Role of the inhibitory receptor LAIR-1 on NK cells in Chronic Hepatitis B

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Statement of originality

I, Navjyot Kaur Hansi, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

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Details of collaboration and publications

Publications:

IL-2^{high} tissue-resident T cells in the human liver: Sentinels for hepatotropic infection.
Pallett LJ, Davies J, Colbeck EJ, Robertson F, ***Hansi N***, Easom NJW, Burton AR, Stegmann KA, Schurich A, Swadling L, Gill US, Male V, Luong T, Gander A, Davidson BR, Kennedy PT, Maini MK.
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Abstract and Poster Presentations:

Transcriptome profiles of NK and T cells regulating immune control in chronic hepatitis B, proximate to patients treated with Pegylated Interferon alpha-Nucleos(t)ide analogue (NA) sequential therapy compared to de novo NA therapy.

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Abstract

There are multiple immune mechanisms identified for persistence of hepatitis B virus (HBV) infection. This thesis considers the vital role that inhibitory receptors play in contributing to impairment of the adaptive immune system in chronic hepatitis B (CHB), and the potential role they play in the innate immune system, focusing on the inhibitory receptor leucocyte-associated immunoglobulin-like receptor (LAIR)-1. The unique aspect of this work is that for the first time LAIR-1 expression has been investigated on natural killer (NK) cells in CHB.

Our striking findings of increased LAIR-1 expression on peripheral NK cells in CHB and an inverse correlation between expression and effector function suggest this inhibitory receptor could have a potential role in exhaustion of NK cells in CHB. We therefore additionally explored the expression of LAIR-1 on circulating NK cells from patients with hepatocellular carcinoma (HCC) and non-alcoholic fatty liver disease (NAFLD).

The particular relevance of LAIR-1 to liver disease is that one of its major ligands is collagen. We demonstrated a downregulation of LAIR-1 expression on intrahepatic NK cells, which we postulate might occur following repetitive engagement with abundant collagen within the liver. In line with this, intrahepatic NK cells with a liver-resident (CXCR6+) phenotype had even lower LAIR-1 expression than liver infiltrating (non-resident, CXCR6-) NK cells. Furthermore, preliminary experiments display attenuation of the cytotoxic degranulation capacity (CD107a) by circulating NK cells from CHB patients upon exposure to plate-bound collagen.

We demonstrate differential expression of LAIR-1 on NK cells in viral hepatitis, HCC and NAFLD and between peripheral and intrahepatic NK cells. Preliminary experiments demonstrate a role in inhibiting NK cell function suggesting this as a novel therapeutic target to harness the capacity of NK cells to control chronic infection and cancer.

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I was a virgin lab member, having never picked up a pipette prior to starting this MD. But I had wonderful Uppy to guide me through, and despite my crankiness about expanding my panels your clever idea for LAIR-1 propelled this project further. Your friendship is golden.

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Finally I was not a natural scientist but I have learnt many new and exciting skills to transfer back to clinical medicine, with a better appreciation of research (perhaps more cynicism) and how challenging it is working at the bench. When an experiment works it is a wonderful exhilarating feeling and I will miss those science highs!

Unnamed person: 'Tell me, exactly why is it so hard to finish a thesis, isn't it just like writing a long paper'

Me: 😑

List of abbreviations

2B4	NK cell surface receptor (CD244)
αSMA	alpha smooth muscle actin
aGVHD	Acute graft versus host disease
AFP	Alpha fetoprotein (tumour marker)
ALT	Alanine transaminase
AML	Acute myeloid leukaemia
APC	Antigen presenting cells
APRI	Aspartate platelet ratio index
BCR	B cell receptor
C4	Complement 4
C5	Complement 5
C1q/C1r/C1s	Components of complement
CCR6	Chemokine (C-C motif) receptor 6
cccDNA	Covalently closed circular DNA
CD	Cluster of differentiation
CD107a	Cluster of differentiation 107a (marker of NK cell function)
CD4+	Helper T cells
CD8+	Effector T cells
CHB	Chronic hepatitis B virus
CLL	Chronic lymphocytic leukaemia
CMV	Cytomegalovirus
CPA	Collagen proportionate area
Csk	c-terminal Src kinase
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTNNB1	gene for beta-catenin
CXCR6	C-X-C- motif chemokine receptor 6
DAA	Direct-acting antiviral
DAMPs	Damage associated molecular patterns
DC	Dendritic cells
DDR	Discoid domain receptor (class of transmembrane receptors that binds to collagen)

DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
DX26	mAb against LAIR-1
EASL	European Association for the Study of the Liver
EBV	Epstein-Barr virus
caEBV	chronic active EBV
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
ECM	Extracellular matrix
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FCAR	Fc receptor for IgA
FMO	Fluorescence minus one
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPO	Glycine-proline-hydroxyproline
GPVI	Glycoprotein VI (class of transmembrane receptors that binds to collagen)
HBV	Hepatitis B virus
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
Anti-HBe	antibody to Hepatitis B e antigen
HBx	Hepatitis B encoded X protein
HBsAg	Hepatitis B surface antigen
HBsAb	Hepatitis B surface antibody
HC	healthy control
HCC	Hepatocellular cancer
HCV	Hepatitis C virus
H&E	Haematoxylin and eosin
HepG2	Human liver cancer cell line
HFRS	Haemorrhagic fever with renal syndrome
HIV	Human immunodeficiency virus
HLA	Human Leucocyte Antigen
HLA-DR	Human Leukocyte Antigen - antigen D Related
HSC	Hepatic stellate cell
HSCs	Hepatic stellate cells
HSV-2	Herpes simplex virus-2
IFNα	Interferon-alpha
IFNγ	Interferon-gamma

Ig	Immunoglobulin
IHL	Intrahepatic lymphocytes
IL	Interleukin
ILT	Immunoglobulin-like transcript
iNKT	Invariant natural killer T
ISG	Interferon-stimulated genes
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAK	Janus kinase
K562	human cell line
KIR	Killer immunoglobulin receptor
KLRG-1	Killer cell lectin-like receptor subfamily G, member 1
KC	Kupffer cells
LAG-3	Lymphocyte activation gene-3
sLAG-3	soluble LAG-3
LAIR-1	Leucocyte associated immunoglobulin-like receptor-1
LAIR-2	Leucocyte associated immunoglobulin-like receptor-2
sLAIR-1	soluble LAIR-1
Anti-hLAIR-1 F(ab')2	Blocking antibody to collagen-LAIR-1 interaction
LAMP-1	Lysosome-associated membrane protein-1
LCMV	Lymphocytic choriomeningitis virus
LIR	Leukocyte immunoglobulin-like receptor
LPS	Lipopolysaccharide
lrNK	Liver resident NK
LSEC	Liver sinusoidal endothelial cells
LSECtin	Liver and lymph node sinusoidal endothelial cell C-type lectin
mAb	Monoclonal antibody
µg	Microgram
µl	Microlitre
MAIT	Mucosal-associated invariant T
MAPK	Mitogen activated protein kinase
MCMV	Mouse cytomegalovirus
MDSC	Myeloid-derived suppressor cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIC-A/B	MHC class 1 polypeptide-related sequence A/B
MMP	Matrix metalloproteinases

MRI	Magnetic resonance imaging
ng	nanogram
NAFLD	Non alcoholic fatty liver disease
NASH	Non alcoholic steatohepatitis
NCAM	Neural cell adhesion molecule
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKT	Natural killer T
NKG2A	Natural-killer group 2, member A
NKG2C	Natural-killer group 2, member C
NKG2D	Natural-killer group 2, member D
NKp30	Natural killer cell p30-related protein
NKp44	Natural killer cell p44-related protein
NKp46	Natural killer cell p46-related protein
NMR	Nuclear magnetic resonance
NTCP	Sodium taurocholate cotransporting polypeptide
NUCs	Nucleos(t)ide analogues
ORF	Open reading frame
P3NP	Pro-collagen type III N-terminal peptide
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PCR	Polymerase chain reaction
PD-1	Programmed death – 1
PD-L1/2	Ligands for Programmed death – 1 receptor
PDGF	Platelet derived growth factor
pgRNA	Pre-genomic RNA
PEG-IFNα	Pegylated Interferon-alpha
pHSC	Primary hepatic stellate cells
PI3K	Phosphoinositide 3-kinase
Pol	Viral polymerase
PRR	Pattern recognition receptor
P40	mAb later recognized as LAIR-1
rcDNA	Relaxed circular DNA
rhIL	Recombinant human interleukin
RAE-1	Retinoic acid early inducible gene-1 (ligand for NKG2D)

RAG	Recombinant associate gene proteins
RNA	Ribonucleic acid
ROC curve	Receiver operating characteristic curve
ROS	Reactive oxygen species
RT PCR	Real time polymerase chain reaction
shRNA	short hairpin RNA
SHP-1/2	Src homology 2 domain-containing phosphatase-1/2
siRNA	silencer RNA
SLE	Systemic lupus erythematous
SNP	Single nucleotide polymorphism
SP-D	Surfactant protein-D
TB	Tuberculosis
TCR	T-cell receptor
TGFβ	Transforming growth factor beta
TIM-3	T cell immunoglobulin mucin-3
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TP53	Tumour suppressor gene
TRAIL	TNF-related apoptosis inducing ligand
TRAIL-R2	TNF-related apoptosis inducing ligand receptor 2
Treg	Natural regulatory T cells
TTFT	Time to first treatment
ULBP	Unique long 16 binding protein
WHO	World health organization
WHV	Woodchuck hepatitis virus

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Chapter 1: Introduction

Our knowledge of Hepatitis B immunology and virology has advanced over the last 50 years since discovery of the antigen first isolated in the serum of an Australian Aboriginal (Blumberg et al., 1965). The 'Australia antigen' is now termed 'surface antigen' and Baruch Blumberg won a Nobel Prize in 1976 for his outstanding efforts in uncovering the hallmark of Hepatitis B virus (HBV).

HBV can cause both acute and chronic infection, for which there is no definitive curative treatment available. A vaccine was introduced in the 1990s, however there is still a considerable burden of chronic HBV infection affecting more than 240 million people worldwide (Ott et al., 2012). The Global Burden of Disease Study highlights the worrying rise in mortality from all viral hepatitides in the last decade compared to other communicable diseases (Stanaway et al., 2016). The highest areas of prevalence include sub-Saharan Africa and East Asia, but due to migration patterns HBV can be branded a global concern. Up to a third of patients with persistent infection will develop cirrhosis and/or hepatocellular carcinoma (HCC). Approximately 780,000 people die each year as a consequence of these complications (Liaw and Chu, 2009), (Kanwal et al., 2015). HCC is the fifth commonest malignancy worldwide, associated with high mortality, and approximately 50% of these patients have HBV (Kim et al., 2016).

The wider implications of these complications include costly long-term secondary care monitoring, including regular surveillance scans, and in some, liver transplantation. Current therapies are sub-optimal and in the majority, long-term viral suppression is the mainstay of management. The goal of current pipeline therapies is functional cure i.e. sustained Hepatitis B surface antigen (HBsAg) loss with undetectable HBV DNA, and whether a 'sterilising cure' is achievable in chronic hepatitis B (CHB) is a subject of debate. The definition of 'sterilising cure' is complete eradication of the virus from the host, which means undetectable HBsAg with eradication of HBV DNA including cccDNA within hepatocytes and integrated HBV DNA. Drugs specifically targeting cccDNA are in

development in experimental models, as well as development of assays to reliably measure intrahepatic cccDNA, however the feasibility of sterilising cure may not be practicable due to the presence of integrated HBV DNA (Lok et al., 2017). Currently, the two existing options employed in the clinic today are pegylated-interferon alpha (PEG-IFNα) or direct-acting antiviral (DAA) therapy. However, the exciting development of DAA therapies in hepatitis C virus (HCV) that offer a cure in more than 95% of chronically infected hepatitis C patients, tantalises clinicians and scientists of further advances within grasp for the CHB field.

1.1 Innate and adaptive immunity are compromised in HBV

Hepatitis B is a hepatotropic, and largely non-cytopathic virus, and liver damage occurs from ongoing immune-mediated damage. Hepatitis B can be cytopathic in some rare circumstances such as fibrosing cholestatic hepatitis, which is a rapidly progressing liver injury that has been reported in liver transplant patients with recurrent hepatitis B.

In CHB both arms of innate and adaptive immunity are impaired (Ferrari, 2015). It has been observed that altered phenotype and function of adaptive and innate immunity contribute to the persistence of viral infection. The immune status of the host is fundamental to viral replication and clearance; in the immune-compromised setting there may be reactivation of the virus. Age of acquisition is critical; young patients with HBV infection are much more likely to develop chronic infection. Age as a host factor is important in other viral infections, for example Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Measles, Mumps, Rubella etc. but most of these viruses do not develop chronicity and will resolve without tissue damage. Although HBV infection is transmitted via blood and body fluids, in highly endemic areas the main routes of transmission are vertical, perinatal or horizontal transmission; exposure from an infected child to an uninfected child during the first 5 years of life. In infants and children 90% will develop chronic infection if infected during their first year of life; conversely HBV acquired in adulthood will progress to chronic infection in less than 5%. Increased susceptibility at a young age is thought to be due to a less mature and less responsive immune system.

There are multiple immune mechanisms identified for persistence of viral infection. This introductory chapter reviews the literature on the vital role inhibitory receptors play in contributing to impairment of the adaptive immune system in CHB, and the potential role they may play in the innate immune system, finally focusing on the inhibitory receptor leucocyte-associated immunoglobulin-like receptor (LAIR)-1.

1.2 HBV Virology

Prior to introducing the immunology of HBV it is fundamental to understand the virology. HBV is a double stranded DNA virus contained within a nucleocaspid formed by core protein (HBcAg) and viral polymerase (pol), surrounded by a lipid envelope composed of hepatitis B surface antigen (HBsAg). Virus particles are commonly referred to as "Dane particles". Original discovery of HBsAg, which facilitates the diagnosis of hepatitis B, was by Baruch Blumberg (1925-2011) in 1963, and later contributions by David Dane (1923-1998) and colleagues identified the infectious virions (Figure 1.1).

Organisation of the genome is compactly packed to contain four open reading frames (ORFs) that overlap and encode viral proteins each with specific roles:

(i) ***'S' ORF*** encodes the envelope protein HBsAg. There are three sizes of HBsAg proteins (small (S), medium (M) and large (L)). The largest, contains a domain, pre-S1, which has an important role in the HBV life cycle. HBsAg is historically described as the hallmark of CHB infection and used as a routine screening marker for the diagnosis of HBV infection. In recent years HBsAg assays have significantly improved so that levels can now be quantified and used to differentiate those considered at risk for disease progression or development of HCC (Chen et al., 2006) and for the assessment of on-treatment response with PEG-IFNα therapy (Martinot-Peignoux et al., 2014). It has been suggested that persistence of HBsAg at high titres tolerises the immune response in infected individuals, with the highest levels seen in childhood, which decrease over time (Winther et al., 2013).

(ii) ***'C' ORF*** encodes the sequences for two proteins with separate functions: HBcAg and hepatitis B e antigen (HBeAg). During chronic HBV infection there is often loss of HBeAg synthesis, but HBeAg-negative variants/mutations may also occur, that are associated with increased viral activity and liver disease (Ganem and Prince, 2004). Whereas HBeAg can be readily detected in the serum,

HBcAg is located in the nucleus and therefore can only be measured quantitatively by immunohistochemical staining. HBcAg is a major component of the nucleocaspid containing the genome. Mason et al studied HBcAg in liver tissue in addition to HBV-DNA integration and clonal hepatocyte expansion, finding interesting differences in the percentage of nuclear HBcAg-positive hepatocytes in different disease phases of CHB (Mason et al., 2016). Although the biological significance of this is unclear, the authors proposed that their findings indicated, in line with other studies, that early phases are not disease free and perhaps merit treatment consideration at an earlier stage of the disease.

(iii) ***'P' ORF*** encodes polymerase protein, which is necessary to complete the cccDNA template.

(iv) ***'X' ORF*** encodes HBV-encoded X protein (HBx) essential for viral replication. It is the smallest of the ORFs. There is an increasing body of evidence pointing to the multifunctional role of HBx in cellular activities and importantly noted to be associated with the pathogenesis of viral HCC (Ng and Lee, 2011). HBx is involved in interfering with innate immunity by down regulating signalling pathways; cytoplasmic signalling and protein kinase C pathways, with a role in cell cycle regulation and repair (Dandri and Petersen, 2016).

The host can generate antibodies to HBsAg, HBeAg and HBcAg, which help delineate which clinical phase a patient is in. This is described in more depth in chapter 2.1.2.

Eight genotypes (A-H) have been identified, each specific to geographic areas. The genotypes are important as they can be utilised as a predictor of response to therapy (Janssen et al., 2005) and identify subgroups of patients who are at higher risk of HCC such as genotype C (Chan et al., 2004).

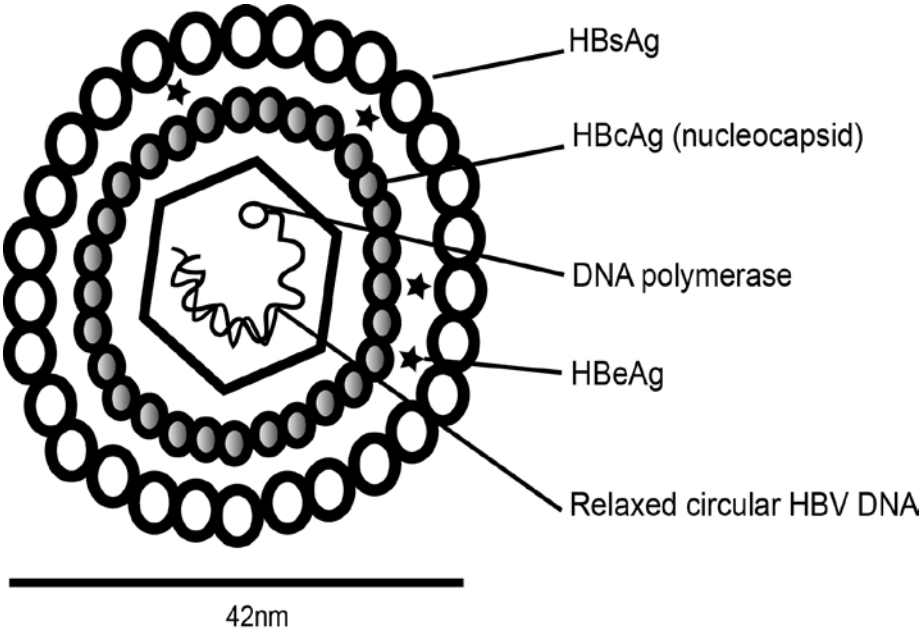


Figure 1.1: Hepatitis B virion

The virus particles are approximately 42nm in diameter and infect hepatocytes. The structure is made up of an outer envelope called hepatitis B surface antigen (HBsAg) and the nucleocaspid core (HBcAg) that contains the viral DNA and DNA polymerase. HBeAg is the secretory form of the hepatitis B core protein.

Hepatocytes are infected with pools of covalently closed circular (ccc) DNA (Cheng et al., 2011). There are a number of steps to the HBV life cycle and each step involves the host machinery. The discovery of the receptor permitting viral entry in to hepatocytes was only made recently in 2012. Hepatitis B virus attaches to hepatocyte-associated heparan sulfate proteoglycans to initiate entry. Yan et al (2012) discovered the functional receptor to be sodium-taurocholate cotransporting polypeptide (NTCP), a bile acid transporter, and elegantly demonstrated binding of the virion to the NTCP via the pre-S1 segment of the HBV envelope protein (Yan et al., 2012).

Simplistically, following viral entry, the HBV virion is uncoated and incorporated in to the nucleus where it is repaired by viral pol protein to form cccDNA enabling a template for transcription. The viral mechanism is sophisticated, enabling the virus to create a highly conserved copy but also cunningly concealing the assembly of cccDNA within the nucleus evading normal host immune defence mechanisms. cccDNA forms a highly stable structure that can lie dormant for years. HBV transcripts are exported in to the cytoplasm to undergo translation, producing a number of proteins and virions that are secreted from the cell in to the bloodstream. Novel therapies are targeted at the individual steps of the life cycle; however, theoretically boosting the immune system would be a useful adjunct. Current treatments for CHB comprise of antiviral agents directly inhibiting DNA polymerase, thereby preventing replication and release of virus particles, in addition to PEG-IFNα, which stimulates the host immune response.

HBV is able to persist via mechanisms that fail to induce and overwhelm the host immune response; in line with this many patients are asymptomatic and don't develop liver disease for many years. Chimpanzees (Wieland et al., 2004b) and human studies (Dunn et al., 2009) have demonstrated why it is often nicknamed a 'stealth virus', observing in early infection minimal activation of the innate response and of interferon-stimulated genes (ISG). The ongoing production of viral antigens has been

reported to induce T cell anergy and exhaustion. Clinically this has been observed in chronically infected patients who persistently have high titres of surface antigen.

1.3 Liver Immunology

The liver is the largest solid organ in the body containing a large number of innate and adaptive immune cells. One of its major functions is involvement in host defence. Despite the continual influx of antigens from the gastrointestinal tract (80% of its blood flow is via the portal vein) it maintains a balance between tolerance to harmless antigens and the means to eliminate pathogens and toxins (Jenne and Kubes, 2013).

Hepatocytes are arranged in a honeycomb pattern woven between hepatic sinusoids, surrounded by immune cells and held together by a collagen structure. A hepatic sinusoid is a type of blood vessel with open fenestrae where blood from hepatic artery and portal vein can mix. The sluggish blood flow allows exposure time to gut derived antigens and clearance of toxins by liver-resident cells, as well as circulating lymphocytes to traverse the hepatic sinusoidal endothelium. The hepatocytes being the major functional cell type of the liver account for two-thirds of the total parenchymal cell population, which are separated from the sinusoids by the space of Disse, where important subsets of immune cells such as liver sinusoidal endothelial cells (LSECs), kupffer cells, hepatic stellate cells (HSCs) and biliary cells reside (Crispe, 2016) (Figure 1.2). HBV replication takes place within hepatocytes, and they are always infected with virus due to their longevity (half-life approximately 6-12 months) as well as the continuous shedding of virions by infected host cells.

Clearance of HBV is a coordinated response between innate, adaptive, humoral and cellular immune responses, however, the difficulty has been determining which is the most favourable to target as a therapeutic strategy.

The introductory section provides an overview of our current understanding of impaired adaptive and innate immunity in CHB, particularly focusing on the role of inhibitory receptors.

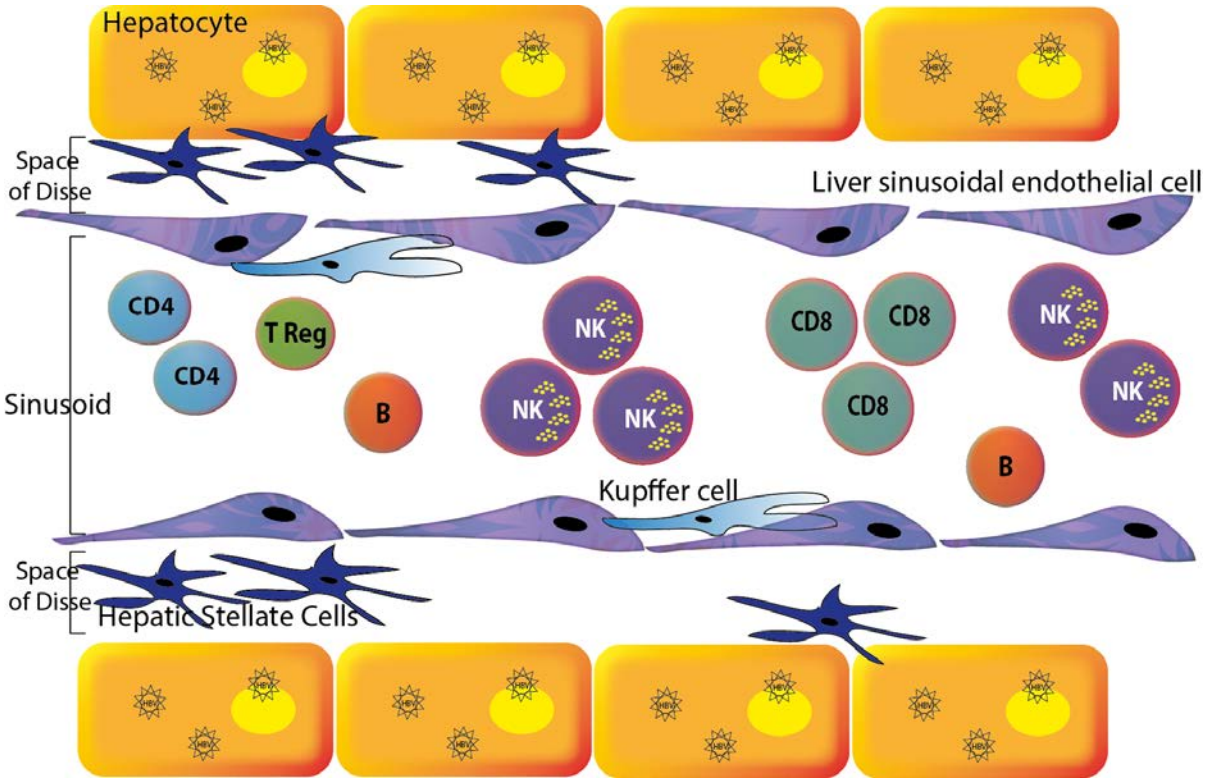


Figure 1.2: Immune cells of the liver

Schematic depicts the microanatomy of the sinusoidal space. Hepatocytes are the host of HBV replication and the major functional cell type of the liver. The space of Disse separates hepatocytes from the sinusoids, which are enriched with lymphocytes. Within the space of Disse important subsets of immune cells such as liver sinusoidal endothelial cells, kupffer cells and hepatic stellate cells reside.

1.4 Adaptive Immunity and HBV

The adaptive immune system is an interplay of various immune cells; lymphocytes such as CD4+ and CD8+ T cells and antibody producing cells like B cells. It is important to point out that research within this field has been challenging because the only host of HBV is humans. Chimpanzees are also susceptible and can be infected with the virus, but most research has been yielded from animal models such as mouse, woodchuck, and duck in addition to cell culture systems.

Due to the limited techniques in B cell analysis we have the most limited knowledge on this subset of immune cells in HBV infected patients, however, there is evidence to suggest that B cells are important in the clearance of HBV and prevention of reinfection (Bertoletti and Ferrari, 2016). Hepatitis B surface antibody (HBsAb) in serum is produced in response to a vaccine or natural infection. Patients with lymphoma and previous exposure to hepatitis B can experience HBV reactivation when treated with Rituximab, a monoclonal antibody (mAb) against CD20 that causes B cell depletion. Furthermore, the importance of B cells can be illustrated in chronic HBV infected patients receiving liver transplantation whereby the donor HBV immunity can clear remaining virus and protect the graft from re-infection (Shouval, 2007)

We have a much greater depth of knowledge of T cell responses in HBV and research to date has emphasised the pivotal role of an effective CD8+ T cell response in controlling and eradicating viral infection (Maini et al., 2000), (Guidotti et al., 1999). Individuals with acute, self-limited HBV infection will mount a T cell response to epitopes of viral proteins, however, patients with CHB exhibit a weak or near undetectable HBV-specific response. Maini et al (2000) were able to show the absolute number of T cells did not impact the control of viral replication but the functionality of HBV-specific T cells was paramount. An elegant study by Thimme et al in chimpanzees examining the relationship between T cell-mediated viral clearance in acute HBV and disease pathogenesis

showed depletion of CD8+ T cells at the peak of infection, altered duration and outcome (Thimme et al., 2003). In the absence of CD8+ T cells, high HBV titres persisted for a longer time span, and viral clearance was prolonged. It was also noted that in infected chimps (Wieland et al., 2004a) and HBV transgenic mice (Guidotti et al., 1995) clearance is mediated by interferon-gamma (IFNγ). Further studies went on to illustrate that in chronic viral infection there is inefficiency of CD8+ effector T cells, resulting in a hierarchal loss of function such as IFNγ production, which was further confirmed as a mechanism also specific to CHB. One of the features noted of these ineffective CD8+ T cells was the overexpression of inhibitory receptors (see Figure 1.3). This panel of receptors has included Programmed death (PD)-1, Cytotoxic T-lymphocyte-associated antigen (CTLA)-4, T cell immunoglobulin- and mucin-domain-containing-molecule (TIM)-3, 2B4, CD160, Killer cell lectin-like receptor subfamily G member (KLRG)-1 and Lymphocyte activation gene (LAG)-3 (Nguyen and Ohashi, 2015) (see Figure 1.4).

The focus has therefore been on how to restore the function of exhausted T cells enabling them to mount an efficient antiviral response. This exhausted phenotype is important, as interestingly even therapeutic vaccines have had limited success in eliminating CHB due to their failure to limit the proliferation of exhausted T cells (Wherry et al., 2005).

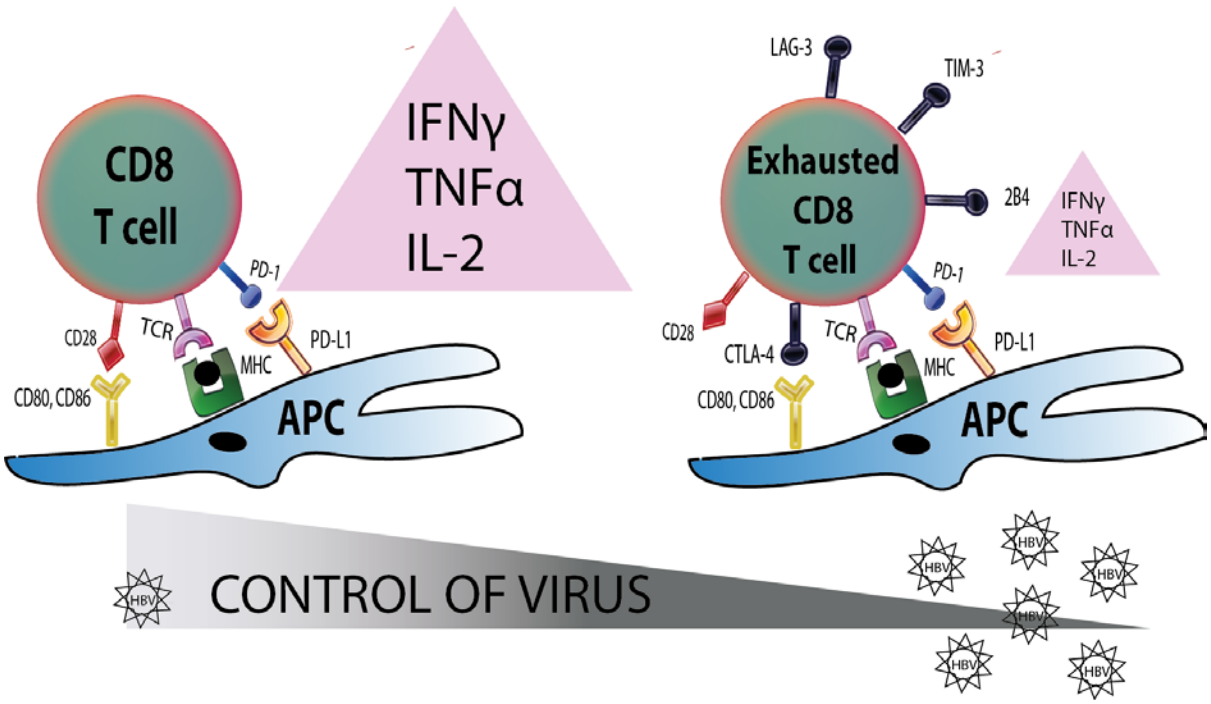


Figure 1.3: Phenotype and function of exhausted T cells in CHB

CD8+ T cells produce effector molecules (left panel). However, in chronic infection, in the presence of persistent viral antigen they can become exhausted. Upregulated signalling through an array of inhibitory receptors such as PD-1, CTLA-4, LAG-3 and TIM-3 has been associated with hierarchical loss of T cell function and inefficient viral control (right panel). CTLA-4 can compete with co-stimulatory molecule CD28 for ligand binding.

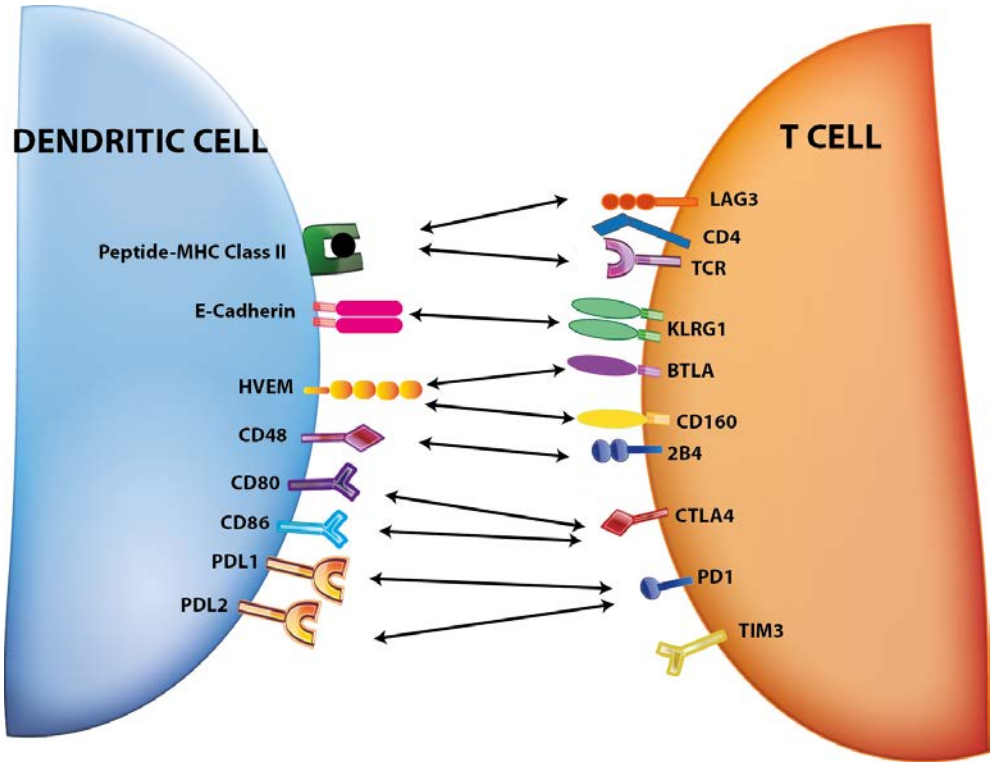


Figure 1.4: Inhibitory receptors on T cells.

Figure adapted from Nguyen et al. Nature reviews immunology 2014.

A panel of receptors known to negatively regulate function of T cells is depicted and their ligands on dendritic cells. These receptors are upregulated after initial interaction between TCR and peptide-MHC class II.

During initial infection a naive T cell requires coordinated signalling from (i) antigen, presented by major histocompatibility complex (MHC)-peptide molecules to stimulate T cell receptor (TCR), (ii) co-stimulation through CD28 and (iii) signals from pro-inflammatory cytokines, to drive activation and differentiation of the cell in to an effector T cell. A subset of these effector T cells will become highly functional memory T cells, long-living and maintained without antigen. Upregulation of inhibitory receptors does not occur until after TCR interaction with antigen presenting cells (APC), clearly due to an evolutionary bias to protect from an excess inflammatory response or autoimmunity. However, in chronic infection in the presence of persistent viral antigen, T cells progress through stages of dysfunction, overexpressing a range of inhibitory receptors. A hierarchical loss of function ensues with reduction in IL-2 production, proliferation and *ex vivo* killing. Severe exhaustion results in failure to produce IFNγ, degranulate and finally deletion of much needed virus-specific T cells. Although the existing regulatory mechanisms are lacklustre for dealing with chronic infection, the evolutionary advantage of such a strategy where exhausted cells engage in a hierarchical switch off of function is the prevention of autoimmunity, with the functions of cytolysis and tumour necrosis factor alpha (TNFα) production switched off first, which have the potential to be more detrimental to the host (Wherry et al., 2003). Factors such as high viral antigen load, longer duration of infection and loss of help from CD4+ T cells contribute to severe exhaustion in CHB (Ye et al., 2015).

Exhaustion of virus-specific T cells was first shown in the lymphocytic choriomeningitis virus (LCMV) mouse model. The major advantage to this choice of animal model is that the two strains used to cause either acute or chronic infection differ in only two amino acids and as neither of these mutations affects any of the known T cell epitopes it is possible to identify the same CD8+ T cell responses after an acute or chronic viral infection enabling T cell mechanisms to be examined in both scenarios. Mouse, woodchuck, squirrel and chimpanzee are all expensive models. Zajac et al studied acute and chronic LCMV infection in wild type and CD4-deficient mice (Zajac et al., 1998). They found that virus-specific CD8+ T cells persist in chronic infection but are functionally unresponsive thereby rendering them ineffective and unable to control the infection. The lack of CD4+ T cell help

resulted in complete loss of CD8 effector function. This study did not explore the molecular mechanisms of exhaustion but was a springboard for further work which has explored the key question regarding whether this phenotype can be altered to restore function, and thereby a potential target for immunotherapy.

A number of studies have shown that inhibitory receptors such as PD-1, TIM-3 and CTLA-4 are highly expressed on CD8+ T cells in CHB patients and blockade of individual receptors or in combination can reinvigorate and restore T cell effector function, as in advanced cancer (Boni et al., 2007), (Schurich et al., 2011), (Nebbia et al., 2012). A great deal of this work *in vitro* and in animal models looking at inhibitory receptors in viral infection has been done at a time in parallel with introduction of CTLA-4 and PD-1 blockade in cancer studies. The rationale for blockade therapy was based on studies demonstrating tumour cells exploit inhibitory mechanisms and evade the host immune response enabling them to expand and proliferate. In the treatment of metastatic melanoma and non-small cell lung cancer PD-1 and CTLA-4 blocking mAb have been approved for clinical use, to enhance anti-tumour responses. However, there is heterogeneity in blocking responses and some tumour types are refractory (Anderson et al., 2016). Similarly this pattern of partial restoration using blockade therapy has also been seen *in vitro* in CHB (Bertoletti and Ferrari, 2016).

The repertoire of inhibitory receptors deemed significant has increased over the years and some of the receptors considered relevant to HBV are discussed further in the next section. The importance of their role in tolerance has been demonstrated in knock-out mouse models that develop lymphoproliferative disorders and autoimmune conditions.

1.4.1 Programmed death (PD)-1

PD-1 is selectively upregulated on exhausted T cells. PD-1 is a member of the immunoglobulin superfamily and can bind two ligands (PD-L1 or PD-L2). The latter is mainly expressed on macrophages and dendritic cells. PD-1 knock-out mice develop glomerulonephritis and arthritis (Nishimura et al., 1999). Barber et al used a mouse model of infection with LCMV and demonstrated that PD-1 was upregulated at both the mRNA and protein level on LCMV-specific CD8+ T cells (Barber et al., 2006). The latter is important, as due to the complexities of transcription and translation, there is not always a corresponding change in both or guaranteed correlation. In summary they found high expression of PD-1 on functionally exhausted CD8+ T cells, and high expression of the ligand on infected cells suggesting a role in T cell dynamics. PD-L1 blockade restored function of CD8+ T cells and clearance of viral infection. The study methods were robust and addressed the issue that enhanced immune responses after blockade treatment might be due to *de novo* T cell responses from naive CD8+ T cells.

These findings can be extended to chronic HBV infection, but it is important to be mindful that T cell experiments are particularly challenging due to the small number of HBV-specific T cells, which require *in vitro* expansion, the large number of HBV epitopes generated by antigen processing and therefore presented by HLA molecules, represent just a number of factors that can affect the results obtained. Boni et al (2007) found *in vitro* blockade of this pathway enhanced the function of HBV-specific T cells, however, perhaps due to the heterogeneity of CHB, blockade enhanced at differing levels (Boni et al., 2007).

