma) and stored at -80°C for 24 hours then moved into liquid nitrogen.

2.2 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) isolated from blood donated from patients with haemochromatosis were stained alongside liver derived MNCs for the first 30 liver samples. Haemochromatosis is an inherited non-inflammatory chronic liver disease characterised by the accumulation of iron in the liver. This patient group was chosen firstly because the condition is managed via venesection, therefore this provides a means of receiving blood without the individual having to undergo an additional intervention. Secondly haemochromatosis patients were later used as a control group for liver disease for patients with PBC. Thereafter paired peripheral blood samples and liver tissue were collected. PBMCs were also isolated from patients with PBC, with a further cohort of individuals with haemochromatosis and healthy volunteers used as controls. Between 30-45ml of blood was drawn from each patient in an EDTA tube. Blood was diluted 1:1 in PBS. PBMCs were isolated by layering 35ml of diluted blood on top of 15ml Ficoll and centrifuged at 2000rpm for

30 minutes (brake / acceleration 1/1). The PBMC layer was harvested and cells washed three times with PBS and counted. 5ml of serum was stored at -80°C for potential ELISA analysis at a later date. Cells required for immediate analysis were handled accordingly, and the remainder were resuspended in FBS supplemented with 10% DMSO and stored at -80°C for 24 hours then moved into liquid nitrogen.

2.3 NK cell surface staining

Hepatic perfusate MNCs and PBMCs were analysed in parallel, in addition to liver parenchyma tissue where cell numbers permitted. Cells were incubated in Zombie Violet Fixable Viability Kit (Biolegend, London, UK) for 15 minutes at room temperature prior to being washed twice in FACS washing buffer (PBS with 1% bovine serum albumin (BSA) (Sigma) and 0.1 % sodium azide (Sigma)) 150µl per $0.5x10^6$ cells, followed by resuspension in blocking buffer (10% human serum (HS) (Sigma) in FACS washing buffer) 100µl per $0.5x10^6$ cells for 30 minutes at 4°C. Cells were then washed twice in FACS washing buffer prior to surface antibody staining for 30 minutes at 4°C. NK cells were identified using the panel shown in **Table 11**. Cells were fixed using 1% paraformaldehyde (Sigma) and analysed using a three laser FACS Aria (BD Biosciences, Oxford, UK) flow cytometer. Compensation beads (BD Biosciences) were used to ensure comparable results on each day of acquisition. Gates were set using fluorescence minus one (FMO) controls. Antibody concentrations were titrated using isotype controls. Data was analysed using FlowJo v.10.0 (Treestar, USA). The gating method used is described in **Fig 2-1** and examples of FMOs used for gating positive and negative populations are shown in **Fig 2-2**.

Table 11 Antibody panel used for cell surface staining

Antibody	Fluorochrome	Company	Clone	Isotype	Cat. No.	Concn
NK cell identification						
Zombie Violet Fixable Viability Kit	BV421	Biolegend			423114	1:100
CD56	PE-Cy7 APC	Biolegend Biolegend	HCD56 HCD56	Ms IgG1κ Ms IgG1κ	318318 318310	1:20 1:20
CD3	Pac Blue BDV450 PerCP BDV500 BV510	BD Biosciences BD Biosciences Biolegend BD Biosciences Biolegend	OKT3 UCHT1 UCHT1 UCHT1 UCHT1	Ms IgG2Aκ Ms IgG1κ Ms IgG1κ Ms IgG1κ Ms IgG1κ	48-0037 560365 300428 561416 344828	1:100 1:100 1:100 1:20 1:33
Maturation						
CD117	PE-CF594	BD Biosciences	YB5.B8	Ms IgG1κ	562407	1:20
CD16	APC-Cy7	Biolegend	3G8	Ms IgG1κ	302018	1:20
CD57	APC PE-CF594	Biolegend BD Biosciences	HNK-1 NK-1	Ms IgMκ Ms IgMκ	359610 562488	1:50 1:100
CD161	BV421	BD Biosciences	DX12	Ms IgG1κ	562615	1:20
Education						
CD158a KIR2DL1/S1/S3/S5	FITC	Biolegend	HP-MA4	Ms IgG2Bκ	562407	1:20
CD158b KIR2DL2/3 KIR2DL2/3 KIR2DL2/L3/S2/S4	 FITC PerCP APC	 BD Biosciences Miltenyi Biotec R&D systems	 CH-L DX27 180704	 Ms IgG2Bκ Ms IgG2a Ms IgG2B	 559784 130-095-285 FAB1848A	 1:50 1:100 1:10
Activation						
NKG2C	PE APC ViobrightFITC	Miltenyi Biotec Miltenyi Biotec Miltenyi Biotec	REA205 REA205 REA205	Ms IgG1 Ms IgG1κ Ms IgG1κ	130-103-635 130-103-636 130-104-778	1:100 1:5 1:20
CD69	APC-Cy7	Biolegend	FN50	Ms IgG1κ	310914	1:20
Others						
CD49a	PE	BD Biosciences	SR84	Ms IgG1κ	559596	1:10
CD49b	FITC	BD Biosciences	AK-7	Ms IgG1κ	555498	1:10
CXCR6	APC PerCP/Cy5.5	Biolegend Biolegend	K041E5 K041E5	Ms IgG2Aκ Ms IgG2Aκ	356006 356010	1:20 1:20

Figure 2-1 Identification of NK cells (CD3-CD56+ lymphocytes) using flow cytometry

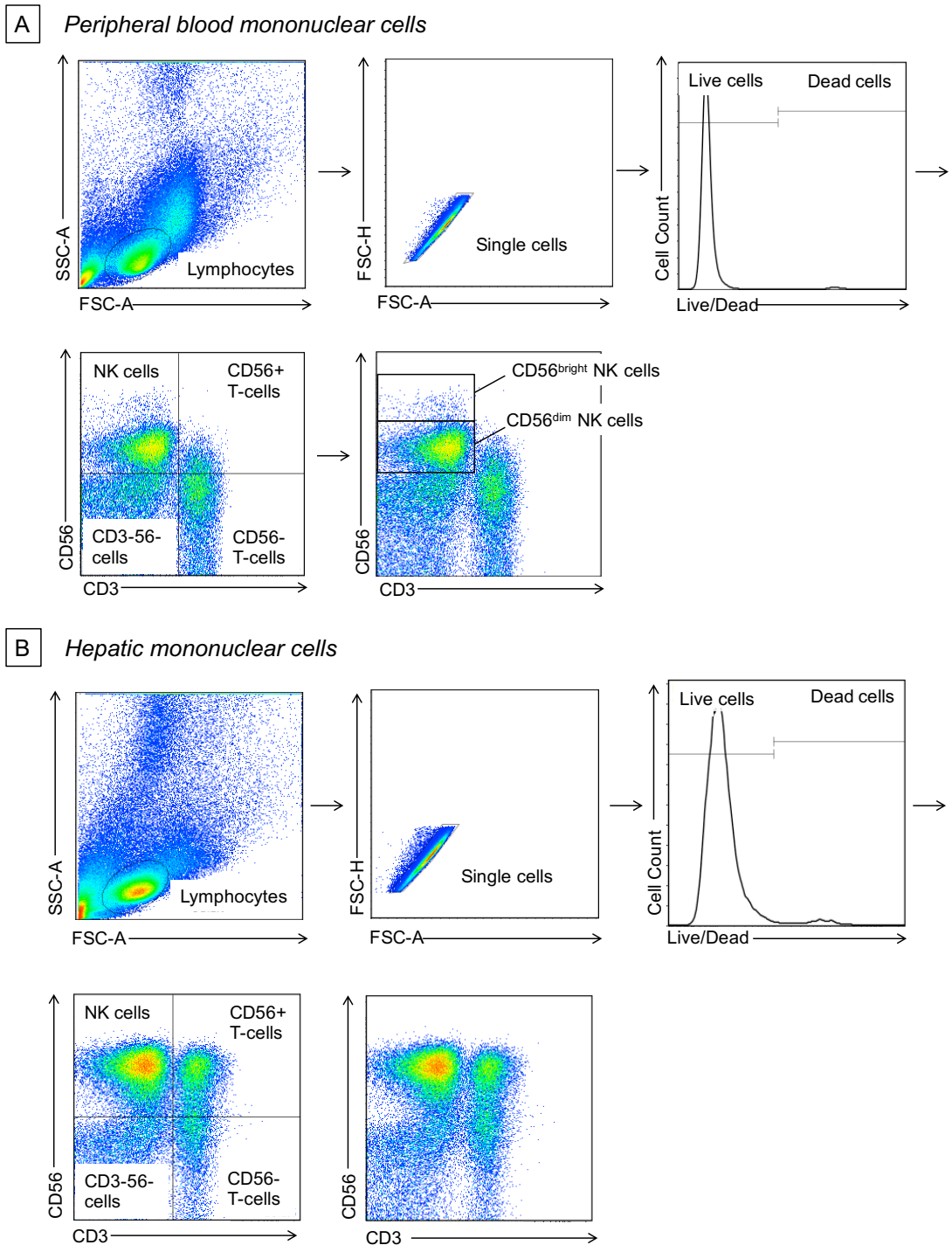
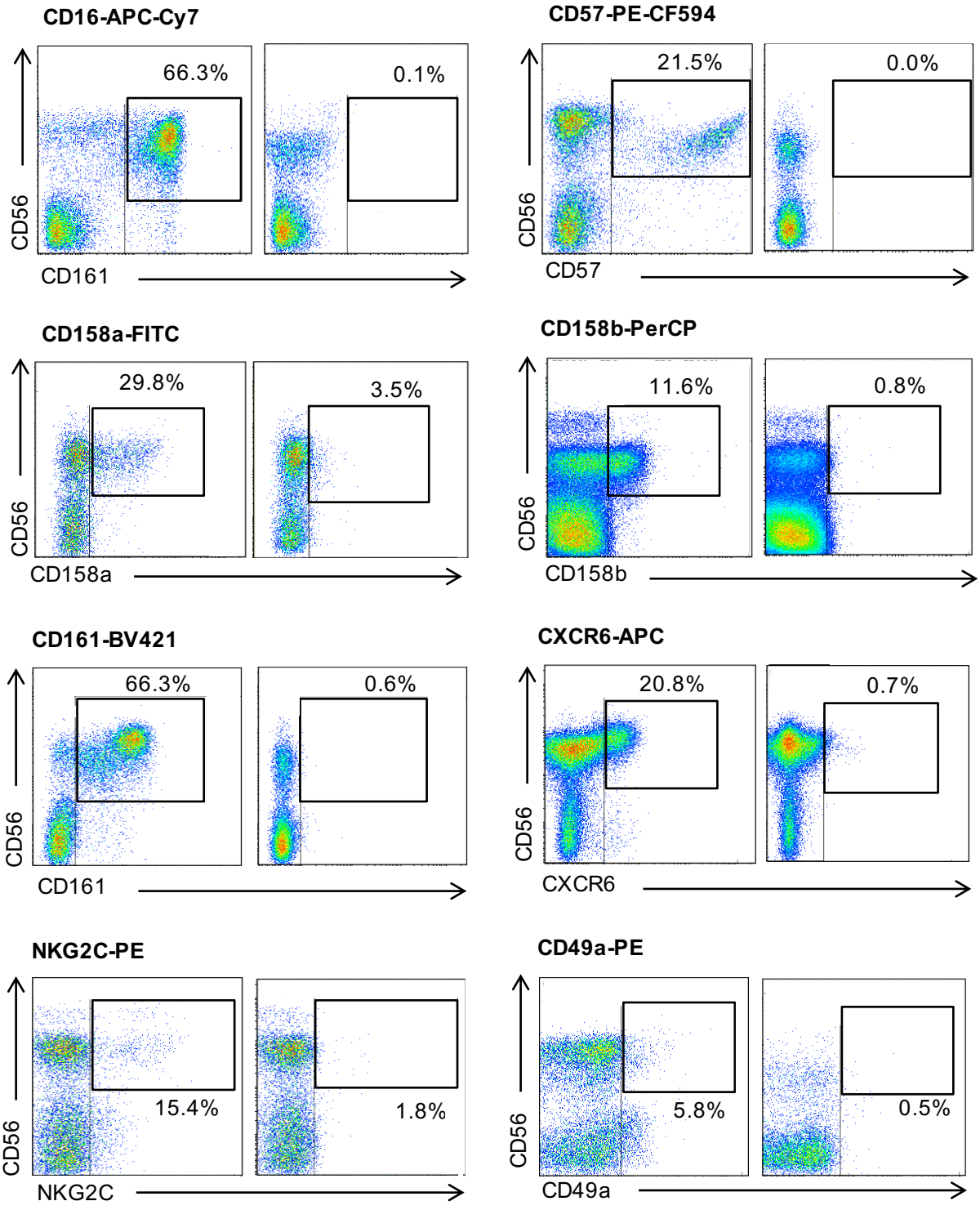


Fig 2-1 Lymphocyte population selected, followed by single cells with proportional width and area, then live cells. CD3/CD56 gating was used to select NK cells (CD3-CD56+), CD56+ T-cells (CD3+CD56+), CD56- T-cells (CD3+CD56-) and CD3-56- lymphocytes (includes B-cells). NK cells were then gated to select $CD56^{bright}$ and $CD56^{dim}$ populations in the blood only where two distinct populations could be seen. The same gating strategy was applied for lymphocytes in **A)** the peripheral blood, and **B)** the liver.

Figure 2-2 Fluorescent minus one gating controls for surface staining (gated on CD3- lymphocytes)



2.4 Fluorescence activated NK cell sorting

Freshly isolated hepatic MNCs cells underwent surface staining for Zombie Violet Live/Dead stain, CD3-PerCP, CD56-PE-Cy7 and CXCR6-APC. Fluorescence-activated cell sorting was used to isolate live CD3-CD56+CXCR6- and live CD3-CD56+CXCR6+ hepatic lymphocytes (**Fig 2-3**). Sorted NK cells were centrifuged and the supernatant discarded. They were stored in Trizol at -80°C 300µl/100,000 cells and shipped on dry ice to the Department of Primate Genetics, Gottingen, Germany for RNA sequencing (**Table 12**).

Table 12 Cells sorted prior to RNA sequencing

Patient ID	Pathology	Cells sorted	No. sorted cells
TH35	Colorectal cancer metastases	CXCR6+ CXCR6-	200,000 cells 200,000 cells
TH50	Colorectal cancer metastases	CXCR6+ CXCR6-	150,000 cells 150,000 cells
TH51	Colorectal cancer metastases	CXCR6+ CXCR6-	110,000 cells 110,000 cells

Figure 2-3 Gating strategy for sorting CXCR6- and CXCR6+ hepatic NK cells

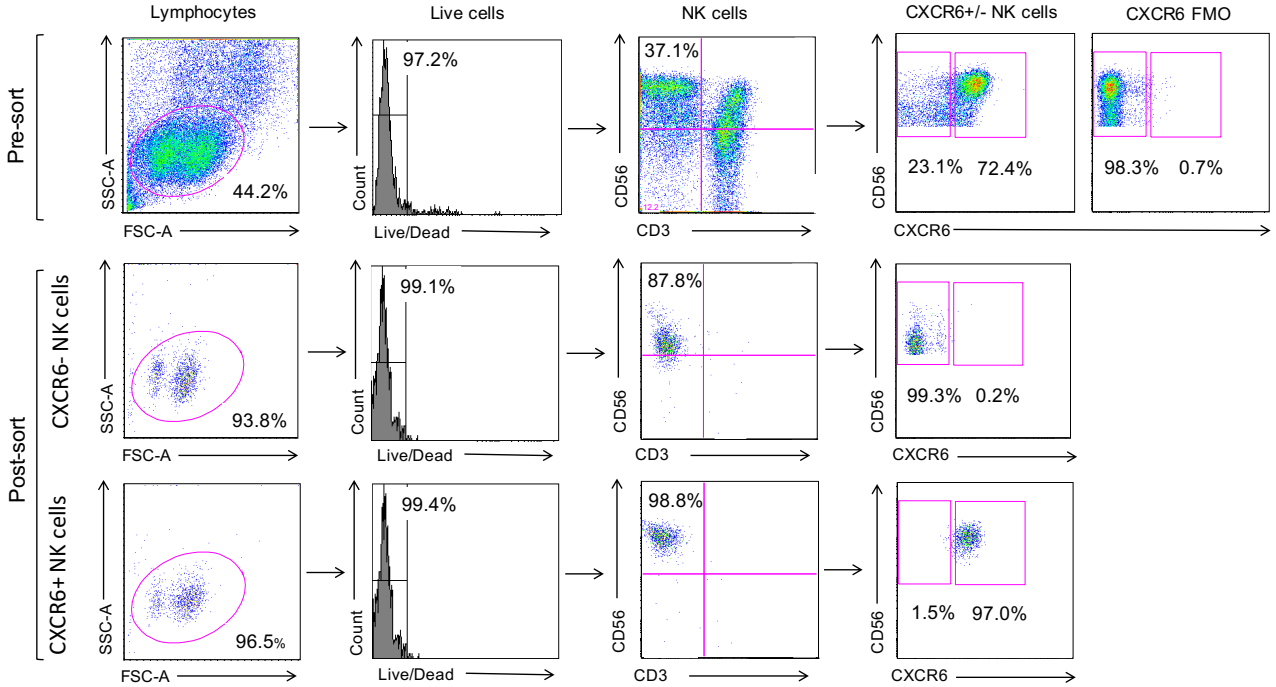


Fig 2-3 Representative flow cytometry plots demonstrating gating strategy for cell sorting. Gated on lymphocytes, live cells, NK cells, CXCR6 status.

2.5 RNA sequencing and analysis

RNA extraction and sequencing was performed by Dr Angela Noll and Professor Lutz Walter (Department of Primate Genetics, German Primate Centre, Göttingen, Germany) and Dr Gabriela Salinas-Riesta (Transcriptome and Genome Analysis Laboratory Göttingen, University Medical Centre Göttingen, Germany).

Total RNA was isolated using TRIzol reagent (Life Technologies) and digested with RNase-Free DNase-I. Quantity and quality of extracted RNA were analysed using the Fragment Analyser (Advanced Analytical) and NanoDrop ND-1000 Spectrophotometer (Thermo Scientific NanoDrop Technologies, USA). 50ng of each total RNA was used as starting material. TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Human/Mouse/Rat (Illumina Cat. N° RS122-2201) was used to prepare samples. Accurate quantitation of cDNA libraries was performed using the QuantiFluorTM dsDNA System (Promega, Germany) and the size range of cDNA libraries determined using the Fragment Analyser (280bp). cDNA libraries were amplified and sequenced using cBot and HiSeq 2000 (Illumina) (SR, 50bp, ca. 30 million reads/sample). Sequence images were transformed with Illumina software BaseCaller, which were demultiplexed with CASAVA (v.1.8.2). Quality checks were performed via FastQC (Babraham Bioinformatics).

Normalization of read counts to the library size, estimation of dispersions and testing for differentially expressed (DE) genes based on a statistical test assuming negative binomial data distribution were computed in the R/Bioconductor environment (v.2.15.2) loading DESeq (1.10.1) and biomaRt (2.14.0) packages.[246,247] DESeq normalized produced the smallest coefficient of variation in terms of diverse library sizes and compositions compared to other common analysis methods. Significant genes were determined as log2 fold change (log2FC) >1 or <−1, base mean <1000, and false discovery rate (FDR)-corrected p-value <0.05 with multiple testing correction according to Benjamini and Hochberg. Data generated conformed to *MIAME* standards and was submitted to the Gene Expression Omnibus (GEO) database.

Gene co-expression was analysed by weighted gene co-expression network analysis (WGCNA) using the WGCNA R package.[248] WGCNA analysis was performed using a power of 12, minimum module size of 200, and merge cut height of 0.15. One module contained 1329 co-expressed genes (including for example CXCR6, NCAM1, KIRs, cytokine receptors, granzymes, EOMES, TBX21) that were used to construct heat maps and principle component analysis using the R package DeSeq2.[249]

2.6 NK cell proliferation assays and expansion of CD49a+ and CXCR6+ NK cell subsets

Freshly isolated paired PBMCs, perfusate and hepatic parenchymal MNCs were counted, then spun down and resuspended in PBS/0.1% BSA to create a 2x cell solution. This was gently resuspended in 10μM CFSE staining solution (CellTrace CFSE Cell Proliferation Kit) (Life Technologies, Paisely, UK) to make a final CFSE concentration of 5μM and incubated for 10 minutes, 37°C. Staining was quenched with 5 volumes ice-cold Roswell Park Memorial Institute Medium (RPMI) 1640 + Glutamax (Gibco, Life Technologies) supplemented with 10% FBS, penicillin, streptomycin and glutamine (Gibco, Life Technologies) (R-10) and incubated for 5 minutes, 4°C. Cells were washed 3 times in R-10 then recounted. PBMCs, perfusate and liver MNCs were incubated in a 96 well plate at a concentration of $0.2x10^6$ cells / 200μl R-10 supplemented with 5% HS (Sigma) in addition to Recombinant Human IL-2 100U/ml (PeproTech, London, UK), IL-12 10ng/ml (PeproTech), IL-15 25ng/ml (R&D Systems, Oxford, UK), IL-18 100ng/ml (Medical and Biological Laboratories, Japan), or a cocktail of all four for six days. Media and cytokines were changed every 2-3 days. A CFSE FMO was included for the initial 8 samples which involved stimulation with IL-15 alone. On day 0 and 6 PBMCs and liver MNCs underwent staining with Zombie Violet Fixable Viability Kit, CD3-BV510, CD56-PECy7, NKG2C-APC, CXCR6-PerCP/Cy5.5, CD49a-PE and CD69-APC-Cy7 to allow analysis of frequencies and absolute cell numbers of different lymphocyte and NK cell subsets. Cells were analysed using a three laser FACS Aria flow cytometer. Compensation beads were used to ensure comparable results on each day of acquisition. Gates were set using FMO controls. Data was analysed using FlowJo v.10.0. The principal of CFSE staining is demonstrated in **Fig 2-4**. For later experiments comparing the induction of CXCR6+ and CD49a+ NK cells in PBMCs in individuals with PBC, haemochromatosis and healthy volunteers, the same protocol was used excluding the CFSE staining step and a using shorter culture period of four days.

Figure 2-4 Principal of CFSE staining.

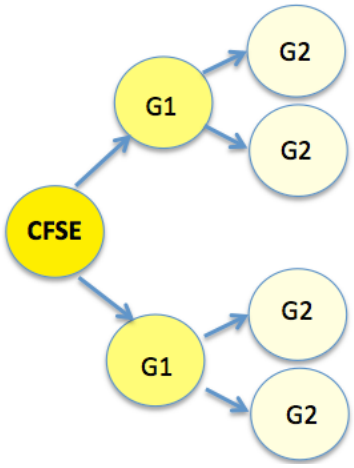


Fig 2-4 CFSE is a fluorescent dye that binds intracellular molecules. When a cell undergoes division to produce the next generation (G1, G2) the fluorescence intensity halves indicating proliferation

2.7 Intracellular staining for interferon gamma and tumour necrosis factor alpha

Frozen PBMCs and liver MNCs were thawed and counted prior to stimulating for 12 hours with IL-12 10ng/ml or IL-15 1ng/ml to examine IFNγ and TNFα release respectively. Cells were plated in a 48 well plate at $0.5x10^6$cells / 500µl R-10 supplemented with 5% HS and cytokines. An unstimulated control was included. BD GolgiStop (BD Cytofix/Cytoperm Plus Fixation / Permeabilisation Kit, BD Biosciences) a protein transport inhibitor containing monensin was added (4µl/6ml culture medium) for the last 4 hours of incubation. Cell surface staining was performed for CD3-BV510, CD56-PE-Cy7, CXCR6-PerCP/Cy5.5 and CD49a-PE as described above. Cells were then washed once and incubated for 20 minutes in 100µl Fixation/Permeabilisation solution (BD Cytofix/Cytoperm Plus Fixation / Permeabilisation Kit, BD Biosciences) per well for 20 minutes. After two washes in 200µl 1x BD Perm/Wash buffer (BD Cytofix/Cytoperm Plus Fixation / Permeabilisation Kit, BD Biosciences), cells underwent further blocking followed by two more washes and prior to incubation with anti-IFNγ 1:10 (B27, APC, Biolegend) or anti-TNFα 1:10 (MAb11, FITC, Biolegend). Following intracellular staining, cells underwent a final wash with FACS washing buffer and were analysed immediately using the BD FACS Aria. Compensation beads were used to ensure comparable results on each day of acquisition. Gates were set using unstimulated controls. Antibody concentrations were titrated using isotype controls. Data was analysed using FlowJo v.10.0. In order to examine IL-12 responsiveness of peripheral blood NK cells in difference aetiologies of chronic liver disease, a dose titration of IL-12 (0.005ng/ml, 0.5ng/ml, 5.0ng/ml and 10ng/ml) was used to examine IFNγ release. Incubation time remained 12 hours.

2.8 Phosphorylated STAT4 intracellular staining

PBMCs from healthy volunteers, individuals with haemochromatosis and individuals with PBC were incubated in a 96 well plate at $0.2x10^6$cells / 200µl R-10 supplemented with 5% HS and stimulated with increasing concentrations of IL-12 (0.005ng/ml, 0.5ng/ml, 5.0ng/ml and 10ng/ml) for 1 hour. An unstimulated control was included. Cell surface staining was performed for CD3-BV510 and CD56-FITC. Cells were washed once and incubated at 37°C for 10-12 minutes in 100µl pre-warmed Cytofix solution (BD Biosciences). Cells were centrifuged at 2000rpm for 2 minutes, the supernatant discarded and the pellet disrupted using vortex, prior to resuspension in 1ml Perm III Buffer (BD Biosciences) and incubated at 4°C for 30 minutes. After two further washes, cells were stained for pSTAT4 1:2.5 (PY693, AF647, BD Biosciences). Following intracellular staining, cells underwent a final wash and were analysed immediately using the BD FACS Aria. Compensation beads were used to ensure comparable results on each day of acquisition. Gates were set using FMO controls. Antibody concentrations were titrated using isotype controls. Data was analysed using FlowJo v.10.0.

2.9 Intracellular staining for T-bet and Eomes

Frozen PBMCs from individuals with haemochromatosis were thawed and counted, prior to cell surface staining for CD3-BV510, CD56-PE-Cy7, CXCR6-PerCP/Cy5.5 and CD49a-PE as described above. Cells were washed in 150µl FACS washing buffer per $0.5x10^6$ cells and incubated with 200µl 1x Foxp3 fixation/perm solution (Foxp3 Staining Buffer Set, eBioscience) at 18°C for 30 minutes, before washing once in 200µl 1x permeabilisation buffer (eBioscience) and incubating with 100µl blocking buffer for 30 minutes at 4°C. Cells were washed twice in 200µl 1x permeabilisation buffer. After the supernatant was discarded in the final wash, cells were resuspended in their residual volume followed by a further 100µl 1x permeabilisation buffer and anti-T-bet 1:100 (4B10, BV421, Biolegend) and anti-Eomes 1:100 (WD1928, efluor660, eBioscience) for 30 minutes at 18°C, prior to one final wash in 200µl 1x permeabilisation buffer. Cells were analysed immediately in 150µl FACS washing buffer using the BD FACS Aria. Compensation beads were used to ensure comparable results on each day of acquisition. Gates were set using FMO controls. Antibody concentrations were titrated using isotype controls. Data was analysed using FlowJo v.10.0. In order to examine expression of T-bet and Eomes on NK cells following culture with IL-12, the above protocol was performed at rest and following stimulation of $0.2x10^6$cells cultured in 200µl R-10 supplemented with 5% HS alone or with IL-12 0.005ng/ml, 0.5ng/ml, 5.0ng/ml and 10ng/ml for 12 and 36 hours.

2.10 NK cell purification and stimulation with IL-12 and IL-15

Freshly isolated PBMCs from individuals with haemochromatosis or PBC were passed through a 70µm filter prior to counting. Cells were then centrifuged at 300g for 10 minutes and resuspended in NK cell isolation buffer (40µl per 10^7 cells) (250mM EDTA 2ml, pH 8, BSA 1.25g, 248ml PBS) and NK cell biotin-antibody cocktail (10µl per 10^7 cells) (Human NK cell isolation kit, Miltenyi Biotec, Woking, UK) and incubated for 5 minutes at 4°C. Cells were then resuspended in a further 30µl of NK cell isolation buffer per 10^7 cells and NK cell micro-bead cocktail (20µl per 10^7 cells) (Human NK cell isolation kit, Miltenyi Biotec) and incubated for 10 minutes at 4°C. The cell solution was made up to a minimum volume of 500µl (maximum 10^8 cells) using NK cell isolation buffer. LS columns (Miltenyi Biotec) were assembled onto the magnetic field of the MACS separator (Miltenyi Biotec) and rinsed with 3ml of NK cell isolation buffer. The cell suspension was placed onto the LS column and unlabelled NK cells collected into a 15ml falcon tube (negative selection). The column was rinsed again with a further 3ml of NK cell isolation buffer and cell suspension collected in the same falcon tube. Cells were spun down then resuspended in R-10 and the purified NK cells counted. Purified NK cells were cultured in a 96 well plate for 12 hours at $0.2x10^6$ cell / 200µl R-10 supplemented with 5% HS and increasing concentrations of IL-12 (0.005ng/ml, 0.5ng/ml, 5.0 ng/ml and 10ng/ml) in addition to IL-15 25ng/ml. The following day NK cells underwent surface staining for CD3-BV510, CD56-PE-Cy7 CXCR6-PerCP/Cy5.5 and CD49a-PE.

2.11 Statistical analysis

Surface marker expression, intracellular staining for IFNγ, TNFα and pSTAT4 and proliferation assay results which express cell percentages and absolute cell numbers are presented using the median and interquartile range (IQR). Comparisons between samples are represented by the Wilcoxon matched pairs signed rank test and Mann Whitney U test for paired and non-paired samples respectively. Friedman's test with Dunn's multiple comparison test was used to compare multiple paired groups and Kruskal-Wallis' test with Dunn's multiple comparison test was used to compare three unpaired sample groups. Regression analysis and the coefficient of determination (r^2) was used to determine correlation between expression levels of pSTAT4 and CXCR6 or CD49a. Chi-squared test was used to compare categorical demographic data.

Analysis of the raw data generated from RNA sequencing was performed by the German Primate Centre using weighted gene co-expression network analysis (WGCNA) and the WGCNA R package (**appendix 7.1.1**) [248]. WGCNA analysis was performed using a power of 12, minimum module

size of 200, and merge cut height of 0.15. One module contained 1329 co-expressed genes (including for example CXCR6, NCAM1, KIRs, cytokine receptors, granzymes, EOMES, TBX21) that were used to construct heat maps and principle component analysis using the R package DeSeq2. All other statistical analysis was performed using GraphPad Prism v7 (GraphPad Prism Software, La Jolla, USA).

2.12 Ethics

Ethical approval to collect paired peripheral blood and liver tissue from patients undergoing hepatic resection, and peripheral blood samples from patients with Primary Biliary Cholangitis was granted by the Research Ethics Committee for Wales (REC No. 13/WA/0329). Ethical approval to collect peripheral blood samples from individuals with haemochromatosis was granted by the Research Ethics Committee for South Central Hampshire (REC No. 06/Q1701/120). Informed consent of all participants was obtained.

3 Chapter 3: Phenotypic analysis of NK cells in the liver compared to the peripheral blood

3.1 Patient demographics

In total I collected liver tissue from 73 patients (43 paired liver and blood samples and 30 unpaired). Overall 30 paired and 23 unpaired samples were phenotyped for NK cell surface markers (n=53). Demographic details of the cohort are described in **Table 13**.

Table 13 Demographic details of patients who donated liver tissue

Demographic Details (n=53)	
Age, years (range)	65 (29-80)
Men, n (%)	33 (54.7%)
Pre-operative chemotherapy, n (%)	29 (54.7)
Reason for resection, n (%)	
Metastases, colorectal	33 (62.2)
Metastases, other	8 (15.1)
Hepatocellular carcinoma	7 (13.2)
Cholangiocarcinoma	2 (3.8)
Gastrointestinal stromal tumour	1 (1.9)
Hepatic cyst	1 (1.9)
Lymphoma	1 (1.9)
Background liver histology, n (%)	
Normal	30 (56.6)
Mild steatosis	16 (30.2)
Moderate steatosis	3 (5.7)
Fibrosis / Cirrhosis	4 (7.5)
Clear resection margin	50 (94.3)

3.2 Isolation of liver mononuclear cells and influence of digestion techniques on NK cell phenotype

3.2.1 Yields of liver MNCs

The average weight of liver sample was 6.74g (range 0.2g – 26.4g). The mean number of MNCs obtained per gram of liver tissue was $2.9x10^6$ for the perfusate (hepatic sinusoids) and $0.9x10^6$ from tissue digestion (liver parenchyma). The combined total number of MNCs acquired per gram of liver

was $3.4x10^6$ (range 0.1 – $12.4x10^6$). Tissue digestion using the tissue homogenizer and enzymatic digestion with collagenase yielded the same average number of MNCs per gram ($0.9x10^6$).

3.2.1.1 Comparison of collagenase vs. mechanical digestion of liver tissue on NK cell phenotype

NK cells were examined from the liver parenchyma in 21 patients, 9 of which were digested using collagenase followed by mechanical disaggregation, and the rest using mechanical disaggregation only. This followed initial concerns that enzymatic digestion may alter NK cell surface marker expression [250]. I performed a direct comparison of the frequencies of NK cells expressing key cell surface receptors, however did not detect any significant differences in NK cell frequency or phenotype using the two techniques (**Fig 3-1**).

Figure 3-1 Comparison of surface receptor expression on NK cells isolated from the liver parenchyma following enzymatic or mechanical digestion

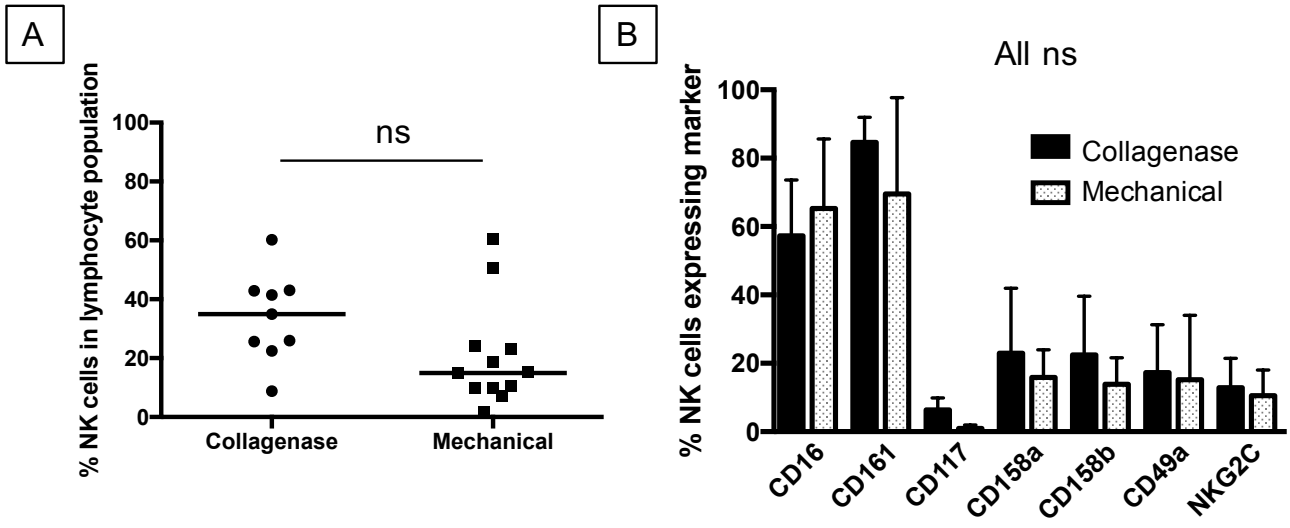


Fig 3-1 **A)** A comparison of the frequency of NK cells within liver tissue digested using collagenase followed by mechanical digestion (34.9%, *n=9*), or mechanical disaggregation alone (15.0%, *n=11*) (non-paired samples). Dot plot shows individual values and median. (*Mann Whitney U test*). **B)** A comparison of the frequency of NK cell surface marker expression following enzymatic and mechanical digestion (non-paired samples). Bar chart displays median & IQR. (*Mann Whitney U test).*

3.2.1.2 Comparison of NK cell phenotype between liver perfusate vs. liver parenchyma

As NK cells were isolated from the perfusate and liver parenchyma, a direct comparison was performed of the frequencies of NK cells expressing each surface marker examined within the two compartments (**Fig 3-2**). The perfusate, thought to contain NK cells flushed from the sinusoids, harboured higher frequencies of CD16- NK cells consistent with findings from Hudspeth et al [15]. Although this did not reach significance, frequencies of CD57- NK cells were also higher in the sinusoids, consistent with more immature NK cells residing here. NK cells expressing all other surface markers were found at similar frequencies between the two compartments and therefore perfusate-derived NK cells appear to accurately represent liver-resident NK cells as described in the literature (**Fig 3-2**) [12,15]. Given the greater yield of MNCs from the perfusate and their superior viability, all comparisons between the peripheral blood and 'liver' shown hereafter were made using NK cells obtained from the hepatic perfusate.

Figure 3-2 Comparison of surface receptor expression on NK cells isolated from the liver perfusate and liver parenchyma

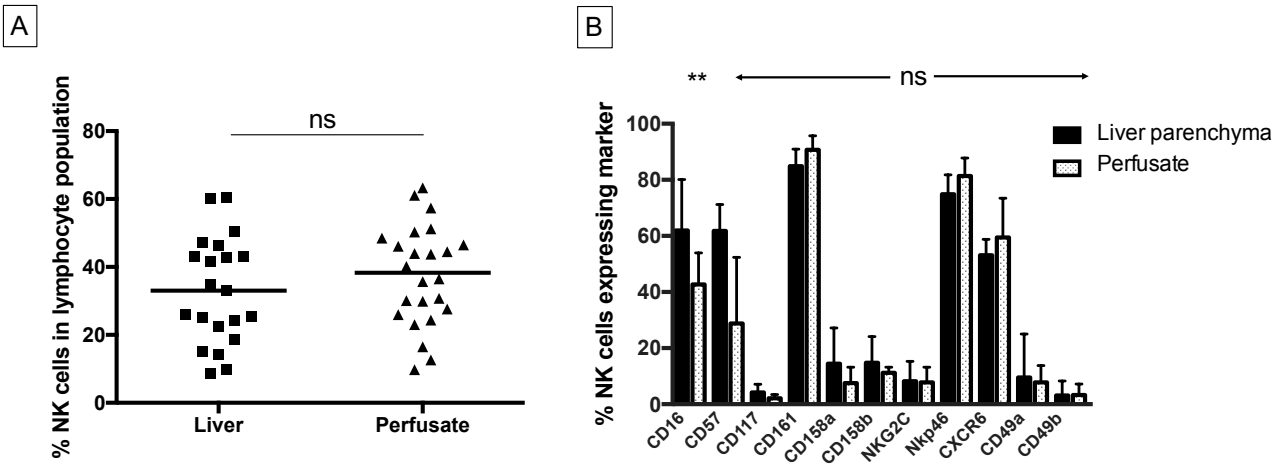


Fig 3-2 **A)** Comparison of the frequency of NK cells within the lymphocyte populations isolated from the liver parenchyma (33.0%, *n=21*) and hepatic perfusate (38.3%, *n=24*). Dot plot shows individual values and median. (*Mann Whitney U test*). **B)** Comparison of frequencies of CD16, CD57, CD117, CD161, CD158a, CD158b, NKG2C, NKp46, CXCR6, CD49a and CD49b expressing NK cells found within the liver parenchyma and perfusate. Bar chart displays median and IQR. *(Mann Whitney U test).* p<0.01**.

3.2.2 Pre-operative chemotherapy does not alter NK cell surface marker expression

Nearly two thirds of the individuals who donated liver tissue to this study had colorectal cancer metastases and had undergone a standard course of neo-adjuvant chemotherapy prior to hepatic resection. This generally consisted of oxaliplatin and capecitabine with additional therapy such as Fluorouracil added on for some patients. Chemotherapy has been implicated in causing a steatohepatitis in this patient group and it was therefore important to exclude any effects this may have on NK cell phenotype as demonstrated below (**Fig 3-3**) [251].

Figure 3-3 Comparison of surface receptor expression on NK cells isolated from the liver of individuals who have undergone pre-operative chemotherapy and those who have not

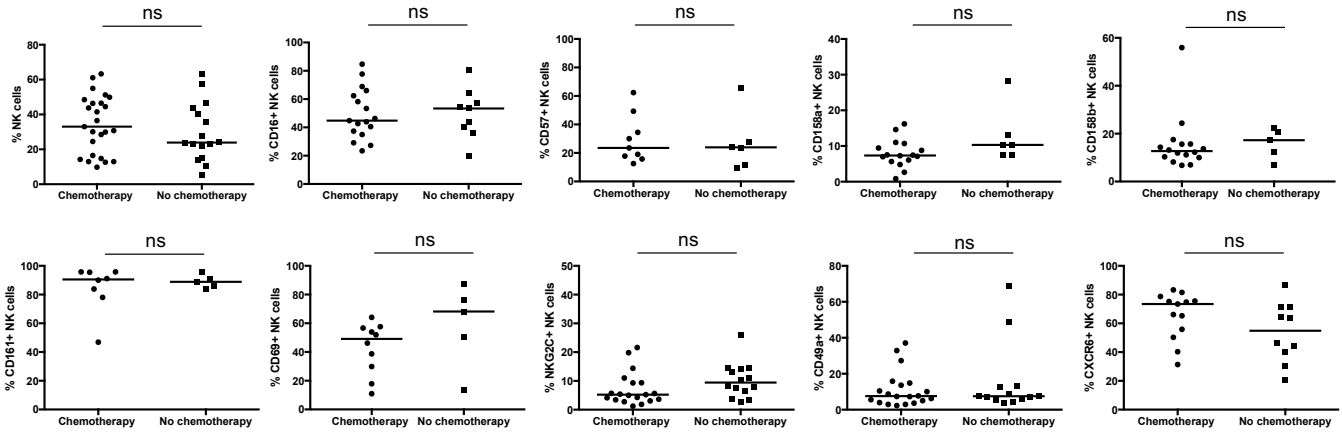


Fig 3-3 Comparison of the frequency of NK cells within the lymphocyte population, and frequencies of CD16 (*n=17, n=9*), CD57 (*n=9, n=6*), CD158a (*n=16, n=6*), CD158b (*n=16, n=5*), CD161 (*n=8, n=5*), CD69 (*n=10, n=5*), NKG2C (*n=19, n=14*), CD49a (*n=20, n=13*) and CXCR6 (*n=13, n=10*) expressing NK cells found within the liver from patients who have received chemotherapy and those who have not. Dot plots display individual values and median. *(Mann Whitney U test).*

3.3 The liver contains a high frequency of NK cells

The liver was found to be rich in innate immune cells, including NK and CD56+ (Natural Killer) T cells compared to the peripheral blood (**Fig 3-4**). The liver perfusate contained the greatest percentage of NK cells within the lymphocyte population (38.3% perfusate [range 9.8% - 66.3%], 33.0% liver parenchyma [range 8.8% - 60.5%],) in line with other studies that have found NK cells to predominantly reside in the sinusoids (**Fig 3-5**). Furthermore the majority of NK cells found in the liver appeared to be $CD56^{bright}$, as opposed to the $CD56^{dim}$ NK cells predominant in the circulation. However as these two populations could not be clearly discriminated on the FACS plots for all individuals, percentages were not examined (**Fig 3-5**).

Figure 3-4 Composition of the lymphocyte population within the peripheral blood and liver

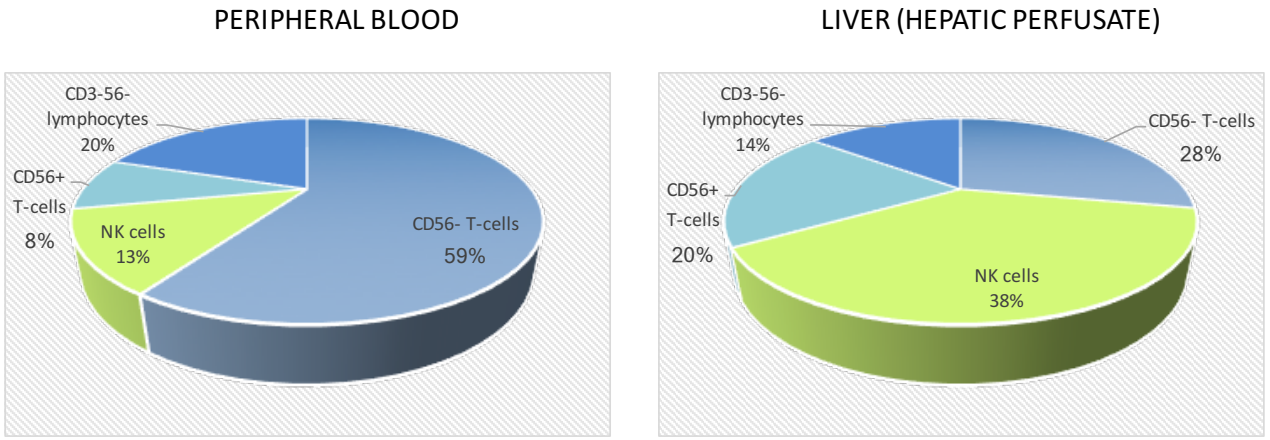


Fig 3-4 Composition of lymphocytes within the peripheral blood (*n=33*) and liver (*n=30*).

Figure 3-5 A comparison of NK cell frequencies between the peripheral blood and liver

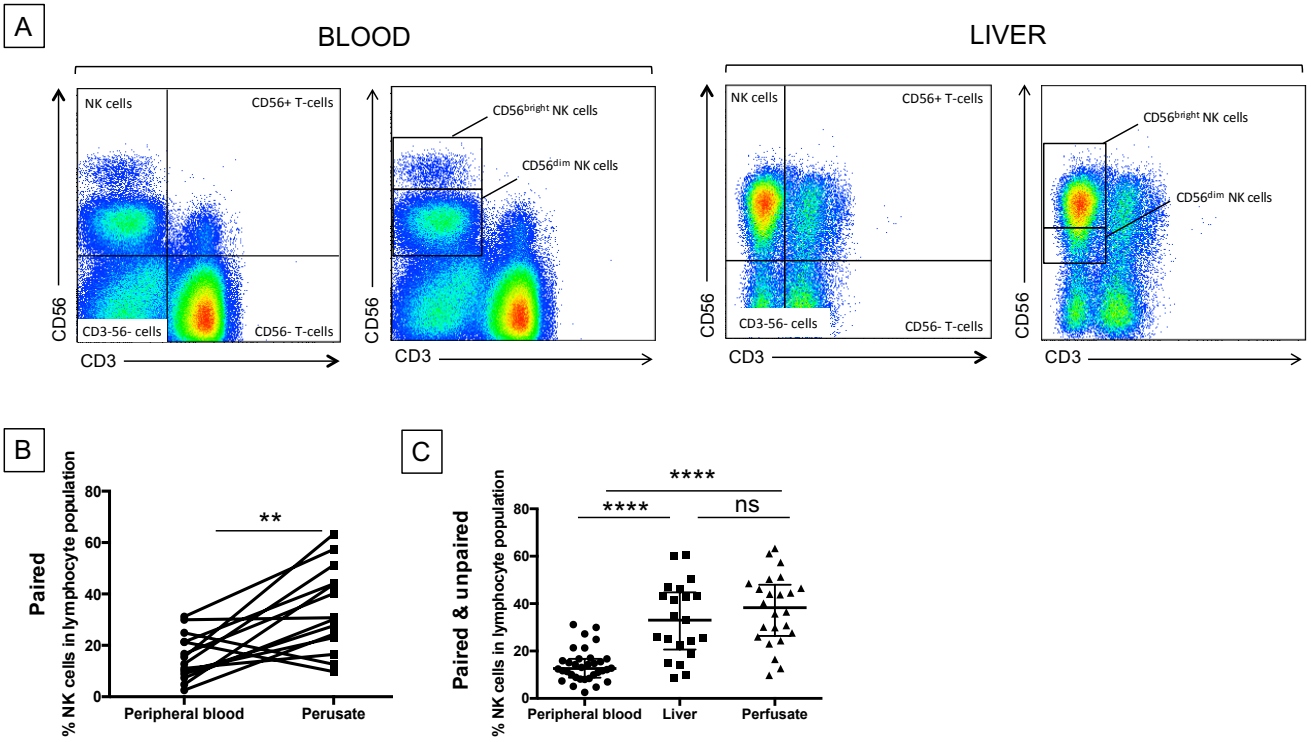


Fig 3-5 **A)** Representative flow cytometry plot displaying lymphocytes subsets, NK cells (CD3-, CD56+), CD56+ T-cells, CD56- T-cells and CD3-CD56- lymphocytes from the peripheral blood and liver, in addition to $CD56^{bright}$ and $CD56^{dim}$ NK cell populations. **B)** A comparison of the frequency of NK cells within the peripheral blood (median 14.1%) and liver perfusate (median 30.4%) (paired samples, *n=14*). Dots represent individual values. (*Wilcoxon matched pairs test).* **C)** A comparison of the frequency of NK cells within the peripheral blood (12.6%), liver parenchyma (33.0%) and liver perfusate (38.3%) (paired & unpaired samples*, n=34, n=21, n=24*). Dot plots display median and IQR. (*Mann Whitney U test).* p<0.001**, p<0.0001****.

3.4 The liver is rich in stage 4 NK cells

NK cell precursors (maturation stage 3, **Fig 1-3**) (CD3-CD56+CD16-CD117+ lymphocytes) were not present in significant frequencies in either the liver or the blood (**Fig 3-6**). Instead the liver contained high frequencies of stage 4 CD16- NK cells and fewer stage 5 CD16+ NK cells and terminally differentiated $CD16^{+}CD57^{+}$ NK cells compared to the peripheral blood. A direct comparison of markers of NK cell maturity between the liver and peripheral blood revealed reduced frequencies of CD16+, CD57+ and KIR+ (KIR2DL1/S1/L2/L3/S2) (CD158a and CD158b) NK cells, with increased percentages of CD161+ NK cells (**Fig 3-7)**. The c-type lectin-like receptor, CD69 was present on 52.0% of hepatic NK cells, compared to 5.0% of circulating NK cells, highlighting this activating receptor as a likely marker of liver-residency (**Fig 3-7**) [15,29,33,35].

Figure 3-6 Frequencies of NK cells at different stages of maturation in the liver and blood

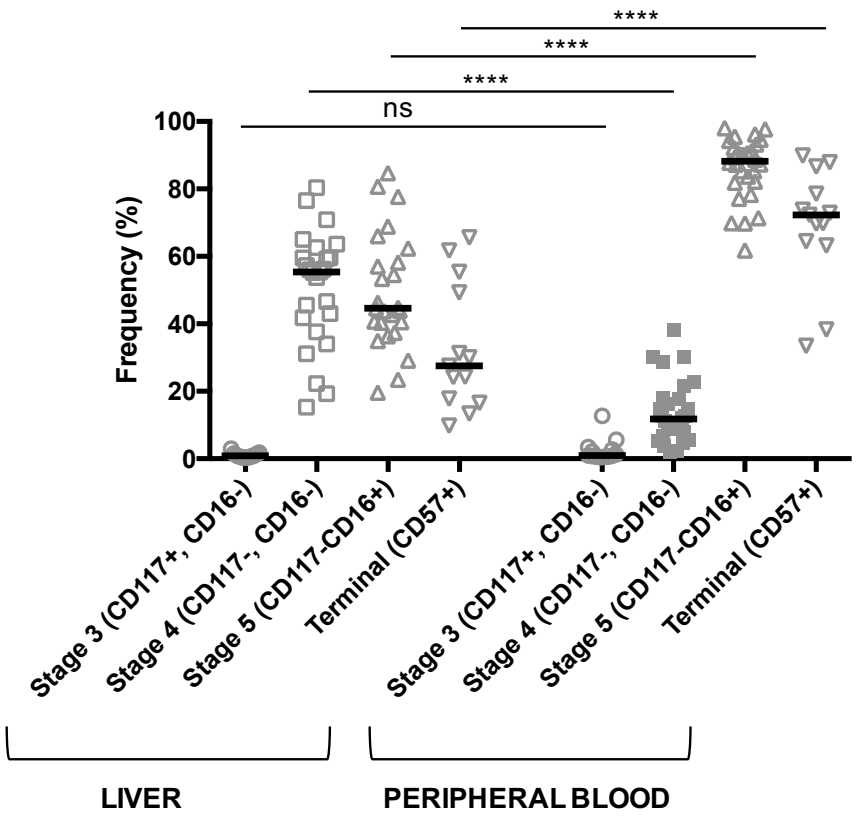


Fig 3-6 A comparison of the frequencies of NK cells at stage 3 (0.9%, 0.9%) (*n=11, n=13*), stage 4 (55.4%, 11.8%) (*n=25, n=28*), stage 5 (44.6%, 88.3%) (*n=25, n=28*) and terminal stages (27.5%, 72.3%) (*n=13, n=13*) of maturation within the liver and peripheral blood. Dot plots display median. (*Mann Whitney U test).* p<0.0001****.

Figure 3-7 Markers of NK cell maturity in the peripheral blood and liver

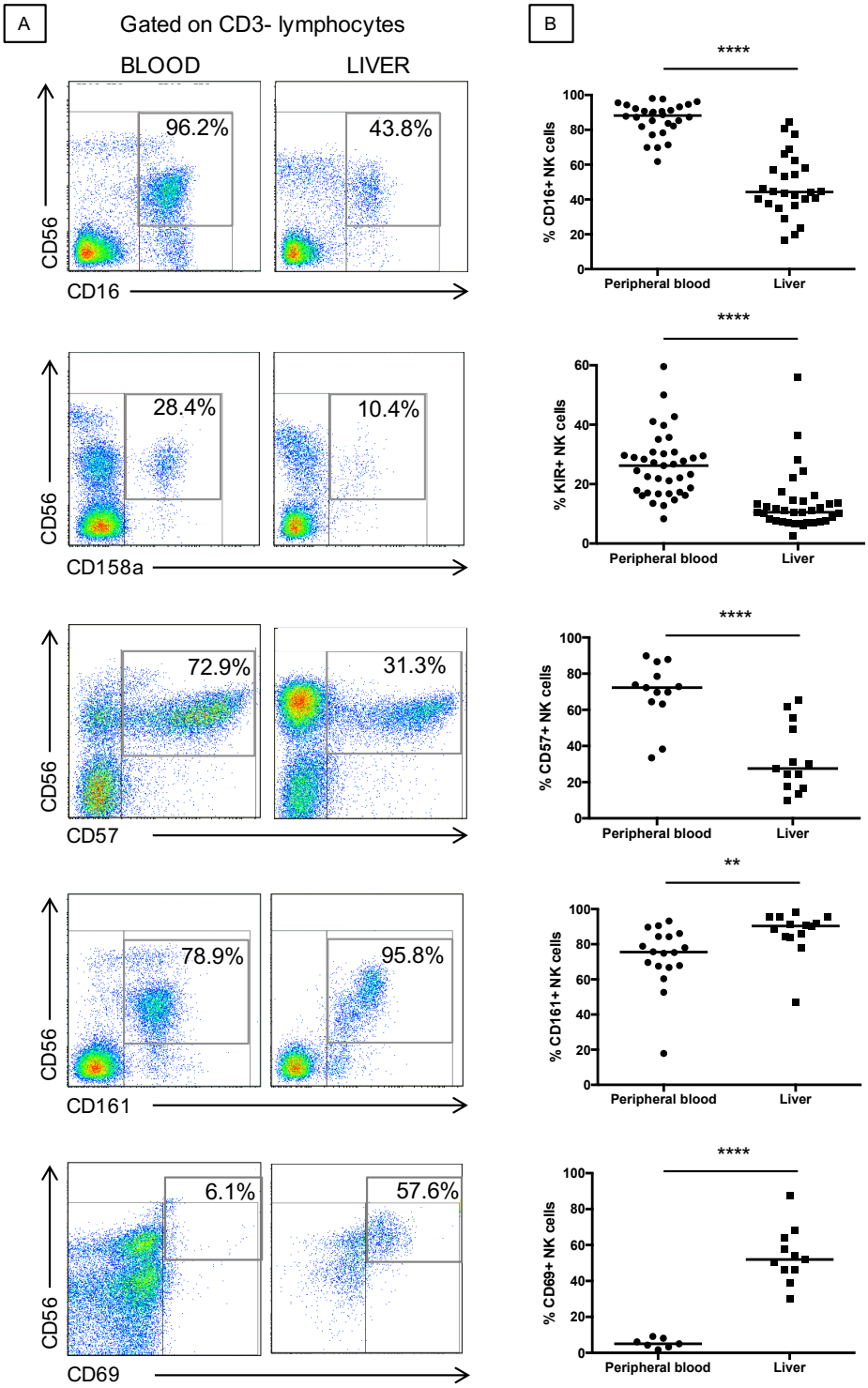


Fig 3-7 **A)** Representative paired flow cytometry plots gated on CD3- lymphocytes comparing CD16+, KIR+, CD57+, CD161+ and CD69+ NK cell frequencies within the peripheral blood and liver. **B)** A comparison of the frequency of CD16+ NK cells (88.3%, 44.8%) (*n=28, n=26*), KIR+ NK cells (26.2%, 10.5%) (*n=37, n=35*), CD57+ NK cells (72.3%, 27.5%) (*n=13, n=13)*, CD161+ NK cells (75.5%, 90.4%) (*n=14, n=18*) and CD69+ (5.0%, 52.0%) (*n=7, n=11*) NK cells within the peripheral blood and hepatic NK cell populations. Dot plots display median values. (*Mann Whitney U test).* p<0.01**, p<0.0001****.