Tumours can upregulate PD-1 on T cells, and a number of tumour cell types express PD-L1. PD-L1 expression can be induced by IFNγ. Although upregulation of PD-L1 on tumour cells has been shown to signify an active response of T cells with production of IFNγ or other such pro-inflammatory

cytokines, equally it is considered to be a mechanism by which tumour cells can advantageously outwit the host response. Assays are in development to determine the potential of quantifying PD-L1 as a biomarker to predict pathway blockade response, as clinical response is variable, as seen in CHB. However, it is more likely that although it can aid prediction it cannot be an absolute marker because expression is dynamic and altered by cytokines and the tumour microenvironment (Zou et al., 2016).

Much of the excitement about inhibitory receptors relates to the clinical success in the cancer field of blocking antibodies targeting PD-1 and CTLA-4. Nivolumab, a human IgG4 mAb, has had favourable success in melanoma, non-small cell lung cancer, renal and bladder cancers and Hodgkin's lymphoma. However, it is important to note that success rates are variable with survival rates superior in some cancers compared to others (Attanasio and Wherry, 2016), (Chinai et al., 2015).

1.4.2 T cell immunoglobulin- and mucin-domain-containing-molecule (TIM)-3

TIM-3 expression on T cells has been linked to a number of inflammatory conditions like multiple sclerosis and systemic lupus erythematous (SLE) (Koguchi et al., 2006), (Wang et al., 2008), bacterial infections such as Tuberculosis (TB) (Jayaraman et al., 2016) as well as viral infections. In LCMV, Human immunodeficiency virus (HIV), HCV and HBV Tim-3+ T cells have the more dysfunctional phenotype (Jin et al., 2010, Jones et al., 2008, Golden-Mason et al., 2009, McMahan et al., 2010, Nebbia et al., 2012). Nebbia et al investigated the relevance of the TIM-3 inhibitory receptor in CHB based on the interesting data presented by Oikawa et al (Oikawa et al., 2006).

Oikawa et al showed in mice with acute graft versus host disease (aGVHD) there was high expression of TIM-3 on hepatic T cells and hypothesised this could play a role in hepatic tolerance and homeostasis as blockade of the receptor with mAb resulted in accelerated aGVHD (Oikawa et al., 2006). The ligand of TIM-3 is galectin-9, which Nebbia et al noted is expressed on kupffer cells, and preferentially expressed in the liver of patients with active CHB. Receptor ligand interaction was suggested to inactivate or delete HBV-specific T cells, the exact mechanism is not clear but *in vitro* blockade of the pathway resulted in recovery of function. However, similar to attempts at pathway blockade of other receptors, the response was not uniform across all patients. The study nicely demonstrates the interaction of immune cells with liver cells. The authors found high levels of the ligand galectin-9 in serum samples from CHB patients versus healthy controls and suggested this could be a tolerance mechanism of T cells that had been exposed to high levels of the ligand in the liver.

1.4.3 Cytotoxic T-lymphocyte-associated antigen (CTLA)-4

CTLA-4 competes with CD28 for CD80/86 binding. Similar to the other inhibitory receptors it is a mechanism that exists to halt further T cell activation and a protective response against an excessive inflammatory response. Knock-out mouse models display severe lymphoproliferative disease and autoimmune phenotypes as a result of both cell-intrinsic and extrinsic effects (Waterhouse et al., 1995). mAb to CTLA-4 (Ipilimumab) was approved for clinical use in malignant melanoma in 2011. Similarly there have been promising developments of blockade eliciting anti-tumour effects to HCC. Promisingly, trials have not shown the development of immune-mediated fulminant hepatitis in patients.

Schurich et al highlighted that although CTLA-4 blockade does allow for some T cell restoration in CHB patients, the lack of uniformity across patients suggests that blocking other receptors may play a role, implying a synergistic effect is needed (Schurich et al., 2011). Many studies have shown that blockade of the pathway can enhance T cell activation and proliferation.

1.4.4 Lymphocyte activation gene (LAG)-3

LAG-3 is expressed on CD4+ and CD8+ T cells and natural killer (NK) cells (Anderson et al., 2016). It structurally resembles CD4 and will bind to MHC class II. Initially it appeared that LAG-3 knock-out mice did not display any T cell defects but further exploration revealed that T cell expansion was affected (Workman et al., 2004). Upregulation only occurs several days after T cell activation (Triebel et al., 1990). Huard et al demonstrated *in vitro* blockade of LAG-3 boosted T cell function and proliferation (Huard et al., 1995). Further *in vitro* and *in vivo* experiments using healthy human cells confirmed the negative regulatory effect of LAG-3 on T cell function (Macon-Lemaitre and Triebel, 2005).

In LCMV, LAG-3 was shown to correlate with severity of infection although blockade of the receptor alone had little effect and synergised blocking with PD-L1 improved T cell response with a fall in viral load (Blackburn et al., 2009). Further studies in LCMV models have shown synergistic blockade of PD-1 and LAG-3 can reverse T cell exhaustion (Richter et al., 2010).

Another ligand for LAG-3 was sought out given it could affect the function of CD8+ T cells and NK cells, both of which do not bind to MHC class II. Subsequently it was suggested liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin) found on tumour or liver cells is another ligand providing a mechanism of action and regulation (Xu et al., 2014).

Similar to LAIR-1, which is the ultimate focus of this thesis, LAG-3 is an inhibitory receptor that also has a natural soluble form (sLAG-3), which has high affinity binding to the same ligand as membrane bound LAG-3. sLAG-3 has been described as functioning as an immune adjuvant (Nguyen and Ohashi, 2015). Triebel et al showed subgroups of patients with breast cancer who had detectable levels of sLAG-3 at time of diagnosis had better disease-free and overall survival rates (Triebel et al.,

2006). Further studies in mice and phase 1 clinical trials have explored the potential of sLAG3-immunoglobulin as an immune adjuvant, indirectly enhancing anti-tumour T cell function in combination with vaccination (Anderson et al., 2016).

There is a breadth of work providing strong evidence implicating the role of inhibitory receptors in the regulation of T cell immune responses, but undoubtedly there are added complexities. Impaired function of CD8+ T cells in CHB is multifactorial and there is emerging evidence of metabolic dysregulation contributing to exhaustion and interaction of other immune cells for example myeloid-derived suppressor cells (MDSC). There are more MDSC in CHB livers and Pallett et al describe a suppressive role of MDSC on T cells contributing to dysfunction of HBV-specific CD8+ T cells (Pallett et al., 2015).

Several challenging issues present themselves with regards to inhibitory pathways:

i. Is modulating a single pathway enough?

ii. How to minimise the side effects when targeting inhibitory pathways, especially of autoimmunity.

iii. Which immune cell populations should be targeted as global targeting of any pathway where the receptor is expressed diversely can have mixed outcomes?

iv. Treatment with oral antivirals or interferon treatment doesn't appear to reprogram the phenotype of these ineffective T cells, hence more targeted approaches have been sought such as blocking these inhibitory pathways.

Up to now, research has tended to focus on the role of inhibitory receptors in the impairment of adaptive immunity in CHB, but what about the innate immune system?

1.5 Innate Immunity and HBV

In CHB it remains unclear which is the superior population of immune cells that would benefit from immunotherapy and we do not have a potential biomarker to predict disease progression.

NK cells are a main component of the innate immune system, originating from the bone marrow but independent of the thymus. NK cells use a different detection strategy to T cells to differentiate pathogen from healthy cells and this occurs via activatory and inhibitory receptors. These are surface receptors with a diverse repertoire originating from germ line, but they are not dynamic and do not undergo recombination (Lanier, 2005).

NK cells are needed to control viral infection and are involved in liver pathogenesis (Tian et al., 2013). They can kill virus by cytokine production and cytotoxic effects. The importance of this cell population is shown in studies of patients with rare genetic disorders with reduced NK cell frequency and function, who are found to be at increased risk of viral infection (Orange, 2002).

As depicted in Figure 1.5 NK cells in CHB have been shown to contribute to viral persistence, shaping the adaptive immune response through various mechanisms (i) by reducing HBV-specific CD8+ T cells via TRAIL-R2 subsequently limiting the T cell response (Peppa et al., 2013), (ii) impaired cytokine response of $CD56^{bright}$ NK cells thereby failing to promote responses of lymphocytes of the adaptive immune system (Peppa et al., 2010), (iii) via crosstalk with other immune cells; cytolytic attacks against HSCs can prevent collagen deposition and fibrosis (Friedman, 2008). Overall, NK cell activity is suppressed by HBV infection (Maini and Gehring, 2016).

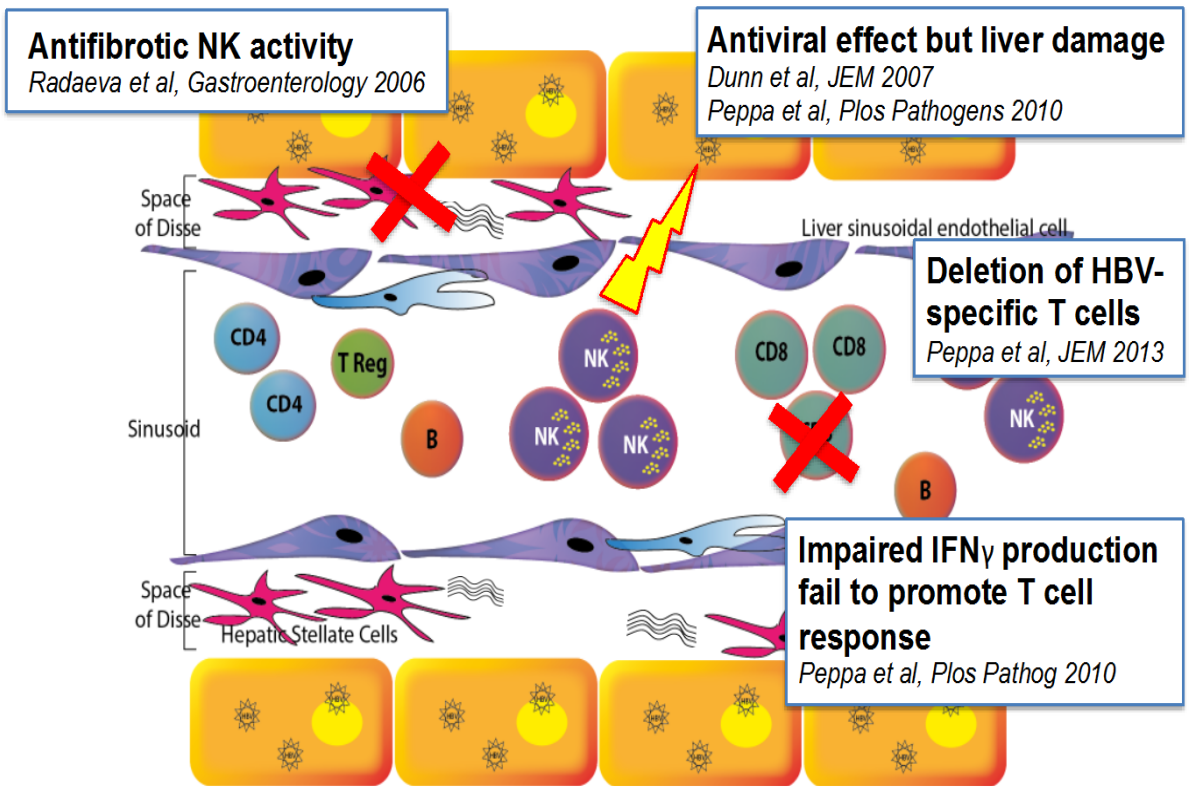


Figure 1.5: Emerging role for NK cells in HBV immunopathology

There are a number of mechanisms by which NK cells in CHB have been identified as important players contributing to viral persistence and immune homeostasis. In summary they have an (i) antifibrotic effect, (ii) antiviral effect, (iii) role in deletion of HBV-specific T cells and (iv) impaired cytokine function thereby failing to promote T cell response.

A normal NK cell will have inhibitory signals that outweigh activatory signals and thereby enable NK cell self-tolerance. In pathological conditions, this balance shifts. NK cells are effective and fast killers as they do not require priming to kill target cells and with no target cell MHC restriction, selective killing of any cells lacking MHC class I are targeted. On encountering their target, pro-inflammatory cytokines are produced to enable recruitment of other innate cells and stimulate response of lymphocytes of the adaptive immune response. Unlike other inhibitory receptors, LAIR-1 employs a human leucocyte antigen (HLA) class-1 independent mechanism (Meyaard et al., 1997).

Since the discovery of NK cells in the mid-1970s, researchers have discovered new depths of the phenotype and evolving contribution of NK cells, and it is now believed that they share receptors and features such as memory and education similar to adaptive immune cells. Similarities begin at the developmental level. It was believed for some time that unlike T and B cells, NK cells do not express recombinant associate gene (RAG) proteins (Mombaerts et al., 1992) which are important for the rearrangement of genes of immunoglobulin and T cell receptor molecules. However, Karo et al showed that although the population of NK cells present in RAG knock-out mice were less competent, with ‘reduced cellular fitness’, when challenged with viral infection these proteins were still important in the developmental stage of NK cells (Karo et al., 2014).

These new insights from a growing body of evidence suggest NK cells can contribute to persistence of infection, and if ‘NK cell fitness’ is impaired it potentially can be reversed.

Importantly, inhibitory and activatory signal balance plays a role in memory and education as discussed below. Education and memory are key aspects of adaptive immunity, defined as cell capacity to learn, retain and then recall a response to a sensitiser. It was believed that as NK cell receptor genes do not undergo rearrangement, they do not have antigen specificity and lack memory. Although differences of opinion still exist, there is compelling data that this memory phenomenon

does exist in NK cells as evidenced using a mouse model of delayed type hypersensitivity (DTH) and enhanced immune responses in secondary challenge with virus. The DTH mouse model is based on exposure to a hapten, a chemical that alters self proteins so they then are recognised as foreign antigen, trigger an allergic response that on first exposure is mild and then on re-exposure generates a more pathological reaction. In a series of experiments in RAG deficient (no T or B cells) mice, NK DTH response was confined to CXCR6+ liver NK cells. CXCR6 is a liver specific adhesion molecule that can be used as a surface marker. These mice were able to recall the sensitising hapten for a few months and discriminate when exposed to a different hapten (Paust et al., 2010). Models of vaccination for herpes simplex virus (HSV)-2 (Abdul-Careem et al., 2012) and vaccinia virus (Gillard et al., 2011) have shown that protection from reinfection can occur independently of T and B lymphocytes. Experimental systems using mouse cytomegalovirus (MCMV) have demonstrated memory NK cells have a distinct transcriptional profile (Sun et al., 2011). These studies have shown NK cells can expand, and contract like T cells to generate memory cells after viral challenge conferring protection in adoptive-transfer models (O'Sullivan et al., 2015). However, the NK cell receptors that mediate these mechanisms have not yet been uncovered. Importantly in CHB, we have recently shown that upon viral suppression, following administration of PEG-IFNα, patients are able to maintain a population of functional NK cells, with long-lived potential, possibly due to the effect of the type-1 interferon exposure (Gill et al., 2016).

1.5.1 Defining the role and activity of NK activatory and inhibitory receptors

In humans, the proportion of NK cells differs between peripheral blood and liver (Doherty and O'Farrelly, 2000). Second only to the lungs and the uterus liver tissue is highly enriched with NK cells (Bjorkstrom et al., 2016). NK cells are characterised via flow cytometry by the expression of CD56 and CD16 but lack of CD3 and accordingly can be subdivided in to $CD56^{bright}$ ($CD56^{hi}CD16^{neg}$) and $CD56^{dim}$ ($CD56^{lo}CD16^{pos}$) NK cells (Caligiuri, 2008). This differentiation is important, as the $CD56^{bright}$ NK cells are believed to be better at producing cytokine compared to the more cytotoxic $CD56^{dim}$ NK cells. As well as differing cytokine and intrinsic cytotoxic capacity they have diversity in NK cell surface receptor repertoire (Figure 1.6). It is unclear whether $CD56^{bright}$ NK cells are a precursor to $CD56^{dim}$ NK cells or a separate lineage. In persistent viral infection there is a marked change in the proportions of these subsets (Oliviero et al., 2009).

What regulates the high proportion of NK cells in the liver is not known but they are placed in a key position for liver immune surveillance (Peng et al., 2016). They are the largest proportion of liver innate immune lymphocytes, which also include NK-T and gamma delta T cells. In addition a unique subset of 'liver-resident NK cells', with different phenotype and functional characteristics, has been described in mouse and human livers. A decade ago Kim et al identified the presence of immature NK cells in the mouse liver that express low amounts of DX5 and Ly49 receptors (markers associated with NK cell maturity) (Kim et al., 2002). In humans these liver resident NK cells are CXCR6+ with a unique transcriptional profile of $TBet^{lo}Eomes^{hi}$ differing to mice and peripheral circulating NK cells (conventional NK cells) (Stegmann et al., 2016). Stegmann et al observed this subset store fewer cytotoxic mediators, display an immature phenotype and upregulate TNF-related apoptosis inducing ligand (TRAIL). The mechanisms relating to the unique characteristics of liver NK cells still requires further probing and more intriguingly it is not known whether these tissue resident NK cells develop from liver hematopoietic progenitor cells.

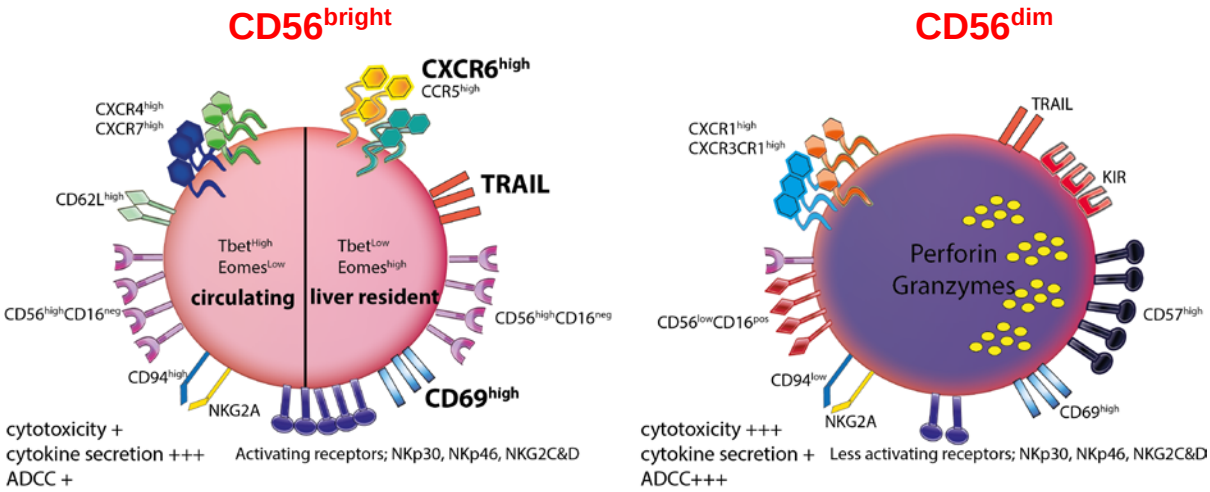


Figure 1.6: Summary of key receptors and function of CD56^{bright} and CD56^{dim} NK cells.

NK cells are characterised by expression of CD56 and CD16. This schematic depicts the main receptors and functions of NK cells subsets. The two subsets have differing surface receptor repertoire and functional ability. The CD56^{bright} NK cells (left panel) are abundant cytokine producers, whereas the CD56^{dim} NK cells have greater cytotoxic potential (right panel).

NK cells have a broad repertoire of activatory (NK cell activatory surface receptors include NKG2D, NKp46 and NKp30) and inhibitory receptors, which tightly regulate their effector functions (Sun et al., 2015a). These are not absolute "on/off" signalling receptors. They are better described in line with a 'see-saw' mechanism whereby cytotoxicity of NK cells against tumour cells for example is determined by effects of opposing NK cell receptors. In reality however, NK cells struggle in maintaining hosts as pathogen and tumour free. Employing theories used to examine inhibitory receptors in the adaptive immune system, it has been suggested boosting NK cell activity by removing the "hand-brake" of inhibitory receptors could be the key to clearance of HBV infection.

There are two families of HLA class-I specific inhibitory receptors (i) CD94/NKG2 receptors - c-type-like lectin family and (ii) killer-cell immunoglobulin-like receptors (KIRs) (Poggi and Zocchi, 2014). The latter recognise polymorphic regions on HLA-A, -B, and -C as opposed to the former that only recognise HLA-E. More recently, immunoglobulin-like transcript (ILT), and leukocyte immunolobulin-like receptors (LIR) have been discovered, all clustered on chromosome 19.

In humans these inhibitory receptors include KIRs and in mice the equivocal receptors as named Ly49. As described earlier NK cells have been shown to share features with adaptive cells such as memory and education and equally are subject to exhaustion/down-modulation through excess inhibitory signalling. Exhaustion of T cells has been extensively studied unlike exhaustion of NK cells. Below, outlined is some of the literature related to the 'exhaustion' of NK cells.

1.5.2 Immunosuppressive cytokines

Peppa et al were the first in this field to describe exhaustion and reversal of exhaustion in NK cells. They showed the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets have marked impaired capacity to produce IFNγ in CHB and immunosuppressive cytokines selectively suppress NK cell IFNγ production (Peppa

et al., 2010). IFNγ is a powerful method by which NK cells would normally be able to clear virus infected hepatocytes (Zhang et al., 2016). Blocking these immunosuppressive cytokines (IL-10 +/- transforming growth factor beta (TGFβ) blockade) restored NK cell capacity *in vitro* to produce IFNγ (Peppa et al., 2010). Heiberg et al (2015) identified $CD56^{dim}$ NK cell subset IFNγ impairment occurs at an early age by comparing IFNγ production in a young HBV infected cohort with healthy age matched cohort (Heiberg et al., 2015).

1.5.3 TIM-3

Some studies have investigated the expression of single surface inhibitory receptors and their function on NK cells. Ndhlovu et al observed high expression of TIM-3 on NK cells from healthy donors and in cord blood, particularly on the mature $CD56^{dim}$ subset (Ndhlovu et al., 2012). Cytokine stimulation induced upregulation of TIM-3. In contrast to the findings on T cells where TIM-3 negatively modulated T cell function, TIM-3 positive NK cells were not found to be dysfunctional or exhausted. Despite this, functional assays demonstrated cross-linking of TIM-3 suppressed NK cell cytotoxicity (Ndhlovu et al., 2012). The authors were unable to shed light on the potential mechanism by which this could occur. It should be noted in the context of metastatic melanoma, TIM-3 on NK cells did mark a dysfunctional phenotype similar to T cells that are exhausted and upregulate inhibitory receptors. TIM-3 blockade was shown to similarly restore function (da Silva et al., 2014).

Increased TIM-3 expression on NK cells has been described in HCV (Golden-Mason et al., 2015) and HIV (Finney et al., 2013). Golden-Mason et al demonstrated higher levels of TIM-3 on NK cells from HCV patients, particularly the $CD56^{dim}$ subset, but high expression of TIM-3 was associated with increased cytotoxicity, contrary to what one would expect, if cell fitness was exhausted by high expression of inhibitory receptor.

Ju et al observed the expression of TIM-3 to be higher on circulating and liver infiltrating lymphocytes in HBV patients. It is obviously difficult to get access to healthy liver tissue and therefore they used non-alcoholic fatty liver disease (NAFLD) patients, as well as healthy controls (HC) as comparators. The authors did not show whether the TIM-3 positive subset were more dysfunctional, however, blockade of TIM-3 pathway led to increased cytotoxicity of NK cells and IFNγ production, however these increases were modest (Ju et al., 2010).

Taken together these functional assays have consistently shown TIM-3 functions as an inhibitory receptor, however, the mechanism needs further investigation as this is difficult to explain when the cytoplasmic domain of the receptor does not have any inhibitory motifs.

1.5.4 PD-1

A great deal is known about PD-1 expression on T cells but little is known about PD-1 expression on NK cells and their function. In humans higher PD-1 expression has been observed in HCV particularly the $CD56^{bright}$ NK cell subset (Golden-Mason et al., 2008) in TB (Alvarez et al., 2010), patients with multiple myeloma (Benson et al., 2010), and in post-transplant lymphoproliferative disease (Wiesmayr et al., 2012). However, these expression levels are relatively low overall. In regards to function, this inhibitory receptor appears to negatively regulate NK cell function. In patients infected with TB, PD-1 blockade *in vitro* resulted in increased NK cell IFNγ production (Alvarez et al., 2010).

NK cell-mediated cytotoxicity is an important response mounted against tumour cells. Tumours can exploit the PD-1 pathway by over expressing the main ligand PD-L1, dampening both T and NK cell responses (Dong et al., 2016). Specific monoclonal antibodies such as Pidilizumab have shown blocking PD-1 *in-vitro* can enhance the activity of NK cells against autologous cancer cells (Benson

et al., 2010). What remains unclear is whether the therapeutic effect is mainly due to reactivation of the exhausted T cells as the effects are not uniform across all patients.

Inhibitory pathways such as PD-1 have been demonstrated to play a critical role in liver tolerance as loss of PD-1 signalling can lead to spontaneous allograft rejection in mice (Morita et al., 2010) and mouse HSCs can suppress T and B cells via PD-L1 to maintain tolerance (Li et al., 2016).

1.5.5 LAG-3

LAG-3 is expressed on CD8+ T cells and NK cells. It binds to LSECtin on tumour or liver cells (Xu et al., 2014). Expression on NK cells and its function is poorly understood on NK cells in chronic infection.

1.5.6 NKG2A

Expression of NKG2A is higher on NK cells in acute HBV compared to acute HCV but this may be a sign of disease activity rather than a feature of HBV infection (Lunemann et al., 2014).

There is evidence of inhibitory receptors overriding the mechanisms of activation to dampen anti-tumour and anti-viral responses. To date, little evidence has been found exploring the role of inhibitory receptor LAIR-1 in chronic viral infection. Our interest was piqued by recent studies revealing the ligand of LAIR-1 to be collagen. Collagen in the liver can be increased four to seven fold in a diseased state (Rojkind et al., 1979). We were interested in looking at LAIR-1 because collagen is abundantly produced in the diseased liver.

1.6 Liver fibrosis and role of HSCs

The liver has a unique capacity to regenerate (Ellis and Mann, 2012). The only other organ with similar features is skin. This particular feature enables orchestration of the complete restoration of architecture and function following acute injury in a short period of time (Fasbender et al., 2016). Fibrosis is universally defined as the deposition of excess extracellular matrix with death of hepatocytes. In fibrosis, initially there is only a slight deficit to hepatocyte function but over time, with repeated insult, this can progress to cirrhosis (Friedman, 2008). There are no approved anti-fibrotic therapies available, although in CHB potent antivirals limit disease progression, with some evidence of fibrosis regression (Chang et al., 2010), (Marcellin et al., 2013), (Calvaruso and Craxi, 2014).

HSCs play a critical role in the pathogenesis of liver fibrosis. They are positioned in the space of Disse and in a healthy liver separated from NK cells by the LSECs, however in a diseased liver this barrier is broken, and HSCs will come in to contact with immune cells (Fasbender et al., 2016). Cell death activates HSCs and transdifferentiation to myofibroblasts (Iwaisako et al., 2012), (Brenner et al., 2012). The inflammatory microenvironment of numerous cytokines such as TGFβ-1, platelet-derived growth factor (PDGF) and other chemokines released in the process of destruction cultivate an environment where activated HSCs can outbalance mechanisms in place to degrade collagen i.e. matrix metalloproteinases (MMPs). In chronic liver damage ongoing stimulation of activated HSC results in excess production of extracellular matrix (ECM) products, particularly type I collagen. Later in this chapter, the crystal structure of collagen and LAIR-1 interaction with different types of collagen are described.

NK cells are a generous proportion of the liver lymphocyte population and the liver can become compromised in function with excess collagen deposition; therefore one might postulate that NK cells may play a role in controlling collagen deposition. In fact several studies have already highlighted a

role for receptor ligand interactions between NK cells and HSCs, driving increased NK cell stimulation and reduced NK cell inhibition, with a shift in the balance of activator and inhibitory signals, thereby reducing liver fibrosis by increasing HSC death (Melhem et al., 2006). NK cells therefore have a role in protecting the liver from fibrosis. Figure 1.7 describes a number of mechanisms by which this can occur.

i. Activated phenotype of NK cell receptor expression

An increase in NK cell activatory receptors NKp30, NKp46 and NKG2D has been described in HCV, and blocking these receptors reduced HSC apoptosis (Radaeva et al., 2006), (Kramer et al., 2012). Although the pathways by which these receptors influence HSC apoptosis is not entirely clear, it is likely that the presence of activated NK cells is protective (Glassner et al., 2012). In general, hepatocyte damage can trigger HSC activation, which results in increased NK cell activity and a key mechanism is the production of retinoic acid by early activated HSCs, giving rise to a higher expression of RAE-1 protein, which was originally isolated from mouse embryonic carcinoma cells and later identified as an NKG2D ligand that activates NK cells (Radaeva et al., 2007). High levels of RAE-1 have been detected in the liver of HBV transgenic mice (Chen et al., 2007). In addition, it is important to note the role of the liver microenvironment influencing regulation of receptor expression. In chronic liver injury the levels of TGFβ released by neighbouring LSECs and kupffer cells is increased and can downregulate NKG2D, inhibiting NK cell activity and allowing survival of HSCs. Studies in mouse models have shown dysregulated NK cell function is associated with worsening liver fibrosis (Muhanna et al., 2011).

ii. MHC class I downregulation on activated HSCs

In mouse models, deletion of the gene encoding Ly49 receptor (inhibitory receptor on mouse NK cells equivalent to KIR in human) increases NK cell killing of HSCs, with fibrosis resolution (Gur et al., 2012). MHC class 1 is downregulated on activated HSCs thereby hindering engagement of inhibitory NK cell receptors and allowing increased killing capacity (Muhanna et al., 2011).

iii. Influence of inflammatory cytokines

In addition to high levels of TGFβ produced by activated HSCs influencing NK cell function (Jeong et al., 2011), the cytokines produced by NK cells themselves can influence cell dynamics. IFNγ produced by NK cells can trigger HSC apoptosis and cell cycle arrest. In addition IFNα can upregulate TRAIL on NK cells (Gill et al., 2016, Micco et al., 2013) binding to upregulated TRAIL-R2 on activated HSCs, thereby propagating NK cell apoptosis (Fasbender et al., 2016).

iv. Activated HSCs express 'death receptors' such as Fas and TRAIL-R

Fas and TRAIL-R are both receptors of the TNF family and initiators of apoptosis. RAE-1 has similarly been shown to upregulate TRAIL-R on activated HSCs (Taimr et al., 2003). Another apoptotic pathway is via Fas expression on activated HSCs.

In order to determine how to reverse the process of fibrosis, it is important to dissect the particular molecular mechanisms and stages of NK cell involvement in this process. Experimentally, the main methods to investigate this interaction would be, *in vivo* via mouse models whereby liver fibrosis can be induced by repeated administration of hepatotoxic substrates or *in vitro* experiments involving culturing HSCs isolated from liver tissue.

NK cells appear to preferentially kill early activated HSCs but not quiescent or fully activated HSCs (Gao and Radaeva, 2013). Recently we, and others, have identified subsets of human liver resident NK (lrNK); their role in liver fibrosis has never been explored.

We were interested to examine the role of LAIR-1 on NK cells interacting with collagen produced by HSCs.

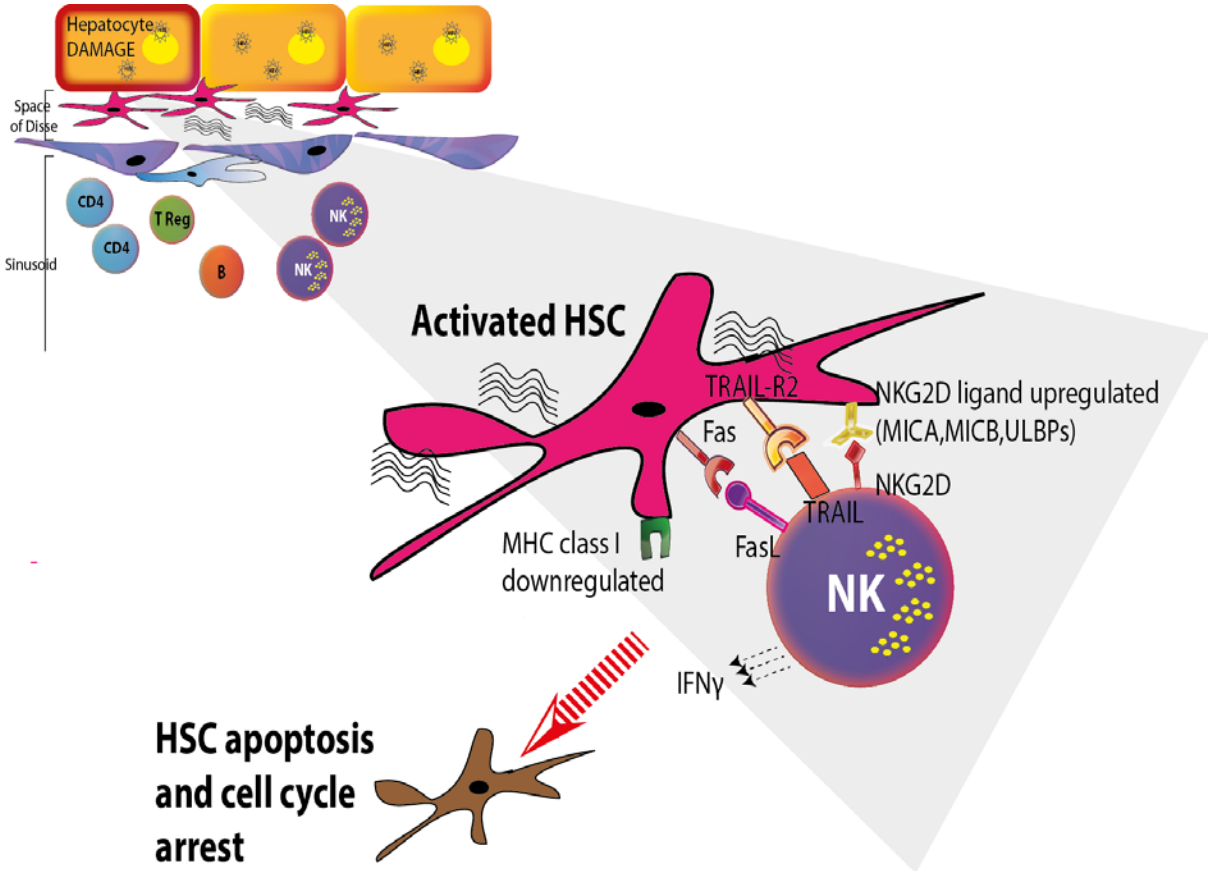


Figure 1.7: NK cells can interact with their respective receptor/ligand on HSCs to induce apoptosis of HSCs and protect the liver from collagen deposition.

In the presence of hepatocyte damage HSCs will become activated. In early activation there is downregulation of NK cell inhibition. Activated HSCs will upregulate NKG2D ligands. Subsequently the HSC will be killed by receptor/ligand interaction between NK cell and HSC. Other mechanisms shown include increased expression of TRAIL-R2 and Fas on HSC surface. MHC class 1 is downregulated on activated HSCs so they are easily recognised by NK cells and IFNγ produced by NK cells can trigger HSC apoptosis and cell cycle arrest.

1.7 LAIR-1

1.7.1. Discovery and function of LAIR molecule

Meyaard et al were the first to describe the inhibitory receptor named LAIR-1 (CD305) by means of a series of experiments isolating the receptor using a mAb (DX26) against LAIR-1 (Meyaard et al., 1997). However, it was Poggi et al who originally reported on a mAb that recognised a molecule that they called p40 but was later recognised as the same molecule as LAIR-1 (Poggi et al., 1995). Like DX26, p40 mAb inhibited NK cell cytotoxicity (Poggi et al., 1997). Meyaard et al in 1997 indicated cross-linking of DX26 and NK cell Fc-receptor (FcR) was essential and this was demonstrated using transfected human FcR-bearing targets by NK cell clones in a mouse compared to a FcR-negative parent line that subsequently had no effect on cytotoxicity. Further functional experiments on human peripheral blood mononuclear cells (PBMC) showed more evidence of the presence of DX26 mAb inhibiting cytolysis in both resting and activated NK cells. The functional role of LAIR-1 is described in more detail later in this chapter.

Generally knock-out models deficient of an inhibitory receptor are used as affirmation of the importance of the receptor. The LAIR-1 knock-out mouse model demonstrated an altered immune cell phenotype but otherwise mice were healthy; normal haematopoiesis was not affected and their longevity unaffected compared with controls (Tang et al., 2012). A major drawback of this mouse model was the failure to consider that an altered immune cell phenotype may only become meaningful when a virus or pathogen stresses these cells. Another problem is it fails to take in to account that inhibitory receptors are hierarchical in their knock-out potential.

1.7.2. LAIR-1 structure

The structure of LAIR-1 consists of an extracellular immunoglobulin (Ig) domain, short stalk, a transmembrane domain and a short cytoplasmic tail containing two immunoreceptor-tyrosine based motifs (ITIMs) (Meyaard et al., 1997). The extracellular domain binds to glycine-proline-hydroxyproline (GPO)-rich segments of collagen. Sophisticated nuclear magnetic resonance (NMR) spectroscopy techniques have illustrated complexities of collagen crystal structure, folding and differential potential binding sites for collagen receptors like LAIR-1.

Structurally it is related to other inhibitory Ig superfamily members mentioned earlier such as KIRs, ILT receptors and the Fc-receptor for IgA (FCAR), which are localised to the same receptor cluster on chromosome 19. However, unlike KIRs it does not recognise HLA class 1 molecules. There are only two members of the LAIR family; LAIR-1 and LAIR-2.

LAIR-1 can be shed from the cell membrane and released when lymphocytes are activated. Ouyang et al observed higher levels of soluble LAIR-1 (sLAIR-1) in patients following kidney transplantation who experienced graft rejection, compared to those who did not and healthy individuals (Ouyang et al., 2004). They hypothesised that LAIR-1 would be similar to other membrane molecules involved in lymphocyte activation which have been seen in the sera of transplant patients, and associated with transplant rejection. The authors were keen to study whether sLAIR-1 could be used as a biomarker for rejection. They found high levels of serum sLAIR-1 in patients with haemorrhagic fever with renal syndrome (HFRS) caused by Hantaan virus, as well as in chronic rejection. LAIR-1 cross-linking *in vitro* inhibited cytotoxicity of CD8+ T cells.

LAIR-2 consists of only an extracellular ectodomain hence no role in signal transduction, but exhibits high affinity for collagen molecules (Lebbink et al., 2008). LAIR-2 is a soluble protein, which

undergoes renal clearance. Study findings by Meyaard et al demonstrated CD4+ T cells as the main producers of LAIR-2 (Olde Nordkamp et al., 2011). It has been suggested that LAIR-2 has a regulatory role by preventing LAIR-1 binding to collagen, and also that it may be a urine biomarker in rheumatoid arthritis (RA) patients with a greater degree of inflammation when compared to a condition with less inflammatory activity, such as osteoarthritis (OA) (Olde Nordkamp et al., 2011).

With the exception of sLAIR-1 these markers have not been investigated in viral infections, where the immune system plays a key role.

1.7.3. LAIR-1 Expression

LAIR-1 is an inhibitory receptor expressed on almost all haematopoietic cells. Several studies have shown upon maturation or activation expression on neutrophils, T cells and B cells can decrease perhaps suggesting a regulatory role of the cell at the level of receptor expression. There is limited published data regarding LAIR-1 expression in tissues, other than that described in a study by Zhang et al. In this study, it was shown that the expression of LAIR-1 was low on CD4+/CD8+ T cells from RA and OA patients (PBMC) but that levels of LAIR-1 from RA patients were higher than OA when assessed by haematoxylin and eosin (H&E) staining of synovial tissue (in a small sample size of six patients). LAIR-1 clustered in areas enriched with CD68 positive macrophages (Zhang et al., 2014). On B cells, LAIR-1 expression varies during B-cell differentiation and is lost on a subset of memory B cells and plasma cells (van der Vuurst de Vries et al., 1999).

1.7.4. LAIR-1 Ligands

In the literature, collagen has been described as the high affinity ligand for LAIR-1. The relatively recent discovery of the ligand of LAIR-1 as collagen (Lebbink et al., 2006), may explain the lack of

literature examining their interaction. There is little known about its role in disease. In a healthy liver, collagens are secreted into the ECM to maintain tissue integrity, however, in disease states there is excess deposition of ECM, mainly type I collagen (Friedman, 2008). As noted, NK cells have the capacity to interact with HSCs through a number of receptor-ligand interactions. HSCs play a central role in ECM deposition in liver fibrosis. To date, there has been no evidence that NK cells interact with HSC-derived collagen and we therefore designed experiments to investigate this possibility.

There are four main classes of transmembrane receptors that specifically bind to collagen; collagen binding integrins, discoid domain receptors (DDR), glycoprotein VI (GPVI), and LAIR-1 (Leitinger, 2011). LAIR-1 resembles GPVI in structure but is an inhibitory receptor. The receptor is interesting for several reasons, highlighted in a review by Meyaard; unlike other inhibitory receptors it does not have an extensive subfamily, and the receptor ligand is not cell bound. For this reason regulation of the receptor must be tightly controlled as it is ubiquitously expressed on all cells of the immune system. Theories for self-regulation include LAIR-2 antagonising collagen-LAIR-1 signalling as a competitive inhibitor, and the LAIR-1 ectodomain being shed, thereby preventing transmembrane signalling.

There is strong experimental evidence that collagen is the ligand of LAIR-1. LAIR-1 can bind multiple types of collagen (Lebbink et al., 2009). Experiments by Lebbink et al illustrate collagen is a 'functional high affinity ligand' for LAIR-1 (Lebbink et al., 2006). They were able to demonstrate this clearly using a number of experimental strategies strengthening their original observations, and showed;

i. Different collagens can act as ligands for LAIR-1 by comparing antibody staining by LAIR-1-IgG in collagen transfected and untransfected cell lines in the presence of blocking antibodies,

ii. Pre-incubating carcinoma cell lines expressing collagen with collagenase, thereby removing the ligand for binding, prevented LAIR-1-IgG binding, with reduced MFI by flow cytometry,

iii. High affinity of the collagen-LAIR interaction by surface plasmon resonance (BIAcore), and finally,

iv. Demonstrating collagen cross-linking directly inhibited degranulation of cells in an *in vitro* model using a LAIR-1 transfected cell line and plate-bound collagen. Pre-incubation with blocking anti-hLAIR-1 $F(ab')_2$ fragments led to recovery of the inhibition.

It has been speculated that the LAIR-1 collagen interaction can be exploited by tumour cells via a mechanism by which they upregulate collagen expression in a number of ways, increasing the availability of ligand to bind to LAIR-1 resulting in negative regulation of effector cells and thereby dampening anti-tumour responses (Meyaard, 2008) (Figure 1.8).

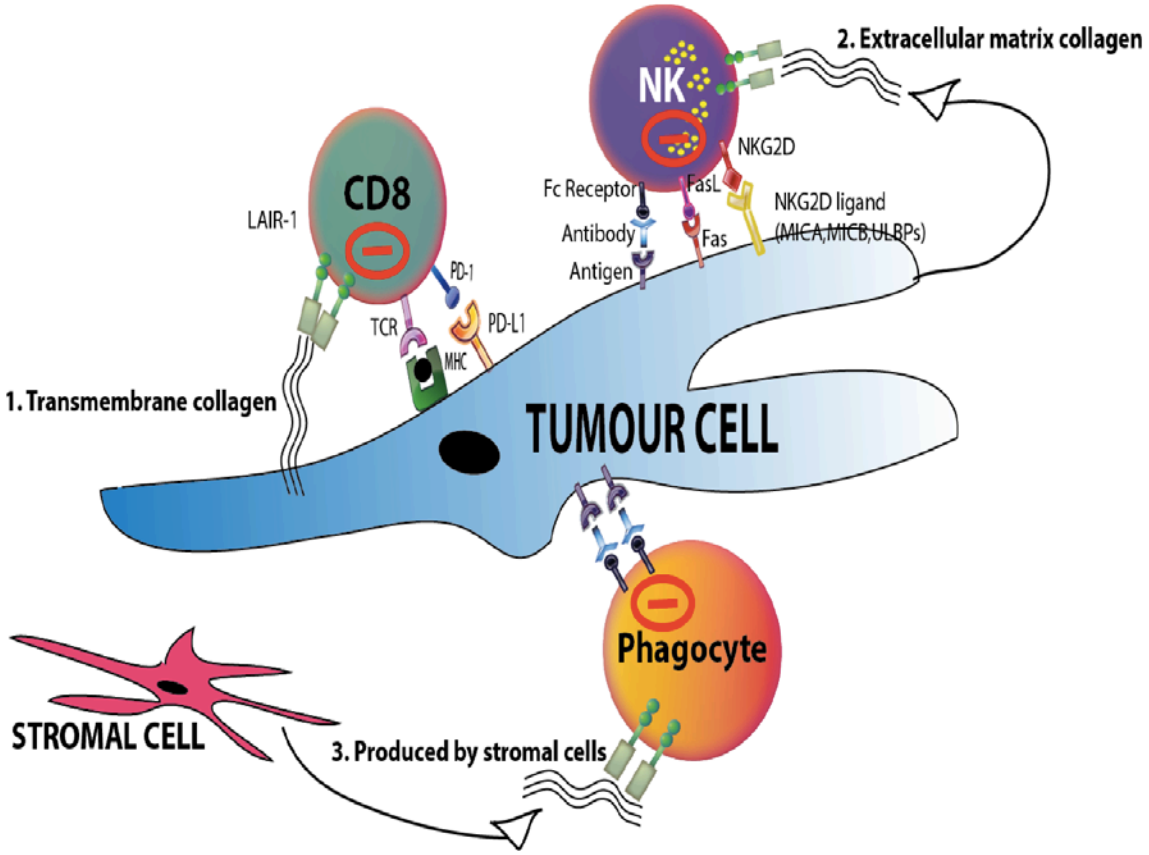


Figure 1.8: Potential mechanism of LAIR-1 described in tumour cells.

Figure adapted from Meyaard et al, J. Leukoc. Biol. 2008.

It has been suggested that a potential mechanism by which tumour cells dampen anti-tumour responses of immune cells is by upregulation of collagen expression in a number of ways i.e. (1) transmembrane collagen, (2) ECM collagen, (3) collagen produced by stromal cells, thereby increasing the availability of ligand to bind to LAIR-1 resulting in negative regulation of effector cells.

The collagen binding properties of LAIR-1 have also been characterised using collagen derived synthetic toolkit peptides and reagents that interfere with binding. An elegant study by Brondijk et al more precisely characterises the collagen binding sites of LAIR-1 by mapping the crystal structure of the receptor using NMR spectroscopy (Brondijk et al., 2010).

More recently C1q (a component of complement) and surfactant protein D (SP-D) have also been suggested as potential ligands for LAIR-1. A major constituent of the innate immune system are the complement components. It is widely accepted that the role of complement is more than the simple 'killing of bacteria'. Complement is involved in opsonisation of pathogens, regulating activity of adaptive immune cells such as T and B cells and also implicated in immunopathogenesis of a number of autoimmune diseases such as SLE (Merle et al., 2015a).

The pathway involves a multifaceted cascade of plasma proteins and the liver produces the majority. These enzymes collaborate together and perhaps due to the complexity of the cascade and the number of proteins involved, it has been a more challenging area for immunologists to research. Historically three activation pathways have been described; classical, alternative and lectin. Each pathway prompts an enzyme cascade that eventually meets at C3.

The classical pathway is triggered via the C1 complex (C1q, C1r and C1s), whereby antigen-antibody immune complexes are formed (Merle et al., 2015b). This is of particular interest in this study, as the complement sub-component C1q has been described as another functional ligand for LAIR-1. Myeloid cells produce C1q and complement activation requires surface bound IgM or IgG molecules nearby. Studies by Son et al demonstrate that the C1q-LAIR-1 interaction can directly inhibit dendritic cell activation and differentiation (Son et al., 2012, Son and Diamond, 2015, Son et al., 2017).

SP-D is a collectin (collagen-containing c-type lectin). Collectins are part of the innate immune system and are soluble pattern recognition receptors (PRRs). They bind pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) to trigger an inflammatory response to eliminate pathogens. Olde Nordkamp et al demonstrated via binding assays that SP-D interacts with LAIR-1 via its collagen domains, inhibits immune activation and production of reactive oxygen species (ROS), suggesting a role in lung homeostasis (Olde Nordkamp et al., 2014). ROS is released by inflammatory cells, along with hydrogen peroxide. Mutations in HBV infection have implicated higher endoplasmic reticulum stress and increased ROS production and inflammatory cytokines (Lee et al., 2015). SP-D expression has been detected in the lung and GI tract (Herias et al., 2007), and their role in the lung has been well established. Saka et al presented immunohistochemistry data showing that SP-D is present in the bile ducts of healthy liver from children and accumulates in hepatocytes of cholestatic livers, however the authors acknowledge the major limitation of small sample sizes (Saka et al., 2015).

The relationship between SP-D and chronic viral infection has not been investigated. If SP-D is present in hepatocytes and the liver, with the collagen domain interaction with LAIR-1, is there a role of this molecule in tolerance in the liver? C1q binding to LAIR-1 could also be highly relevant to CHB because of circulating immune complexes potentially binding C1q.

1.8 LAIR-1 Function

1.8.1. Signalling

The downstream molecular signalling pathways of LAIR-1 are not known but *in-vitro* activation of LAIR-1 by monoclonal antibodies or binding to collagen induces phosphorylation of ITIM motifs and recruitment of Src-homology-2-domain-containing phosphatases (SHP-1 or −2) (Meyaard, 2008) and the c-terminal Src kinase (Csk) (Verbrugge et al., 2006b) (Figure 1.9). An experimental demonstration of this effect was first carried out by Meyaard et al using sodium pervanadate (induces tyrosine phosphorylation) to activate NK cells (Meyaard et al., 1997). Cells were lysed and LAIR-1 proteins immune-precipitated with cross-linking mAb DX26. Western blot analysis showed binding of SHP-1 and SHP-2 phosphatases to LAIR-1 lysates. Many inhibitory receptors have ITIM in their cytoplasmic domain triggering a cascade of downstream signalling events that abrogate effector functions of the cell. In general there is limited knowledge of LAIR-1 signalling pathway, with little *in vivo* data.

It has been suggested that given the abundant expression of LAIR-1 on most immune cells, regulation can occur at cell receptor level with downregulation upon activation/maturation or via shed receptors or soluble proteins that interfere with receptor ligand binding.

1.8.2. Effector functions affected by LAIR-1

Not all inhibitory receptors have a role in exhaustion or compromising the function of cells, for example PD-1 has a role in cell activation (Nguyen and Ohashi, 2015). The study of the role of LAIR-1, has been important, given that it is abundantly expressed on immune cells. In the 1990s several studies demonstrated that LAIR-1 cross-linking can inhibit target cell lysis by NK cells, cytotoxic activity of effector T cells and downregulate B cell receptor (BCR) signalling (van der Vuurst de

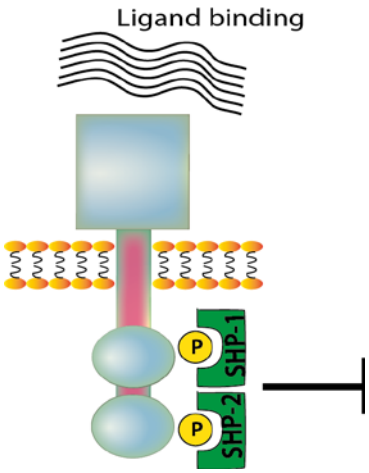
Vries et al., 1999). Van der Vuurst were able to demonstrate that *in vitro* prolonged BCR stimulation forces the downregulation of LAIR-1 on naive B cells A limitation of most of these studies is that they were performed using cross-linking of LAIR-1 by monoclonal antibody, as the ligand was only subsequently identified. Later Poggi et al were able to show Chronic lymphocytic leukaemia (CLL) cells that did not have LAIR-1 expression had no inhibitory capacity (Poggi et al., 2008).

Poggi et al showed p40 (later known as LAIR-1) is able to inhibit the cytolytic activity mediated by both $CD3^{neg}CD16^{pos}$ NK cells and $CD3^{neg}TCR^{pos}$ T cells against tumour target cells (Poggi et al., 1995). Lebbink et al found simultaneous cross-linking of hLAIR-1 using plate-bound anti–hLAIR-1 mAb or collagen I or III caused marked inhibition of degranulation (they looked at release of b-glucuronidase) (Lebbink et al., 2006). Experiments by Jansen et al involved healthy PBMC and IFNγ-Elispot analysis on T cells, which was found to be inhibited by antibody-induced cross-linking of LAIR-1 and by collagen (Jansen et al., 2007). Meyaard et al showed that cross-linking can downregulate NK cell-mediated cytotoxicity on resting and activated NK cells (Meyaard et al., 1997).

Baseline LAIR-1 expression is low on NK cells in chronic Epstein–Barr virus (EBV) (Aoukaty et al., 2003) but with high expression on B cells in HIV infection (De Milito et al., 2004). Maasho et al (Maasho et al., 2005) and Jansen et al (Jansen et al., 2007) described high levels of LAIR-1 expression on naive T cells which is in contrast to the expression of other inhibitory receptors, which are only expressed upon activation of T cells as a feedback loop to 'switch off' T cells preventing excess inflammation and immune pathology. However, Maasho et al (Maasho et al., 2005) pointed out LAIR-1 cross-linking could inhibit TCR-mediated signals in naive T cells and hypothesised LAIR-1 is also a mechanism for inhibiting initiation of immune responses. There is, however inconsistency with this theory, as demonstrated by Jansen et al who showed *in vitro* stimulation of T cells resulted in decreased surface expression of LAIR-1. However these two studies were performed in the presence of different stimulation protocols.

It has been speculated that cells that encounter more ligand have a higher threshold for activation and therefore comparatively higher expression of inhibitory receptor, and the presence of such high levels of LAIR-1 maintains homeostasis and prevents autoimmunity, but this theory requires further substantiation.

We were interested to see whether higher LAIR-1 expression correlates with functional impairment of immune cells in viral infection.



- **Downregulates cell activation**
  Poggi et al, Frontiers in Imm 2014
- **Reduced cytotoxicity of NK cells**
  Meyaard et al, Immunity 1997
- **Inhibits cytotoxic activity of effector T cells**
  Jansen et al, Eur J of Imm 2007

Figure 1.9: Structure of LAIR-1 molecule and function.

Ligand binding triggers a cascade of signalling events. LAIR-1-mediated inhibiting signal occurs via the recruitment of immunoreceptor-tyrosine inhibiting motif (ITIM) in its cytoplasmic tail, with SHP-1 phosphatase.

1.8.3 Role in disease

LAIR-1 captured our interest, as the ligand is abundant in the diseased liver in CHB. Collagen is implicated to play a role in several rheumatological diseases and autoimmune conditions. Researchers have speculated that the LAIR-1-collagen interaction may have a role in these diseases; however, little has been published regarding this. Previously published studies do not show the interaction of immune cells directly with collagen. Much of the work to-date has either related to the function of ligand binding or the potential of sLAIR-1 or LAIR-2 as biomarkers, speculating but not confirming the role of LAIR-1 in disease.

Despite a background of *in-vivo* and *in-vitro* data regarding the potent inhibitory potential of LAIR-1 on cross-linking with collagen there has not been significant progression towards the clinical relevance of the role of LAIR-1. Interestingly, there has been limited focus on the role of LAIR-1 in organs where there is collagen deposition.

Simone et al attempted to explore the role of LAIR-2 in organ specific autoimmunity, looking at the thyroid gland, which in autoimmune conditions can undergo remodelling (changes in matrix structure) and increased inflammatory infiltrate. They found LAIR-2 levels were seen to be increased in serum from patients with autoimmune thyroid disease (Simone et al., 2013). Increased LAIR-2 levels in synovial fluid had been seen in patients with RA and ankylosing spondylitis. Olde Nordkamp et al showed synovial fluid from RA patients had higher levels of LAIR-2 compared with OA patients (OA patients were used as a control group as this is not an autoimmune disease). The major criticism of this study would be that they had a small group of patients, analysing only sixteen subjects (Lebbink et al., 2008). They further studied in this disease the role of secreted soluble LAIR-1 and LAIR-2 as potential biomarkers (Zhang et al., 2014), (Olde Nordkamp et al., 2011). Study findings demonstrated CD4+ T cells were the main producers of LAIR-2 and urine LAIR-2 correlated with

markers of inflammation. However, whether these markers reflect the level of disease activity and damage in patients has yet to be investigated. Similarly high levels of LAIR-2 have been seen in ankylosing spondylitis (Diaz-Pena et al., 2012). Theoretically it has been suggested that high levels of LAIR-2 might contribute to pathogenesis of disease, acting as a pro-inflammatory mediator, with ongoing activation of immune cells as a result of preventing LAIR-1-collagen interaction and thereby inhibitory effects to the cell (Olde Nordkamp et al., 2011), (Simone et al., 2013).

There is evidence of inhibitory receptors overriding the mechanisms of activation to dampen anti-tumour and anti-viral responses. NK cells have a role in controlling tumour cell growth. NK cells reside in the liver, as do bone marrow stromal cells that are reported to have a role in haemopoietic malignancies and stem cell proliferation (Poggi et al., 2005). The close interaction of NK cells with cells that may express collagen has been explored looking at LAIR-1 in Acute myeloid leukaemia (AML) and Chronic lymphocytic leukaemia (CLL). Kang et al used a LAIR-1 knock-down model achieving deletion of LAIR-1 by silencing expression using Lentivirus-encoded short-hairpin-RNA (shRNA) (Kang et al., 2015). Knock-out models remove the gene whereas knock-down models will target specific RNA and reduce expression of protein in cells. Limitations of the latter technique can result in mice not showing the same phenotype as knock-outs, however Kang et al showed the LAIR-1 knock-down did not alter the cell cycle state or normal haematopoiesis, consistent with findings from knock-out models described earlier. The study identified that the knock-down model in mouse and human AML cells markedly increased apoptosis and inhibited growth of leukaemia cells. Given inhibition of this receptor does not affect normal haematopoiesis, potentially makes it a more interesting therapeutic target for treating leukaemia. CLL is a heterogeneous disease with a variable clinical course, akin to CHB, also being heterogeneous and dynamic in nature. Studies have consistently shown higher expression of LAIR-1 in early stages of disease and lower expression in high-risk CLL patients (Poggi et al., 2008). A simple study of a large cohort of 311 CLL patients showed its potential role as a marker to predict advanced disease, using time to first treatment (TTFT) as a main parameter (Perbellini et al., 2014). Unfortunately the follow-up of the study was too short to

investigate the role of expression and survival of patients, which would be a more robust end point. TTFT, however, is a commonly used validated prognostic marker related to tumour progression. Interestingly they found only ~60% of patients were LAIR-1 positive (however, the cut-off value of 30% was used to differentiate LAIR-1 positivity, which was determined as an initial observation from the spread of values and then validated using a receiver operating characteristic (ROC) curve for each cut-off value), and the 'negative' patients had more advanced disease with a tendency for autoimmune haemolytic anaemia at presentation. The observation of lower expression of LAIR-1 on B cells showing a more aggressive clinical phenotype, could lead us to speculate that these cells are less inhibited and cause "more harm to self" in the process of attack against disease. This also provides further evidence advocating the important role that LAIR-1 potentially plays in dampening down immune responses to maintain haemostasis.

Kennedy et al measured LAIR-1 on global T cells as a marker of exhaustion in a population of young adults with CHB (age range 10-30 years) (Kennedy et al., 2012). They found no difference in LAIR-1 expression between patients and healthy controls on CD4+ or CD8+ T cells. We are the first to explore expression of LAIR-1 on NK cells in CHB. Perhaps this receptor has been neglected due to the lack of tantalising data on T cells, which have been the main focus of the CHB literature for some time.

However, with respect to viral infection, patients with chronic active Epstein-Barr virus (CAEBV), which has a more severe and prolonged disease course profile of infectious mononucleosis, had much lower expression of LAIR-1 (Aoukaty et al., 2003). Aoukaty et al were able to demonstrate the functional role of LAIR-1 in cytotoxicity with addition of mAb (DX26) to human CAEBV NK cells, which reduced their cytotoxicity. The degree of impairment was significant, and an isotope control did not elicit the same effect. Interestingly NK cells from CAEBV patients also had impaired IFNγ secretion, similar to what has been described in CHB, however, they did not differentiate whether this

was the $CD56^{bright}$ NK cell subset. These cells had a phenotype that displayed low expression of LAIR-1 compared with healthy controls.

1.9 Hypothesis and Aims

1.9.1 Hypothesis

Engagement of LAIR-1-expressing NK cells by collagen in the liver drives their impaired function in CHB.

1.9.2 Aims

1.9.2.1 Aim 1

Investigate expression of LAIR-1 on NK cells in the periphery and liver of CHB patients.

1.9.2.2 Aim 2

Investigate effect of LAIR-1 expression on NK cell function in CHB.

1.9.2.3 Aim 3

Investigate whether there is cross-talk between collagen producing cells in the liver (HSCs) and LAIR-1 on NK cells.

Chapter 2: Materials and Methods

2.1 Study ethics

Written informed consent was obtained from all patients (study cohorts included those with CHB, HCC and NAFLD) and also a cohort of HC's (Figure 2.1 summarises the samples used within this thesis).

PBMC

HC	n=21
CHB	n=69
HCC	n=8

Paired PBMC and intrahepatic lymphocytes (IHL)

CHB	n=17
NAFLD	n=7

Figure 2.1: Summary of study cohorts

Summary of the number of patients and HC included in this study. Patients included had CHB, NAFLD and HCC.

The study has been approved by the local ethics committee (Barts and The London NHS Trust Ethics Review Board, REC references pertinent to this study include: P/01/23, 07/Q0604/20, 09/H0717/32, 10/H0715/39). All sample and data storage comply with the requirements of Data Protection Act 1998.

2.1.1. Study cohorts

Sixty-nine CHB treatment naive patients attending outpatient clinics and seventeen CHB patients in attendance for liver biopsy were screened and invited to participate in the study. These patients receive hepatology specialist secondary care at the Royal London Hospital. All patients were seropositive for HBsAg and negative for anti–hepatitis D virus, anti-HCV, anti-HIV-1/2, and autoantibodies. In order to characterise disease phase, data was collected retrospectively from laboratory biochemistry and serology tests. Clinical parameters were repeated at every clinic attendance and over the course of a year this data was aggregated in order to clarify the patients' disease profile. At the time of inclusion in the study, blood sampling was performed. The mean HBV DNA value was calculated using three values from the preceding year. HBV DNA was quantified by real-time PCR (Roche COBAS AmpliPrep/COBAS Taqman HBV test v2.0-dynamic range 20 to $1.7x10^8$ IU/ml-Roche molecular diagnostics, Pleasanton, CA). HBsAg titre (Abbott Architect) was also quantified. Serum was tested for HBeAg and Hepatitis B e Antigen antibodies (anti-HBe) with a chemiluminescent microparticle immunoassay (Abbott Architect, Abbott Diagnostics, Abbot Park, IL). HBV genotype was recorded where available, along with serum transaminases, Ishak fibrosis stage and necroinflammatory scores where paired liver biopsies were performed.

The details of study participants with CHB from whom PBMC and paired data was collected is summarised in tables 2.1 and 2.2. These tables also summarise data from eight patients with HCC who were screened and invited to participate in the study. These patients were HCC treatment naive. In addition seven patients with abnormal liver functions in attendance for liver biopsy were screened and invited to participate in the study. These patients had NAFLD and were negative for HBsAg, anti–hepatitis D virus, anti-HCV as well as anti-HIV-1/2 negative. PBMC were also taken from twenty-one healthy, uninfected volunteers and described as the HC cohort within this study as detailed in table 2.1.

	Age (years) Median (range)	**Sex (%)** Male:Female	**HBeAg (%)** Positive:Negative **Disease phase (n)**	**HBsAg (IU/ml)** Median (range)	**HBV DNA (log)** Median (range)	**ALT (IU/L)** Median (range)
CHB n= 69	29 (5-68)	55:45	43:57 IT (n=13) HBeAg+ IA (n= 17) IC (n=20) HBeAg- IA (n=19)	9,176 (0.03-1,249,250)	5.25 (0-9.52)	32 (12-918)
HC n= 21	35 (23-52)	57:43	n/a	n/a	n/a	n/a
HCC n= 8	57 (38-67)	90:10	n/a	n/a	n/a	34 (10-109)

Table 2.1: Summary characteristics of study cohorts (PBMC).

Summary characteristics of PBMC taken from study cohorts of CHB and HCC patients, as well as HC.

Abbreviations: Hepatitis B e Antigen (HBeAg), Hepatitis B surface Antigen (HBsAg), serum alanine transaminase (ALT), not applicable (n/a), immune tolerant (IT), HBeAg positive immune active (HBeAg+ IA), inactive carrier (IC), HBeAg negative immune active (HBeAg- IA).

	Age (years) Median (range)	**Sex (%)** Male:Female	**HBeAg (%)** Positive:Negative **Disease phase (n)**	**HBsAg (IU/ml)** Median (range)	**HBV DNA (log)** Median (range)	**ALT (IU/L)** Median (range)	**Fibrosis score** (n)	**NI score** (out of 18) (n)
CHB n= 17	29 (25-52)	65:35	41:59 IT (n=1) HBeAg+ IA (n= 6) IC (n=2) HBeAg- IA (n=8)	11,842 (533.5-40,354)	5.25 (2.17-8.54)	51 (15-918)	0 (n=7) 1 (n=2) 2 (n=3) 3 (n=2) 4 (n=1) 5 (n=2) 6 (n=0)	1 (n=4) 2 (n=4) 3 (n=3) 4 (n=4) 7 (n=1) 9 (n=1)
NAFLD n= 7	40 (19-79)	43:57	n/a	n/a	n/a	105 (37-246)	All patients had steatosis but no cirrhosis	n/a

Table 2.2: Summary characteristics of the study cohorts (paired data – CHB and NAFLD)

Summary characteristics of the study participants for paired data (blood and liver) in CHB and NAFLD patients.

Abbreviations: Hepatitis B e Antigen (HBeAg), Hepatitis B surface Antigen (HBsAg), serum alanine transaminase (ALT), not applicable (n/a), immune tolerant (IT), HBeAg positive immune active (HBeAg+ IA), inactive carrier (IC), HBeAg negative immune active (HBeAg- IA), Necroinflammatory (NI) score.

2.1.2 Defining disease phases

CHB is a dynamic disease and patients progress through phases as illustrated in Figure 2.2 (Liaw and Chu, 2009). These distinct disease phases are characterised by serum ALT, HBV DNA and HBeAg status (detailed in Table 2.3). These clinical parameters are repeated at every clinic attendance and over a course of a year these data were aggregated in order to clarify the patients' disease profile. Upon determining these phases treatment decisions are made.

Historically CHB is thought to progress through four disease phases; immune tolerant (IT), HBeAg positive immune active (IA), inactive carrier (IC) and in a proportion of patients, immune escape and viral reactivation leading to HBeAg negative CHB.

Early infection in childhood is generally associated with high levels of virus, normal ALT, minimal inflammation and mild or no fibrosis on liver biopsy. This has been described as the 'IT' disease phase.

The IA phase is thought to represent an awakening of the immune response with more marked immune activity reflected in fluctuation of the serum ALT. Persistence of this disease phase is thought to result in progressive liver damage and thus treatment is indicated to prevent the development of fibrosis and cirrhosis. HBeAg seroconversion combined with low viral load (<2,000 IU/ml) and normal serum ALT represents patients who become IC and have an increased degree of immune control. If this occurs before the age of 30 there is reduced risk for the development of HCC.

A proportion of IC will develop disease reactivation with the emergence of viral escape mutants and elevated HBV DNA levels and serum ALT reflect this. This is referred to as HBeAg negative CHB as HBeAg is not secreted owing to mutations in the pre-core or core promoter areas of the virus.

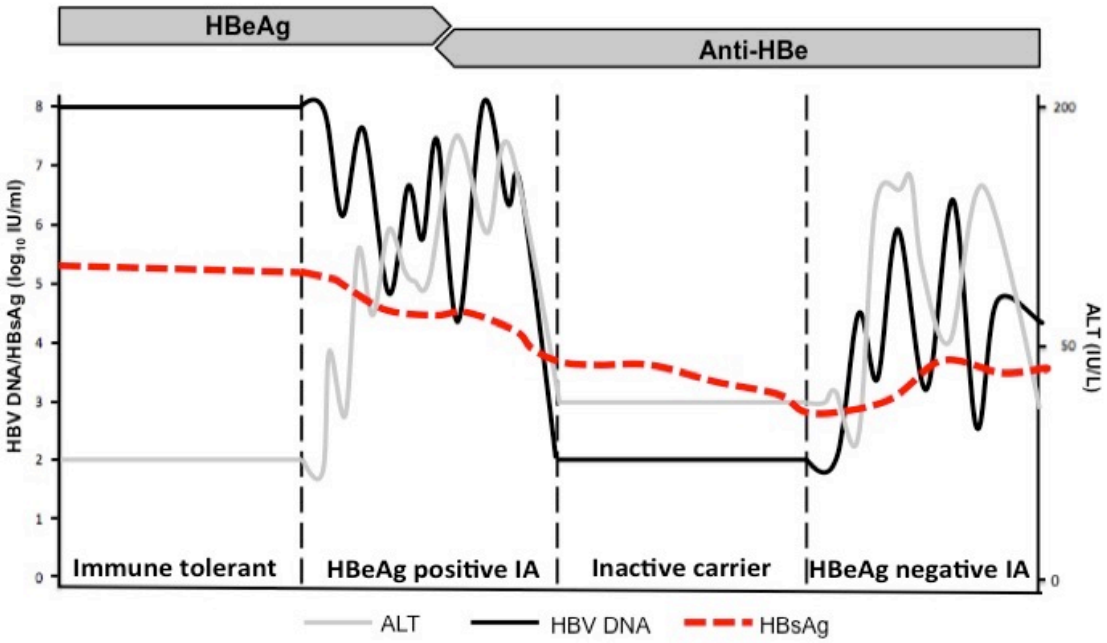


Figure 2.2: Diagram of disease phases.

Adapted from Gill & Kennedy, Clin Med 2015

CHB is a dynamic disease with distinct disease phases; immune tolerant (IT), HBeAg positive immune active (IA), inactive carrier (IC) and in a proportion of patients, reactivation of the virus occurs leading to HBeAg negative CHB. These phases can be characterised by expression of HBeAg, levels of viral load, qHBsAg levels and ALT.

Phase	***HBeAg***	***ALT***	***HBV DNA***
Immune tolerant	Positive	<40 IU/L	HBV DNA > 8 log IU (100,000,000 IU/ml)
HBeAg positive immune active	Positive	>40 IU/L	Any
HBeAg negative immune active	Negative	Any	>3.3 log IU (2,000 IU/ml)
Inactive carrier	Negative	<40 IU/L	<3.3 log IU (2,000 IU/ml)

Table 2.3: CHB disease phases

CHB disease phases are characterised by biochemical and virological markers. For the purpose of this study patients clinical parameters were aggregated in order to clarify the patients' disease profile and categorised in to disease phases in keeping with guidelines.

2.2 Sample preparation and isolation

2.2.1 Isolation of peripheral blood mononuclear cells

PBMC were isolated from whole blood using Ficoll-plaque (GE Healthcare, Buckinghamshire, UK) density gradient centrifugation. Blood was taken from patients and healthy controls in 9ml BD vacutainer CPT tubes containing sodium heparin. In a 50ml falcon tube the blood was diluted in RPMI 1640 (Sigma Aldrich) in a 1:2 ratio and then carefully layered over 12.5ml Ficoll-Plaque. This was then centrifuged at 2500 rpm at 20°C for 20 minutes without brakes. Into a new 50ml falcon tube, the PBMC layer was aspirated using 2ml Pasteur pipettes whilst leaving the Ficoll-plaque layer undisturbed at the interphase. The mononuclear cell layer was diluted with RPMI and washed at 1500 rpm at 20°C for 5 minutes. The supernatant was removed and the PBMC pellet re-suspended in RPMI to be counted prior to a final wash. Cells were diluted 1:9 in trypan blue (Sigma-Aldrich, St Louis, USA) and were counted using Neubauer counting chamber under a light microscope. The number of cells was determined using the following formula:

Number of cells/ml = number of cells counted x dilution factor x 10^4

The cells were washed and either used fresh for staining or re-suspended in heat inactivated foetal calf serum (FCS) (Invitrogen™, California, USA) with 10% dimethylsulphoxide (DMSO) (Sigma-Aldrich, St Louis, USA) and transferred into cryovials (Corning, New York, USA). Cryovials were frozen at -80°C in Mr Frosty® (Fisher Scientific, Waltham, USA) before transfer to gas-phase nitrogen storage tanks. Cryopreserved PBMC were used later for overnight stimulation experiments.

2.2.2 Serum

For the purposes of serum extraction blood was taken in tubes containing a clot activator. These underwent direct centrifugation to remove red blood cells from the serum layer. Serum was removed and immediately frozen at 80°C in 500µl volumes in cryovials for later use.

2.2.3 Urine

Urine was aseptically collected from HC and patients. Individuals were asked to void directly in to a sterile container. Urine was directly aliquoted into 500µl aliquots and stored at -80°C for later use ELISA experiments (described in section 2.7).

2.2.4 Liver biopsy sample acquisition

The ratio of liver parenchymal to non parenchymal cells is approximately 70:30 and IHL constitute approximately 25% of the non-parenchymal cells (Gao et al., 2008). Although there has been a movement towards non-invasive diagnostic tests, the gold standard to diagnose and assess liver fibrosis remains liver biopsy. The technique was first reported in 1923, and subsequently modified but is widely used and associated with low mortality (Grant and Neuberger, 1999). At the Royal London Hospital, the hepatology department continues to perform percutaneous liver biopsies. Access to this tissue is an opportunity to explore the unique phenotype and functional properties of IHL and liver resident IHL in diseased liver states. Patients attended the endoscopy unit for this outpatient procedure and were consented for complications of bleeding, perforation, infection and pain. A nurse was present to assist with the procedure and sample collection. The biopsy site is usually located in the seventh or eighth intercostal space in the midaxillary line, and was confirmed using a bedside portable ultrasound machine. A sterile field was prepared, and the skin cleaned and infiltrated with local anaesthesia administered in both superficial and deep planes extending to the capsule of the liver. A

small skin incision aids the biopsy needle to pass more easily through the skin. In our department we use the Menghini needle to sample hepatic tissue. The Menghini needle employs a suction technique and has a feature with a small stylet that occludes and prevents aspiration of tissue in to the syringe preventing disruption of tissue morphology. The Menghini needle is attached to a 10 ml syringe filled with 5 mls saline as it is advanced in to the intercostal space with the plunger retracted. On patient expiration and with maintained plunger retraction of the syringe a quick movement advancing the needle forward in to the liver and then quickly removing it is performed. No more than two passes through the liver were taken. The contents of the needle was assessed, and split. At least 20mm of liver tissue would be sent to histopathology in a formalin-containing pot and the excess tissue was transferred to a saline pot for acquisition of intrahepatic lymphocytes. After the procedure the patient is asked to lie in a supine position and has regular observations of pulse and blood pressure as per local procedural policy prior to discharge home later that day.

2.2.5 Isolation of intrahepatic lymphocytes

Our lab has expertise in processing liver tissue based on more than ten years of experience. Classically techniques involve gentle mechanical dissociation of tissue, which can affect the yield from biopsy samples (Morsy et al., 2005). Enzymes for the digestion of tissue were not used in my protocol.

In a petri-plate (Sarstedt, Numbrecht, Germany), liver biopsy tissue was suspended in RPMI and gently mashed using the back of a 5ml syringe plunger. The cell suspension was then passed through a 70µm cell strainer (BD Biosciences) and centrifuged at 1500 rpm for 15 minutes to discard cell debris. The cell pellet was resuspended in RPMI for counting. The cells were then used immediately for characterisation by flow cytometry and *in vitro* functional experiments.

2.3 Culturing K562 cell line

Although NK cells produce cytokine their principle effector function is via target cell lysis (Caligiuri, 2008). This can be assessed using a degranulation assay measuring lysosome-associated membrane protein (LAMP)-1 or CD107a expressed on the activated cell surface or a cytotoxicity assay. Previous experiments have demonstrated antibody induced cross-linking of LAIR-1 can downregulate NK cell-mediated cytotoxicity (Meyaard et al., 1997) and degranulation (Lebbink et al measured release of b-glucuronidase) (Lebbink et al., 2006). The K562 cell line has attained widespread use as an *in vitro* target for flow cytometry based NK cell cytotoxicity assays (Bryceson et al., 2012). K562 cells belong to an immortalised myeologenous leukaemia line. They lack MHC complex required to inhibit NK cell activity and therefore are sensitive to killing by NK cells.

For the purposes of NK cell degranulation, the K562 cell line was utilised as the target. Previously cryopreserved K562 were thawed slowly within a 37°C waterbath. These were resuspended in 10ml RPMI with 10% FCS and Penicillin/Streptomycin (complete RPMI; cRPMI). This solution was then centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the cellular pellet resuspended in 10ml of the cRPMI and transferred into a small ($25cm^3$) cell culture flask and incubated at 37°C. These cells progress into log phase in 5 to 7 days. Counting was therefore initiated at day 3 when the density had reached 0.7 to $0.8x10^6$/ml. The cells were then split into approximately 0.4 million cells per ml with fresh cRPMI. From this point the cell count doubles every 24 hours and therefore these were split and the media replenished every other day to a volume, which contained 300,000 cells. These were then used at the appropriate concentration for the degranulation assay as described in section 2.5.2. When the total number of cells was more than $3x10^6$/ml, they were frozen down as stock in liquid nitrogen at 3 million cells/ml in freeze medium.

2.4 Flow cytometry

Flow cytometry is the principal tool used in this thesis to interrogate the phenotype and characteristics of cells. Results are based upon light scattering properties of the cells being analysed. Below is a discussion of the limitations of fluorescence compensation and non-specific binding, and methods incorporated in the experiment protocol to overcome these.

Fluorescence compensation is the concept by which amounts of spectral overlap are estimated and subtracted from the total detected signals to generate an estimate of the actual amount of each dye (Herzenberg et al., 2006). In order to measure the spectral overlaps, 'compensation control' samples are used (Hulspas et al., 2009). These control samples are labelled with only one of the fluorochromes used in the panel of colours. They are used to estimate the contribution of the spectral overlap to the overall positive signal.