Furthermore CD16– NK cells isolated from the liver contain a lower percentage of NK cells expressing classical markers of maturity (CD57, KIR) compared to CD16- NK cells found in the peripheral blood, in addition to a greater frequency of CD161+ NK cells (**Fig 3-8**). Frequencies of CD117+ NK cells were comparable for CD16- NK cells within the two compartments. This suggests the existence of an 'immature stage 4 CD16-' NK cell subset in the human liver.

Figure 3-8 CD16- NK cells in the liver harbour reduced frequencies of NK cells expressing markers of maturity compared to those in the peripheral blood

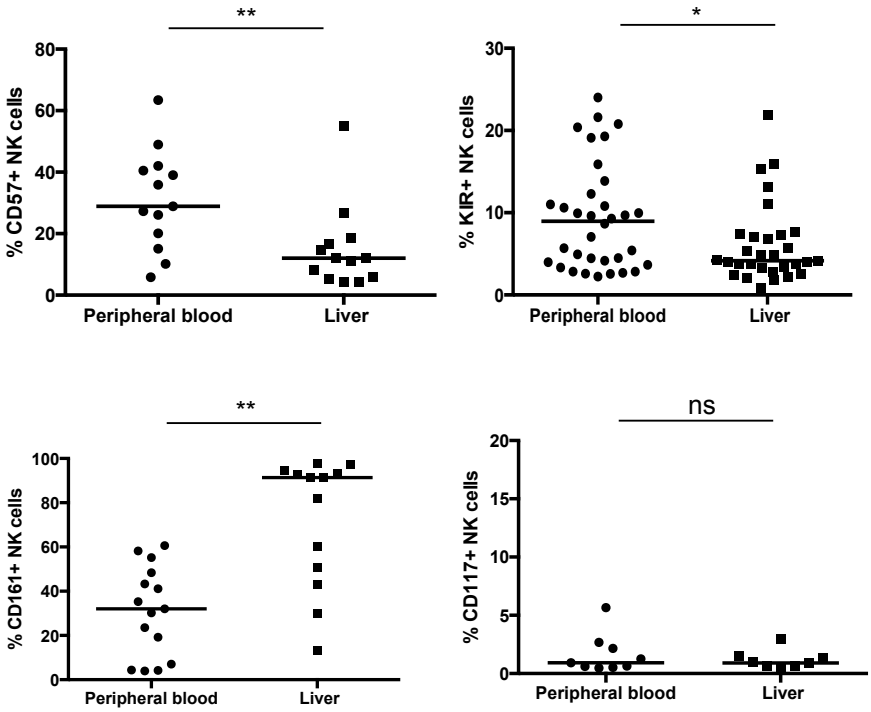


Fig 3-8 A comparison of the frequency of CD57+ NK cells (28.9%, 12.0%) (*n=13, n=13*); KIR+ NK cells (9.0%, 4.2%) (*n=34, n=30*), CD161+ NK cells (32.0%, 91.4%) (*n=15, n=13*) and CD117+ NK cells (0.9%, 0.9%) (*n=9, n=8*) found within the peripheral blood and hepatic CD16- NK cell populations respectively. Dot plots display median values. (*Mann Whitney U test).* $p<0.05$*, $p<0.01$**.

3.5 Mature innate-like lymphocytes are not found in high frequencies in the human liver

Following the formal identification of ILCs in mucosal tissue there has been much interest in their role and residence in other organs. There are three distinct subsets [252]. ILC1s express the transcription factor T-bet and release IFNγ and TNFα in response to IL-12 and IL-18. They closely resemble NK cells but are CD56-CD94-Eomes-. ILC2s express the transcription factors GATA3 and RORα and release Th2 cytokines IL-5 and IL-13. Finally ILC3s are RORγt+ and release IL-22 and IL-17. A subset of these express natural cytotoxicity receptors, in particular NKp44. I retrospectively analysed my phenotyping data to assess whether any of these populations could be found in the human adult liver. As this analysis was reliant on previously selected cell surface markers it was only possible to look for ILC1 and ILC3 populations. These were defined as CD3-CD56-CD117-CD161+(ILC1) and CD3-56-CD117+CD161+NKp46+ (ILC3) lymphocytes. Frequencies of both ILC subsets were extremely low within the hepatic lymphocyte population (1.2% ILC1, 0.06% ILC3) and were comparable to those observed in the peripheral blood (**Fig 3-9**). Therefore the human adult liver does not appear to be enriched in ILCs.

Figure 3-9 Frequencies of ILC1 and ILC3 subsets within the hepatic and peripheral blood lymphocyte populations

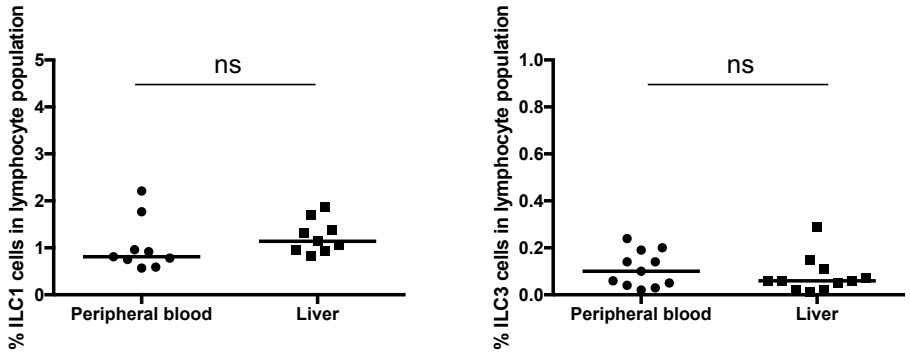


Fig 3-9 A comparison of the frequency of ILC1 (CD3-CD56-CD117-CD161+) (*n=9, n=9*) and ILC3 CD3-56-CD117+CD161+NKp46+ (*n=11, n=12*) subsets within the hepatic and peripheral blood lymphocyte populations. Dot plots display median values. (*Wilcoxon-matched pairs test*).

3.6 The human liver contains CXCR6+ and CD49a+ NK cells

Following phenotypic analysis of paired peripheral blood and liver samples I identified both CD49a+ and CXCR6+ NK cell populations in humans. Hepatic CXCR6+ NK cells were found in all individuals, with a median frequency of 59.5% (range 17.4 – 91.1%), whereas CD49a+ NK cell frequencies were lower, median frequency 7.8% (range 2.3 - 69.0%) (**Fig 3-10**). Both subsets were virtually absent from the peripheral blood; CXCR6+ NK cells 4.0% (range 0.5 – 29.5%) and CD49a+ NK cells 2.0% (range 0.3 – 12.0%) (**Fig 3-10**). Frequencies of CXCR6+ and CD49a+ NK cells were similar whether perfusion or tissue digestion isolation techniques were used (CD49a 7.8%, 9.6%, $p>0.05$; CXCR6 59.5%, 53.1%, $p>0.05$ respectively) **(Fig 3-10**).

Only 4/36 individuals (11%) had high frequencies of hepatic CD49a+ NK cells (defined as greater than 30%), compared to 32/35 (91.4%) for CXCR6+ NK cells **(Fig 3-10**). Thus CXCR6+ NK cells represent the dominant liver-specific NK cell sub-population. Separation of hepatic NK cell subsets into CD49a+CXCR6+, CD49a+CXCR6-, CD49a-CXCR6+ and CD49a-CXCR6- populations demonstrated that only 4.1% of hepatic NK cells were CD49a+CXCR6+ 'double-positive' (range 1.5 – 41.7%) with a third of individuals (9/28) displaying higher frequencies (6.3 - 41.7%), dictated by CD49a expression (**Fig 3-10**). The majority of NK cells in the human liver were CXCR6+CD49a- (41.9%) or CXCR6-CD49a- (31.7%) (**Fig 3-10**). Out of the four patients with significant enrichment of hepatic CD49a+ NK cells, two had hepatocellular carcinoma (i.e. 50%, compared to 10% (5/49) for individuals with < 30% hepatic CD49a+ NK cells), one had an aggressive colorectal cancer with synchronous lesions and bi-lobar liver metastases and the forth had colorectal metastases extending to the resection margins (**Table 14)**. Therefore more aggressive disease associated with an inflammatory cytokine environment may lead to expansion of CD49a+ NK cells in the liver. All these patients were also over the age of 70 however and it is possible that age or CMV status may also play a role in the generation of CD49a+ NK cells in these individuals.

Figure 3-10 CD49a+ and CXCR6+ NK cells are found in the human liver

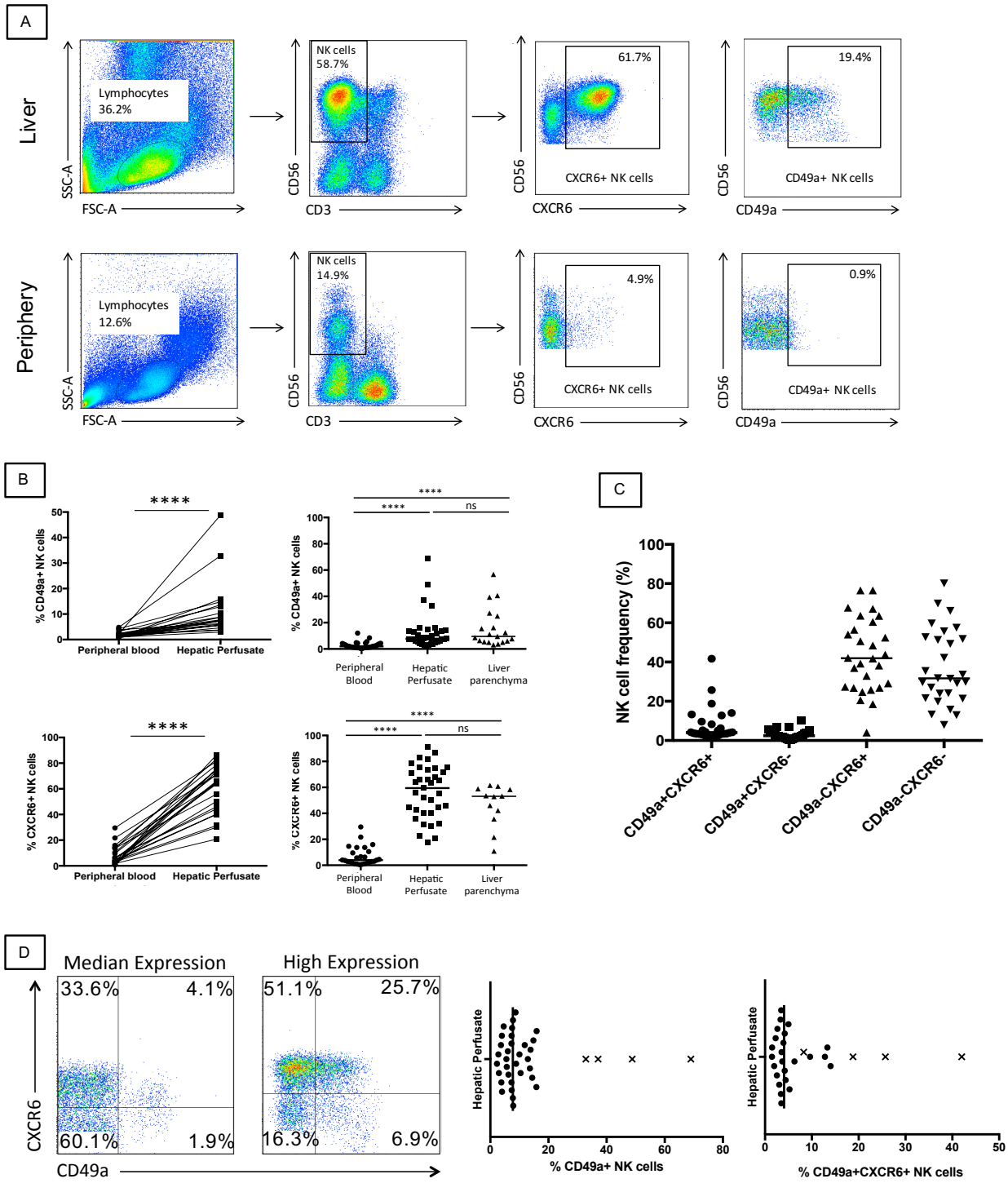


Fig 3-10 **A)** Representative flow cytometry plots showing gating strategy and individual frequencies of CD49a+ and CXCR6+ NK cell populations within the peripheral blood and hepatic perfusate. **B)** A comparison of the frequency of CXCR6+ (*n=23*) and CD49a+ (*n=21*) NK cells within the peripheral blood and hepatic perfusate (paired samples), and peripheral blood, hepatic perfusate and liver parenchymal (paired and unpaired samples, CD49a *n=36, n=36, n=19;* CXCR6 *n=27, n=35, n=12)*. Dot plots display individual values (*Wilcoxon matched pairs test*) for paired samples, and median (*Mann Whitney U test) for non-paired samples.* **C)** Frequencies of CD49a+CXCR6+, CD49a+CXCR6-, CD49a-CXCR6+ and CD49a-CXCR6- NK cell subsets in the human liver (*n=28*). Dot plot displays median. **D)** Representative flow cytometry plots gated on NK cells showing examples of individuals with average and high frequencies of CD49a+CXCR6+ NK cells. Distribution of frequencies of CD49a+ (*n=36*) and CD49a+CXCR6+ (*n=28*) NK cells within the hepatic lymphocyte population. Individuals with high frequencies of CD49a+ NK cells are plotted with a cross. Dot plot displays individual values and median. p<0.0001****.

Table 14 Phenotypic description of patients with high levels of CD49a+ hepatic NK cells

ID	Age	Sex	Underlying pathology	Background liver histology	Operation	Co-morbidities	Pre-op Chemotherapy	% CD49a NK cells
A20	76	Male	HCC (grade 2/3), vascular invasion, clear of resection margin, pT2 pN0,	Normal	Left hemi-hepatectomy & cholecystectomy	Aortic aneurysm T2 diabetes	No	69.0%
A24	79	Male	CRC, right lobar metastases, focally extends to margin	Minimal steatosis and mild nodular regenerative hyperplasia	Right posterior sectionectomy, wedge excision of the liver, cholecystectomy	High cholesterol Hypertension	Oxaliplatin / Capecitabine / Bevacizumab	37.1%
TH10	76	Male	Moderately differentiated HCC, completely excised.	Steatohepatitis & macronodular cirrhosis		Obesity	No	48.8%
TH34	72	Male	Synchronous colon cancers (splenic flexure & sigmoid), liver metastases in both lobes, local excision complete	Normal	Extended right hepatetomy & cholecystectomy (2 lesions remained requiring radio-frequency ablation)	T2 diabetes Gout Hypertension Ischaemic stroke	Folfox / Cetuximan	32.9%

3.7 Hepatic CD49a+ and CXCR6+ NK cells are phenotypically and functionally distinct

Both CD49a and CXCR6 are markers of NK cell memory in mice. Therefore to determine whether CD49a+ and CXCR6+ NK cells might represent 'memory-like' NK cells in humans, I compared their expression of markers of maturity and function. Liver-resident CXCR6+ NK cells have an immature phenotype. They are $CD56^{bright}$ (**Fig 3-10**), and contain low frequencies of CD16+ (25.9%) and CD57+ (6.4%) NK cells. They nearly all express CD161+ (97.2%) however, a marker associated with stage 4 of maturation. CXCR6+ NK cells have higher frequencies of cells expressing surface markers associated with liver-residency, such as CD69 (69.8%) and CD49a (8.9%) compared to CXCR6- NK cells (39.3%, 7.1% respectively). However, there is significant donor variability (**Fig 3-11**). NK cell surface receptors are known to display considerable variation between individuals and in the same individual overtime[253]. This can be influenced by disease status, previous CMV infection, gender and age, with the greatest variability being observed for the percentage of cells expressing a receptor, rather than cell surface density of a particular marker, i.e. the MFI [253]. Frequencies of KIR+ NK cells (4.9%) were significantly lower in comparison to CXCR6- NK cells (14.7%) (**Fig 3-11**).

Conversely the liver-resident CD49a+ subpopulation contained similar frequencies of CD16+ (44.5%) and reduced percentages of CD161+ (74.8%) NK cells compared to hepatic CD49a- NK cells (50.5%, 91.5% respectively). This suggests they are relatively immature compared to NK cells found in the peripheral blood, but more mature than the majority of liver-resident CXCR6+ NK cells. Similarly CD49a+ populations contained fairly comparable frequencies of NK cells expressing the traditional marker of terminal differentiation, CD57 compared to CD49a- NK cells (18.4% vs. 21.8%) (**Fig 3-11**). Interestingly despite over 50% of CD49a+ NK cells being CD16-, CD49a+ NK cells were more likely to be KIR+ (18.3% vs 10.1%, $p<0.05$) or NKG2C+ (10.3% vs 4.8%, $p<0.001$) in comparison to CD49a- NK cells, suggesting a subset of these cells may be licenced and products of previous clonal expansion (**Fig 3-11**). Frequencies of CD69+ (65.4%) and CXCR6+ NK cells (70.8%) were both high within the CD49a+ NK cell population, consistent with a liver-resident phenotype **(Fig 3-11).** Finally, human CD49a+ NK cells found in both the liver and blood did not express CD49b (3.2% vs. 2.9%).

Figure 3-11 Phenotypic analysis of liver-resident CXCR6+ and CD49a+ NK cells

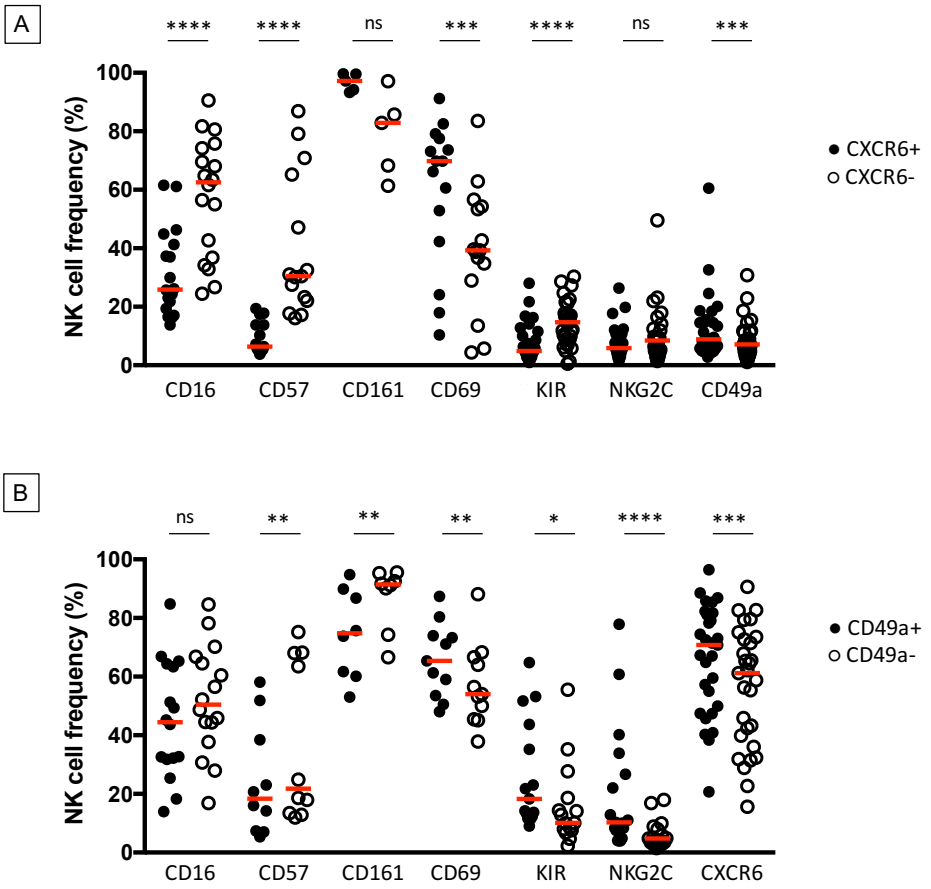


Fig 3-11 **A)** Comparison of the frequency of CD16+ 25.9%, 62.5% (*n=18*), CD57+ 6.4%, 30.5% (*n=15*), CD161+ 97.2%, 82.9% (*n=5*), CD69+ 69.8%, 39.3% (*n=15*), KIR+ 4.9%, 14.7% (*n=36*), NKG2C+ 5.9%, 8.5% (*n=27*) and CD49a+ 8.9%, 7.1% (*n=29*) NK cells within the hepatic CXCR6+ and CXCR6- NK cell populations. Dot plot displays median values. (*Wilcoxon matched pairs test*). **B)** Comparison of the frequency of CD16+ 44.5%, 50.5% (*n=16*), CD57+ 18.4%, 21.8% (*n=10*), CD161+ 74.8%, 91.5% (*n=8*), CD69+ 65.4%, 54.1% (*n=11*), KIR+ 18.3%, 10.1% (*n=15*), NKG2C+ 10.3%, 4.8% (*n=17*) and CD49a+ 70.8%, 61.2% (*n=29*) NK cells within the hepatic CXCR6+ and CXCR6- NK cell populations. Dot plot displays median values. (*Wilcoxon matched pairs test*). p<0.05*, p<0.01**, p<0.001***, p<0.0001****.

Analysis of CD49a+CXCR6+, CD49a+CXCR6-, CD49a-CXCR6+ and CD49a-CXCR6- hepatic NK cell subsets demonstrated that CXCR6+ populations harboured low frequencies of CD16+ and CD57+ NK cells, and high frequencies of CD69+ NK cells, independent of CD49a status (**Figure 3-12**). CD49a+ NK cells were more likely to be NKG2C+ or KIR+ compared to CD49a- NK cells, irrelevant of CXCR6 expression. Therefore, CD49a+CXCR6+ NK cells were CD69+CD16^{low}CD57^{low} with a higher frequency of KIR+ and NKG2C+ NK cells compared to CD49a- NK cells (**Figure 3-12**).

Figure 3-12 Phenotypic comparison of CD49a+CXCR6+, CD49a+CXCR6-, CD49a-CXCR6+ and CD49a-CXCR6- NK cells

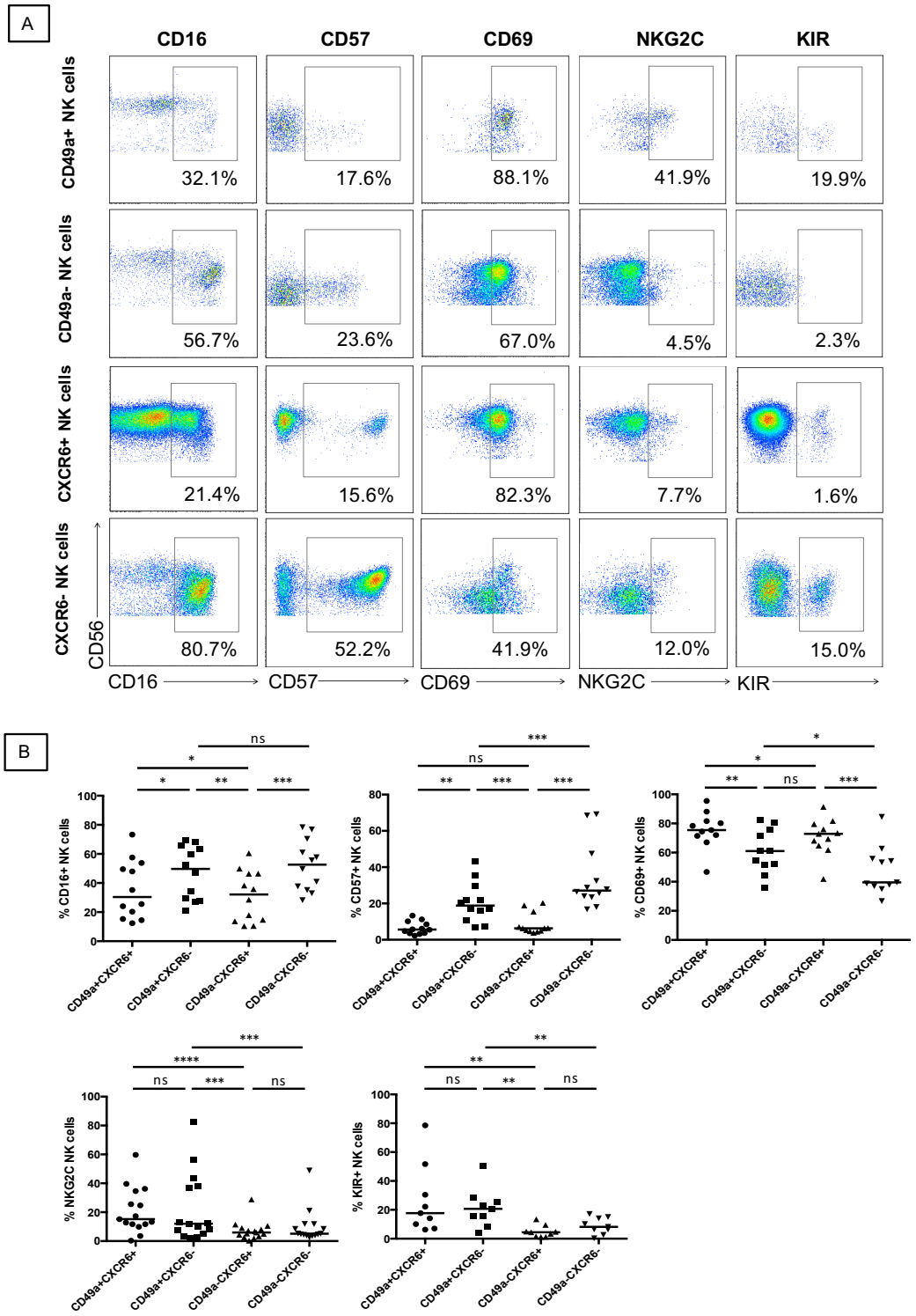


Fig 3-12 **A)** Representative flow cytometry plots gated on CD49a+/- and CXCR6+/- NK cells showing frequency of CD16, CD57, CD69, NKG2C and KIR+ NK cells. **B)** Comparison of frequency of CD16 (*n=12*), CD57 (*n=12*), CD69 (*n=11*), NKG2C (*n=22*) and KIR+ (*n=9*) NK cells between liver-resident subpopulations CD49a+CXCR6+, CD49a+CXCR6-, CD49a-CXCR6+ and CD49a-CXCR6- (*Wilcoxon matched pairs test*). p<0.05*, p<0.01**, p<0.001***, p<0.0001****.

Following stimulation with IL-12 10ng/ml or IL-15 1ng/ml, overnight, hepatic NK cells expressing CD49a produced greater quantities of IFNγ (7.5% vs. 1.6%, $p<0.05$) and TNFα (22.5 vs. 2.4%, $p<0.05$) compared to CD49a- NK cells (**Fig 3-13**). In CD49a+ NK cells IFNγ production remained high independent of CXCR6 expression. TNFα expression was particularly high in CD49a+CXCR6+ NK cells (**Fig 3-13**). Thus double-positive CD49a+CXCR6+ cells behave more like single-positive CD49a 'adaptive' NK cells, than single-positive 'liver-resident' CXCR6+ NK cells.

Figure 3-13 Th1 cytokine profile of CD49a+ and CXCR6+ NK cells

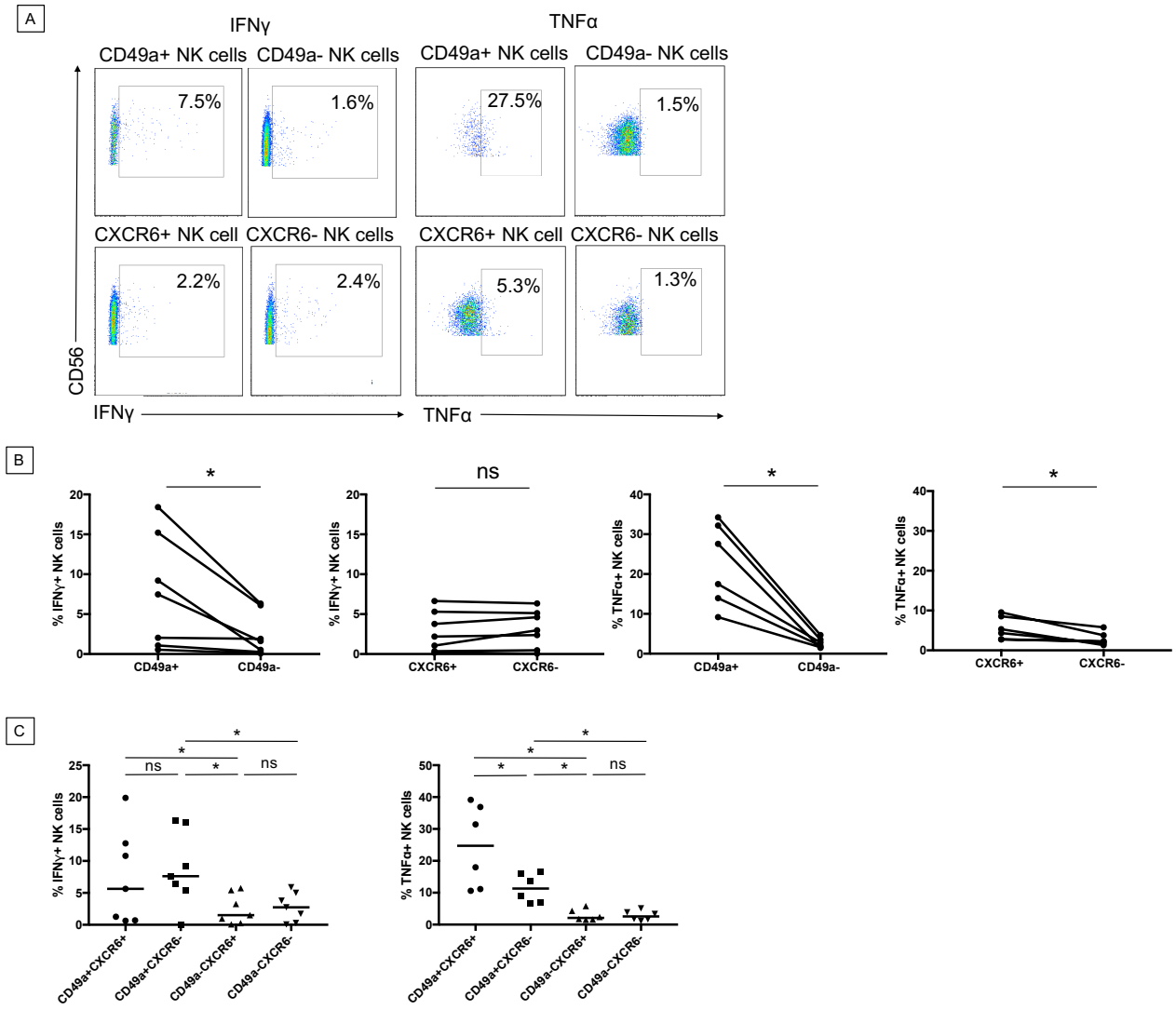


Fig 3-13 **A)** Representative flow cytometry plots gated on CD49a+/- and CXCR6+/- NK cells showing IFNγ and TNFα expression. **B)** Percentage of IFNγ+ (*n=7*) and TNFα+ (*n=6*) NK cells within the hepatic CD49a+, CD49a-, CXCR6+ and CXCR6- NK cell subsets following stimulation with IL-12 10ng/ml and IL-15 1ng/ml for 12 hours respectively. Dot plots display individual values. (*Wilcoxon matched pairs test).* **C)** Percentage of IFNγ+ (*n=7*) and TNFα+ (*n=6*) NK cells within the hepatic CD49a+CXCR6+, CD49a+CXCR6-, CD49a-CXCR6+ and CD49a-CXCR6- NK cell subsets following stimulation with IL-12 10ng/ml and IL-15 1ng/ml for 12 hours respectively. Dot plots display median. (*Wilcoxon matched pairs test).* p<0.05*.

3.8 Transcriptomic analysis of CXCR6+ and CXCR6- NK cells

To investigate the transcriptional profile of the CXCR6+ and CXRCR6- NK cells, I sorted liver-resident CXCR6+ and CXCR6- NK cell subsets from three individuals and collaborated with the Department of Primate Genetics, Göttingen, who performed RNA sequencing analysis on these samples (**Table 12, Fig 2-3**, **Fig 3-14, Appendix Fig 1 and 2**). My findings were consistent with those recently reported by Cuff et al comparing $Eomes^{high}$ (CXCR6+) and $Eomes^{low}$ (CXCR6-CD16+) liver-resident NK cells [33].

Transcriptional profile

Consistent with the current literature the transcriptional profile of CXCR6+ NK cells was confirmed as T-bet-Eomes+ [29]. Transcription factors GATA3 (required for Th2 and ILC2 differentiation) and FOXP3 (controls T-regulatory cell development) were also more highly expressed in comparison to CXCR6- NK cells. The Hobit-Blimp1 transcriptional module has recently been identified as a universal regulator of transcription in mice that instructs retention of tissue-resident memory lymphocytes, including liver-resident CD49a+ NK cells [254]. Conventional liver-resident and circulating NK cells were dependent on Blimp-1 (PRDM1), whereas CD49a+ liver-resident NK cells were dependent on Hobit (ZNF683) [254]. In humans CXCR6+ NK cells are the dominant liver-resident population, however we did not detect any expression of Hobit in CXCR6+ NK cells from any of the three patients examined, and only small amounts in CXCR6- NK cells. Expression of Blimp-1 was downregulated in CXCR6+ compared to CXCR6- NK cells. Therefore murine and human transcriptional regulators of tissue retention appear to differ.

NK cell surface receptors

Consistent with my flow cytometry findings, CXCR6+ NK cells were immature ($CD56^{bright}CD16^{low}CD57^{low}$) compared to liver-resident CXCR6- NK cells, with reduced expression of both inhibitory and activating KIR. They also had low expression of both activating (NKp30, NKp44, CD94, DNAM1) and inhibitory receptors (siglec-7, siglec-9, LILRB1).

Liver-residency

CXCR6+ NK cells highly expressed markers CD69 and CD103 known to be associated with tissue-residency [27,255]. They were also CD49e-, a phenotype recently described to denote liver-resident NK cells by the Yokoyama group [256], and expressed high levels of the TNF-receptor family member, CD27. This surface marker appears to be associated with tissue residency, and has been identified on NK cells in the decidua [257], and those in primary and secondary lymphoid tissue [167,258] but is absent from the peripheral blood. It has been associated with reduced cytotoxicity and is regulated by IL-15 [258]. CXCR6+ NK cells showed upregulation of genes which support their migration towards, and long-term residence in the liver, including preferential expression of the chemotactic receptors CC-chemokine receptor-like (CCRL) 2 and CCR5, for which the ligands include CCL3 (MIP-1α), CCL4 (MIP-1β), CCL3L1 and CCL5 (RANTES). This suggests there may be further recruitment of CXCR6+ NK cells to the liver in the presence of inflammation during which these ligands are upregulated on kupffer cells (CCL3) and T-cells (CCL5) [259]. CXCR6+ NK cells also displayed upregulation of adhesion (Intracellular Adhesion Molecule (ICAM1), Neural Cell Adhesion Molecule (NCAM), CD49a, neogenin1, cadherin1, actin4, PALS1-associated TJ protein (PATJ)) and nectin-like molecules (CD96, T-cell Immunoreceptor with Immunoglobulin and ITIM domains (TIGIT), Cytotoxic and Regulatory T-cell Molecule (CRTAM)), which may contribute to their long-term residence within the liver. Furthermore, CXCR6+ NK cells showed downregulation of signalling proteins which promote migration of lymphocytes out of tissue into the circulation (CCR7, S1PR1, PXN (paxillin), ITGAM) (**Fig 3-14**). Of note I did not observe any over-expression of CD2, recently identified to be critical for the adaptive behaviour of the NKG2C+CD57+ NK cells in hCMV [260].

Cytokine profile

CXCR6+ NK cells were found to have low expression of IFNγ and TNFα, consistent with my flow cytometry data, in addition to markers of degranulation (perforin, granzyme A and B, granulysin) other than TRAIL. This was confirmed at a protein level by Stegmann et al [29]. The CXCR6+ NK cell population did however show expression of GM-CSF and CCL3. This suggests this subgroup may be able to recruit other immune cells to the liver during disease. Enhanced expression of Th2 cytokines IL-4 and IL-10 was also seen, consistent with the immature status of CXCR6+ NK cells. However, the quantity of gene transcripts was too low to allow a definitive conclusion. Liver-resident CXCR6+ NK cells highly expressed Leukaemia Inhibitory Factor (LIF), known to play a role in mediating tolerance following transplantation and during embryonic implantation [261,262].

Finally, to determine the potential for CXCR6+ liver-resident NK cells to respond to cytokines used to generate 'memory-like NK cells' in the blood, we assessed signalling pathways for IL-2, IL-12, IL-15 and IL-18. We observed upregulation of IL-12RB2 and IL-23R gene transcripts, however IL-12RB1, which pairs with IL-23R was downregulated compared to CXCR6- NK cells. While liver-resident CXCR6+ NK cells appear to have a tolerant phenotype, RNA levels of STAT4 and transcripts coding for STAT4 associated signalling proteins, JAK2, Tyk2 and SH2B3 were not downregulated as seen in tolerant NK cell phenotypes in the peripheral blood following liver transplantation [46]. There was no consistent significant differential expression of other receptors or downstream signalling molecules within any of the other cytokine pathways examined (**Figure 3-14**).

Figure 3-14 Summary of RNA-sequencing data of liver-resident CXCR6+ and CXCR6- NK cells

	Gene	Expression in liver resident CXCR6+ vs CXCR6- NK cells
NK surface receptors and signalling	CD57 (B3GAT1)	0.00
	CD16 (FCGR3A)	0.10
	CD117 (KIT)	0.16
	CD161 (KLRB1)	2.34
	Nkp46 (NCR1)	1.53
	Nkp44 (NCR2)	0.17
	Nkp30 (NCR3)	0.44
	NKG2C (KLRC2)	0.87
	DNAM-1 (CD226)	0.18
	CD94 (KLRD1)	0.63
	LILRB1	0.01
	SIGLEC-7	0.54
	SIGLEC-9	0.06
	KIR2DL1	0.02
	KIR2DL3	0.13
	KIR3DL1	0.05
	KIR2DS4	0.03
	KIR3DL2	0.19
	SYNJ2	0.04
	LAIR1	0.08
	AKT3	0.21
	CD2	1.30
Adhesion panel	ICAM1	3.94
	CD56 (NCAM1)	1.56
	CD103 (ITGAE)	2.73
	Neo1	4.92
	CDH1	5.07
	CDHR1	6.28
	PDZD (PATJ)	4.14
	ACTN4	1.62
	CD96	1.69
	TIGIT	3.15
	CRTAM	3.97
Lymphocyte tissue regression	NCF1	2.59
	TCF7	2.39
	ACTN1	4.53
	CCR7	0.04
	KLF2	0.31
	S1PR1	0.01
	PDGFRB	0.04
	PXN	0.02
	VCL	0.05
	SELPGP	0.07
	ITGAM	0.36
	TIMP1	0.41
Upregulation <----------> Downregulation		

	Gene	Expression in liver resident CXCR6+ vs CXCR6- NK cells
Cytokine panel	IFNγ	0.77
	TNFα	0.86
	IL-4	4.59
	IL-10	1.38
	IL-13	0.70
	MIP-1α (CCL3)	1.95
	GM-CSF (CSF2)	6.64
	LIF	7.98
Cytotoxicity panel	Perforin1 (Prf1)	0.38
	GranzymeA	0.89
	GranzymeB	0.03
	GranzymeK	6.19
	Granulysin (GNLY)	0.02
	TRAIL (TNFSF10)	1.41
Transcription Factors	T-bet (TBX21)	0.32
	Eomes	2.51
	HOBIT (ZNF683)	0.00
	Blimp-1 (PRDM1)	0.44
	GATA3	1.92
	HELIOS (IKZF2)	0.45
	FOXP3	6.75
Cytokine signalling	IL-12 RB1	0.27
	IL-12 RB2	2.49
	IL-23 R	6.39
	JAK2	0.76
	Tyk2	1.00
	SH2B3	1.33
	STAT4	1.03
	IL-15RA	1.07
	IL-2 RB	3.31
	JAK1	0.78
	JAK3	0.74
	STAT1	0.75
	STAT3	0.75
	STAT5A	1.32
	STAT5B	1.00
	IL-18R1	0.70
	IL-18 RAP	0.48
	IRAK1	1.05
Tissue residency	CD69	2.05
	CD27	17.63
	CD49a (ITGA1)	1.98
	CD49e (ITGA5)	0.02
Chemotactic receptors	CCR5	6.67
	CCRL2	4.20
	CX3CR1	0.00
	CXCR1	0.03
	CCR7	0.04

Fig 3-14 RNA-sequencing data displaying differential expression of genes in CXCR6+ compared to CXCR6- NK cells isolated from the liver perfusate from paired samples (*n=3*). All 3 patients underwent resection for colorectal metastases and had either a normal background liver or mild steatosis. All modules are included. Genes are grouped according to function. Up or down-regulation is displayed using red and blue according the fold difference in the mean quantity of gene transcripts between the CXCR6+ and CXCR6- samples (≤ 0.5 blue, > 0.5-0.90 light blue, > 0.90-1.1 white, ≥ 1.1-1.9 light red, ≥ 2.0 red).

3.9 Discussion

This chapter provides a comprehensive phenotypic and functional comparison of NK cells isolated from the human liver and peripheral blood. Analysis of over 50 tissue samples (30 paired) revealed the liver to be rich in both NK cells (38% of lymphocytes isolated from the hepatic perfusate and 33% of lymphocytes isolated from the liver parenchyma) and innate CD56+ T-cells, compared to the peripheral blood. Surface expression of markers of maturation, activation and potential 'memory' markers were generally comparable for NK cells isolated from the sinusoids (hepatic perfusate) and parenchyma, although the sinusoids were found to contain a higher frequency of CD16- NK cells. These findings are consistent with the current literature [11,15].

The majority of liver-resident NK cells did not express the classical markers of maturation and terminal differentiation, CD16 and CD57, in contrast to NK cells isolated from the peripheral blood. Expression of KIR (KIR2DL1, 2DS1, 2DL2, 2DL3) was also low. Despite being rich in more immature stage 4 CD16- NK cells, high frequencies of NK cell precursors were not detected in the liver. It is likely that relatively immature $CD56^{bright}CD16^{-}$ NK cells may be shaped by the hepatic environment to differentiate into phenotypically distinct tissue-resident NK cells. Nearly all liver-resident NK cells were found to be CD161+ and just over half expressed the activation receptor CD69.

While NK cells are classically members of the innate immune system, selected populations in mice and macaques display memory towards haptens [19,34,170] and viral antigens [170,174,176,180]. The chemokine receptor, CXCR6 [34] and adhesion molecule CD49a [19] have been identified as surface markers of memory NK cells in mice capable of mediating antigen-specific delayed-type hypersensitivity reactions. Interestingly these features were generally limited to liver-resident NK cells. In this chapter I provide a description of CD49a+ [35] and CXCR6+ NK cells [11,15,29] in the human liver and define two distinct phenotypes. Both populations are generally absent from the peripheral blood in people.

Liver-resident CXCR6+ NK cells are immature ($CD56^{bright}CD16^{low}CD57^{low}$), $KIR^{low}CD69^{high}$ and are found in all individuals, comprising nearly two thirds of hepatic NK cells. They harbour similarly low frequencies of NKG2C+ NK cells to that of liver-resident CXCR6- NK cells. Overall CXCR6+ NK cells do not contain high percentages of IFNγ or TNFα-producing cells, however they do express GM-CSF, IL-4, CCL3 and LIF. While human CXCR6+ NK cells do not display phenotypic or functional features consistent with 'memory' as in mice, CXCR6 may play a role retaining NK cells

within the liver via its interaction with the chemokine ligand 16 (CXCL16), expressed on HSECs, hepatocytes and cholangiocytes [32]. RNA sequencing revealed co-expression of CCR5 and a large number of adhesion molecules, which may promote translocation to, and long-term residence in the liver. Deep sequencing results also show reduced expression of granzymes and perforin, suggesting CXCR6+ NK cells are likely to have impaired cytotoxicity. These findings has since been confirmed at a protein level using flow cytometry [29]. Transcriptomic and functional data presented here therefore support the role of CXCR6+ innate NK cells in establishing hepatic immunotolerance and homeostasis with minimal expression of activating and inhibitory KIR, markers of degranulation and Th1 cytokines. RNA sequencing data confirmed the transcriptional profile of liver-resident CXCR6+ NK cells as $Eomes^{high}T\text{-}bet^{low}$ [11,29].

Conversely, hepatic CD49a+ NK cells were only found at substantial frequencies in 10% of individuals, suggesting a genetic or environmental influence may lead to the emergence of this population. It may therefore be the case that this subset is not found in completely healthy individuals with no history of significant disease. The CD49a+ NK cell subset contains higher frequencies of KIR+ and NKG2C+ NK cells compared to the CD49a- population, suggesting some of these cells may be licensed and have undergone previous clonal expansion. While I did not stratify for CMV, no correlation was demonstrated between seropositivity and hepatic CD49a+ NK cell frequencies by Marquardt el at [35]. Although they are not terminally differentiated (CD16+/-$CD57^{low}$), CD49a+ NK cells are more mature than the majority of CXCR6+ liver-resident NK cells. CD49a+ NK cells also produced high quantities of both IFNγ and TNFα. Of the four patients in this cohort with significant enrichment of hepatic CD49a+ NK cells, two had hepatocellular carcinoma, one aggressive colorectal cancer with synchronous lesions and bi-lobar liver metastases and the forth had colorectal metastases extending to the resection margins. This suggests exposure to an inflammatory cytokine microenvironment may lead to expansion of CD49a+ NK cells. Liver-resident CD49a+ NK cells therefore appear to express phenotypic and functional features suggestive of memory; including evidence of possible clonal expansion following priming through cytokine exposure, or possibly antigen presentation, and enhanced function. However further assays looking at enhanced proliferation, and a boosted functional response on secondary exposure to an activating stimulus would be required to demonstrate this more definitely. While I did not examine transcription factor expression for this subset, liver-resident CD49a+ NK cells have been found to express a unique transcriptional profile (T-bet+Eomes-)[35] compared to conventional liver-resident NK cells (Eomes+T-bet-) [11,29]. Liver-resident CD49a+ NK cells may therefore be generated from T-bet+Eomes- NK cell precursors either in situ, or elsewhere, or may be a product of differentiation of conventional liver-resident NK cell which have developed this unique transcriptional signature in response to local environmental stimuli.

Double-positive CD49a+CXCR6+ NK cells comprise less than 5% of the overall NK cell population in the human liver (range 1.5% - 41.7%). While they have some features of immaturity ($CD16^{low}CD57^{low}$), CD49a+CXCR6+ populations contain similar frequencies of KIR+ and NKG2C+ NK cells to single positive liver-resident CD49a+ NK cells, and importantly produce similar quantities of IFNγ and TNFα. They therefore function more like single positive CD49a+ NK cells than single positive CXCR6+ NK cells. It is possible that the double-positive CD49a+CXCR6+ population represents a developmental intermediary population between CXCR6+CD49a- and CXCR6-CD49a+ NK cells.

This chapter identifies two distinct liver-resident NK cell subpopulations. CXCR6+ NK cells are the predominant NK cell population in the human liver, and may play a role in maintaining hepatic immunotolerance and liver-homing. CD49a+ NK cells are highly functional and appear to represent a liver-resident adaptive population of NK cells.

This work has some limitations however. Firstly it was not possible to sample completely healthy liver tissue. While every effort was made to take tissue as distal from the tumour margin as possible, the presence of metastases could have affected the phenotype and function of the NK cells examined. Furthermore just less that 10% of patients had fibrosis or cirrhosis. It would also have been extremely beneficial to have assessed the CMV serological status of all individuals examined, particularly to establish if this is associated with higher frequencies of CD49a+ NK cells. Finally given the variability between patient samples it would have been worthwhile to have sorted CXCR6+ and CXCR6- NK cells from a larger number of patients and to have calculated a p-value representing the differential expression of transcript levels between NK cell subpopulations.

4 Chapter 4: The influence of cytokines on liver-resident and peripheral blood NK cells

I hypothesised that given the potential 'memory-like' properties of CD49a+ NK cells, they would proliferate better than their negative counterparts in response to IL-15 and other activating cytokines. Given the potential residence or priming of 'memory-like' NK cells in the liver, I hypothesised that expansion might be greatest in this compartment. Finally I was interested in whether the cytokine cocktail (IL-2, IL-12, IL-15, IL-18), shown to 'prime' human peripheral blood and murine splenic NK cells in vitro [182,183] would be superior to individual cytokines at inducing the proliferation of potential liver-resident memory-like NK cells. I therefore stained paired PBMCs and hepatic MNCs with CFSE and performed cell surface staining on day 0 and day six following incubation with IL-2, IL-12, IL-15, IL-18 and a cocktail of all four cytokines. Samples were donated from individuals who had undergone hepatic resection for primary or secondary liver cancer.

4.1 The differential influence of cytokines on NK cells isolated from the peripheral blood and liver

Proliferation assays of paired peripheral blood and liver MNCs were performed on 17 individuals following IL-15 stimulation, and 9 individuals following stimulation with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail. Incubation of PBMCs with both IL-2, IL-15 and the cytokine cocktail led to a significant expansion of NK cell frequencies within the lymphocyte population and absolute numbers (**Fig 4-1, Fig 4-2**). This appeared to be driven by expansion of the $CD56^{bright}$ compartment, perhaps due to the increased capacity of more immature NK cells to divide (**Fig 4-2, Fig 4-4**). Conversely IL-18 resulted in contraction of the NK cell population (**Fig 4-1, Fig 4-2).** Both IL-15 and the cytokine cocktail also stimulated expansion of CD56+ T-cells relative to other lymphocyte populations, and IL-18 led to an increase in CD56- T-cell frequencies.

Surprisingly liver-resident NK cells failed to expand in both absolute number, and frequency, following stimulation with any of the activating cytokines examined (**Fig 4-1, Fig 4-3)**. While further expansion of the $CD56^{bright}$ population was observed in the presence of IL-2 and IL-15, this was less compared to the peripheral blood (**Fig 4-3, Fig 4-4)**. Cytokine culture of liver MNCs did not result in a significant change in the composition of the lymphocyte population in terms of NK cell, CD56+ T-cell and CD3-56- lymphocyte frequencies, but a slight increase in the frequncy of CD56- T-cells

following culture with IL-18 was found (**Fig 4-1, Fig 4-3**). Hepatic lymphocyte populations were therefore more stable in vitro in their response to activating cytokines.

Figure 4-1 NK cells isolated from the peripheral blood, but not the liver expand in response to IL-2, IL-15 and the cytokine cocktail

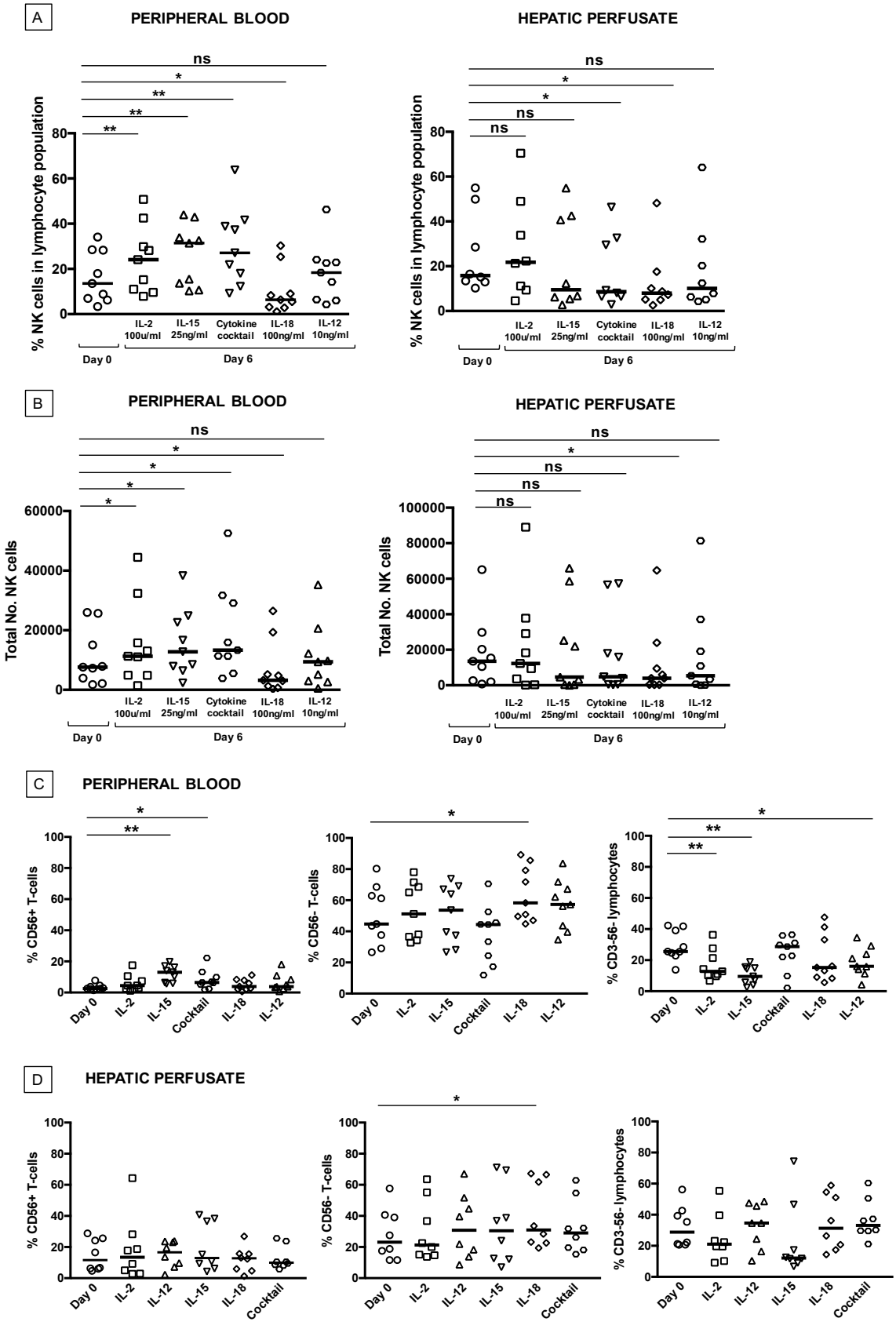


Fig 4-1 **A)** % NK cells within the lymphocyte population in the peripheral blood at rest, 13.6% and following culture with IL-2 24.1%, IL-15 31.5%, cytokine cocktail 27.1%, IL-18 6.4% and IL-12 18.4% (*n=9*); and the liver at rest, 15.9%, and following stimulation with IL-2 21.8%, IL-15 9.5%, cytokine cocktail 8.6% IL-18 8.0% and IL-12 10.1% (*n=8*). **B)** Absolute number of NK cells in the peripheral blood (*n=9*) and liver (*n=8*) at rest and following stimulation with IL-2, IL-15, cytokine cocktail, IL-18 and IL-12. **C)** % CD56+ T-cells, CD56- T-cells and CD3-56- lymphocytes at rest and following stimulation with IL-2, IL-15, cytokine cocktail, IL-18 and IL-12 in the peripheral blood (*n=9*) and **D)** the liver (*n=8*). Significant t-test values only shown when comparing lymphocyte frequencies at rest and post stimulation. Dot plots display median values. (*Wilcoxon matched pairs test).* $p<0.05$*, $p<0.01$**.

Figure 4-2 The influence of activating cytokines on NK cells, CD56+ T-cell, CD56- T-cells and CD3-56- lymphocytes in the peripheral blood

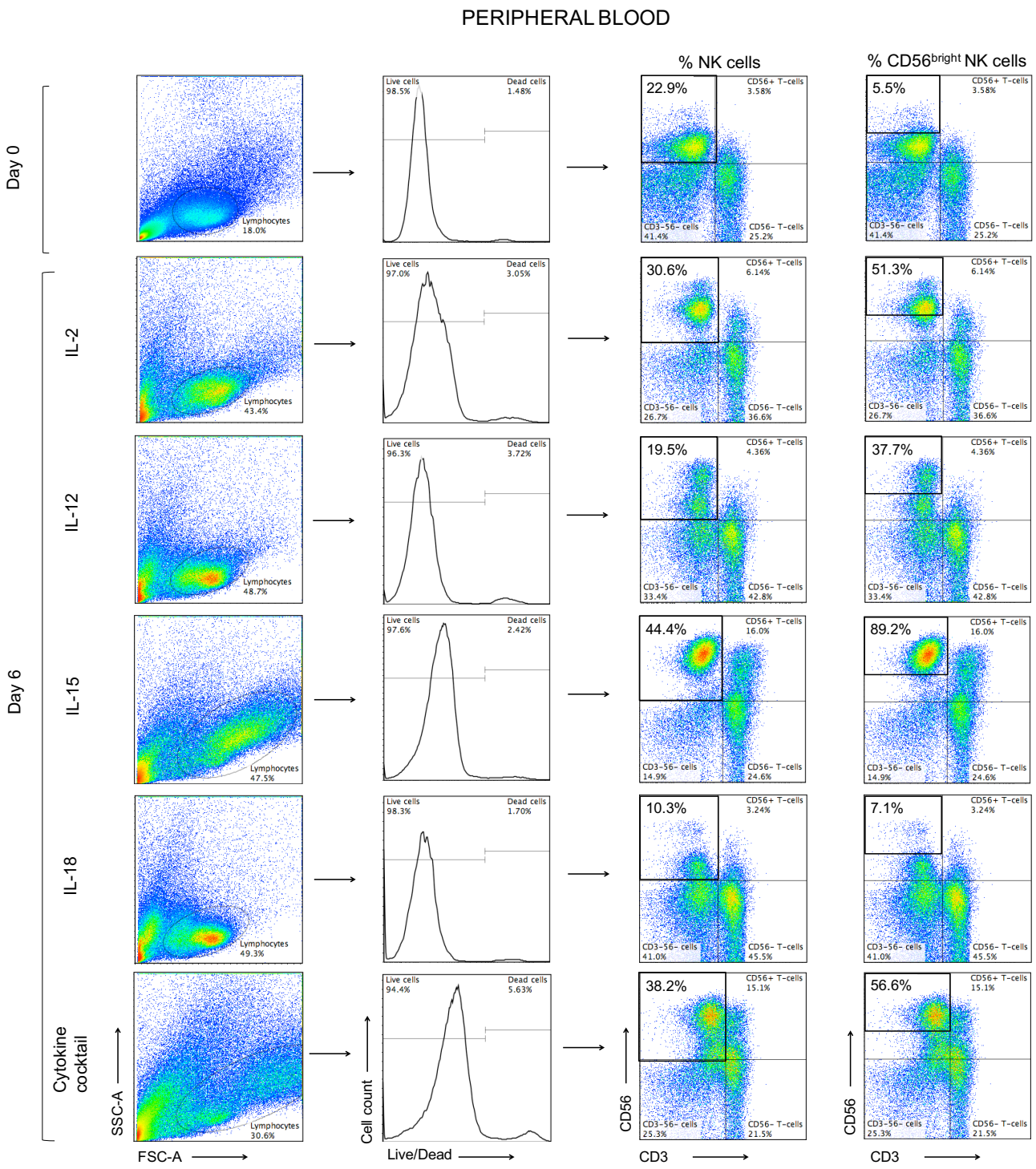


Fig 4-2 Representative flow cytometry plots from donor 'TH45' displaying the frequencies of CD56+ T-cells, CD56- T-cells, CD3-56- lymphocytes, NK cells and CD56^{bright} NK cells within the peripheral blood at rest and following stimulation with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail.