Non-specific binding of mAb-fluorochrome conjugates can arise from interactions between probes and the cell surface. To overcome this limitation and improve the standard to make the FACS data interpretable, control samples are essential in flow cytometry (Kim et al., 2007). We have used 'fluorescence minus one' (FMO) for all antibodies for which the threshold between unstained and less fluorescent cells is unclear. The data collected for cells stained with FMO control is compensated and gated consistently in the same way as cells stained with the full complement stain set. These control samples have been used to check for spectral overlap. An isotope has not been used, as often these are more helpful for demonstrating where there is poor blocking of the cells. In addition, the protocol for functional experiments has involved an Fc-FcR-blocking step, to reduce background non-specific binding.

All stains for flow cytometry analysis were performed in 96 U-bottomed plates (Sarstedt, Numbrecht, Germany) at 4ºC.

2.4.1 Extracellular staining of PBMC and IHL

Cells were washed in PBS (Invitrogen™, California, USA) and centrifuged at 1500 rpm for five minutes. The supernatant was discarded and cell pellets re-suspended and divided between staining wells. The cells were stained with Live/Dead® Cell viability stain (Invitrogen™, California, USA) for ten minutes at 4ºC in the dark. Cells were then washed and FcR blocking reagent (Miltenyi) was added to the cells to avoid non-specific binding of antibodies at a concentration of 1:10 for a further ten minutes, after which the relevant anti-human monoclonal antibodies in PBS were added for thirty minutes at 4ºC in the dark, with no washing step in between. Appropriate FMO were used where necessary. Post incubation the cells were washed by centrifugation and fixed with 75µl/well of BD™ Cytofix-Cytoperm (Beckton Dickinson, Oxford, UK). Cells were transferred to 500µl polystyrene tubes for acquisition. Data was acquired on BD™ LSR II flow cytometer using Diva (Beckton Dickinson, New Jersey, USA) and analysed using FlowJo (Treestar Oregon, USA) version X.0.7.

The gating strategy for identification of NK cells within the peripheral and intrahepatic compartment is shown in Figure 2.3 and 2.4 respectively. Figure 2.5 shows the gating strategy for CD8+ T cells.

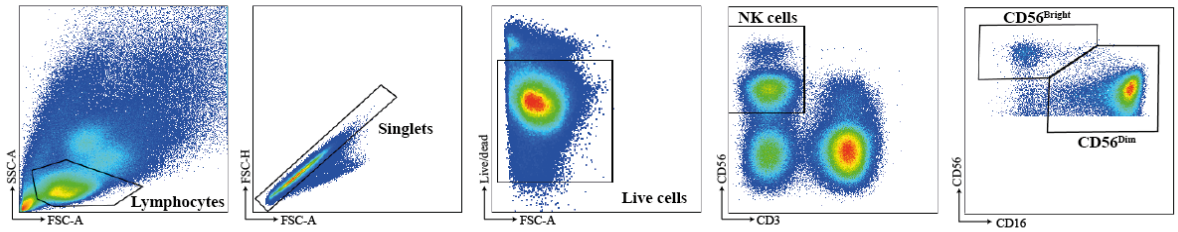


Figure 2.3: Gating strategy for identification of NK cells from PBMC.

PBMC were stained for $CD56^{hi}CD3^{neg}$ NK cells after exclusion of doublets and dead cells. CD16 was used as a marker to differentiate between $CD56^{bright}$ ($CD56^{hi}CD16^{neg}$) and $CD56^{dim}$ ($CD56^{lo}CD16^{pos}$) $CD3^{neg}$ NK cell subsets.

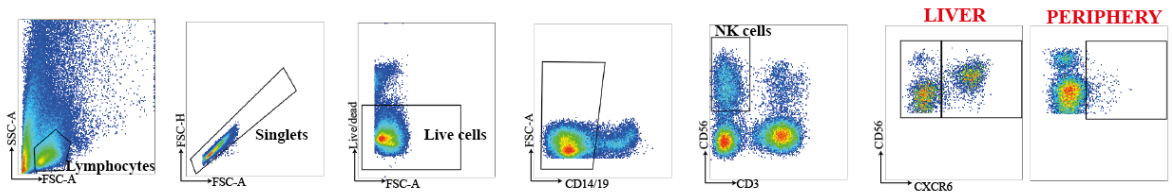


Figure 2.4: Gating strategy for identification of NK cells and lrNK cells from IHL

IHL were stained for $CD56^{hi}CD3^{neg}$ NK cells after exclusion of doublets, dead cells and CD14/19 positive cells. CXCR6 was used as a marker to differentiate between $CD56^{bright}$ ($CD56^{hi}CXCR6^{pos}$) and $CD56^{dim}$ ($CD56^{lo}CXCR6^{neg}$) $CD3^{neg}$ NK cell subsets. CXCR6+ NK cells are enriched in the liver.

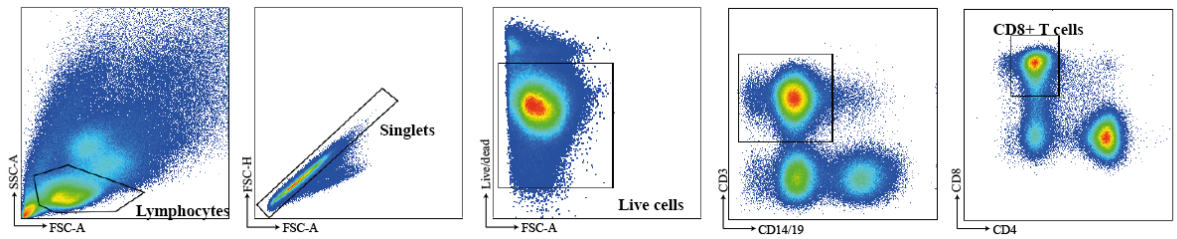


Figure 2.5: Gating strategy for identification of CD8+ T cells from PBMC.

PBMC were surface stained with mAb CD8 and CD4 to identify CD8+ T cells after excluding doublets, dead cells, B cells (CD19) and monocytes (CD14).

2.4.2 Intracellular staining of PBMC and IHL

Cells requiring an intracellular stain were surface stained as described above, following which they were washed and re-suspended in BD^{TM} Cytofix-Cytoperm for thirty minutes at 4ºC in the dark. After permeabilisation and fixation the cells were stained intracellularly using the relevant anti-human antibodies in 0.1% saponin (in PBS with 1% FCS) for an additional thirty minutes at 4ºC in the dark. Cells were there transferred to tubes for acquisition on LSR II flow cytometer as described earlier in section 2.4.1.

The antibodies used for both extra- and intra-cellular staining are listed below in Table 2.4.

Surface Marker	Fluorochrome	Clone	Manufacturer	Dilution
Live/Dead	UV	n/a	Fisher Scientific UK Ltd	1.5ul/ml
CD3	PE-Cy7	UCHT1	eBioscience	1:100
CD3	BV605	OKT3	Biolegend	
CD56-ECD	FITC	A82943	Beckman Coulter	2.5:100
CD56	ECD	A82943	Beckman Coulter	3:100
CD56	PE-Cy7	NCAM16	Becton Dickinson UK	
CD38	PETexasRed	HIT2	Becton Dickinson UK	1:200
CD16	APC-CY7	3G8	Becton Dickinson UK	2:100
TIM-3	APC	F38-2E2	eBioscience	1:100
LAG-3	APC	3DS223H	eBioscience	1:100
HLADR	V500	G46-6	Becton Dickinson UK	1:100
CD14	V500	M5E2	Becton Dickinson UK	1:100
CD19	BV510	SJ25C1	Becton Dickinson UK	1:100
CD4	BV421	OKT4	Biolegend	1:100
CXCR6	BV421	K041E5	Biolegend	5:100
CD57	BV605	NK-1	Becton Dickinson UK	2:100
CD57	FITC	TB01	eBioscience	
PD1	PerCP/Cy5.5	EH12.2H7	Biolegend	1:50
CD8	AF700	OKT8	eBioscience	1:200
LAIR-1	PE	NKTA255	eBioscience	1:100
CD107a	PE	H4A3	Becton Dickinson UK	1:100
CD107a	APC	H4A3	Becton Dickinson UK	
Intracellular Marker				
IFNγ	V450	B27	Becton Dickinson UK	3:100

Table 2.4: Directly conjugated anti-human mAb

This table identifies the directly conjugated anti-human mAb against extracellular and intracellular antigens used in identification and phenotyping of cells.

2.5 Functional studies

In order to assess NK cell function the following were measured;

a) Cytokine production by intracellular staining

b) CD107a degranulation assay

2.5.1 Cytokine production by intracellular staining:

PBMC were incubated overnight with 5 ng/mL of recombinant human interleukin (rhIL)-12 and rhIL-18 (R&D Systems) for a total of twenty-one hours at 37°C. The biology of NK cells is regulated by interleukins (IL) and IL-12 is a major stimulatory cytokine that is essential for NK cell activation and proliferation (Zwirner and Domaica, 2010). Monensin (Golgistop) (4µl for 6ml of cell culture) and Brefeldin A (Golgiplug) (BD Biosciences) (1µl for 1ml cell culture) were added for the final three hours of incubation. The addition of Monensin prevents CD107a breakdown (see below CD107a degranulation assay) and Brefeldin A blocks exocytosis of IFNγ. Cells were stained, thereafter for cell surface markers, fixed and permeabilised and then stained for intracellular expression of IFNγ. The readout of IFNγ was chosen as it represents the effector cytokine which NK cells secrete and can promote engagement of other immune cells (Vivier et al., 2008).

Preliminary experiments used phorbol myristate acetate (PMA) (3ng/ml) and ionomycin (100ng/ml) as a positive control to check cytokine and cytotoxic responses from NK cells (example shown in Figure 2.6). PMA activates protein kinase C, whilst ionomycin is a calcium ionophore and stimulation while these compounds bypasses receptor/ligand interaction and results in activation of NK cells.

Unstimulated samples (i.e. media alone) have been used to define a positive/negative threshold for a cytokine response. Functional measures were corrected by subtracting background signals obtained

from medium alone sample/cytokine samples. PBMC without stimulation would create an environment less artificial but unfortunately has been previously shown to yield minimal amounts of cytokine and would be insensitive to discriminate functional change.

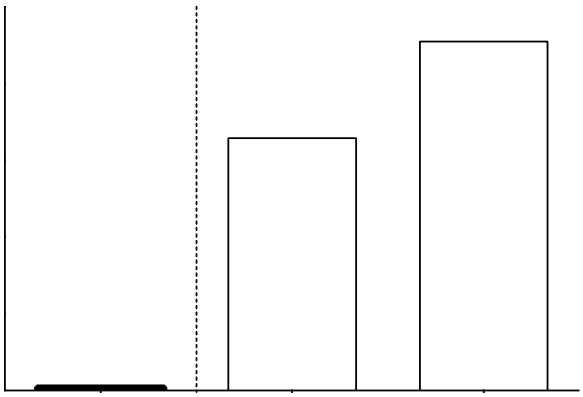


Figure 2.6: Optimisation experiment using PMA and ionomycin

PMA and ionomycin was used as a positive control to verify that cytokine stimulation with rhIL-12 and rhIL-18 generated a readout for effector cytokine production (n=1).

2.5.2 CD107a degranulation assay

The main assay traditionally used to measure lymphocyte-mediated cytotoxicity included chromium (51Cr) release assays, however flow cytometry based assays have become more popular (Zaritskaya et al., 2010). Chromium release assays had a number of disadvantages including biohazard and disposal problems for the isotope, low sensitivity and poor labelling. The method was based upon internalisation and binding of the isotope by target cells, which on lysis released the radioactive isotype, allowing it to be quantitatively detected (Brunner et al., 1968). However, unlike the flow cytometry assays they are not as robust in reproducibility or as easy to conduct. Betts et al presented a novel technique demonstrating CD107a expression as a sensitive marker of cytotoxic CD8+ T cell degranulation and this was shortly shown to be similarly as effective as a marker for NK cells (Betts et al., 2003),(Alter et al., 2004).

Preliminary experiments on healthy controls were done to check the dose response of interleukin stimulation, to avoid overstimulating cells with high dose interleukins (Figure 2.7) and to ascertain an optimal effector (E) to target (T) cell ratio for CD107a production (Figure 2.8). A dose of 5ng/ml rhIL-12 and rhIL-18 cytokines did not over stimulate NK cells and percentage IFNγ and CD107a are in keeping with published data. As seen in Figure 2.7a, a media only well was used as the negative control for gating of cytokines and CD107a expression and subtracted in final calculations. Media alone, as expected produced none or very minimal amounts of IFNγ and CD107a. No significant difference as noted between CD107a expression with effector to target cell ratios of 5:1 versus 10:1. A ratio of 5:1 was consistently used for the remainder of experiments as it allowed fewer effector cells to be used.

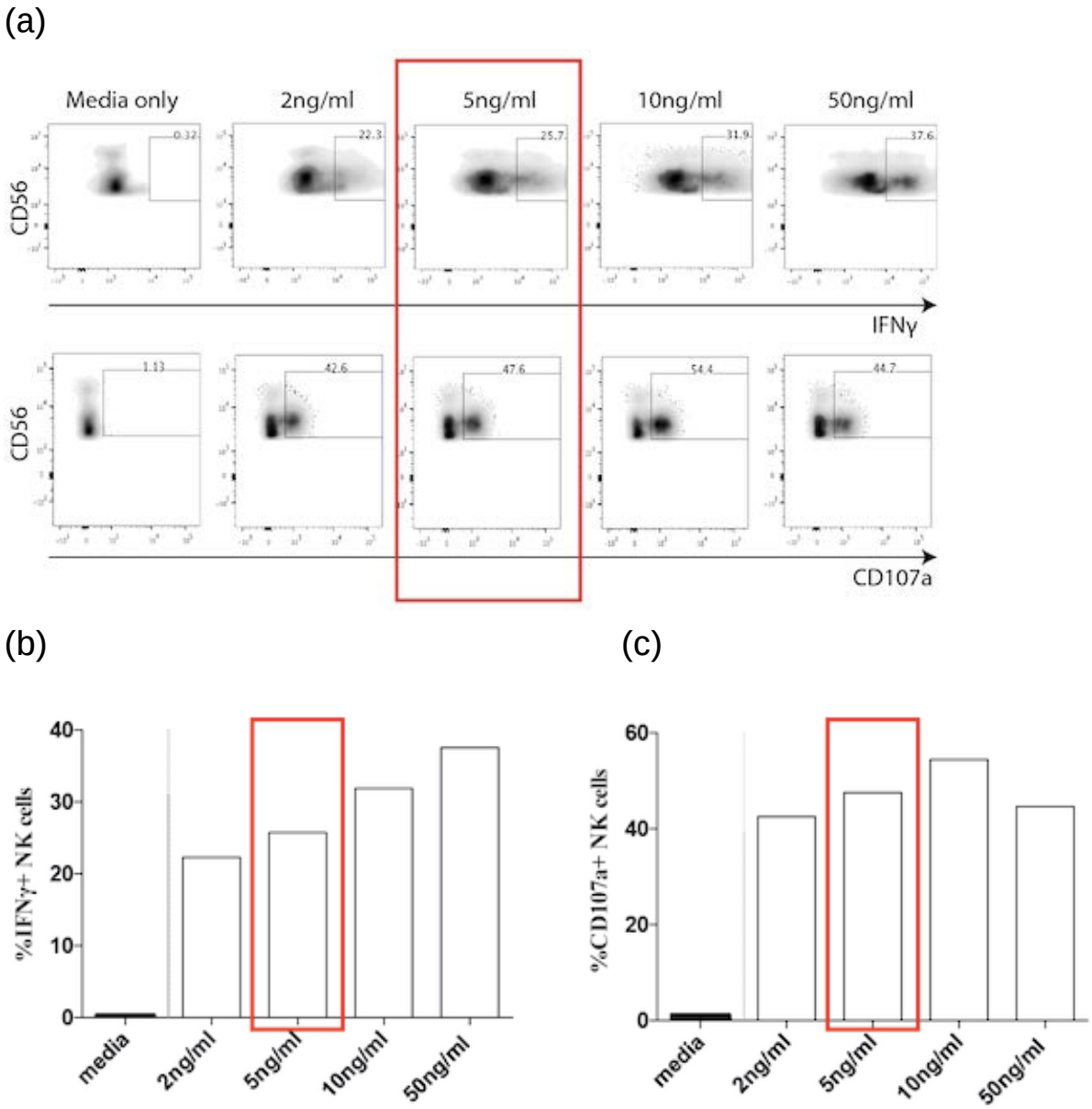


Figure 2.7: Cytokine stimulation with serial titration of rhIL-12 and rhIL-18 in HC PBMC.

(a) Representative FACS plots from one HC showing %IFNγ production and %CD107a expression of PBMC unstimulated and with stimulation with rhIL-12 and rhIL-18 at varying doses (2ng/ml, 5ng/ml, 10ng/ml and 50ng/ml). Dose response is shown in graph format for (b) %IFNγ and (c) %CD107a with serial titration of cytokines. N=1 therefore unable to show a dose response curve and descriptive statistics have been shown.

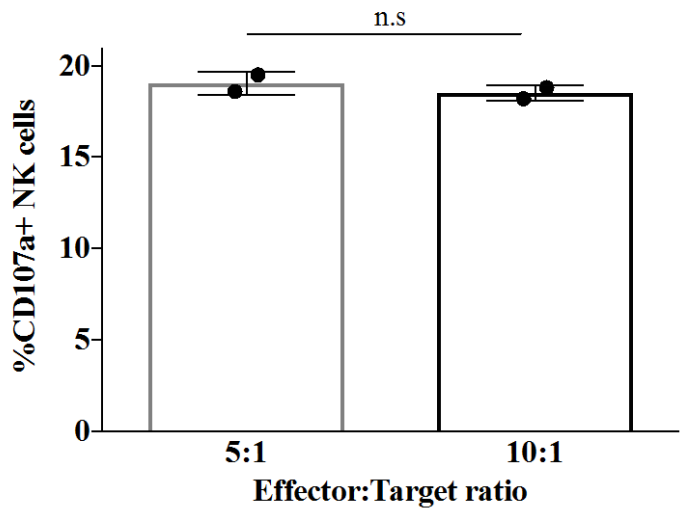


Figure 2.8: Optimising Effector:Target ratio for degranulation assay.

Degranulation of NK cells was measured using CD107a, which is extracellularly expressed by NK cells and a sensitive marker of cell activity. HC PBMC (n=2) were combined with target K562 cells at two different effector to target cell ratios of 5:1 and 10:1. Significance testing was carried out using Wilcoxon paired test.

PBMC were incubated with K562 cells (5:1, E:T ratio) for 3 hours at 37°C following overnight stimulation with a combination of rhIL-12 (5ng/ml) and rhIL-18 (5ng/ml). The effector cells need to be activated by rhIL-12, rhIL-18 prior to addition of target cells. Activated NK cells have significant killing activity compared with basal NK cells. Flow cytometry allows discrimination between effector and target cells, as well as identification of live versus dead cells. This differentiation is on the basis of marker expression. CD107a-PE antibody (BD Biosciences), Monensin (4µl for 6ml of cell culture) and Brefeldin A (1µl for 1ml cell culture) were added at the same time as the target cells. The flow cytometry based NK cell assay for cytotoxicity activity is well described within this field (Bryceson et al., 2012) (experiment summarised schematically in Figure 2.9).

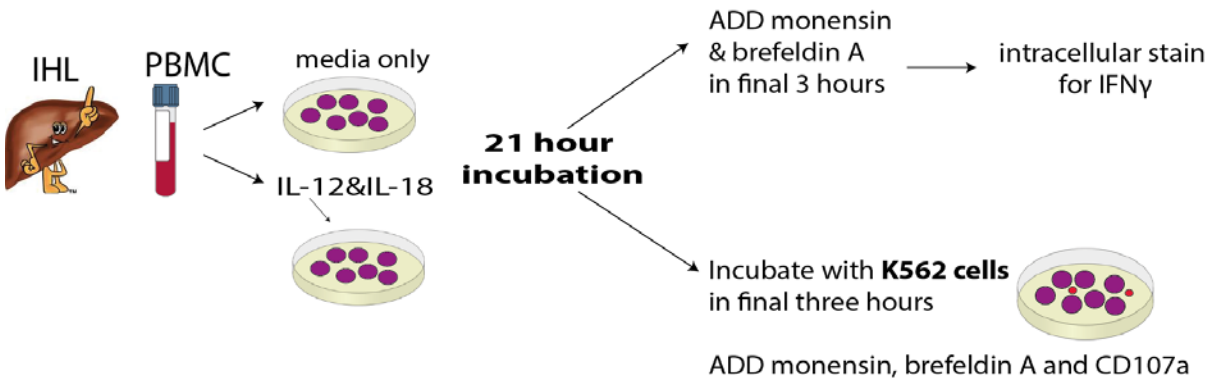


Figure 2.9: Functional study to assess NK cell function

The functionality of NK cells can be measured by cytokine production and cytotoxicity. This diagram depicts NK cell assays, which are well described in the literature.

2.5.3 Experiments involving collagen plates

The above functional experiments were done in the presence and absence of cells incubated on pre-coated collagen plates (Thermo-Fisher Ltd). Different collagens can act as functional ligands for LAIR-1 with high affinity to collagens I and III (Meyaard, 2008, Lebbink et al., 2006). Collagen I, III and V are predominant within the liver. In order to measure functional inhibition of NK cells we used a two-dimensional (2D) system with plate bound collagen. We chose to use commercial pre-coated collagen plates as these are ready to use and quality tested to help ensure consistency. Self-coating protocols can be inconsistent. These 96-well plates were pre-coated with collagen I at 5mg/ml concentration and can be used with human cells. The disadvantage of commercial bought collagen plates is a dosing assay was not performed. A dosing assay would have been optimal but was not performed within the limits of time. $1x10^6$ cells were pre-incubated on 96 well plates (with or without collagen) for 2 hours prior to adding cytokine and 21 hour incubation at 37°C (experiment summarised schematically in Figure 2.10).

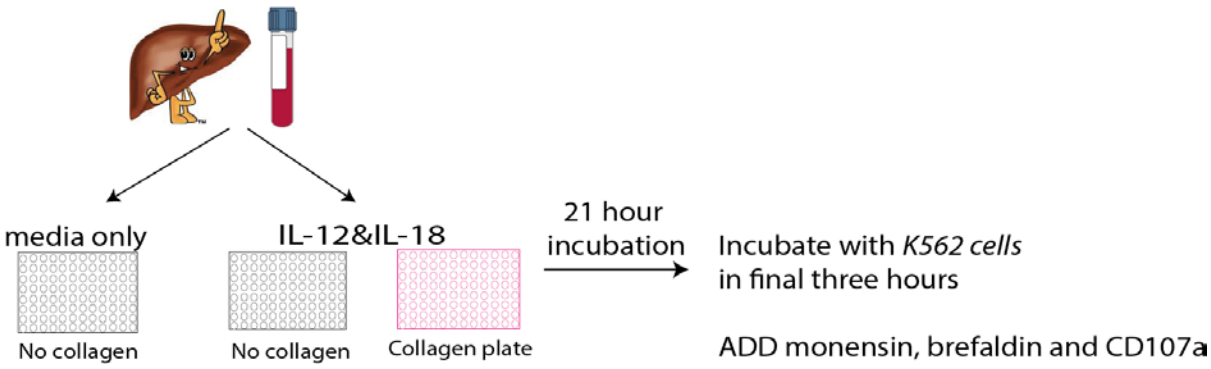


Figure 2.10: Functional study to assess NK cell function on collagen plates

To measure functional inhibition of NK cells we used a 2D system with plate bound collagen. Isolated PBMC and IHL were incubated with media, cytokine and cytokine in a collagen plate. In the final three hours of incubation target cells were added and cytotoxicity marked by degranulation CD107a measured.

2.6 Examine impact of stellate cells or their supernatant on LAIR-1 expression

2.6.1 Isolation and co-culture with pHSC

Experiments using primary human hepatic stellate cells (pHSC) were done in conjunction with Dr Laura Pallett, who has expertise in the isolation and culture of these primary cells. Prior to experimental use pHSC were cultured by Dr Laura Pallett from frozen cells previously isolated by her.

Briefly these cells were isolated and cultured as follows:

Step 1: Liver digestion and cell suspension

Healthy margins of metastatic liver tissue were obtained from the Royal Free Hospital from consented patients during surgery. Liver tissue was both mechanically and enzymatically digested using 0.001% DNAse I (Roche) and 0.01% collagenase IV (Life Technologies) at 37°C for thirty minutes. The suspension of liver tissue was filtered under pressure through a 70µM filter (BD) and centrifuged at low speeds to remove the hepatocytes and any residual enzymes (500 rpm, two minutes).

Step 2: Gradient selection

Stellate cells are the least dense of the non-parenchymal cells (NPC) and unlike the other NPC (Kupffer cells and endothelial cells) they can be effectively purified to 90-95% purity using a simple density barrier or gradient process (Weiskirchen and Gressner, 2005).

The remaining pellet comprising NPC were subjected to density centrifugation using OptiPrep® (Sigma-Aldrich, St Louis, USA). The pellet was re-suspended and carefully layered in a double density gradient; 10 ml of 17% Optiprep® at the bottom (density gradient diluted in PF4) and 10 ml of 11.5% of Optiprep® was carefully layered on top of it. An additional 4ml of PF4 was layered on top and centrifuged at 2700 rpm for 17 minutes at 20°C without any breaks. The hepatic stellate cell fraction on top of the 11.5% optiprep layer was aspirated using a 2ml Pasteur pipette. An equal

volume of PF4 was added to it and centrifuged at 1500 rpm for 10 minutes. After isolation pHSC pellet was re-suspended in Stellate cell media (ScienCell Research Laboratories).

Step 3: pHSC culture and activation

Before freezing, isolated pHSC were washed once in PBS and plated at a density of $5x10^4$ cells/cm^2 in tissue culture flasks and cultured at 37°C in a humidified atmosphere at 5% CO_2 for expansion. Previous lab experience in this technique had observed to fully optimise this protocol freshly isolated pHSC need to be cultured in flasks rather than plates, which was attributed to plausible reasons of surface area available of flask versus plate and the plastic material differing between the two. The cells were left for 24 hours to attach, after which they were washed with media and fresh media added. The quiescent hepatic stellate cells start differentiating into an activated state approximately 7 to 10 days post culture. The cells are then passaged twice before freezing them down using FBS and 10% DMSO as previously described. Previously, our group have shown that the HSC isolated and cultured were uniformly positive for the activated myofibroblast-specific marker anti-smooth muscle actin (αSMA, flow cytometric staining and RT-PCR), and microscopically show a characteristic 'star-like' myofibroblast phenotype (Singh et al., 2017).

When used for experimentation, cells were thawed and cultured in $25cm^2$ tissue culture flasks in Stellate Cell Media to approximately 80% confluency. Cells were then detached with trypsin and re-plated in 24 well plates at 25,000 cells/well and left for 24 hours to reattach. Prior to use in co-cultures the Stellate cell media was removed and replaced with cRPMI without disturbing the adherent pHSC layer.

Fresh PBMC were cultured overnight with rhIL-12 and rhIL-18. Approximately ten million cells per patient were prepped overnight either in media or with cytokine. These were then co-cultured with supernatant from activated pHSC alone or in direct contact with activated pHSC. Co-culture experiments were undertaken using twenty-four well plates. Approximately 25,000 pHSC were incubated with one million PBMC overnight in 500µl of fresh cRPMI with or without cytokine. After

twenty-four hours the cells were removed by pipetting and transferred to ninety-six round well plates for surface staining protocol. This experiment has been shown schematically in Figure 2.11.

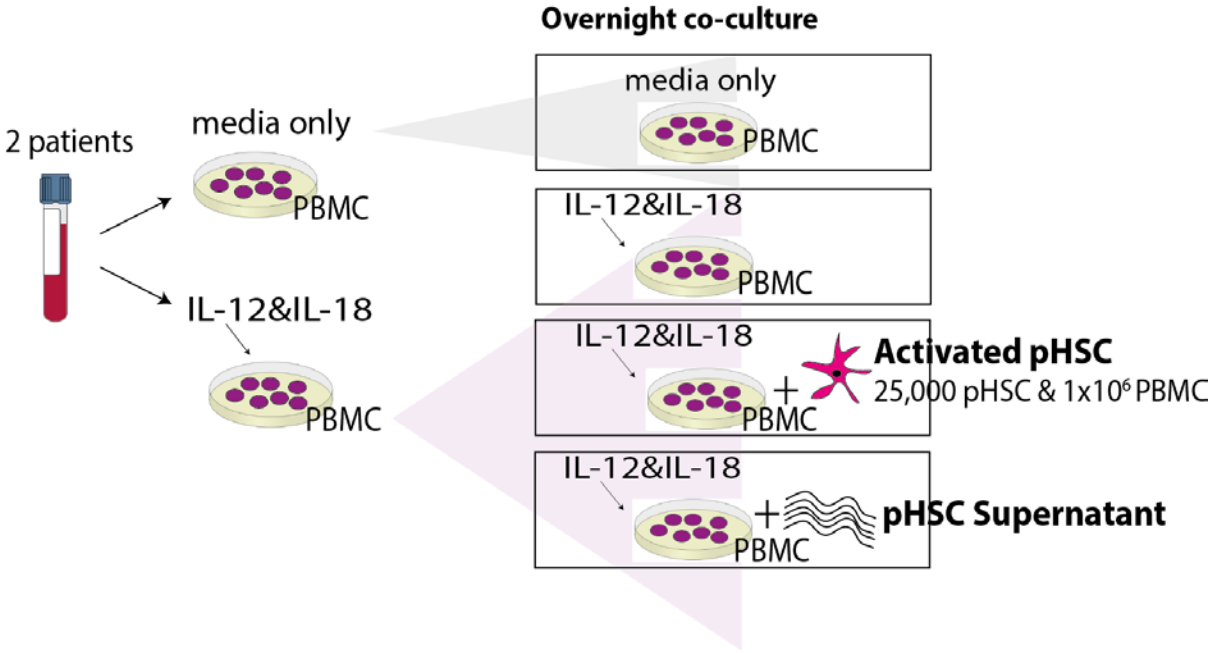


Figure 2.11: NK cells and pHSC co-culture

NK cells were activated and observed as to whether LAIR-1 expression was affected by pHSC or their supernatant.

2.7 LAIR-2 ELISA

Paired urine and serum were utilised from fifteen patients with CHB, with an additional eight samples (n=23) for serum without paired urine. These patients (n=23) were categorised by disease phase; of which thirteen were immune active and ten inactive carriers. Paired samples of urine and serum were also utilised from HC subjects (n=5) for comparison.

On the day of ELISA (Enzyme-Linked Immunosorbent Assay) experiments; aliquots of sera were thawed to room temperature. Urine aliquots were thawed and centrifuged for ten minutes at 1000 rpm to remove particulates and the supernatant was used for assaying. Human LAIR-2 (CD306) ELISA kit (LifeSpan BioSciences. Inc.) assay has been manufactured for use with serum and urine samples. The ELISA was used as per the manufacturers instructions. Briefly, for the ELISA a two-step protocol was undertaken; standard preparation and then sample preparation. Preparation of the standard dilution series as per manufacturers protocol was used to generate the standard curve. Standard stock solution 1,000pg/ml was resuspended with 1ml of sample diluent and incubated at room temperature for ten minutes. Eight standards including zero standard were made up as per the manufacturers instructions.

Initial optimisation was to perform a sample dilution series to identify optimal dilution. Sample dilutions were made up to 1:1, 1:100, 1:10,000, and 1:1,000000. All samples were run in duplicate to provide adequate data for statistical validation of the results. No interpretable concentration was elicited at dilutions less than 1:1 (see Figure 2.12.1).

The samples were diluted in the same buffer as the standard used to generate the standard curve (see Figure 2.12.2). The standard curve was used to determine the concentration of target antigen in unknown samples. The standards were also undertaken in duplicate, and average of standards absorbency values taken for the known concentrations as prepared earlier with the average zero

standard optical density subtracted. The concentrations of each of these standards were in pg/ml. A standard curve was created by reducing the data using PRISM software capable of generating a four parameter logistic (4-PL) curve-fit as per the manufacturers instructions.

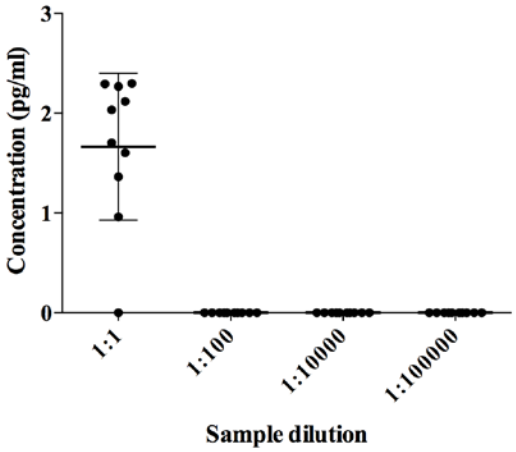


Figure 2.12.1: ELISA assay optimisation – sample dilution

Concentrations of samples at varying sample dilutions made up to 1:1, 1:100, 1:10,000, 1:1,000000. No interpretable concentration was elicited at dilutions less than 1:1.

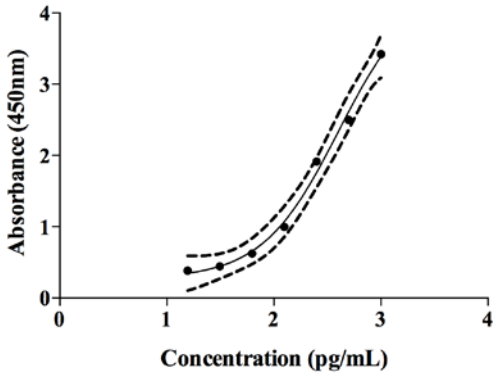


Figure 2.12.2: Standard curve for ELISA assay

Sample dilutions of less than 1:1 did not generate an interpretable concentration and therefore all samples were run as 1:1 dilution.

2.8 Reagents

As outlined in Table 2.5, a complete list of the full details of manufacturers and catalogue numbers for reagents used throughout the study.

Product	Manufacturer	Catalogue No.
BD ™ compensation beads (anti mouse IgG)	BD Biosciences	51-90-9001229
Brefeldin-A (Golgiplug)	BD Biosciences	555029
Collagen I, Coated Plate, 24 well	Life Technologies™	A1142802
Collagen I, Coated Plate, 96 well	Life Technologies™	A1142803
Cryovials	Nunc	V7509
DMSO	Sigma-Aldrich	D2650
FcR Blocking Reagent, human, 2ml	Miltenyi Biotec	130-059-901
Fetal Calf Serum (FCS) – Heat inactivated	Life Technologies ™	10108-165
Ficoll-Plaque	GE Healthcare	17-1440-03
Fixation/Permeabilization Solution 125mL	Becton Dickinson Uk Ltd	554722
Human IL-2	PeproTech EC Ltd	200-02-10
Human IL-8	PeproTech EC Ltd	200-08
Human LAIR ELISA kit	Lifespan Biosciences Ltd	LS-F6502
Ionomycin	Molecular Probes (Life Tech)	I24222
Monensin (Golgistop)	BD Biosciences	554724
PBS	Life Technologies™	15575-020
Penicillin/Streptomycin	Life Technologies ™	15140-122
PMA	Sigma-Aldrich	P8139
Recombinant Human IL-12 Protein	R & D Systems Europe Ltd	219-IL-005/CF
Recombinant Human IL-15 Protein, CF, 5ug	R & D Systems Europe Ltd	247-ILB-005/CF
Recombinant Human IL-18 Protein	R & D Systems Europe Ltd	B001-5
Recombinant Human TGF-beta, CF	Cambridge Bioscience	580702
RPMI 1640	Life Technologies ™	21875-054
Saponin	Sigma-Aldrich	47036-50G-F
Stellate cell media	ScienCell Research Laboratories	5301
Trypan Blue	Life Technologies ™	15250-061

Table 2.5: Details of reagents

This table lists the reagents used throughout the study.

2.9 Statistical analysis

Statistical analysis was performed using GraphPad prism software. Normal distribution was tested with the Kolmogorov–Smirnov test. Non-parametric statistical tests were used since the assumption of normally distributed data was not met within all cohorts being compared. For all tests p values less than 0.05 were considered significant. Significance levels were defined as *p<0.05, **p<0.01, ***p<0.001 and **** p<0.0001.

Mann–Whitney test was used for comparison between independent data groups/unpaired sample groups.

Wilcoxon paired tests was used for comparison between different NK cell subsets of the same patient and used for paired liver and PBMC samples from the same individual.

Kruskal–Wallis (one-way ANOVA) with Dunn's/Tukey's multiple comparison groups test used to compare two or more unpaired sample groups.

Friedman with Dunn's multiple comparison test was used to compare repeated measures and matched data. i.e. multiple cytokines or conditions within the same patient.

Two-way ANOVA with Tukey's multiple comparison test was used to analyse incubation intervals with and without collagen.

Non-parametric Spearman's rank correlation test was used to determine correlations.

Results - Chapter 3: Frequency and phenotype of NK cells in CHB

3.1 Introduction

Co-inhibitory receptors and blockade of their pathways, both individually and synergistically, have been described extensively in the literature. However, the dominant focus of research has been on T cells, where these receptors have been widely studied.

Some of these inhibitory receptors and the role they play are described in the introduction. These immune cell surface receptors act as rheostats in maintaining balance and facilitating the recognition of damaged and healthy cells. Inhibitory receptors are an important mechanism to prevent autoimmunity and an exaggerated inflammatory response. In viral infection it has been shown that these receptors can be upregulated on effector T cells, dampening down their overall response. Previous studies have shown that exhausted T cell responses are defined by the complex array of inhibitory receptors. There is limited evidence to suggest NK cells are also subject to exhaustion/inhibition but this has not been examined thoroughly in any persistent viral infection.

This chapter focuses on a comprehensive investigation of the expression of co-inhibitory receptors on NK cells in healthy and HBV-infected subjects.

3.2 NK cell frequency and subsets

Before exploring the concept of NK exhaustion/inhibition through receptor expression we analysed peripheral NK cell frequencies and subsets, in a cohort of twenty-one healthy controls and sixty-nine CHB patients. PBMC were isolated and stained for $CD56^{hi}CD3^{neg}$ NK cells. On analysing frequencies

of NK cells in the periphery, a wide variability was noted in both healthy controls and patients with CHB, with no significant differences between them (Figure 3.1). The percentage of circulating NK cells varied from 2.4% to a maximum of 25.5% amongst patients with CHB. To see if this large spread of frequencies was related to clinical correlates such as age or disease activity we analysed the data according to age, and repeated measurements of viral load and liver inflammation (ALT). The frequency of circulating NK cells showed no significant association with age (Figure 3.2a), ALT (Figure 3.2b) or viral load (Figure 3.2c).

We then analysed the frequency of $CD56^{bright}$ and $CD56^{dim}$ NK cells in the same cohort of twenty-one controls and sixty-nine patients with CHB. NK cells were divided into subsets based on expression of CD56 and CD16, allowing them to be differentiated as $CD56^{bright}$ ($CD56^{hi}CD16^{neg}$) and $CD56^{dim}$ ($CD56^{lo}CD16^{pos}$) NK cells. As shown in Figures 3.3a and 3.3b, there was no difference in the proportion of these subsets between controls and patients. However a small subset of patients with CHB (highlighted on Figure 3.3a) had a higher frequency of circulating $CD56^{bright}$ NK cells; further analysis according to ALT (Figure 3.3c) and viral load (Figure 3.3d) did not reveal any clinical factors driving an expansion of $CD56^{bright}$ NK cells.

Analysis of the activation marker human leucocyte antigen- antigen D related (HLA-DR) on circulating NK cells revealed higher frequencies of HLA-DR-expressing NK cells in CHB patients (mean 6.102%) than healthy controls (mean 4.058%) (Figure 3.4a). To explore this increase further, we analysed the expression of NK cell HLA-DR according to viral load and ALT within the CHB cohort. As shown in Figures 3.4b and 3.4c, there was no clear association between these disease parameters.

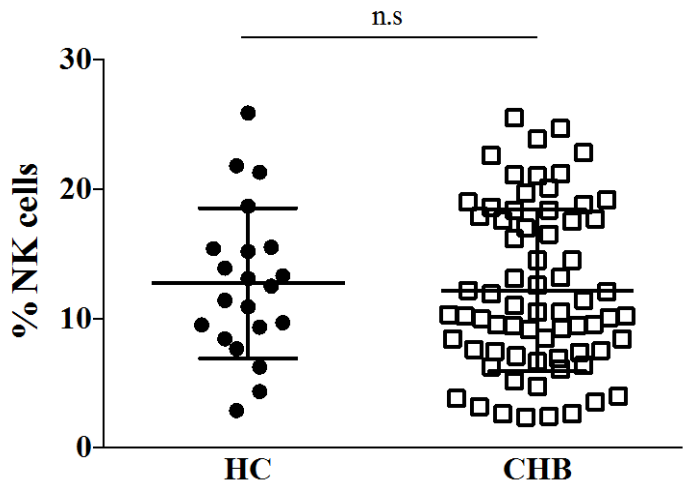
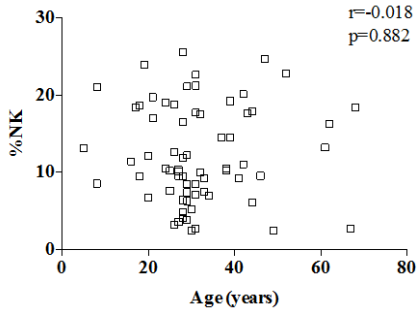


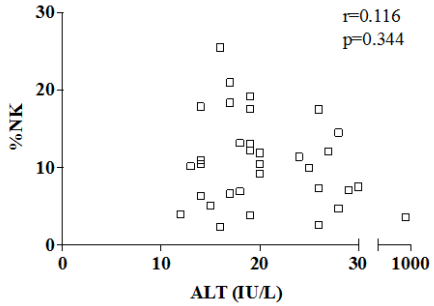
Figure 3.1: Comparison of proportion of circulating NK cells between HC and CHB patients.

PBMC were isolated and stained from twenty-one HC and sixty-nine CHB patients. PBMC were stained for $CD56^{hi}CD3^{neg}$ NK cells after exclusion of doublets and dead cells. Error bars represent the mean ± SEM. HC; 12.72 ± 1.269, CHB; 12.17± 0.753. Significance testing was carried out using Mann Whitney test and deemed non significant.

(a)



(b)



(c)

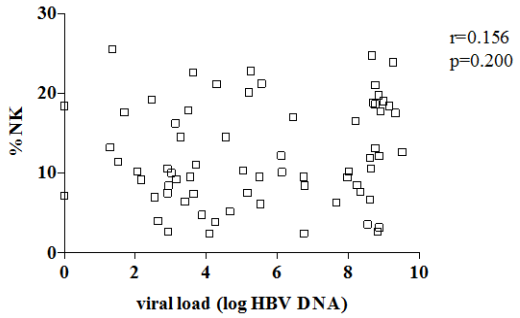


Figure 3.2: Analysis of CHB data to explore heterogeneity of NK cell frequency in CHB

PBMC were isolated and stained from sixty-nine CHB patients. PBMC were stained for $CD56^{hi}CD3^{neg}$ NK cells after exclusion of doublets and dead cells. The percentage of NK cells was correlated with clinical variables (a) age (b) ALT and (c) viral load. Spearman correlation analysis was performed between variables and deemed non significant.

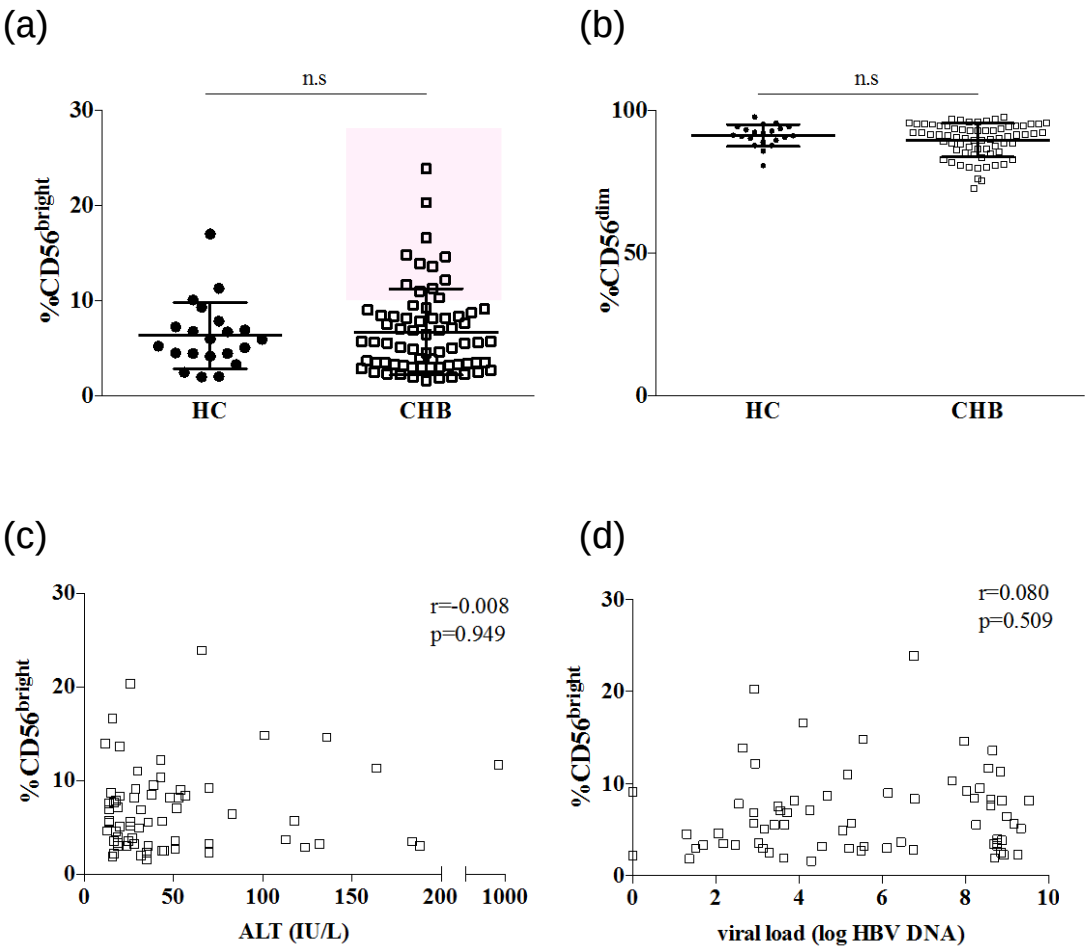


Figure 3.3: Comparison of percentage of peripheral (a) CD56^{bright} and (b) CD56^{dim} NK cells between HC and CHB patients, with correlations of CD56^{bright} NK cell subset in CHB with (c) ALT and (d) viral load.

PBMC were isolated and stained from twenty-one HC and sixty-nine CHB patients. NK cells were divided into subsets based on expression of CD56 and CD16, allowing them to be differentiated as (a) CD56^{bright} (CD56^{hi}CD16^{neg}) CD3^{neg} NK cells and (b) CD56^{dim} (CD56^{lo}CD16^{pos}) CD3^{neg} NK cells. Error bars represent the mean ± SEM. (a) CD56^{bright} HC; 6.724 ± 0.542, CHB; 6.731 ± 0.568. (b) CD56^{dim} HC; 91.2 ± 0.823, CHB; 89.54 ± 0.708. Significance testing was carried out using Mann Whitney test and deemed non significant.

The %CD56^{bright} NK cell subset was correlated with clinical variables (c) ALT and (d) viral load. Spearman correlation analysis was performed between variables and deemed non significant.

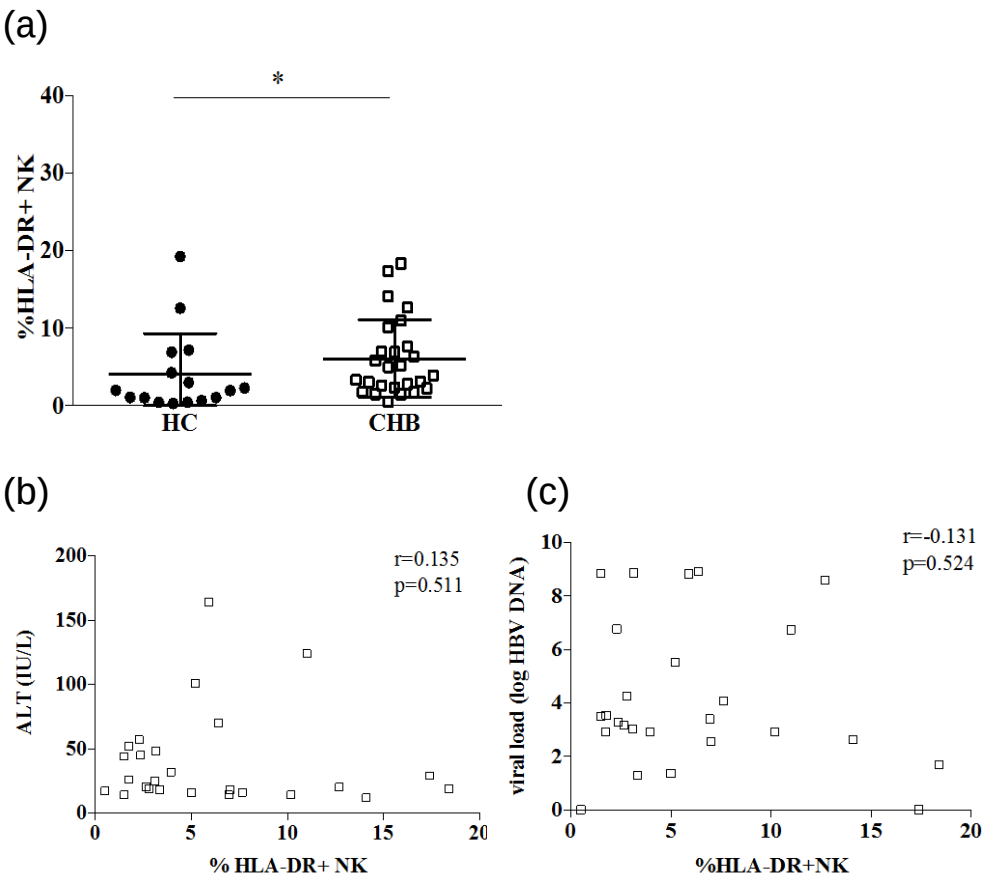


Figure 3.4: Comparison of expression of activation marker HLA-DR on total NK cells between HC and CHB patients and the relationship of HLA-DR with clinical correlates in CHB patients.

PBMC were isolated and stained for HLA-DR from sixteen HC and twenty-six CHB patients. HLA-DR expressing NK cells were gated as positive for HLA-DR staining on $CD56^{hi}CD3^{neg}$ NK cells after exclusion of doublets and dead cells.

(a) Comparison of expression of HLA-DR on total NK cells between HC and CHB patients. Error bars represent the mean ± SEM. HC; 4.058 ± 1.31, CHB; 6.102 ± 0.981. Significance testing was carried out using Mann Whitney test. Significance is indicated as * $p<0.05$.

Correlation of expression of HLA-DR on total NK cells with clinical correlate (b) ALT and (c) viral load in CHB patients.

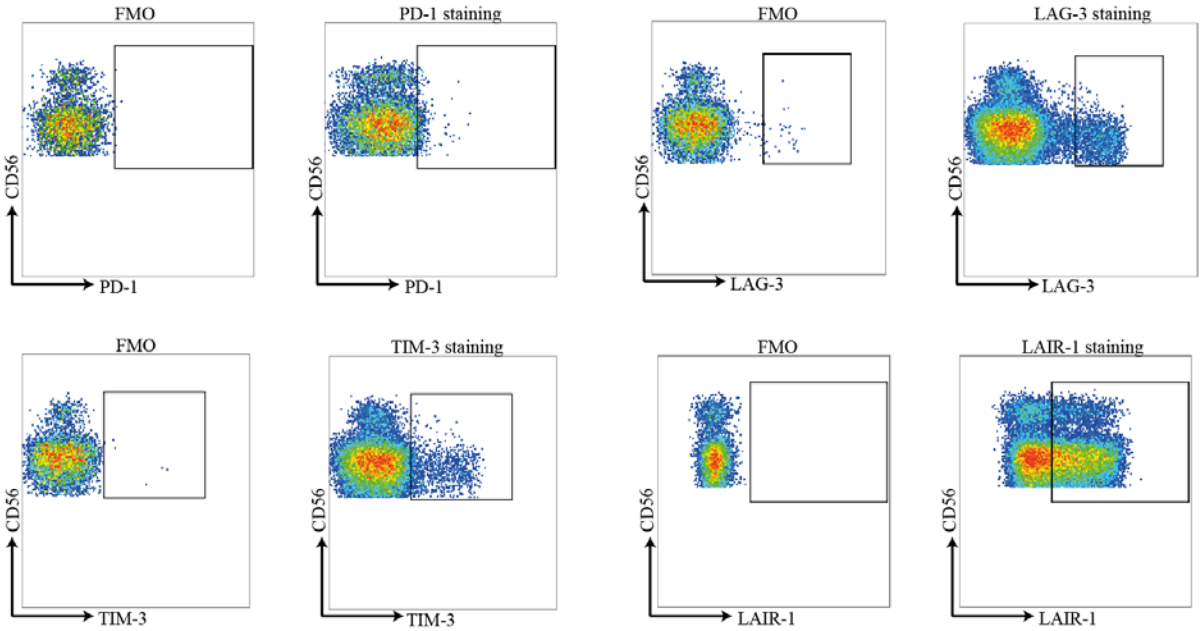
Expression of HLA-DR was correlated with ALT and viral load level in twenty-six CHB patients. Spearman correlation analysis was performed between variables and deemed non significant.

3.3 Co-inhibitory receptors on NK cells

NK cells express an array of receptors but there is limited literature regarding the expression of these 'classical' co-inhibitory or exhaustion receptors. Initially, we explored the repertoire of co-inhibitory receptors on global NK cells: PD-1, TIM-3, LAG-3 and LAIR-1 in HC versus CHB patients. The NK cell expression of the receptor LAIR-1 in CHB has never been investigated previously.

For the surface staining of some receptors, FMO was required as a control to help identify and gate the positive cells for respective antibodies where spread/shift can occur due to multiple fluorochromes in the panel. An example of gating using FMO for LAIR-1 is illustrated in Figure 3.5a. As LAIR-1 has not been explored in CHB previously, staining was checked on both fresh and frozen samples. Summary data in Figure 3.5b shows no significant difference in levels of LAIR-1 expression when the same sample was analysed before and after cryopreservation.

(a)



(b)

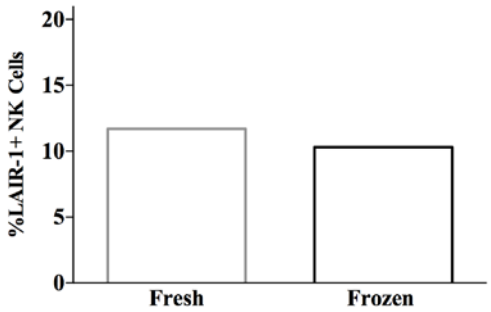


Figure 3.5: Optimisation of staining.

(a) ***Representative plots of inhibitory receptors PD-1, TIM-3, LAG-3 and LAIR-1 on NK cells from PBMC***.

FMO used for gating. PBMC were stained for $CD56^{hi}CD3^{neg}$ NK cells after exclusion of doublets and dead cells.

(b) ***Descriptive statistic comparing %LAIR-1 NK cell positive (LAIR-1+NK) expression between freshly isolated PBMC sample and frozen PBMC sample.*** n=1.

3.3.1 PD-1

PBMC were isolated and stained from thirteen HC and thirty CHB patients. There was no significant difference in PD-1 expression on NK cells between HC and CHB patients (Figure 3.6). However, there was greater variability of expression amongst CHB patients with a range between 0.28% and maximum 7.65%. PD-1 expression also varied on $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets (Figure 3.6). To see if this variability was related to clinical correlates such as disease activity we analysed the data according to CHB disease phase. The expression of PD-1 on circulating NK cells in CHB showed no significant association with viral load or ALT (data not shown).

3.3.2 TIM-3

PBMC were isolated and stained from thirteen HC and twenty-four CHB patients. There was no significant difference in TIM-3 expression on NK cells between HC and CHB patients (Figure 3.7). There was variability of expression amongst HC and CHB patients (range for HC between 0.22% and 7.16% and in CHB between 0.3% and 13.9%). TIM-3 expression also varied within $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets. To see if this variability was related to clinical correlates such as disease activity we re-analysed the data according to repeated measurements of viral load and liver inflammation (ALT). The expression of TIM-3 on circulating NK cells in CHB showed no significant association with viral load or ALT (data not shown).

3.3.3 LAG-3

PBMC were isolated and stained from thirteen HC and thirty CHB patients. There was a non-significant trend towards an increase in LAG-3 expression on NK cells in CHB patients compared to HC (Figure 3.8). LAG-3 expression on HC was negligible with a mean expression of 0.53%. However, there was greater variability of expression amongst CHB patients with a range between

2.07% and maximum 20.1%. This variability was also seen in when analysing NK cells by subset. To see if this variability was related to clinical correlates such as disease activity we re-analysed the data according to repeated measurements of viral load and liver inflammation (ALT). The expression of LAG-3 on circulating NK cells in CHB showed no significant association with viral load or ALT (data not shown).

In summary no difference between PD-1, TIM-3 or LAG-3 expression was seen between HC and CHB patients

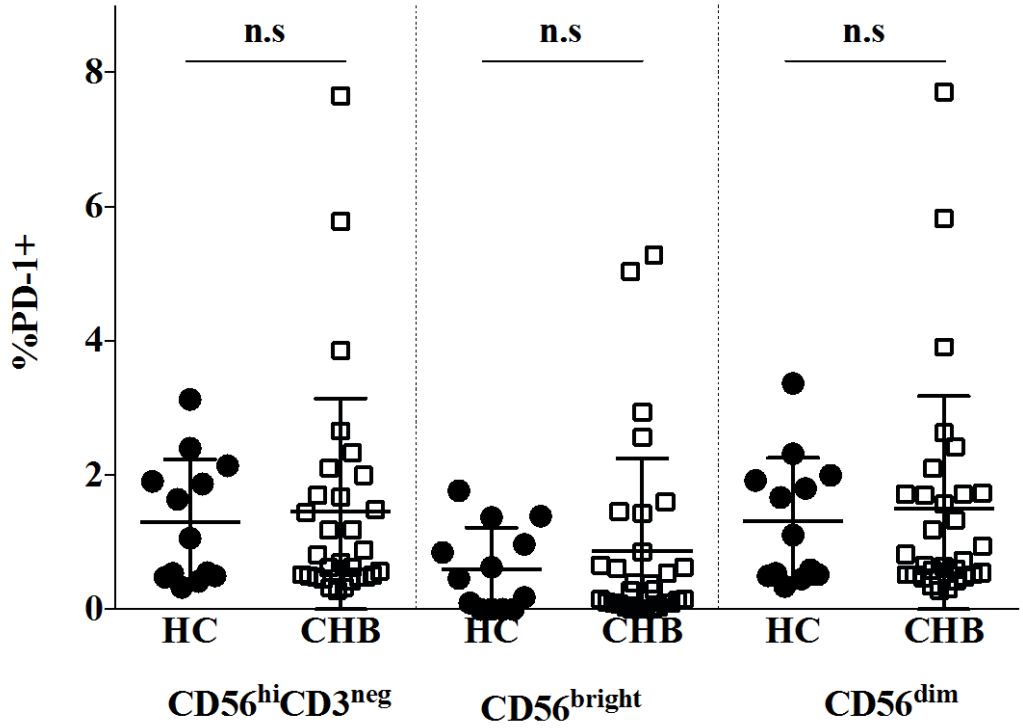


Figure 3.6: Comparison of percentage PD-1 positive (PD-1+) on total NK cells and within $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets between HC and CHB patients.

PBMC were isolated and stained from thirteen HC and thirty CHB patients. PBMC were stained for PD-1 expression on $CD56^{hi}CD3^{neg}$ NK cells, $CD56^{bright}$ and $CD56^{dim}$ NK cells. HC vs CHB patients, error bars represent the mean ± SEM, $CD56^{hi}CD3^{neg}$; 1.305±0.257 vs 1.469±0.305, $CD56^{bright}$; 0.594±0.173 vs 0.866±0.253, $CD56^{dim}$; 1.318±0.261 vs 1.499±0.306. Significance testing was carried out using Mann Whitney test.

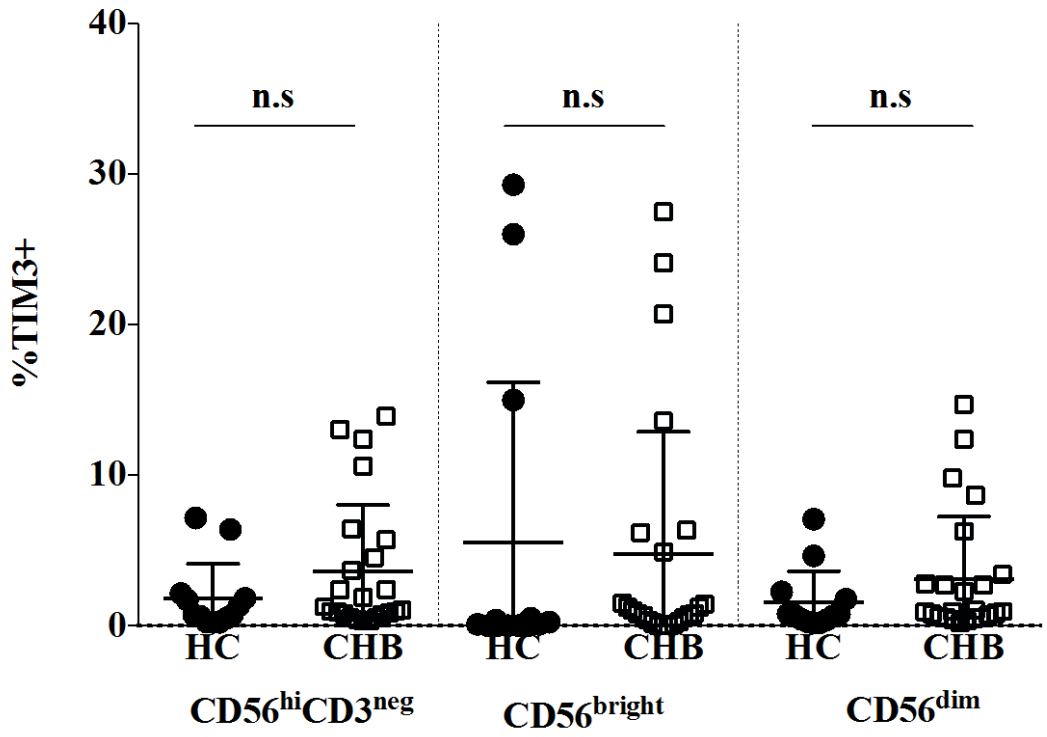


Figure 3.7: Comparison of percentage TIM-3 positive (TIM-3+) on total NK cells and within $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets between HC and CHB patients.

PBMC were isolated and stained from thirteen HC and twenty-four CHB patients. PBMC were stained for TIM-3 expression on $CD56^{hi}CD3^{neg}$ NK cells, $CD56^{bright}$ and $CD56^{dim}$ NK cells.

HC vs CHB patients, error bars represent the mean ± SEM, $CD56^{hi}CD3^{neg}$; 1.825±0.636 vs 3.605±0.9, $CD56^{bright}$; 5.508±2.959 vs 4.760±1.66, $CD56^{dim}$; 1.548±0.573 vs 3.124±0.843. Significance testing was carried out using Mann Whitney test.

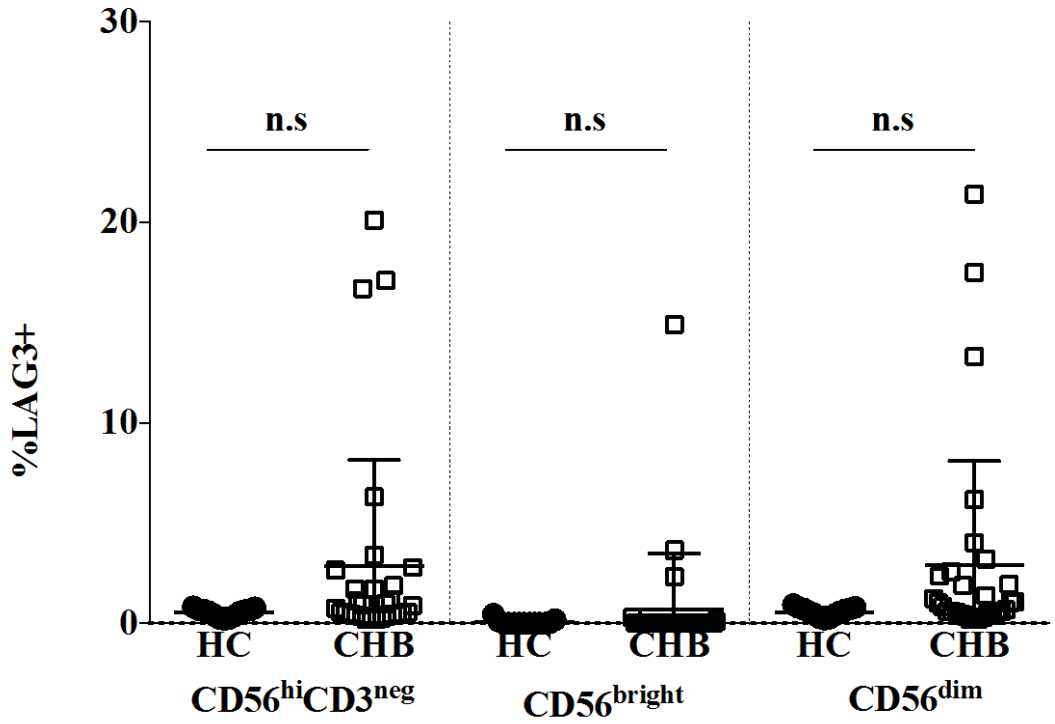


Figure 3.8: Comparison of percentage LAG-3 positive (LAG-3+) on total NK cells and within $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets between HC and CHB patients.

PBMC were isolated and stained from thirteen HC and thirty CHB patients. PBMC were stained for LAG-3 expression on $CD56^{hi}CD3^{neg}$ NK cells, $CD56^{bright}$ and $CD56^{dim}$ NK cells. HC vs CHB patients, error bars represent the mean ± SEM, $CD56^{hi}CD3^{neg}$; 0.53±0.057 vs 2.858±0.967, $CD56^{bright}$; 0.06±0.036 vs 0.71±0.509, $CD56^{dim}$; 0.573±0.063 vs 2.913±0.948. Significance testing was carried out using Mann Whitney test.

3.3.4 LAIR-1

Figure 3.9 illustrates that NK cells from all patients with CHB infection express LAIR-1 and the mean expression is higher in CHB than HC. PBMC were isolated and stained from twenty-one HC and sixty-nine CHB patients. Circulating NK cells revealed higher frequencies of LAIR-1-expressing NK cells in CHB patients (mean 40%) than healthy controls (mean 30%). Compared to the other inhibitory receptors investigated, the mean expression of LAIR-1 is much higher on both HC and CHB patients. In CHB patients there is heterogeneity and some overlap, with a range between 7.68% and maximum 74.9%. On further analysis in CHB, $CD56^{bright}$ NK cells have significantly higher mean expression of LAIR-1 of 36% compared to 24% in HC (Figure 3.9). CHB $CD56^{dim}$ NK cells also had significantly higher mean expression of LAIR-1 of 40% compared to 31% in HC (Figure 3.9).

Given the striking finding of higher LAIR-1-expressing NK cells in CHB patients we compared expression of LAIR-1 on other immune cells. In a smaller cohort of patients, PBMC were isolated and stained from thirty-four CHB patients. Within the same donors PBMC were surface stained with mAb CD8 and CD4 to identify CD8+ T cells. LAIR-1 expression was gated on CD8+ T cells and NK cells (Figure 3.10a). CD8+ T cells were chosen due to their key role in controlling and eradicating viral infection. A similar high expression and variability was observed on CD8+ T cells with a range between 9.42% and 63.1%, however mean LAIR-1 expression of 46% was higher on NK cells than CD8+ T cells (mean 35%). Notably as shown in Figure 3.10b there was no difference in LAIR-1 expression on CD8+ T cells in CHB compared to HC.

Traditionally $CD56^{bright}$ NK cells are functionally distinct and potent cytokine producers and on further probing as depicted in Figure 3.11 there was a weak but significant inverse correlation showing that NK cells with higher LAIR-1 expression displayed impaired cytokine production. To conduct this experiment, baseline ex-vivo LAIR-1 expression was compared to the amount of IFNγ production by NK cells after whole PBMC were incubated with rhIL-12 and rhIL-18 for 21 hours. No

correlation was seen with cytotoxicity (data not shown) in keeping with the observation that in CHB NK cells have impaired cytokine production but maintain cytotoxicity (Peppa et al., 2010).

Expression of inhibitory receptors has been widely explored but there is limited data regarding the co-expression patterns of these receptors on NK cells. Blackburn et al observed that in an LCMV mouse model there was the presence of increased expression of inhibitory receptors 2B4 and CD160 in conjunction with PD-1, especially on the more terminally exhausted CD8+ T cells (Blackburn et al., 2009). Figure 3.12 illustrates the analysis of co-expression of NK cell PD-1 and LAIR-1. PBMC was stained for PD-1 and LAIR-1 expression in the same cohort of 30 CHB patients. Although there was no significant correlation between PD-1 and LAIR-1 co-expression, there is a weak trend for NK cells with high expression of PD-1 to also co-express high LAIR-1.

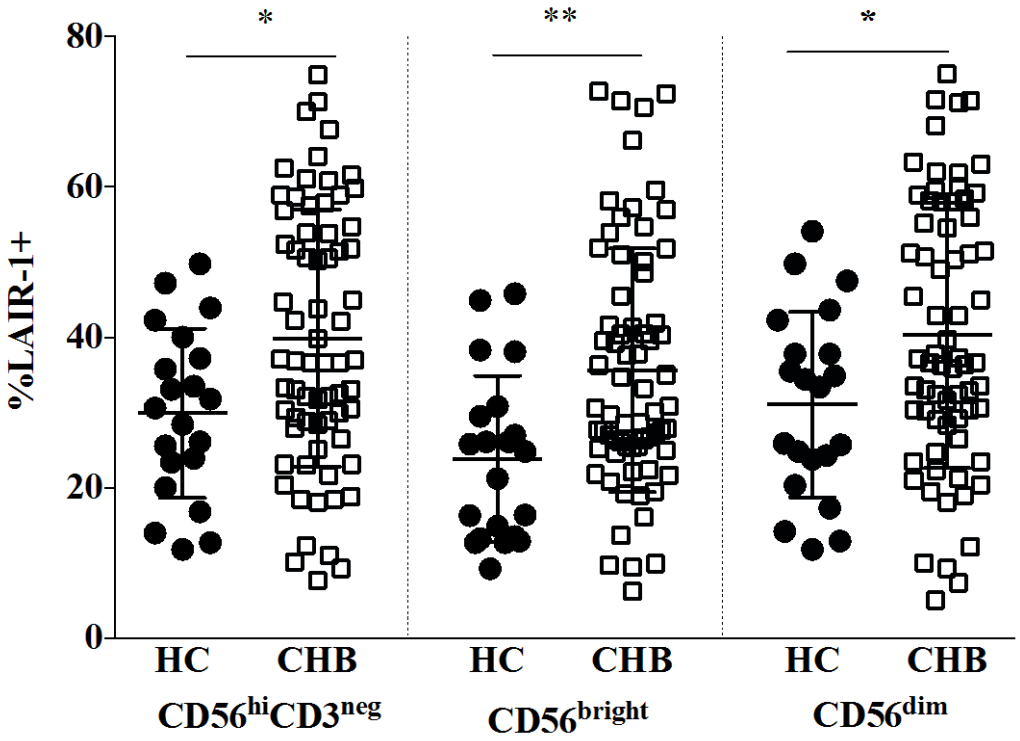


Figure 3.9: Comparison of percentage LAIR-1 positive (LAIR-1+) on total NK cells and within CD56^{bright} and CD56^{dim} NK cell subsets between HC and CHB patients.

PBMC were isolated and stained from twenty-one HC and sixty-nine CHB patients. PBMC were stained for LAIR-1 expression on CD56^{hi}CD3^{neg} NK cells, CD56^{bright} and CD56^{dim} NK cells.

HC vs CHB patients, error bars represent the mean ± SEM, CD56^{hi}CD3^{neg}; 29.9±2.45 vs 39.87±2.06, CD56^{bright}; 23.8±2.41 vs 35.64±1.95, CD56^{dim}; 31.05±2.7 vs 40.27±2.1. Significance testing was carried out using Mann Whitney test. Significance is indicated as * $p<0.05$, ** $p<0.01$.

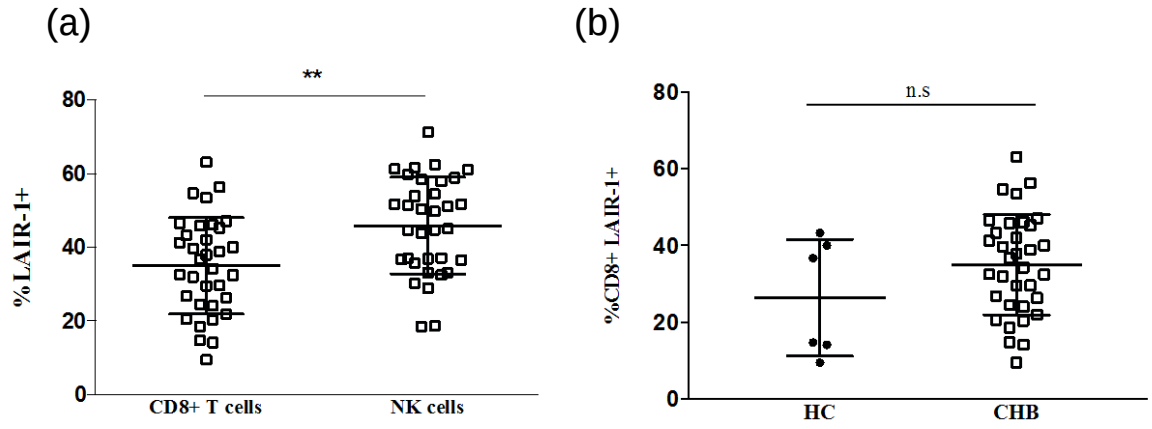


Figure 3.10: Comparison of percentage LAIR-1+ on (a) CD8+ T cells versus total NK cells in CHB patients (b) CD8+ T cells in HC and CHB patients.

PBMC were isolated and stained from thirty-four CHB patients and six healthy controls. (a) Within the same donors PBMC from CHB patients were surface stained with mAb CD8 and CD4 to identify CD8+ T cells (after excluding doublets, dead cells and monocytes) and NK cells were identified as $CD56^{hi}CD3^{neg}$ NK cells (after exclusion of doublets and dead cells). LAIR-1 expression was gated on CD8+ T cells and NK cells. (a) CD8+ T cells vs NK cells in CHB, error bars represent the mean ± SEM, 34.99±2.24 vs 45.9±2.24. (b) HC vs CHB patients, error bars represent the mean ± SEM, CD8+ T cells; 26.37±6.2 vs 34.99±2.247. Significance testing was carried out using Mann Whitney test. Significance is indicated as ** $p<0.01$.

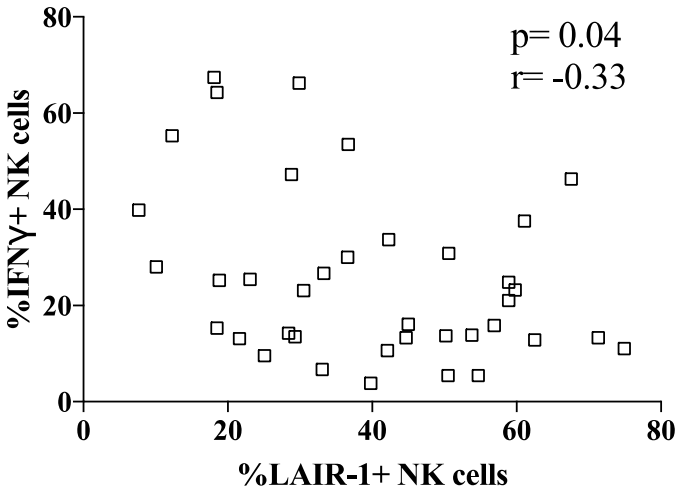


Figure 3.11: Investigation of the expression of LAIR-1 on NK cells and cytokine production in CHB.

PBMC from thirty-eight CHB patients were incubated overnight with rhIL-12 and rhIL-18 for a total of 21 hours. Cells were thereafter fixed and permeabilised and then stained for intracellular expression of IFNγ. Baseline surface LAIR-1 expression was compared to the amount of IFNγ expression of NK cells. Significance testing was carried out using Spearman correlation coefficient. Significance was measured at $p<0.05$.

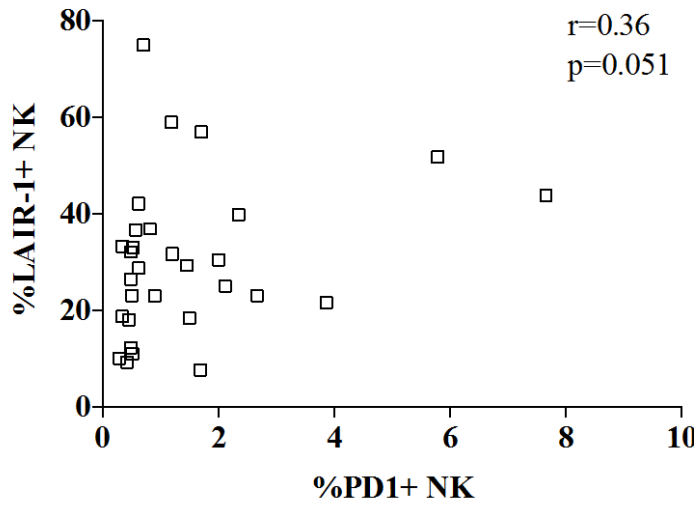


Figure 3.12: Correlation between PD-1 and LAIR-1 co-expression on NK cells in CHB

PBMC were isolated and stained from thirty CHB patients. NK cells were identified as $CD56^{hi}CD3^{neg}$ NK cells after exclusion of doublets and dead cells. The same cohort of PBMC were stained for PD-1 and LAIR-1 expression. Significance testing was carried out using Spearman correlation. Significance was measured at $p<0.05$.

3.4 LAIR-1 expression according to disease phase

All patients with CHB express LAIR-1 with the mean being higher within this group compared to HC. We sought to investigate whether heterogeneity of expression was caused by different disease phases of CHB or clinical correlates. CHB as mentioned earlier is a dynamic disease and patients progress through phases, illustrated in Figure 2.2 (Liaw and Chu, 2009). These distinct disease phases are characterised by ALT, HBV DNA and HBeAg status.