Figure 4-3 The influence of activating cytokines on NK cells, CD56+ T-cells, CD56- T-cells and CD3-56- lymphocytes in the liver

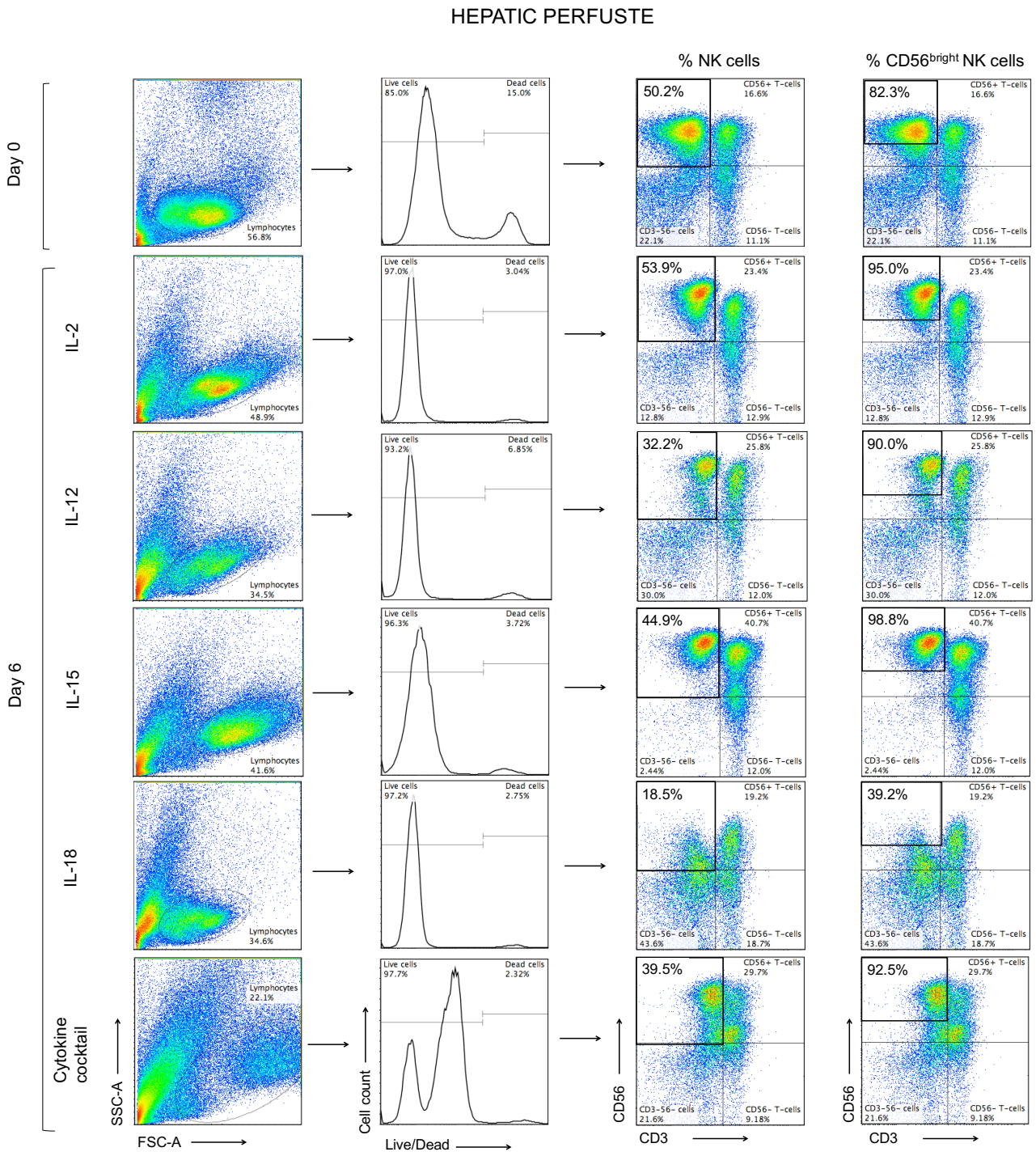


Fig 4-3 Representative flow cytometry plots from donor 'TH45' displaying the frequencies of CD56+ T-cells, CD56- T-cells, CD3-56- lymphocytes, NK cells and CD56^{bright} NK cells within the liver at rest and following stimulation with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail.

Figure 4-4 NK cell expansion in the peripheral blood is driven by expansion of the CD56^{bright} compartment

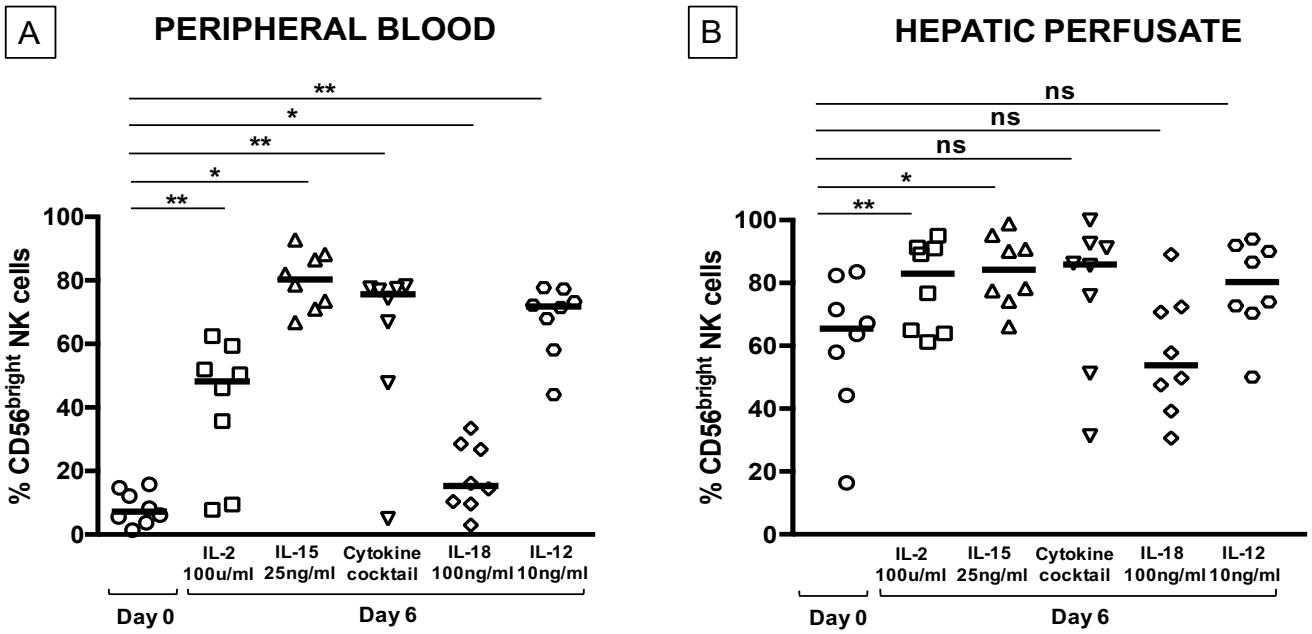
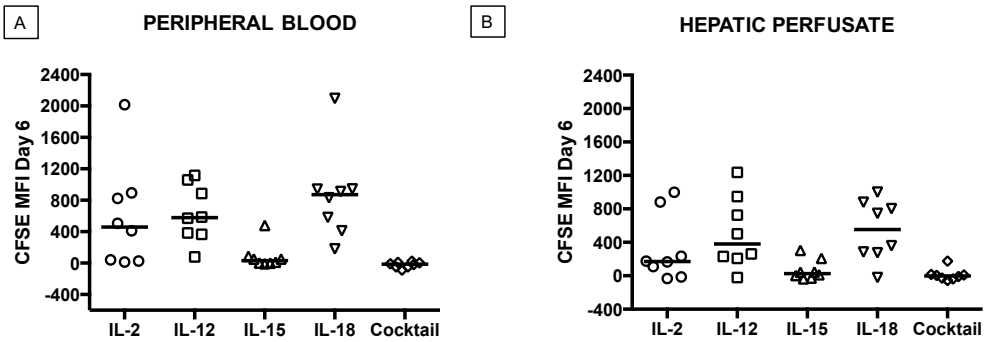


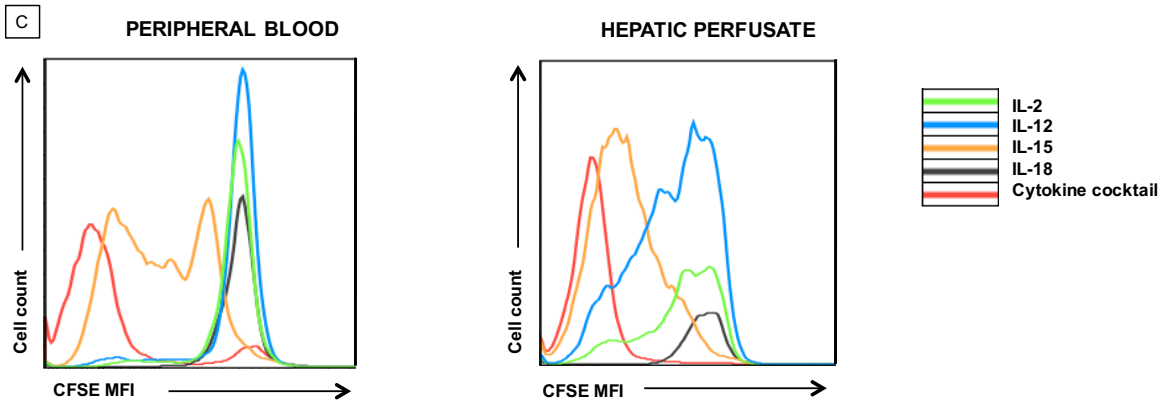
Fig 4-4 Comparison of the % CD56^{bright} NK cells within the NK population in **A)** the peripheral blood at rest, 7.2% and following culture with IL-2 48.2%, IL-15 80.3%, the cytokine cocktail 75.6%, IL-18 15.3% and IL-12 71.8% (*n=8)*; and **B)** the liver at rest 65.3%, and following culture with IL-2 82.9%, IL-15 84.2%, the cytokine cocktail 85.9%, IL-18 53.8% and IL-12 80.3% (*n=8)*. Dot plots display median values. (*Wilcoxon matched pairs test).*

CFSE staining suggested that the increase in absolute numbers of NK cells seen following stimulation of PBMCs with IL-15 and the cytokine cocktail occurs as a result of their enhanced proliferation (**Fig 4-5**). Culture of hepatic MNCs under the same conditions however, did not result in any increase in either NK cell frequencies within the lymphocyte population or absolute numbers. Despite this, CFSE fluorescent intensities of NK cells stained at day 0 decreased to similar extent as observed in the peripheral blood, with IL-15 and the cytokine cocktail resulting in the lowest values. These results suggest that a subpopulation of hepatic NK cells have proliferated under these conditions, however the population as a whole may have failed to expand due to poor viability of hepatic MNCs in culture compared to the peripheral blood. Total numbers of MNCs counted decreased significantly from day three and failed to recover at day six (**Fig 4-6**). Taken together these results suggest that hepatic MNCs decrease in number in vitro, however the liver may contain a sub-population of NK cells which are highly proliferative.

Figure 4-5 IL-15 and the cytokine cocktail induce the strongest proliferation of NK cells isolated from both the liver and peripheral blood



	Day 0		Day 6				
	Lymphocytes	Unstained	IL-2	IL-12	IL-15	IL-18	Cocktail
Peripheral Blood	147100	0.8065	459.6	579.1	30.52	870.9	-13.11
Liver	147100	-1.765	170	381.3	26.91	553.1	-2.58



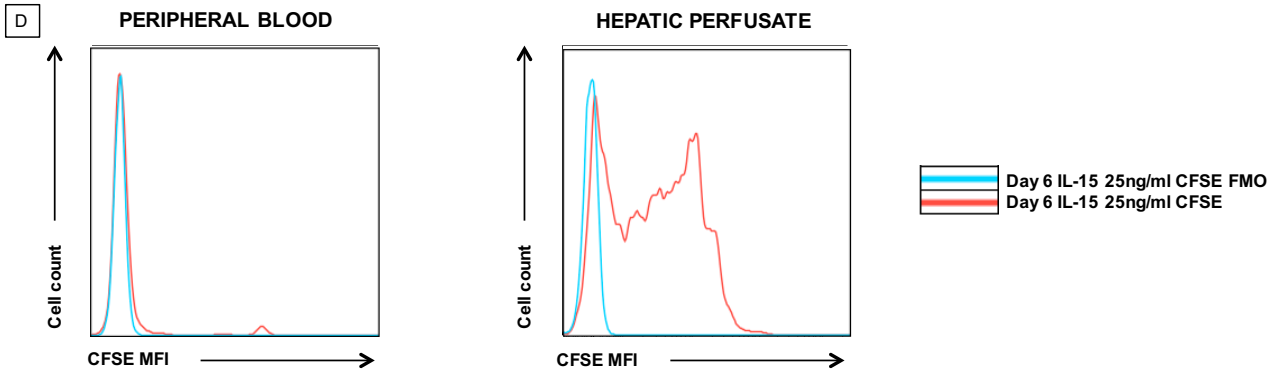


Fig 4-5 Day six CFSE MFI of **A)** peripheral blood and **B)** hepatic NK cells following culture with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail. Median values displayed below (*n=8*). Dot plots display median. **C)** Representative flow cytometry histograms from one individual show CFSE expression at day six following culture with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail. **D)** Representative flow cytometry plot from second individual showing CFSE expression at day six following culture with IL-15 with CFSE FMO.

Figure 4-6 Absolute numbers of hepatic MNCs decrease in vitro

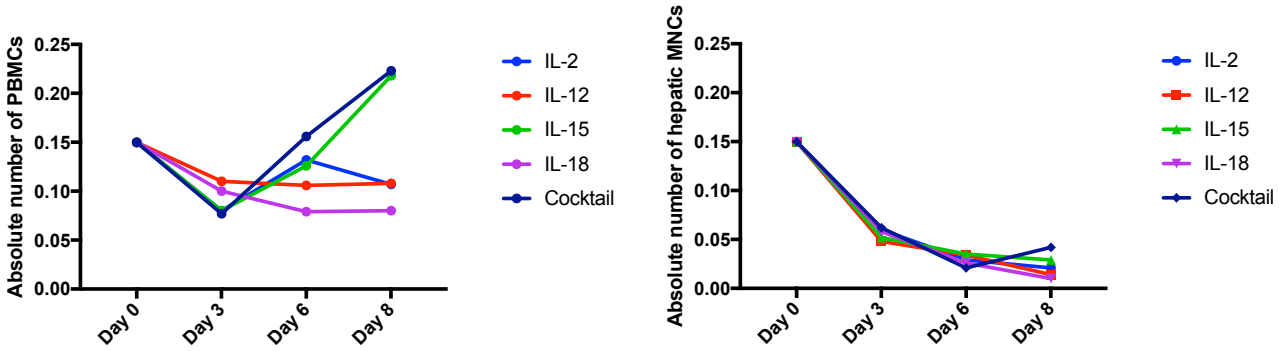


Fig 4-6. A representative count of the absolute number of PBMCs and hepatic MNCs at day 0, 3, 6 and 8 in culture with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (paired sample).

4.2 The influence of cytokines on the induction of liver-homing memory-like NK cell phenotypes in the liver

4.2.1 Culture of hepatic MNCs with activating cytokines leads to an increase in CD49a+ NK cell frequencies, with no further enrichment of the CXCR6+ NK subset

Having identified both CXCR6+ and CD49a+ NK cells in the human liver, I investigated the influence of activating cytokines, particularly the 'adaptive phenotype-inducing' cytokine cocktail (IL-12, IL-15, IL-18 and IL-2) on the induction of potential liver-homing and memory-like NK cells within the liver. Following culture with IL-2 100U/ml, IL-12 10ng/ml, IL-15 25ng/ml, IL-18 100ng/ml or a cytokine cocktail, hepatic NK cells in vitro preferentially expressed CD49a rather than CXCR6 (**Fig 4-7**). The frequency of CD49a+ NK cells increased from a median resting frequency of 8.7%, to 77.1% (IL-2), 55.7% (IL-12), 83.9% (IL-15), 85.7% (IL-18) and 88.9% (cytokine cocktail) following six days of cytokine stimulation (**Fig 4-7**).

Frequencies of hepatic CXCR6+ NK cells did not increase significantly beyond their resting levels under the same conditions, with a negligible change in the frequency of CXCR6+ NK cells from 65.1% at rest to 65.5% (IL-2), 64.2% (IL-15) and 56.7% (IL-18) after six days of culture (**Fig 4-7**). IL-12 generated the highest frequency of CXCR6+ NK cells by day six (74.1%), and can be seen to consistently enrich the CXCR6+ NK cell population where resting frequencies are low, however this increase failed to reach significance (**Fig 4-7**). Culture with the cytokine cocktail led to a decrease in the percentage of NK cells expressing CXCR6 (to 24.2% of total NK cells), in sharp contrast to its ability to enrich the CD49a+ population (**Fig 4-7**).

The generation of high frequencies of CD49a+ NK cells following culture with activating cytokines, resulted in a significant increase in the percentage of 'double-positive' CD49a+CXCR6+ NK cells seen within the hepatic NK cell population in vitro (**Fig 4-8**). This was seen following culture with IL-2, IL-12, IL-15 and IL-18 individually, but not the cytokine cocktail as a result of its negative influence on the frequency of CXCR6+ NK cells (**Fig 4-8**). Therefore, consistent with my functional data, hepatic CD49a+ but not CXCR6+ liver-resident NK cells appear to be reactive towards cytokines, particularly the cytokine cocktail.

As a result of the high death rate of liver-resident NK cells in culture, particularly within the first few days (**Fig 4-6**), absolute numbers of CD49a+ NK cells failed to increase by day six, unless cultured with the cytokine cocktail, which led to the fastest recovery of NK cell numbers along with IL-15 (**Fig 4-9**).

Figure 4-7 Proliferating NK cells upregulate CD49a, but not CXCR6

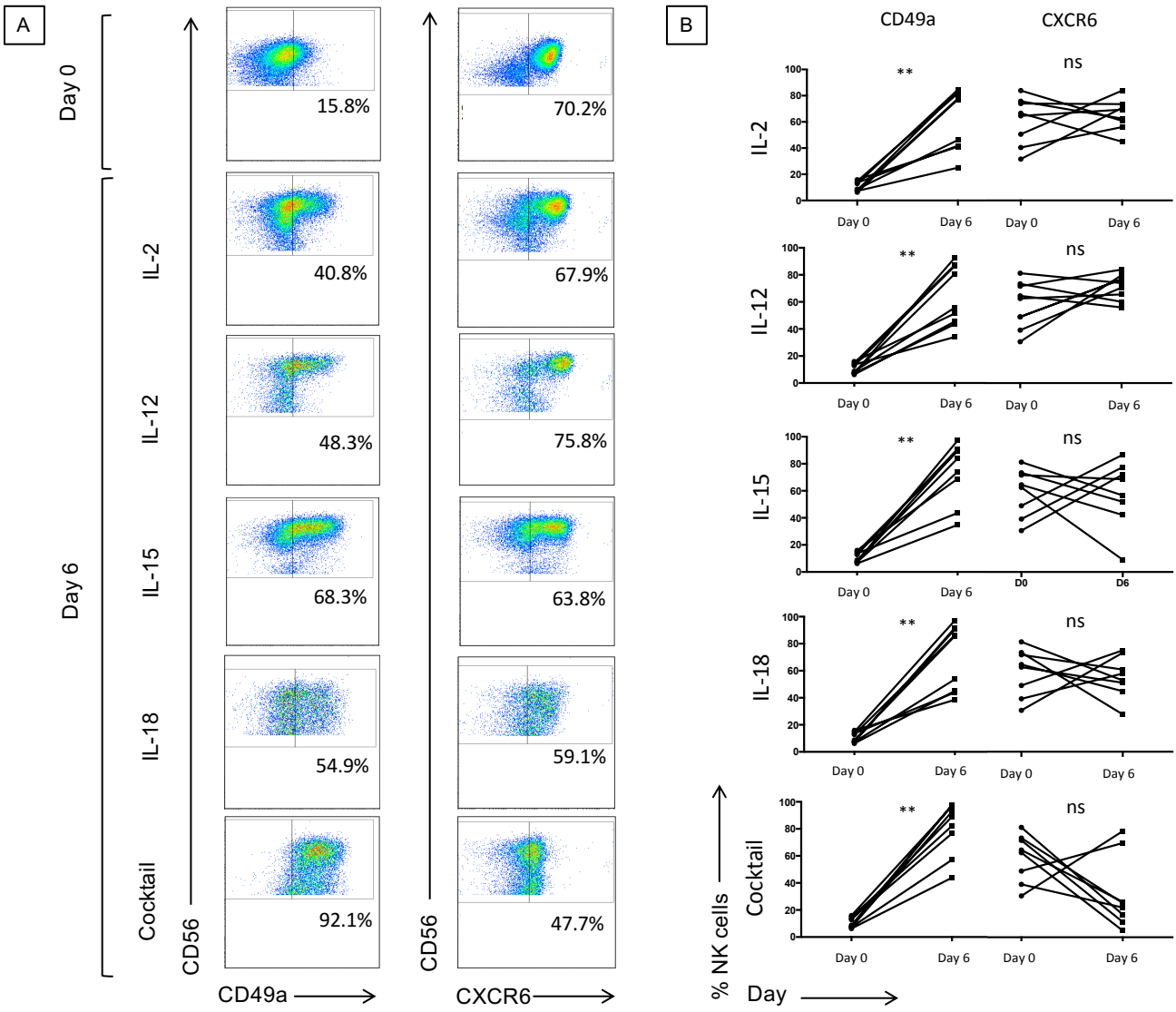


Fig 4-7 **A)** Representative flow cytometry plots gated on NK cells. **B)** Percentage of CD49a+ and CXCR6+ NK cells isolated from the liver at rest (day 0) and following incubation of hepatic MNCs with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (*n=8*). Dot plots display individual values. (*Wilcoxon matched pairs test).* p<0.01**.

Figure 4-8 Activating cytokines can generate increased frequencies of double-positive CD49a+CXCR6+ NK cells within the hepatic NK cell population

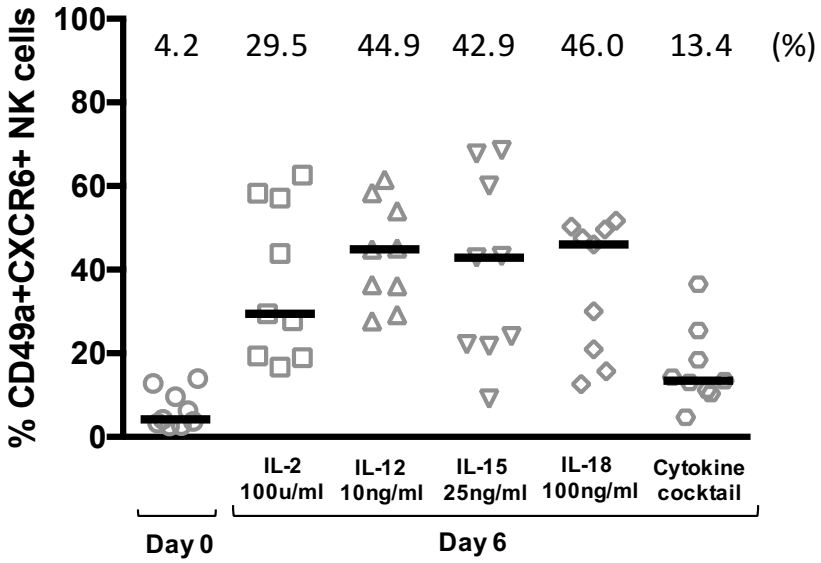
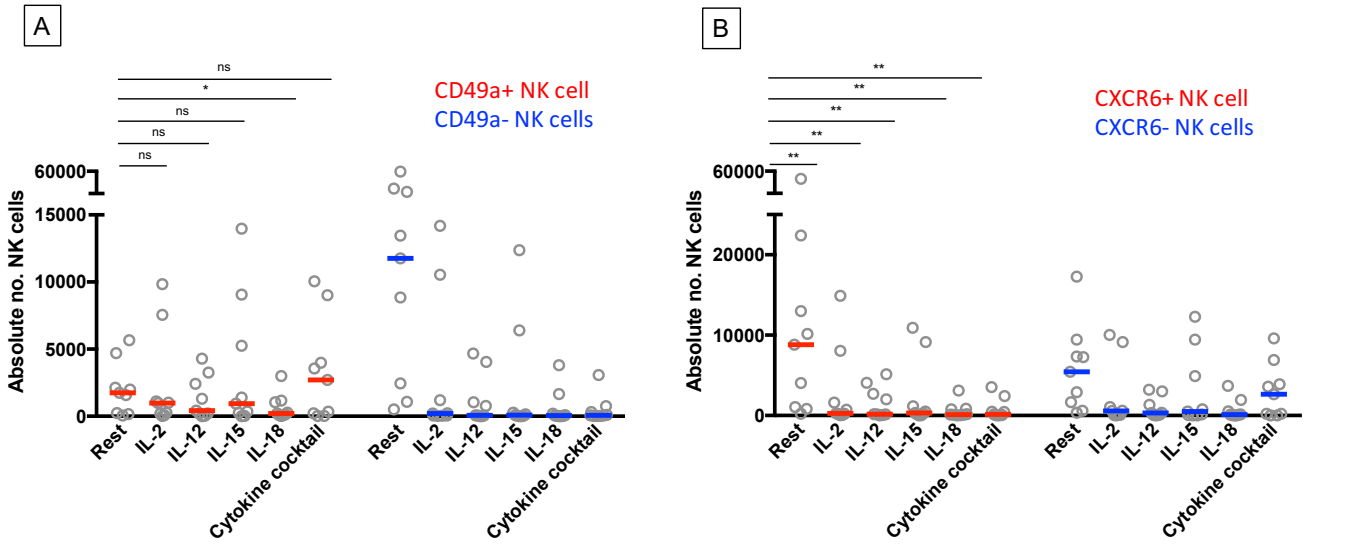


Fig 4-8. Percentage of CD49a+CXCR6+ NK cells isolated from the liver at rest (day 0) and following incubation of hepatic MNCs with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (*n=8*). Median percentages are shown for each condition. Dot plot displays median.

Figure 4-9 Absolute numbers of CD49a+/- and CXCR6+/- NK cells isolated from the liver at rest and following six days of cytokine stimulation

HEPATIC PERFUSATE



Absolute number cells	*Day 0*	*Day 6 IL-2*	*Day 6 IL-12*	*Day 6 IL-15*	*Day 6 IL-18*	*Day 6 Cocktail*
CD49a+ NK cells	1745	985	414	941	206	2711
CD49a- NK cells	11760	202	65	90	54	69

Absolute number cells	*Day 0*	*Day 6 IL-2*	*Day 6 IL-12*	*Day 6 IL-15*	*Day 6 IL-18*	*Day 6 Cocktail*
CXCR6+ NK cells	8817	261	172	309	126	140
CXCR6- NK cells	5430	558	328	492	126	2640

Fig 4-9 **A)** Absolute number of CD49a+ and CD49a- NK cells at rest (day 0) and following incubation with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (*n=8*). **B)** Absolute number of CXCR6+ and CXCR6- NK cells at rest (day 0) and following incubation with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (*n=8*). Dot plots display median. Median absolute cell numbers shown in table below. (*Wilcoxon matched pairs test).* p<0.05*, p<0.01**.

4.2.2 Liver-resident CD49a+ NK cells display features consistent with enhanced proliferation compared to CD49a- and CXCR6+ NK cells following stimulation with IL-15 and the cytokine cocktail

The cytokine cocktail and IL-15 induced the strongest proliferation of liver-resident NK cells, including both CD49a+ and CXCR6+ subpopulations (**Fig 4-10**). While NK cells carrying the marker CD49a displayed features consistent with enhanced proliferation over CD49a- and CXCR6+ NK cells with both IL-15 and the cytokine cocktail, differences in day six CFSE MFIs between NK cell groups were small compared to differences observed between the different cytokines themselves (**Fig 4-10**). Furthermore CD49a- and CXCR6+ NK cells also proliferated well with IL-15 and the cytokine cocktail (**Fig 4-10**). I did not observe a significant increase in the overall number of CD49a+ NK cells at day six, which may reflect the poor viability of liver NK cells in vitro in the short term. However absolute numbers of CD49a- NK cells decreased dramatically despite those remaining showing evidence of proliferation. Taken together this suggests that expansion of the CD49a+ NK cell population in vitro may occur through upregulation of CD49a on previously CD49a- NK cells, and not solely due to the enhanced proliferative properties of an existing liver-resident CD49a+ subset (**Fig 4-9, Fig 4-10**).

There were no features suggestive of a proliferative advantage of CXCR6+ NK cells over CXCR6- NK cells, and the absolute number of CXCR6+ NK cells decreased significantly, particularly in the presence of the activating cytokine cocktail. In common with the decrease in frequency of CXCR6+ NK cells post stimulation with the cytokine cocktail, CXCR6+ NK cells appeared to demonstrate impaired proliferation, compared to CXCR6- NK cells under these conditions (**Fig 4-10**).

Figure 4-10 CD49a+ NK cells display features suggestive of enhanced proliferation over CD49a- and CXCR6+ NK cells following culture with both IL-15 and the cytokine cocktail

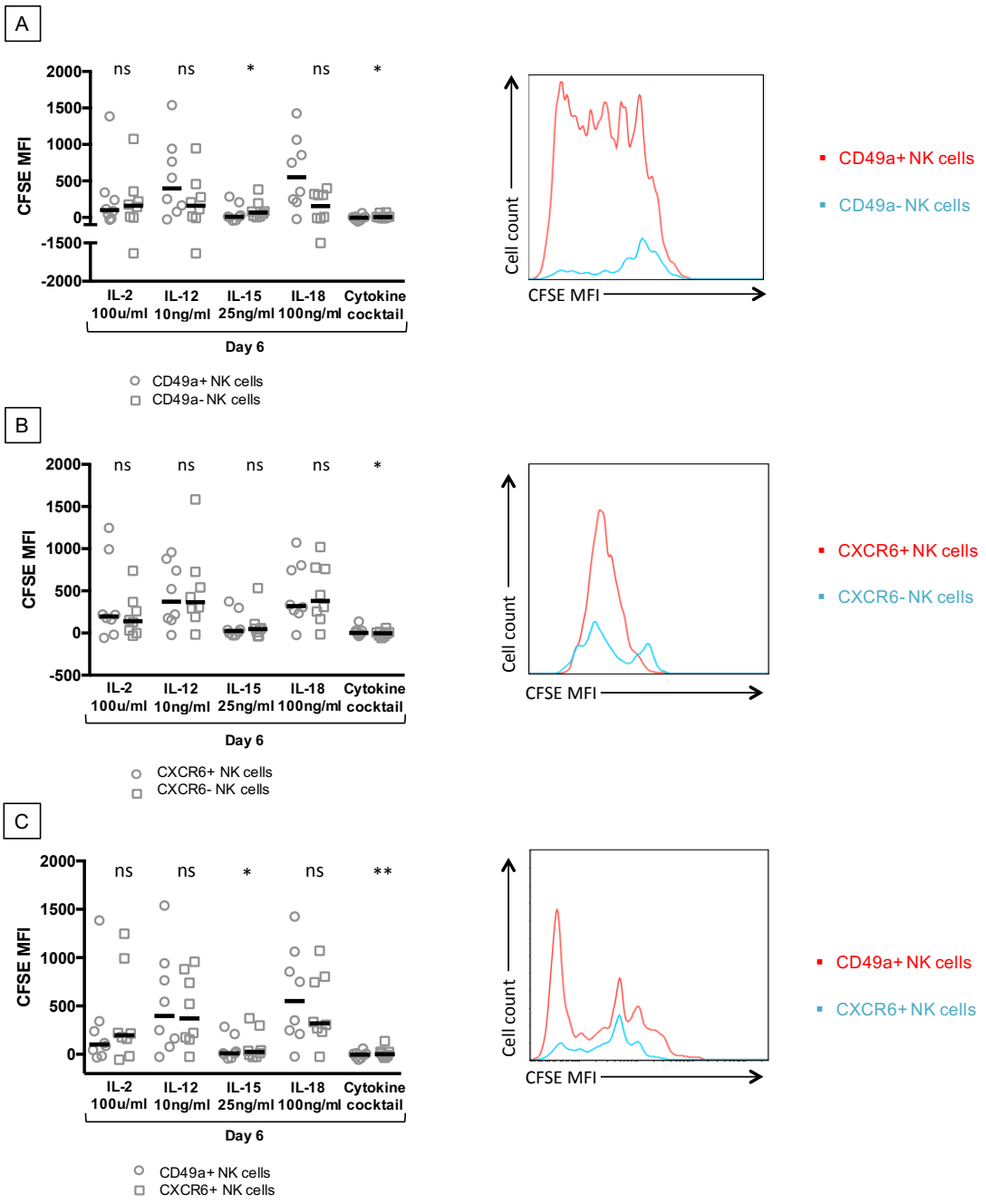


Fig 4-10 **A)** Day six CFSE MFI of hepatic CD49a+ and CD49a- NK cells following culture with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (*n=8*). Representative CFSE MFI histogram at day six comparing CD49a+ and CD49a- NK cells following culture with IL-15. **B)** Day six CFSE MFI of hepatic CXCR6+ and CXCR6- NK cells following culture with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (*n=8*). Representative CFSE MFI histogram at day six comparing CXCR6+ and CXCR6- NK cells following culture with IL-15. **C)** Day six CFSE MFI of hepatic CD49a+ and CXCR6+ NK cells following culture with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (*n=8*). Representative CFSE MFI histogram at day six comparing CD49a+ and CXCR6+ NK cells following culture with IL-15. Dot plots display median. (*Wilcoxon matched pairs test).* p<0.05*, p<0.01**.

4.3 CXCR6 and CD49a expression can be induced on NK cells isolated from the peripheral blood

4.3.1 Both CD49a+ and CXCR6+ NK cells can easily be induced in the peripheral blood using activating cytokines

Proliferation assays were performed on paired peripheral blood and liver samples. Despite resting frequencies being low, cytokines were able to induce extensive expansion of CD49a+ NK cells in the peripheral blood from a median frequency of 2.1% to 98% (cytokine cocktail), 83.3% (IL-18), 71.9% (IL-15), 71.3% (IL-12) and 66.9% (IL-2) (**Fig 4-11**). Furthermore, the absolute number of CD49a+ NK cells isolated from the peripheral blood can be seen to significantly increase following culture with all activating cytokines, particularly the 'adaptive-phenotype' inducing cytokine cocktail (**Fig 4-12**). The increase in the absolute number of CD49a+ NK cells was accompanied by a decrease in absolute numbers of CD49a- NK cells (**Fig 4-12**). In common with the liver, measurement of CFSE intensity at day six suggests IL-15 leads to enhanced proliferation of CD49a+ NK cells over CD49a- NK cells, although both can be seen to proliferate well (**Fig 4-13**). The fact that the CD49a- population is nearly lost under these conditions (**Fig 4-11**) also supports the premise that CD49a+ NK cell expansion occurs both secondary to enhanced proliferation of this subgroup, and de novo expression of this integrin on CD49a- NK cells. CD49a is also known as very-late antigen 1 and may therefore be upregulated through activation. NK cells isolated from the liver and peripheral blood therefore respond similarly to all activating cytokines in terms of CD49a induction, with the cytokine cocktail being the most effective stimulus in both compartments.

In contrast to the liver, CXCR6 could be upregulated on peripheral blood NK cells, with an increase in median frequency from 3.1% to 10.9% (IL-2), 21.4% (IL-12) and 23.3% (IL-15) CXCR6+ NK cells (**Fig 4-11**). Frequencies remained unchanged with IL-18 (4.1%) and the cytokine cocktail (5.6%) (**Fig 4-11**). CXCR6 was predominantly induced on CD49a+ NK cells **(Fig 4-11).** Expansion of CXCR6+ NK cell frequencies was accompanied by an increase in absolute CXCR6+ NK cell numbers following culture with IL-15 only (**Fig 4-12**). This may reflect an initial decrease in cell number in culture, with IL-15 resulting in the fastest recovery (**Fig 4-6**).

Peripheral blood NK cells expressing CXCR6 at day six displayed features consistent with enhanced proliferation over CXCR6- NK cells at day six following stimulation with IL-2, IL-12 and IL-18 and

proliferated at similar rates to CD49a+ NK cells following culture with all activating cytokines, other than the cytokine cocktail (**Fig 4-13**). Again, this contrasts with CXCR6+ NK cells isolated from the liver and suggests that peripheral blood CXCR6+ NK cells may be more cytokine-responsive than those which are liver-resident, in terms of either their enhanced proliferation or ability to upregulate CXCR6 on their surface (**Fig 4-12, 4-13**).

Figure 4-11 CD49a and CXCR6 can be upregulated on NK cell in the peripheral blood

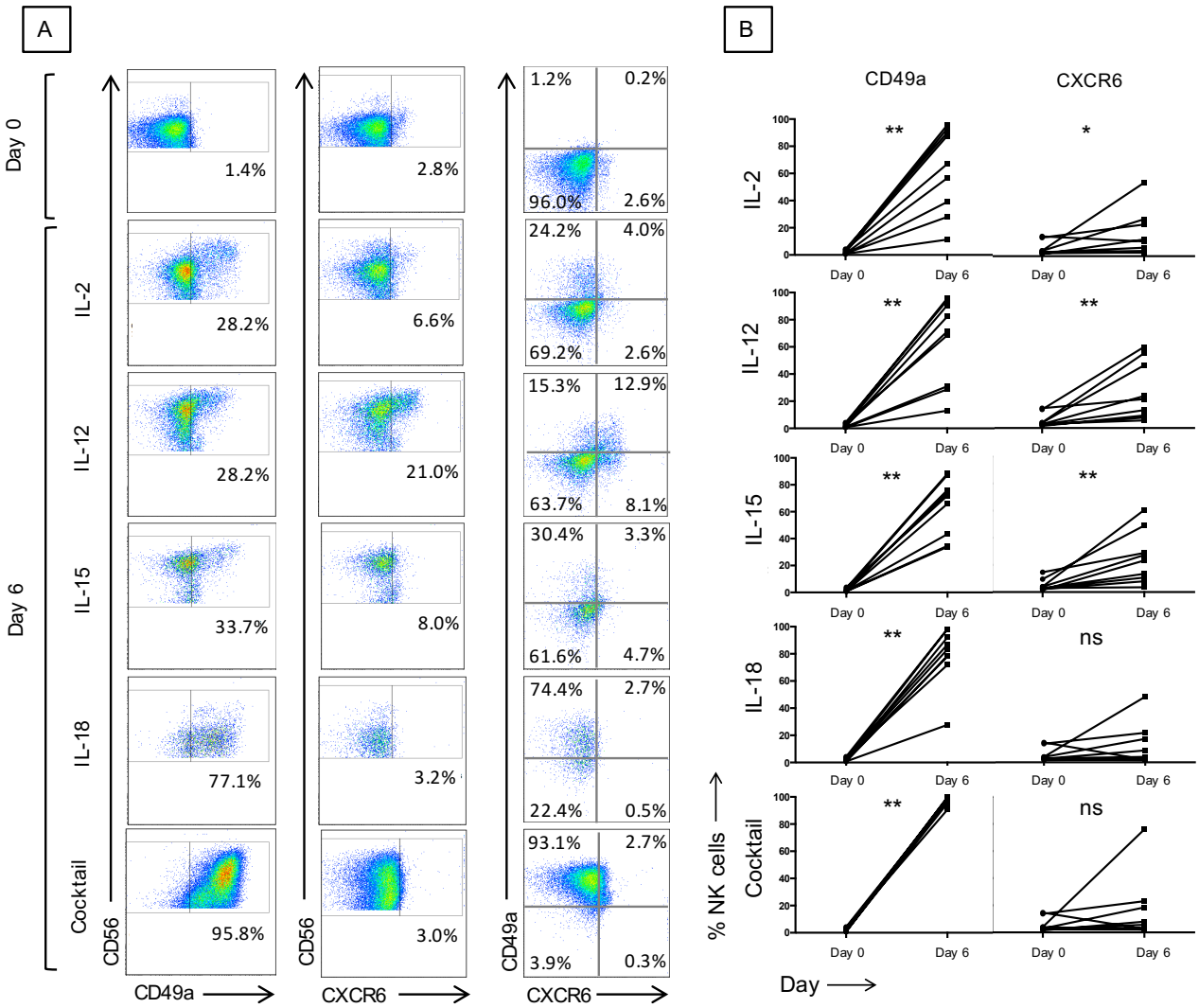
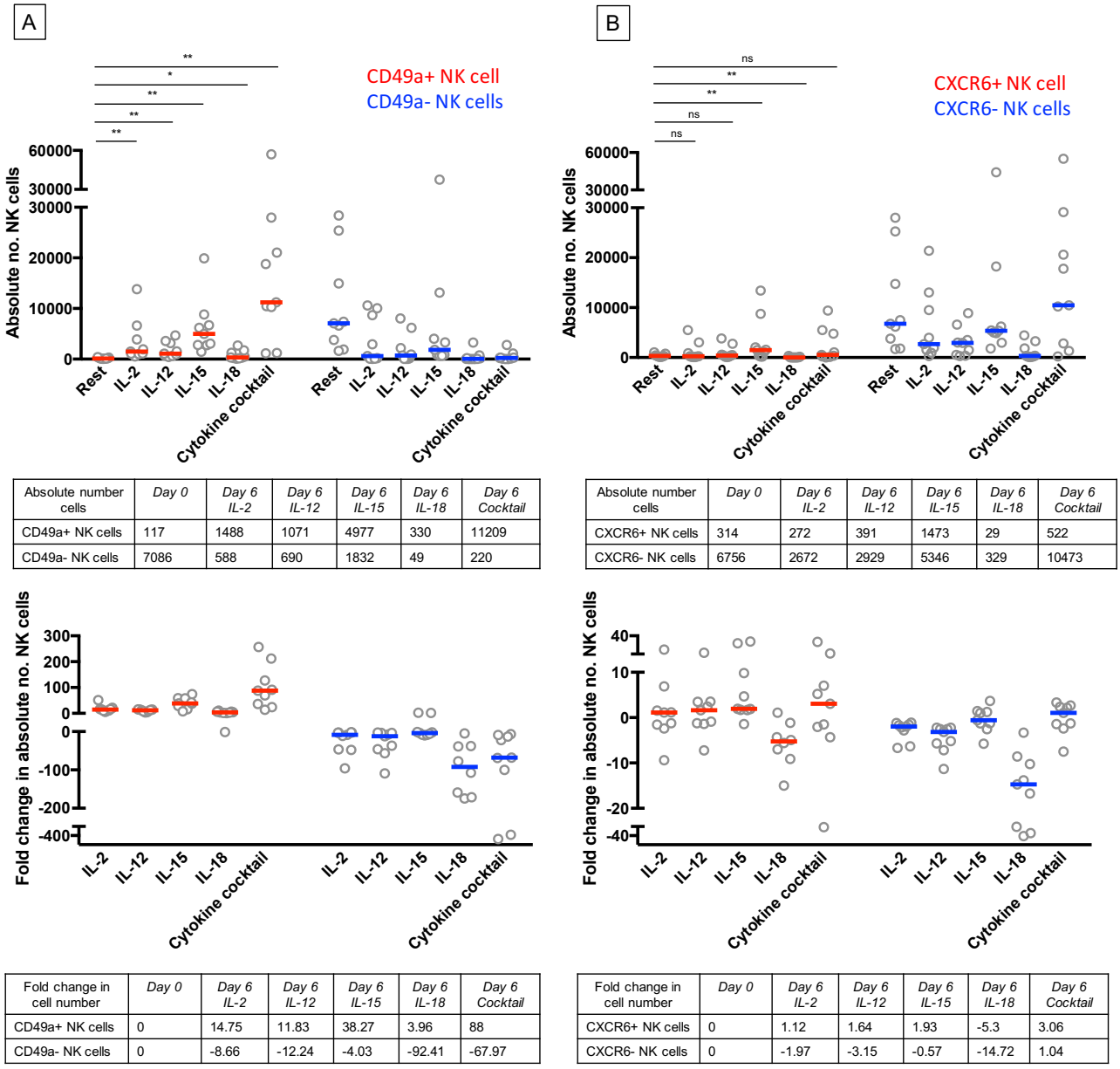


Fig 4-11 **A)** Representative flow cytometry plots gated on NK cells. **B)** Percentage of CD49a+ and CXCR6+ NK cells isolated from the peripheral blood at rest (day 0) and following incubation with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (*n=9*). Dot plots display individual values. (*Wilcoxon matched pairs test).* p<0.05*, p<0.01**.

Figure 4-12 Absolute numbers of CD49a+/- and CXCR6+/- NK cells isolated from the peripheral blood at rest and following six days of cytokine stimulation

PERIPHERAL BLOOD



Absolute number cells	*Day 0*	*Day 6 IL-2*	*Day 6 IL-12*	*Day 6 IL-15*	*Day 6 IL-18*	*Day 6 Cocktail*
CD49a+ NK cells	117	1488	1071	4977	330	11209
CD49a- NK cells	7086	588	690	1832	49	220

Absolute number cells	*Day 0*	*Day 6 IL-2*	*Day 6 IL-12*	*Day 6 IL-15*	*Day 6 IL-18*	*Day 6 Cocktail*
CXCR6+ NK cells	314	272	391	1473	29	522
CXCR6- NK cells	6756	2672	2929	5346	329	10473

Fold change in cell number	*Day 0*	*Day 6 IL-2*	*Day 6 IL-12*	*Day 6 IL-15*	*Day 6 IL-18*	*Day 6 Cocktail*
CD49a+ NK cells	0	14.75	11.83	38.27	3.96	88
CD49a- NK cells	0	-8.66	-12.24	-4.03	-92.41	-67.97

Fold change in cell number	*Day 0*	*Day 6 IL-2*	*Day 6 IL-12*	*Day 6 IL-15*	*Day 6 IL-18*	*Day 6 Cocktail*
CXCR6+ NK cells	0	1.12	1.64	1.93	-5.3	3.06
CXCR6- NK cells	0	-1.97	-3.15	-0.57	-14.72	1.04

Fig 4-12 **A)** Absolute number of CD49a+ and CD49a- NK cells at rest (day 0) and following incubation with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail. Median absolute cell numbers shown in table below. Fold change in absolute number of CD49a+ and CD49a- NK cells at rest (day 0) and following incubation with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail. Median fold change in absolute cell numbers shown in table below. (*n=9).* **B)** Absolute number of CXCR6+ and CXCR6- NK cells at rest (day 0) and following incubation with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail. Median absolute cell numbers shown in table below. Fold change in absolute number of CXCR6+ and CXCR6- NK cells at rest (day 0) and following incubation with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail. Median fold change in absolute cell numbers shown in table below. (*n=9).* Dot plots display median. (*Wilcoxon matched pairs test).* p<0.05*, p<0.01**.

Figure 4-13 NK cells isolated from PBMCs expressing CD49a+ and CXCR6+ at day six following in vitro culture with cytokines, display features consistent with enhanced proliferation over CD49a- and CXCR6- NK cells

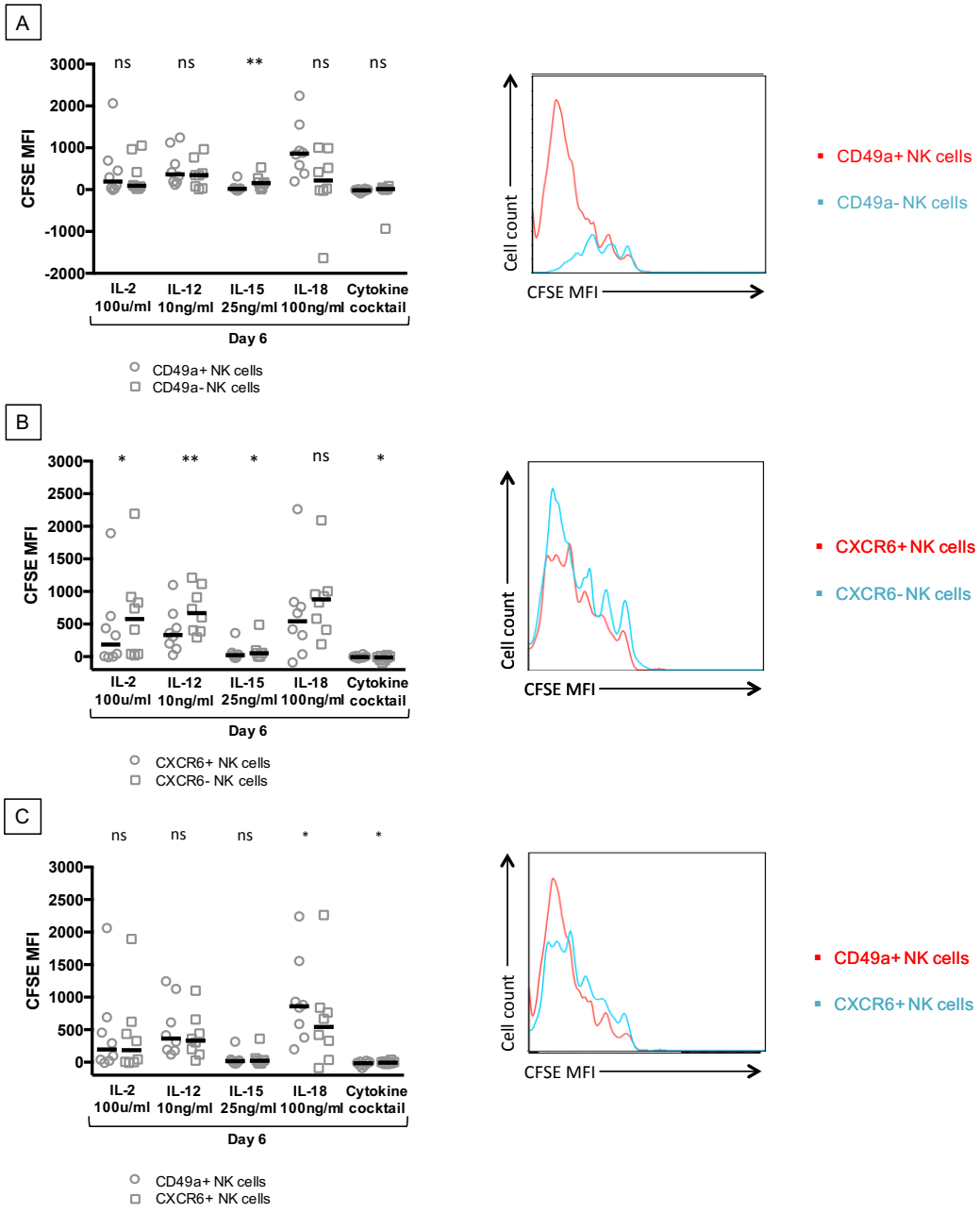


Fig 4-13 **A)** Day six CFSE MFI of peripheral blood CD49a+ and CD49a- NK cells following culture with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (*n=9*). Representative CFSE MFI histogram at day six comparing CD49a+ and CD49a- NK cells following culture with IL-15. **B)** Day six CFSE MFI of peripheral blood CXCR6+ and CXCR6- NK cells following culture with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (*n=9*). Representative CFSE MFI histogram at day six comparing CXCR6+ and CXCR6- NK cells following culture with IL-15. **c)** Day six CFSE MFI of peripheral blood CD49a+ and CXCR6+ NK cells following culture with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (*n=9*). Representative CFSE MFI histogram at day six comparing CD49a+ and CXCR6+ NK cells following culture with IL-15. Dot plots display median. (*Wilcoxon matched pairs test).* p<0.05*, p<0.01**.

Importantly IL-15 and IL-12 were the most effective cytokines at inducing the highest frequencies of CD49a+CXCR6+ double-positive NK cells from PBMCs in vitro (15.7% and 10.8% respectively), compared to IL-2 (5.5%), IL-18 (3.1%) and the cytokine cocktail (4.5%) following six days of culture (**Fig 4-14**). As a result of their enhanced ability to promote NK cell proliferation overall, IL-15 followed by the cytokine cocktail generated the greatest increase in absolute number of CD49a+CXCR6+ NK cells (**Fig 4-14**). Despite the cytokine cocktail containing IL-12 and IL-15, the combination of IL-2, IL-12, IL-15 and IL-18 failed to increase frequencies of CD49a+CXCR6+ NK cells to the same degree as IL-12 and IL-15 individually, due to the negative influence of the cytokine cocktail on CXCR6+ NK cell frequencies and absolute numbers (**Fig 4-11, 4-12, 4-14**).

Figure 4-14 IL-12 and IL-15 induce the highest frequencies of double-positive CD49a+CXCR6+ NK cells from PBMCs in vitro

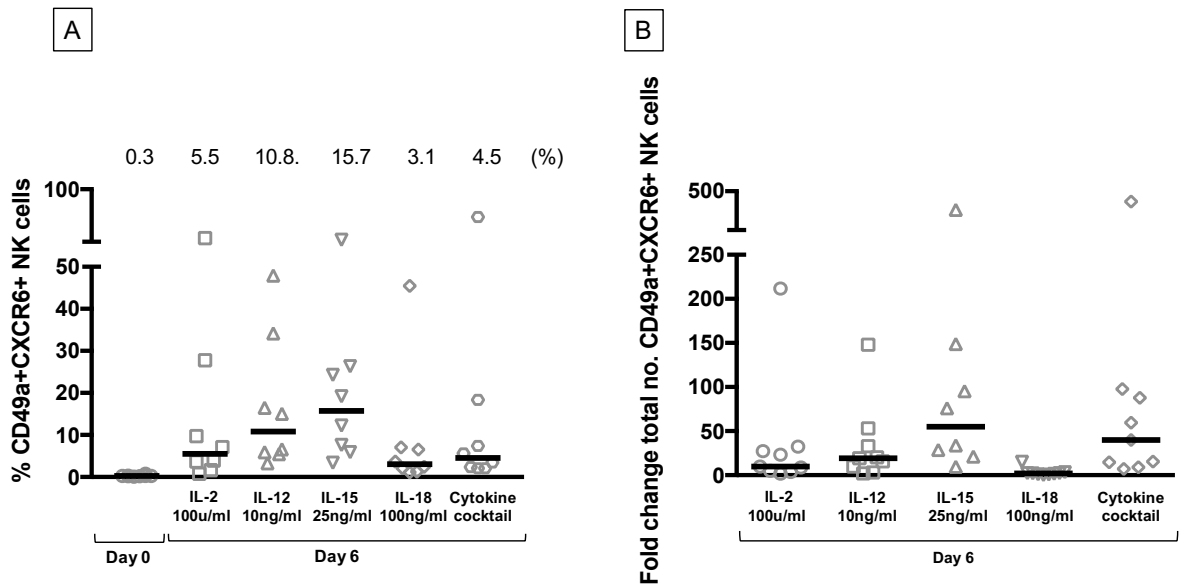


Fig 4-14 **A)** Frequency of CD49a+CXCR6+ NK cells isolated from the peripheral blood at rest (day 0) and following incubation of PBMCs with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (*n=9*). Median percentages are shown for each condition. **B)** Fold change in absolute number of CD49a+CXCR6+ NK cells from the peripheral blood at rest (day 0) and following incubation of PBMCs with IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail (*n=9*). Dot plots display median.

4.3.2 IL-12 is important for the induction of CXCR6+ NK cells and IL-15 for CD49a+ NK cells

To better differentiate the effect of different cytokines on the induction of potential 'memory-like' 'liver-homing' NK cell phenotypes in the peripheral blood, PBMCs donated from individuals with haemochromatosis were stimulated with IL-12 and IL-15 for the shorter time of four days. These cytokines were chosen as they were most effective at enriching the CD49a+CXCR6+ population in peripheral blood NK cells (**Fig 4-14**). IL-12 led to the greatest induction of CXCR6+ NK cells (IL-12 31.9% [range 11.4 – 43.0] vs. IL-15 29.8% [range 12.8 – 50.3]) and IL-15 led to the greatest induction of CD49a+ NK cells (IL-15 51.6% [range 32.1 – 67.3] vs. IL-12 39.6 [range 29.3 – 54.6]) (**Fig 4-15**).

Figure 4-15 A comparison of the influence of IL-12 and IL-15 on the induction of CD49a+ and CXCR6+ NK cell populations following four days of culture

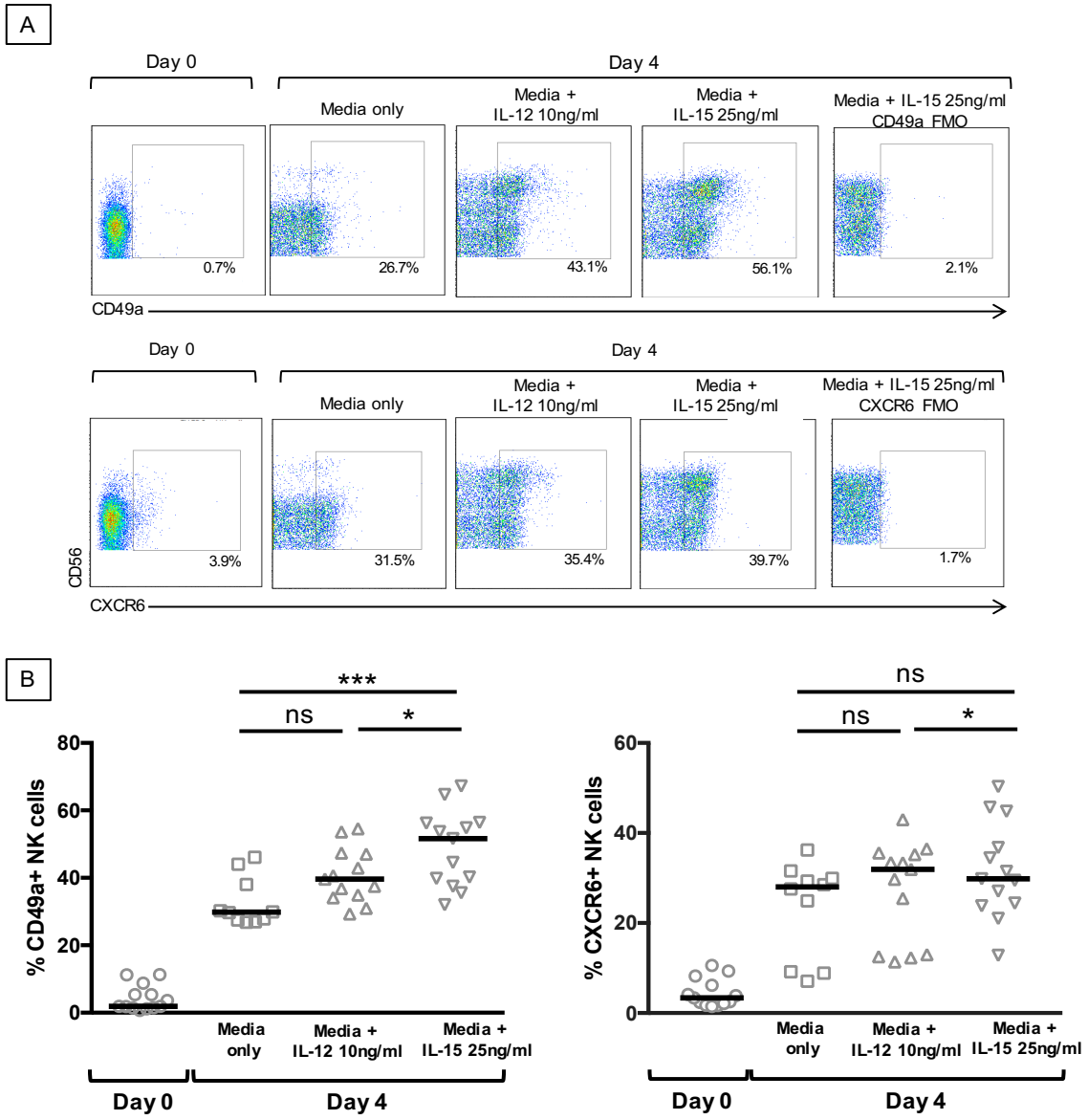


Fig 4-15 **A)** Representative flow cytometry plots displaying gating for CD49a+ and CXCR6+ NK cell populations (gated on NK cells). **B)** Frequencies of CD49a+ and CXCR6+ NK cell populations at rest and following a four day culture with media only (*n=10*), IL-12 (*n=13*) or IL-15 (*n=13*). (*Mann Whitney U test and Wilcoxon matched pairs test*). p<0.05*, p,<0.01**, p<0.001***.

Induction of both CD49a+ and CXCR6+ NK cells could be seen by day four without the addition of exogenous cytokines, i.e. following culture of PBMCs in media only (29.8% and 28.0% respectively) (**Fig 4-15**). I hypothesised this occurred as a result of release of IL-12 and IL-15 from monocytes and dendritic cells in culture. It is also possible that the cytokines are acting extrinsically, for example through the activation of dendritic cells. To more definitively assess the effects of IL-12 and IL-15 on NK cells, I purified NK cells using MACS beads and performed a time response experiment. This showed that a small increase in both CD49a+ and CXCR6+ NK cells could be seen as early as 12 hours in culture (**Fig 4-16**). CD49a+ NK cells could be induced early in the peripheral blood using both IL-12 (4.1%) and IL-15 (9.0%), with greater sensitivity towards IL-15. In contrast CXCR6+ NK cells could only be induced at this time point using IL-12, from a resting frequency of 2.1% to 4.0% (12 hours of IL-15, frequency 2.1%), highlighting this signalling axis as a key pathway in the generation of NK cells with this phenotype (**Fig 4-16**). Interestingly CD49a+ NK cells generated at this time point were generally CD56^{bright}, whereas CXCR6+ NK cells were CD56^{dim} but became CD56^{bright} by day four (**Fig 4-15)**.

Figure 4-16 A comparison of the influence of IL-12 and IL-15 on the induction of CXCR6+ and CD49a+ NK cells following 12 hours of culture using purified NK cells isolated from the peripheral blood

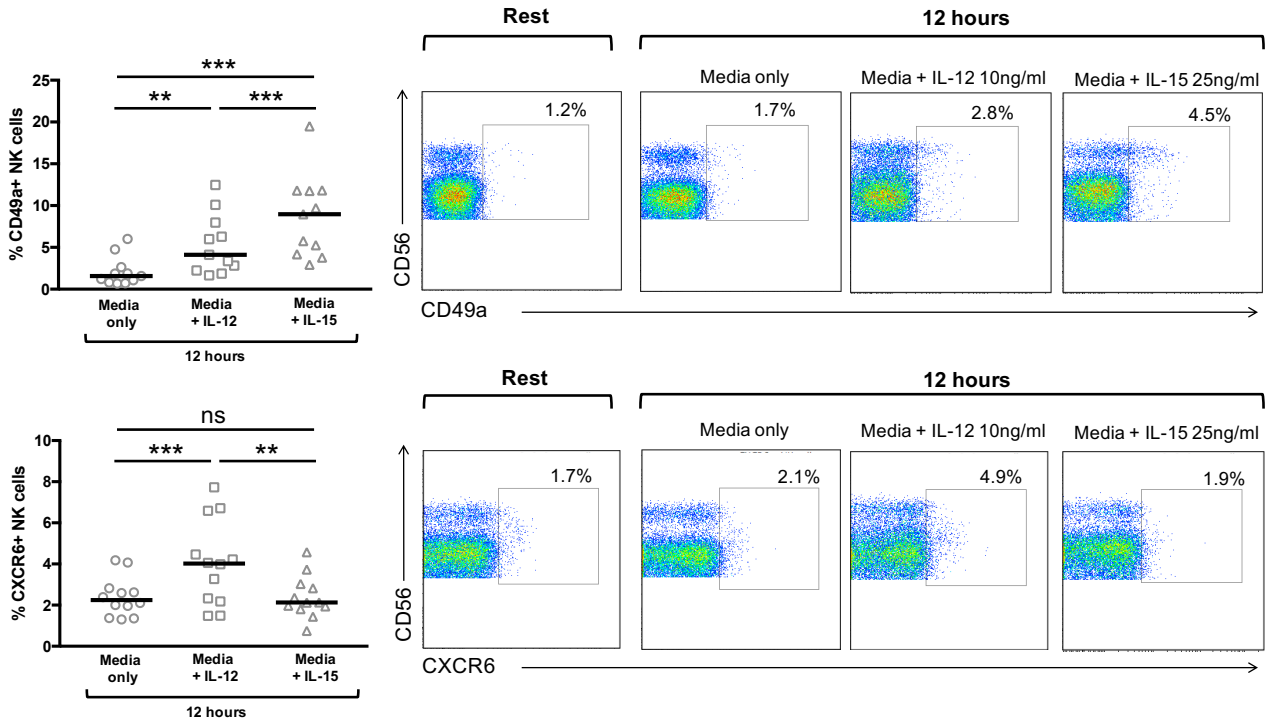


Fig 4-16 **A)** Representative flow cytometry plots gated on NK cells. **B)** Frequency of CD49a+ and CXCR6+ NK cells at rest and following a 12 hours culture with media only, IL-12 10ng/ml or IL-15 25ng/ml using purified NK cells (*n=12*). Box plots display median. (*Wilcoxon matched pairs test).* p<0.01**, p<0.001***

4.3.3 Cytokine-induced CD49a+CXCR6+ peripheral blood NK cells are CD56^{bright}CD69+ with a higher frequency of NKG2C+ NK cells compared to other NK subsets

Cytokine-induced CD49a+ NK cells generated from PBMCs in vitro displayed a similar phenotype to liver-resident CD49a+ NK cells, i.e. they were CD56^{bright} (68.8%), CD69+ (83.0%) following IL-15 stimulation (**Fig 4-17**). While peripheral blood CD49a- NK cells can also have this phenotype following culture, cytokine-induced CD49a+ NK cell contained a greater frequency of NKG2C+ NK cells compared to NK cells that remained CD49a- (37.7% vs 19.6%, $p<0.01$) (**Fig 4-17**) and CD49a+ liver-resident NK cells (37.7% vs 10.3%, $p<0.05$). Similarly CXCR6 expression was higher within cytokine-generated CD49a+ NK cells, compared to those that remained CD49a- (32.5% vs 13.7%, $p<0.01$), although CXCR6+ NK cell frequencies were not as high as found in populations of liver-resident CD49a+ NK cells (32.5% vs 70.8%, $p<0.01$) (**Fig 4-17**).

The majority of cytokine-induced CXCR6+ NK cells generated in the peripheral blood were CD56^{bright} (73.3%) CD69+ (81.4%) (**Fig 4-17**), indicating that they display similar levels of markers of maturation and liver-residency as those found in the liver tissue. However, cytokine-induced CXCR6+ NK cells contained higher frequencies of NKG2C+ NK cells compared to peripheral NK cells that remained CXCR6- in the presence of IL-15 (47.5% vs 25.0%, $p<0.01$), and resting liver-resident CXCR6+ NK cells (47.5% vs 5.9%, $p<0.0001$) (**Fig 4-17**). Cytokine-induced CXCR6+ NK cells also harboured higher frequencies of CD49a+ NK cells, compared to peripheral NK cells that remained CXCR6- (83.0% vs 63.0%, $p<0.01$) and liver-resident CXCR6+ NK cells (83.0% vs 8.9%, $p<0.0001$) (**Fig 4-17**).

Frequencies of double-positive CD49a+CXCR6+ NK cells were higher following six days of cytokine stimulation of PBMCs, than found in the liver at rest (15.7% vs 4.1%, $p<0.05$ (IL-15)). A direct comparison of the phenotype of resting liver-resident CD49a+CXCR6+ NK cells and those generated through IL-15 stimulation in the peripheral blood, revealed the latter to be CD56^{bright} (71.1%), with high levels of CD69+ NK cells (83.4% vs 75.4%, $p>0.05$), and a significantly higher frequency of NK cells expressing the activating receptor NKG2C (48.0% vs 13.5%, $p<0.001$) (**Fig 4-18**). A similar phenotype was generated using IL-2, IL-12, IL-18 and the cytokine cocktail, the latter resulting in particularly high frequencies of NKG2C+ NK cells within the CD49a+CXCR6+ population (64.2%) (**Fig 4-19**). Upregulation of NKG2C on cytokine-generated CXCR6+ NK cells, does not appear to be fully dictated by CD49a expression, as seen for liver isolated NK cells, as CD49a-CXCR6+ NK cells induced by IL-15 contained higher frequencies of NKG2C+ NK cells (35.0%) compared to CD49a-CXCR6- NK cells (18.9%, $p<0.001$) (**Fig 4-19**).

Figure 4-17 Phenotype of IL-15-induced CD49a+ and CXCR6+ NK cells generated from PBMCs in vitro

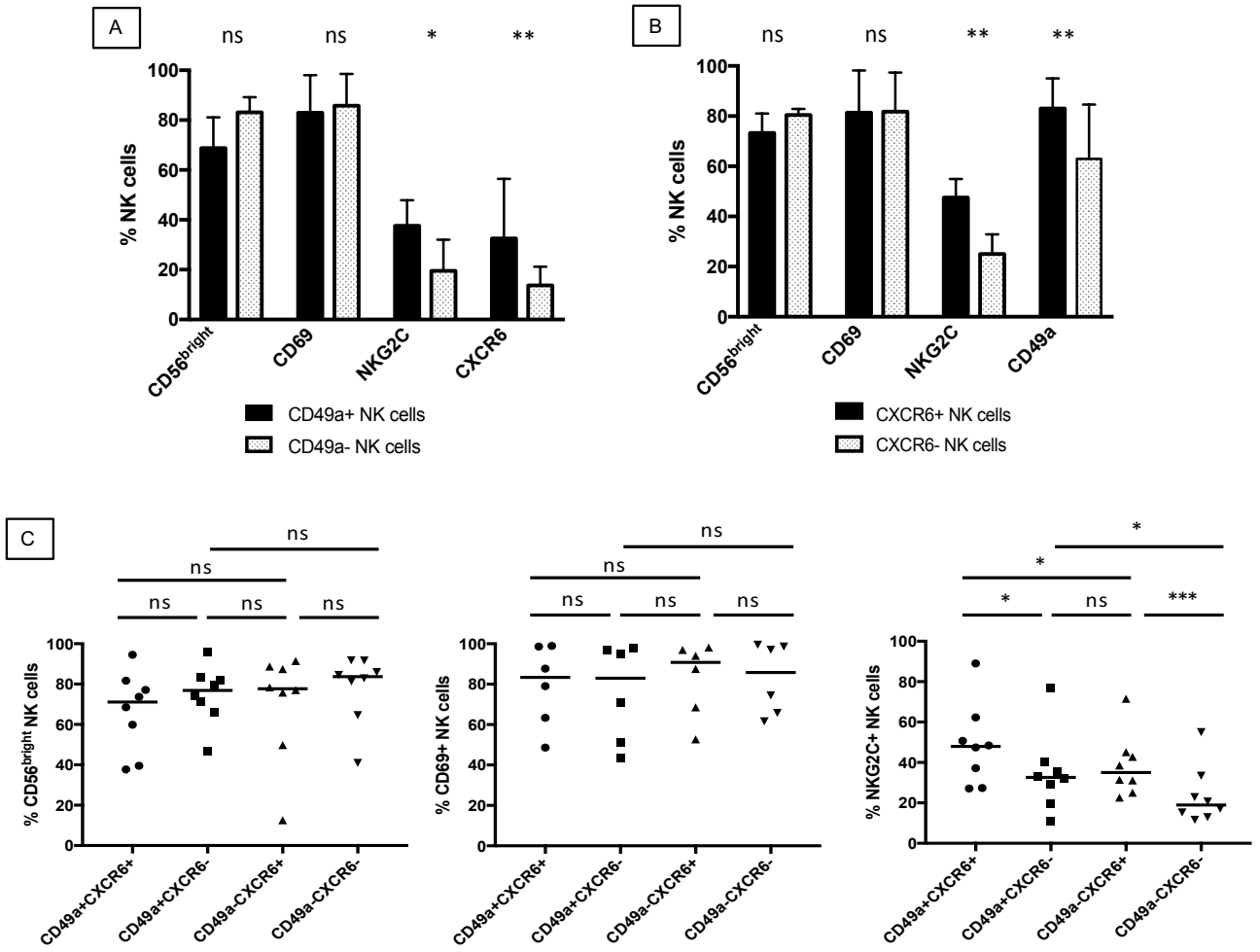


Fig 4-17 A comparison of the frequency of $CD56^{bright}$, CD69+, NKG2C+ and CXCR6+ NK cells between **A)** CD49a+ and CD49a- NK subsets, and **B)** CXCR6+ and CXCR6- subsets, generated in the peripheral blood following six days of culture with IL-15 (*n=9*). Bar chart displays median and interquartile range. (*Wilcoxon matched pairs test).* **C)** A comparison of the frequency of $CD56^{bright}$ (*n=8*), CD69+ (*n=6*) and NKG2C+ (*n=8*) between CD49a+CXCR6+, CD49a+CXCR6-, CD49a-CXCR6+, CD49a-CXCR6- NK subsets generated in the peripheral blood following six days of culture with IL-15, Dot plots display median. (*Wilcoxon matched pairs test).* p<0.05*, p<0.01**, p<0.001***

Figure 4-18 Comparison of the phenotype of CD49a+CXCR6+ NK cells induced in PBMCs in vitro using IL-15, and liver-resident CD49a+CXCR6+ NK cells

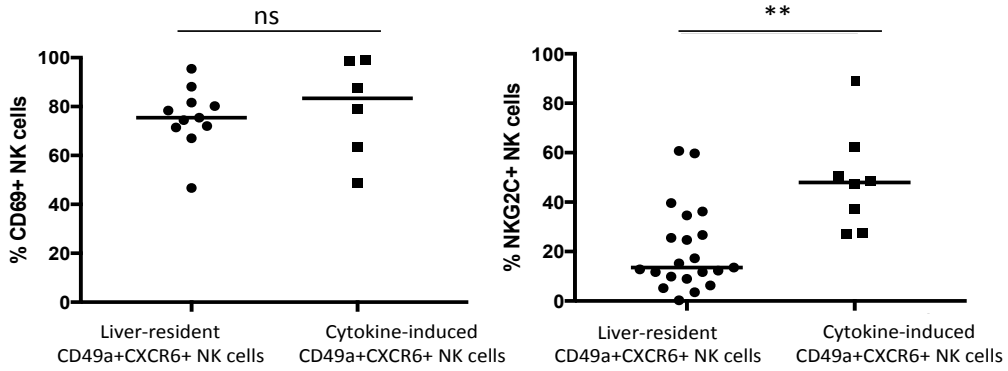


Fig 4-18 A comparison of CD69+ (*n=11* liver, *n=6* cytokine-induced) and NKG2C+ (*n=21* liver, *n=8* cytokine-induced) NK cell frequencies between CD49a+CXCR6+ found in situ in the liver and those generated from PBMCs in vitro following six days of culture with IL-15. Dot plots display median. (*Mann Whitney U test)*. p<0.01**.

Figure 4-19 Phenotype of CD49a+CXCR6+, CD49a+CXCR6-, CD49a-CXCR6+ and CD49a-CXCR6- NK cells generated from PBMCs in vitro using IL-2, IL-12, IL-15, IL-18 and the cytokine cocktail

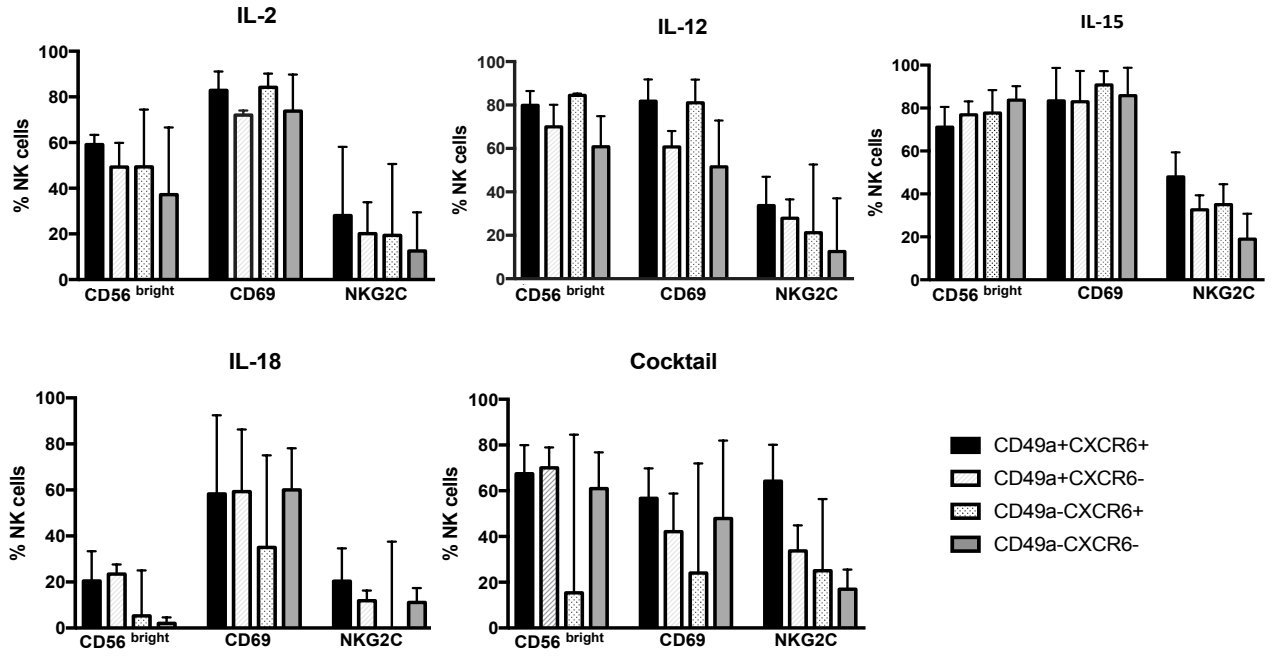


Fig 4-19 A comparison of CD56^{bright}, CD69+ and NKG2C+ NK cell frequencies between CD49a+CXCR6+, CD49a+CXCR6-, CD49a-CXCR6+, CD49a-CXCR6- NK subsets generated from PBMCs in vitro following six days of culture with IL-2, IL-12, IL-15, IL-18 and a cocktail of all four cytokines (*n=9*). Bar chart displays median and interquartile range.

4.3.4 Cytokine-induced CD49a+CXCR6+ NK cells produce high quantities of IFNγ

Following culture of PBMCs for 12 hours with IL-12 10ng/ml, a small increase in the percentages of CD49a+ (2.2% to 4.0%, $p<0.001$) and CXCR6+ (5.6% to 26.2%, $p<0.0001$) NK cells from rest can be seen, although again in the absence of NK cell purification these could also be induced without the addition of exogenous cytokines (**Fig 4-20**). Consistent with their comparable phenotype to liver-resident CD49a+ NK cells, the presence of CD49a on peripheral blood NK populations following IL-12 10ng/ml stimulation, was associated with high IFNγ production (**Fig 4-21**). Cytokine-generated peripheral blood CXCR6+ NK cells failed to produce significant quantities of IFNγ unless they co-expressed CD49a (**Fig 4-21**). This may also reflect the fact that the CD49a+ NK cells induced at 12 hours contained a higher frequency of $CD56^{bright}$ NK cells in comparison to CXCR6+ NK cells, which were nearly all $CD56^{dim}$ at this time point (**Fig 4-20**). However, after six days of culture, CXCR6+ NK cells contained a higher frequency of the $CD56^{bright}$ subset (**Fig 4-11, 4-19**). Furthermore over 80% of CXCR6+ NK cells were CD49a+ at day six (unlike in the liver) (**Fig 4-16**), and cells with this phenotype were found to release similar quantities of IFNγ compared to CD49a+CXCR6- NK cells (12.8% vs 15.8%, $p>0.05$) (**Fig 4-21)**. Therefore, cytokine stimulation of peripheral blood NK cells can generate high frequencies of CD49a+CXCR6+ NK cells which are activated, express CD69 and produce high quantities of IFNγ (**Fig 4-21)**.

Figure 4-20 Induction of CD49a+ and CXCR6+ NK cells following culture of PBMCs for 12 hours

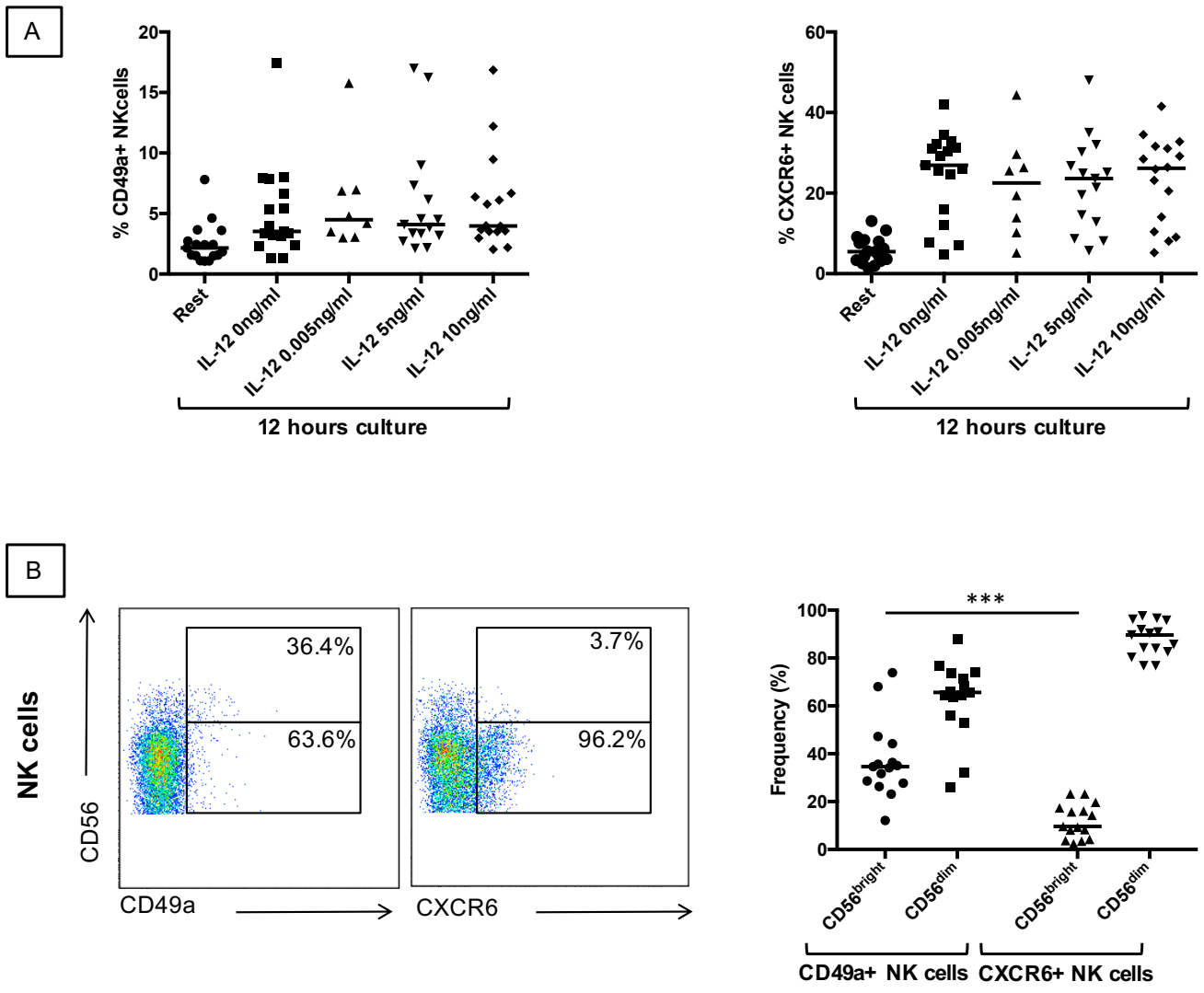


Fig 4-20 **A)** Frequency of CXCR6+ and CD49a+ NK cells found at rest and following 12 hours of culture with increasing concentrations of IL-12 (rest *n=17*, no media *n=17*, IL-12 0.005ng/ml *n=8*, IL-12 5ng/ml *n=15*, IL-12 10ng/ml *n=16).* **B)** Representative flow cytometry plots gated on NK cells showing gating of $CD56^{bright}$ and $CD56^{dim}$ subsets. Frequency of $CD56^{bright}$ and $CD56^{dim}$ NK cell populations within CD49a+ and CXCR6+ NK cell subsets following culture in IL-12 10ng/ml for 12 hours (*n=16*). (*Wilcoxon matched pairs test)*. p<0.001***.

Figure 4-21 IFNγ production from peripheral blood CD49a+ and CXCR6+ NK cells following stimulated with IL-12 10ng/ml for 12 hours

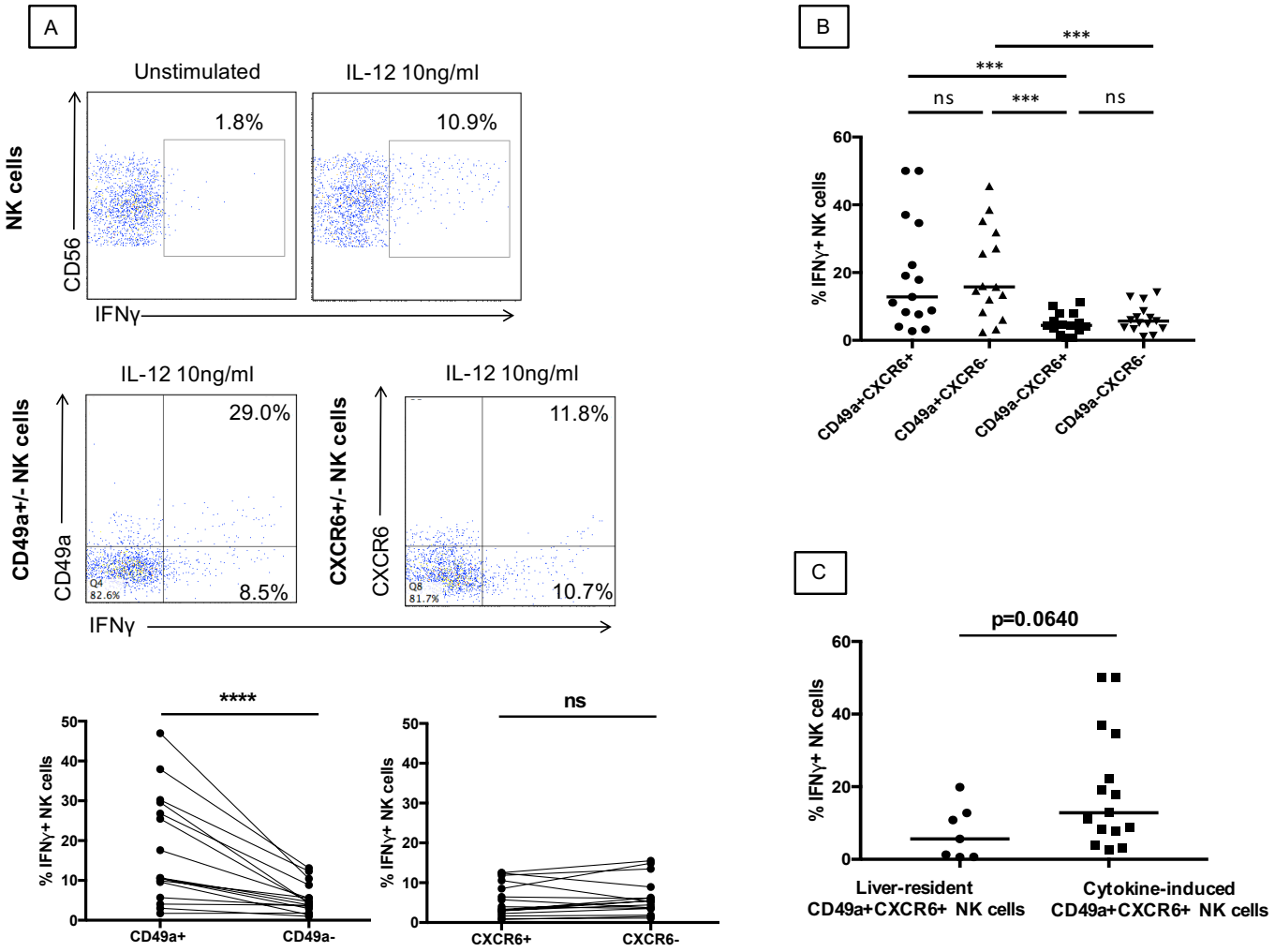


Fig 4-21 **A)** Representative flow cytometry plots gated on NK cells showing IFNγ+ production for all NK cells, CD49a+/- and CXCR6+/- NK cells**. %** IFNγ+ NK cells within CD49a+ (10.6%), CD49a- (4.4%), CXCR6+ (3.7%) and CXCR6- (5.3%) NK cells populations (*n=16*) following stimulation of PBMCs with IL-12 10ng/ml for 12 hours. Dot plots display individual values. **B) %** IFNγ+ NK cells within CD49+CXCR6+ (12.0%), CD49a-CXCR6+ (4.3%), CD49a+CXCR6- (15.2%) and CD49a-CXCR6- (5.4%) populations following stimulation of PBMCs with IL-12 10ng/ml for 12 hours. Dot plots display median. (*n=15).* (*Wilcoxon matched pairs test*). **C)** Comparison of **%** IFNγ+ NK cells between CD49a+CXCR6+ NK cells in the liver (5.6%, *n=7)* and those generated from PBMCs in vitro following stimulation with IL-12 10ng/ml for 12 hours (12.8%, *n=15*). p<0.001***, p<0.0001****.

4.4 T-bet and Eomes expression in CD49a+ and CXCR6+ NK cells isolated from the peripheral blood

Recently published studies have demonstrated a distinct transcriptional profile for human liver-resident CD49a+ (T-bet+Eomes-) [35] and CXCR6+ (Eomes^{high}T-bet^{low}) NK cells [29]. Findings from the latter paper by Stegmann et al are supported by the RNA sequencing data presented above (**Fig 3-14**). I therefore tested transcription factor expression in CD49a+ and CXCR6+ NK cells in the peripheral blood at rest. This has not been previously examined for CD49a+ NK cells, although Stegmann et al reported circulating CXCR6+ NK cells to be T-bet^{high}Eomes^{low}, i.e. more closely related to liver-resident CXCR6- NK cells. I was also keen to examine whether cytokine-induced CD49a+ and CXCR6+ NK cells display altered expression of either transcription factor following culture with IL-12 (known to upregulate both proteins through pSTAT4 signalling in T-cells [263]), as this may provide insights into whether cytokine-induced CXCR6+ and CD49a+ NK cells are generated as a result of changes at a transcriptional level.

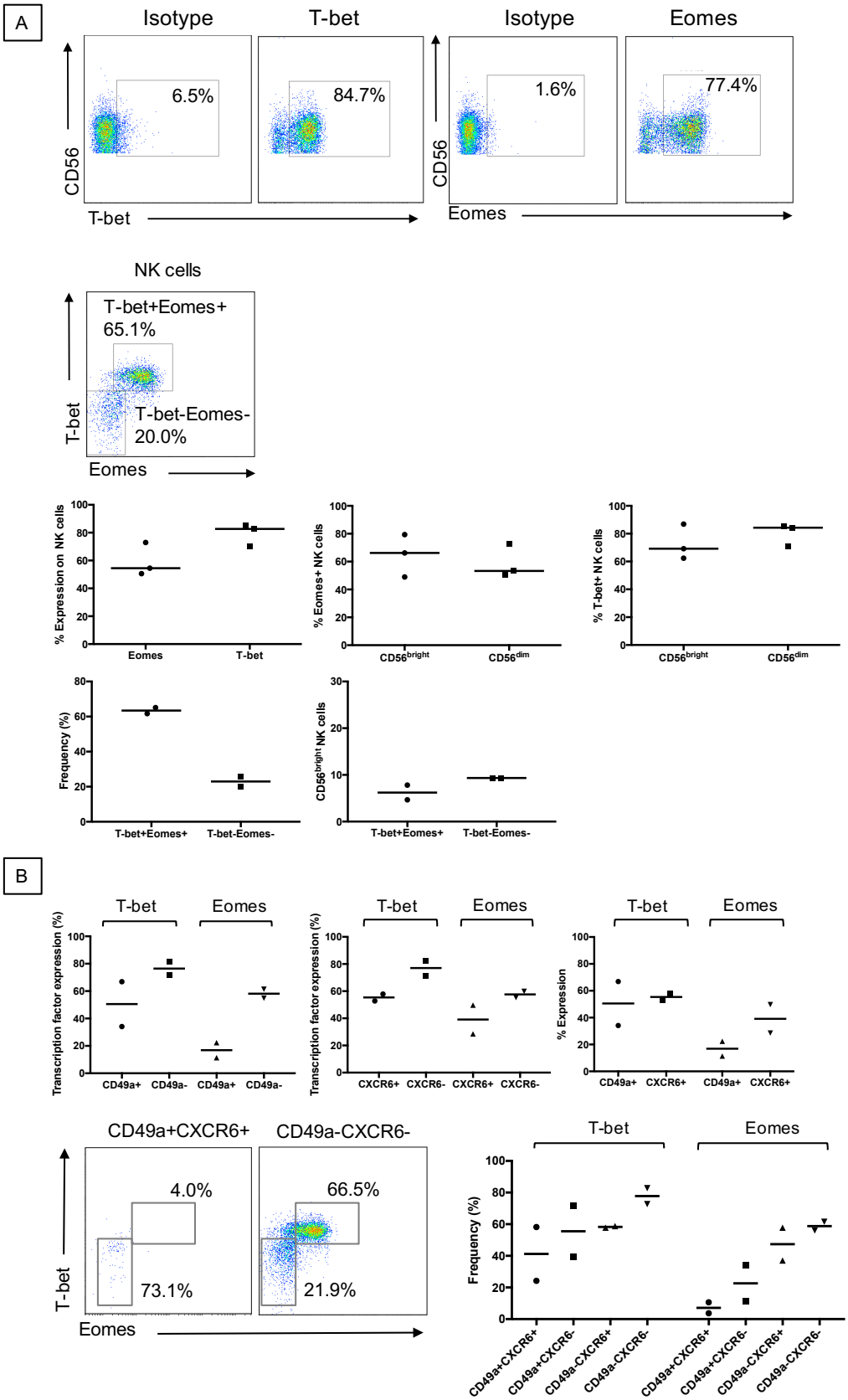
The majority of NK cells in the peripheral blood were T-bet+ (82.7%), and just over half were Eomes+ (54.5%) (**Fig 4-22**). CD56^{bright} NK cells had higher levels of Eomes+ (66.2%) and lower levels of T-bet+ (69.2%) NK cells, compared to CD56^{dim} NK cells (Eomes 53.3%, T-bet 84.3%) consistent with previously published data (**Fig 4-22**) [30]. NK cells could be divided into two subpopulations according to transcription factor expression: T-bet+Eomes+ (63.4%) and T-bet-Eomes- (23.0%) NK cells. However there was no significant difference in CD56^{bright} NK cell frequencies between the two populations (6.3% and 9.3% respectively) (**Fig 4-22**).

CXCR6+ NK cells isolated from the peripheral blood were T-bet^{low} (55.4%), Eomes^{low} (39.1%), compared to the overall NK cell population and CXCR6- NK cells (**Fig 4-22**). In contrast to the liver, circulatory CD49a+ NK cells had a similar transcriptional profile to CXCR6+ NK cells in the same compartment, and were also T-bet^{low} (50.5%), Eomes^{low} (16.9%), compared to the overall NK cell population and CD49a- NK cells (**Fig 4-22**). Double positive CD49a+CXCR6+ circulatory NK cells were therefore T-bet^{low} (41.2%), Eomes^{low} (7.2%) (**Fig 4-22**).

Following culture of PBMCs in media with and without IL-12, frequencies of both T-bet+ and Eomes+ NK cells increased within the NK cell population as a whole as early as 12 hours to 94.5% and 79.4% respectively (**Fig 4-22**). While frequencies of T-bet+ and Eomes+ NK cells did increase within the

double-positive CD49a+CXCR6+ population following IL-12 stimulation, frequencies were significantly lower compared to those observed in all other NK cell subsets (**Fig 4-22**). Of note Cuff et al recently demonstrated that Eomes^{low} NK cells can become Eomes^{high} NK cells following culture with IL-15, IL-15 and TGF-β or IL-15 and IL-12 [33]. While induced Eomes^{high} NK cells displayed a generally similar phenotype to those in the liver, interestingly they did not upregulate CXCR6 [33]. Therefore, it appears unlikely that cytokine-induced CD49a+CXCR6+ NK cells are generated through a unique transcriptional change in T-bet and Eomes expression, and other mechanistic pathways are likely to be important. For this reason, this experiment was only performed on 2 individuals with haemochromatosis and therefore no statistics are shown, however results were extremely comparable between the two patients.

Figure 4-22 Expression of transcription factors T-bet and Eomes on CD49a+ and CXCR6+ circulatory NK cells



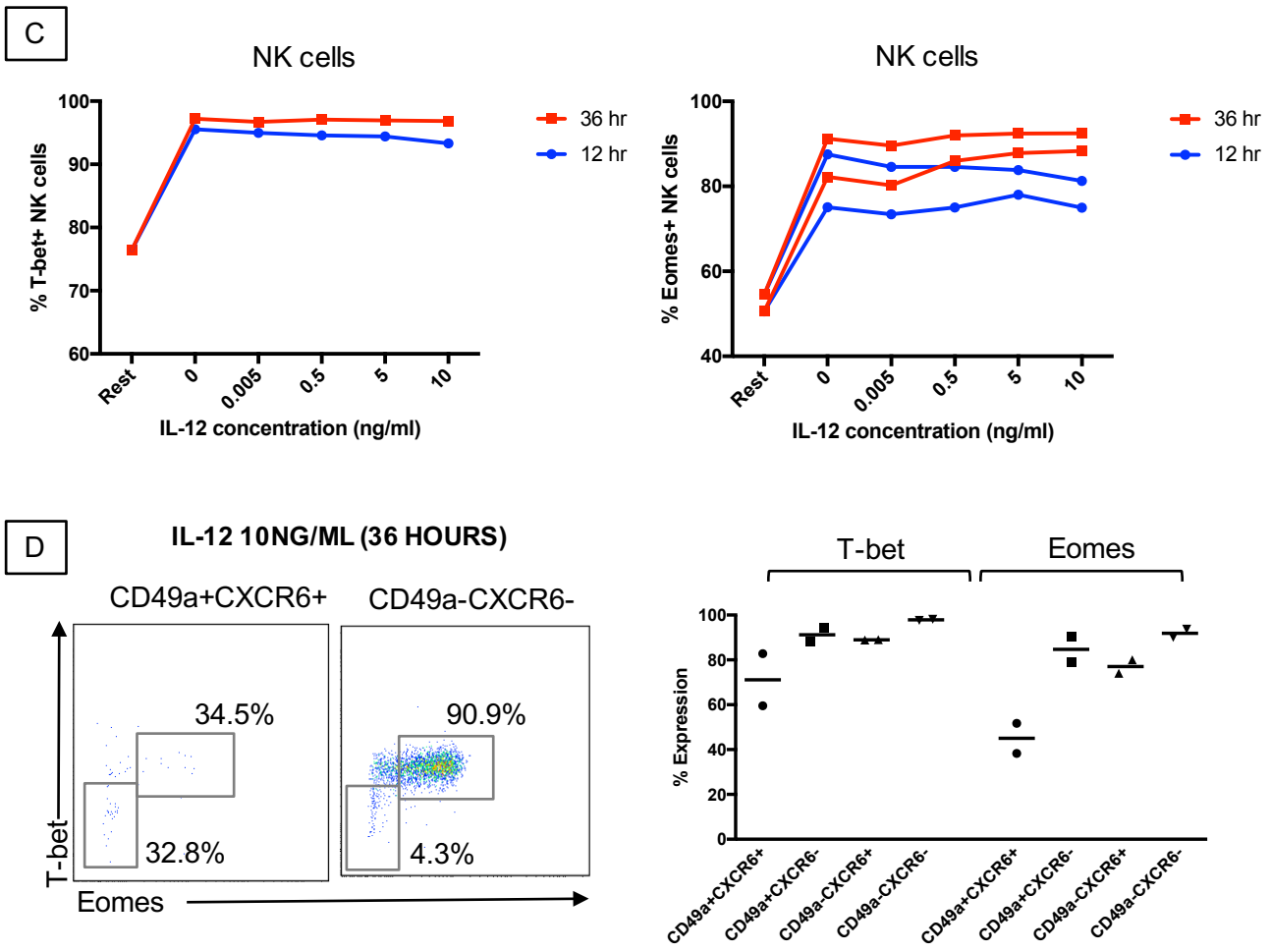


Fig 4-22 **A)** Representative flow cytometry plots gated on NK cells showing gating of T-bet+, Eomes+ T-bet+Eomes+ and T-bet-Eomes- NK cell populations in the peripheral blood at rest. Frequency of T-bet+ and Eomes+ NK cells within the overall, $CD56^{bright}$ and $CD56^{dim}$ NK cell populations in the peripheral blood at rest (*n=3*). Frequency of T-bet+Eomes+ and T-bet-Eomes- NK cell populations in the peripheral blood at rest *(n=2)*. Dot plots display median. **B)** Representative flow cytometry plots showing gating of T-bet+Eomes+ and T-bet-Eomes- NK cell populations within CD49a+CXCR6+ and CD49a-CXCR6- NK cell subsets at rest. Frequency of T-bet+ and Eomes+ NK cells within CD49a+/- and CXCR6+/- NK cell populations within the peripheral blood at rest (*n=2*). Dot plots display median. **C)** Frequency of T-bet+ and Eomes+ NK cells in the overall NK cell populations at rest and following stimulation with increasing concentrations of IL-12. Line chart displays median for each condition. **D)** Representative flow cytometry plots showing gating of T-bet+Eomes+ and T-bet-Eomes- within CD49a+CXCR6+ and CD49a-CXCR6- NK cell subsets following culture with IL-12 10ng/ml for 36 hours. Frequency of T-bet+ and Eomes+ NK cells within the CD49a+/-, CXCR6+/- NK cell populations following culture with IL-12 10ng/ml for 36 hours. Dot plots dispay median.

4.5 Discussion

The unique hepatic cytokine microenvironment may drive maturation of non-terminally differentiated NK cells towards functionally distinct liver-resident subsets [33,38]. This may promote tolerance in health in the face of large volumes of non-self antigens from the portal vein, and an activated 'hyperfunctional' phenotype during disease. It is therefore important to understand the influence of cytokines on liver-resident NK cells, in particular the cytokine cocktail (IL-12, IL-15, IL-18 and IL-2) which has been shown to induce memory-like NK cells in the peripheral blood [182,183]. How cytokines can influence NK cells with potential liver-homing (CXCR6) and memory-like properties (CD49a) is also an important area of interest. Peripheral blood cytokine-induced memory-like NK cells have already demonstrated therapeutic benefit for haematological malignancies [185,264] and the generation of these in, or recruitment to, the liver may open doors for novel immunological therapies for viral hepatitis and liver cancer.

Six days of culture with IL-2, IL-15 or the cytokine cocktail led to expansion of NK cell frequencies and absolute numbers within PBMCs in vitro, with evidence of proliferation which was strongest following supplementation with IL-15 or the cocktail. These cytokines also led to the greatest expansion of the $CD56^{bright}$ population within NK cells isolated from the peripheral blood, which may be a feature of their more immature phenotype. Despite my hypothesis that the human liver may contain memory-like NK cells with superior proliferative capabilities, there was no increase in NK cell frequencies within the hepatic lymphocyte population following culture with any activating cytokines and absolute numbers of NK cells were seen to decrease. This is likely to be a result of the poor viability of NK cells isolated from the liver in vitro. However, the CFSE experiments suggest the presence of live cells that undergo proliferation, with IL-15, the cytokine cocktail and also IL-2, resulting in the lowest CFSE intensities at day six. The liver may therefore contain a sub-population of NK cell with enhanced proliferative capabilities. While IL-15 and the cytokine cocktail appear to drive the greatest proliferation of NK cells in both the peripheral blood and liver, IL-2 promoted the greatest expansion of the $CD56^{bright}$ population in the liver along with IL-15, perhaps a result of the fact that they both signal through the common gamma chain receptor. The cytokine cocktail led to contraction of the overall NK cell frequency in this compartment, highlighting that cytokines have different influences on NK cells isolated from the liver and peripheral blood.

Culture of hepatic MNCs with any activating cytokine (IL-2/-12/-15/-18 or the cytokine cocktail) led to a significant increase in the frequencies of NK cells which express CD49a, from a median resting frequency of 8.7% to 55.7% with IL-12 and up to 88.9% with the cytokine cocktail. CFSE staining

suggests both CD49a+ and CD49a- NK cells undergo proliferation with all activating cytokines, particularly IL-15 and the cytokine cocktail. However NK cells expressing CD49a at day six do appear to have a proliferative advantage under both conditions. Furthermore, an increase in the absolute number of CD49a+ NK cells can be observed following culture with the cytokine cocktail. Without sorting of CD49a+/- NK cells prior to culture it is difficult to conclude whether the enrichment of this subset occurs as a result of superior proliferative capacities of existing CD49a+ NK cells, or through the upregulation of CD49a de novo on CD49a- NK cells. This experiment was recently performed by Marquardt et al, who sorted liver-resident CD49a+ and CD49a- NK cells and observed an 800 fold increase in CD49a+ NK cells over 3 weeks, but also upregulation of CD49a on CD49a- NK cells following stimulation with IL-15 and feeder cells [35]. It is therefore likely that both mechanisms operate.

Conversely frequencies of CXCR6+ NK cell did not increase following culture with any activating cytokines. In contrast to its influence on the CD49a+ NK cell population, the cytokine cocktail led to a dramatic reduction in frequencies of CXCR6+ NK cells in the majority of patients. Again CFSE plots show proliferation of all liver-resident NK cells, which was strongest with IL-2, IL-15 and the cytokine cocktail, however CXCR6+ NK cells displayed slightly higher CFSE intensities at day six compared to both CXCR6- and CD49a+ NK cells with the cytokine cocktail suggesting their proliferative capabilities are marginally reduced.

I have demonstrated that cytokines can generate hyperfunctional CD49a+ NK cells from PBMCs in vitro despite extremely low resting frequencies. Again, it is not clear if this is a result of enhanced proliferation of existing populations, as both CD49a+/- NK cells can be seen to proliferate, however an increase in absolute numbers of CD49a+ NK cells can be observed under all conditions, mirrored by a decrease in CD49a- NK cells which suggests de novo expression may play a role. Cytokine-generated CD49a+ NK cells display a comparable phenotype to those found in the resting liver ($CD56^{bright}$ $CD69^{high}$IFNγ+, with higher percentages of NKG2C+ NK cells compared to CD49a- NK subsets). The 'adaptive phenotype-inducing' cocktail led to the greatest upregulation of CD49a. This may mimic events in vivo in which an inflammatory hepatic cytokine microenvironment, found in hepatitis or cancer, dominated by IL-1, IL-2, IL-6, IL-12, IL-15, IL-18, IFNα, IFNγ and TNFα/β [48,49], leads to the expansion of CD49a+ NK cells. The cytokine hyper-responsive behaviour observed in this subset may therefore occur as a result of previous cytokine-priming, and the adaptive qualities of CD49a+ NK cells may be driven through similar mechanisms to cytokine-induced memory-like NK cells generated in the blood [183].

I have also shown that IL-2, IL-12 and IL-15 can upregulate CXCR6 on peripheral NK cells, which is likely to induce their homing and residence within the liver. NK cells expressing CXCR6 at day six of culture show similar proliferation to that of CD49a+ NK cells, and appear to have a proliferative advantage over CXCR6- NK cells with IL-2, IL-12 and IL-15. While CD49a+ NK cells generated using cytokines in the periphery have many similarities to those which are liver-resident, cytokine-induced CXCR6+ NK cells appear more activated than their hepatic counterparts, in terms of NKG2C expression, cytokine-induction and ability to produce high quantities of IFNγ where CD49a is co-expressed.

The main exception occurs with the cytokine cocktail. Culture with IL-2, IL-12, IL-15 and IL-18 in combination leads to loss of the CXCR6+ population within the hepatic NK cell population, and fails to expand the CXCR6+ population within PBMCs, in contrast to the individual influences of all four cytokines. The contraction of the frequencies of CXCR6+ NK cells within the hepatic NK cell population in the presence of the cytokine cocktail is difficult to explain, as there is no increase in the number of dead cells in this setting compared to other cytokines. CFSE experiments suggest CXCR6- NK cells isolated from both the liver and the blood proliferate better than CXCR6+ NK cells under these conditions, and absolute numbers of CXCR6- NK cells are significantly higher at the end of culture in comparison to stimulation IL-2, IL-12, IL-15 or IL-18 individually. However CXCR6+ NK cells display lower CFSE intensities at day six following culture with the cytokine cocktail than with IL-12 for example, where frequencies remain stable or increase in the liver, and increase in the blood. This raises the possibility that in addition to CXCR6- NK cells 'out-proliferating' CXCR6+ NK cells following stimulation with the cytokine cocktail, CXCR6+ NK cells may also down-regulate CXCR6 as surface receptor. A possible explanation for this is that stimulation with the cytokine cocktail leads to NK cell exhaustion due to persistent exposure to multiple activating cytokines, and this leads to an inability to upregulate CXCR6 on the cell surface, activation-induced NK cell apoptosis or impaired proliferation. It would therefore be interesting to examine markers of apoptosis within CXCR6+ NK cells in culture. It is not clear however why this phenomenon is not observed for CD49a+ NK cells.

By performing NK cell purification prior to culture with activating cytokines, I have identified IL-12 as a key cytokine for the induction of CXCR6+ NK cells, and IL-15 as a strong stimulus for the induction of CD49a+ NK cells in the peripheral blood. In order to demonstrate this definitively however it would be ideal to perform a blocking experiment for IL-12 and IL-15 respectively. RNA sequencing identified upregulation of transcripts encoding the IL-12Rβ2 and IL-23 receptors on CXCR6+ NK cells which support my functional data, however this was not accompanied by upregulation of the

gene encoding IL-12Rβ1. Through culture with IL-12 or IL-15 it is possible to generate high frequencies of activated double-positive CD49a+CXCR6+ NK cells in the peripheral blood in vitro, which display both markers of tissue-residency (CD69) and markers associated with adaptive behavior (NKG2C, IFNγ). This data also suggests cytokine signalling, in addition to CMV infection, can lead to expansion of NK cells expressing the adaptive marker NKG2C [186,187,204].

I hypothesized this transition may be supported by changes at a transcription factor level, as in the human liver CD49a+ NK cells have been found to be T-bet+Eomes- [35] and CXCR6+ NK cells are T-bet^{low}Eomes^{high} [29]. Furthermore IL-15 and TGFβ have recently been shown to induce transition of Eomes^{low} to Eomes^{high} NK cells, although CXCR6 was not upregulated under these conditions [33]. While frequencies of both T-bet+ and Eomes+ NK cells increased following culture with IL-12, the majority of CD49a- and CXCR6- NK cells within the peripheral blood already expressed these transcription factors at rest, and it was the CD49a- and CXCR6- subsets which showed the greatest upregulation of both transcription factors in culture. Therefore neither T-bet or Eomes showed any specificity towards the development of cytokine-induced CD49a+ or CXCR6+ phenotypes in my experiments.

The induction of double-positive CD49a+CXCR6+ NK cells within PBMCs in vitro suggests that in addition to clonal expansion of adaptive-like CD49a+ liver-resident NK cells in liver disease, there may be hepatic recruitment of newly generated CD49a+CXCR6+ NK cells, capable of releasing high levels of Th1 cytokines, induced by systemic inflammation in the peripheral blood including high levels of IL-12/IL-15. This process may be driven through IFNγ upregulation of CXCL16 in the liver. Insurgence of CXCR6+ NK cells may lead to recruitment and activation of neutrophils and macrophages via GM-CSF and CCL3 [32]. These findings may have important therapeutic applications. The generation of activated CXCR6+ NK cells in the peripheral blood that co-express CD49a and adopt an ‘adaptive’ phenotype, may allow hyperfunctional NK cells to preferentially enrich the liver, boosting the hepatic innate immune response to fight viruses and cancer, providing a basis for novel, locally acting immunotherapies for common hepatic disease.

The main limitations of this work are that the experiments performed were not fully optimised to understand the influence of individual cytokines. Firstly it would have been beneficial to examine the dose response and kinetic curves for the influence of increasing concentrations of IL-2, IL-12, IL-15 and IL-18 on NK cell proliferation in the human liver and peripheral blood. This was originally performed by another member of the Khakoo lab in mice, however, it is not clear that these concentrations are optimal for human NK cells, or that the same concentrations are required to induce proliferation of circulatory and liver-resident NK cells. Variation in survival signals and cytokine receptor expression may mean that differences exist between liver- and circulatory-derived

NK cell in terms of their response towards varying concentrations and combinations of cytokines in vitro. In addition, a negative control in which cells PBMCs were cultured in media alone should have been included to understand the additional influence of exogenous cytokines. Considerable variation was observed between individuals for the induction of CXCR6 and CD49a+ NK cells, and therefore greater patient numbers and stratification for disease would have been worthwhile.