To determine if the expression of LAIR+ NK cells, with the wide frequency range noted, was related to disease activity we analysed the data after dividing patients into distinct clinical stages based on repeated measurements of expression of HBeAg, liver inflammation (ALT) and viral load. Patients were categorised as defined in Table 2.3, according to European Association for the Study of the Liver (EASL) clinical practice guidelines (EASL, 2012)

***Since the writing of this thesis updated clinical practice guidelines have been published by EASL on the management of HBV infection (EASL, 2017). The most relevant change is the disease phases have been renamed. The new nomenclature takes into account the presence or absence of liver inflammation and defines this as hepatitis versus infection. Table 2.3 has been amended to demonstrate the new changes in nomenclature and shown in Table 3.1.*

There was uniformity in the spectrum of LAIR-1 expression across disease phases (Figure 3.13a). Similarly there was no correlation of LAIR-1 with other clinical correlates such as age, ALT, HBV DNA or HBsAg (Figure 3.13b).

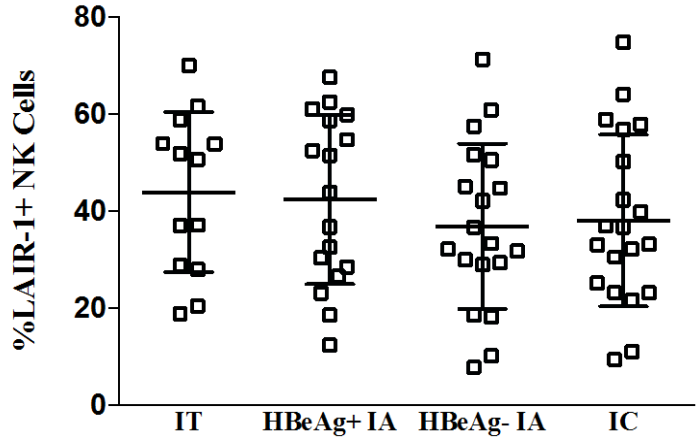
We were interested in the expression pattern of LAIR-1 and other inhibitory molecules during disease phases. NK cells most frequently expressed significantly higher LAIR-1 in all disease phases in

contrast to PD-1, TIM-3 and LAG-3 (Figure 3.14). There was a trend for HBeAg- IA and IC phases exhibiting a profile with uniformly higher burden of inhibitory receptor expression.

Phase	*EASL 2017 - New nomenclature*	*HBeAg*	*ALT*	*HBV DNA*
Immune tolerant	HBeAg positive chronic infection	Positive	<40 IU/L	HBV DNA > 8 log IU (100,000,000 IU/ml)
HBeAg positive immune active	HBeAg positive chronic hepatitis	Positive	>40 IU/L	Any
HBeAg negative immune active	HBeAg negative chronic hepatitis	Negative	Any	>3.3 log IU (2,000 IU/ml)
Inactive carrier	HBeAg negative chronic infection	Negative	<40 IU/L	<3.3 log IU (2,000 IU/ml)

Table 3.1: CHB disease phases (as per new EASL 2017 guidelines)

(a)



(b)

	LAIR-1 vs. Age	LAIR-1 vs. mean ALT	LAIR-1 vs. log mean HBV DNA	LAIR-1 vs. HBsAg
r	-0.1630	0.1824	0.1697	0.1963
P (two-tailed)	0.1810	0.1337	0.1634	0.1115
No. of XY pairs	69	69	69	67

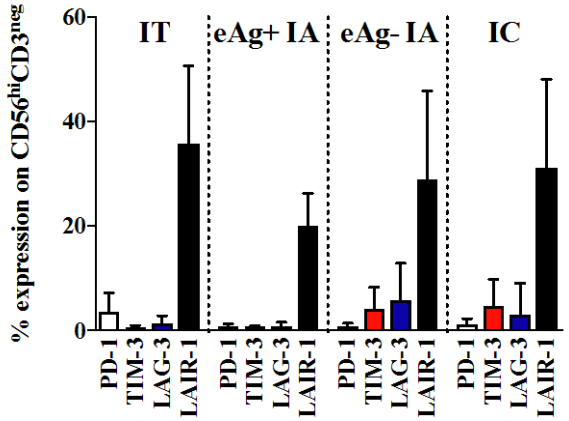
Figure 3.13: Analysis of LAIR-1 expression with clinical correlates

PBMC were isolated and stained from sixty-nine CHB patients. PBMC were stained for LAIR-1 expression on $CD56^{hi}CD3^{neg}$ NK cells.

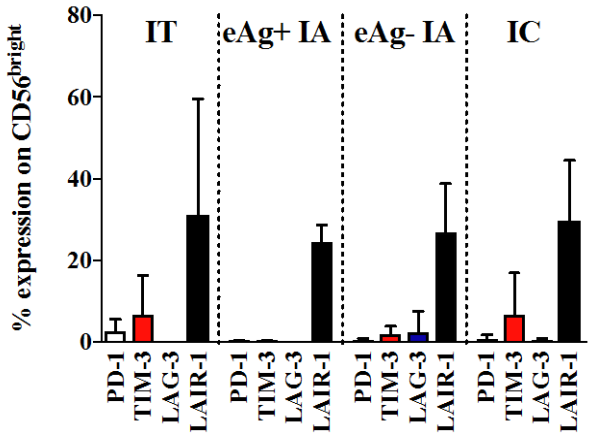
(a) LAIR-1 expression during the different disease phases of CHB was measured. There is no significant difference between disease phases (p=0.51). Error bars represent the mean ± SEM. Kruskal-Wallis statistical test was used.

(b) LAIR-1 expression with clinical correlates such as age, ALT, HBV DNA and HBsAg was measured. Significance testing was carried out using Spearman correlation coefficient (Age: n=69, ALT: n=69, HBV DNA: n=69, qHBsAg n=67, qHBsAg not known for two patients). Significance was measured at $p<0.05$.

(a)



(b)



(c)

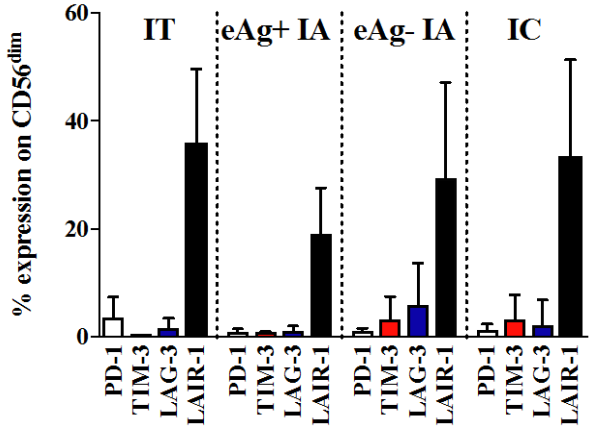


Figure 3.14: Comparison of percentage LAIR-1+ with other inhibitory receptors on (a) $CD56^{hi}CD3^{neg}$ and within (b) $CD56^{bright}$ and (c) $CD56^{dim}$ NK cell subsets in disease phases of CHB.

PBMC were isolated and stained from a total of thirty-two CHB patients: four IT, four HBeAg positive immune active (eAg+ IA), eight HBeAg negative immune active (eAg- IA) and sixteen IC. PBMC were stained at the same time for PD-1 (white bars), TIM-3 (red bars), LAG-3 (blue bars) and LAIR-1 (black bars) expression on $CD56^{hi}CD3^{neg}$, $CD56^{bright}$ and $CD56^{dim}$ NK cells. Multiple comparisons were made between the different groups with Kruskal-Wallis testing and deemed non significant.

3.5 LAIR-1 on intrahepatic NK cells

Staining experiments thus far had exclusively been done on peripheral blood but unique access to surplus liver tissue from percutaneous liver biopsies enabled us to undertake paired analysis of tissue and blood. This is advantageous as it enables the investigation of tissue NK cells that have a distinct phenotype and function compared to those in the periphery and allows closer evaluation of the site of CHB infection. Paired PBMC and IHL were isolated and stained from seventeen patients with CHB.

The $CD56^{bright}$ NK cell subset, which are efficient cytokine producers are described as more preferentially enriched within the liver compared to the periphery and our data was comparable with this as illustrated in Figure 3.15 the mean expression of $CD56^{bright}$ NK cells in the liver was 51.79% compared to 10.77% of $CD56^{bright}$ NK cells in the periphery. In the liver the percentage of $CD56^{bright}$ and $CD56^{dim}$ NK cells is equal with no significant difference between the two (Figure 3.15b).

Bonorino et al observed an altered phenotype of intrahepatic NK cells and showed HBV patients had reduced intrahepatic NKG2A+ NK cells (Bonorino et al., 2009). Paired analysis data shown in Figure 3.16 depicts LAIR-1 expression on total NK cells is significantly lower in the liver (mean 22.76%) compared to the periphery (mean 40.58%). The range in the periphery is much wider between 10.1% and 70% maximum compared to the intrahepatic compartment with a range of 3.12% and 49%.

Paired analysis data shown in Figure 3.17 illustrates there is significantly higher expression of LAIR-1 on $CD56^{bright}$ NK cells in the periphery (mean 35.8%) compared to intrahepatic compartment (mean 15.21%) mirroring the data seen for global NK cells.

We were interested to observe whether LAIR-1 is associated with liver residency. The homing marker CXCR6 was used as a marker for liver residency, based on recent studies (Stegmann et al., 2016,

Harmon et al., 2016). A smaller subset of paired samples (n=5) was stained with this marker. Figure 3.18 depicts LAIR-1 expression is also lower on lrNK cells (mean 18.74%).

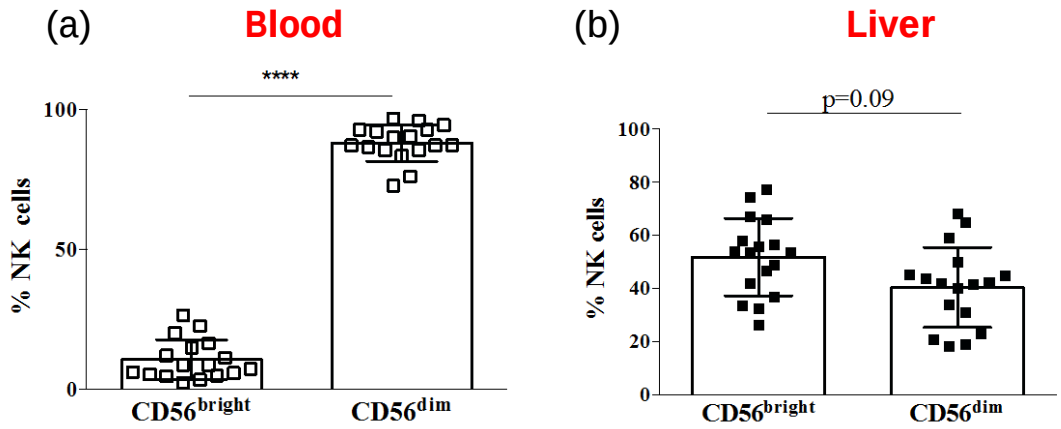


Figure 3.15: Comparison of frequency of circulating versus intrahepatic NK cell subsets.

Paired PBMC and IHL were isolated from seventeen patients with CHB. $CD56^{hi}CD3^{neg}$ NK cells were analysed after exclusion of doublets and dead cells. NK cells were divided into subsets based on expression of CD56 and CD16, allowing them to be differentiated as $CD56^{bright}$ and $CD56^{dim}$ NK cells. Paired Wilcoxon test used. Significance indicated as: **** p=<0.0001.

(a) $CD56^{bright}$ and $CD56^{dim}$ NK cells in blood, mean ± SEM, 10.77±1.732 and 88.11±1.570 and (b) $CD56^{bright}$ and $CD56^{dim}$ NK cells in liver, mean ± SEM, 51.79±3.539 and 40.38±3.665.

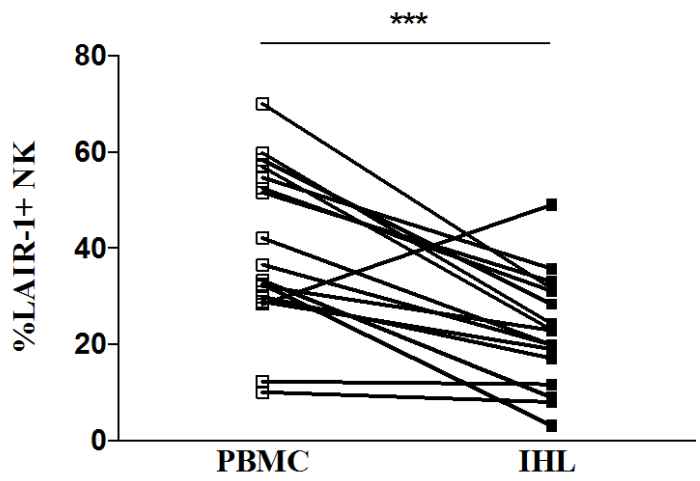


Figure 3.16: Comparison of percentage LAIR-1+ on total circulating versus intrahepatic NK cells in CHB.

Paired PBMC and IHL were isolated from seventeen patients with CHB. PBMC and IHL were stained for LAIR-1 on $CD56^{hi}CD3^{neg}$ NK cells.

Paired Wilcoxon test used. Significance indicated as: *** p=<0.001.

LAIR-1+ NK PBMC vs IHL, mean ± SEM, 40.58±4.118 and 22.76±2.793

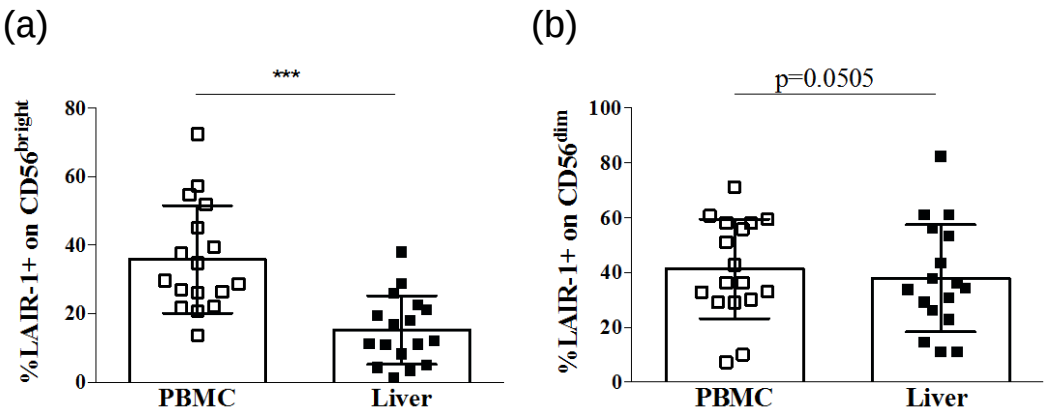


Figure 3.17: Comparison of percentage LAIR-1+ on circulating versus intrahepatic NK cell subsets in CHB.

Paired PBMC and IHL were isolated and stained from seventeen patients with CHB. NK cells were gated for LAIR-1 positive cells on (a) $CD56^{bright}$ (b) $CD56^{dim}$ NK cells. Paired Wilcoxon test used. Significance indicated as: *** p=<0.001.

(a) LAIR-1+ $CD56^{bright}$ NK cells; PBMC vs liver, mean ± SEM, 35.85±3.817 and 15.21±2.430.

(b) LAIR-1+ $CD56^{dim}$ NK cells; PBMC vs liver, mean ± SEM, 41.3±4.383 and 37.96±4.757.

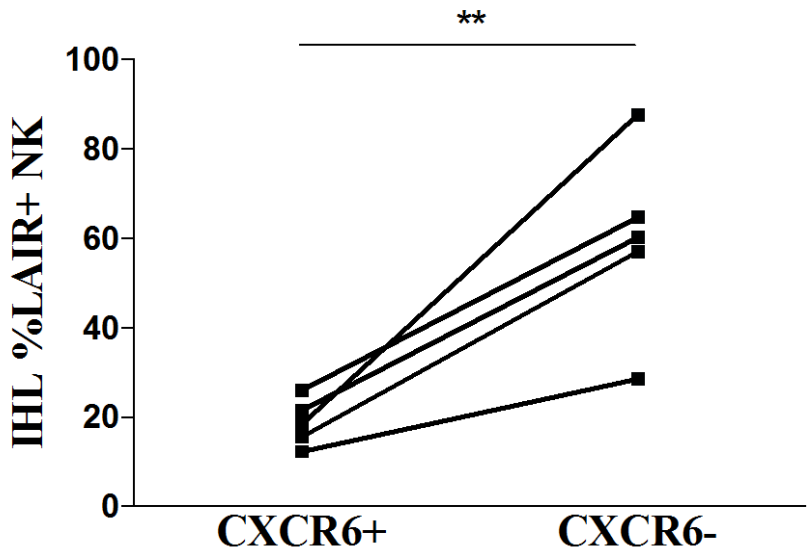


Figure 3.18: Comparison of percentage LAIR-1+ IHL with liver residency marker CXCR6 in CHB.

IHL were isolated and stained from five patients with CHB. The homing marker CXCR6 was used as a marker for liver residency.

Wilcoxon paired test used. Significance indicated as: ** p=<0.01.

CXCR6+ vs CXCR6-, mean ± SEM, 18.74±2.371 and 59.62±9.46.

3.6 Discussion

Significant evidence from NK cell descriptive phenotypic/functional studies support a significant role for NK cells in HBV infection. There are many comprehensive analyses of NK cell frequency and subset distribution. In this study, the frequencies of total NK cells in the periphery and respective $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets showed no significant difference in CHB patients compared to HC. Bonorino et al also observed no frequency differences in $CD56^{hi}CD3^{neg}$ NK cells and differentiation subsets in a much smaller cohort of nineteen patients (Bonorino et al., 2009).

HBV is a dynamic disease, NK activity is reported to be different in various phases of infection, and a number of studies have analysed the impact of HBV on NK cell frequency and phenotype with polarised results. Oliviero et al found the percentage of peripheral NK cells in HBV was significantly lower compared to HC (Oliviero et al., 2009). In children no difference was seen between the proportions of NK cells (Heiberg et al., 2015). A recent meta-analysis aimed to tease out these inconsistencies, attributed them to differing patient cohorts, and disease phases (Zhang et al., 2016). Zhang et al reviewed 1237 studies, of which 19 were deemed methodologically appropriate to include in the meta-analysis. It is a comprehensive review that summarises peripheral NK cells in CHB exist at a lower frequency with a more activated phenotype compared with HC, with a higher proportion of peripheral $CD56^{bright}$ NK cells.

In keeping with the literature, we show HLA-DR expression was higher in CHB patients compared with HC. HLA-DR molecules are upregulated in response to signalling and therefore a marker for immune stimulation. We chose to analyse the expression of HLA-DR to assess the activation state of NK cells, however frequencies of other markers such as CD38 and CD69 can also be used. The increased expression of activation markers on NK cells in CHB patients may be more suggestive of an

‘inflammatory phenotype’ in these patients, however no correlation was seen with ALT, used as a crude marker of liver inflammation, or viral load.

In chronic viral infection the upregulation of inhibitory receptors is believed to be a mechanism contributing to cell exhaustion and impaired function. Previously, great efforts have been undertaken to examine the expression of various individual inhibitory receptors on T cells. The most cited receptor is PD-1. In several inflammatory and chronic viral infections NK cell PD-1 expression (PD-1+ NK cells) is higher than in HC and higher on the $CD56^{bright}$ NK cell subset (Alvarez et al., 2010), (Golden-Mason et al., 2008), (Norris et al., 2012). The levels are variable and can be near negligible with the lowest expression levels described in HIV positive treatment naive patients with median 0.1%, range 0.02–1.7%, although this is higher than seronegative controls (median 0.05%, range 0.01–0.6%) (Norris et al., 2012). In TB the percentage of PD-1+ NK cells in peripheral blood had a higher mean of 11%. In our analysis, a low range of PD-1 expression was seen on NK cells, with no difference observed between HC and CHB patients, and no significant change within CD56 subsets.

Increased TIM-3 expression on NK cells has been described in HCV (Golden-Mason et al., 2015), HIV (Finney et al., 2013) and HBV (Ju et al., 2010). However, we did not note any significant difference of TIM-3 expression on total NK cells between HC and CHB patients. The mean expression levels of TIM-3 in our cohort; 1.825% in HC vs 3.605% in CHB patients, were marginally higher than previously displayed in the published literature. Ju et al described mean TIM-3 expression of 0.89% in HC and 1.40% in CHB. This disparity may be due to differences in patient cohort composition and staining with a different clone of TIM-3.

The expression of LAG_3, directly ex-vivo, has been shown by Juno et al, demonstrating that it is expressed at low frequency on all NK cell subsets in healthy individuals. LAG-3 levels were expressed at higher levels on invariant NKT cells than T cells or NK cells in HIV infection (Juno et

al., 2015). There is limited literature on LAG-3 expression in CHB. Similar to Juno et al our data showed no difference between LAG-3 expression on total NK Cells between HC and CHB patients, nor between $CD56^{bright/dim}$ NK cell subsets.

Of the array of inhibitory receptors that have been shown to play an important role in T cell exhaustion, interestingly the least described, LAIR-1, revealed its increased frequency in CHB compared to healthy controls suggesting a potential role in CHB. LAIR-1 has not previously been studied on NK cells in CHB. Kennedy et al measured LAIR-1 on global T cells as a marker of exhaustion in a population of young adults with CHB (age range 10-30 years) (Kennedy et al., 2012). They found no difference in LAIR-1 expression between patients and healthy controls on CD4+ or CD8+ T cells. We found higher mean expression of LAIR-1 on NK cells compared with CD8+ T cells, however we did not look at HBV-specific CD8+ T cells as the LAIR-1 expression was not as striking on global CD8+ T cells as on NK cells. LAIR-1 expression was higher on NK cells and this piqued our interest in exploring the role of this receptor, further on NK cells. This had never been described or evaluated previously.

In this study HC and CHB patients were not matched for age and we were aware this could be a potential confounder and might affect co-inhibitory receptor expression. The role of NK cell senescence and associations with a change in the repertoire of receptors, despite percentage of NK cells increasing with age is described in the literature (Naumova et al., 2016). We did not observe a similar correlation in the frequency of circulating NK cells with age, however this may be explained by the lack of older patients in our study cohorts; the maximum age range for HC was fifty-two years and in CHB patients was sixty-eight years. LAIR-1 expression did not correlate with age. Noting the potential confounder of age and change in the repertoire of receptors, the isolated increase of NK cell LAIR-1 expression (not seen with other inhibitory receptors PD-1, LAG-3 and TIM-3) in CHB patients indicates a genuine difference.

Meyaard et al describe LAIR-1 as a broadly expressed antigen on ninety-five to one hundred percent of $CD56^{hi}CD3^{neg}$ NK cells from healthy donors. In our population there is a broader expression of LAIR-1 on PBMC from HC, than suggested by Meyaard et al however higher expression in their study could be attributed to a gating strategy that did not exclude dead cells and a different clone of the LAIR-1 antibody (Meyaard et al., 1997).

Despite the variability in expression, LAIR-1 is an inhibitory receptor that is highly expressed on NK cells in both HC and CHB patients, especially when compared to the restricted expression of other inhibitory receptors. Although we do not have a positive control, as seen further on in chapter 5 LAIR-1 expression was also high on NK cells in NAFLD and HCC.

Another potential confounder explored was regarding whether increased LAIR-1 expression could be attributed to human CMV serostatus. In a healthy person CMV will be latent but persist life-long. The particular relationship between CMV and CHB is not completely understood but CMV infection has been shown to induce the expression of NKG2C, an activatory receptor, in HC and trigger NK cell expansion (Guma et al., 2004). Beziat et al showed similar results in chronic hepatitis patients but there was heterogeneity in expression (Beziat et al., 2012). In this study data was not available on CMV status for HC. However, ninety-nine percent of a cross-sectional cohort of patients with CHB had evidence of CMV seropositivity implying it is unlikely to account for the wide variability in LAIR-1 expression within the CHB cohort.

It is possible to speculate that perhaps such high levels of expression of LAIR-1 regulate peripheral NK cells when they enter tissues such as the liver on exposure to high levels of the ligand, collagen. Thus these expression levels may represent a mechanism of tolerance, to prevent cellular activation and regulate immune responsiveness.

Amongst the CHB patients there was heterogeneity and some overlap of LAIR-1 expression in HC. To evaluate whether this heterogeneity may be explained by the phases of CHB infection, patients were classified by disease phase, and no clear relationship was observed. There was uniformity in the spectrum of LAIR-1 expression across disease phases. Similarly there was no correlation of LAIR-1 with other clinical correlates such as age, ALT, HBV DNA or HBsAg.

The literature demonstrates an association between the profile of receptors on immune cells and cell exhaustion. For example, Bengsch et al observed in their analysis of thirty-eight patients with chronic hepatitis C infection a subset of HCV-specific T cells, identified using CD127 as a marker, expressed a greater array of inhibitory receptors and were more functionally impaired *in vivo* (Bengsch et al., 2010). Kennedy et al found PD-1 was the only inhibitory receptor with increased expression on T cells in young patients compared to age-matched HC, and they exhibited an exhausted profile, although not to the same degree of impairment as in older patients (Kennedy et al., 2012). We looked at whether within disease phases NK cells have a differing inhibitory profile, specifically in regards to LAIR-1. Although LAIR-1 expression was significantly higher than PD-1, TIM-3 and LAG-3 within disease phases, there was no difference of LAIR-1 expression across the disease phases.

In CHB the changes in NK cell phenotype are associated with altered function. NK cells are described as functionally impaired in CHB (Peppa et al., 2010) and we had hypothesised there would be higher expression of LAIR-1, as evident in collective studies observing expression of other inhibitory receptors such as PD-1, TIM-3 and NKG2A. TIM-3 was found to be increased on peripheral and intrahepatic NK cells in forty CHB patients compared with HC and patients with NAFLD (Ju et al., 2010).

In line with studies showing higher expression of LAIR-1 on immature and naive cells (Meyaard, 2008), we also found mean LAIR-1 expression was also higher on $CD56^{bright}$ NK cells. High cell surface expression of LAIR-1 has been reported to be associated with a phenotype of cells that are

less differentiated. Naïve B cells express high levels of LAIR-1 with absent expression in at least half of memory B cells and plasma cells (van der Vuurst de Vries et al., 1999). Similarly in T cells, LAIR-1 expression is high on naïve cells (Jansen et al., 2007).

The literature indicates that $CD56^{bright}$ NK cells express high levels of CD94/NKG2A but virtually no KIRs, raising the possibility that perhaps LAIR-1 is another potential mechanism of NK cell regulation. In CHB, cytokine production from $CD56^{bright}$ NK cells is diminished, and are therefore indirectly believed to contribute to persistence of infection by preventing promotion of T cell responses (Maini and Gehring, 2016). There was a weak but significant inverse correlation showing that those NK cells with higher LAIR-1 expression have impaired cytokine production.

The phenotype of intrahepatic NK cells varies from circulating NK cells in CHB. The difficulty in analysing the intrahepatic compartment stems from the limited yield and viability of these cells. In the absence of infection, inflammation or disease peripheral NK cells will express a higher level of inhibitory receptors, as there is limited requirement for their on-going activation (Rehermann, 2015). A number of studies demonstrate higher expression of activatory receptors (NKp30, NKp46 and NKG2C) and lower inhibitory receptor expression on peripheral NK cells in CHB compared with HCV-infected patients and healthy controls (Rehermann, 2013). Although NK cells are activated, this does not correlate with inducing effector function as would be expected. Largely, intrahepatic NK cells are more activated, and show higher cytotoxic capacity compared to peripheral NK cells, despite a higher proportion of $CD56^{bright}$ NK cells in the liver. Interestingly, we observed much lower LAIR-1 expression on total NK cells in the liver compared to the periphery.

We were interested to observe whether LAIR-1 is associated with liver residency. Initially described in mice, a population of lrNK cells has recently been identified in humans (Marquardt et al., 2015). Stegmann et al found the homing marker CXCR6 could identify a unique subset of lrNK that use a different transcriptional profile that is eomesodermin (EOMES) predominant rather than T-bet.

Anderson et al proposed inhibitory receptors are hierarchical in order of importance, with certain receptors having a more dominant function (Anderson et al., 2016). In keeping with this theory we could speculate liver resident cells that encounter their ligand more readily have a higher threshold for activation and therefore comparatively have a higher expression of inhibitory receptors, and the presence of such high levels of LAIR-1 maintains a balanced/tolerant environment and prevents autoimmunity. Generally, liver NK cells are perceived to be more activated with greater cytotoxicity than peripheral NK cells but lrNK cells are only weakly cytotoxic, perhaps adapted for a role in the maintenance of liver tolerance. In order to establish whether there was a high predominance of LAIR-1 on cells that are thought to have a role in hepatic tolerance we stained a smaller subset of paired samples with the CXCR6 marker and found LAIR-1 expression was also lower on liver resident cells. This is an interesting observation given other inhibitory receptors such as TIM-3 are enriched on the liver infiltrating subset. There is no previous data with regards to LAIR-1 expression in collagen-rich organs. In this regard, we went on to further hypothesise that hepatic NK cells downregulate LAIR-1 in response to continuous exposure to collagens & pro-inflammatory cytokines.

Results – Chapter 4: Function of LAIR-1 in CHB

4.1 Introduction

Important summary findings from the first results chapter indicated:

i. Higher expression of LAIR-1 on NK cells in CHB patients compared with HC.

ii. Downregulation of LAIR-1 expression on NK cells in the liver compared with the periphery.

We hypothesised that the lower NK cell expression of LAIR-1 in the liver may be due to hepatic NK cells being continuously exposed to collagens, antigens and pro-inflammatory cytokines, thereby downregulating LAIR-1. A series of functional experiments were designed to investigate this further.

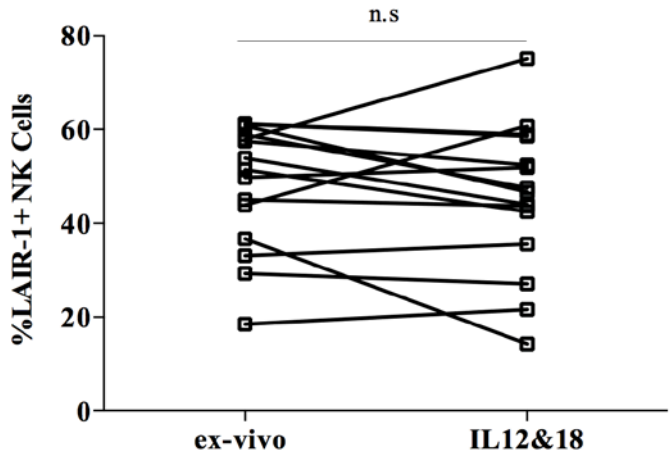
4.2 LAIR-1 expression in the presence of different cytokines

The microenvironment of the liver plays a key role in maintenance of homeostasis as a metabolic and immunological organ. The environment is cytokine and chemokine rich (Heymann and Tacke, 2016). Liver resident cells produce these as do circulating immune cells. To evaluate whether the cytokine milieu of the liver may play a role in the lower expression of LAIR-1 on NK cells from the intrahepatic compartment compared with peripheral NK cells, whole PBMC were incubated overnight, in combination or individually with other liver-relevant cytokines of interest for 21 hours and then stained for surface LAIR-1. Both activatory and immunosuppressive cytokines were investigated.

Figure 4.1a summarises findings of incubation with pro-inflammatory cytokines IL-12 and IL-18, cytokines used commonly to activate NK cells. There was no significant change in surface LAIR-1

expression with standard rhIL-12 and rhIL-18 cytokine stimulation. However, the liver has a unique cytokine milieu so further investigation was carried out to assess the surface expression of LAIR-1 in the presence of different cytokines. These were all liver relevant cytokines, including immunosuppressive cytokines such as TGFβ and IL-10, which are abundant in the liver. IL-10 is produced by Kupffer cells (the local resident macrophage population) and TGFβ by HSCs. IL-15 similar to IL-12 and IL-18 is an activator cytokine used to prime NK cells. IL-2 levels are high in inflammation. IL-8 expression has been reported to be higher in CHB patients, with serum and hepatic IL-8 expression correlating with severe inflammation and fibrosis (Yang et al., 2014). Figure 4.1b demonstrates no change was seen with other liver relevant cytokines.

(a)



(b)

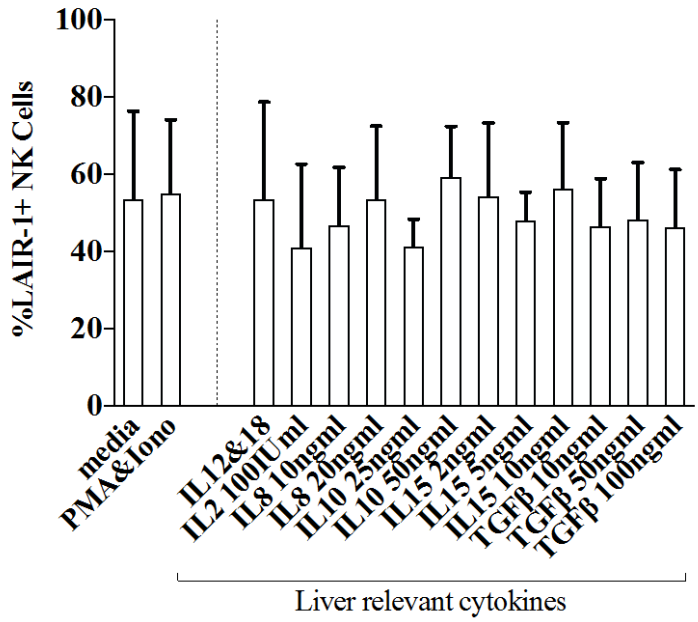


Figure 4.1: Surface expression of NK cell LAIR-1 in the presence of different cytokines

Whole PBMC were incubated overnight in combination with liver relevant cytokines before extracellular staining for surface LAIR-1 on $CD56^{hi}CD3^{neg}$ NK cells

(a) Expression of NK cell LAIR-1 in the presence of rhIL-12 and rhIL-18.

PBMC were isolated and stained from fifteen CHB patients. Paired Wilcoxon test used.

(b) Expression of NK cell LAIR-1 in the presence of other liver-relevant cytokines.

PBMC were isolated and stained from three CHB patients. Analysed with Friedman test and deemed non significant. p=0.3050.

4.3 LAIR-1 expression and collagen

4.3.1 LAIR-1 expression and fibrosis

We postulated that since the ligand of LAIR-1 is collagen, LAIR-1 might correlate with fibrosis stage and act as a crude biomarker. To test this, CHB patients were divided on the basis of their fibrosis stage (Ishak score 0 or ≥1). Patients were analysed within these cohorts, as patients with fibrosis stage greater than one would be considered for closer monitoring or further investigation and if LAIR-1 correlated with fibrosis, this could be used to further guide management, i.e. where an invasive test such as liver biopsy should be readily used to assess the degree of fibrosis and progression of disease.

The Ishak score is a histological scoring system used in chronic viral hepatitis to methodically assess the degree of inflammation and scarring of liver biopsies (Goodman, 2007). A score is given to separate components (periportal or periseptal interface hepatitis, necrosis, focal inflammation and portal inflammation) and the stage of fibrosis (Table 4.1). Fibrosis is graded on a scale from zero to six.

There was no difference in LAIR-1 expression on total NK cells within the periphery or liver by these divisions according to fibrosis level (Figure 4.2).

Ishak stage	*Score*
No fibrosis	0
Fibrous expansion of some portal areas	1
Fibrous expansion of most portal areas	2
Fibrous expansion of most portal areas with occasional portal to portal bridging	3
Fibrous expansion of portal areas with marked bridging	4
Marked bridging with occasional nodules	5
Cirrhosis	6

Table 4.1: Ishak stage scoring system for fibrosis.

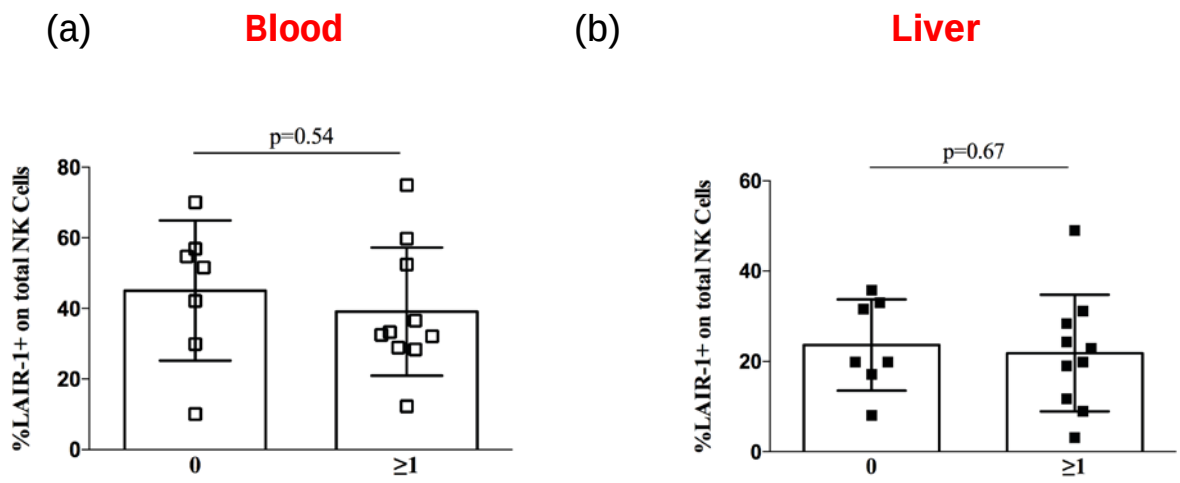


Figure 4.2: Comparison of LAIR-1 expression on NK cells with fibrosis stage.

Paired PBMC and IHL were isolated from seventeen patients with CHB. PBMC and IHL were stained for LAIR-1 expression on $CD56^{hi}CD3^{neg}$ NK cells. These patients were divided on the basis of their fibrosis stage (Ishak score 0 or ≥1) established by histopathological staging. Mann-Whitney statistical test has been used.

4.3.2 LAIR-1 expression and collagen

In order to investigate the outcome of the interaction between LAIR-1 and whether collagen binding effected surface expression, we examined the effect of collagen-coated plates on LAIR-1. Several types of collagen exist that can be used, but there is convincing experimental evidence of type 1 collagen, as one of the high affinity collagen ligands for LAIR-1 (Lebbink et al., 2006), (Brondijk et al., 2010). In the diseased liver the most predominant collagens are collagen I, III, IV and V (Rojkind et al., 1979), and they have been seen to be localised to different areas. Immunohistochemical studies from the 1970s isolated collagen I to the liver capsule, portal stroma, sinusoidal wall and in Disse's space where there is direct contact with the plasma membrane of hepatocytes, whereas collagen IV is highly detected in basement membranes (Martinez-Hernandez, 1984). To analyse the effect of collagen on LAIR-1 we chose to use type I collagen in our experimental design.

Figure 4.1 depicts the experiments that demonstrated NK cell surface expression levels of LAIR-1 did not change on incubation with different cytokines. Therefore to investigate whether collagen binding affects surface expression we performed a series of experiments to examine the effect of collagen-coated plates on incubation with PBMC. PBMC from four CHB patients were used rather than healthy controls. As a consequence of earlier experiments that had shown CHB patients have higher mean expression of NK cell LAIR-1, an apparent downregulation would have been easier to observe. PBMC were also incubated for two hours versus six hours to determine whether duration of collagen exposure made a difference. 96-well pre-coated collagen plates were used. Figure 4.3a shows there was no change in NK cell surface LAIR-1 on pre-coated collagen plates. The PBMC did not show any difference at six-hour incubation either.