Blocking experiments for IL-2, IL-12, IL-15 and IL-18 would have been beneficial to definitively establish the influence of each of these cytokines. As discussed above sorting NK cells according to CD49a and CXCR6 status would be ideal to understand whether these subpopulations are proliferating, or upregulating CD49a and CXCR6 in culture. Furthermore it would be interesting to more comprehensively examine the phenotype of CD49a+ and CXCR6+ NK cells in culture, particularly to examine whether cytokine-induced CD49a+ NK cells in the peripheral blood express KIR as seen in the liver at rest.

It would also be worthwhile to examine the viability of liver-resident NK cells, particularly CXCR6+ NK cells in culture at an earlier time point, for example two days, to understand if the contraction of the liver-resident CXCR6+ NK cells seen in vitro occurs as a result of cell death. Finally given the differential influence of cytokines demonstrated here on CXCR6+ and CD49a+ populations it would be useful to stain both these populations for cytokine receptors, for example to see if IL-2, IL-15 and IL-18 receptors are more highly expressed on CD49a+ NK cells and if the IL-12 receptor is more highly expressed on CXCR6+ NK cells.

5 Chapter 5: IL-12 Hypersensitivity in Primary Biliary Cholangitis

5.1 Introduction

The previous two results chapters provide a description of the differences between two distinct liver-resident NK cell subpopulations and the influence of cytokines on inducing these phenotypes in the liver and peripheral blood. In this final chapter I aim to apply these findings to understand some of the immunological mechanisms behind the autoimmune liver disease, Primary Biliary Cholangitis (PBC). This is the most common of the autoimmune liver diseases, affecting 1 in 1000 women in the UK over 40 and is the third most common indication for a liver transplant [131]. Antibodies are formed against the pyruvate dehydrogenase complex found within the mitochondria in BECs and this, accompanied by activation of other components of the immune response leads to progressive destruction of the biliary tree. PBC strongly impacts on quality of life causing severe fatigue and pruritus, and is associated with increased mortality [131]. The only approved treatment in the UK is currently UDCA, however up to 40% of individuals fail to respond biochemically to this and these patients can go onto to develop cirrhosis which may require liver transplantation [265,266]. In order to address this unmet therapeutic need, a number of GWAS studies have been performed, revealing three affected immunological pathways (**Table 5**). Of these the IL-12/STAT4 signalling pathway was found to be associated with increased susceptibility to PBC in all populations studied. (**Table 5**).

Previous work from the Khakoo lab identified transcriptional changes post liver transplantation, resulting in deficiencies in STAT4 and a tolerant NK cell phenotype [46]. Furthermore I have identified IL-12 as an important cytokine involved in the upregulation of the liver-homing adhesion marker, CXCR6 (**Fig 4-16**). I therefore hypothesised that overactivation of the IL-12/STAT4 axis may result in hyperfunctional NK cells and asked the following questions: (1) Is the IL-12/STAT4 pathway upregulated in NK cells in patients with PBC, (2) Does upregulation of the IL-12/STAT4 pathway influence the generation or function of adaptive-like (CD49a+) and liver-homing (CXCR6+) NK cell phenotypes in the peripheral blood. Therefore, I aimed to investigate a novel association of adaptive-like NK cells in causing autoimmunity and suggest a possible mechanism for this, whilst also providing a functional correlate for the published GWAS studies.

5.2 Patient demographics

Peripheral blood was collected from 35 individuals with PBC, 31 patients with the chronic liver disease haemochromatosis and 9 healthy controls (HC). Demographic details are described below (**Table 15**). Haemochromatosis is a genetic condition caused predominantly by mutations in the HFE gene, leading to increased intestinal absorption of iron and iron deposition in multiple organs including the liver. While this eventually may result in hepatic fibrosis, the mechanism is unknown as haemochromatosis is thought to be a non-inflammatory liver disease. It was not possible to match the two disease groups in terms of gender as PBC predominantly affects women and haemochromatosis men, however the groups were matched for age and rates of cirrhosis. Recruited individuals with PBC had a median ALP of 173 U/L (range 64-1487 U/L).

Table 15 Demographic details from individuals with PBC and controls

	Primary Biliary Cholangitis (n=35)	**Haemochromatosis (n=31)**	**Healthy Controls (n=9)**	**p-value**		
				PBC vs NALD	PBC vs HC	NALD vs HC
Age, median (range)	63.0 (44 - 83)	56.5 (20 - 73)	31.2 (20-40)	0.0710	<0.0001	0.0009
Men, n (%) **Women, n (%)**	3 (8.6) 32 (91.4)	24 (77.4) 7 (22.6)	0 (0) 9 (100)	<0.0001	0.3629	<0.0001
Cirrhosis, n (%)	3 (8.6)	7 (22.6)	0 (0)	0.1132	0.3629	0.1165
UDCA, n (%)	28 (80.0)	0 (0)	0 (0)	<0.0001	<0.0001	-
Liver Transplant, n (%)	0 (0)	0 (0)	0 (0)			

5.3 CXCR6+ and CD49a+ NK cells are found at higher frequencies in the peripheral blood in patients with PBC compared to controls

I tested the frequencies of CD49a+ and CXCR6+ NK cells in the circulation at rest between the three cohorts. Frequencies of NK cells within the lymphocyte population in the peripheral blood were comparable between patients with PBC (10.7%), haemochromatosis (11.3%) and healthy controls (11.5%) (**Fig 5-1**). Frequencies of $CD56^{bright}$ NK cells within the NK cell population in the peripheral blood were also similar between the groups (PBC 8.7%, haemochromatosis 6.4%, HCs 5.3%) (**Fig 5-1**). Interestingly, there was significant enrichment of both CD49a+ and CXCR6+ NK cells at rest in PBC compared to both control groups (**Fig 5-1**). This was specific to NK cells, with no increase in frequencies of CXCR6+ or CD49a+ CD56- T-cells seen in the same PBC cohort compared to patients with haemochromatosis (**Fig 5-1**).

Figure 5-1 The peripheral blood NK cell population in individuals with PBC contains higher frequencies of CXCR6+ and CD49a+ NK cells compared to control groups

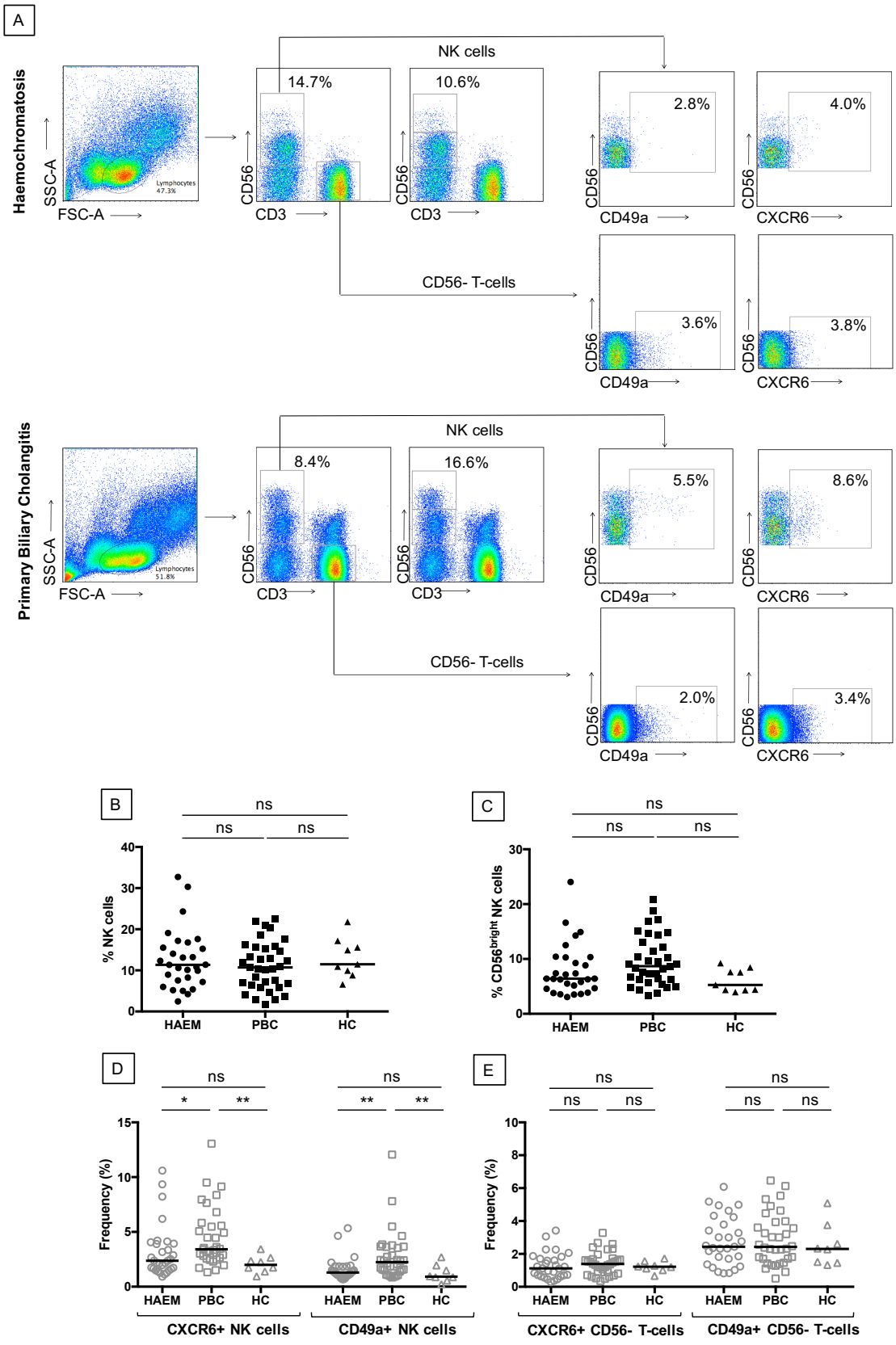


Fig 5-1 **A)** Representative flow cytometry plots showing gating on NK cells, $CD56^{bright}$, $CD56^{dim}$, CXCR6+ and CD49a+ NK cell subpopulations within PBMCs for patients with PBC and haemochromatosis. **B)** Percentage of NK cells within the lymphocyte population for individuals with haemochromatosis (HAEM) (11.3%, *n=29*), PBC (10.7%, *n=35*) and HC (11.5%, *n=9*). Dot plots display median. **C)** Percentage of $CD56^{bright}$ NK cells within the NK cell population for individuals with haemochromatosis (6.4%, *n=29*), PBC (8.7%, *n=35*) and HC (5.3%, *n=9*). Dot plots display median. **D)** Percentage of CXCR6+ and CD49a+ NK cells within the NK cell population for individuals with haemochromatosis (*n=31*), PBC (*n=34*) and HC (*n=8*). Dot plots display median. **E)** Percentage of CXCR6+ and CD49a+ CD56- T-cells within the CD56- T-cell population for individuals with haemochromatosis (*n=29*), PBC (*n=34*) and HC (*n=8*). Dot plots display median. (*Kruskal-Wallis with Dunn's multiple comparison test*). $p<0.05$*, $p<0.01$**.

5.4 Increased frequencies of CXCR6+ NK cells can be induced in patients with PBC, and increased frequencies of CD49a+ NK cells can be induced in patients with chronic liver disease in general

Having identified increased frequencies of both CXCR6+ and CD49a+ NK cells in the peripheral blood at rest, I tested whether 'liver-homing' and 'adaptive' phenotypes could be more easily induced in individuals with PBC as a result of cytokine hyper-responsiveness. I cultured PBMCs from individuals with PBC, haemochromatosis and healthy volunteers for four days in media only and increasing concentrations of IL-12 and IL-15. I found that culture in media alone provided sufficient stimulus to increase the frequencies of CXCR6+ NK cells from rest in patients with PBC (5.1% to 28.3%, $p<0.0001$), haemochromatosis (2.5% to 9.7%, $p=0.0003$) and healthy controls (2.0% to 5.2%, $p=0.0156$) (**Fig 5-2**). This is likely to be secondary to the release of IL-12 and IL-15, along with other activating cytokines, in the culture media by monocytes, B-cells and dendritic cells.

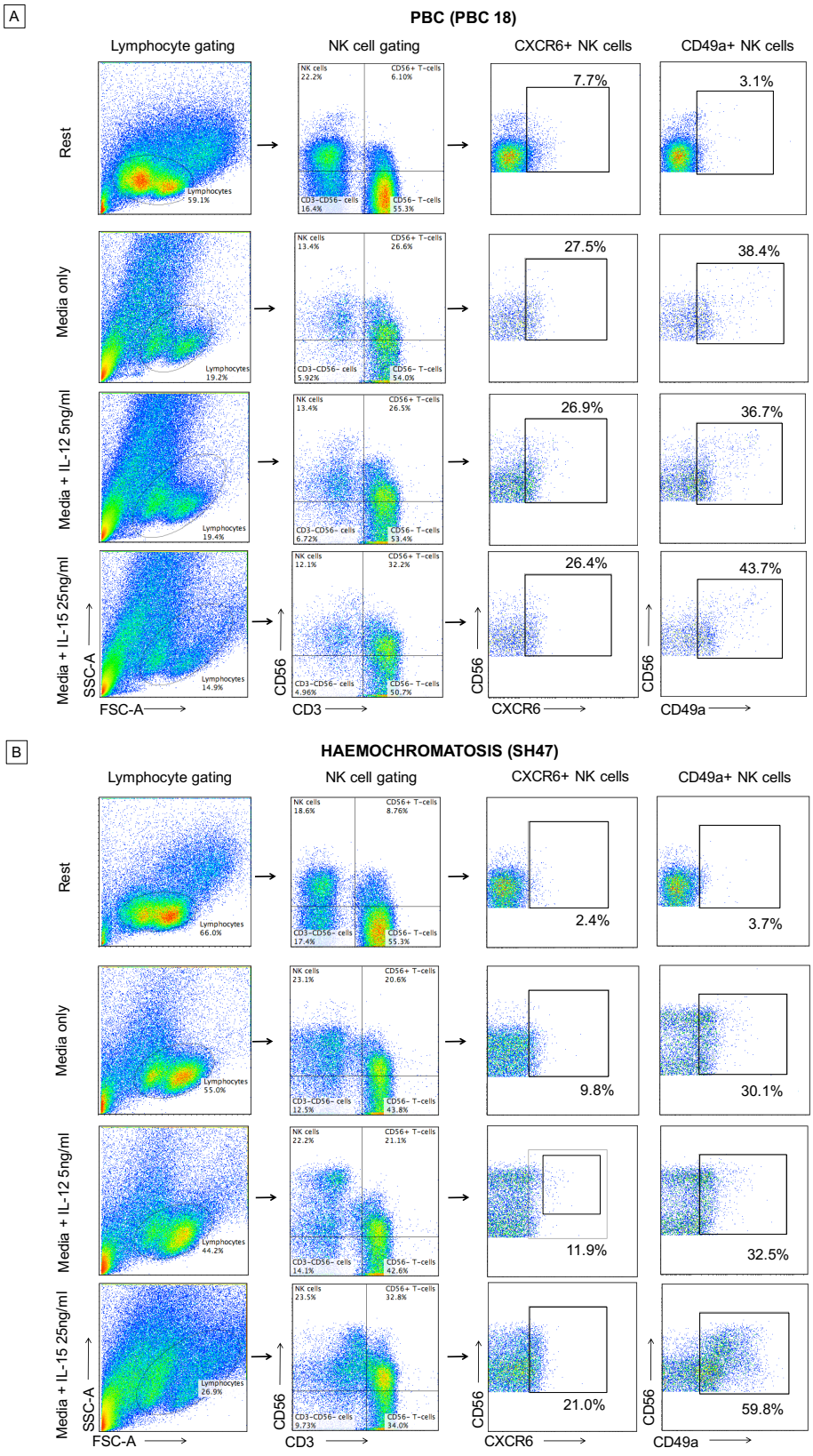
A greater frequency of CXCR6+ NK cells was observed when culturing PBMCs in unsupplemented media from patients with PBC compared to both control groups. Furthermore a small increase was seen on addition of increasing concentrations of IL-12 ($p=0.0079$ Friedman with Dunn's multiple comparison test), but not IL-15 ($p=0.3202$ Kruskal-Wallis with Dunn's multiple comparison test) for PBC patients (**Fig 5-2**). Frequencies of CXCR6+ NK cells increased steadily with increasing concentrations of IL-12 (haemochromatosis: $p=0.4749$ Kruskal-Wallis with Dunn's multiple comparison test; HC: $p=0.0005$ Friedman with Dunn's multiple comparison test) and IL-15 (haemochromatosis: $p=0.3080$ Kruskal-Wallis with Dunn's multiple comparison test; HC: $p<0.0001$ Friedman with Dunn's multiple comparison test) for healthy controls, however no statistically significant increase was seen for patients with haemochromatosis, perhaps due to greater individual variation within the group. Frequencies of CXCR6+ NK cells induced in both patients with haemochromatosis and healthy controls with exogenous cytokines were still lower than those induced in PBC with media alone (**Fig 5-2**).

CD49a could also be more readily induced on NK cells on culturing PBMCs in unsupplemented media from patients with PBC (2.3% to 30.6%, $p<0.0001$), but in contrast to CXCR6+ NK cells, similarly high frequencies were seen in individuals with haemochromatosis (1.8% to 27.2%, $p<0.0001$) (**Fig 5-2**). The increase in CD49a+ NK cell frequencies seen for healthy volunteers was much smaller (0.9% to 7.6%, $p=0.0078$). Further increases in frequencies could be generated on

adding IL-15 (PBC: p=0.0120 Kruskal-Wallis with Dunn's multiple comparison test; haemochromatosis: p=0.0081 Kruskal-Wallis with Dunn's multiple comparison test), but not IL-12 (PBC: p=0.0786, Friedman with Dunn's multiple comparison test; haemochromatosis: p=0.1012 Kruskal-Wallis with Dunn's multiple comparison test), for patients with PBC and haemochromatosis. A lower dose-dependent induction of CD49a+ NK cells was seen in healthy controls for IL-12 (p=0.0120 Friedman with Dunn's multiple comparison test) and IL-15 (p<0.0001 Friedman with Dunn's multiple comparison test) (**Fig 5-2**).

These results show that CXCR6+ NK cells are more easily induced in culture in PBC, whereas CD49a+ NK cells are more easily induced in chronic liver disease in general. I have previously demonstrated through NK cell purification that IL-12 is important for the induction of CXCR6+ NK cells (**Fig 4-17**). However it was not possible to definitively conclude that the induction of CXCR6+ NK cells seen in patients with PBC when culturing PBMCs in media alone occurred as a result of IL-12 hyper-responsiveness without NK cell purification prior to culture.

Figure 5-2 CXCR6+ NK cells are more easily induced in individuals with PBC, and CD49a+ NK cells are more easily induced in individuals with chronic liver disease in general (non-purified NK cells, four days of culture)



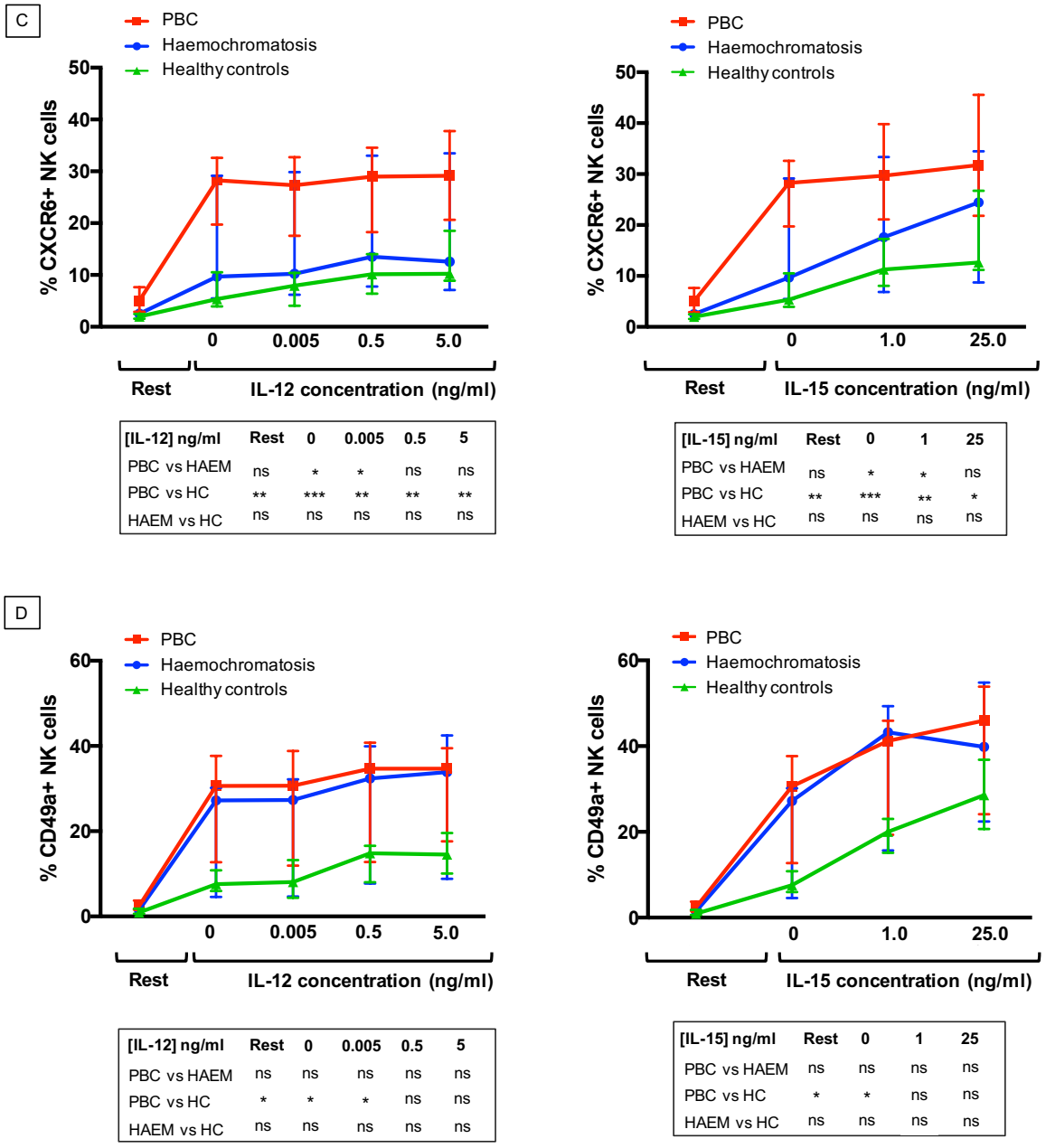
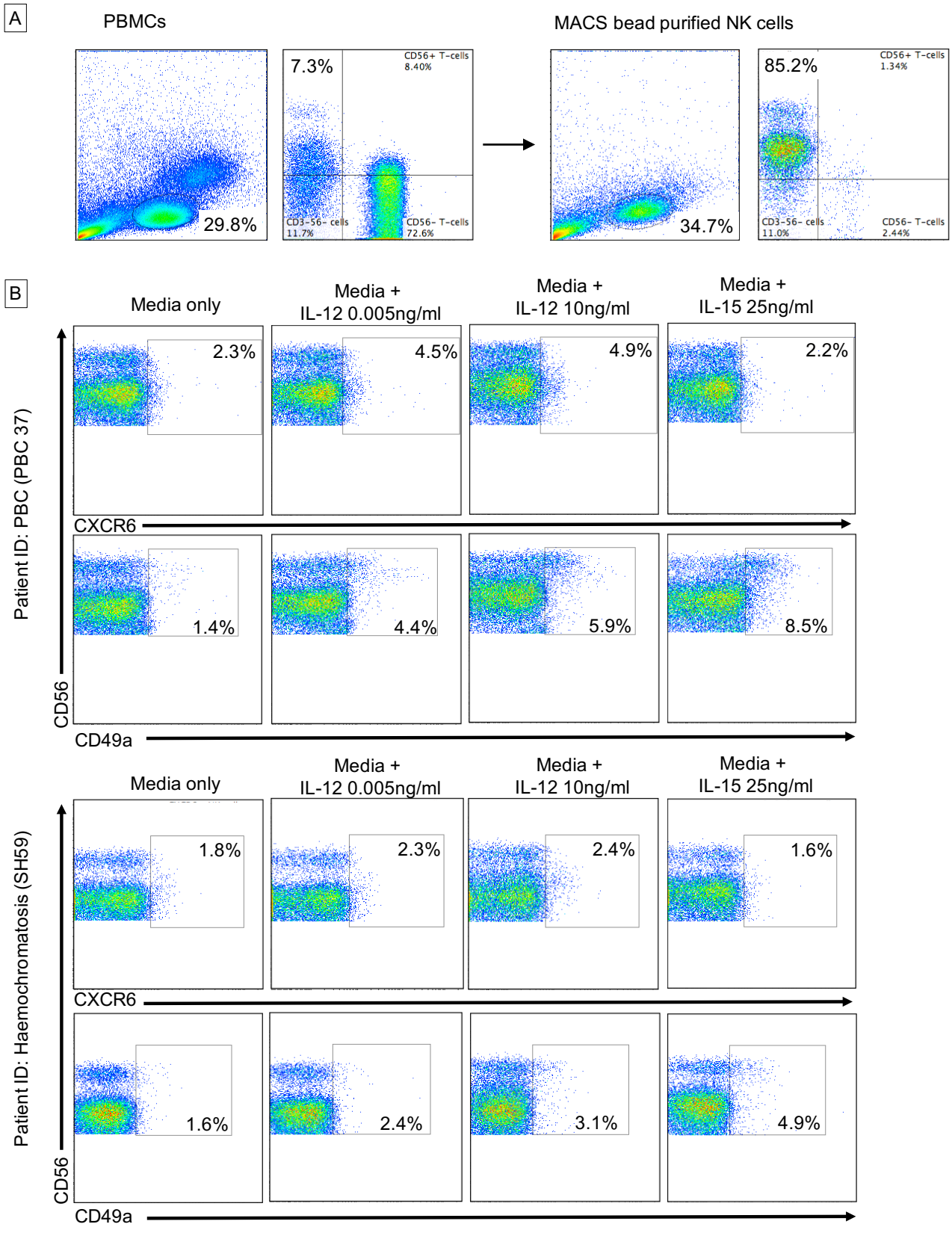


Fig 5-2 **A)** Representative flow cytometry plots from an individual with PBC showing gating of lymphocytes, NK cells and CXCR6+ and CD49a+ NK cells at rest and following culture with media only, media supplemented with IL-12 5ng/ml and media supplemented with IL-15 25ng/ml. **B)** Representative flow cytometry plots from an individual with haemochromatosis showing gating of lymphocytes, NK cells and CXCR6+ and CD49a+ NK cells at rest and following culture with media only, media supplemented with IL-12 5ng/ml and media supplemented with IL-15 25ng/ml. **C)** Percentage of CXCR6+ NK cells within the peripheral blood NK cell population of individuals with haemochromatosis (*n=16-19*), PBC (*n=18-19)* and HC (*n=8*) at rest and following stimulation of PBMCs for four days in media only and with increasing concentrations of IL-12 and IL-15. **D)** Percentage of CD49a+ NK cells within the peripheral blood NK cell population of individuals with haemochromatosis (*n=16-19*), PBC (*n=20-21)* and HC (*n=8*) at rest and following stimulation of PBMCs for four days in media only and with increasing concentrations of IL-12 and IL-15. Line charts display median for each condition. (*Kruskal-Wallis with Dunn's multiple comparison test*). p<0.05*, p<0.01**, p<0.001***, p<0.0001****

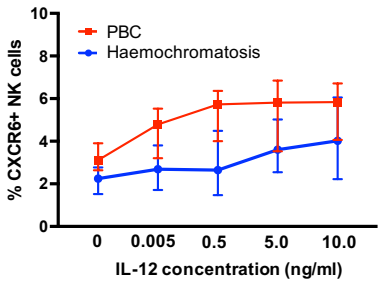
In order to determine the influence of IL-12 and IL-15, NK cells were purified from freshly isolated PBMCs using magnetic separation. Purified NK cells were then cultured with increasing concentrations of IL-12 and IL-15 for 12 hours, as my previous results have demonstrated it is possible to induce CXCR6+ and CD49a+ NK cells at this time point (**Fig 4-16**). Stimulation with just IL-12 0.005ng/ml led to a small, but significant enrichment of the CXCR6+ subset compared to individuals with haemochromatosis, and compared to culture with media alone (**Fig 5-3**). Higher concentrations of IL-12 are required to induce similar frequencies of CXCR6+ NK cells for individuals with haemochromatosis. At this short time point and following the removal of other leukocytes from the culture media, IL-15 did not lead to any increase in the frequency of CXCR6+ NK cells. Therefore, IL-12 hyper-responsiveness is associated with augmented induction of CXCR6+ NK cells in individuals with PBC.

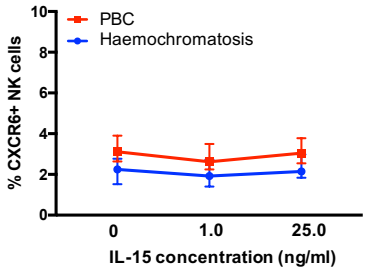
As demonstrated for non-purified NK cells in culture, CD49a+ NK cells from individuals with both haemochromatosis and PBC were sensitive to induction using both IL-12 and IL-15. An increase in the frequency of CD49a+ NK cells was detected in both patient cohorts at this short time point (**Fig 5-3**). As reported in results chapter 2, IL-15 is able to trigger a greater increase in frequencies of CD49a+ NK cells, compared to IL-12 (**Fig 5-3**). No significant difference was detected in the percentage of CD49a+ NK cells following stimulation with low dose IL-12 between the two patient cohorts (**Fig 5-3**).

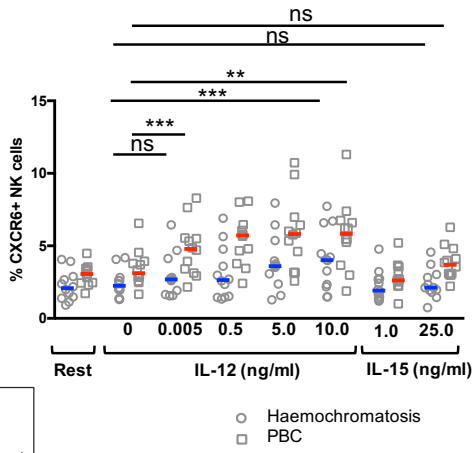
Figure 5-3 CXCR6+ and CD49a+ NK cells are more easily induced in individuals with PBC (purified NK cells, 12 hours of culture)



C



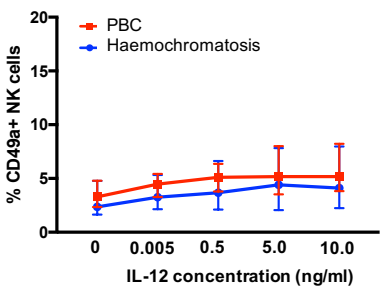


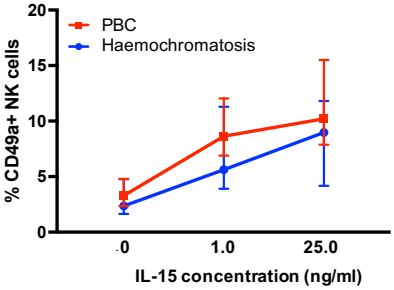


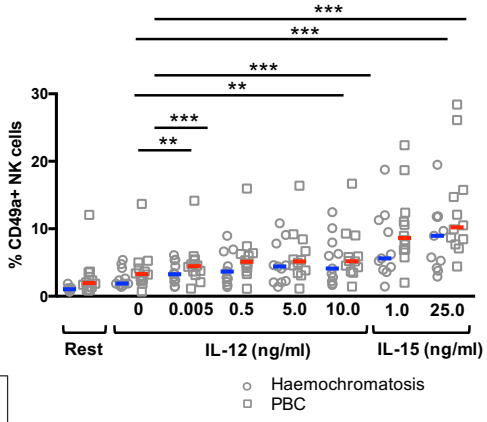
[IL-12] ng/ml	0	0.005	0.5	5	10
PBC vs HAEM	*	**	**	ns	ns
HAEM	***	(Friedmans with Dunn's multiple comparison)			
PBC	****	(Friedmans with Dunn's multiple comparison)			

[IL-15] ng/ml	0	1	25
PBC vs HAEM	*	ns	*
HAEM	ns	(Friedmans with Dunn's multiple comparison)	
PBC	*	(Friedmans with Dunn's multiple comparison)	

D



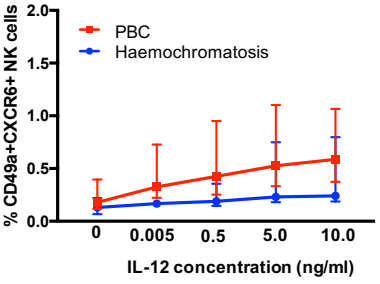


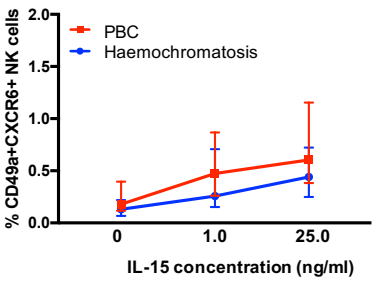


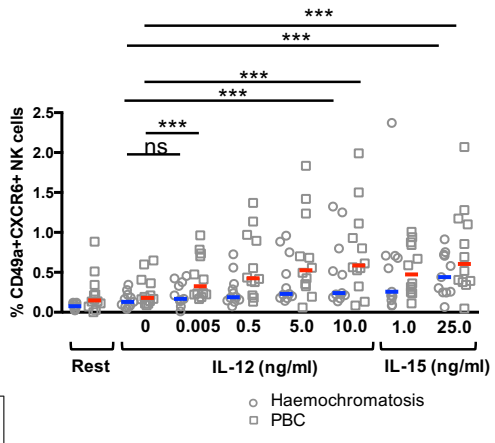
[IL-12] ng/ml	0	0.005	0.5	5	10
PBC vs HAEM	ns	ns	ns	ns	ns
HAEM	**	(Friedmans with Dunn's multiple comparison)			
PBC	****	(Friedmans with Dunn's multiple comparison)			

[IL-15] ng/ml	0	1	25
PBC vs HAEM	ns	ns	ns
HAEM	***	(Friedmans with Dunn's multiple comparison)	
PBC	****	(Friedmans with Dunn's multiple comparison)	

E







[IL-12] ng/ml	0	0.005	0.5	5	10
PBC vs HAEM	ns	*	ns	*	ns
HAEM	***	(Friedmans with Dunn's multiple comparison)			
PBC	****	(Friedmans with Dunn's multiple comparison)			

[IL-15] ng/ml	0	1	25
PBC vs HAEM	ns	ns	ns
HAEM	***	(Friedmans with Dunn's multiple comparison)	
PBC	***	(Friedmans with Dunn's multiple comparison)	

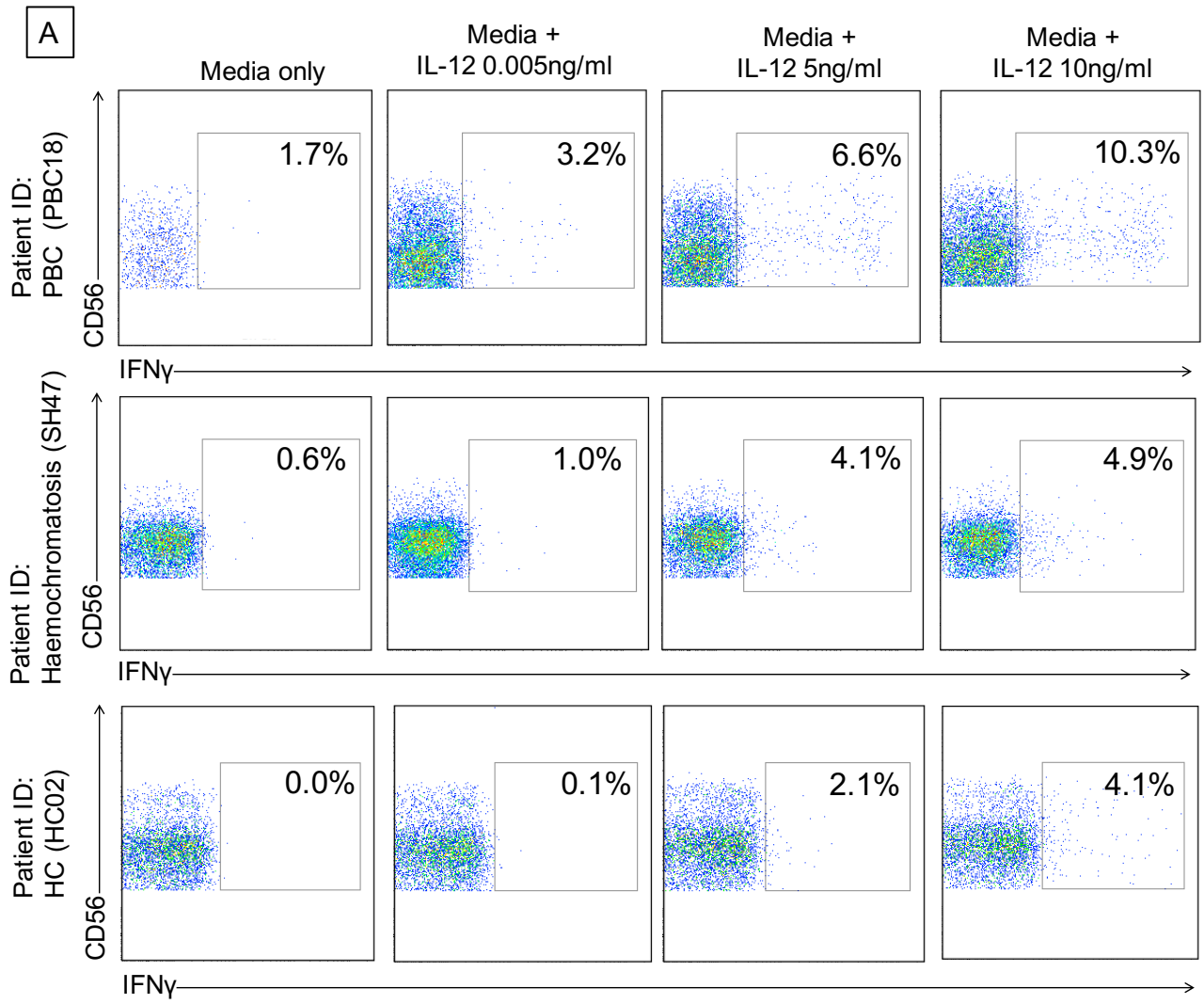
Fig 5-3 **A**) Representative flow cytometry plots displaying lymphocyte and NK cell gating before and after NK cell purification using MACS beads. **B)** Representative flow cytometry plots gated on NK cells showing percentage of CXCR6+ and CD49a+ NK cells following culture in media alone and following the addition of low and high dose IL-12 and high dose IL-15 for individuals with PBC and haemochromatosis. **C)** Percentage of CXCR6+ NK cells within the peripheral blood NK cell population of individuals with haemochromatosis (*n=12*) and PBC (*n=12)* following stimulation of purified NK cells for 12 hours in media only and with increasing concentrations of IL-12 and IL-15. **D)** Percentage of CD49a+ NK cells within the peripheral blood NK cell population of individuals with haemochromatosis (*n=11*) and PBC (*n=12)* following stimulation of purified NK cells for 12 hours in media only and with increasing concentrations of IL-12 and IL-15. **E)** Percentage of CD49a+CXCR6+ NK cells within the peripheral blood NK cell population of individuals with haemochromatosis (*n=11*) and PBC (*n=12)* at rest and following stimulation of purified NK cells for 12 hours in media only and with increasing concentrations of IL-12 and IL-15. Line charts display median and IQR for each condition. (*Mann Whitney U test compares patient groups for each condition, Friedman with Dunn's multiple comparison test compares differences between multiple conditions within each patient group*). Dot plots show individual values and median. (*Wilcoxon matched pairs test*). $p<0.05$*, $p<0.01$**

5.5 NK cells in individuals with PBC display IL-12 hypersensitivity

Having demonstrated that IL-12 hypersensitivity can generate higher frequencies of CXCR6+ NK cells in the peripheral blood, I examined IL-12 responsiveness in the three patient groups using IFNγ production as an end point (**Fig 5-4**). Overall NK cells from the peripheral blood of individuals with PBC produced greater quantities of IFNγ in response to stimulation with only IL-12 0.005ng/ml (2.9%, range 1.0-14.9%) compared to individuals with haemochromatosis (1.2%, range 0.1-2.9%) and healthy controls (0.4%, range 0.1-1.2%) (**Fig 5-4**). Frequencies of IFNγ+ NK cells were however similar between patient populations when stimulating NK cells with higher concentrations of IL-12 (**Fig 5-4**). While there was a slight increase in frequency of IFNγ+ CD56- T-cells in PBC patients compared to control groups, no increase was seen on addition of IL-12 0.005ng/ml to the media, and percentages were negligible compared to NK cells (< 1.5% of total CD56- T-cells) (**Fig 5-4**).

CD49a+ NK cells isolated from patients with PBC contained the highest frequencies of IFNγ+ NK cells (8.6%, range 0 – 43.0%) in response to just IL-12 0.005ng/ml. This was significantly higher than both control groups (**Fig 5-4**). Interestingly CD49a- NK cells from individuals with PBC did not express greater quantities of IFNγ compared to CD49a- NK cells from patients with haemochromatosis, suggesting CD49a+ NK cells may be particularly sensitive to IL-12 in PBC (**Fig 5-4**). Surprisingly CXCR6+ NK cells from patients with PBC also contained a greater frequency of IFNγ+ NK cells (3.6%, range 0.3 – 15.7%) compared to healthy controls. Therefore, both CD49a+ and CXCR6+ NK cells in individuals with PBC appear to demonstrate hyper-responsiveness towards IL-12.

Figure 5-4 NK cells from individuals with PBC produce high quantities of IFNγ in response to low concentrations of IL-12



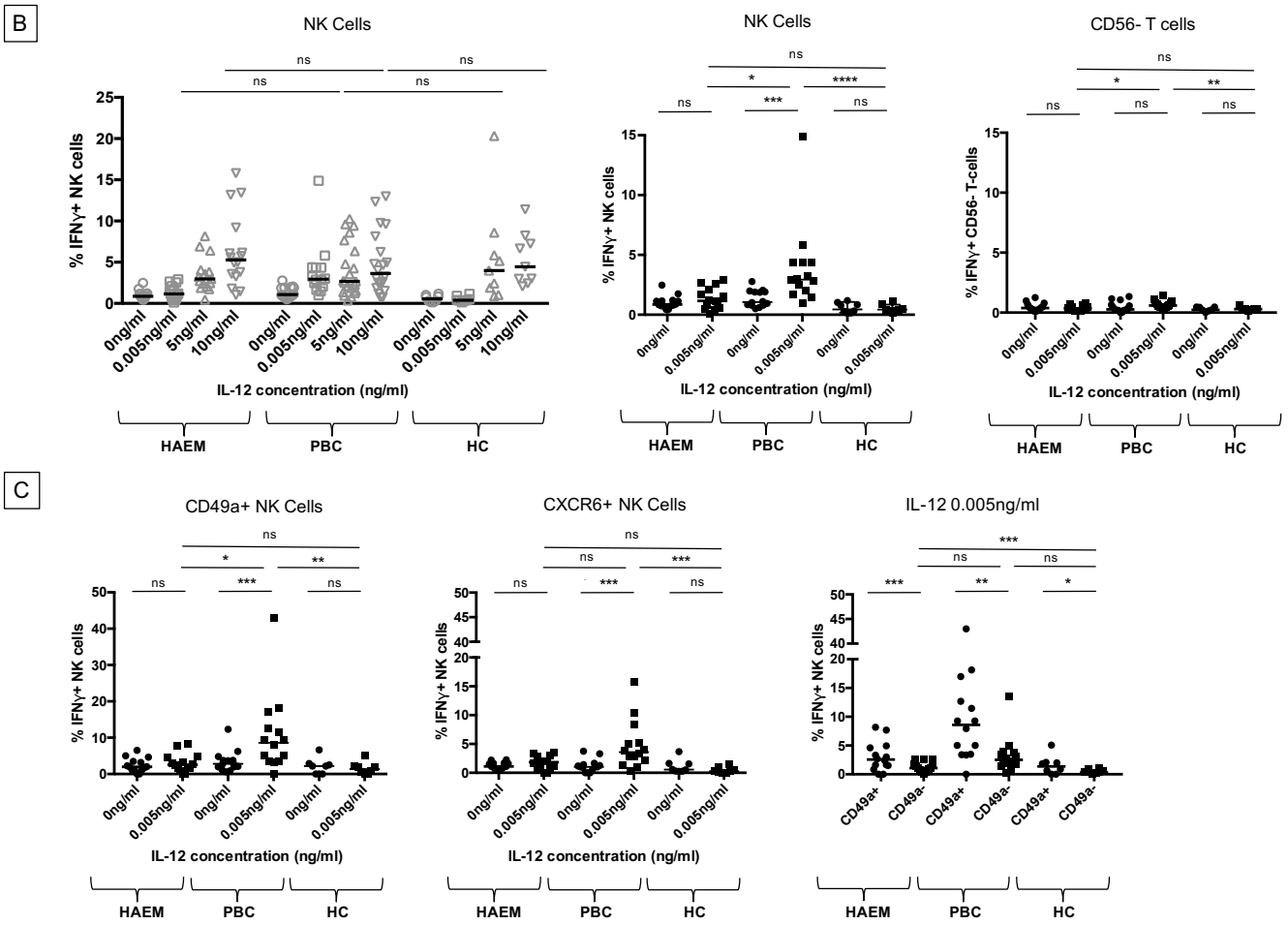
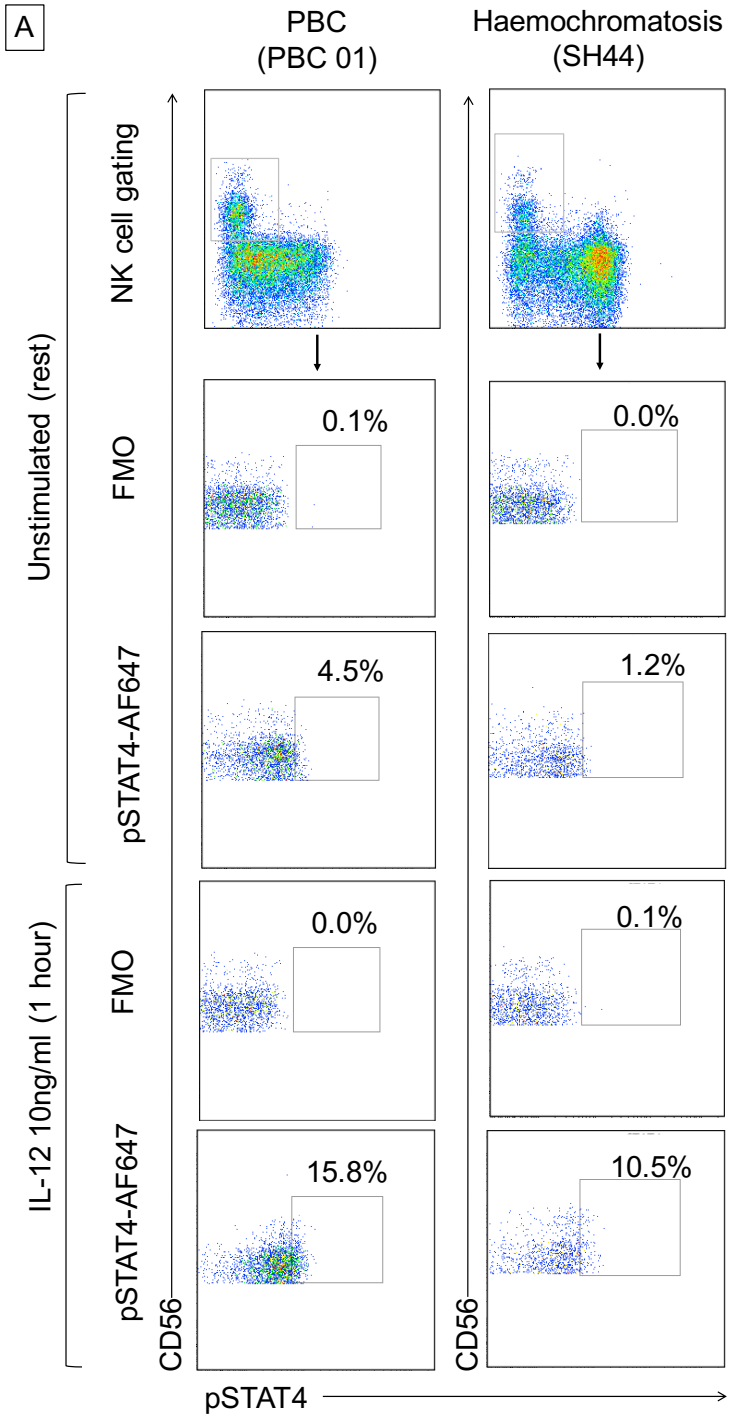


Fig 5-4 **A)** Representative flow cytometry plots of individuals with PBC and haemochromatosis and a healthy volunteer, gated on NK cells, showing IFNγ+ expression following stimulation with increasing concentrations of IL-12 for 12 hours. **B)** Percentage of IFNγ+ NK cells from the peripheral blood of individuals with haemochromatosis (*n=14-16*), PBC (*n=14-21*) and healthy controls (*n=8*) following stimulation with increasing concentrations of IL-12 for 12 hours. Percentage of IFNγ+ NK cells and IFNγ+ CD56- T cells from the peripheral blood of individuals with haemochromatosis (*n=14*), PBC (*n=14*) and healthy controls (*n=8*) following stimulation with media only or IL-12 0.005ng/ml for 12 hours. Dot plots display median. **C)** Percentage of IFNγ+ NK cells within CD49a+ and CXCR6+ NK cell populations from the peripheral blood of individuals with haemochromatosis (*n=14*), PBC (*n=14*) and healthy controls (*n=8*) following stimulation with IL-12 0.005ng/ml for 12 hours and media only. Percentage of IFNγ+ NK cells within CD49a+/- CD49a+ NK cell populations from the peripheral blood of individuals with haemochromatosis (*n=14*), PBC (*n=14*) and healthy controls (*n=8*) following stimulation with IL-12 0.005ng/ml for 12 hours. Dot plots display median. (*Kruskal-Wallis with Dunn's multiple comparison test compares patient groups, Wilcoxon matched pairs test compares IL-12 0ng/ml and IL-12 0.005ng/ml within the same patient group*). p<0.05*, p<0.01**, p<0.001***, p<0.0001****.

5.6 NK cells in individuals with PBC have baseline activation of the IL-12/pSTAT4 axis

Having identified evidence of IL-12 hypersensitivity in NK cells in patients with PBC I went on to examine pSTAT4 expression. A greater percentage of NK cells from individuals with PBC were found to express pSTAT4 at rest compared to patients with haemochromatosis and healthy controls. Patients with PBC and haemochromatosis had similar frequencies of pSTAT4+ CD56- T-cells (**Fig 5-5**). Of note individuals with haemochromatosis also had elevated baseline frequencies of pSTAT4+ NK cells and pSTAT4+ CD56- T-cells compared to healthy controls (**Fig 5-5**). On stimulation with low doses of IL-12, frequencies of pSTAT4+ NK cells did not increase further in either patient group until concentrations of IL-12 5ng/ml or above were used, suggesting that the raised levels of pSTAT4 seen in patients with PBC at rest may be a result of baseline activation of the pathway in vivo (**Fig 5-5**). Differences in frequencies of pSTAT4+ NK cells between patient groups and the heathy volunteers were lost following stimulation with high concentrations of IL-12 (**Fig 5-5**).

Figure 5-5 NK cells from individuals with PBC display elevated frequencies of pSTAT4+ NK cells at rest indicating an activated status



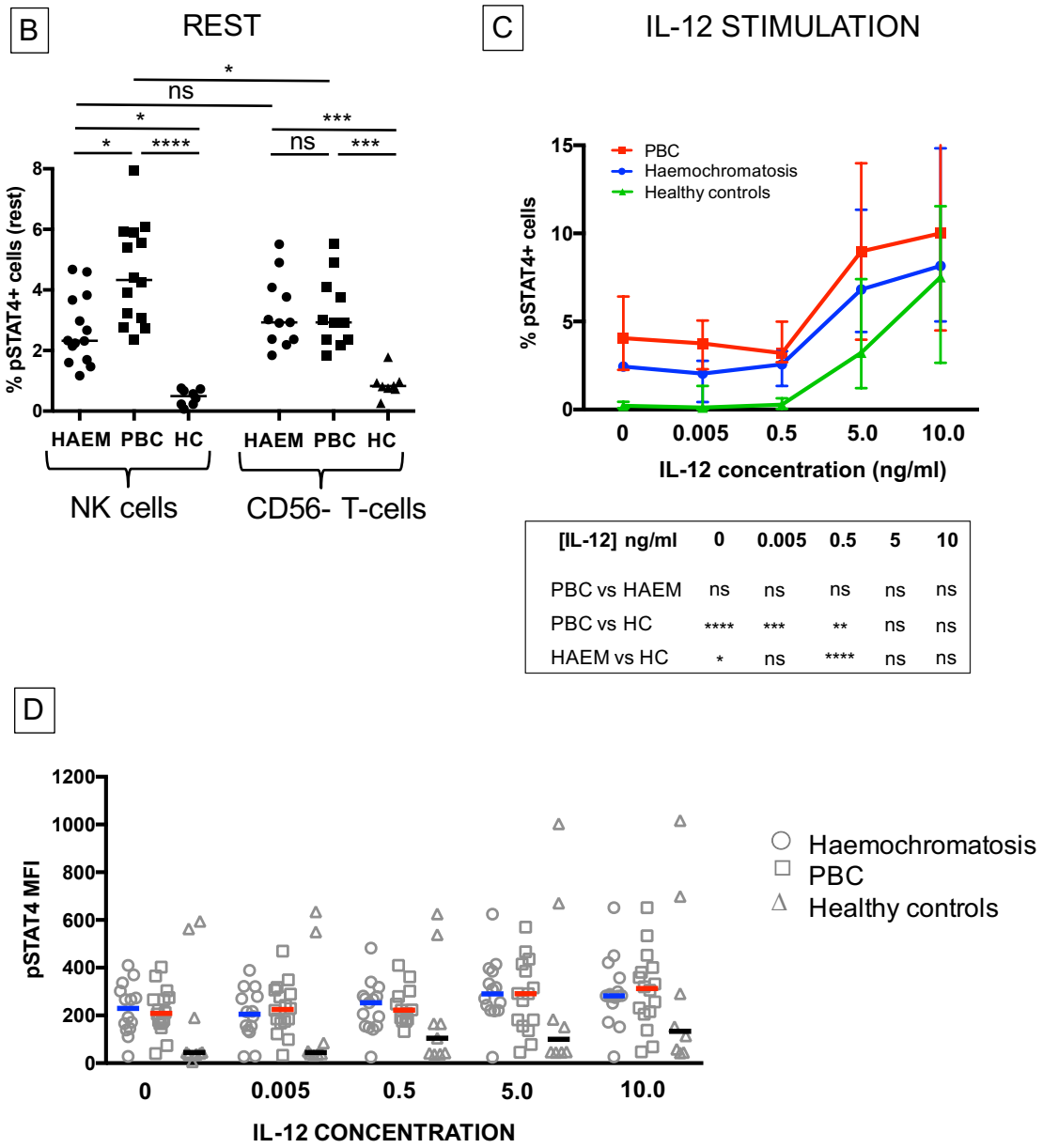


Fig 5-5 **A)** Representative flow cytometry plots of individuals with PBC and haemochromatosis showing gating strategy for pSTAT4+ NK cells **B)** Percentage of pSTAT4+ NK cells from the peripheral blood of individuals with haemochromatosis (2.3%, *n=14*), PBC (4.3%, *n=14*) and healthy controls (0.5%, *n=8*) at rest. Percentage of pSTAT4+ CD56- T-cells from the peripheral blood of individuals with haemochromatosis (2.9%, *n=11*), PBC (2.9%, *n=11*) and healthy controls (0.7%, *n=8*) at rest. Dot plots display median. (*Kruskal-Wallis with Dunn's multiple comparison test compares patient groups and Mann Whitney U test compares NK cells and CD56- T-cells within the same patient group*). **C)** Comparison of the percentage of pSTAT4+ NK cells in the peripheral blood from individuals with haemochromatosis, PBC, and healthy controls following stimulation with increasing concentrations of IL-12 for 1 hour. Line chart displays median and IQR. (*Kruskal-Wallis with Dunn's multiple comparison test*). D) pSTAT4 MFI for NK cells from individuals with haemochromatosis, PBC and healthy volunteers with increasing concentrations of IL-12. Dot plot displays median. p<0.05*, p<0.01**, p<0.001***, p<0.0001****.

Having demonstrated baseline activation of the IL-12/STAT4 pathway and increased responsiveness towards IL-12 for the induction of CXCR6+ NK cells in individuals with PBC, I was interested in whether there was a correlation between expression of the transcription factor STAT4 and CXCR6 surface expression. A linear regression model demonstrated a positive correlation between resting frequencies of pSTAT4+ NK cells and CXCR6+ NK cells in individuals with PBC, but not those with haemochromatosis or healthy controls (**Fig 5-6**). No significant correlation was seen between resting percentages of pSTAT4+ NK cells and CD49a+ NK cells for all patient groups (**Fig 5-6**). Furthermore there was no correlation of pSTAT4+ NK cell frequencies, and frequencies of CD49a+ or CXCR6+ NK cells following stimulation with exogenous IL-12 (**Appendix Fig 3**). This data suggests that IL-12 driven generation of NK cells expressing the liver homing marker CXCR6 may occur through downstream signalling of pSTAT4 in patients with PBC, although it is not clear why this correlation is lost following IL-12 stimulation.