Although there was no difference in the surface expression of LAIR-1 on peripheral NK cells, we tested whether intrahepatic NK cells might downregulate NK cell surface LAIR-1 on exposure to

collagen (Figure 4.3b). IHL from five CHB patients were incubated for two hours with or without collagen. After the two-hour incubation period they were surface stained for LAIR-1. The IHLs were not exposed to collagen for longer than two hours, as attempted with PBMC. This is due to the lower number of IHL obtained from liver biopsy tissues, which limits the experimental conditions that can be undertaken. Only two patients showed downregulation of intrahepatic NK cell LAIR-1.

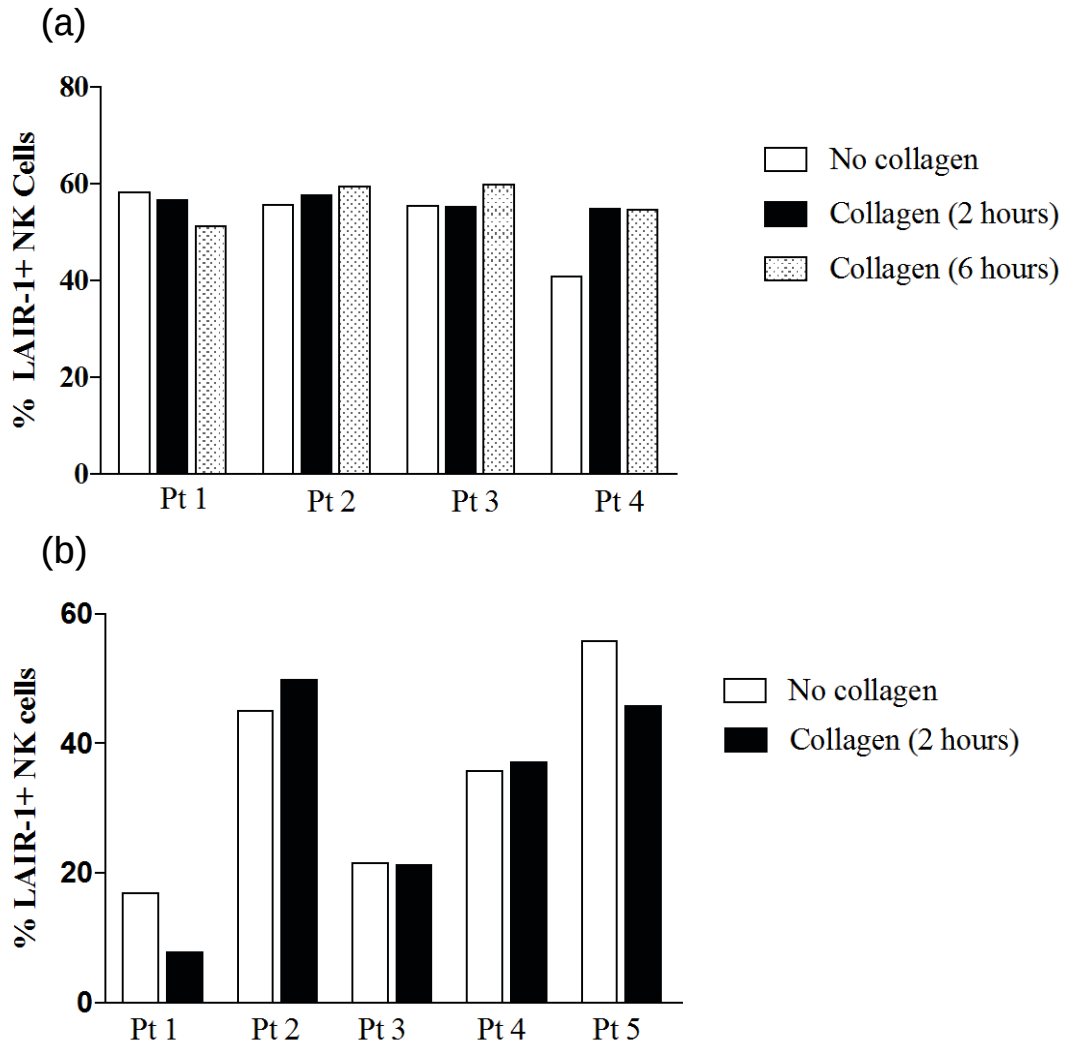


Figure 4.3: Investigating effect on (a) PBMC and (b) IHL NK cell LAIR-1 surface expression in the presence of collagen.

PBMC and IHL were incubated in the absence and presence of collagen (96-well pre-coated collagen plates) and surface stained for LAIR-1 expression on $CD56^{hi}CD3^{neg}$ NK cells.

(***a) PBMC;*** PBMC from four CHB patients were incubated in the presence and absence of collagen for 2 hours vs 6 hours to determine whether duration of collagen exposure made a difference. Analysed with two-way ANOVA (Tukey's multiple comparison test) deemed not significant.

(b) IHL; IHL from five CHB patients were incubated in the presence and absence of collagen for 2 hours. Paired Wilcoxon test used and deemed not significant.

4.4 NK cell function in CHB

Before investigating mechanism and functional role of LAIR-1 we wanted to ascertain NK cell activity in our study cohort of CHB patients and compare to findings elsewhere in the literature. NK cell function can be measured by cytokine and cytotoxicity of NK cells and subsets.

NK cells are potent producers of IFNγ, which can mediate non-cytolytic antiviral effects. The percentage of IFNγ expression of NK cells was calculated after whole PBMC from twenty-nine CHB patients were incubated with rhIL-12 and rhIL-18 for 21 hours. There is variability amongst patients and IFNγ production from circulating NK cells varied from 3.85% to a maximum of 68.2% (Figure 4.4). Although the $CD56^{bright}$ NK cell subset is the principal IFNγ producing subset (mean 64.23%), the $CD56^{dim}$ NK cell subset does also contribute to producing IFNγ (mean 14.39%).

The cytotoxic potential of NK cells was measured by determining degranulation capacity evident by CD107a expression following stimulation of PBMC with rhIL-12, rhIL-18 and K562 target cells. As shown in Figure 4.5 PBMC from thirty-four CHB patients were used. Circulating NK cell CD107a expression varied between 12% and 70% (mean 44.64%). Differential NK cell subset analysis showed both the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets have cytotoxic potential. There was a significant difference in the proportion of CD107a expression between subsets. $CD56^{bright}$ NK cells had a higher mean expression of 49.68% versus 32.57% in the $CD56^{dim}$ NK cell subset. This is surprising as the literature shows that the $CD56^{bright}$ NK cell subset as the more efficient cytokine producer with less cytotoxic capacity.

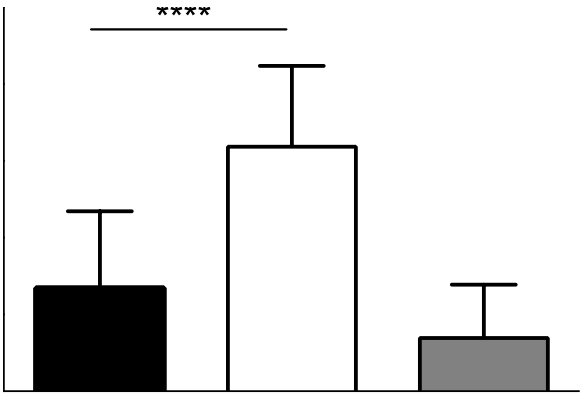


Figure 4.4: Comparison of percentage of IFNγ expression of peripheral NK cells and NK cell subsets ($CD56^{bright}$ and $CD56^{dim}$) in CHB patients.

PBMC from twenty-nine CHB patients were incubated overnight with rhIL-12 and rhIL-18 for a total of 21 hours. Cells were thereafter fixed and permeabilised and then stained for $CD56^{hi}CD3^{neg}$ NK cells divided into subsets based on expression of CD56 and CD16, allowing them to be differentiated as $CD56^{bright}$ and $CD56^{dim}$ NK cells and intracellular expression of IFNγ. $CD56^{hi}CD3^{neg}$ NK cells vs $CD56^{bright}$ vs $CD56^{dim}$ NK cells, mean ± SEM, 27.6±3.568 vs 64.2±3.813 vs 14.39±2.494. Analysed with Kruskal-Wallis (one-way ANOVA) test. Significance was indicated at * $p<0.05$, **** $p<0.0001$.

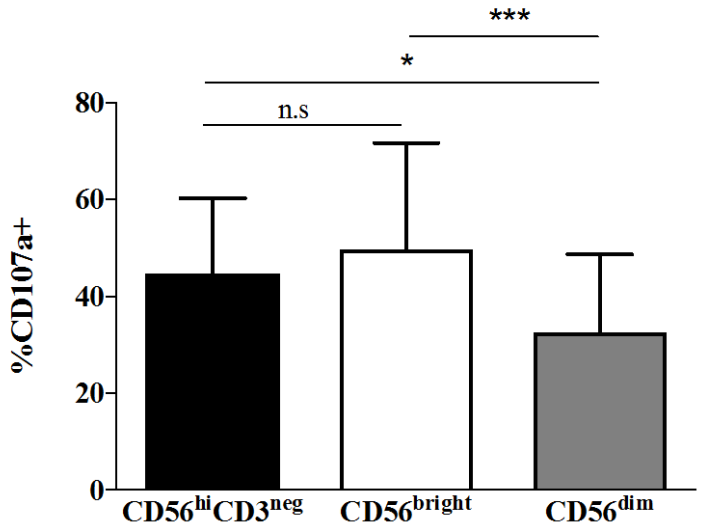


Figure 4.5: Comparison of percentage of CD107a expression of peripheral NK cells and NK cell subsets (CD56^{bright} and CD56^{dim}) in CHB patients.

PBMC from thirty-four CHB patients were incubated overnight with rhIL-12 and rhIL-18 for a total of 21 hours and in the last three hours of incubation target cells added. Cells were thereafter fixed and stained for CD56^{hi}CD3^{neg} NK cells divided into subsets based on expression of CD56 and CD16, allowing them to be differentiated as CD56^{bright} (CD56^{hi}CD16^{neg}) and CD56^{dim} (CD56^{lo}CD16^{pos}) NK cells and expression of CD107a. CD56^{hi}CD3^{neg} NK cells vs CD56^{bright} vs CD56^{dim} NK cells, mean ± SEM, 44.64±2.691 vs 49.68±3.775 vs 32.57±2.755. Analysed with Kruskal-Wallis (one-way ANOVA) test. Significance was indicated at * $p<0.05$, *** $p<0.001$.

4.5 Function of LAIR-1 / Function of NK cells on collagen

The next sets of experiments performed were to investigate the function of LAIR-1. A number of studies described in detail in the main introduction demonstrate that LAIR-1 cross-linking with collagen can downregulate NK cell-mediated cytotoxicity (Lebbink et al., 2006), (Meyaard et al., 1997), as well as production of IFNγ by T cells (Jansen et al., 2007). We carried out cytokine production and CD107a degranulation assays in the presence and absence of collagen I coated plates to assess whether there was a functional change in NK cells when exposed to collagen.

Despite no change in surface LAIR-1 expression with a differing cytokine milieu or on incubation with collagen plates we further investigated whether NK cell function changed in the presence of collagen. Lebbink et al had previously demonstrated collagens can inhibit immune cell function by binding to LAIR-1 and this effect was pertinent to the LAIR-1-collagen interaction as pre treating the cells with blocking anti-hLAIR-1 $F(ab')_2$ (8A8) fragments reversed this inhibition (Lebbink et al., 2006). In particular it was cell degranulation that was inhibited and reversed.

To measure this effect NK cell degranulation was assessed by measuring CD107a as a readout on the surface of NK cells. Whole PBMC from six CHB patients were incubated on pre-coated collagen plates or uncoated plates for two hours and thereafter incubated overnight with cytokine before a three-hour co-incubation with target cell line K562. Preliminary experiments on PBMC from CHB patients, indeed demonstrated downregulation of CD107a on NK cells in CHB upon collagen pre-coated plates (Figure 4.6). The range of inhibition was between 10.7% and maximum 22.4% (mean 14.7%). however there was no correlation between degree of inhibition and surface expression of LAIR-1 on NK cells (Figure 4.7).

In parallel the percentage of IFNγ expression of NK cells was also evaluated. These data are from whole PBMC from five of the above CHB patients that had shown change in NK cell degranulation. Whole PBMC were incubated on pre-coated collagen plates or uncoated plates for two hours and thereafter incubated overnight with cytokine. Figure 4.8 demonstrates variability, with only two patients showing downregulation in function (6.5% in patient 2 and 7.48% in patient 3). Thus whilst the cytotoxicity capacity of NK cells was significantly altered by collagen stimulation, cytokine production did not significantly change.

As previously noted, we demonstrated a weak but significant inverse correlation showing that NK cells with higher LAIR-1 expression have impaired cytokine production. However, in keeping with the lack of significant change in NK cells producing IFNγ on incubation with collagen, no correlation was noted between the expression of LAIR-1 on NK cells and cytokine production in the presence of collagen (Figure 4.9).

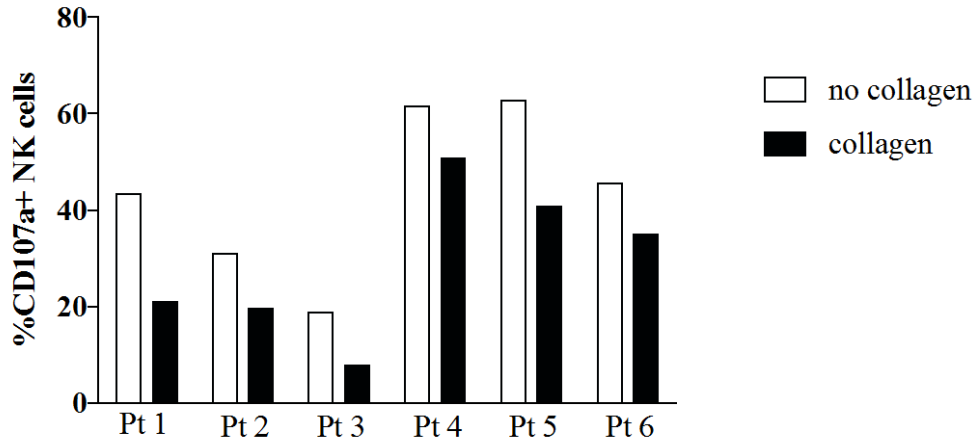


Figure 4.6: Investigation of NK cell CD107a expression in the presence of collagen.

PBMC from six CHB patients were pre-incubated on 96 well plates (with or without collagen) for 2 hours prior to adding rhIL-12 and rhIL-18 and incubated overnight for a total of 21 hours and in the last three hours of incubation target cells added. Cells were thereafter fixed and stained for $CD56^{hi}CD3^{neg}$ NK cells and expression of CD107a. Paired Wilcoxon test used. Significance was measured at $p<0.05$. $p= 0.03$

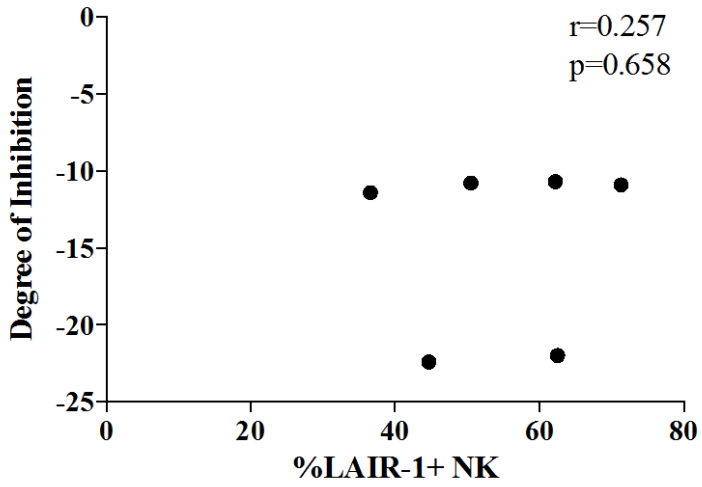


Figure 4.7: Investigation of the expression of LAIR-1 on NK cells and degree of inhibition.

PBMC from six CHB patients were incubated overnight with rhIL-12 and rhIL-18 for a total of 21 hours and in the last three hours of incubation target cells added. Cells were thereafter fixed and stained for $CD56^{hi}CD3^{neg}$ NK cells and expression of CD107a. The degree of inhibition was correlated against surface expression of LAIR-1. Significance testing was carried out using Spearman correlation coefficient. Significance was measured at $p<0.05$. p=0.658

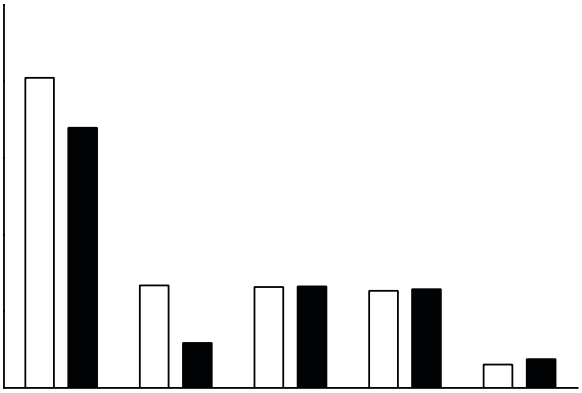


Figure 4.8: Investigation of NK cell IFNγ expression in the presence of collagen.

PBMC from five CHB patients were pre-incubated on 96 well plates (with or without collagen) for 2 hours prior to adding rhIL-12 and rhIL-18 and incubated overnight for a total of 21 hours. Cells were thereafter fixed and stained for $CD56^{hi}CD3^{neg}$ NK cells and intracellular expression of IFNγ. Paired Wilcoxon test used. Significance was measured at $p<0.05$. $p= 0.81$

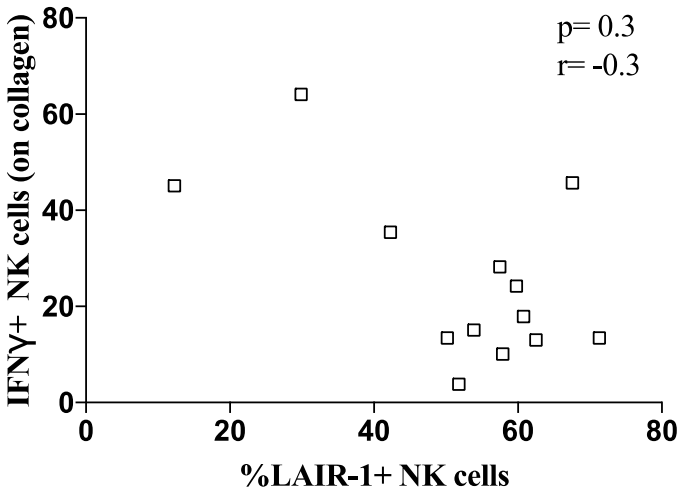


Figure 4.9: Investigation of the expression of LAIR-1 on NK cells and cytokine production in the presence of collagen.

Baseline surface LAIR-1 expression was compared to the % IFNγ expression of NK cells after whole PBMC were incubated with rhIL-12 and rhIL-18 for 21 hours in the presence of collagen. PBMC from thirteen CHB patients were used. Cells were thereafter fixed and permeabilised and then stained for intracellular expression of IFNγ. Significance testing was carried out using Spearman correlation coefficient. Significance was measured at $p<0.05$. $p=0.3$.

4.6 LAIR-1 and HSCs

Several studies have highlighted a role for receptor-ligand interactions between NK cells and HSCs, demonstrating increased NK cell stimulation and reduced NK cell inhibition, with a shift in the balance of activating and inhibitory signals, thereby reducing liver fibrosis by increasing HSC death (Melhem et al., 2006). There are several strategies by which HSCs can interact with NK cells but it is not a straightforward mechanism involving one particular pathway. It is also not known which population of liver NK cells is responsible for curtailing fibrosis and whether this is a potential role of the more recently identified lrNK cells.

Results have shown that collagen-LAIR-1 interactions can inhibit cell activation and potentially dampen immune cell responses (Lebbink et al., 2006). In chronic liver damage ongoing stimulation of activated HSCs results in excess production of ECM products, particularly type I collagen. Activated HSCs in this situation can persist for longer periods surrounded by type I collagen, which may prevent access of NK cells to their target (Fasbender et al., 2016).

We had hypothesised that a possible mechanism that protects activated HSCs from clearance is via collagen produced by over activated HSCs inhibiting NK activity by directly binding with LAIR-1. To analyse whether HSCs might be capable of cross-linking LAIR-1 PBMC were co-cultured with media alone, cytokine alone, and cytokine with activated pHSC or pHSC supernatant. These experiments were done in conjunction with Dr Laura Pallett (Mala Maini group, UCL). There was no convincing downregulation of LAIR-1 upon co-culture with pHSC or pHSC supernatant (Figure 4.10), but it is possible that the function of NK cells could be altered if there was ligand engagement. This potential mechanism is of interest and warrants further exploration.

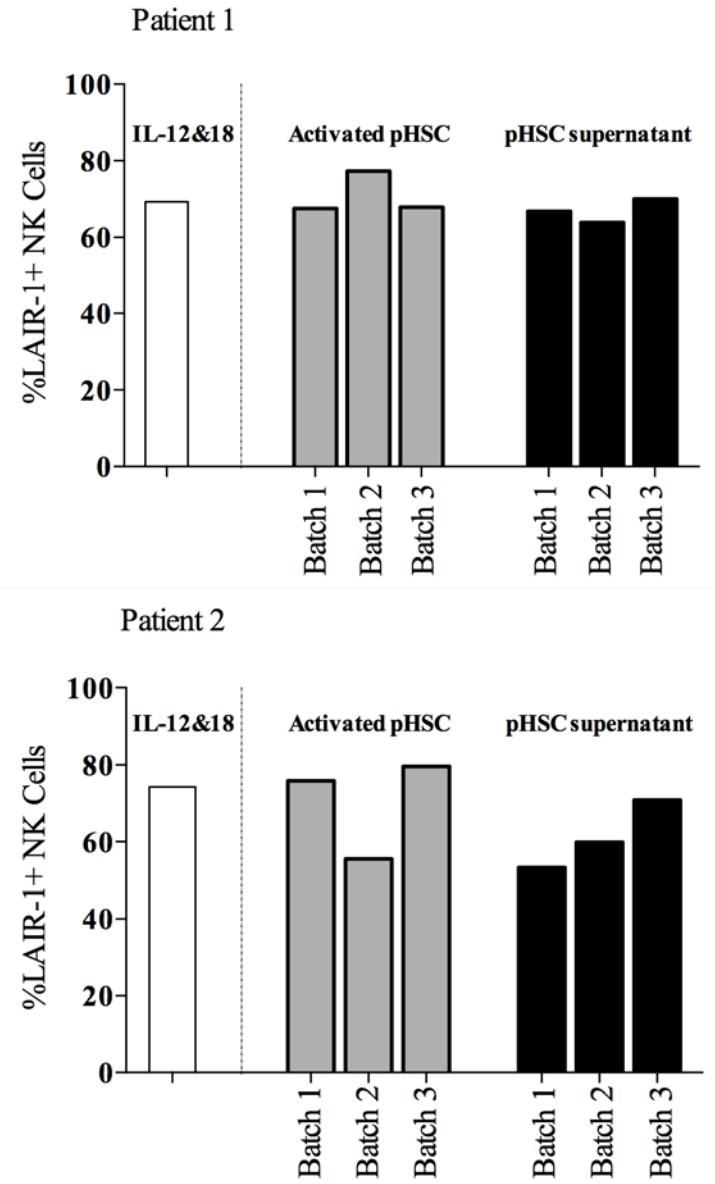


Figure 4.10: Analysis of surface NK cell LAIR-1 expression after co-culture with pHSC or pHSC supernatant.

PBMC (n=2 CHB) were incubated overnight with media as negative control or cytokine to activate the NK cells. They were then co-cultured with media alone, activated pHSC or pHSC supernatant. Three batches of each of these were used.

4.7 Discussion

The function of NK cells in CHB patients was verified using assays to measure their cytokine production and cytotoxic potential. Here, we were able to reproduce results similar to those reported in the literature. In the periphery the $CD56^{bright}$ NK cell subset were more IFNγ producing but findings also showed them to be capable of degranulation, marked by CD107a. There was variability amongst patients and although the mean for CD107a expression was higher on $CD56^{bright}$ NK cells than in other studies this can be easily explained by differences in patient cohorts, different E:T ratios and a new batch of rhIL-12 and rhIL-18 cytokines that were used.

Given other studies have proposed that on maturation or activation LAIR-1 expression is reduced on neutrophils, T cells and B cells as a mechanism for regulation at the level of the receptor, we were keen to investigate the effect on NK cells. Results from previous studies have been contradictory, with regards to change in LAIR-1 expression upon stimulation (van der Vuurst de Vries et al., 1999, Verbrugge et al., 2006a). Jansen et al (2007) summarised that *in vitro* stimulation of T cells resulted in decreased surface expression of LAIR-1 (human T cells stimulated with anti-CD3 (aCD3) and anti-CD28 antibodies), whereas Maasho et al (2005) had shown TCR stimulation increases cell surface expression of LAIR-1 (cells stimulated with aCD3 and rhIL-12) (Jansen et al., 2007, Maasho et al., 2005). Our results showed no significant change in surface NK cell LAIR-1 expression with standard rhIL-12 and rhIL-18 cytokine stimulation. We had hypothesised that perhaps as a consequence of the cytokine milieu in the liver being different to the periphery (liver resident cells can produce immunosuppressive cytokines e.g. Kupffer cells produce IL-10 and TGFβ, which can tolerise local NK cells) this could explain the downregulation of LAIR-1 on liver NK cells. However, there was also no change with other liver relevant cytokines. One of the reasons for this may be that NK cells are more sensitive to membrane bound cytokine e.g. cell surface transpresentation of IL-15 or perhaps it could be due to differing circulating cytokine profile in chronic hepatitis B patients. Bertoletti et al

were able to show that different cytokine profiles exist within the HBV infected liver (Bertoletti et al., 1997).

Although there was no change in LAIR-1 expression with cytokine stimulation we wanted to further dissect why there was evidence of downregulation of LAIR-1 in the liver compared to the periphery and postulated this may be due to encountering of the ligand collagen which is more abundant in the liver. However, we discovered there was no change in surface LAIR-1 expression on incubation with pre-coated collagen plates. The duration of exposure of PBMC to collagen also did not show any difference.

We wondered whether LAIR-1 might have useful clinical significance, as a potential non-invasive test that could represent a crude biomarker indicating severity of fibrosis, in the knowledge the ligand is collagen. However, there was no difference within fibrosis groups or NK cell LAIR-1 expression in the periphery or liver. Limitations include the lack of normal controls and small patient numbers, however another major limitation of this analysis is that most patients who underwent biopsy in this study displayed only minimal to moderate levels of fibrosis and thus it is difficult to ascertain such a correlation without a more broad range of patients, to obtain a normal distribution. The potential use of collagen proportionate area (CPA) for fibrosis assessment in the patients detailed in Table 2.2 could be explored further. CPA is a well-validated method of fibrosis quantification, and offers increased accuracy in calculating the parenchymal fibrous tissue collagen (Tsochatzis et al., 2014). Additional work looking at non-invasive serum biomarkers of fibrosis such as hyaluronic acid, pro-collagen III N-peptide or aspartate platelet ratio index (APRI), and transient elastography of the patient cohorts in Table 2.1 and 2.2 would be another strategy to determine whether LAIR-1 can be used as an adjunct to tools used to assess fibrosis.

Peppa et al were the first in this field to describe the functional impairment and reversal of exhaustion of NK cells in CHB. They showed the $CD56^{bright/dim}$ NK cell subsets have marked impaired capacity to produce IFNγ in CHB and immunosuppressive cytokines selectively suppress NK cell IFNγ production, whilst cytotoxicity is maintained (Peppa et al., 2010). Blocking of inhibitory receptors such as TIM-3 on NK cells has shown enhancement of cytotoxicity (Ju et al., 2010). When we explored whether NK cell function changed in the presence of collagen, preliminary experiments on PBMC from CHB patients showed downregulation of CD107a on NK cells in CHB upon collagen pre-coated plates. In these experimental designs collagen was coated on plastic plates and cells were incubated together with an activating stimulus. Other authors investigating the function of LAIR-1 had previously used this method. Of course it is contentious as to whether this represents how immune cells interact with collagen *in vivo* and the response of immune cells in the presence of ECM collagens or transmembrane collagens would need to be studied in much greater detail to address this. In this experimental design there was overall downregulation of NK cell degranulation in six patients (PBMC). Future studies are underway utilising liver scaffolds containing ECM in a more physiological three-dimensional (3D) structure to investigate its impact on LAIR-expressing NK cells (see future directions chapter 7).

These data are promising as they are consistent with previous studies by Lebbink et al demonstrating collagens can inhibit immune cell function by binding to LAIR-1 (Lebbink et al., 2006). Further work would be to look at other aspects of cytotoxicity. NK cell cytotoxicity is a stepwise combined process including adhesion, activation and secretion of lytic granules, thus CD107a as a readout, is not the only correlate of cytotoxicity. We would also want to look specifically at whether LAIR-1 positive NK cells tend to degranulate less. We would repeat these experiments using intrahepatic lymphocytes. These experiments used whole PBMC rather than sorted fractions of NK cells as this provides a more physiological environment. Moving forward, experiments would involve observing whether pre-incubation with blocking anti-hLAIR-1 $F(ab')_2$ (8A8) fragments reverses inhibition.

Results – Chapter 5: Expression of LAIR-1 in patients with liver disease from NAFLD and in HCC

5.1 Introduction

5.1.1 HBV and HCC

HCC is an aggressive disease with rising incidence and mortality. An estimated 600,000 deaths per year are attributed to cirrhosis and HCC complicating HBV (Liaw and Chu, 2009), (Stanaway et al., 2016). There are a number of liver diseases that can predispose to HCC, however the majority require architectural change in the form of advanced fibrosis or cirrhosis to have already occurred. Chronic viral infection is an important risk factor for HCC development, with HBV accounting for 50% of cases (Bosetti et al., 2008), (Parkin, 2006). The new developments in oral antivirals for HCV may reduce incidence of HCC from HCV, however in areas such as Asia and Africa where HBV is highly endemic, it is the primary cause of HCC. Some recent studies have demonstrated that the newer more potent NUCs for HBV may prevent the development of HCC but this needs to be substantiated in large robust clinical trials (Hosaka et al., 2013), (Lai and Yuen, 2013). Hepatitis B is unique in that it is pro-oncogenic and patients can develop HCC despite not having cirrhosis.

A meta-analysis of studies in China showed the risk for HCC in HBV is 15 to 20 times more than the general population (Shi et al., 2005). Other risk factors for HCC are genetic and environmental. In HBV, factors that increase risk include demographics (male gender, older age, family history of HCC), viral (co-infection, genotypes C&D, longer duration of infection) and lifestyle factors (exposure to Aflatoxin, alcohol excess, smoking) (Chen et al., 2006). HBV integration was shown to drive oncogenic change in a large study of patients with HCC (Zhao et al., 2016). Approximately one

third of patients who acquire HBV perinatally or in early childhood are at risk of death due to HBV related liver cirrhosis or HCC (Beasley et al., 1981).

5.1.2 Treatment and screening for HCC in HBV

Current treatments for HCC are medical, a combination of locoregional therapies; radiofrequency ablation and transarterial chemoembolization; in addition to systemic targeted chemotherapy like Sorafenib and surgical approaches. The median survival following diagnosis is between 6 and 20 months. Although surgical resection can result in 5-year survival rates of better than 70%, patients with cirrhosis are at high risk of decompensation and therefore this option is restricted to a small number of patients. Surgical resection and liver transplantation are the only curative options. European and international guidelines agree screening should occur in high risk patients with CHB defined as Asian/African origin, age >40 years and male, age >50 years and female (EASL, 2012), (Sarri et al., 2013), (Terrault et al., 2016). Screening is carried out using a combination of using serum alpha fetoprotein (AFP) tumour marker and interval radiology imaging, using ultrasound as the first-line, but often other modalities such as computed tomography (CT) or magnetic resonance imaging (MRI) are employed. AFP is used but suboptimal in isolation, as it has a sensitivity of only 60% (Singal et al., 2009). Specificity of diagnosing HCC increases with AFP greater than 400ng/ml (cut-off is 20ng/ml) (Taketa et al., 1993). Currently we do not have an accurate biomarker that can be used alone to identify patients who have HCC (Forner and Bruix, 2012).

5.1.3 Immunology of HCC

HCC development is believed to be a multistep complex process involving altered growth and survival signalling pathways, activation of inflammatory pathways, formation of free radicals and mutations within the tumour microenvironment and the tumour cells (Forner et al., 2012). The main mutations are in tumour suppressor gene TP53 and the gene for beta-catenin CTNNB1. Key

oncogenes are altered. HCC is a highly vascular tumour and angiogenesis pathways are altered. Studies in a mouse model of HBV-related HCC have shown a role for platelet-mediated hepatic T cell accumulation in driving pro-inflammatory carcinogenesis (Sitia et al., 2012). Microarray gene studies are being used to help provide prognostic information and guide therapeutic intervention.

There is heterogeneity within the stromal tumour microenvironment but it overall consists of immune cells and several cell types including HSCs, macrophages and endothelial cells (Heindryckx and Gerwins, 2015). Tumour and stromal cells exploit the host immune response creating an environment rich in immunosuppressive molecules dampening effector cell function. Cancer cells can evade recognition by CTLs by downregulating or loss of MHC class I, although this can be a double edged sword resulting in killing by NK cells, which play a key role in cancer immune surveillance. However, tumour production of ligands such as soluble MHC class I polypeptide-related sequence A (MIC-A) and unique long 16 binding protein (ULBPs) can result in downregulation of NKG2D and impaired NK cell function (Prieto et al., 2015). Inhibitory pathways are altered. There have been a number of studies looking at the PD-1-PD-L1 pathway as described earlier with interest in anti-PD-1 and anti-CTLA-4 mAbs. Tumours upregulate PD-L1 and high expression of the ligand in HCC has been associated with tumour aggressiveness and post resection recurrence (Gao et al., 2009).

It has been speculated that the LAIR-1 collagen interaction can be orchestrated to the advantage of tumour cells via a mechanism by which they upregulate collagen expression in a number of ways, increasing the availability of ligand to bind to LAIR-1, resulting in negative regulation of effector cells and thereby dampening anti-tumour responses (Meyaard, 2008). A study by Mailloux et al showed that the collagen matrix protects tumour cells from death. They observe the HepG2 HCC cell line, which does not produce any collagen in the absence of activated HSCs, is susceptible to killing by NK cells. Their results suggest collagen derived from HSCs may contribute to the dysfunction of NK cells in HCC and LAIR-1 plays a key role in this process. They found bioartificial collagen matrix significantly inhibited human NK cell proliferation, however, NK cells isolated from LAIR-1

germline knock-out mice were not inhibited by collagen (Mailloux and Epling-Burnette, 2013). The abstract is however a stand alone meeting abstract and due to this it is difficult to tease out methodology and other studies are needed to confirm the role of LAIR-1 as a potential mediator of immune suppression by the local collagen environment in HCC and fibrosis. These are however very interesting and striking observations.

Thus far we have seen LAIR-1 expression on total NK cells in patients with CHB is significantly lower in the liver compared to the periphery. We did not have access to 'healthy' liver tissue for comparison, and thus to investigate whether the effects of LAIR-1 downregulation in the intrahepatic compartment in CHB were unique to viral infection, we utilised samples (blood and liver) from patients without evidence of chronic viral infection. For this purpose, we utilised paired blood and liver samples from patients with NAFLD. In addition to this we also utilised samples from the periphery of HCC patients to determine the expression of LAIR-1 in advanced liver disease (some of these patients were infected with HBV or HCV). There is a clear unmet need for a predictive biomarker within this field. The predictive value of negative PD-L1 staining is not precise enough to be able to exclude patients from treatment with checkpoint inhibitors (Postow et al., 2015). We therefore focused our attention on LAIR-1 and its expression on NK cells in NAFLD and HCC patients.

5.2 NK cell LAIR-1 expression in NAFLD patients

Thus far we have seen in LAIR-1 expression on total NK cells in CHB patients is significantly lower in the liver compared to the periphery. We further analysed paired blood and liver samples from patients with fatty liver disease. These samples were stained for LAIR-1 and analysed. The full characteristics are shown in Table 5.1. PBMC and IHL were analysed from seven paired samples. Summary of patient data is shown in Table 5.1.1

Sex	**Age** (years)	**mean ALT** (IU/L)
F	52	111
F	35	37
M	54	59
F	19	246
M	25	105
M	40	140
F	79	63

Table 5.1: Full details of the study participants with NAFLD

Full details of the study participants with NAFLD for paired data (blood and liver) n=7

	Age (years) Median (range)	**Sex (%)** Male:Female	**ALT (IU/L)** Median (range)
NAFLD n= 7	40 (19-79)	43:57	105 (37-246)

Table 5.1.1: Summary of NAFLD cohort paired data

LAIR-1 was increased on circulating NK cells in NAFLD (mean 57%) compared to healthy controls (29.9%) (Figure 5.1). The higher frequencies of LAIR-1-expressing NK cells in NAFLD were also significantly higher than CHB patients (mean 39.5%). In NAFLD LAIR-1 expression on both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets displayed mean values of 45.16% and 58.01% respectively. LAIR-1+ $CD56^{bright}$ NK cells were significantly higher in NAFLD (mean 45.16%) patients compared with those with CHB (mean 35.6%) and HC (mean 23.8%). The highest mean expression of LAIR-1 was observed on $CD56^{dim}$ NK cells in NAFLD.

Paired analysis of blood and liver shown in Figure 5.2 depicts LAIR-1 expression on total NK cells in patients with NAFLD, which is significantly lower in the liver (mean 34.63%) compared to the periphery (mean 57.04%). The range in the periphery is between 45.1% to 75.6%, compared to the intrahepatic compartment with a range of 16.2% and 46.8%. Interestingly, although not significant there is a trend for higher LAIR-1 expression on intrahepatic NK cells in NAFLD (mean 34.63%) than in CHB (mean 22.76%) (Figure 5.3).

In CHB patients, LAIR-1 expression was also lower on lrNK cells (Figure 3.18 - mean 18.74%). Samples from patients with NAFLD were also analysed using CXCR6, and similar to that seen in CHB, there was a significant downregulation of LAIR-1 on lrNK cells (Figure 5.4). Mean expression on lrNK cells was 28.84% versus 51.93% on liver infiltrating cells.