Figure 5-6 Baseline pSTAT4+ NK cell frequencies positively correlate with resting frequencies of CXCR6+ NK cells in individuals with PBC

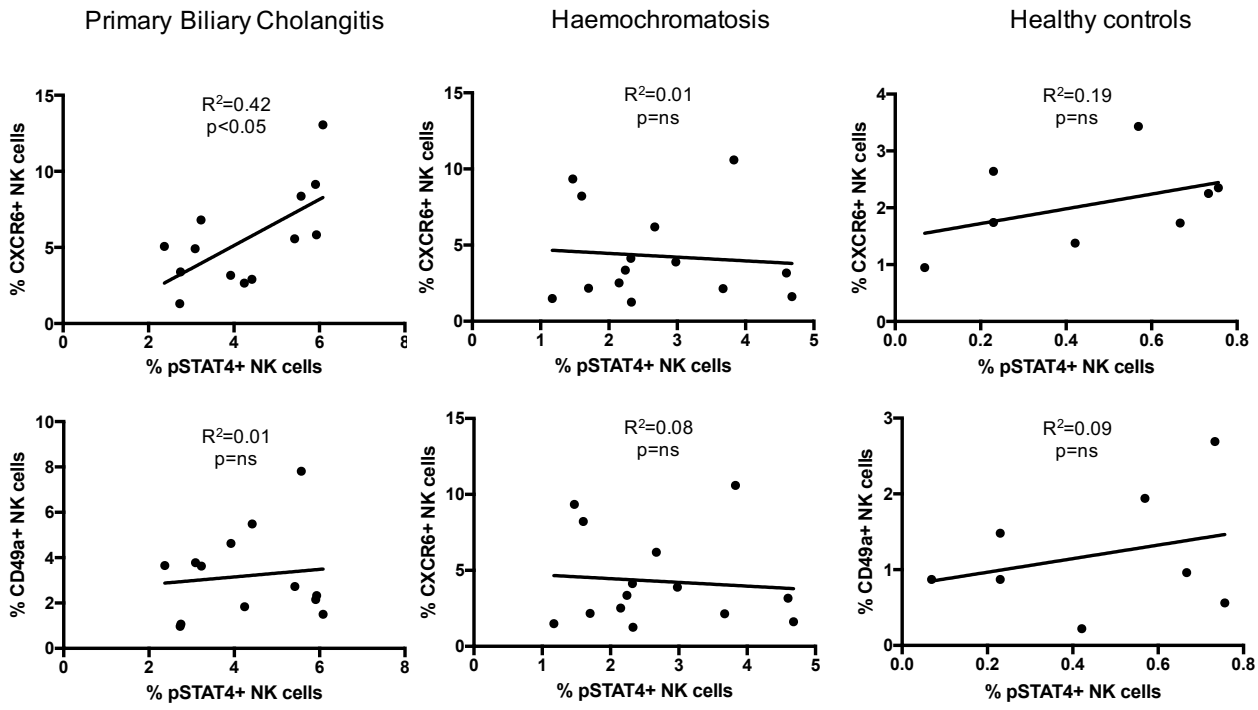


Fig 5-6 Linear regression model of pSTAT4+, CXCR6+ and CD49a+ NK cell frequencies in NK cells from paired individuals with PBC (*n=14*), haemochromatosis (*n=14*) and HCs (*n=8*).

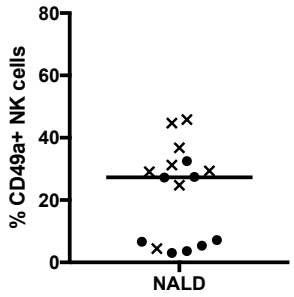
5.7 High frequencies of induced-CD49a+ NK cells are associated with more advanced liver disease

While analysing the induction of CD49a+ NK cells during culture with IL-12 or IL-15, I observed that within both the PBC and haemochromatosis patient cohorts, individuals separated into two distinct groups according to the frequency of cytokine-induced CD49a+ NK cells at day four, i.e. CD49a^{rich} (> 20% CD49a+ NK cell) and CD49a^{poor} individuals (< 10% CD49a+ NK cells) (**Fig 5-7**). A comparison of their demographic data revealed that within the haemochromatosis cohort, high frequencies of cytokine-induced CD49a+ NK cells were associated with more advanced liver disease, i.e. advanced fibrosis or cirrhosis (**Fig 5-7**). This is consistent with our finding that CD49a+ NK cells are induced by all activating cytokines, and therefore are likely to be seen more frequently in the end-stages of hepatitis.

Whilst a similar dichotomy was seen within the PBC cohort, fewer individuals had advanced disease overall, and no association between demographic characteristics and induced CD49a frequencies was seen (**Fig 5-7**). It is possible that polymorphisms within the IL-12/STAT4 pathway may account for these phenotypic differences in PBC. We have therefore contacted the study organisers for 'PBC UK' to access data on polymorphisms within the IL-12/pSTAT4 signalling pathway for dual recruited patients to investigate this further.

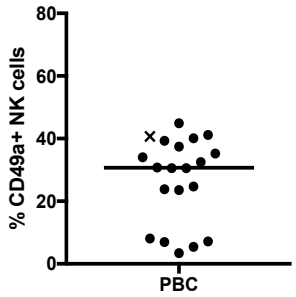
Figure 5-7 High frequencies of cytokine-induced CD49a+ NK cells are associated with more advanced liver disease in individuals with haemochromatosis

A



HAEMOCHROMATOSIS			
	CD49a^{rich} (n=10)	CD49a^{poor} (n=6)	
Male, n (%)	8 (80.0)	5 (83..3)	p=0.8686
Age, mean	56.7	53.8	p=0.6546
Cirrhosis / severe fibrosis, n (%)	7 (70.0)	1 (16.7)	***p=0.0389***
Metabolic syndrome, n (%)	7 (70.0)	3 (50.0)	p=0.4237
Alcohol excess, n (%)	1 (10)	1 (16.7)	p=0.6963

B



PRIMARY BILIARY CHOLANGITIS			
	CD49a^{rich} (n=15)	CD49a^{poor} (n=5)	
Male, n (%)	2 (13.3)	0 (0.0)	p=0.3894
Age, mean	64.1	61.4	p=0.5980
Cirrhosis / severe fibrosis, n (%)	1 (6.7)	0 (0)	p=0.5536
UDCA, n (%)	13 (86.7)	5 (100)	p=0.3894
ALP, mean	198.1	432.2	p=0.7354
Other autoimmune condition	2 (13.3)	2 (40)	p=0.2487

x Cirrhosis / severe fibrosis

Fig 5-7 **A)** Distribution of frequencies of CD49a+ NK cell induced following four days of culture with IL-12 0.005ng/ml in individuals with haemochromatosis. Dot plot shows median. (*n=16).* Table displays demographic details for CD49a^{rich} and CD49a^{poor} individuals. **B)** Distribution of frequencies of CD49a+ NK cell induced following four days of culture with IL-12 0.005ng/ml in individuals with PBC. Dot plot shows median. (*n=20*). Table displays demographic details for CD49a^{rich} and CD49a^{poor} individuals.

5.8 Hypersensitivity of the IL-12/pSTAT4 axis is not affected by UDCA

Overall 80% of individuals recruited with PBC were taking the naturally occurring bile acid, UDCA. The numbers of patients not prescribed this medication were too small to perform a comprehensive stratification, however using the numbers available UDCA did not appear to significantly influence any of the key outcomes examined (**Fig 5-8**).

Figure 5-8 Markers of IL-12 hypersensitivity and UDCA prescription

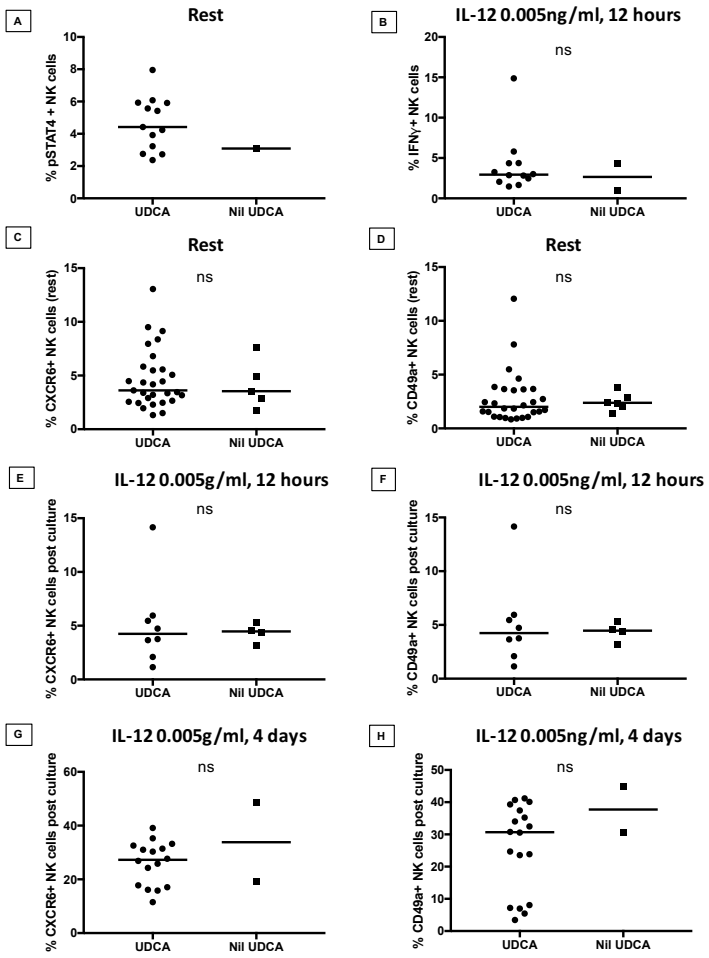


Fig 5-8 **A)** Frequency of pSTAT4+ NK cells at rest in individuals with PBC according to UDCA prescription**.** (*n=13, n=1).* **B)** Frequency of IFNγ+ NK cells following stimulation with IL-12 0.005ng/ml for 12 hours in individuals with PBC according to UDCA prescription. (*n=12, n=2).* **C)** Frequency of CXCR6+ NK cells at rest in individuals with PBC according to UDCA prescription. (*n=29, n=5).* **D)** Frequency of CD49a+ NK cells at rest in individuals with PBC according to UDCA prescription. (*n=29, n=6).* **E)** Frequency of CXCR6+ NK cells following stimulation with IL-12 0.005ng/ml for 12 hours in individuals with PBC according to UDCA prescription. (*n=8, n=4).* **F)** Frequency of CD49a+ NK cells following stimulation with IL-12 0.005ng/ml for 12 hours in individuals with PBC according to UDCA prescription. (*n=8, n=4).* **G)** Frequency of CXCR6+ NK cells following stimulation with IL-12 0.005ng/ml for four days in individuals with PBC according to UDCA prescription. (*n=16, n=2).* **H)** Frequency of CD49a+ NK cells following stimulation with IL-12 0.005ng/ml for four days in individuals with PBC according to UDCA prescription. (*n=18, n=2).*

5.9 Discussion

This chapter presents the first functional correlate in humans of the PBC GWAS studies which identified components IL-12/pSTAT4 signalling cascade as susceptibility loci. These were identified across a range of countries including the UK (**Table 5**) [135–140]. I describe baseline activation of the IL-12/pSTAT4 pathway in NK cells in individuals with PBC, demonstrated by elevated resting levels (measured without exogenous exposure to IL-12) of pSTAT4 in NK cells in PBC patients compared to patients with the non-autoimmune liver disease, haemochromatosis and healthy volunteers. While resting levels of p-STAT4 were also raised in CD56- T-cells in both patient groups compared to healthy controls, the frequency of pSTAT4+ NK cells was greater than that of pSTAT4+ CD56- T-cells within the PBC group only. However it is likely that overactivation of this pathway is a feature of both NK cells and CD56- T-cells in PBC. I also noted that patients with haemochromatosis had elevated resting levels of pSTAT4 compared to healthy controls. This has not been described before and its clinical significance is unclear, however this for this reason this patient cohort provides a robust control group for these experiments.

Furthermore, I have demonstrated IL-12 hyper-responsiveness in NK cells in patients with PBC, resulting in enhanced effector function. Stimulation of PBMCs with just 0.005ng/ml (5 picograms / ml) of IL-12 can lead to production of significant quantities of IFNγ by NK cells, which is not seen in patients with haemochromatosis or healthy controls. I show in chapter 4 that culture of PBMCs with IL-12 overnight can lead to the induction of small frequencies of CD49a+ and CXCR6+ NK cells with upregulation of markers of clonal expansion (NKG2C) and tissue-residency (CD69). Peripheral blood NK cells expressing CD49a at the end of culture harboured the highest frequency of IFNγ+ NK cells, mirroring the inflammatory cytokine profile of adaptive liver-resident CD49a+ NK cells. Here, I report that CD49a+ NK cells, but not CD49a- NK cells, generated in vitro from PBMCs isolated from patients with PBC show significant IL-12 hyper-responsiveness in terms of IFNγ production compared to patients with haemochromatosis. Interestingly small percentages of CXCR6+ NK cells in patients with PBC, but not the two control groups, also expressed IFNγ in response to minute concentrations of IL-12, suggesting this ‘tolerant’ NK cell subset may be more cytokine responsive in PBC. Differences between patient groups were lost following stimulation with higher concentrations of IL-12, probably due to saturation of the signalling pathway.

In addition to demonstrating that IL-12 hyper-responsiveness can lead to an enhanced IFNγ production in PBC, I have shown that activation of this pathway can lead to the induction of higher frequencies of NK cells expressing the liver-homing marker CXCR6 in the peripheral blood. Greater

resting frequencies of CXCR6+ NK cells are observed in the circulation of patients with PBC, perhaps due to their egress from the liver where they have been found to be particularly enriched in this condition [149]. Furthermore I have shown following NK cell purification, the addition of extremely low concentrations of IL-12 to the media can lead to an increase in the frequency of CXCR6+ NK cells in the peripheral blood in vitro even after just 12 hours. This phenomenon was not observed in patients with haemochromatosis or healthy controls, where higher concentrations of IL-12 were required to induce a change in CXCR6+ NK cell frequency. Interestingly linear regression analysis revealed that resting levels of pSTAT4 correlate with CXCR6 surface expression in patients with PBC, suggesting that overactivation of this axis may directly lead to CXCR6 upregulation.

It would be extremely worthwhile to compare expression of the IL-12 and IL-23 receptors between patients with PBC and those with other liver disease. RNA sequencing of CXCR6+ NK cells from individuals undergoing resection for metastases highly expressed IL-12RB2 and IL-23R. Both proteins are able to interact with IL-12Rβ1 to initiate Th1 (IFNγ) and Th17 (IL-17) signalling respectively. Upregulation of these receptors on NK cells from patients with PBC may contribute towards IL-12 hyper-responsiveness. Interestingly it was the IL-12RB2 gene that was linked to PBC susceptibility in the recent GWAS studies. This is not constitutively expressed, but upregulated by IFNγ. It is therefore likely that the Th1 cytokine environment found in the livers of patients with PBC reinforces high expression of this receptor on CXCR6+ and CD49a+ NK cells.

Similarly significantly higher frequencies of CD49a+ NK cells could be induced in patients with PBC (approximately 30% of the total NK cell population) compared to healthy controls (less than 10% of the total NK cell population) following culture of PBMCs with media alone for four days. However, in contrast to the generation of CXCR6+ NK cells, similar frequencies of CD49a+ NK cells could also be induced from PBMCs isolated from patients with haemochromatosis under the same conditions. I propose that this observation supports my findings in chapter 4, where I demonstrate that following six days of culture with any activating cytokine (IL-2, IL-12, IL-15, IL-18), nearly 100% of NK cells are found to be CD49a+, either through de novo expression or proliferation of existing populations; i.e. CD49a+ NK cells are extremely responsive to a wide range of inflammatory cytokines. Furthermore the greatest frequencies of CD49a+ NK cells observed at day six were generated following culture with the cytokine cocktail (IL-2, IL-12, IL-15, IL-18). A significant proportion of the haemochromatosis patients recruited as controls, had additional risk factors associated with hepatic inflammation, including severe fibrosis or cirrhosis, alcohol excess or components of the metabolic syndrome.

These clinical conditions were associated with higher frequencies of CD49a+ NK cells generated in culture. I therefore hypothesise that NK cells in individuals with haemochromatosis are exposed to an inflammatory cytokine environment and develop CD49a+ NK cells in vivo which are 'primed', with small frequencies detected in the circulation. Exposure to exogenous cytokines can then lead to a rapid expansion of these population in vitro. NK cell purification assays demonstrate that while both exogenous IL-12 and IL-15 can induce these populations, IL-15 is a particularly effective stimulus. CD49a+ NK cell induction in people is therefore likely to occur through multiple cytokine-stimulated STAT signalling pathways.

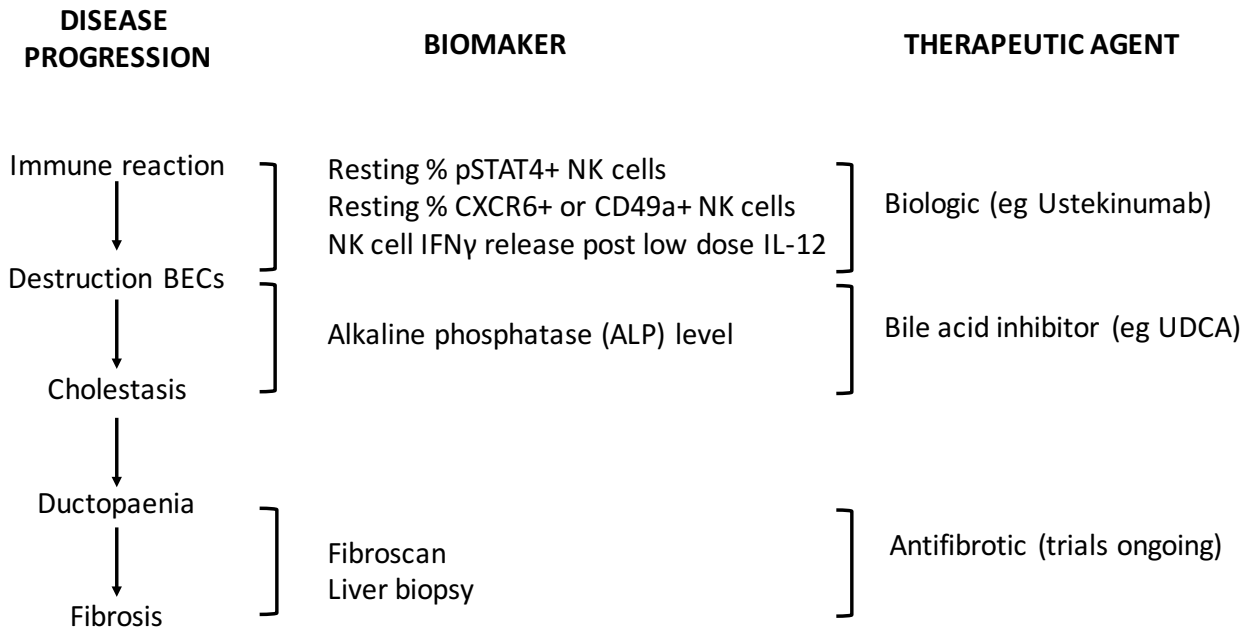
It is possible that the Th17 signalling pathway may also play a role in PBC. IL-23 shares the IL-12p40 subunit and signals via the heterodimeric receptor composed of IL-23R and IL-12Rβ1. IL-23 signals via a similar pathway to IL-12, although its ability to activate STAT4 is weaker. IL-23 is secreted by dendritic cells and macrophages, but has also been shown to be released from degenerating hepatocytes and therefore may be found in higher quantities in the intrahepatic environment in PBC [10]. Importantly IL-23 can induce CD8+ T-cells and ILC-3 lymphocytes to produce IL-17 which is a driver of tissue inflammation. Th17 lymphocytes have been implicated in a number of autoimmune diseases (**Table 10**). In PBC elevated levels of the IL-23p19 protein, IL-12/IL-23 receptor expressing lymphocytes and BECs, in addition to IL-17 secreting cells have been found next to damaged bile ducts [11,10]. Serum levels of IL-17 and IL-23 are also elevated, particularly in the early stages of disease [267]. Furthermore IL-17, along with TLR-ligands has been shown to induce BECs and inflamed hepatocytes to secrete IL-23p19. Activation of the IL-17 signalling pathway has also been associated with more advanced disease. The IL-23/Th17 cascade may therefore act alongside IL-12 mediated immunopathology in PBC, despite neither the IL-23R or IL-12Rβ1 being associated with susceptibility in the gene association studies. Our RNA sequencing data identifies strong upregulation of the IL-23R on liver-resident CXCR6+ NK cells however, and therefore these liver-homing NK cells may also display sensitivity towards IL-23 and a strong Th17 cytokine profile in PBC.

Data presented in this thesis suggests that inhibition of the IL-12/STAT4 pathway may provide a novel therapeutic target for PBC. Following the identification of an association between the IL-12 signalling cascade and PBC susceptibility, a multicentre, open-label, proof of concept study using Ustekinumab, an anti-IL-12/IL-23 monoclonal antibody (IgG1κ) was performed [12]. Ustekinumab binds to the shared p40 subunit of IL-12 and IL-23 and prevents their interaction with the cell surface receptor protein, IL-12Rβ1. This biologic is currently licenced for the treatment of psoriasis and was recently approved by the US Food and Drug Administration as a treatment option for severe Crohn's

disease. The drug was delivered subcutaneously at 90ng at week 0 and week 4, then every 8 weeks until week 20. Only patients with an inadequate response to UDCA (ALP > 1.67x upper limit of normal (ULN) after at least 6 months) were included and the cohort had fairly advanced disease with a median Enhanced Liver Fibrosis (ELF) score of 9.8 (moderate to severe fibrosis), high levels bile acid concentrations and the majority had a baseline ALP > 3x ULN. Steady-state serum Ustekinumab concentrations were achieved, associated with a modest reduction in ALP levels at week 28 (12.1%), and minor decrease in total serum bile acid concentrations and ELF scores. However no patients achieved a definitive ALP response, defined by a decrease of > 40% from baseline, or ALP remission.

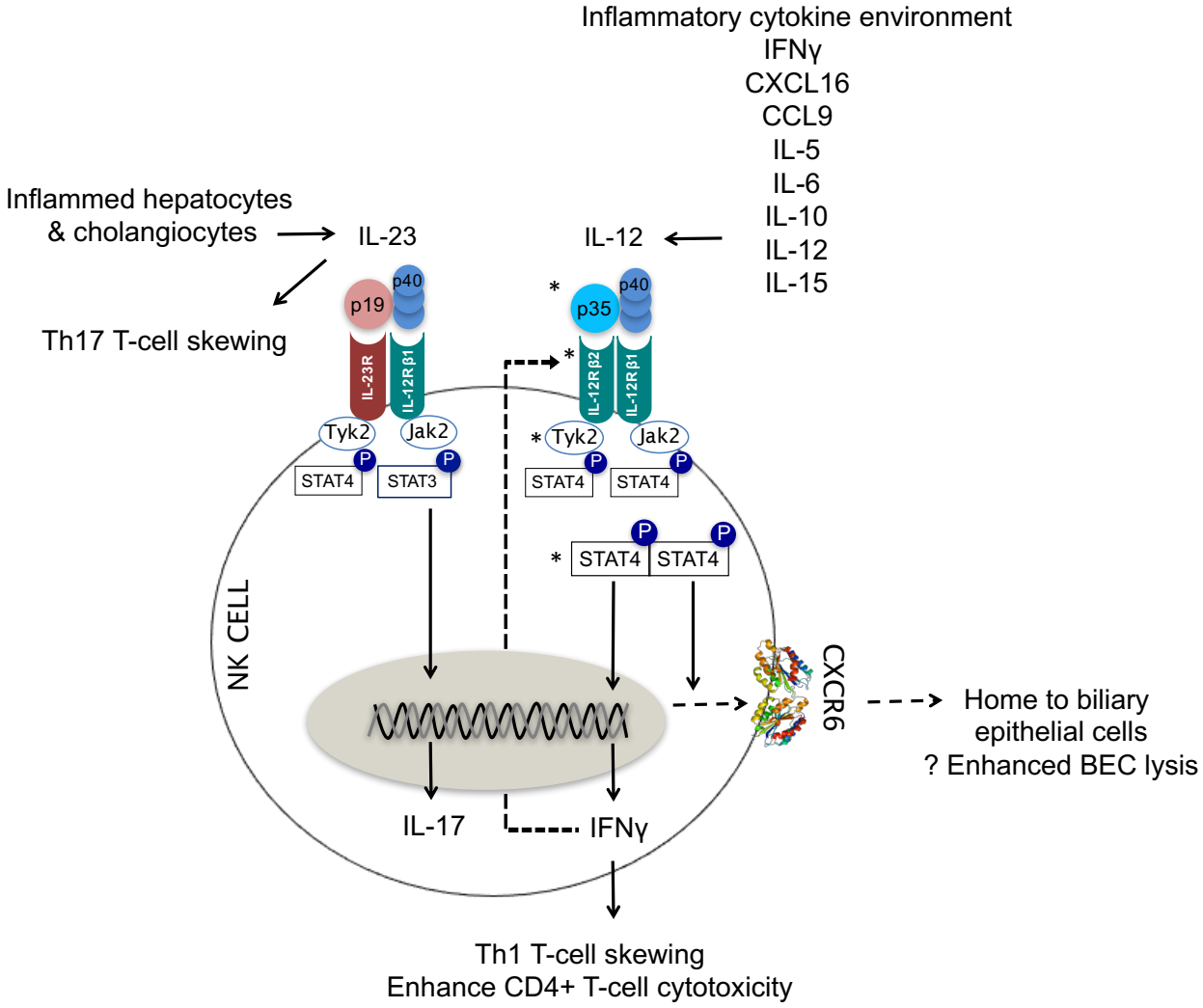
This study provides an example of some of the challenges faced when translating basic biological insights into a clinical trial. The study authors noted that following administration of Ustekinumab a reduction in serum IL-17A and IFNγ was seen, i.e. IL-12p40 inhibition led to modulation of this biological pathway in vivo. These changes were seen for patients who achieved a greater than 20% reduction in ALP. Therefore analysis of immunological endpoints may be more informative in this type of clinical trial. It may also be the case that as cholestasis advances (as in this patient cohort), and becomes the dominant pathological pathway over immune-mediated injury, combined treatment modalities for example with UDCA may be required to modulate this stage of the disease. Furthermore, Ustekinumab may be effective in patients with aggressive PBC in the early 'immune' phases of the disease, a currently 'difficult to treat' patient group (**Fig 5-9**). Stratification of patients most likely to respond to Ustekinumab treatment may also be required. Classical risk stratification markers including ALP and liver biopsy, may identify those with advanced cholestasis requiring additional treatment. The optimal biomarker for this trial however would select individuals with evidence of overactivation of the IL-12/STAT4 signalling cascade. The final part of this thesis identifies NK cell IL-12 hypersensitivity as a potential disease-modulating immunological pathway in PBC and suggests a role for adaptive-like CD49a+ and liver-homing CXCR6+ NK cells in mediating this. The identification of elevated resting frequencies of CXCR6+ or CD49a+ NK cells, high frequencies of pSTAT4+ NK cells or analysis of IFNγ release in response to low dose IL-12 by these subsets are all potential novel biomarkers which could predict response to Ustekinumab and be used to define inclusion criteria for future trials. Indeed it may be that the use of biologics in autoimmune disease will need to take on a personalised approach based on phenotyping, genomics, and biomarkers in order to target the dominant aberrant immunological pathway.

Figure 5-9 Different treatment modalities may be required for different pathological stages of PBC



In summary I have identified overactivation of the IL-12/STAT4 axis in NK cells in patients with PBC (**Fig 5-10**). This can lead to the generation of higher frequencies of liver-homing (CXCR6+) and adaptive (CD49a+) NK cells which produce greater quantities of IFNγ in response to low doses of IL-12. Hyperfunctional NK cells may home to the biliary tree via their interaction with CXCL16 and potentially lyse autologous BECs. Assessment of the cytotoxic capacity of CD49a+CXCR6+ NK cells in PBC is therefore essential. Furthermore upregulation of the IL23R, in addition to IL-12Rβ2, on CXCR6+ NK cells may lead to enhanced IL-17 production (**Fig 5-10**). As descried by Shimoda et al, NK cell-mediated destruction of BECs can lead to the release of the PDC-E2 peptide within microparticles and upregulation of HLA-II on BECs promoting activation of autoreactive CD8+ and CD4+ T-cells [144]. The pro-inflammatory cytokine intrahepatic milieu found in PBC, rich in IFNγ, IL-5, IL-6, IL-12, IL-15 and IL-23 may help maintain cytokine-responsive liver-homing adaptive-like NK cells in this activated state, particularly through the upregulation of IL-12Rβ2, the inducible subunit of the IL-12 receptor (**Fig 5-10**). [268,269]. This final chapter therefore implicates overactivation of the IL-12/STAT4 signalling cascade in NK cells in PBC and suggests a role for the newly discovered CXCR6+ and CD49a+ liver-resident NK cell subsets in autoimmunity. Inhibition of this pathway in correctly selected patients using NK cell-related novel biomarkers may provide an effective new therapeutic target for PBC.

Figure 5-10 Model for IL-12 hypersensitivity in NK cells in the immunopathology of PBC



* Proteins coded for by genes associated with PBC susceptibility in PBC GWAS studies

My original hypothesis was that activation of the STAT4 signalling pathway could lead to the generation of 'hyperfunctional', potentially memory-like NK cells, mirroring the finding that reduced levels of STAT4 are associated with a tolerant NK cell phenotype [46]. While PBC provides a model for this, in which with elevated resting levels of pSTAT4 are found in a patient cohort with raised resting levels of CXCR6+ and CD49a+ NK cells with a strong Th1 cytokine profile, it was not possible to specifically investigate pSTAT4 expression in liver-resident or cytokine-induced CD49a+ or CXCR6+ NK cells using FACS, as the requirement to permeabilise NK cells using Perm Buffer III (BD Biosciences) to examine pSTAT4 prevented the analysis of a wide range of cell surface markers. Purification of CXCR6+/- and CD49a+/- NK cells would therefore be required prior to permeabilisation and pSTAT4 staining to address this question.

Other limitations of this work include low patient numbers. As PBC has varying phenotypes it would have been ideal to have stratified patients according to those who have early onset aggressive disease, in comparison to those with more indolent progression, in addition to the presence of cholestasis and fibrosis. Furthermore in order to definitively establish if NK cells demonstrate IL-12 hypersensitivity, leading to the generation of elevated levels of CXCR6 and CD49a and IFNγ release, a blocking experiment would be required. Finally it would be interesting to examine CXCR6+ and CD49a+ NK cells in the livers of individuals with PBC and to use frozen sections to establish if these are found in high densities next to the biliary epithelial cells. It would also be worthwhile to measure the concentrations of cytokines found within the liver in these patients, particularly IL-12, IL-23 and IL-17. These could be measured from hepatic perfusates using ELISA.

6 Chapter 6: Final Discussion

6.1 Characterisation of liver-resident NK cells

NK cells comprise approximately 40% of the hepatic lymphocyte population and are enriched in the sinusoids. Approximately 60% are $CD56^{bright}$CXCR6+($Eomes^{high}$) (**Fig 6-1**) [11,29,33]. This NK subset differs phenotypically from the $CD56^{bright}$CXCR6-($Eomes^{high}$) NK cells found in the peripheral blood, suggesting $CD56^{bright}$CXCR6+ NK cells represent a large tissue-resident population [15]. I have shown that liver-resident $CD56^{bright}$CXCR6+ NK cells are $CD16^{low}CD57^{low}$ and highly express CD161. This subset are likely to remain liver-resident through the interaction of CXCR6 with CXCL16 (expressed by cholangiocytes, hepatocytes and HSECs) [32]. RNA sequencing data has shown that CXCR6+ NK cells also highly express CCR5 which interacts with CCL3 and CCL5 expressed by kupffer cells and liver-resident T-cells respectively. Furthermore over two thirds of CXCR6+ NK cells expressed the surface marker CD69 known to inhibit S1PR1 which promotes lymphocyte egress into the circulation [15,25–27]. Indeed examination of NK cells from donor livers before and after an HLA-mismatched liver transplant, revealed that only $Eomes^{low}$ NK cells exit the liver [33]

In terms of function, I have shown that while CXCR6+ NK cells do not produce lower quantities of IFNγ and TNFα compared to liver-resident CXCR6- NK cells, they release lower quantities than CD49a+ NK cells. Furthermore I would have expected a stronger Th1 cytokine profile from this group given their $CD56^{bright}$CD16- phenotype. Harmon et al also confirmed lower production of IFNγ by liver-resident $CD56^{bright}$ NK cells compared to peripheral $CD56^{bright}$ NK cells [11]. Instead RNA sequencing suggests they release GM-CSF, LIF and IL-4. I did not directly examine the cytotoxic capacity of CXCR6+ NK cells, however expression of granzymes A and B, in addition to granulysin and perforin were reduced at a transcript level. Furthermore their lack of surface expression of CD16 suggests ADCC will be impaired. While hepatic NK cells have been shown to demonstrate enhanced cytotoxicity against HepG2 and K562 target cells the capacity of CXCR6+ NK cells to kill these targets has not been assessed [11,14,24,25,28]. Interestingly despite it generally being accepted that TRAIL is not found on NK cells in the healthy liver [15,28], I found this death-ligand to be expressed in CXCR6+ NK cells at a RNA level. This may have been induced through an inflammatory microenvironment associated with colorectal metastases, consistent with previously reported upregulation of TRAIL in response to pro-inflammatory cytokines [28]. Stegmann et al have suggested that CXCR6+ NK cells may mediate cell death in a TRAIL-dependent manner during disease using HBV infection as an example [29]. CXCR6+ NK cells are therefore the dominant tissue-resident subpopulation in the adult human liver. This subset is immature and express markers associated with tissue-residency and adhesion, but do not release high quantities of Th1 cytokines

or cytotoxic granules. They may however be able to mediate target cell death in a TRAIL dependent manner during disease states.

An interesting point for discussion is how CXCR6+ (Eomes^{high}) NK cells come to reside in the liver. Cuff et al have shown that liver-resident Eomes^{high} NK cells can be replenished from the circulation post liver transplantation [33]. Given that nearly all liver-resident Eomes^{high} NK cells are CD56^{bright} [11], and the fact that CD56^{dim} NK cells appear to circulate unchanged between the two compartments, it is likely that the Eomes^{high} population in the liver is replenished by circulatory CD56^{bright} NK cells (**Fig 6-1**). Hudspeth et al have shown however that peripheral blood CD56^{bright} NK cells generally do not express CXCR6, CCR5 and CD69 [15]. While these surface markers may be upregulated within the tissue, the majority of CD56^{bright} NK cells would not in fact home to the liver through the expression of CXCR6 or CCR5. Alternatively, hepatic CXCR6+ population may be replenished by the small population (< 5% NK cells) of circulatory CXCR6+ NK cells described here. While Stegmann et al reported peripheral CXCR6+ NK cells to be T-bet^{high}Eomes^{low} [29], I found that approximately 40% expressed Eomes, and Knox et al reported that nearly all peripheral CD56^{bright} NK cells expressed Eomes [30]. It is more likely however that CXCR6+CD56^{bright} NK cells migrate from the phenotypically related CXCR6+CD69+ population identified in human lymphoid tissue, particularly as peripheral blood and liver-resident CD56^{bright} NK cells have been found to differ significantly from each other (**Fig 6-1**) [167,181]. Finally liver-resident CXCR6+ NK cells may arise from CD34+ liver-resident stem cells (as demonstrated for CD49a+DX5- NK cells in the murine liver [19]), or NK cell precursors. However myself and others have found these to be present in only very small frequencies in the human liver [23].

I have shown that liver-resident CD49a+ NK cells represent a unique NK cell subset with distinct phenotypic and functional properties that distinguish them from both liver-resident CXCR6+CD56^{bright} NK cells and 'conventional' CXCR6-CD56^{dim} NK cells which move freely between the liver and circulation. Interestingly CD49a+ NK cells were only found at significant frequencies, of more than 30%, in 10% of individuals. While patient numbers were not large enough to make any definitive conclusions, this group appeared to have more advanced disease. Given that patients had a mixture of pathologies (HCC (secondary to steatohepatitis and spontaneous) and colorectal metastases), one hypothesis is that CD49a+ NK cells are generated in response to an inflammatory cytokine environment, rather than a specific antigen (**Fig 6-1**).

I have demonstrated that expression of CD49a is associated with higher frequencies of NKG2C+ and KIR+ NK cells. While not all of this subset were NKG2C+KIR+, it is likely that a proportion of CD49a+ NK cells may be KIR+ NK cells that have undergone clonal expansion. This is supported by findings by Marquardt et al who demonstrated than KIR expansion on CD49a+ NK cells occurred in an oligoclonal pattern [35]. It is still unclear whether KIR is upregulated on CD49a+ NK cells as a result of clonal expansion of NKG2C+ subsets, which express KIR, or because they represent antigen-experienced, licensed NK cells. Data presented here suggests that the liver-resident CD49a+ subset share some similarities to cytokine-induced memory-like NK cells in the peripheral blood [183]. I have shown this population are highly sensitive to all activating cytokines (IL-2, IL-12, IL-15 and IL-18), but particularly the cytokine cocktail, leading to a rapid expansion of CD49a+ NK cell frequencies within the overall NK cell population. This appears to be driven through both enhanced proliferative capabilities of CD49a+ NK cells and upregulation of CD49a de novo [35]. Furthermore stimulation with IL-12 results in enhanced production of IFNγ and TNFα from CD49a+ NK cells compared to CD49a- NK cells. I hypothesise that CD49a+ NK cells have been previously primed by a cocktail of pro-inflammatory cytokines found within the liver during periods of inflammation (IL-1, IL-2, IL-6, IL-12, IL-15, IL-18, IFNα/γ and TNFα/β) and therefore demonstrate an enhanced response towards secondary exposure to exogenous cytokines. This is supported by the demonstration here that CD49a+ NK cells are easily induced using IL-12 and IL-15 in both patients with haemochromatosis and PBC compared to healthy controls, and may account for the observed association between frequencies of CD49a+ NK cells generated in vitro and cirrhosis in haemochromatosis patients.

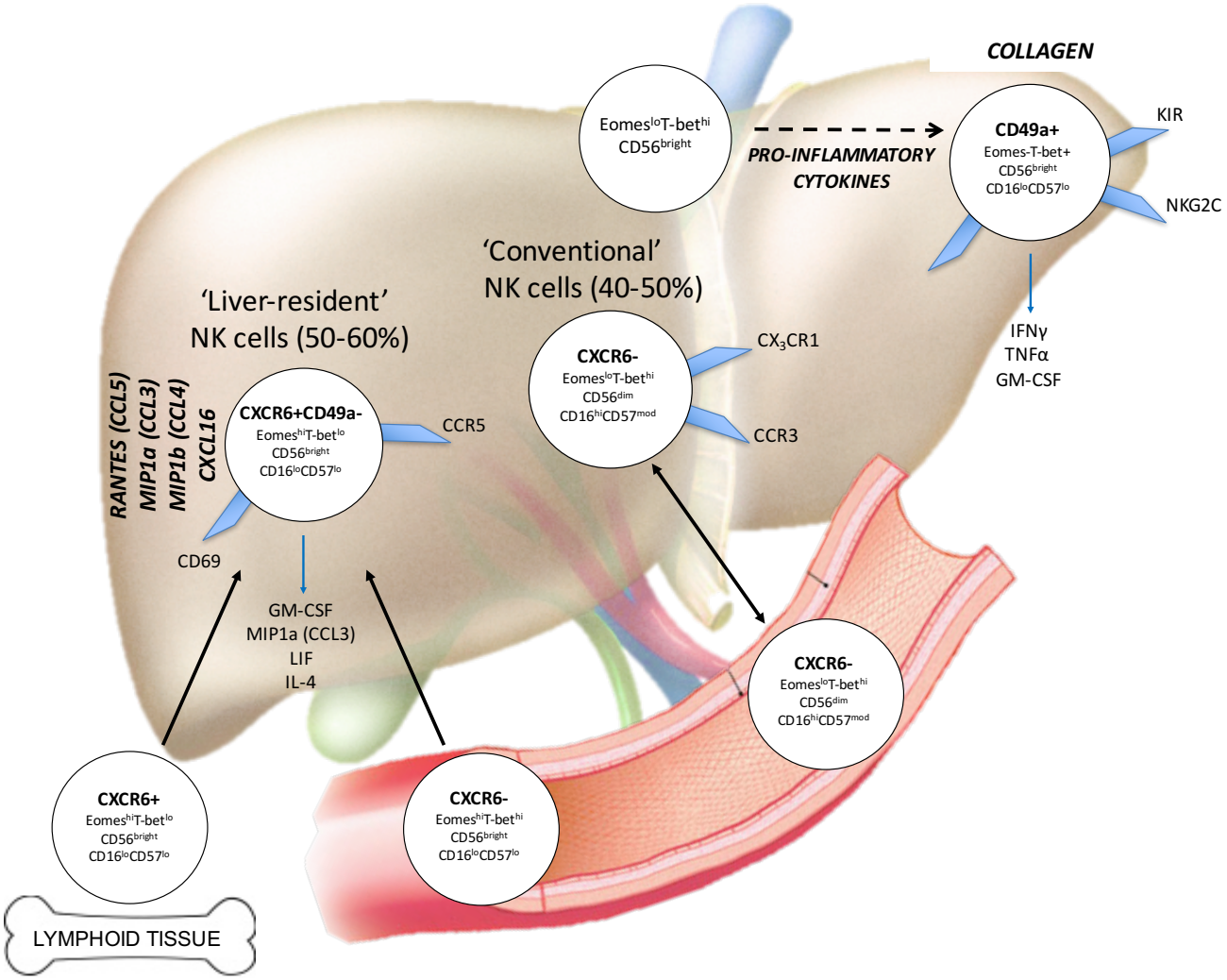
Liver-resident CD49a+ NK cells have been found to be T-bet+Eomes- [35]. Furthermore, nearly 50% express CD16, suggesting this subset is more mature than liver-resident CXCR6+ NK cells. Together this would suggest CD49a+ NK cells differentiate from $CD56^{dim}$ NK cells; yet both myself and Marquardt et al have found this subset to be $CD56^{bright}$. I therefore propose that CD49a+T-bet+Eomes- NK cells develop from the small population of $CD56^{bright}$ $T\text{-}bet^{high}Eomes^{low}$ NK cells (approximately 10% of all NK cells) found in the liver by Harmon et al (**Fig 6-1**) [11].

In summary liver-resident CD49a+ NK cells demonstrate features suggestive of adaptive-like behaviour, including enhanced proliferation, evidence of clonal expansion and enhanced effector function in terms of Th1 cytokine release. They may not however be highly cytotoxic as they express low levels of perforin and granzyme A and B and degranulate poorly [35]. TRAIL expression has not as yet been examined. I have demonstrated that this population are highly cytokine responsive, and in common with peripheral cytokine-induced memory-like NK cells are most reactive towards a

cocktail of inflammatory cytokines. This presents an important finding as it highlights combinations of cytokines as a means of 'boosting' NK cell function through the development of adaptive-like features. Whether CD49a+ NK cells are also capable of mounting antigen-specific memory behaviour in humans remains an area for investigation. Furthermore the underlying mechanisms leading to the adaptive-like behaviour and the role of CD49a itself are still unclear.

This work provides the first description of 'double-positive' CD49a+CXCR6+ NK cells. The frequencies of these are dependent on the frequency of CD49a+ NK cells and therefore vary hugely between individuals (range 1.5 – 41.7%). Approximately 70% of liver-resident CD49a+ NK cells are CXCR6+, however I have shown that adaptive-like properties, including enhanced IFNγ release and evidence of clonal expansion are not altered by CXCR6 expression. Therefore while CXCR6+ NK cells and CD49a+ NK cells are shown here to represent functionally distinct NK cell subsets, it would be more accurate to define the first group as 'CXCR6+CD49a-'. In conclusion 'CXCR6+CD49a-' NK cells define a large tolerant liver-resident population found in nearly all individuals in health, whereas CD49a+ NK cells represent a cytokine-responsive adaptive-like subset of NK cells found in 10% of the population (**Fig 6-1**).

Figure 6-1 Proposed model for the development, phenotype and function of CXCR6+ and CD49a+ NK cells in the liver



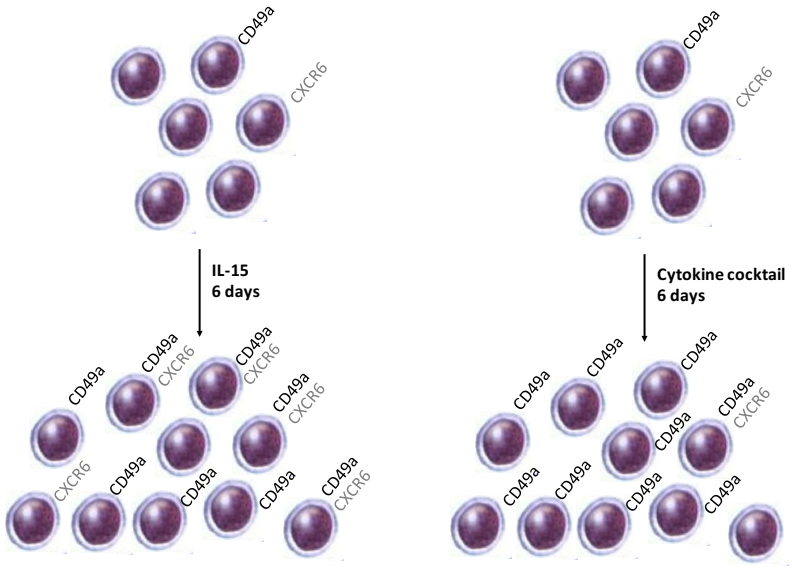
6.2 Liver-homing and memory-like NK cells can be induced in the peripheral blood in vitro using cytokines

We have shown that it is possible to use cytokines to increase the frequencies of CXCR6+ and CD49a+ NK cells within the peripheral blood in vitro. Frequencies of CD49a+ NK cells can be induced using any activating cytokine (IL-2, IL-12, IL-15 and IL-18) and are highest following culture with the 'adaptive phenotype-inducing' cocktail. While CXCR6+ NK cell percentages cannot be increased further on culturing hepatic MNCs, increases were seen within PBMCs in vitro using IL-2, IL-12 and IL-15. Elevated frequencies of 'double-positive' CD49a+CXCR6+ NK cells could be generated most effectively using IL-12 and IL-15. Sorting of CD49a+/- and CXCR6+/- subsets would

be required to understand to what degree these changes occur as a result of enhanced proliferation of existing subsets or upregulation of markers on previously CD49a- or CXCR6- cells. It is clear that both CD49a+/- and CXCR6+/- NK cells proliferate in response to both IL-15 and the cytokine cocktail. While CD49a+ NK cells expressed slightly lower CFSE MFIs at day six under both conditions, the differences were minimal. I therefore propose that in addition to CD49a+ NK cells proliferating strongly, CD49a is also upregulated as a surface marker under these conditions, in keeping with it being a very late antigen (VLA-1) activation receptor. This would account for the increase in absolute number and frequency of CD49a+ NK cells and decrease of CD49a- NK cells (**Fig 6-2**). The same is likely to apply for CXCR6+ NK cells in the presence of IL-15 although they are less cytokine responsive than the CD49a+ subset.

Interestingly the two NK cell subsets respond dramatically differently to the cytokine cocktail (**Fig 6-2**). While this leads to the strongest induction of CD49a+ NK cells, CXCR6+ NK cell populations within the peripheral blood fail to increase, and frequencies decrease when culturing hepatic MNCs. Yet proliferation is only marginally reduced on NK cells expressing CXCR6 at day 6, compared to CXCR6- NK cells and is still stronger under these conditions than with IL-12 for example. I therefore expect that in to addition CXCR6- NK cells proliferating more strongly under these conditions, CXCR6 is being downregulated as a surface marker both on hepatic NK cells and on proliferating peripheral blood NK cells. It is possible that this occurs as a result of NK cell exhaustion. These events would both account for the increase in absolute numbers of CXCR6- NK cells observed under these conditions.

Figure 6-2 Depiction of CD49a+/- and CXCR6+/- NK cell numbers and frequencies in culture



Cytokine-induced CD49a+CXCR6+ NK cells display features consistent with both a hyperfunctional, adaptive-like phenotype (enhanced expression of NKG2C and IFNγ, and enhanced proliferation) and liver-residency (CXCR6+CD69+), although further experiments would be required to more definitively demonstrate adaptive behaviour. The fact that they can be generated in vitro using a peripheral blood sample only and then directed towards the liver through the upregulation of CXCR6, offers an attractive therapeutic option for generating NK cells with 'boosted' function which can act locally.

These findings therefore suggest that cytokine-induced 'CD49a+CXCR6+' memory-like NK cells generated in the peripheral blood may provide a novel therapeutic target for liver disease. Specifically they may be able to induce clearance of HBV or HCV, or reduce the HBV viral load. Of particular interest is their potential to limit growth or induce regression of HCC. Cytokine-induced memory-like NK cells have shown clinical effectiveness against AML and therefore harnessing these in the liver presents a promising opportunity to develop a new cell-based immunological therapy for HCC. Cytokine-induced 'CD49a+CXCR6+' memory-like NK cells may also have potential activity as a preventative therapeutic agent against HCC in individuals at particularly high risk, for example those with HBV and cirrhosis, depending on their longevity.

These findings also raise some interesting questions regarding the nature of NK memory. As previously discussed the mechanisms leading to the generation of memory NK cells have not been established. In T-cells this occurs through re-arrangement of the RAG gene, however NK cell receptors are encoded in the germ-line and are therefore fixed. It has been suggested that memory-like behaviour occurs at an epigenetic level following exposure to a combination of cytokines, in the case of cytokine-induced memory-like NK cells, leading to high levels of IFNγ production [183]. However how these modifications bring about enhanced proliferation and cell killing is unclear, as is the mechanism by which memory is passed onto daughter cells if changes do not occur at a DNA level. Furthermore g- NK cells (NKG2C+) NK cells seen to undergo expansion following hCMV infection, were found to have a vigorous antibody-dependent functional response towards hCMV and were deficient in multiple transcription factors including SYK, suggesting epigenetic modifications of the SYK promoter may induce a stably maintained pool of antigen experienced NK cells, continuously re-shaped following repeat exposure to a related antigen in the presence of autologous immunoglobulins [198].

This project demonstrates that liver-resident CD49a+ NK cells have features to suggest they may be the memory-like equivalent of CD49a+ NK cells found in mice, with evidence that they have undergone clonal expansion and have enhanced IFNγ production. Furthermore I have shown that they can be generated in the peripheral blood using activating cytokines, with the strongest induction following culture with a cocktail of cytokines. Therefore CD49a+ NK cells may represent a group of cytokine-induced memory like NK cells as described by Romee et al [183,185], which home towards the liver. They therefore also display longevity and pass their enhanced effector function onto future generations, increasing their duration of action. In terms of taking this work forward into translational studies it will be very important to establish the stability of this population, or to determine if this is just a transitional state. This could be done in vitro, however maintaining viability is likely to be a problem, or by sampling NK cell populations in the peripheral blood and liver tissue over time from patients who have undergone an HLA-mismatched liver transplant. It would also be beneficial to establish if memory-like features of CD49a+ NK cells are passed onto daughter cells. CFSE staining to identify the progeny of cytokine-induced CD49a+ NK cells, followed by FACS sorting and examination of NKG2C/KIR status and Th1 cytokine release would be way of investigating this. Determining the longevity of CD49a+ cytokine-induced memory like NK cells is likely to significantly impact their therapeutic potential as this would allow an expanded population of hyper-functional NK cells to persist within the liver for sustained period of time to allow a clinical effectiveness. The use of cytokine-induced memory-like NK cells will hopefully be able to move translational use of autologous NK cells forward, particularly for the treatment of solid tumours, where the expansion of NK cells using IL-2 only has not been successful [270].

6.3 Peripheral blood NK cells play a role in primary biliary cholangitis

GWAS studies have identified components of the IL-12/pSTAT4 signalling cascade to be associated with susceptibility to the autoimmune liver disease, PBC. I identify elevated resting levels of pSTAT4 in NK cells in the peripheral blood in this condition and hypersensitivity of NK cells towards picogram doses of IL-12 leading to enhanced production of IFNγ compared to NK cells from individuals with the genetic liver disease, haemochromatosis and healthy volunteers. This thereby implicates NK cells in the pathogenesis of PBC through hyperactivation of this axis. Interestingly I have also shown that IL-12 hypersensitivity in PBC can lead to induction of CXCR6+ NK cells in the peripheral blood using much lower concentrations of IL-12 compared to control groups. Therefore overactivation of the IL-12/pSTAT4 signalling axis in NK cells in PBC can lead to the generation of NK cells with enhanced Th1 cytokine production and upregulation of markers of liver-homing.

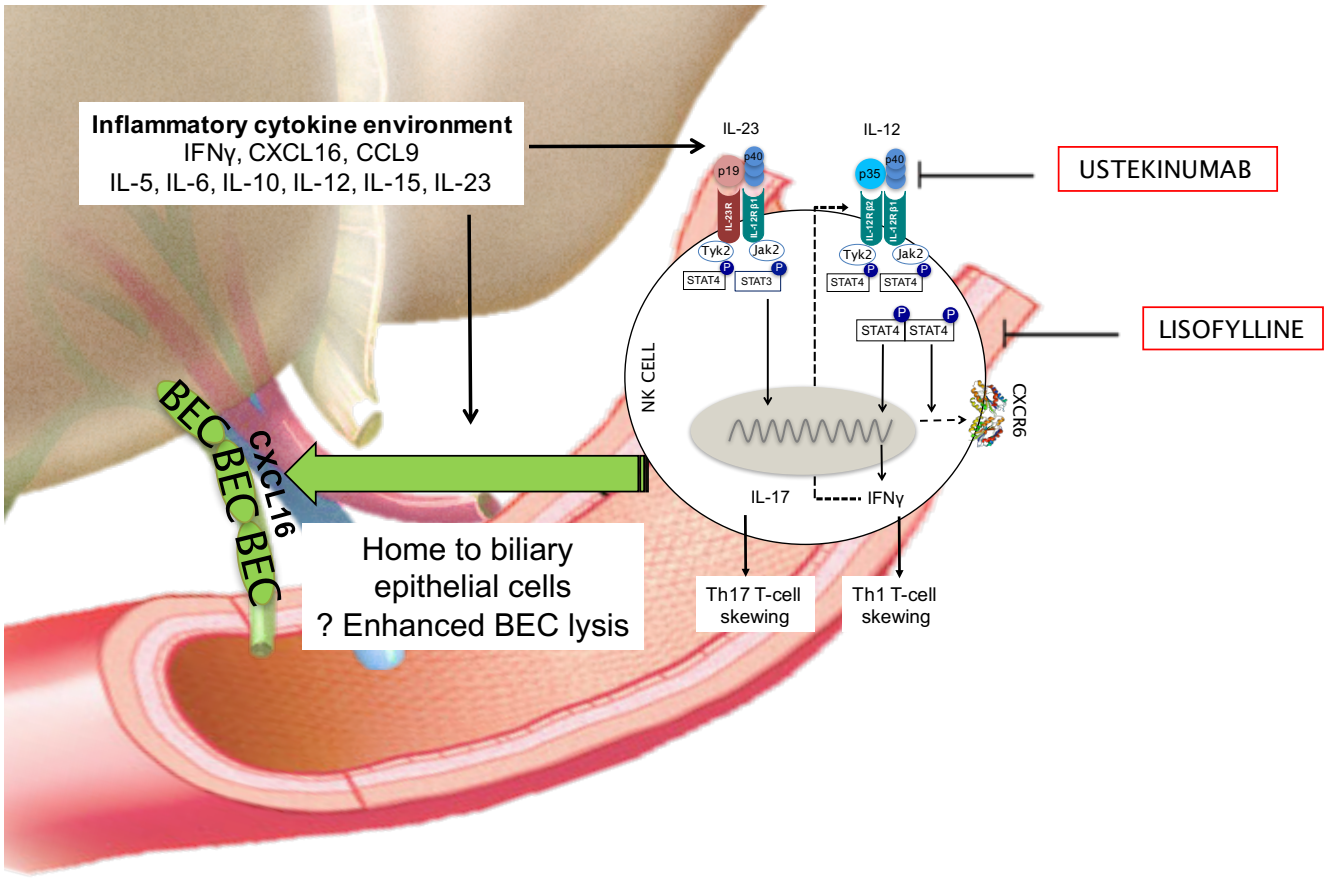
A number of polymorphisms along this pathway have been linked to PBC including IL-12A, IL-12 receptor B2, STAT4, SH2B3 and Tyk [135,137–139]. RNA sequencing results presented here identify upregulation of transcripts encoding for the IL-12RB2 and IL-23 receptors on liver-resident CXCR6+ NK cells. I therefore propose that genetic variants encoding IL-12Rβ2 may be one mechanism through which IL-12 hypersensitivity, and elevated resting levels of pSTAT4 arise in NK cells in PBC. Activation or overexpression of this receptor in NK cells would result in higher levels of IFNγ production enhancing the autoreactivity of CD4+ and CD8+ T-cells (**Fig 6-3**). Furthermore the IL-23R is also over-expressed on liver-resident CXCR6+ NK cells and this may result in NK cell-mediated IL-17 production and Th17 T-cell differentiation. It would therefore be interesting to investigate activation of the IL-23/IL-17 signaling pathways in NK cells in PBC. The β2 subunit of the IL-12 receptor is induced by IFNγ and therefore its expression may be maintained by release of IFNγ from autoreactive NK cells and T-cells (**Fig 6-3**). I would expect that induction of CXCR6 through IL-12 sensitivity would lead a subpopulation of hyperfunctional NK cells to home to the liver, particularly the biliary tree, and it would be interesting to know whether this subset also upregulate CCR5. Investigating the cytotoxic capacity of NK cells with raised resting levels of pSTAT4 is the key experiment here however.

Inhibiting this signalling cascade therefore presents on attractive therapeutic target for PBC. Options include Ustekinumab, an IL-12p40 antibody which is known to have a favourable side effect profile, being currently used for the treatment of psoriatic arthritis, and has the advantage of inhibiting both the IL-12 and IL-23 signalling pathways. While this was not shown to reduce ALP levels, i.e. improve cholestasis, in the group of patients in who were not responsive to UDCA, it is likely that this biologic should instead be targeted at young patients with aggressive PBC still in the ‘immunological’ rather than ‘cholestatic’ phase of the disease and a repeat clinical trial may be of benefit [271]. The use of novel biomarkers to direct therapy to patients most likely to be responsive may also be worthwhile, and the identification of IL-12 hyper-responsive NK cells may be useful in this setting. Another option is Lisofylline, a small synthetic molecule that inhibits STAT4 activation and has been used in phase II trials for T1 diabetes [272]. These biologics have the potential to alter the course of more rapidly advancing PBC, and prevent young people from requiring a liver transplant (**Fig 6-3**).

While NK cells as a whole were found to produce IFNγ in response to low concentrations of IL-12, not seen in control groups, CD49a+ NK cells produced much greater quantities than other NK cell subsets. Furthermore differences in IFNγ production between patients with PBC and

haemochromatosis were only seen for CD49a+ NK cells and not CD49a- NK cells. Elevated resting levels of CD49a+ NK cells were also found in the peripheral blood of patients with PBC, although similar frequencies could be induced using exogenous IL-12 in patients with both PBC and haemochromatosis. It is therefore possible that adaptive-like CD49a+ NK cells may also play a role in PBC, and it would be fascinating to investigate further possible links between memory-like NK cells and autoimmune disease.

Figure 6-3 Model for the role of IL-12 hypersensitivity in NK cells in the pathogenesis of PBC and potential therapeutic targets



6.4 Future Directions

6.4.1 PBC project

In PBC anti-mitochondrial antibodies are directed against the PDC-$E2_{212-226}$ epitope. It would therefore be interesting to stimulate potential adaptive-like NK cells with a peptide sequence from PDC-E2 to look for evidence of enhanced functional response and peptide specificity. I have previously demonstrated high expression of the activating receptor NKG2C on liver-resident, and cytokine-induced CD49a+ NK cells and hypothesise that this may play an important role in promoting recognition of the PDC-E2 protein through HLA-E. Using netMHCpan software it would be possible to identify peptides from the PDC-E2 protein with potential to bind HLA-E*0101. If these peptides are able to stabilise HLA-E on the cell surface of target cells, for example, MHC-II TAP deficient 721.174 cells, it would be worthwhile assessing NK cell effector function and markers of adaptive behaviour following stimulation of peptide-loaded target cells. This work would further investigate an association between adaptive-like NK cells and autoimmune disease, and look for evidence of antigen specificity using other peptides known to activate NKG2C as controls.

Interestingly hCMV along with a handful of other viruses contain microbial sequences (V120 in the case of hCMV) which shares a high degree of similarity with PDC-E2 [273]. This suggests microbial infection may play a role in the induction of anti-mitochondrial antibodies against PDC-E2, breaking down immunological tolerance via microbial mimicry. It would therefore be interesting to examine the sera of patients with PBC to look for evidence of an association with CMV. This could help establish if viral infection may predispose patients to developing this autoimmune condition.

CD49a+ and CXCR6+ NK cells may mediate disease progression in PBC via IL-12 hypersensitivity leading to adaptive-like NK cells with biliary-homing markers. It would therefore be useful to sort CD49a+/- and CXCR6+/- NK cells from patients with PBC for RNA sequencing analysis. Genes of particular interest include other cytokine signalling pathways, particularly IL-23/IL-17 and markers associated with liver homing.

To move these findings forward into translational work it will be important to establish if IL-12 activated CD49a+CXCR6+ NK cells found PBC can mediate enhanced killing of BECs. This could be achieved using a BEC cell line, or using techniques described by Shimoda et al, in which they

are able to isolate BECs from human liver samples. Secondly it would be useful as a proof of concept study to attempt to block markers of IL-12 hypersensitivity reported in CXCR6+ and CD49a+ NK cells using Lisofylline or Ustekinumab. A number of mouse models of PBC do exist, however as discussed this disease is complex in that its aetiology is likely to be multifactorial, involving genetic and environmental factors, and it has a number of pathological stages. Therefore current animal models may not be sufficiently optimised to investigate these findings further [274].

Finally a further important translational opportunity exists in undertaking a second clinical trial using Ustekinumab in a stratified patient cohort to allow examination of its effectiveness in individuals with rapidly progressing disease who have significant inflammation, but have not developed severe cholestasis or fibrosis as yet. This would also permit the assessment of NK cell reactivity towards low dose IL-12 as a novel biomarker of treatment response, and allow an opportunity to validate the results of this work.

6.4.2 Liver-homing cytokine-induced memory-like NK cells

This work identifies that IL-12 and IL-15 can be used to generate hyperfunctional CD49a+CXCR6+ NK cells that express biliary-homing markers. These NK subsets may also demonstrate adaptive-like features including enhanced proliferation and longevity. To investigate their therapeutic potential a mouse model would be the next step. Work is currently ongoing in the Khakoo lab in which peripheral blood NK cells are stimulated with a cocktail of cytokines to see if they can prevent or slow the progression of disease in a mouse model of liver cancer.

Appendices

A.1 Information sheet and consent form for patients undergoing hepatic resection for cancer

PARTICIPANT INFORMATION SHEET

PATIENTS UNDERGOING HEPATIC RESECTION FOR CANCER

Title of Project: Understanding the role of natural killer cell tolerance in the liver in determining outcomes for chronic liver disease

REC Submission No: 137013

Version No & date of issue: Version 4, 20/01/14

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take your time to read the following information and discuss it with friends, relative and your GP if you wish. 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 participate. You are not under any obligation to take part and your decision to enter this project will not affect the care you receive.

What's is the purpose of this study?

Liver disease is the fastest growing cause of death in the under 65s. If not treated it can result in liver failure and cancer of the liver, yet unfortunately very few treatments exist for these conditions. The Clinical and Experimental Sciences (CES) academic unit at the University of Southampton is investigating a group of cells, called Natural Killer (NK) cells which are vital components of the body's immune defence against viruses and cancers. They are also important in a number of liver diseases. We are always looking to improve the treatments we provide. To do this we need to understand more about NK cells, particularly NK cells found inside the liver of patients both with and without chronic liver disease as we suspect they may differ to those found in the blood. This difference may

be an important target for new treatments. We shall be using a number of detailed laboratory techniques to study these samples to gain a better understanding of how NK cells mature in normal livers and how their development is affected in chronic liver disease. This greater understanding will allow us to develop better treatments for patients in the future and may help generate new ideas for vaccines.

Why have I been invited?

You have been asked to take part because you are due to have an operation on your liver to remove cancer that either originated in the liver or has spread to the liver. In order to understand the changes that occur in NK cells in liver disease it is important to understand how they develop in the livers of patients without a primary liver disease as well as those with liver cancer. If you are willing to consent to our study, we will take a small portion of liver tissue that the surgeon has removed at the time of your operation that is surplus to requirement. This will not require any extra tissue to be removed as we would use part of the tissue that would be taken from your liver anyway. We will also ask your permission to take an additional sample of blood prior to your surgery.

Do I have to take part?

No, it is up to you to decide whether you wish to take part. We will describe the study and go through this information sheet which we will then give you to keep. If you agree to take part, we will ask you to sign a consent form. You will be free to withdraw at anytime without giving a reason. Your decision will not affect your medical care in any way.

What are the possible benefits of taking part?

The study will have no direct benefit to you, but the information we get from this study will help our understanding of chronic liver disease. It is hoped the information we get from this study will help us develop better treatments for liver disease.

Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept strictly confidential by your clinical carers and the research team. The handling, processing, storage and destruction of study information and data will be conducted in accordance with the Data Protection Act 1998. We will collect data relevant only to our study. Data recorded may include your date of birth, gender, age of surgery, and whether you are known to have an underlying primary liver condition. This data will be coded anonymously so that you cannot be identified. It will then be stored securely on a password protected database and made available only to members of the research team. On occasions other research groups may ask to access data that we have collected as part of this study. The researchers will review each case in detail and share data only to parties with sound ethically

approved projects where the research question is clearly defined and judged to be of adequate clinical importance. In all circumstances a data sharing agreement will be signed which will prohibit secondary parties from attempting to identify study participants.

What will happen to me if I take part?

If you agree to take part we will collect a sample of blood from you (about four teaspoonfuls) before your operation. During your operation a small piece of liver tissue will be taken from the surgical specimen removed. Once the surgical specimen and blood have been obtained, you will not be required to undergo any further tests or follow up for this research study. The scientific procedures used in the study are not diagnostic and will not be used to determine further treatment. They are purely to aid our understanding of NK cell maturation. You will therefore not be exposed to any extra risk in addition to the risks of your treatment. No other inconvenience is anticipated.

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time. If you withdraw from the study, we will destroy all the identifiable samples, but we will use the data up to your withdrawal. Any stored blood or tissue samples that can still be identified as yours will be destroyed if you wish. If you participate in the study, you will be free to withdraw at any time without giving a reason. The standard of care which you receive will be the same whether or not you take part in this study.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (Dr Theresa Hydes 07799141947 and Professor Khakoo 023 8120 4004). If you remain unhappy and wish to complain formally, you can do this through the NHS complaints procedure (or private institution). Details can be obtained from Southampton General Hospital.

What will happen to any samples I give?

A part of your liver containing the cancer will be removed as part of your planned operation. A small piece of the liver section which has been removed, will be taken and examined as part of this study. Only liver tissue required for your clinical care will be removed. The blood sample can be collected at a point convenient to you up until the end of your operation. All samples will be coded for storage and their use in any experiments so that you cannot be easily identified. Blood and tissue samples will be processed in a secure laboratory. The samples will be available for researchers in the Clinical and Experimental Sciences Academic Unit at the University of Southampton School of Medicine to study. The liver and blood samples will be closely analysed to try to establish how the liver affects NK cell development. The information obtained from the samples is unlikely to yield any information

that will be individually significant however. We will also ask your permission to store the blood and liver samples you have donated (1) for further study in this research project, and (2) for use in future research projects investigating liver disease that are granted approval from a local Research Ethics Committee. If future researchers are granted ethical approval then any tissue used will be made anonymous so you cannot be identified.

What will happen to results of the research study?

The results of any research will be published in the scientific literature. There will be no means of identifying individuals within this.

Who has reviewed and given ethical approval for the study?

All research in the NHS is looked at by an independent group of people called a Research Ethics Committee, to protect your safety, right, wellbeing and dignity.

Further information

If you have any questions please feel free to ask the study doctors directly at Southampton General Hospital:

Dr. Theresa Hydes 07799141947

Professor Salim Khakoo 023 8120 4004

Alternatively for independent advice you can contact 'Involve', a national advisory group, funded by the National Institute for Health Research (NIHR), whose role is to support and promote active public involvement in NHS, public health and social care research. Website: www.invo.org.uk/index.asp. Tel 02380 651088.

Thank you for taking the time to read this leaflet

Title of Project: Understanding the role of natural killer cell tolerance in the liver in determining outcomes for chronic liver disease

REC Submission No: 137013

Name of Researcher: Salim Khakoo

Version No & date of issue: Version 4, 20/01/14

Patient Identification Number for this research:

CONSENT FORM – PATIENTS UNDERGOING HEPATIC RESECTION FOR CANCER

Please initial box

1. I confirm that I have read and understand the information sheet (Version 4, dated 20/01/14) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the Clinical and Experimental Sciences Academic Unit at the University of Southampton, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I give permission to have an additional sample of blood taken and examined before my operation to be used as part of this study. ☐

5. I give permission for left-over samples of my liver taken at surgery to be examined as part of this study. ☐

6. I give permission for anonymised blood and liver samples, in addition to clinical data to be stored securely for the purpose of this research in the Clinical and Experimental Sciences Academic Unit at the University of Southampton for the duration of this study. ☐

7. I give permission for anonymised blood and liver samples donated for this study to be stored in a Human Tissue Authority approved tissue bank at the University of Southampton following the completion of this study, with the understanding that these may be used in future research projects related to the investigation of liver disease, providing that specific study protocols have received local Research Ethics Committee approval. ☐

8. I give permission for DNA analysis to be carried out on the collected samples. ☐

9. I agree to take part in the above study. ☐

_____	_____	_____
Name of Participant	Date	Signature

_____	_____	_____
Name of Person taking consent.	Date	Signature

1 for patient, 1 for researcher, 1 to be kept with the hospital notes

A.2 Information sheet and consent form for patients with Primary Biliary Cholangitis

PARTICIPANT INFORMATION SHEET

PATIENTS WITH PRIMARY BILIARY CIRRHOSIS

Title of Project: Understanding the role of natural killer cell tolerance in the liver in determining outcomes for chronic liver disease

REC Submission No: 137013

Version No & date of issue: Version 3, 01/08/16

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take your time to read the following information and discuss it with friends, relative and your GP if you wish. 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 participate. You are not under any obligation to take part and your decision to enter this project will not affect the care you receive.

What's is the purpose of this study?

Primary Biliary Cirrhosis (PBC) is a chronic liver disease in which the patient's own immune system becomes 'overactive' and starts targeting some of the normal cells in the bile ducts which drain the liver. This leads to inflammation of the bile ducts and if the disease is more aggressive, a gradual destruction of the bile ducts over time. Most patients with PBC have no symptoms and will live to a normal life expectancy, however a subpopulation of patients have a more aggressive form of PBC and in the worst case scenario can go on to develop liver cirrhosis (scarring of the liver) and cancer of the liver. Treatment options for PBC are limited and little progress has been made in the development of new drugs over the last ten years. Furthermore, currently available treatment options for PBC are often not effective in patients with the more severe form, resulting in such patients often requiring a liver transplant. Current estimates suggest 13 per 100,000 people in the UK are affected by PBC, and the incidence is rising steadily. There is therefore an urgent need to develop new treatment targets in order to holt disease progression and prevent the development of

cirrhosis and liver cancer.

The Clinical and Experimental Sciences (CES) academic unit at the University of Southampton is investigating a group of cells called Natural Killer (NK) cells, which are vital components of the body's immune system. They are important in a number of autoimmune diseases, however their function in PBC is yet to be determined. Furthermore recent large gene mapping studies have highlighted variations in a gene expressed by NK cells which is known to be important in determining outcomes in PBC. We would therefore like to examine the behavior of NK cells in PBC and to further define the role of this signaling gene in disease progression. To do this we need to collect blood samples from patients with PBC and analyse these using a number of detailed laboratory techniques. A greater understanding of the behaviour of NK cells in PBC will allow us to develop better treatments for patients in the future.

Why have I been invited?

You have been asked to take part because you have been previously diagnosed with PBC. We would like to examine the behaviour of NK cells in PBC as we believe they may be dysfunctional in this condition and could be targets for future treatments. If you are willing to consent to our study we will collect one sample of blood from you for research purposes.

Do I have to take part?

No, it is up to you to decide whether you wish to take part. During your research clinic appointment we will describe the study to you and go through this information sheet. If you agree to take part, we will ask you to sign a consent form. You will be free to withdraw at anytime without giving a reason. Your decision will not affect your medical care in any way.

What are the possible benefits of taking part?

The study will have no direct benefit to you, but the information we get from this study will help our understanding of PBC. It is hoped the information we get from this study will help us develop better treatments for this condition.

Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept strictly confidential by your clinical carers and the research team. The handling, processing, storage and destruction of study information and data will be conducted in accordance with the Data Protection Act 1998. We will collect data relevant only to our study. Data recorded may include your date of birth, gender, age of diagnosis, current medication and the stage of your PBC. This data will be coded anonymously so that you cannot be identified. It will then be stored securely on a password protected database and

made available only to members of the research team. On occasions other research groups may ask to access data that we have collected as part of this study. The researchers will review each case in detail and share data only to parties with sound ethically approved projects where the research question is clearly defined and judged to be of adequate clinical importance. In all circumstances a data sharing agreement will be signed which will prohibit secondary parties from attempting to identify study participants.

What will happen to me if I take part?

If you agree to take part we will arrange for you to attend a research clinic at a time convenient to you. During the clinic appointment, we will ask you to sign a consent form and then collect a sample of blood from you (about three tablespoonfuls). Once the blood sample has been collected you will not be required to undergo any further tests or follow up for this research study. The scientific procedures used in the study are not diagnostic and will not be used to determine further treatment. They are purely to aid our understanding of PBC. You will therefore not be exposed to any extra risk. No other inconvenience is anticipated. Each donation is valuable to us and we may therefore approach you in the future to ask if you would like to provide a further blood sample for this study. The donation of any further samples is wholly voluntary and you do not have to provide this if you do not wish to. Choosing not to provide any additional samples will not affect any future participation in this study or your future medical care. We will ensure a minimum of 4 weeks between donations. We will ask for your written consent prior to each donation you give.

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time. If you withdraw from the study, we will destroy all the identifiable samples, but we will use the data up to your withdrawal. Any stored blood samples that can still be identified as yours will be destroyed if you wish. If you participate in the study, you will be free to withdraw at any time without giving a reason. The standard of care which you receive will be the same whether or not you take part.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (Dr Theresa Hydes 02381 206671). If you remain unhappy and wish to complain formally, you can do this through the NHS complaints procedure (or private institution). Details can be obtained from Southampton General Hospital.

What will happen to any samples I give?

A blood sample will be collected during your clinic appointment. All samples will be coded for storage and their use in any experiments so that you cannot be easily identified. Blood samples will be

processed in a secure laboratory. The samples will be available for researchers in the Clinical and Experimental Sciences Academic Unit at the University of Southampton School of Medicine to study. The blood sample will be closely analysed to try to establish how NK cells behave in PBC. The information obtained from the samples is unlikely to yield any information that will be individually significant however. We will also ask your permission to store the blood sample you have donated (1) for further study in this research project, and (2) for use in future research projects investigating liver disease that are granted approval from a local Research Ethics Committee. If future researchers are granted ethical approval then any blood samples used will be made anonymous so you cannot be identified.

What will happen to results of the research study?

The results of any research will be published in the scientific literature. There will be no means of identifying individuals within this.

Who has reviewed and given ethical approval for the study?

All research in the NHS is looked at by an independent group of people called a Research Ethics Committee, to protect your safety, right, wellbeing and dignity.

Further information

If you have any questions please feel free to ask the study doctors directly at Southampton General Hospital:

Dr. Theresa Hydes	02381 206671
Professor Salim Khakoo	023 8120 4004

Alternatively for independent advice you can contact 'Involve', a national advisory group, funded by the National Institute for Health Research (NIHR), whose role is to support and promote active public involvement in NHS, public health and social care research. Website: www.invo.org.uk/index.asp. Tel 02380 651088.

Thank you for taking the time to read this leaflet

Title of Project: Understanding the role of natural killer cell tolerance in the liver in determining outcomes for chronic liver disease

REC Submission No: 137013

Name of Researcher: Salim Khakoo

Version No & date of issue: Version 2, 09/09/16

Patient Identification Number for this research:

CONSENT FORM – PRIMARY BILIARY CIRRHOSIS

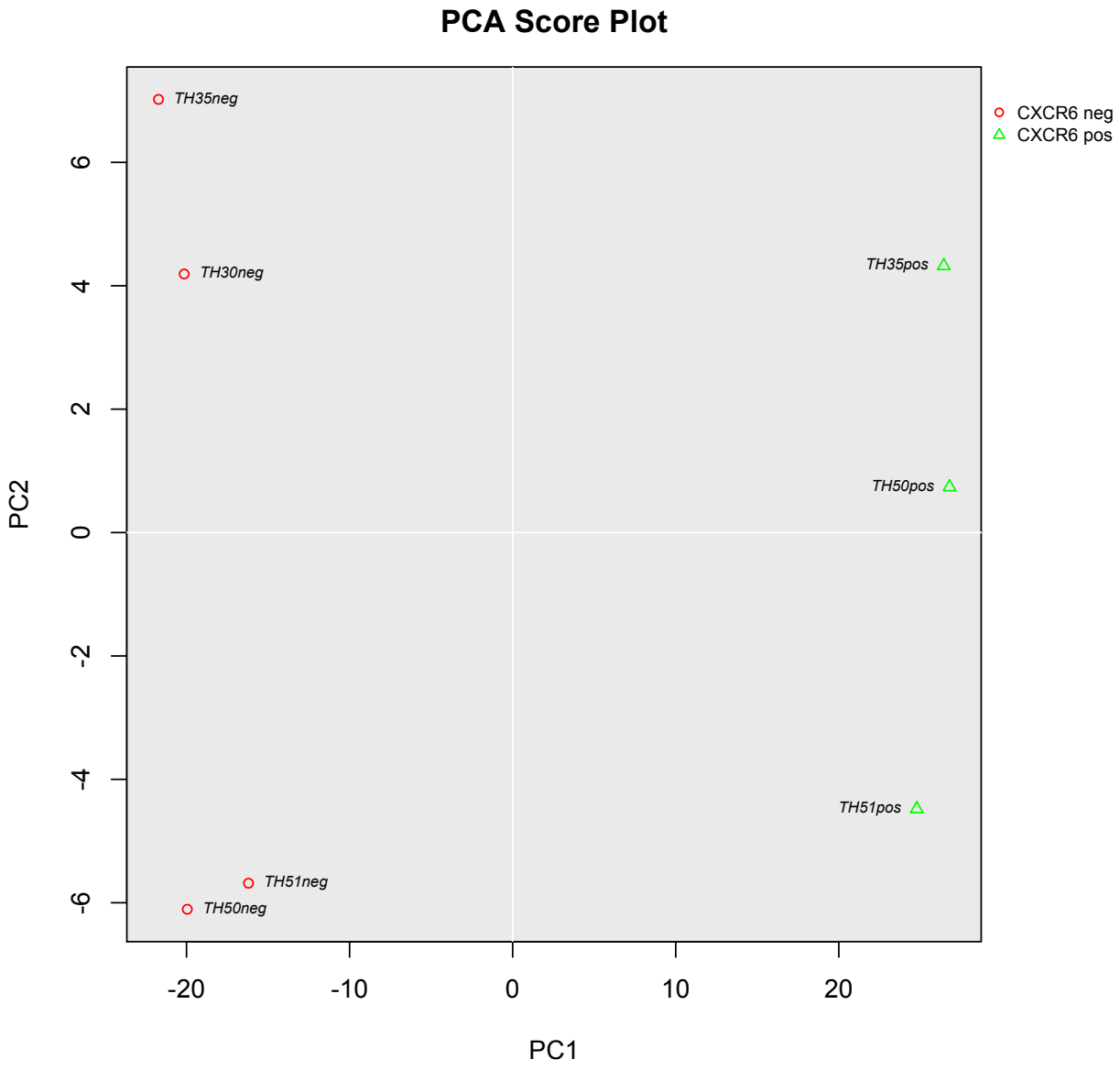
	Please initial box
1. I confirm that I have read and understand the information sheet (Version 3, dated 01/08/16) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the Clinical and Experimental Sciences Academic Unit at the University of Southampton, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
4. I give permission to have an additional sample of blood taken and examined as part of this study.	☐
5. I give permission for anonymised blood samples, in addition to clinical data to be stored securely for the purpose of this research in the Clinical and Experimental Sciences Academic Unit at the University of Southampton for the duration of this study	☐
6. I give permission for anonymised blood samples donated for this study to be stored in a Human Tissue Authority approved tissue bank at the University of Southampton following the completion of this study, with the understanding that these may be used in future research projects related to the investigation of liver disease, providing that specific study protocols have received local Research Ethics Committee approval.	☐
7. I give permission for DNA analysis to be carried out on the collected samples.	☐
8. I agree to take part in the above study.	☐

Name of Participant	Date	Signature
Name of Person taking consent.	Date	Signature

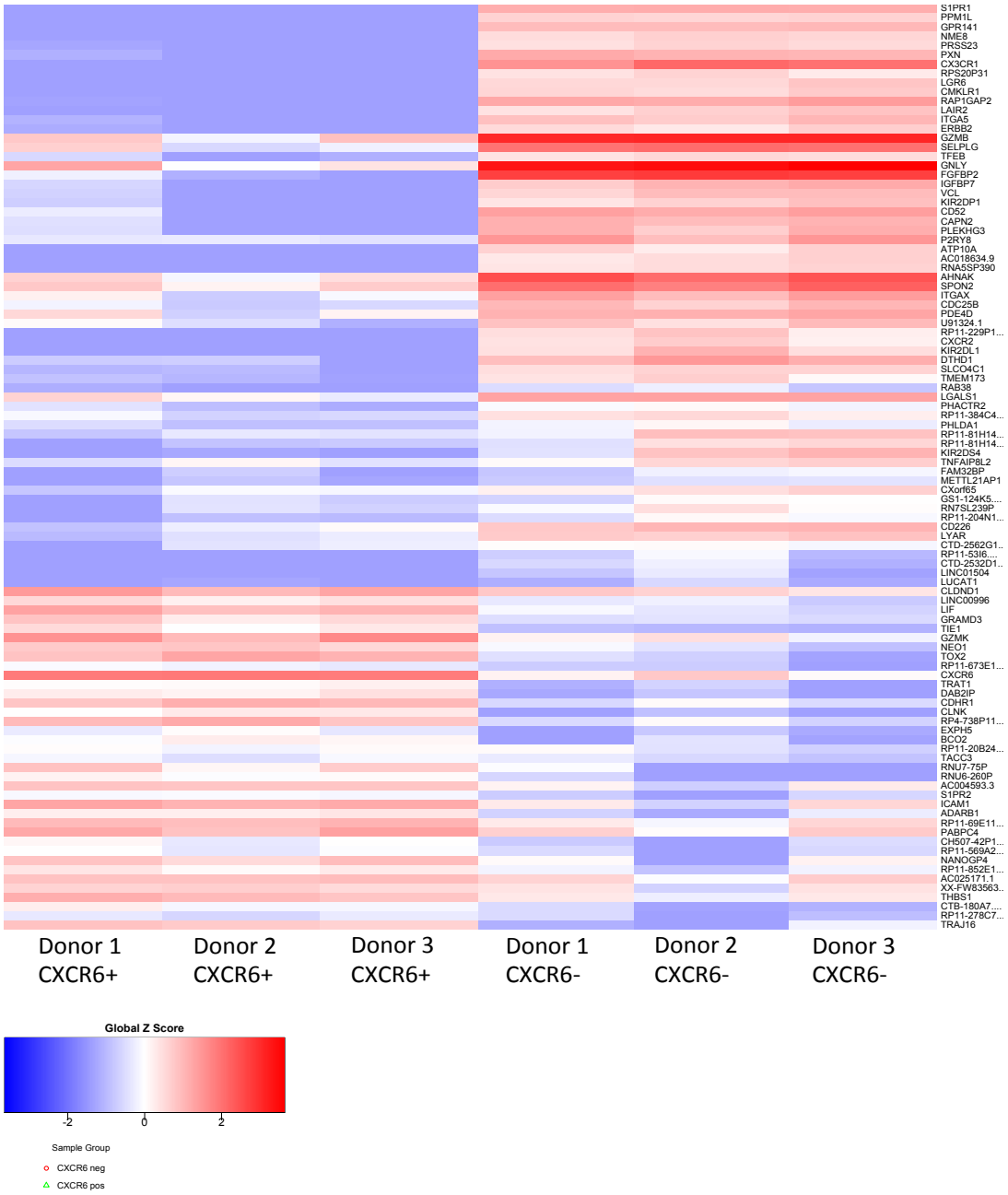
1 for patient, 1 for researcher, 1 to be kept with the hospital notes

A.3 Additional results

Supplementary results from RNA sequencing data of liver-resident CXCR6+ and CXCR6- NK cells



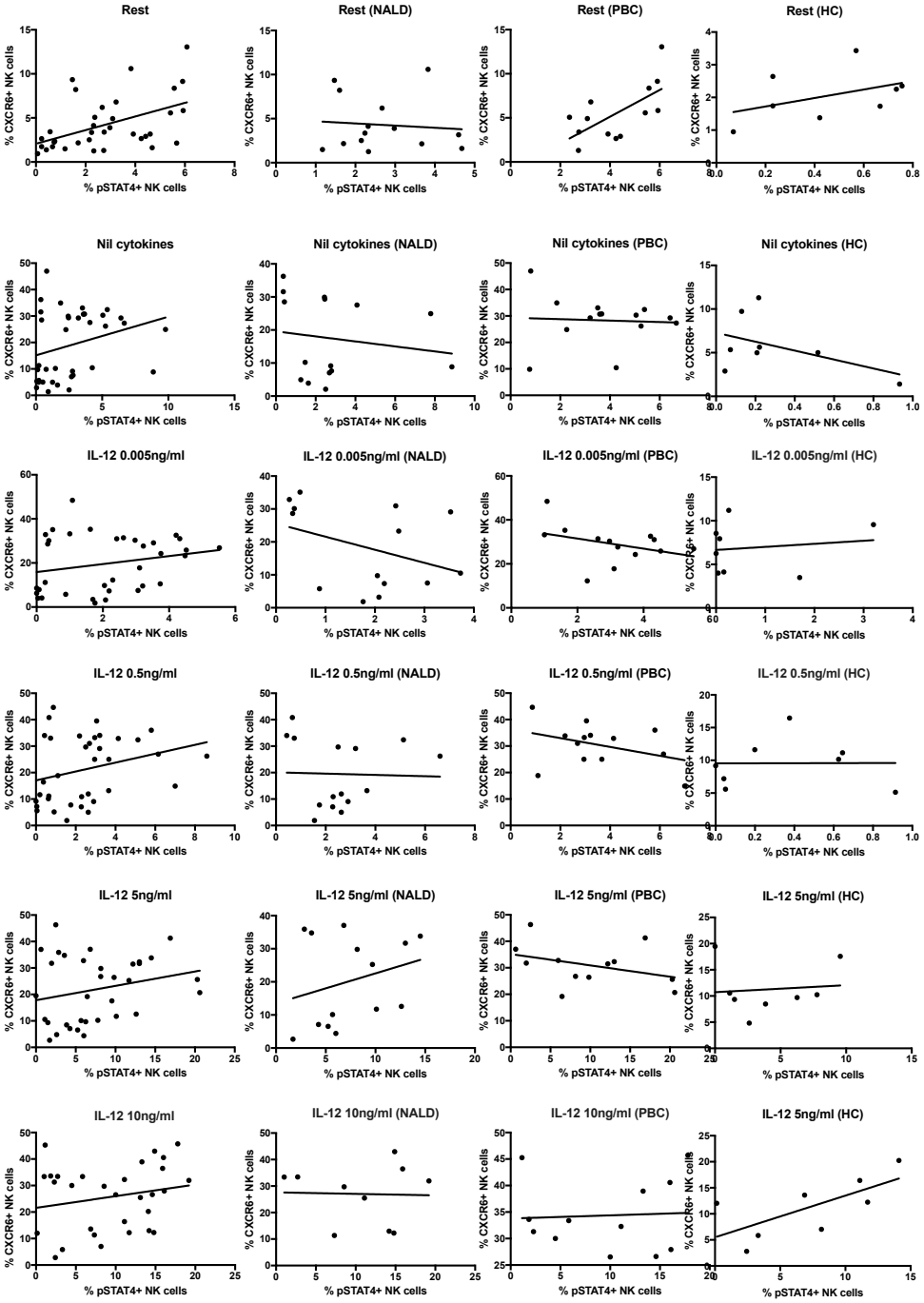
Appendix Fig 1 Primary Component Analysis plots of CXCR6+ (n=3) and CXCR6- (n=4) samples examined.

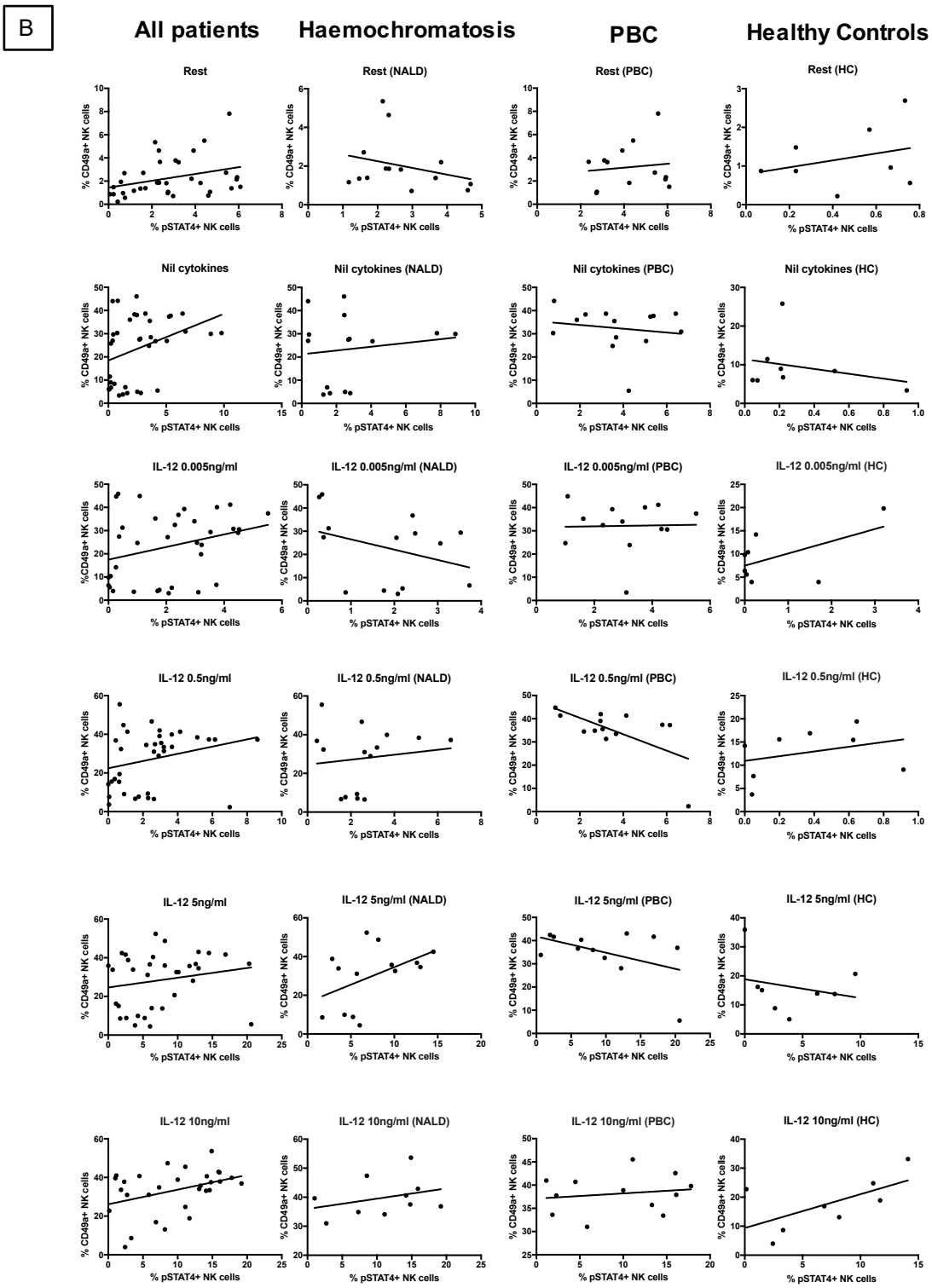


Appendix Fig 2 Heat map generated using RNA-seq data displaying the top 100 differentially expressed genes between liver-resident CXCR6+ and CXCR6- NK cells.

Linear regression for levels of pSTAT4 and surface expression of CXCR6 and CD49a

A





Appendix Fig 3 Linear regression for levels of pSTAT4 and surface expression of CXCR6 and CD49a for individuals with haemochromatosis, PBC and healthy controls at rest and following stimulation with increasing concentrations of IL-12

A.4 List of Reagents

1. Phosphate Buffered Saline, 0.0067 M PO_4 without Ca + Mg, BioWhittaker, Lonza, Belgium, Cat BE17-516F
2. Roswell Park Memorial Institute Medium 1640 (1X) + GlutamMAX-I, Gibco, Life Technologies, UK, Cat. 61870-044
3. Dulbecco's Modified Eagle Medium (1X) + 4.5g/l D-Glucose, -L-Glutamine, -Pyruvate, Gibco, Life Technologies, UK, Cat. 11960-085
4. AIM-V Medium, +L-Glutamine, + 50µg/ml Streptomycin sulfate, + 10µg/ml Gentamicin sulfate, Gibco, Life Technologies, Lot No. 1722762, Cat. 12055-083
5. Fetal Bovine Serum, Hyclone, South American origin, ThermoScientific, Northumberland, Cat SV30180-03
6. Penicillin, Streptomycin, Glutamine, Gibco, Life Technologies, UK, Cat. 10378-016
7. Dimethly sulfoxide, Sigma, Cat. D8418
8. Ficoll-Paque PREMIUM, GE Healthcare Life Sciences, Sweden, 17-5442-03
9. Bovine Serum Albumin, Sigma, Cat. A2153
10. Sodium Azide, Sigma, Cat. S2002-100G
11. Human Serum, Lot No. SLBK155OV, from male AB clotted whole blood, USA origin, Sigma, Cat. H6914
12. HEPES ≥99.5% titration, Sigma, Cat. H3375
13. EGTA, Sigma, Cat. 34596
14. TIV Collagenase from Clostridium histolyticum, ≥125CDU/mg, Sigma, Cat. C5138
15. Calcium Chloride, Sigma, Cat. 223506
16. UltraPure 0.5M EDTA, pH 8.0, Invitrogen, Cat. 15575-020
17. LS Columns, Miltenyi Biotec, Cat. 130-042-401
18. TRI Reagent Solution, RNA isolation reagent, Ambion, Cat. AM9738
19. BD Cytofix, Fixation Buffer (4.2% formaldehyde), Lot No. 5163547, BD Biosciences, Cat. 554655
20. BD Phosphoflow, Perm Buffer III (87.68% Methanol), Lot No. 5075568-1, BD Biosciences, Cat. 558050
21. BD Cytofix/Cytoperm Plus, Fixation/Permeabilisation Kit with BD GolgiStop, Lot No. 5103563, BD Biosciences, Cat. 554715
    - BD Cytofix/Cytoperm, Fixation and Permeabilisation solution (4.2% formaldehyde), BD Biosciences, Cat. 51-2090KZ
    - BD Perm/Wash, 10X, Buffer, BD Biosciences, Cat. 51-2091KZ

- BD GolgiStop, Protein transport inhibitor, BD Biosciences, Cat. 51-2092KZ

22. Foxp3 Staining Buffer Set, eBioscience, Cat. 00-5523-00
    - Fixation/Permeabilisation Concentrate, eBioscience, Cat. 00-5123-43
    - Fixation Permeabilisation Diluent, eBioscience, Cat. 00-5223-56
    - Permeabilisation Buffer, 10X, eBioscience, Cat. 00-8333-56
23. CellTrace CFSE Cell Proliferation Kit, Molecular probes, Life Technologies, Cat. C34554
24. Intracellular antibodies
    - Anti-human IFNγ-APC, 1:10, Clone B27, Lot No. B200216, Biolegend Cat No. 506510
    - Anti-human pSTAT4-AF647, 1:2.5, Clone pY693, Lot No. 5247620, BD Biosciences, Cat No. 558137
    - Anti-human T-bet-BV421, 1:100, Clone 4B10, Lot No. B204996, Biolegend, Cat. 644815
    - Anti-human Eomes-efluor660, 1:100, Clone WD1928, Lot No.4278546, eBioscience, Cat. 50-4877
24. Human Cytokines
    - Recombinant human IL-2, Lot No. 051212, PeproTech, UK, Cat No. 200-02
    - Recombinant human IL-12 (p70), Lot No. 0707S96-4, PeproTech, UK, Cat No. 200-12
    - Recombinant human IL-15, Lot No. EA5412071, R&D Systems, Oxford, UK, Cat No. 285-IF-100
    - Recombinant human IL-18, Lot No. 114, Medical and Biological Laboratories Ltd, Japan, Cat No. B001-5
25. BD CompBeads, Anti-Mouse Ig, κ / Negative Control, Compensation Particles Set, BD Biosciences, Lot. 4101845, Cat. 552843

A.5 Accompanying Material

NK 2016 (2016)

ABSTRACT BOOK

A comparison of CD49a+ and CXCR6+ Natural Killer cell populations in the human liver reveals distinct phenotypes suggestive of NK cell memory and migration

Theresa Hydes[1,2], Jennie Naftel[1], Mohammed AbuHilal[1], Thomas Armstrong[1], Zaed Hamady[1], John Primrose[1], Arjun Takhar[1], Salim Khakoo[1,2]

1. University of Southampton, Southampton, UK
2. University Hospital Southampton NHS Foundation Trust, Southampton, UK

Background: Selected NK cell populations exhibit 'memory' against haptens and viruses in mice, with liver-specific adhesion molecules CXCR6 and CD49a acting as surface markers. NK cells expressing both markers have been identified in the human liver. In both species cytokines can generate NK cells with adaptive properties in the peripheral blood.

Aim: To compare liver resident CXCR6+ and CD49a+ NK cells in humans in terms of phenotype, expansion and function and to assess the role of cytokines in generating NK cells with this phenotype.

Methods: Liver tissue was sampled from 52 patients with metastases. Paired peripheral blood samples were obtained from 30 patients. Mononuclear cells were stained for surface markers and IFNγ. To examine cytokine-driven expansion paired blood and liver mononuclear cells were incubated with IL-2/12/15/18 or all four.

Results: CD49a+ and CXCR6+ NK cells comprised 7.6% and 65.3% of intrahepatic NK cells (<5% circulation). Unlike the universal presence of CXCR6+ NK cells, CD49a+ NK cells were present in only half of individuals. While not terminally differentiated, CD49a+ NK cells maintained high levels of KIR and NKG2C suggestive of clonal expansion and licensing, whereas CXCR6+ NK cells were CD16-CD161+CD69+$KIR^{low}NKG2C^{low}$. CD49a+ NK cells displayed enhanced IFNγ production and expansion following stimulation with all activating cytokines, including the cytokine cocktail compared to CXCR6+ NK cells. Interestingly expression of CD49a and CXCR6 could be induced on circulatory NK cells following culture with IL-15 and IL-12 respectively. Cytokine-generated CD49a+ NK cells were $CD56^{bright}NKG2C^{high}IFN\gamma^{high}$ consistent with an activated memory-like phenotype, whereas CXCR6+ NK cells were $CD56^{bright}NKG2C^{low}IFN\gamma^{low}$.

Conclusion: Human hepatic CD49a+ NK cells display features consistent with memory, whereas CXCR6 is a likely marker of liver residency. Cytokine stimulation may allow the generation of memory-like NK cells in the blood and induce their migration to the liver with therapeutic implications for viral hepatitis and liver cancer.

Basic Science

Natural Killer cell hypersensitivity in Primary Biliary Cholangitis: a novel mechanism for autoimmunity

THERESA HYDES [1]

[1] University of Southampton

Introduction

GWAS studies have identified polymorphisms in the IL-12/STAT4 pathway as being significantly associated with developing Primary Biliary Cholangitis (PBC). Natural Killer (NK) cells express the IL-12 receptor and respond to IL-12 stimulation through the STAT4 pathway. The healthy human liver is rich in CXCR6+ NK cells and "adaptive" NK cells have been characterised in mice as being liver resident and, expressing CD49a and the liver homing marker CXCR6. Human CD49a+ NK cells are mature and express NKG2C. They constitute a clonally expanded population which display enhanced proliferative capabilities, a Th1 cytokine profile and hyper-responsiveness to inflammatory cytokines. They may thus represent an "adaptive" NK cell population.

Aim

To assess the role of the IL-12-pSTAT4 axis in NK cells in PBC.

Method

PBMCs were isolated from 23 patients with PBC and age-matched haemochromatosis controls. NK cells were analysed for surface markers (CXCR6, CD49a, NKG2C), pSTAT4 and IFNγ by flow cytometry at rest and following stimulation with IL-12 and IL-15.

Results

PBC patients had more $CD56^{bright}$ NK cells in the peripheral blood than controls (8.9% vs 6.4%, $p<0.05$). Their circulating NK cells expressed greater levels of CD49a (2.4% vs 1.4%, $p<0.05$), CXCR6 (5.7% vs 2.4%, $p<0.01$) and higher resting levels of pSTAT4 compared to controls (5.4% vs 2.3%, $p<0.05$) indicating an activated status. CXCR6 could be readily induced in PBC patients using small concentrations of IL-12 (0.005ng/ml), increasing the frequency of CXCR6+ NK cells from 5.2 to 11.2% (PBC) vs 2.8 to 3.4% (controls), $p<0.001$. Consistent with increased baseline levels of pSTAT4, CD49a+ NK cells from PBC patients responded better to low levels of IL-12 by secreting IFNγ (4.3% vs 1.6%, $p<0.01$). Furthermore CD49a+ and NKG2C+ NK cells could be generated in the peripheral blood in PBC more easily using IL-15 (24.3% vs 12.5%, $p<0.05$) and IL-12 (11.5% vs 7.7%, p

Conclusion

Our data provide a functional correlate to the GWAS studies. NK cells from patients with PBC have baseline activation of the IL-12/STAT4 pathway and exhibit hyper-responsiveness to IL-12 leading to the generation of "adaptive" NK cells that express the liver homing marker CXCR6. Hypersensitivity of peripheral NK cells to cytokines in PBC may lead to activated CXCR6+ NK cells homing to the liver and causing biliary destruction. Blocking this pathway may provide a novel therapeutic target for PBC.

FRI-264

DISTINCT NATURAL KILLER CELL POPULATIONS IN THE HUMAN LIVER EXPRESS MARKERS OF NK CELL MEMORY AND MIGRATION

T.J. Hydes[1], M. AbuHilal[2], T. Armstrong[2], Z. Hamady[2], J. Primrose[2], A. Takhar[2], S. Khakoo[1]. [1]*University of Southampton;* [2]*University Hospital Southampton NHS Foundation Trust, southampton, United Kingdom*
E-mail: therasa@doctors.org.uk

Background and Aims: The liver is thought to be a site of cytokine-driven Natural Killer (NK) cell differentiation & memory-like NK cell residence. Liver-specific adhesion molecules CXCR6 & CD49a act as surface markers of memory–like NK cells in mice capable of mounting a vigorous antigen specific response on re-exposure to haptens & viruses. We aim to determine the existence CXCR6 & CD49a + NK cells in humans, define their maturation status & examine the role of cytokines in driving their differentiation & proliferation.

Methods: Liver tissue was sampled from the margin of 40 patients with metastases & flushed with chelating buffer followed by collagenase or mechanical digestion. Paired blood samples were taken from 16 patients. Mononuclear cells were isolated by ficoll density separation & cell surface staining performed. To assess proliferation paired peripheral blood & liver mononuclear cells were stained with 5 μM CFSE & incubated with IL-2/-12/-15/-18 or a cytokine cocktail for 6 days (n = 9). Surface staining was performed on day 0 & 6. Statistical analysis included median, IQR, Mann-Whitney *U* & Wilcoxon signed rank test.

Results: CD49a + & CXCR6 + NK cells comprised 6.5% & 68.3% of intrahepatic NK cells with < 5% of each in the peripheral blood. CD49a + NK cells were present in only 50% of individuals. While most were immature (CD16-) they maintained high levels of KIR (21.8%) & NKG2C (12.9%) suggestive of clonal expansion & antigen experience. CD49a + NK cells demonstrated other adaptive qualities including enhanced proliferation post exposure to IL-15 & a cocktail of IL-2/12/15/18. Interestingly CD49a expression could be induced by cytokines on both hepatic & peripheral blood NK cells. Conversely CXCR6 + NK cells were found in the liver at significant levels in all individuals & were less mature than the CD49a + population. They expressed low levels of KIR (4.7%) & NKG2C (6.5%) & did not proliferate in the liver in response to cytokines. The expression of CXCR6 could however be induced on peripheral blood NK cells following culture with IL-12 & IL-15. CD49a expression did not differ between CXCR6 ± liver NK cells.

Conclusions: Human hepatic CD49a + NK cells display features which suggest their potential to exert memory-like behavior, whereas CXCR6 appears to be a marker of liver residency. Cytokine stimulation may be a key mechanism for generating NK cells with a memory phenotype & inducing their migration to the liver. This has therapeutic implications for the management of viral hepatitis & liver cancer.

Natural killer cell maturation markers in the human liver and expansion of an NKG2C+KIR+ population

Theresa Hydes, Mohammed Abuhilal, Thomas Armstrong, John Primrose, Arjun Takhar, Salim Khakoo

Abstract

Background Selected populations of murine natural killer (NK) cells possess memory features to haptens, cytokines, and viruses. Liver-specific adhesion molecules CXCR6 and CD49a have been identified as surface markers in mice. In people, expansion of long-lived terminally differentiated NKG2C+ populations occur in the blood after viral infection. We aimed to compare intrahepatic and blood NK cell receptor expression to determine the existence of CD49a+ and CXCR6+ NK cells in human liver and define the maturation status of NKG2C+ NK cells at this site.

Methods Tissue samples were taken from the liver margin of 39 patients with hepatic metastases and flushed with chelating buffer followed by collagenase or mechanical digestion. Paired peripheral blood samples were taken from 15 patients, the remainder being unpaired. Mononuclear cells were isolated by ficoll separation and cell surface staining performed for CD3, CD56, CD16, CD57, CD117, CD161, CD158a, CD158b, CD49a, CD49b, CXCR6, NKG2C, and NKp46. Statistical analysis to compare intrahepatic and blood NK cell receptor expression included the median, IQR, and Mann-Whitney *U* test.

Findings Frequencies of NK cell precursors were similar in the liver and the blood (0·91% [0·62–3·26] *vs* 0·87 [0·41–1·52]); however, expression of all later markers of maturity were reduced including CD16 (47% [40·4–61·4] *vs* 88·7 [82·2–93·2], p<0·0001), CD57 (30·7% [25·0–53·9] *vs* 73·4 [70·4–87·6], p=0·0003), and KIR (11·2% [7·5–14·5] *vs* 26·7 [17·3–30·8], p<0·0001). Expanded hepatic CD16– NK cells were particularly immature with reduced CD57 and increased CD161 compared with the blood. NKG2C+ NK cells were found in similar frequencies in liver and blood. The hepatic NKG2C+ population was terminally differentiated, as in the circulation, but demonstrated a three-fold increase in KIR expression compared with NKG2C– counterparts, which was not seen in the blood. As in previously published research in mice, CD49a+ and CXCR6+ NK cells were liver resident (6·5% [3·9–14·6] liver *vs* 2·1 [1·3–4·3] blood, p<0·0001, and 65·3 [48·1–75·2] *vs* 4·5 [1·43–12·12], p=0·0039, respectively). Both populations were immature, with reduced KIR expression.

Interpretation We have shown that the liver contains an expanded population of immature CD16– NK cells. These cells might traffic from the blood and then differentiate into hepatic-specific CD49a+ and CXCR6+ NK cells. The function of these subsets is unknown but their immaturity hints against memory. Terminally differentiated NKG2C+ cells show KIR expansion in the human liver and probably represent an antigen-experienced population, raising the question of whether the liver is a site of NK cell memory acquisition.

Funding MRC Clinical Research Fellowship.

Contributors
TH was the principal investigator for project, carried out all laboratory procedures, analysed the data, and wrote the abstract. SK was the supervisor for TH, reviewed the abstract, and supervised laboratory work and data analysis. MA, TA, JP and AT provided liver specimens.

Declaration of interests
We declare no competing interests.

Published Online
February 26, 2015

Poster 68

University of Southampton, Southampton, UK (T Hydes MBBS, S Khakoo MD); and Southampton University Hospital, Southampton, UK (M Abuhilal MD, T Armstrong MBBS, J Primrose MBBS, A Takhar MBBS)

Correspondence to:
Dr Theresa Hydes, University of Southampton, Mailpoint 811, Level E, South Academic Block, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, UK
theresa@doctors.org.uk

List of References

1. Lanier LL. NK Cell Recognition. *Annu Rev Immunol*. Annual Reviews; 2005;23(1):225–74.
2. Ljunggren HG, Kärre K. In search of the “missing self”: MHC molecules and NK cell recognition. *Immunol Today*. 1990;11(7):237–44.
3. Kärre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*. 319(6055):675–8.
4. Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science*. 2004;305(5685):872–4.
5. Knapp S, Warshow U, Hegazy D, Brackenbury L, Guha IN, Fowell A, et al. Consistent beneficial effects of killer cell immunoglobulin-like receptor 2DL3 and group 1 human leukocyte antigen-C following exposure to hepatitis C virus. *Hepatology*. 2010;51(4):1168–75.
6. Fadda L, Borhis G, Ahmed P, Cheent K, Pageon S V, Cazaly A, et al. Peptide antagonism as a mechanism for NK cell activation. *Proc Natl Acad Sci U S A*. 2010;107(22):10160–5.
7. Cassidy S, Mukherjee S, Myint TM, Mbribindi B, North H, Traherne J, et al. Peptide selectivity discriminates NK cells from KIR2DL2- and KIR2DL3-positive individuals. *Eur J Immunol*. 2014;45(2):492–500.
8. Brodin P, Kärre K, Höglund P. NK cell education: not an on-off switch but a tunable rheostat. *Trends Immunol*. 2009;30(4):143–9.
9. Hoglund P, Brodin P. Current perspectives of natural killer cell education by MHC class I molecules. *Nat Rev Immunol*. 2010;10(10):724–34.
10. Norris S, Collins C, Doherty DG, Smith F, McEntee G, Traynor O, et al. Resident human hepatic lymphocytes are phenotypically different from circulating lymphocytes. *J Hepatol*. 1998;28(1):84–90.
11. Harmon C, Robinson MW, Fahey R, Whelan S, Houlihan DD, Geoghegan J, et al. Tissue-resident Eomes hi T-bet lo CD56 bright NK cells with reduced proinflammatory potential are enriched in the adult human liver. *Eur J Immunol*. 2016;46(9):2111–20.
12. Jonsson JR, Hogan PG, Balderson GA, Ooi LL, Lynch S V, Strong RW, et al. Human liver transplant perfusate: An abundant source of donor liver-associated leukocytes. *Hepatology*. 1997;26(5):1111–4.
13. Hata K, Zhang XR, Iwatsuki S, Van Thiel DH, Herberman RB, Whiteside TL. Isolation, phenotyping, and functional analysis of lymphocytes from human liver. *Clin Immunol Immunopathol*. 1990;56(3):401–19.
14. Doherty DG, Norris S, Madrigal-Estebas L, McEntee G, Traynor O, Hegarty JE, et al. The human liver contains multiple populations of NK cells, T cells, and CD3+CD56+ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. *J Immunol*. 1999;163(4):2314–21.
15. Hudspeth K, Donadon M, Cimino M, Pontarini E, Tentorio P, Preti M, et al. Human liver-resident CD56(bright)/CD16(neg) NK cells are retained within hepatic sinusoids via the engagement of CCR5 and CXCR6 pathways. *J Autoimmun*. 2016;66:40–50.
16. Lysakova-Devine T, O’Farrelly C. Tissue-specific NK cell populations and their origin. *J Leukoc Biol*. 2014;96(6):981–90.
17. Shi F-D, Ljunggren H-G, La Cava A, Van Kaer L. Organ-specific features of natural killer cells. *Nat Rev Immunol*. 2011;11(10):658–71.
18. Lim AI, Li Y, Lopez-Lastra S, Stadhouders R, Paul F, Casrouge A, et al. Systemic Human ILC Precursors Provide a Substrate for Tissue ILC Differentiation. *Cell*. 2017;168(6):1086–1100.e10.
19. Peng H, Jiang X, Chen Y, Sojka DK, Wei H, Gao X, et al. Liver-resident NK cells confer adaptive immunity in skin-contact inflammation. *J Clin Invest*. 2013;123(4).

20. Grégoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E, et al. The trafficking of natural killer cells. *Immunol Rev*. 2007;220:169–82.
21. Moroso V, Famili F, Papazian N, Cupedo T, van der Laan LJW, Kazemier G, et al. NK cells can generate from precursors in the adult human liver. *Eur J Immunol*. 2011;41(11):3340–50.
22. Jaleco AC, Blom B, Res P, Weijer K, Lanier LL, Phillips JH, et al. Fetal liver contains committed NK progenitors, but is not a site for development of CD34+ cells into T cells. *J Immunol*. 1997;159(2):694–702.
23. Freud AG, Yokohama A, Becknell B, Lee MT, Mao HC, Ferketich AK, et al. Evidence for discrete stages of human natural killer cell differentiation in vivo. *J Exp Med*. 2006;203(4):1033–43.
24. Hata K, Van Thiel DH, Herberman RB, Whiteside TL. Natural killer activity of human liver-derived lymphocytes in various liver diseases. *Hepatology*. 1991;14(3):495–503.
25. Moroso V, Metselaar HJ, Mancham S, Tilanus HW, Eissens D, van der Meer A, et al. Liver grafts contain a unique subset of natural killer cells that are transferred into the recipient after liver transplantation. *Liver Transpl*. 2010;16(7):895–908.
26. Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJC, et al. Distribution and Compartmentalization of Human Circulating and Tissue-Resident Memory T Cell Subsets. *Immunity*. 2013;38(1):187–97.
27. Shiow LR, Rosen DB, Brdičková N, Xu Y, An J, Lanier LL, et al. CD69 acts downstream of interferon-α/β to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature*. 2006;440(7083):540–4.
28. Ishiyama K, Ohdan H, Ohira M, Mitsuta H, Arihiro K, Asahara T. Difference in cytotoxicity against hepatocellular carcinoma between liver and periphery natural killer cells in humans. *Hepatology*. 2006;43(2):362–72.
29. Stegmann KA, Robertson F, Hansi N, Gill U, Pallant C, Christophides T, et al. CXCR6 marks a novel subset of T-betloEomeshi natural killer cells residing in human liver. *Sci Rep*. 2016;6:26157.
30. Knox JJ, Cosma GL, Betts MR, McLane LM. Characterization of T-bet and eomes in peripheral human immune cells. *Front Immunol*. 2014;5:217.
31. Wehr A, Baeck C, Heymann F, Niemietz PM, Hammerich L, Martin C, et al. Chemokine Receptor CXCR6-Dependent Hepatic NK T Cell Accumulation Promotes Inflammation and Liver Fibrosis. *J Immunol*. 2013;190(10):5226–36.
32. Heydtmann M, Lalor PF, Eksteen JA, Hübscher SG, Briskin M, Adams DH. CXC chemokine ligand 16 promotes integrin-mediated adhesion of liver-infiltrating lymphocytes to cholangiocytes and hepatocytes within the inflamed human liver. *J Immunol*. 2005;174(2):1055–62.
33. Cuff AO, Robertson FP, Stegmann KA, Pallett LJ, Maini MK, Davidson BR, et al. Eomes hi NK Cells in Human Liver Are Long-Lived and Do Not Recirculate but Can Be Replenished from the Circulation. *J Immunol*. 2016;197(11):4283–91.
34. Paust S, Gill HS, Wang B-Z, Flynn MP, Moseman EA, Senman B, et al. Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. *Nat Immunol*. 2010;11(12):1127–35.
35. Marquardt N, Béziat V, Nyström S, Hengst J, Ivarsson MA, Kekäläinen E, et al. Cutting Edge: Identification and Characterization of Human Intrahepatic CD49a+ NK Cells. *J Immunol*. 2015;194(6):2467–71.
36. Yamagiwa S, Kamimura H, Ichida T. Natural killer cell receptors and their ligands in liver diseases. *Med Mol Morphol*. 2009;42(1):1–8.
37. Gao B, Jeong W-I, Tian Z. Liver: An organ with predominant innate immunity. *Hepatology*. 2008;47(2):729–36.
38. Lassen MG, Lukens JR, Dolina JS, Brown MG, Hahn YS. Intrahepatic IL-10 maintains NKG2A+Ly49- liver NK cells in a functionally hyporesponsive state. *J Immunol*. 2010;184(5):2693–701.
39. Jinushi M, Takehara T, Tatsumi T, Yamaguchi S, Sakamori R, Hiramatsu N, et al. Natural killer cell and hepatic cell interaction via NKG2A leads to dendritic cell-

mediated induction of CD4 CD25 T cells with PD-1-dependent regulatory activities. *Immunology*. 2007;120(1):73–82.
40. Hintermann E, Bayer M, Pfeilschifter JM, Luster AD, Christen U. CXCL10 promotes liver fibrosis by prevention of NK cell mediated hepatic stellate cell inactivation. *J Autoimmun*. 2010;35(4):424–35.
41. Michel T, Hentges F, Zimmer J. Consequences of the crosstalk between monocytes/macrophages and natural killer cells. *Front Immunol*. 2012;3:403.
42. Daar AS, Fuggle S V, Fabre JW, Ting A, Morris PJ. The detailed distribution of HLA-A, B, C antigens in normal human organs. *Transplantation*. 1984;38(3):287–92.
43. Eisenberger CF, Viebahn R, Lauchart W, de Groot H, Becker HD. MHC antigen presentation on the surface of hepatocytes: modulation during and after hypoxic stress. *Transpl Int*. 1994;7 Suppl 1:S163-6.
44. Franco A, Barnaba V, Natali P, Balsano C, Musca A, Balsano F. Expression of class I and class II major histocompatibility complex antigens on human hepatocytes. *Hepatology*. 1988;8(3):449–54.
45. Jinushi M, Takehara T, Tatsumi T, Kanto T, Miyagi T, Suzuki T, et al. Negative regulation of NK cell activities by inhibitory receptor CD94/NKG2A leads to altered NK cell-induced modulation of dendritic cell functions in chronic hepatitis C virus infection. *J Immunol*. 2004;173(10):6072–81.
46. Jamil KM, Hydes TJ, Cheent KS, Cassidy SA, Traherne JA, Jayaraman J, et al. STAT4-associated natural killer cell tolerance following liver transplantation. *Gut*. 2016;doi 10.113.
47. Wald O, Pappo O, Safadi R, Dagan-Berger M, Beider K, Wald H, et al. Involvement of the CXCL12/CXCR4 pathway in the advanced liver disease that is associated with hepatitis C virus or hepatitis B virus. *Eur J Immunol*. 2004 Apr;34(4):1164–74.
48. Tilg H. Cytokines and liver diseases. *Can J Gastroenterol*. 2001;15(10):661–8.
49. Ramadori G, Armbrust T. Cytokines in the liver. *Eur J Gastroenterol Hepatol*. 2001;13(7):777–84.
50. Baroni GS, D'Ambrosio L, Curto P, Casini A, Mancini R, Jezequel AM, et al. Interferon gamma decreases hepatic stellate cell activation and extracellular matrix deposition in rat liver fibrosis. *Hepatology*. 1996;23(5):1189–99.
51. Melhem A, Muhanna N, Bishara A, Alvarez CE, Ilan Y, Bishara T, et al. Anti-fibrotic activity of NK cells in experimental liver injury through killing of activated HSC. *J Hepatol*. 2006;45(1):60–71.
52. Radaeva S, Sun R, Jaruga B, Nguyen VT, Tian Z, Gao B. Natural killer cells ameliorate liver fibrosis by killing activated stellate cells in NKG2D-dependent and tumor necrosis factor-related apoptosis-inducing ligand-dependent manners. *Gastroenterology*. 2006;130(2):435–52.
53. Tu Z, Bozorgzadeh A, Pierce RH, Kurtis J, Crispe IN, Orloff MS. TLR-dependent cross talk between human Kupffer cells and NK cells. *J Exp Med*. 2008;205(1):233–44.
54. Hou X, Zhou R, Wei H, Sun R, Tian Z. NKG2D-retinoic acid early inducible-1 recognition between natural killer cells and kupffer cells in a novel murine natural killer cell-dependent fulminant hepatitis. *Hepatology*. 2009;49(3):940–9.
55. Cheng C-W, Duwaerts CC, Rooijen N van, Wintermeyer P, Mott S, Gregory SH. NK cells suppress experimental cholestatic liver injury by an interleukin-6-mediated, Kupffer cell-dependent mechanism. *J Hepatol*. 2011;54(4):746–52.
56. Okazaki A, Hiraga N, Imamura M, Hayes CN, Tsuge M, Takahashi S, et al. Severe necroinflammatory reaction caused by natural killer cell-mediated Fas/Fas ligand interaction and dendritic cells in human hepatocyte chimeric mouse. *Hepatology*. 2012;56(2):555–66.
57. Chen Y, Wei H, Sun R, Dong Z, Zhang J, Tian Z. Increased susceptibility to liver injury in hepatitis B virus transgenic mice involves NKG2D-ligand interaction and natural killer cells. *Hepatology*. 2007;46(3):706–15.
58. Miethke AG, Saxena V, Shivakumar P, Sabla GE, Simmons J, Chougnet CA. Post-

natal paucity of regulatory T cells and control of NK cell activation in experimental biliary atresia. *J Hepatol*. 2010;52:718–26.
59. Jeong W-I, Park O, Suh Y-G, Byun J-S, Park S-Y, Choi E, et al. Suppression of innate immunity (natural killer cell/interferon-γ) in the advanced stages of liver fibrosis in mice. *Hepatology*. 2011;53(4):1342–51.
60. Wang J, Sun R, Wei H, Dong Z, Gao B, Tian Z. Poly I:C prevents T cell-mediated hepatitis via an NK-dependent mechanism. *J Hepatol*. 2006;44:446–54.
61. Zhang Z, Zhang S, Zou Z, Shi J, Zhao J, Fan R, et al. Hypercytolytic activity of hepatic natural killer cells correlates with liver injury in chronic hepatitis B patients. *Hepatology*. 2011 Jan;53(1):73–85.
62. Peppa D, Gill US, Reynolds G, Easom NJW, Pallett LJ, Schurich A, et al. Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell–mediated deletion. *J Exp Med*. 2013;210(1):99–114.
63. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science*. 1989;244(4902):359–62.
64. Lavanchy D. The global burden of hepatitis C. *Liver Int*. 2009;29 Suppl 1:74–81.
65. Hoofnagle JH. Course and outcome of hepatitis C. *Hepatology*. 2002;36(S1):S21–9.
66. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature*. 2009;461(7265):798–801.
67. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna K V, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature*. 2009;461(7262):399–401.
68. Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, Dickensheets H, et al. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nat Genet*. 2013;45(2):164–71.
69. Hydes TJ, Moesker B, Traherne JA, Ashraf S, Alexander GJ, Dimitrov BD, et al. The interaction of genetic determinants in the outcome of HCV infection: evidence for discrete immunological pathways. *Tissue Antigens*. 2015;86(4):267–75.
70. Romero V, Azocar J, Zúñiga J, Clavijo OP, Terreros D, Gu X, et al. Interaction of NK inhibitory receptor genes with HLA-C and MHC class II alleles in Hepatitis C virus infection outcome. *Mol Immunol*. 2008;45(9):2429–36.
71. Vidal-Castiñeira JR, López-Vázquez A, Díaz-Peña R, Alonso-Arias R, Martínez-Borra J, Pérez R, et al. Effect of killer immunoglobulin-like receptors in the response to combined treatment in patients with chronic hepatitis C virus infection. *J Virol*. 2010;84(1):475–81.
72. Dring MM, Morrison MH, McSharry BP, Guinan KJ, Hagan R, Hcv I, et al. Innate immune genes synergize to predict increased risk of chronic disease in hepatitis C virus infection. *Proc Natl Acad Sci U S A*. 2011;108(14):5736–41.
73. Rivero-Juarez A, Gonzalez R, Camacho A, Manzanares-Martin B, Caruz A, Martinez-Peinado A, et al. Natural killer KIR3DS1 is closely associated with HCV viral clearance and sustained virological response in HIV/HCV patients. *PLoS One*. 2013;8(4):e61992.
74. Nozawa Y, Umemura T, Joshita S, Katsuyama Y, Shibata S, Kimura T, et al. KIR, HLA, and IL28B variant predict response to antiviral therapy in genotype 1 chronic hepatitis C patients in Japan. *PLoS One*. 2013;8(12):e83381.
75. Umemura T, Ota M, Katsuyama Y, Wada S, Mori H, Maruyama A, et al. KIR3DL1-HLA-Bw4 combination and IL28B polymorphism predict response to Peg-IFN and ribavirin with and without telaprevir in chronic hepatitis C. *Hum Immunol*. 2014;75(8):822–6.
76. Vidal-Castiñeira JR, López-Vázquez A, Martínez-Borra J, Martínez-Camblor P, Prieto J, López-Rodríguez R, et al. Diversity of Killer Cell Immunoglobulin-Like Receptor (KIR) Genotypes and KIR2DL2/3 Variants in HCV Treatment Outcome. *PLoS One*.

2014;9(6):e99426.
77. Askar M, Avery R, Corey R, Lopez R, Thomas D, Pidwell D, et al. Lack of killer immunoglobulin-like receptor 2DS2 (KIR2DS2) and KIR2DL2 is associated with poor responses to therapy of recurrent hepatitis C virus in liver transplant recipients. *Liver Transpl*. 2009;15(11):1557–63.
78. De Maria A, Fogli M, Mazza S, Basso M, Picciotto A, Costa P, et al. Increased natural cytotoxicity receptor expression and relevant IL-10 production in NK cells from chronically infected viremic HCV patients. *Eur J Immunol*. 2007;37(2):445–55.
79. Ahlenstiel G, Titerence RH, Koh C, Edlich B, Feld JJ, Rotman Y, et al. Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon-alfa-dependent manner. *Gastroenterology*. 2010;138(1):325-35-2.
80. Edlich B, Ahlenstiel G, Zabaleta Azpiroz A, Stoltzfus J, Noureddin M, Serti E, et al. Early changes in interferon signaling define natural killer cell response and refractoriness to interferon-based therapy of hepatitis C patients. *Hepatology*. 2012;55(1):39–48.
81. Miyagi T, Takehara T, Nishio K, Shimizu S, Kohga K, Li W, et al. Altered interferon-alpha-signaling in natural killer cells from patients with chronic hepatitis C virus infection. *J Hepatol*. 2010;53(3):424–30.
82. Gonzalez VD, Falconer K, Michaëlsson J, Moll M, Reichard O, Alaeus A, et al. Expansion of CD56- NK cells in chronic HCV/HIV-1 co-infection: reversion by antiviral treatment with pegylated IFNalpha and ribavirin. *Clin Immunol*. 2008;128(1):46–56.
83. Gonzalez VD, Falconer K, Björkström NK, Blom KG, Weiland O, Ljunggren H-G, et al. Expansion of functionally skewed CD56-negative NK cells in chronic hepatitis C virus infection: correlation with outcome of pegylated IFN-alpha and ribavirin treatment. *J Immunol*. 2009;183(10):6612–8.
84. Mavilio D, Lombardo G, Benjamin J, Kim D, Follman D, Marcenaro E, et al. Characterization of CD56-/CD16+ natural killer (NK) cells: A highly dysfunctional NK subset expanded in HIV-infected viremic individuals. *Proc Natl Acad Sci*. 2005;102(8):2886–91.
85. Bonorino P, Ramzan M, Camous X, Dufeu-Duchesne T, Thélu M-A, Sturm N, et al. Fine characterization of intrahepatic NK cells expressing natural killer receptors in chronic hepatitis B and C. *J Hepatol*. 2009;51(3):458–67.
86. Pembroke T, Christian A, Jones E, Hills RK, Wang ECY, Gallimore AM, et al. The paradox of NKp46+ natural killer cells: drivers of severe hepatitis C virus-induced pathology but in-vivo resistance to interferon α treatment. *Gut*. 2013;1–10.
87. Varchetta S, Mele D, Mantovani S, Oliviero B, Cremonesi E, Ludovisi S, et al. Impaired intrahepatic natural killer cell cytotoxic function in chronic hepatitis C virus infection. *Hepatology*. 2012;56(3):841–9.
88. Krämer B, Körner C, Kebschull M, Glässner A, Eisenhardt M, Nischalke H-D, et al. Natural killer p46High expression defines a natural killer cell subset that is potentially involved in control of hepatitis C virus replication and modulation of liver fibrosis. *Hepatology*. 2012;56(4):1201–13.
89. Yamagiwa S, Matsuda Y, Ichida T, Honda Y, Takamura M, Sugahara S, et al. Sustained response to interferon-alpha plus ribavirin therapy for chronic hepatitis C is closely associated with increased dynamism of intrahepatic natural killer and natural killer T cells. *Hepatol Res*. 2008;38(7):664–72.
90. Boisvert J, Kunkel EJ, Campbell JJ, Keeffe EB, Butcher EC, Greenberg HB. Liver-infiltrating lymphocytes in end-stage hepatitis C virus: subsets, activation status, and chemokine receptor phenotypes. *J Hepatol*. 2003;38(1):67–75.
91. Corado J, Toro F, Rivera H, Bianco NE, Deibis L, De Sanctis JB. Impairment of natural killer (NK) cytotoxic activity in hepatitis C virus (HCV) infection. *Clin Exp Immunol*. 1997;109(3):451–7.
92. Kawarabayashi N, Seki S, Hatsuse K, Ohkawa T, Koike Y, Aihara T, et al. Decrease of CD56(+)T cells and natural killer cells in cirrhotic livers with hepatitis C may be involved in their susceptibility to hepatocellular carcinoma. *Hepatology*.

2000;32(5):962–9.
93. Nattermann J, Feldmann G, Ahlenstiel G, Langhans B, Sauerbruch T, Spengler U. Surface expression and cytolytic function of natural killer cell receptors is altered in chronic hepatitis C. *Gut*. 2006;55(6):869–77.
94. Sène D, Levasseur F, Abel M, Lambert M, Camous X, Hernandez C, et al. Hepatitis C virus (HCV) evades NKG2D-dependent NK cell responses through NS5A-mediated imbalance of inflammatory cytokines. *PLoS Pathog*. 2010;6(11):e1001184.
95. Meier U-C, Owen RE, Taylor E, Worth A, Naoumov N, Willberg C, et al. Shared alterations in NK cell frequency, phenotype, and function in chronic human immunodeficiency virus and hepatitis C virus infections. *J Virol*. 2005;79(19):12365–74.
96. Morishima C, Paschal DM, Wang CC, Yoshihara CS, Wood BL, Yeo AET, et al. Decreased NK cell frequency in chronic hepatitis C does not affect ex vivo cytolytic killing. *Hepatology*. 2006;43(3):573–80.
97. Golden-Mason L, Madrigal-Estebas L, McGrath E, Conroy MJ, Ryan EJ, Hegarty JE, et al. Altered natural killer cell subset distributions in resolved and persistent hepatitis C virus infection following single source exposure. *Gut*. 2008;57(8):1121–8.
98. Oliviero B, Varchetta S, Paudice E, Michelone G, Zaramella M, Mavilio D, et al. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. *Gastroenterology*. 2009;137(3):1151–60, 1160-7.
99. Dessouki O, Kamiya Y, Nagahama H, Tanaka M, Suzu S, Sasaki Y, et al. Chronic hepatitis C viral infection reduces NK cell frequency and suppresses cytokine secretion: Reversion by anti-viral treatment. *Biochem Biophys Res Commun*. 2010;393(2):331–7.
100. Bozzano F, Picciotto A, Costa P, Marras F, Fazio V, Hirsch I, et al. Activating NK cell receptor expression/function (NKp30, NKp46, DNAM-1) during chronic viraemic HCV infection is associated with the outcome of combined treatment. *Eur J Immunol*. 2011;41(10):2905–14.
101. Yoon JC, Lim J-B, Park JH, Lee JM. Cell-to-cell contact with hepatitis C virus-infected cells reduces functional capacity of natural killer cells. *J Virol*. 2011;85(23):12557–69.
102. Maini MK, Peppa D. NK Cells: A Double-Edged Sword in Chronic Hepatitis B Virus Infection. *Front Immunol*. 2013;4:57.
103. Dunn C, Brunetto M, Reynolds G, Christophides T, Kennedy PT, Lampertico P, et al. Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell–mediated liver damage. *J Exp Med*. 2007;204(3):667–80.
104. Jeong W-I, Park O, Radaeva S, Gao B. STAT1 inhibits liver fibrosis in mice by inhibiting stellate cell proliferation and stimulating NK cell cytotoxicity. *Hepatology*. 2006;44(6):1441–51.
105. Kamimura H, Yamagiwa S, Tsuchiya A, Takamura M, Matsuda Y, Ohkoshi S, et al. Reduced NKG2D ligand expression in hepatocellular carcinoma correlates with early recurrence. *J Hepatol*. 2012;56(2):381–8.
106. Peppa D, Micco L, Javaid A, Kennedy PTF, Schurich A, Dunn C, et al. Blockade of Immunosuppressive Cytokines Restores NK Cell Antiviral Function in Chronic Hepatitis B Virus Infection. *PLoS Pathog*. 2010;6(12):e1001227.
107. Dunn C, Peppa D, Khanna P, Nebbia G, Jones M, Brendish N, et al. Temporal analysis of early immune responses in patients with acute hepatitis B virus infection. *Gastroenterology*. 2009;137(4):1289–300.
108. Gao X, Jiao Y, Wang L, Liu X, Sun W, Cui B, et al. Inhibitory KIR and specific HLA-C gene combinations confer susceptibility to or protection against chronic hepatitis B. *Clin Immunol*. 2010;137(1):139–46.
109. Lu Z, Zhang B, Chen S, Gai Z, Feng Z, Liu X, et al. Association of KIR genotypes and haplotypes with susceptibility to chronic hepatitis B virus infection in Chinese Han population. *Cell Mol Immunol*. 2008;5(6):457–63.
110. Cancer Research UK - Cancer Statistics 2014.
111. Guo C-L, Yang H-C, Yang X-H, Cheng W, Dong T-X, Zhu W-J, et al. Associations

between infiltrating lymphocyte subsets and hepatocellular carcinoma. *Asian Pac J Cancer Prev*. 2012;13(11):5909–13.
112. Fathy A, Eldin MM, Metwally L, Eida M, Abdel-Rehim M. Diminished absolute counts of CD56dim and CD56bright natural killer cells in peripheral blood from Egyptian patients with hepatocellular carcinoma. *Egypt J Immunol*. 2009;16(2):17–25.
113. Cai L, Zhang Z, Zhou L, Wang H, Fu J, Zhang S, et al. Functional impairment in circulating and intrahepatic NK cells and relative mechanism in hepatocellular carcinoma patients. *Clin Immunol*. 2008;129(3):428–37.
114. Chew V, Chen J, Lee D, Loh E, Lee J, Lim KH, et al. Chemokine-driven lymphocyte infiltration: an early intratumoural event determining long-term survival in resectable hepatocellular carcinoma. *Gut*. 2012;61(3):427–38.
115. Hoechst B, Voigtlaender T, Ormandy L, Gamrekelashvili J, Zhao F, Wedemeyer H, et al. Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor. *Hepatology*. 2009;50(3):799–807.
116. Jinushi M, Takehara T, Tatsumi T, Hiramatsu N, Sakamori R, Yamaguchi S, et al. Impairment of natural killer cell and dendritic cell functions by the soluble form of MHC class I-related chain A in advanced human hepatocellular carcinomas. *J Hepatol*. 2005;43(6):1013–20.
117. Chuang WL, Liu HW, Chang WY. Natural killer cell activity in patients with hepatocellular carcinoma relative to early development and tumor invasion. *Cancer*. 1990;65(4):926–30.
118. Kumar V, Kato N, Urabe Y, Takahashi A, Muroyama R, Hosono N, et al. Genome-wide association study identifies a susceptibility locus for HCV-induced hepatocellular carcinoma. *Nat Genet*. 2011;43(5):455–8.
119. Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, Xiong N, et al. NKG2D-Deficient Mice Are Defective in Tumor Surveillance in Models of Spontaneous Malignancy. *Immunity*. 2008;28(4):571–80.
120. Stegmann KA, Björkström NK, Veber H, Ciesek S, Riese P, Wiegand J, et al. Interferon-alpha-induced TRAIL on natural killer cells is associated with control of hepatitis C virus infection. *Gastroenterology*. 2010;138(5):1885–97.
121. Sun C, Sun H, Xiao W, Zhang C, Tian Z. Natural killer cell dysfunction in hepatocellular carcinoma and NK cell-based immunotherapy. *Acta Pharmacol Sin*. 2015;36(10):1191–9.
122. Laso FJ, Almeida J, Torres E, Vaquero JM, Marcos M, Orfao A. Chronic alcohol consumption is associated with an increased cytotoxic profile of circulating lymphocytes that may be related with the development of liver injury. *Alcohol Clin Exp Res*. 2010;34(5):876–85.
123. O'Connell J, Lynch L, Cawood TJ, Kwasnik A, Nolan N, Geoghegan J, et al. The Relationship of Omental and Subcutaneous Adipocyte Size to Metabolic Disease in Severe Obesity. *PLoS One*. 2010;5(4):e9997.
124. O'Shea D, Cawood TJ, O'Farrelly C, Lynch L. Natural Killer Cells in Obesity: Impaired Function and Increased Susceptibility to the Effects of Cigarette Smoke. *PLoS One*. 2010;5(1):e8660.
125. Kahraman A, Schlattjan M, Kocabayoglu P, Yildiz-Meziletoglu S, Schlensak M, Fingas CD, et al. Major histocompatibility complex class I-related chains A and B (MIC A/B): a novel role in nonalcoholic steatohepatitis. *Hepatology*. 2010;51(1):92–102.
126. Schleinitz N, Vély F, Harlé J-R, Vivier E. Natural killer cells in human autoimmune diseases. *Immunology*. 2010;131(4):451–8.
127. Jie H-B, Sarvetnick N. The role of NK cells and NK cell receptors in autoimmune disease. *Autoimmunity*. 2004;37(2):147–53.
128. Oo YH, Hubscher SG, Adams DH. Autoimmune hepatitis: new paradigms in the pathogenesis, diagnosis, and management. *Hepatol Int*. 2010;4(2):475–93.
129. Kaneda K, Kurioka N, Seki S, Wake K, Yamamoto S. Pit cell-hepatocyte contact in autoimmune hepatitis. *Hepatology*. 1984;4(5):955–8.
130. Chen Y, Wei H, Gao B, Hu Z, Zheng S, Tian Z. Activation and function of hepatic NK

cells in hepatitis B infection: an underinvestigated innate immune response. *J Viral Hepat*. 2005;12(1):38–45.
131. Prince M, Chetwynd A, Newman W, Metcalf J V., James OFW. Survival and symptom progression in a geographically based cohort of patients with primary biliary cirrhosis: Follow-up for up to 28 years. *Gastroenterology*. 2002;123(4):1044–51.
132. EASL Clinical Practice Guidelines: Management of cholestatic liver diseases. *J Hepatol*. 2009;51(2):237–67.
133. Corpechot C, Abenavoli L, Rabahi N, Chrétien Y, Andréani T, Johanet C, et al. Biochemical response to ursodeoxycholic acid and long-term prognosis in primary biliary cirrhosis. *Hepatology*. 2008;48(3):871–7.
134. Kaplan MM, Gershwin ME. Primary biliary cirrhosis. *N Engl J Med*. 2005;353(12):1261–73.
135. Hirschfield GM, Liu X, Xu C, Lu Y, Xie G, Lu Y, et al. Primary Biliary Cirrhosis Associated with HLA, IL12A, and IL12RB2 Variants. *N Engl J Med*. 2009;360(24):2544–55.
136. Hirschfield GM, Liu X, Han Y, Gorlov IP, Lu Y, Xu C, et al. Variants at IRF5-TNPO3, 17q12-21 and MMEL1 are associated with primary biliary cirrhosis. *Nat Genet*. 2010;42(8):655–7.
137. Liu X, Invernizzi P, Lu YY, Kosoy R, Lu YY, Bianchi I, et al. Genome-wide meta-analyses identify three loci associated with primary biliary cirrhosis. *Nat Genet*. 2010;42(8):658–60.
138. Mells GF, Floyd JAB, Morley KI, Cordell HJ, Franklin CS, Shin S-Y, et al. Genome-wide association study identifies 12 new susceptibility loci for primary biliary cirrhosis. *Nat Genet*. 2011;43(4):329–32.
139. Nakamura M, Nishida N, Kawashima M, Aiba Y, Tanaka A, Yasunami M, et al. Genome-wide association study identifies TNFSF15 and POU2AF1 as susceptibility loci for primary biliary cirrhosis in the Japanese population. *Am J Hum Genet*. 2012 Oct;91(4):721–8.
140. Liu JZ, Almarri MA, Gaffney DJ, Mells GF, Jostins L, Cordell HJ, et al. Dense fine-mapping study identifies new susceptibility loci for primary biliary cirrhosis. *Nat Genet*. 2012;44(10):1137–41.
141. Joshita S, Umemura T, Nakamura M, Katsuyama Y, Shibata S, Kimura T, et al. STAT4 gene polymorphisms are associated with susceptibility and ANA status in primary biliary cirrhosis. *Dis Markers*. 2014;2014(7):727393.
142. Chuang Y-H, Lian Z-X, Tsuneyama K, Chiang B-L, Ansari AA, Coppel RL, et al. Increased killing activity and decreased cytokine production in NK cells in patients with primary biliary cirrhosis. *J Autoimmun*. 2006;26(4):232–40.
143. Shimoda S, Harada K, Niiro H, Yoshizumi T, Soejima Y, Taketomi A, et al. Biliary epithelial cells and primary biliary cirrhosis: the role of liver-infiltrating mononuclear cells. *Hepatology*. 2008;47(3):958–65.
144. Shimoda S, Hisamoto S, Harada K, Iwasaka S, Chong Y, Nakamura M, et al. Natural killer cells regulate T cell immune responses in primary biliary cirrhosis. *Hepatology*. 2015;62(6):1817–27.
145. Shimoda S, Harada K, Niiro H, Shirabe K, Taketomi A, Maehara Y, et al. Interaction between Toll-like receptors and natural killer cells in the destruction of bile ducts in primary biliary cirrhosis. *Hepatology*. 2011;53(4):1270–81.
146. Gao B, Bertola A. Natural killer cells take two tolls to destruct bile ducts. *Hepatology*. 2011 Apr;53(4):1076–9.
147. Liang Y, Yang Z, Li C, Zhu Y, Zhang L, Zhong R. Characterisation of TNF-related apoptosis-inducing ligand in peripheral blood in patients with primary biliary cirrhosis. *Clin Exp Med*. 2008;8(1):1–7.
148. Takeda K, Kojima Y, Ikejima K, Harada K, Yamashina S, Okumura K, et al. Death receptor 5 mediated-apoptosis contributes to cholestatic liver disease. *Proc Natl Acad Sci*. 2008;105(31):10895–900.
149. Parker R, Weston CJ, Adams DH. CXCR6 and CXCL16 in liver disease. *Lancet*.

2013;381:S83.
150. Hov J-R, Boberg K-M, Karlsen T-H. Autoantibodies in primary sclerosing cholangitis. *World J Gastroenterol*. 2008;14(24):3781–91.
151. Saarinen S, Olerup O, Broome U. Increased frequency of autoimmune diseases in patients with primary sclerosing cholangitis. *Am J Gastroenterol*. 2000;95(11):3195–9.
152. Silvain C, Zeevi A, Saidman S, Duquesnoy RJ, Van Thiel DH. Phenotypic and functional characteristics of colonic lymphocytes isolated from patients with primary sclerosing cholangitis and inflammatory bowel disease. *Hepatogastroenterology*. 1995;42(3):250–8.
153. Chuang Y-H, Lian Z-X, Cheng C-M, Lan RY, Yang G-X, Moritoki Y, et al. Increased levels of chemokine receptor CXCR3 and chemokines IP-10 and MIG in patients with primary biliary cirrhosis and their first degree relatives. *J Autoimmun*. 2005;25(2):126–32.
154. Isse K, Harada K, Zen Y, Kamihira T, Shimoda S, Harada M, et al. Fractalkine and CX3CR1 are involved in the recruitment of intraepithelial lymphocytes of intrahepatic bile ducts. *Hepatology*. 2005;41(3):506–16.
155. Steinle A, Li P, Morris DL, Groh V, Lanier LL, Strong RK, et al. Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family. *Immunogenetics*. 53(4):279–87.
156. Van Steenbergen W, De Goede E, Emonds M-P, Reinders J, Tilanus M, Fevery J. Primary sclerosing cholangitis in two brothers: report of a family with special emphasis on molecular HLA and MICA genotyping. *Eur J Gastroenterol Hepatol*. 2005;17(7):767–71.
157. Caligiuri MA. Human natural killer cell development. 2006;214:56–72.
158. Chan A, Hong D-L, Atzberger A, Kollnberger S, Filer AD, Buckley CD, et al. CD56bright human NK cells differentiate into CD56dim cells: role of contact with peripheral fibroblasts. *J Immunol*. 2007;179(1):89–94.
159. Golden-Mason L, Curry MP, Nolan N, Traynor O, McEntee G, Kelly J, et al. Differential expression of lymphoid and myeloid markers on differentiating hematopoietic stem cells in normal and tumor-bearing adult human liver. *Hepatology*. 2000;31(6):1251–6.
160. Vanderkerken K, Bouwens L, De Neve W, Van den Berg K, Baekeland M, Delens N, et al. Origin and differentiation of hepatic natural killer cells (pit cells). *Hepatology*. 1993;18(4):919–25.
161. Leong JW, Wagner JA, Ireland AR, Fehniger TA. Transcriptional and post-transcriptional regulation of NK cell development and function. *Clin Immunol*. 2016;
162. Boos MD, Yokota Y, Eberl G, Kee BL. Mature natural killer cell and lymphoid tissue–inducing cell development requires Id2-mediated suppression of E protein activity. *J Exp Med*. 2007;204(5):1119–30.
163. Gordon SMM, Chaix J, Rupp LJJ, Wu J, Madera S, Sun JCC, et al. The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity*. 2012;36(1):55–67.
164. Cichocki F, Miller JS, Anderson SK, Bryceson YT. Epigenetic regulation of NK cell differentiation and effector functions. *Front Immunol*. 2013;4:55.
165. Jiang X, Chen Y, Peng H, Tian Z. Single line or parallel lines: NK cell differentiation driven by T-bet and Eomes. *Cell Mol Immunol*. 2012;9(3):193–4.
166. Stegmann KA, Robertson F, Pallant C, Christophides T, Fusai G, Davidson BR, et al. CXCR6 marks a novel subset of human liver NK cells with a T-bet(lo)Eomes(hi) transcriptional profile. 4th European Congress of Immunology. 2015.
167. Lugthart G, Melsen JE, Vervat C, van Ostaijen-ten Dam MM, Corver WE, Roelen DL, et al. Human Lymphoid Tissues Harbor a Distinct CD69 + CXCR6 + NK Cell Population. *J Immunol*. 2016;197(1):78–84.
168. Hoffmann JA. Phylogenetic Perspectives in Innate Immunity. *Science (80-)*. 1999;284(5418):1313–8.
169. Rölle A, Pollmann J, Cerwenka A. Memory of infections: an emerging role for natural

killer cells. *PLoS Pathog*. 2013;9(9):e1003548.
170. O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat Immunol*. 2006;7(5):507–16.
171. Vermijlen D, Seynaeve C, Luo D, Kruhøffer M, Eizirik DL, Ørntoft TF, et al. High-density oligonucleotide array analysis reveals extensive differences between freshly isolated blood and hepatic natural killer cells. *Eur J Immunol*. 2004;34(9):2529–40.
172. Majewska-Szczepanik M, Paust S, von Andrian UH, Askenase PW, Szczepanik M. Natural killer cell-mediated contact sensitivity develops rapidly and depends on interferon-α, interferon-γ and interleukin-12. *Immunology*. 2013;140(1):98–110.
173. Ray SJ, Franki SN, Pierce RH, Dimitrova S, Koteliansky V, Sprague AG, et al. The collagen binding alpha1beta1 integrin VLA-1 regulates CD8 T cell-mediated immune protection against heterologous influenza infection. *Immunity*. 2004;20(2):167–79.
174. Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. *Nature*. 2009;457(7229):557–61.
175. Min-Oo G, Lanier LL. Cytomegalovirus generates long-lived antigen-specific NK cells with diminished bystander activation to heterologous infection. *J Exp Med*. 2014;211(13):2669–80.
176. Sun JC, Madera S, Bezman NA, Beilke JN, Kaplan MH, Lanier LL. Proinflammatory cytokine signaling required for the generation of natural killer cell memory. *J Exp Med*. 2012;209(5):947–54.
177. Nabekura T, Kanaya M, Shibuya A, Fu G, Gascoigne NRJ, Lanier LL. Costimulatory molecule DNAM-1 is essential for optimal differentiation of memory natural killer cells during mouse cytomegalovirus infection. *Immunity*. 2014;40(2):225–34.
178. O'Sullivan TE, Johnson LR, Kang HH, Sun JC. BNIP3- and BNIP3L-Mediated Mitophagy Promotes the Generation of Natural Killer Cell Memory. *Immunity*. 2015;43(2):331–42.
179. Gillard GO, Bivas-Benita M, Hovav A-H, Grandpre LE, Panas MW, Seaman MS, et al. Thy1+ NK [corrected] cells from vaccinia virus-primed mice confer protection against vaccinia virus challenge in the absence of adaptive lymphocytes. *PLoS Pathog*. 2011;7(8):e1002141.
180. Reeves RK, Li HH, Jost S, Blass E, Li HH, Schafer JL, et al. Antigen-specific NK cell memory in rhesus macaques. *Nat Immunol*. 2015;16(9):927–32.
181. Hudspeth K, Pontarini E, Tentorio P, Cimino M, Donadon M, Torzilli G, et al. The role of natural killer cells in autoimmune liver disease: a comprehensive review. *J Autoimmun*. 2013;46:55–65.
182. Cooper MA, Elliott JM, Keyel PA, Yang L, Carrero JA, Yokoyama WM. Cytokine-induced memory-like natural killer cells. *Proc Natl Acad Sci U S A*. 2009;106(6):1915–9.
183. Romee R, Schneider SE, Leong JW, Chase JM, Keppel CR, Sullivan RP, et al. Cytokine activation induces human memory-like NK cells. *Blood*. 2012;120(24):4751–60.
184. Leong JW, Chase JM, Romee R, Schneider SE, Sullivan RP, Cooper MA, et al. Preactivation with IL-12, IL-15, and IL-18 Induces CD25 and a Functional High-Affinity IL-2 Receptor on Human Cytokine-Induced Memory-like Natural Killer Cells. *Biol Blood Marrow Transplant*. 2014;20(4):463–73.
185. Romee R, Rosario M, Berrien-Elliott MM, Wagner JA, Jewell BA, Schappe T, et al. Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Sci Transl Med*. 2016;8(357).
186. Gumá M, Angulo A, Vilches C, Gómez-Lozano N, Malats N, López-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood*. 2004;104(12):3664–71.
187. Gumá M, Cabrera C, Erkizia I, Bofill M, Clotet B, Ruiz L, et al. Human Cytomegalovirus Infection Is Associated with Increased Proportions of NK Cells That Express the CD94/NKG2C Receptor in Aviremic HIV-1–Positive Patients. *J Infect Dis*. 2006;194(1):38–41.

188. Petitdemange C, Becquart P, Wauquier N, Béziat V, Debré P, Leroy EM, et al. Unconventional repertoire profile is imprinted during acute chikungunya infection for natural killer cells polarization toward cytotoxicity. *PLoS Pathog*. 2011;7(9):e1002268.
189. Bjorkstrom NK, Lindgren T, Stoltz M, Fauriat C, Braun M, Evander M, et al. Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. *J Exp Med*. 2010;208(1):13–21.
190. Béziat V, Liu LL, Malmberg J-A, Ivarsson MA, Sohlberg E, Björklund AT, et al. NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. *Blood*. 2013;121(14):2678–88.
191. Braud VM, Allan DS, O'Callaghan CA, Söderström K, D'Andrea A, Ogg GS, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature*. 1998;391(6669):795–9.
192. Thomas R, Low HZ, Kniesch K, Jacobs R, Schmidt RE, Witte T. NKG2C deletion is a risk factor of HIV infection. *AIDS Res Hum Retroviruses*. 2012;28(8):844–51.
193. Nattermann J, Nischalke HD, Hofmeister V, Kupfer B, Ahlenstiel G, Feldmann G, et al. HIV-1 infection leads to increased HLA-E expression resulting in impaired function of natural killer cells. *Antivir Ther*. 2005;10(1):95–107.
194. Prod'homme V, Tomasec P, Cunningham C, Lemberg MK, Stanton RJ, McSharry BP, et al. Human cytomegalovirus UL40 signal peptide regulates cell surface expression of the NK cell ligands HLA-E and gpUL18. *J Immunol*. 2012;188(6):2794–804.
195. Muntasell A, López-Montañés M, Vera A, Heredia G, Romo N, Peñafiel J, et al. NKG2C zygosity influences CD94/NKG2C receptor function and the NK-cell compartment redistribution in response to human cytomegalovirus. *Eur J Immunol*. 2013;43(12):3268–78.
196. Hwang I, Zhang T, Scott JM, Kim AR, Lee T, Kakarla T, et al. Identification of human NK cells that are deficient for signaling adaptor FcRγ and specialized for antibody-dependent immune functions. *Int Immunol*. 2012;24(12):793–802.
197. Zhang T, Scott JM, Hwang I, Kim S. Cutting edge: antibody-dependent memory-like NK cells distinguished by FcRγ deficiency. *J Immunol*. 2013;190(4):1402–6.
198. Lee J, Zhang T, Hwang I, Kim A, Nitschke L, Kim M, et al. Epigenetic Modification and Antibody-Dependent Expansion of Memory-like NK Cells in Human Cytomegalovirus-Infected Individuals. *Immunity*. 2015;42(3):431–42.
199. Petersen L, Petersen CC, Møller-Larsen A, Hokland ME. Short-term exposure to human cytomegalovirus-infected fibroblasts induces a proportional increase of active CD94/NKG2A(+) natural killer cells. *Hum Immunol*. 2010;71(1):29–35.
200. Wu Z, Sinzger C, Frascaroli G, Reichel J, Bayer C, Wang L, et al. Human Cytomegalovirus-Induced NKG2Chi CD57hi Natural Killer Cells Are Effectors Dependent on Humoral Antiviral Immunity. *J Virol*. 2013;87(13):7717–25.
201. Paust S, Senman B, von Andrian UH. Adaptive immune responses mediated by natural killer cells. *Immunol Rev*. 2010;235(1):286–96.
202. Monsiváis-Urenda A, Noyola-Cherpitel D, Hernández-Salinas A, García-Sepúlveda C, Romo N, Baranda L, et al. Influence of human cytomegalovirus infection on the NK cell receptor repertoire in children. *Eur J Immunol*. 2010;40(5):1418–27.
203. Noyola DE, Fortuny C, Muntasell A, Noguera-Julian A, Muñoz-Almagro C, Alarcón A, et al. Influence of congenital human cytomegalovirus infection and the NKG2C genotype on NK-cell subset distribution in children. *Eur J Immunol*. 2012;42(12):3256–66.
204. Lopez-Vergès S, Milush JM, Schwartz BS, Pando MJ, Jarjoura J, York VA, et al. Expansion of a unique CD57^{+}NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proc Natl Acad Sci U S A*. 2011;108(36):14725–32.
205. Foley B, Cooley S, Verneris MR, Pitt M, Curtsinger J, Luo X, et al. Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function. *Blood*. 2012;119(11):2665–74.
206. Mela CM, Goodier MR. The contribution of cytomegalovirus to changes in NK cell receptor expression in HIV-1-infected individuals. *J Infect Dis*. 2007;195(1):158-9-60.

207. Brunetta E, Fogli M, Varchetta S, Bozzo L, Hudspeth KL, Marcenaro E, et al. Chronic HIV-1 viremia reverses NKG2A/NKG2C ratio on natural killer cells in patients with human cytomegalovirus co-infection. *AIDS*. 2010;24(1):27–34.
208. Béziat V, Dalgard O, Asselah T, Halfon P, Bedossa P, Boudifa A, et al. CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. *Eur J Immunol*. 2012;42(2):447–57.
209. Saghafian-Hedengren S, Sohlberg E, Theorell J, Carvalho-Queiroz C, Nagy N, Persson J-O, et al. Epstein-Barr Virus Coinfection in Children Boosts Cytomegalovirus-Induced Differentiation of Natural Killer Cells. *J Virol*. 2013;87(24):13446–55.
210. Della Chiesa M, Falco M, Bertaina A, Muccio L, Alicata C, Frassoni F, et al. Human cytomegalovirus infection promotes rapid maturation of NK cells expressing activating killer Ig-like receptor in patients transplanted with NKG2C-/- umbilical cord blood. *J Immunol*. 2014;192(4):1471–9.
211. D. Frucht, M. Aringer, J.Galon, C. Danning, M. Brown, S. Fan, M. Centola JO. STAT4 is expressed in activated peripheral blood monocytes, dendritic cells, and macrophages at sites of Th1-mediated inflammation. *J Immology*. 2000;164:4659–64.
212. Yamamoto K, Quelle FW, Thierfelder WE, Kreider BL, Gilbert DJ, Jenkins NA, et al. Stat4, a novel gamma interferon activation site-binding protein expressed in early myeloid differentiation. *Mol Cell Biol*. 1994;14(7):4342–9.
213. Kaplan MH. STAT4: A Critical Regulator of Inflammation In Vivo. *Immunol Res*. 2005;31(3):231–42.
214. Wang KS, Ritz J, Frank DA. IL-2 induces STAT4 activation in primary NK cells and NK cell lines, but not in T cells. *J Immunol*. 1999;162(1):299–304.
215. Watford WT, Hissong BD, Bream JH, Kanno Y, Muul L, O'Shea JJ. Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. *Immunol Rev*. 2004;202(1):139–56.
216. Thierfelder WE, van Deursen JM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, et al. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature*. 1996;382(6587):171–4.
217. Sarasin-Filipowicz M, Oakeley EJ, Duong FHT, Christen V, Terracciano L, Filipowicz W, et al. Interferon signaling and treatment outcome in chronic hepatitis C. *Proc Natl Acad Sci U S A*. 2008;105(19):7034–9.
218. Wubetu GY, Utsunomiya T, Ishikawa D, Yamada S, Ikemoto T, Morine Y, et al. High STAT4 expression is a better prognostic indicator in patients with hepatocellular carcinoma after hepatectomy. *Ann Surg Oncol*. 2014;21 Suppl 4:S721-8.
219. Yoshida K, Yang G-X, Zhang W, Tsuda M, Tsuneyama K, Moritoki Y, et al. Deletion of interleukin-12p40 suppresses autoimmune cholangitis in dominant negative transforming growth factor beta receptor type II mice. *Hepatology*. 2009;50(5):1494–500.
220. Elaine F. Remmers, Robert M. Plenge et al. STAT4 and the Risk of Rheumatoid Arthritis and Systemic Lupus Erythematosus. *N Engl J Med*. 2007;357(10):977–86.
221. Migita, M. Nakamura, S. Abiru, Y. Jiuchi KN. Association of STAT4 Polymorphisms with Susceptibility to Type-1 Autoimmune Hepatitis in the Japanese Population. *PLoS One*. 2013;8(8):e71382.
222. Finnegan A, Grusby MJ, Kaplan CD, O'Neill SK, Eibel H, Koreny T, et al. IL-4 and IL-12 regulate proteoglycan-induced arthritis through Stat-dependent mechanisms. *J Immunol*. 2002;169(6):3345–52.
223. Cargill M, Schrodi SJ, Chang M, Garcia VE, Brandon R, Callis KP, et al. A Large-Scale Genetic Association Study Confirms IL12B and Leads to the Identification of IL23R as Psoriasis-Risk Genes. *Am J Hum Genet*. 2007;80(2):273–90.
224. Tsunemi Y, Saeki H, Nakamura K, Sekiya T, Hirai K, Fujita H, et al. Interleukin-12 p40 gene (IL12B) 3'-untranslated region polymorphism is associated with susceptibility to atopic dermatitis and psoriasis vulgaris. *J Dermatol Sci*. 2002;30(2):161–6.
225. Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, et al. A genome-

wide association study identifies IL23R as an inflammatory bowel disease gene. *Science*. NI; 2006;314(5804):1461–3.
226. Zingoni A, Ardolino M, Santoni A, Cerboni C. NKG2D and DNAM-1 activating receptors and their ligands in NK-T cell interactions: role in the NK cell-mediated negative regulation of T cell responses. *Front Immunol*. 2012;3:408.
227. Roy S, Barnes PF, Garg A, Wu S, Cosman D, Vankayalapati R. NK cells lyse T regulatory cells that expand in response to an intracellular pathogen. *J Immunol*. 2008;180(3):1729–36.
228. Ottaviani C, Nasorri F, Bedini C, de Pità O, Girolomoni G, Cavani A. CD56brightCD16- NK cells accumulate in psoriatic skin in response to CXCL10 and CCL5 and exacerbate skin inflammation. *Eur J Immunol*. 2006;36(1):118–28.
229. Wolk K, Witte E, Warszawska K, Schulze-Tanzil G, Witte K, Philipp S, et al. The Th17 cytokine IL-22 induces IL-20 production in keratinocytes: A novel immunological cascade with potential relevance in psoriasis. *Eur J Immunol*. 2009;39(12):3570–81.
230. Batista MD, Ho EL, Kuebler PJ, Milush JM, Lanier LL, Kallas EG, et al. Skewed distribution of natural killer cells in psoriasis skin lesions. *Exp Dermatol*. 2013;22(1):64–6.
231. Dalbeth N, Callan MFC. A subset of natural killer cells is greatly expanded within inflamed joints. *Arthritis Rheum*. 2002;46(7):1763–72.
232. Ren J, Feng Z, Lv Z, Chen X, Li J. Natural Killer-22 Cells in the Synovial Fluid of Patients with Rheumatoid Arthritis Are an Innate Source of Interleukin 22 and Tumor Necrosis Factor-. *J Rheumatol*. 2011;38(10):2112–8.
233. Soderstrom K, Stein E, Colmenero P, Purath U, Muller-Ladner U, Teixeira de Matos C, et al. Natural killer cells trigger osteoclastogenesis and bone destruction in arthritis. *Proc Natl Acad Sci*. 2010;107(29):13028–33.
234. Nedvetzki S, Sowinski S, Eagle RA, Harris J, Vely F, Pende D, et al. Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses. *Blood*. 2007;109(9):3776–85.
235. Rabinovich BA, Li J, Shannon J, Hurren R, Chalupny J, Cosman D, et al. Activated, but not resting, T cells can be recognized and killed by syngeneic NK cells. *J Immunol*. 2003;170(7):3572–6.
236. Kanai T, Mikami Y, Sujino T, Hisamatsu T, Hibi T. RORγt-dependent IL-17A-producing cells in the pathogenesis of intestinal inflammation. *Mucosal Immunol*. 2012;5(3):240–7.
237. Spits H, Di Santo JP. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat Immunol*. 2011;12(1):21–7.
238. Benczur M, Petrányl GG, Pálffy G, Varga M, Tálas M, Kotsy B, et al. Dysfunction of natural killer cells in multiple sclerosis: a possible pathogenetic factor. *Clin Exp Immunol*. 1980;39(3):657–62.
239. Kastrukoff LF, Lau A, Wee R, Zecchini D, White R, Paty DW. Clinical relapses of multiple sclerosis are associated with “novel” valleys in natural killer cell functional activity. *J Neuroimmunol*. 2003;145(1–2):103–14.
240. Chanvillard C, Jacolik RF, Infante-Duarte C, Nayak RC. The role of natural killer cells in multiple sclerosis and their therapeutic implications. *Front Immunol*. 2013;4:63.
241. Perini P, Wadhwa M, Buttarello M, Meager A, Facchinetti A, Thorpe R, et al. Effect of IFNbeta and anti-IFNbeta antibodies on NK cells in multiple sclerosis patients. *J Neuroimmunol*. 2000;105(1):91–5.
242. Putzki N, Baranwal MK, Tettenborn B, Limmroth V, Kreuzfelder E. Effects of Natalizumab on Circulating B Cells, T Regulatory Cells and Natural Killer Cells. *Eur Neurol*. 2010;63(5):311–7.
243. Bielekova B, Catalfamo M, Reichert-Scrivner S, Packer A, Cerna M, Waldmann TA, et al. Regulatory CD56bright natural killer cells mediate immunomodulatory effects of IL-2R -targeted therapy (daclizumab) in multiple sclerosis. *Proc Natl Acad Sci*. 2006;103(15):5941–6.
244. Anolik JH, Campbell D, Felgar RE, Young F, Sanz I, Rosenblatt J, et al. The

relationship of Fc?RIIIa genotype to degree of B cell depletion by rituximab in the treatment of systemic lupus erythematosus. *Arthritis Rheum*. 2003;48(2):455–9.
245. Hu Y, Turner MJ, Shields J, Gale MS, Hutto E, Roberts BL, et al. Investigation of the mechanism of action of alemtuzumab in a human CD52 transgenic mouse model. *Immunology*. 2009;128(2):260–70.
246. Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biol*. 2010;11(10):R106.
247. Durinck S, Spellman PT, Birney E, Huber W. Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. *Nat Protoc*. 2009;4(8):1184–91.
248. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics*. 2008;9(1):559.
249. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550.
250. Curry MP, Norris S, Golden-Mason L, Doherty DG, Deignan T, Collins C, et al. Isolation of lymphocytes from normal adult human liver suitable for phenotypic and functional characterisation. *J Immunol Methods*. 2000;242(1):21–31.
251. Vauthey JN1, Pawlik TM, Ribero D, Wu TT, Zorzi D, Hoff PM, Xiong HQ, Eng C, Lauwers GY, Mino-Kenudson M, Risio M, Muratore A, Capussotti L, Curley SA AE. Chemotherapy regimen predicts steatohepatitis and an increase in 90-day mortality after surgery for hepatic colorectal metastases. *J Clin Oncol*. 2006;24(13):2065–72.
252. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells--a proposal for uniform nomenclature. *Nat Rev Immunol*. 2013;13(2):145–9.
253. Angelo LS, Banerjee PP, Monaco-Shawver L, Rosen JB, Makedonas G, Forbes LR, et al. Practical NK cell phenotyping and variability in healthy adults. *Immunol Res*. 2015;62(3):341–56.
254. Mackay LK, Minnich M, Kragten NAM, Liao Y, Nota B, Seillet C, et al. Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. *Science (80-)*. 2016;352(6284):459–63.
255. Mackay LK, Rahimpour A, Ma JZ, Collins N, Stock AT, Hafon M-L, et al. The developmental pathway for CD103+CD8+ tissue-resident memory T cells of skin. *Nat Immunol*. 2013;14(12):1294–301.
256. Yeang HXA, Lin Y, Malkova ON, Yang L, Piersma SJ, Miner C, et al. Human liver contains two populations of NK cells based on CD49e expression. *J Immunol*. 2016;196(1 Supplement):194.7-194.7.
257. Fu B, Wang F, Sun R, Ling B, Tian Z, Wei H. CD11b and CD27 reflect distinct population and functional specialization in human natural killer cells. *Immunology*. 2011;133(3):350–9.
258. Vossen MTM, Matmati M, Hertoghs KML, Baars PA, Gent M-R, Leclercq G, et al. CD27 defines phenotypically and functionally different human NK cell subsets. *J Immunol*. 2008;180(6):3739–45.
259. Maurer M, von Stebut E. Macrophage inflammatory protein-1. *Int J Biochem Cell Biol*. 2004;36(10):1882–6.
260. Rölle A, Halenius A, Ewen E-M, Cerwenka A, Hengel H, Momburg F. CD2-CD58 interactions are pivotal for the activation and function of adaptive natural killer cells in human cytomegalovirus infection. *Eur J Immunol*. 2016;46(10):2420–5.
261. Metcalfe SM. Axotrophin and leukaemia inhibitory factor (LIF) in transplantation tolerance. *Philos Trans R Soc B Biol Sci*. 2005;360(1461):1687–94.
262. Salleh N, Giribabu N. Leukemia inhibitory factor: roles in embryo implantation and in nonhormonal contraception. *ScientificWorldJournal*. 2014;2014:201514.
263. Lazarevic V, Glimcher LH, Lord GM. T-bet: a bridge between innate and adaptive immunity. *Nat Rev Immunol*. 2013;13(11):777–89.
264. Berrien-Elliott MM, Wagner JA, Fehniger TA. Human Cytokine-Induced Memory-Like Natural Killer Cells. *J Innate Immun*. 2015;7(6):563–71.

265. Corpechot C, Chazouillères O, Poupon R. Early primary biliary cirrhosis: Biochemical response to treatment and prediction of long-term outcome. *J Hepatol*. 2011;55(6):1361–7.
266. Carbone M, Mells GF, Pells G, Dawwas MF, Newton JL, Heneghan MA, et al. Sex and Age Are Determinants of the Clinical Phenotype of Primary Biliary Cirrhosis and Response to Ursodeoxycholic Acid. *Gastroenterology*. 2013 Mar;144(3):560–569.e7.
267. Qian C, Jiang T, Zhang W, Ren C, Wang Q, Qin Q, et al. Increased IL-23 and IL-17 expression by peripheral blood cells of patients with primary biliary cirrhosis. *Cytokine*. 2013;64(1):172–80.
268. Krams SM, Cao S, Hayashi M, Villanueva JC, Martinez OM. Elevations in IFN-γ, IL-5, and IL-10 in Patients with the Autoimmune Disease Primary Biliary Cirrhosis: Association with Autoantibodies and Soluble CD30. *Clin Immunol Immunopathol*. 1996;80(3):311–20.
269. Harada K, Shimoda S, Sato Y, Isse K, Ikeda H, Nakanuma Y. Periductal interleukin-17 production in association with biliary innate immunity contributes to the pathogenesis of cholangiopathy in primary biliary cirrhosis. *Clin Exp Immunol*. 2009;157(2):261–70.
270. Parkhurst MR, Riley JP, Dudley ME, Rosenberg SA. Adoptive Transfer of Autologous Natural Killer Cells Leads to High Levels of Circulating Natural Killer Cells but Does Not Mediate Tumor Regression. *Clin Cancer Res*. 2011;17(19):6287–97.
271. Hirschfield GM, Gershwin ME, Strauss R, Mayo MJ, Levy C, Zou B, et al. Ustekinumab for patients with primary biliary cholangitis who have an inadequate response to ursodeoxycholic acid: A proof-of-concept study. *Hepatology*. 2016;64(1):189–99.
272. Yang Z, Chen M, Fialkow LB, Ellett JD, Wu R, Nadler JL. Inhibition of STAT4 Activation by Lisofylline Is Associated with the Protection of Autoimmune Diabetes. *Ann N Y Acad Sci*. 2003;1005(1):409–11.
273. Bogdanos D-P, Baum H, Grasso A, Okamoto M, Butler P, Ma Y, et al. Microbial mimics are major targets of crossreactivity with human pyruvate dehydrogenase in primary biliary cirrhosis. *J Hepatol*. 2004;40(1):31–9.
274. Katsumi T, Tomita K, Leung PSC, Yang G, Gershwin ME, Ueno Y. Animal Models of Primary Biliary Cirrhosis. *Clin Rev Allergy Immunol*. 2015;48(2–3):142–53.