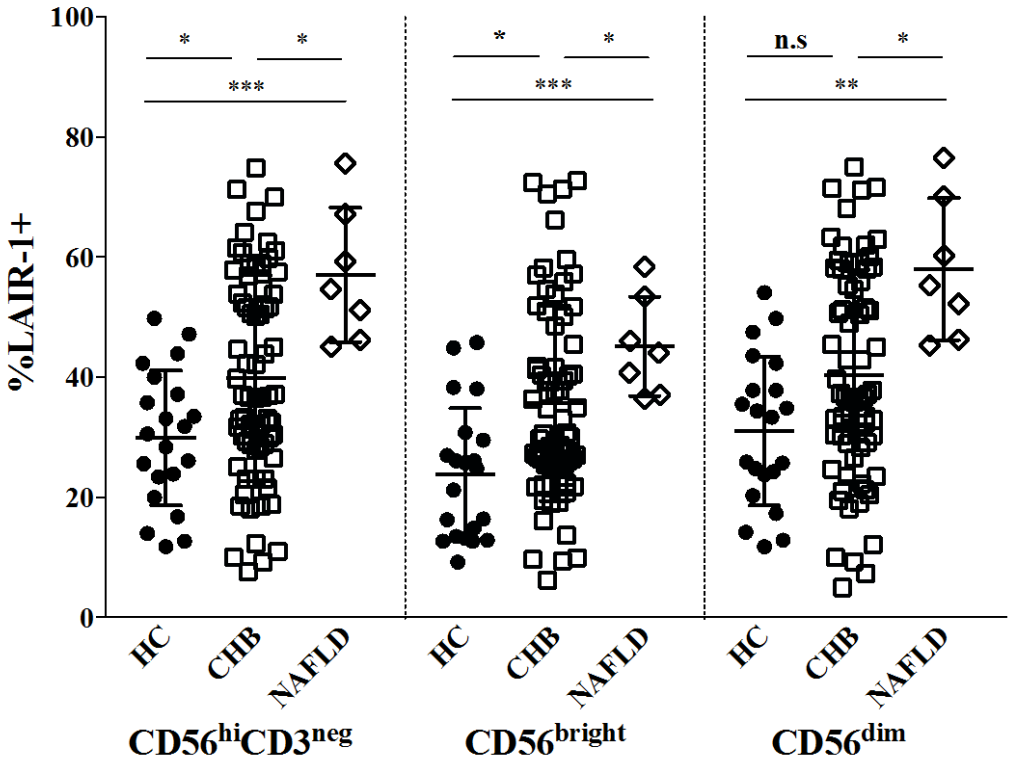


Figure 5.1: Comparison of percentage LAIR-1+ on total peripheral NK cells and within CD56^{bright} and CD56^{dim} NK cell subsets between HC, CHB and NAFLD patients.

PBMC were isolated and stained from twenty-one HC, sixty-nine patients with CHB and seven patients with NAFLD. PBMC were stained for LAIR-1 expression on CD56^{hi}CD3^{neg} NK cells, CD56^{bright} and CD56^{dim} NK cells. HC vs CHB vs NAFLD, patients mean ± SEM, CD56^{hi}CD3^{neg}; 29.9±2.45 vs 39.87±2.06 vs 57.04±4.23, CD56^{bright}; 23.8±2.41 vs 35.6±1.95 vs 45.16±3.11, CD56^{dim}; 31.05±2.7 vs 40.27±2.1 vs 58.01±4.46. Error bars represent the mean ± SEM. Analysed with Kruskal-Wallis (one-way ANOVA) test. Significance is indicated as * p<0.05, ** p<0.01, ***p<0.001.

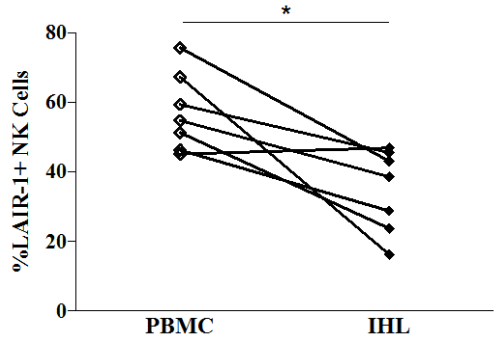


Figure 5.2: Comparison of percentage LAIR-1+ on total circulating versus intrahepatic NK cells in NAFLD patients.

Paired PBMC and IHL were isolated and stained from seven patients with liver disease from NAFLD. PBMC and IHL were stained for LAIR-1 expression on $CD56^{hi}CD3^{neg}$ NK cells. Paired Wilcoxon test used. Significance indicated as * p=<0.05. LAIR-1+ NK PBMC vs IHL, mean ± SEM, 57.04±4.233 and 34.63±4.487.

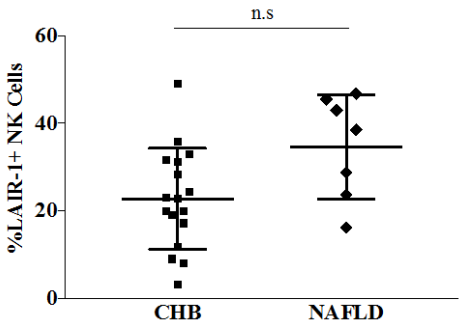


Figure 5.3: Comparison of percentage LAIR-1+ on intrahepatic NK cells in NAFLD versus CHB patients.

IHL were isolated and stained for LAIR-1 expression on $CD56^{hi}CD3^{neg}$ NK cells from seven patients with liver disease from NAFLD and compared to the cohort of IHL that were isolated and stained from seventeen patients with CHB. Significance testing was carried out using Mann Whitney test. Significance indicated as * p=<0.05. LAIR-1+ NK CHB vs NAFLD, mean ± SEM, 22.76±2.793 and 34.63±4.487.

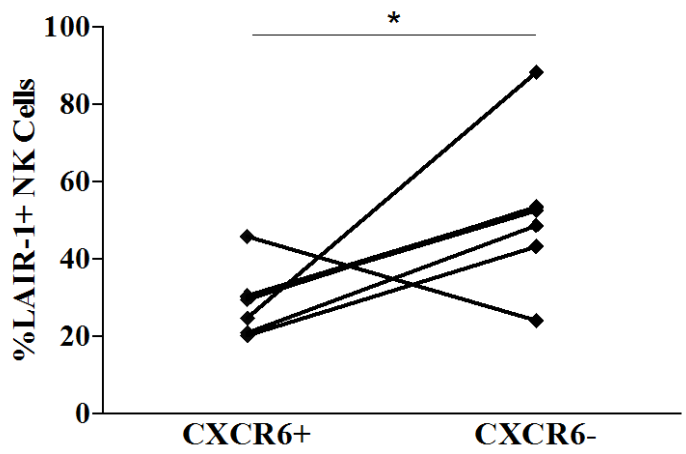


Figure 5.4: Comparison of percentage LAIR-1+ intrahepatic NK cells with liver residency marker CXCR6, in NAFLD patients.

IHL were isolated and stained from seven patients with NAFLD. Paired Wilcoxon test used. Significance indicated as: * p=<0.05. CXCR6+ vs CXCR6-, mean ± SEM, 28.84±3.254 and 51.93±7.218.

5.3 NK cell LAIR-1 expression in HCC

PBMC from eight treatment naive HCC patients were used. The aetiology of HCC differed between patients and is shown in Table 5.2. Patients were HCC treatment naive, as treatment could have been a confounding factor. Patient characteristics are summarised in Table 5.2.1.

Aetiology	HBeAg	ALT	Segment & Size	AFP	Background	Viral suppression
HBV	neg	109	segment 6, 90mm	3	cirrhotic	on entecavir
HBV	neg	10	segment 4b, 20mm	22.8	cirrhotic	on entecavir
HBV	neg	23	segment 8, 34mm	1.5	not cirrhotic (Ishak 2/6)	not on treatment
HBV	neg	83	segment 8, 25mm	201	not cirrhotic	on tenofovir
HCV	n/a	35	segment 6, 16mm	3.5	cirrhotic	not on treatment
HCV	n/a	77	segment 4a, 30mm	16.6	cirrhotic	no viral load
HCV	n/a	33	segment 5, 33mm	7	cirrhotic	not on treatment
ALD	n/a	24	multifocal HCC	not done	cirrhotic	n/a

Table 5.2: Details of the study participants with HCC

PBMC from 8 patients with HCC were used and disease characteristics are detailed in this table.

	Age (years) Median (range)	**Sex (%)** Male:Female	**ALT (IU/L)** Median (range)
HCC n= 8	57 (38-67)	90:10	34 (10-109)

Table 5.2.1: Summary of study participants with HCC

We compared peripheral NK cell frequencies in eight patients with HCC against the previous cohorts shown for CHB, NAFLD and HC. PBMC were isolated and stained for $CD56^{hi}CD3^{neg}$ NK cells. The percentage of circulating NK cells in HCC varied from 4.9% to a maximum of 37.8%, with a mean expression of 21.4%, which was not found to be significantly higher than HC, CHB and patients with NAFLD (Figure 5.5a).

Earlier analysis using the activation marker HLA-DR on circulating NK cells revealed higher frequencies of HLA-DR-expressing NK cells in CHB patients (mean 6%) than healthy controls (mean 4%) (Figure 3.4a). Although we noted variability in the expression of HLA-DR+ NK cells amongst HCC patients with a range between 1.6% and 21.8% maximum, the mean expression of ~8% was not significantly different from CHB patients (mean 6.8%) (Figure 5.6).

PBMC from eight patients with HCC were isolated and stained for LAIR-1 on NK cells. Figure 5.7 demonstrates all patients with HCC express LAIR-1 and circulating NK cells display higher frequencies of LAIR-1-expressing NK cells in HCC (mean 59.2%) than CHB patients (mean 39.5%) and healthy controls (mean 29.9%). In HCC both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets had high mean expression of 51.93% and 64.45% respectively. $CD56^{bright}$ NK cells had significant differences in LAIR-1 expression between HC (mean 23.8%), CHB (mean 35.6%) and HCC (mean 51.93%) patients. The highest mean expression of LAIR-1 was observed on $CD56^{dim}$ NK cells in HCC. LAIR-1 expression on NK cells in HCC patients was marginally higher than those seen on circulating NK cells from those patients with NAFLD (Figure 5.1).

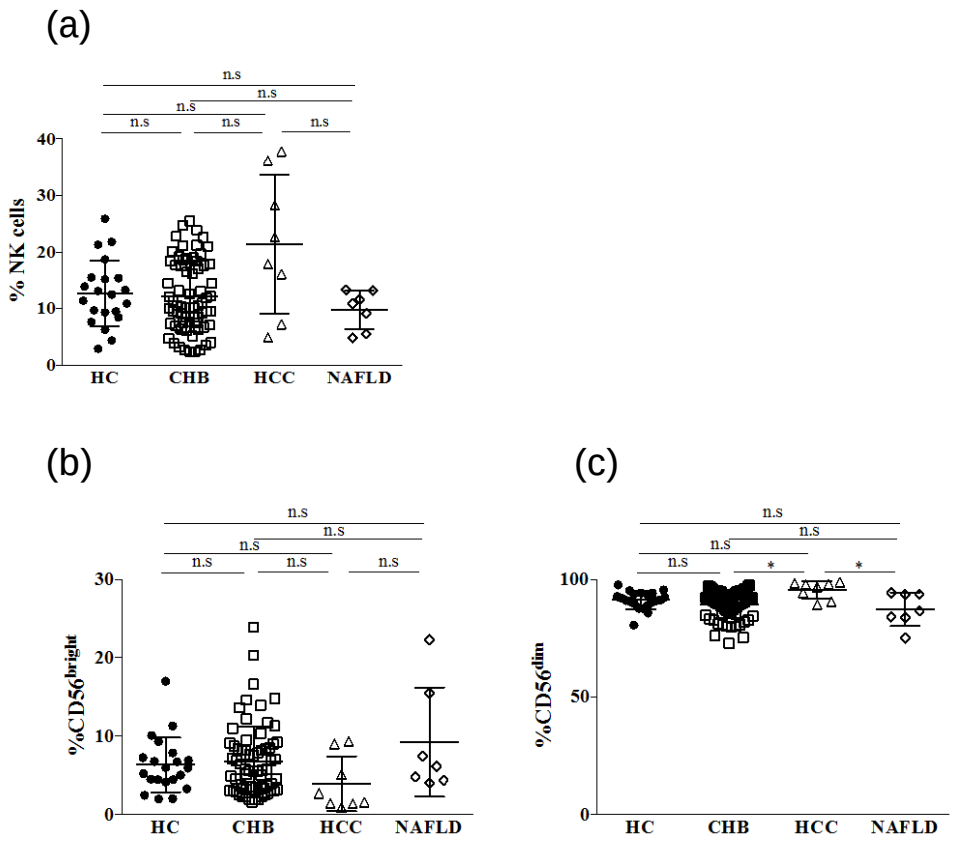


Figure 5.5: Comparison of proportion of (a) total circulating NK cells and subsets (b) $CD56^{bright}$ and (c) $CD56^{dim}$ NK cells between HC, CHB, HCC and NAFLD patients.

PBMC were isolated and stained from twenty-one HC, sixty-nine CHB patients, eight HCC patients and seven NAFLD patients. PBMC were stained for (a) $CD56^{hi}CD3^{neg}$ NK cells after exclusion of doublets and dead cells. NK cells were further divided in to subsets based on expression of CD56 and CD16, allowing them to be differentiated as (b) $CD56^{bright}$ and (c) $CD56^{dim}$ NK cells. Error bars represent the mean ± SEM. (a) HC; 12.72±1.269, CHB; 12.17±0.753, HCC; 21.4±4.33, NAFLD; 9.79±1.304. (b) HC; 6.32±0.764, CHB; 6.72±0.543, HCC; 3.92±1.238, NAFLD; 9.24±2.63. (c) HC; 91.2±0.823, CHB; 89.54±0.708, HCC; 95.45±1.336, NAFLD; 87.29±2.684. Analysed with Kruskal-Wallis (one-way ANOVA) test. Significance is indicated as *p<0.05.

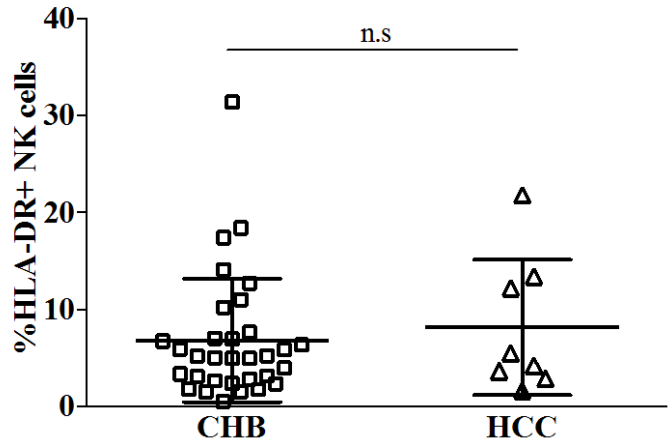


Figure 5.6: Comparison of expression of activation marker HLA-DR on total NK cells between CHB and HCC patients.

PBMC were isolated from thirty-two CHB patients and eight HCC patients. PBMC were stained for HLA-DR. HLA-DR expressing NK cells were gated as positive for HLA-DR staining on $CD56^{hi}CD3^{neg}$ NK cells after exclusion of doublets and dead cells. Error bars represent the mean ± SEM. CHB; 6.809±1.124, HCC 8.155±2.477. Significance testing was carried out using Mann Whitney test.

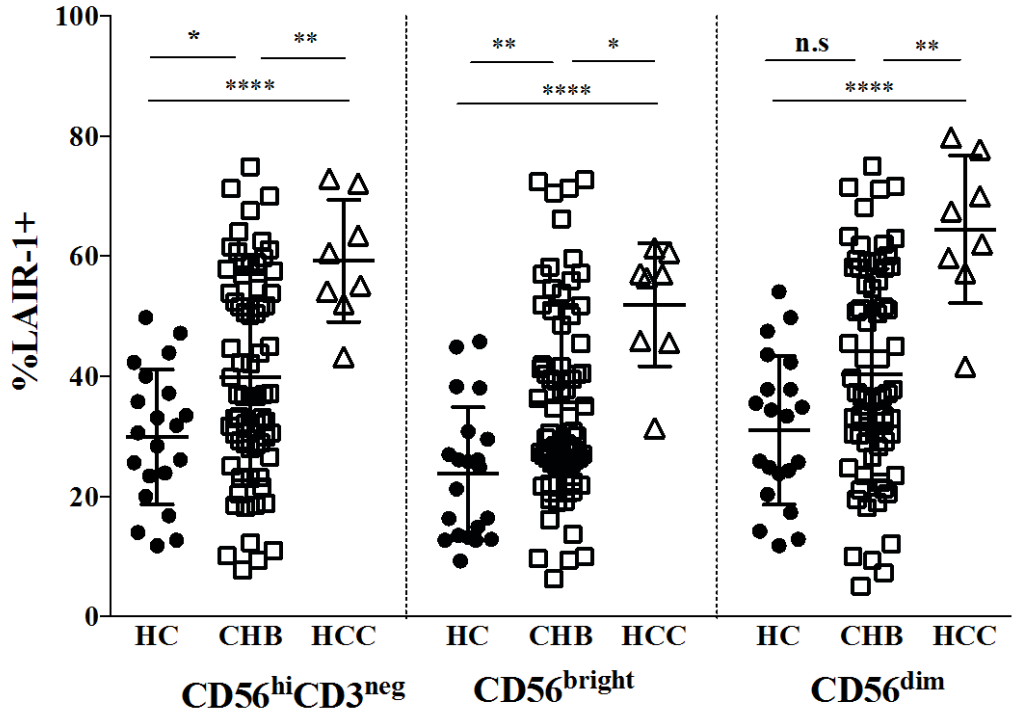


Figure 5.7: Comparison of percentage LAIR-1+ on total peripheral NK cells and within CD56^{bright} and CD56^{dim} NK cell subsets between HC, CHB and HCC patients.

PBMC were isolated from twenty-one HC, sixty-nine patients with CHB and eight patients with HCC. PBMC were stained for LAIR-1 expression on CD56^{hi}CD3^{neg} NK cells, CD56^{bright} and CD56^{dim} NK cells. HC vs CHB vs HCC, patients mean ± SEM, CD56^{hi}CD3^{neg}; 29.9±2.45 vs 39.87±2.06 vs 59.21±3.59, CD56^{bright}; 23.8±2.41 vs 35.6±1.95 vs 51.93±3.62, CD56^{dim}; 31.05±2.7 vs 40.27±2.1 vs 64.45±4.34. Error bars represent the mean ± SEM. Analysed with Kruskal-Wallis (one-way ANOVA) test. Significance is indicated as * p<0.05, ** p<0.01, ***p<0.001 and **** p=<0.0001.

5.4 Discussion

Thus far we have seen LAIR-1 expression on total NK cells in CHB patients is significantly lower in the liver compared to the periphery. We further investigated the expression of LAIR-1 on PBMC and IHL in patients with NAFLD was studied. LAIR-1 was increased on circulating NK cells in NAFLD compared to HC and significantly higher than expression in CHB. Similar to CHB, in NAFLD there was downregulation of LAIR-1 on liver resident (CXCR6+) NK cells, indicating downregulation of LAIR-1 in the liver may be organ specific rather than disease specific.

To explore the expression of NK cell LAIR-1 further, we also analysed PBMC from patients with HCC. There are a number of key associations that link NK cells and progression of HCC. Firstly, NK cells are the dominant immune cell accounting for 25-50% of IHL suggesting they have a role in liver immunity (Gao et al., 2008). Secondly, the frequency of NK cells in the circulation and tissue of HCC patients is positively correlated with survival and prognosis (Chew et al., 2012). Thirdly, adoptive transfer of genetically modified 'enhanced' NK cells has shown promise in preventing recurrence and regression of HCC (Sun et al., 2015b). However, the mechanisms linking NK cells and HCC remain unclear.

Here, we showed total NK cell frequency is higher in HCC patients but does not reach significance, likely due to the wide spread of percentage frequency within this group. The heterogeneity within the group could possibly be explained by unknown variables, such as different stages of disease progression in HCC. Cai et al observed varying frequencies of circulating NK cells with lower frequencies in patients with more advanced HCC disease (Cai et al., 2008). Interestingly Cai et al also observed, on analysing 110 HCC patients at different disease stages, a reduction in peripheral $CD56^{dim}$ NK cells. In our cohort of patients this subset is increased compared to CHB and NAFLD patients, however this difference might be associated with a different disease stage.

There is strong evidence that in addition to the abnormal frequency of NK cells in HCC they are also functionally impaired. The activation of NK cells is governed by the balance of activatory and inhibitory receptors. Increased levels of the latter skewing the balance can limit tumour immune surveillance by NK cells in HCC patients. Previous studies have shown upon maturation or activation, LAIR-1 expression on immune cells reduces (Olde Nordkamp et al., 2011). Compared to HC, in CHB and HCC patients there was significantly higher LAIR-1 expression on $CD56^{dim}$ NK cells, could potentially be a mechanism by which they are constrained. However, it is important to note that there was significant heterogeneity of the clinical features of the HCC population of patients and therefore limitations on HCC-specific interpretations of LAIR-1 on NK cells as a result.

It is believed downregulation of the receptor is a mechanism of autoregulation. However, if NK cells are poorly functioning in CHB and HCC perhaps this mechanism is impaired. Olde Nordkamp et al. were able to show the levels of soluble LAIR-1 were increased in patients with chronic inflammation. We demonstrate high levels of LAIR-1 on total NK cells and their respective NK cell subsets in HCC. In HCC, the inflammatory microenvironment can stimulate and exacerbate injury. Most HCC occur in the context of chronic inflammation and liver injury, which has led to cirrhosis. In HBV the virus is non-cytopathic and the oncogenic role is understood to be a change of direct and indirect effects of the virus as HCC can occur without underlying cirrhosis (Hanahan and Weinberg, 2011). Unfortunately the cohort of HCC patients was not large enough to determine if LAIR-1 expression is different in those of HBV aetiology and without cirrhosis, however, the two patients with HBV without cirrhosis expressed the lowest LAIR-1 expression in the cohort of 8 patients.

LAIR-1 is preferentially expressed on $CD56^{dim}$ NK cells at higher levels in HCC. This is contrary to literature that suggests LAIR-1 is higher on $CD56^{bright}$ NK cells and other such immature immune cells. However, LAIR-1 has only been shown to have a role in inhibiting cytotoxicity, and $CD56^{dim}$ NK cells are traditionally viewed as those with more cytotoxic potential. Several studies in animals

and humans have demonstrated abnormal NK cytolytic function and NK cell exhaustion in advanced stages of HCC. There is a large body of evidence that NK cells from PBMCs in HCC patients are defective in cytotoxic potential and cytokine production compared to healthy donors (Hoechst et al., 2009). Hoechst et al provided an interesting insight in to the role of MDSCs inhibiting NK cells, which was mediated via the receptor NKp30 requiring direct cell-to-cell contact. Paradoxically NKp30 is an activating receptor, and co-culture resulted in inhibition of NK cell function. In further work it would be interesting to probe whether LAIR-1 and NKp30 are co-expressed on NK cells and if LAIR-1 contributes to diminishing the response against clearance of tumour cells.

The cohort of patients with HCC stained with LAIR-1 is small and would need to be expanded; so far preliminary results indicate that LAIR-1 is higher in HCC, suggesting a potential role in pathogenesis. HSCs are important in the stromal tumour microenvironment and impairment of NK cell function in fibrosis and HCC could be due to collagen deposition by HSCs and NK cell LAIR-1-collagen interaction. Tumour cells themselves are also known to upregulate collagen and may use the interaction to dampen anti-tumour responses. It would be interesting to look at tumour progression in these HCC patients and survival, and to investigate the correlation with LAIR-1. Evaluation of the intrahepatic compartment and HCC tissue would be interesting to see whether there is any correlation or mirroring of data from the periphery.

Along with the increase of LAIR-1+ NK cells in HCC subjects, we also noted an increase in this cell population in patients with NAFLD. We did not have the opportunity to sample NAFLD cirrhotic patients with HCC, which would be of interest. However, the markedly elevated frequency of LAIR-1 expression on NK cells in NAFLD compared to patients with CHB and healthy controls is intriguing.

The reason for this expression is not entirely clear and there may be multiple factors contributing to increased LAIR-1 in NAFLD, in relation to the pathogenesis of the disease. Of note, none of the patients analysed had any evidence of fibrosis on liver biopsy and only steatohepatitis. Thus, the

overall degree of collagen may be low and not present to bind to its receptor, resulting in higher circulating levels of LAIR-1. It would be interesting to determine the expression of other NK cell receptors, especially activating C-type lectins or natural cytotoxicity receptors (NCRs) in relation to LAIR-1. A recent study showed that the increased expression of the NCR, NKp46 was associated with the attenuation of liver fibrosis in metabolic liver disease by regulating macrophage activation in mice (Tosello-Trampont et al., 2016). The co-expression of NKp46 with LAIR-1 would be worth pursuing further. It is possible that NK cell NKp46 may be low in these patients, and negatively correlate with LAIR-1 which may be a poor prognostic disease feature. In this regard it would therefore be interesting to analyse the expression of LAIR-1 in patients with NAFLD cirrhosis.

In line with this it would also be interesting to analyse serum levels of pro-collagen type III N-terminal peptide (P3NP) as this has been shown to be elevated in certain populations of patients with NAFLD (Hamza et al., 2016) and observe if there is a correlation with LAIR-1 that could further aid in disease management. As NAFLD is an inflammatory liver disease, it is possible that the cytokine/chemokine milieu in the circulating environment is markedly different to that seen in CHB, which is not reported, and this may be another reason for the variation in LAIR-1 expression in these liver diseases. The metabolic/hypoxic environment in non alcoholic steatohepatitis (NASH) may also be a contributory factor and thus the further investigation of metabolic components (fatty acid markers, glucose metabolic etc.) along with hypoxia would be an interesting avenue of study in relation of NK cell LAIR- expression and other NK cells receptors.

Results – Chapter 6: LAIR-2 in CHB

6.1 Introduction

Unlike other inhibitory receptors that belong to a family of several receptors, there are only two members of the LAIR family; LAIR-1 and LAIR-2. As mentioned earlier in the introduction LAIR-2 consists of only an extracellular ectodomain and therefore has no role in signal transduction, but exhibits high affinity for collagen molecules (Lebbink et al., 2008). LAIR-2 is a soluble protein, cleared from the body by the kidneys. Study findings by Meyaard et al demonstrated CD4+ T cells were the main producers of LAIR-2 (Olde Nordkamp et al., 2011) and it has been suggested that LAIR-2 has a regulatory role by preventing LAIR-1 binding to collagen, and that it may be a urine biomarker in rheumatoid arthritis patients with a greater degree of inflammation (Olde Nordkamp et al., 2011).

Another inhibitory receptor that also has a natural soluble form is LAG-3, which has high affinity binding to the same ligand as membrane bound LAG-3. Triebel et al showed subgroups of patients with breast cancer who had detectable levels of sLAG-3 at time of diagnosis had better disease-free and overall survival rates (Triebel et al., 2006). Further studies in mice and phase 1 clinical trials have explored the potential of sLAG3-immunoglobulin as an immune adjuvant, indirectly enhancing anti tumour T cell function in combination with vaccination (Anderson et al., 2016).

There is no known role for sLAIR-2 in directly inhibiting immune cells but it is possible that sLAIR-2 can affect the interaction of LAIR-1 with collagen and indirectly affect NK cell function. However, initially we explored whether LAIR-2 is differentially expressed in CHB patients compared with healthy controls, as seen with LAIR-1.

6.2 Serum and Urine LAIR-2

In order to determine whether LAIR-2 has a possible role in the pathogenesis of CHB or use as a biomarker, we investigated the presence of LAIR-2 in a smaller number of HC and CHB patients (Table 6.1). ELISA can measure LAIR-2 concentrations in both serum and urine. LAIR-2 was detected in the serum and urine of all healthy controls (n=5). LAIR-2 was also detectable in serum (n=23) and urine (n=15) from CHB patients. Although there was no significant difference between LAIR-2 concentration in serum (Figure 6.1) or urine (Figure 6.2) of HC and CHB patients, the circulating levels of LAIR-2 in CHB patients were higher than the urine (serum mean concentration of 2.091 pg/ml vs urine mean concentration of 1.567 pg/ml, p=0.0049).

To investigate whether LAIR-2 is related to inflammation in CHB patients, we sought to investigate whether heterogeneity of expression segregated with different disease phases of CHB. Serum from thirteen IA and ten IC patients was analysed for LAIR-2. These two phases were chosen as they have opposing level of circulating virus and ALT. No significant difference was seen between these phases (Figure 6.3a). A trend towards lower serum LAIR-2 concentration was found in IA HBeAg negative CHB when compared to IA HBeAg positive patients, however, this difference was not significant (Figure 6.3b). Similarly there was no difference between urine LAIR-2 concentrations of IA and IC patients (Figure 6.4).

(a)

	Age (years) Median (range)	**Sex (%)** Male:Female	**HBeAg (%)** Positive:Negative **Disease phase (n)**	**HBsAg (IU/ml)** Median (range)	**HBV DNA (log)** Median (range)	**ALT (IU/L)** Median (range)	**LAIR-2 (Serum)** Median (range)
HC n= 5	37 (34-40)	40:60	n/a	n/a	n/a	n/a	2.22 (1.7-2.49)
CHB n=23	34 (25-68)	57:43	26:74 IT (n=0) HBeAg+ IA (n= 6) IC (n=10) HBeAg- IA (n=7)	7,686 (0.03-8,376,687)	3.3 (0-9.06)	29 (11-918)	2.05 (1.57-2.295)

(b)

	Age (years) Median (range)	**Sex (%)** Male:Female	**HBeAg (%)** Positive:Negative **Disease phase (n)**	**HBsAg (IU/ml)** Median (range)	**HBV DNA (log)** Median (range)	**ALT (IU/L)** Median (range)	**LAIR-2 (Urine)** Median (range)
HC n= 5	37 (34-40)	40:60	n/a	n/a	n/a	n/a	1.36 (1.13-2.53)
CHB n=15	32 (25-62)	60:40	33:67 IT (n=0) HBeAg+ IA (n= 5) IC (n=5) HBeAg- IA (n=5)	8,619 (31.76-8,376,687)	4.62 (0-9.06)	32 (11-155)	1.57 (0-2.73)

Table 6.1: Summary details of the study cohort for (a) LAIR-2 serum and (b) LAIR-2 urine

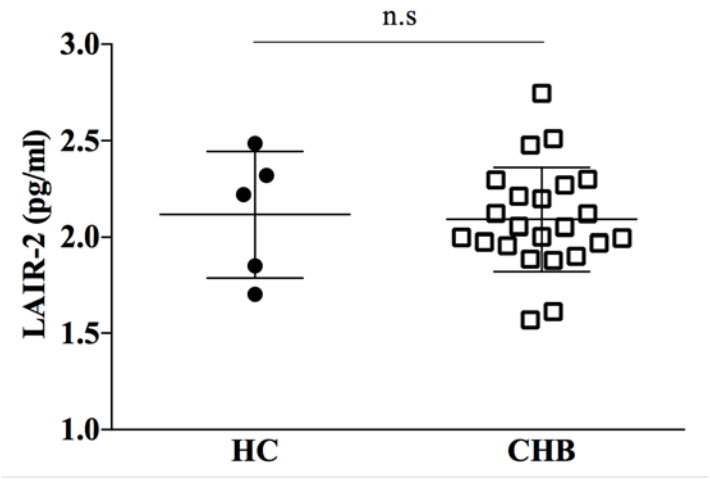


Figure 6.1: Comparison of serum LAIR-2 concentration between HC and CHB patients.

ELISA was performed on sera from five HC and twenty-three CHB patients. Error bars represent the mean ± SEM. HC; 2.116 ± 0.1466, CHB; 2.091± 0.056. Significance testing was carried out using Mann Whitney test.

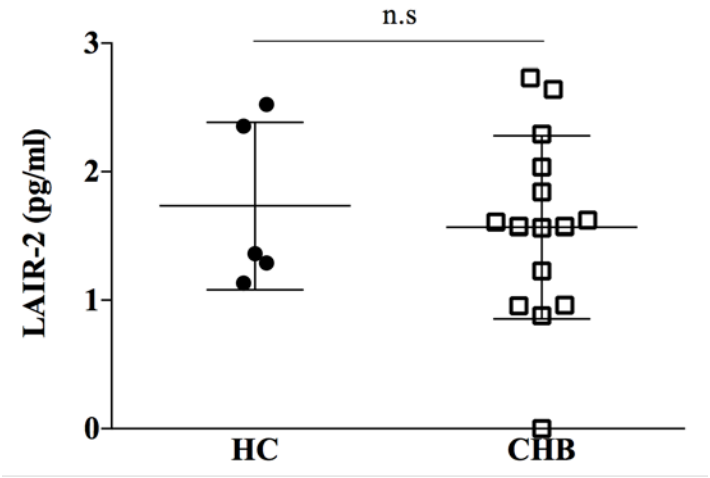
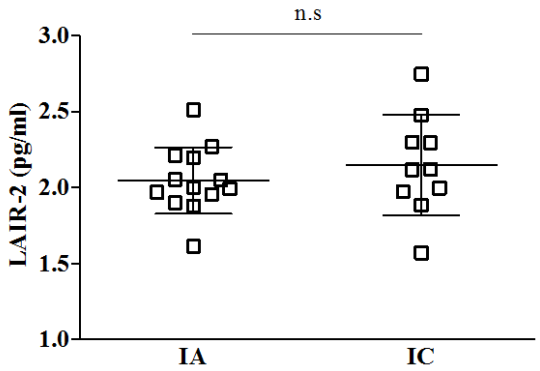


Figure 6.2: Comparison of urine LAIR-2 concentration between HC and CHB patients.

ELISA was performed on urine from five HC and fifteen CHB patients. Error bars represent the mean ± SEM. HC; 1.734 ± 0.292, CHB; 1.567± 0.184. Significance testing was carried out using Mann Whitney test.

(a)



(b)

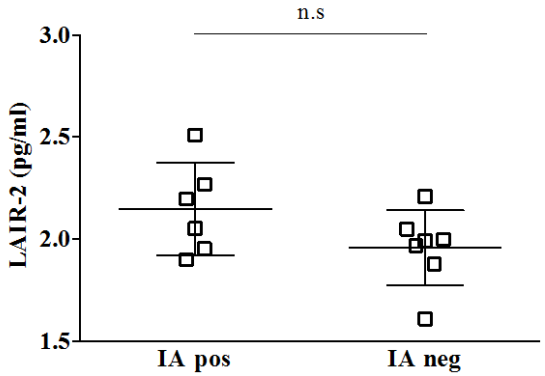


Figure 6.3: Comparison of serum LAIR-2 concentration between disease phases in CHB patients.

ELISA to quantify LAIR-2 were performed on serum from patients in different disease phases of CHB. Error bars represent the mean ± SEM. Significance testing was carried out using Mann Whitney test.

(a) Comparison of serum LAIR-2 concentration between IA and IC disease phase. ELISAs were performed on serum from thirteen IA and ten IC patients. IA; 2.046 ± 0.06, IC; 2.149± 0.104.

(b) Comparison of serum LAIR-2 concentration between HBeAg positive and negative IA disease phase. ELISAs were performed on serum from six HBeAg IA positive and seven HBeAg negative IA patients. HBeAg+ve IA; 2.148 ± 0.09, HBeAg-ve IA; 1.959± 0.069.

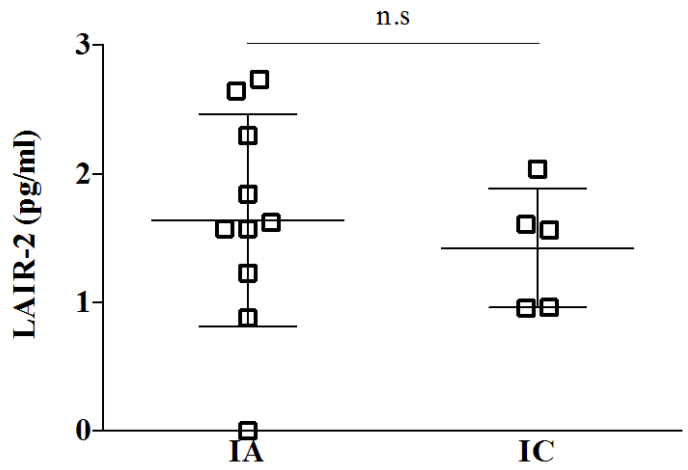


Figure 6.4: Comparison of urine LAIR-2 concentration between disease phases in CHB patients.

ELISA to quantify LAIR-2 were performed on urine from patients in different disease phases of CHB. Error bars represent the mean ± SEM. Significance testing was carried out using Mann Whitney test. ELISAs were performed on urine from ten IA and five IC patients. Error bars represent the mean ± SEM. IA; 1.639 ± 0.261, IC; 1.424± 0.207. Significance testing was carried out using Mann Whitney test.

6.3 Discussion

There is limited data on soluble LAIR-1 and LAIR-2 production *in vivo*. However, some studies do suggest that LAIR-2 levels are significantly elevated in inflammatory conditions. The expression of LAIR-2 has been described in inflammatory arthritis and autoimmune thyroid disease. Both are autoimmune disorders resulting in destruction and remodelling of a localised affected area. LAIR-2 levels were compared in patients with inflammatory RA and non-inflammatory OA. Olde Nordkamp et al concluded in their study that LAIR-2 has a regulatory role by preventing LAIR-1 binding to collagen, and also that it may be a urine biomarker in RA patients with a greater degree of inflammation (Olde Nordkamp et al., 2011). Previously the group had shown synovial fluid from RA patients had higher levels of LAIR-2, and noted increased levels of LAIR-2 in pregnant women (Lebbink et al., 2008). Serum LAIR-2 levels have also been demonstrated to be higher in autoimmune thyroid disease patients compared to healthy donors, and this effect was not thought to be attributed to change in thyroid function as LAIR-2 levels in non autoimmune hyperthyroid patients were similar to healthy subjects (Simone et al., 2013).

We were the first to look at LAIR-2 as a biomarker in the urine and serum of CHB patients. Although the cohorts of patients are small, similar to previous studies, LAIR-2 was detected in healthy individuals. However, there was no difference in serum or urine LAIR-2 concentrations between CHB and HC. In addition serum and urine LAIR-2 concentrations in the IA and IC disease phases were no different suggesting no role for LAIR-2 as a meaningful biomarker in distinguishing these two phases. These phases were chosen as IA patients are characterised by fluctuating levels of liver enzymes and viral load indicative of on going immune activity and inflammation. Perhaps there was no apparent difference between phases as the inflammation was not significant enough to show a true difference. ALT is often considered a suboptimal surrogate for inflammation and the mean ALT of this group of CHB patients did not exceed 85. Perhaps CHB is an indolent disease and although inflammation is chronic, it is not significant enough to cause raised levels of LAIR-2.

Chapter 7: Final Conclusions and future directions

7.1 Summary and overall discussion of work presented

In CHB both innate and adaptive immunity are impaired (Ferrari, 2015). It has been observed that altered function of adaptive and innate immune cells contribute to the persistence of viral infection. NK cells have been implicated in the control of HBV in mouse models and are involved in liver pathogenesis (Tian et al., 2013). Specific to CHB, NK cells may contribute to viral persistence by deleting HBV-specific T cell responses. Peppa et al observed upregulation of TRAIL-R2 receptor on intrahepatic virus-specific CD8+ T cells rendering the virus-specific T cells susceptible to apoptosis by TRAIL-expressing NK cells (Peppa et al., 2013). TRAIL-expressing NK cells were also shown to be capable of killing TRAIL-R2-expressing hepatocytes in CHB, thereby potentially exacerbating liver damage (Dunn et al., 2007). Despite preserved NK cell cytotoxicity, IFNγ production by NK cells in HBV infection is suppressed which may impair viral control (Maini and Gehring, 2016). There has been significant interest in the role of inhibitory receptors. Conventionally NK cells are activated when there is a reduction in the signal from inhibitory receptors. There are arrays of potential receptors that have shown to be overexpressed on functionally impaired immune cells. On blockade these receptors have demonstrated reversal of exhaustion. This work predominately focuses on the role of LAIR-1, an inhibitory receptor, in CHB. To our knowledge, this is the first investigation of LAIR-1 on NK cells in CHB.

7.1.1. Aim 1: Investigate expression of LAIR-1 on NK cells in periphery and liver of CHB patients.

In Chapter three we reviewed some of the most commonly investigated inhibitory receptors and explored the expression of co-inhibitory receptors on NK cells in CHB. Interestingly, the least studied inhibitory receptor is LAIR-1 but LAIR-1 prompted our interest, as the ligand is abundant in the diseased liver in CHB. Collagen is implicated in several rheumatological diseases i.e. RA, SLE and

autoimmune conditions, and the receptor has been explored in this context but has received limited focus in liver disease. We show in LAIR-1 expression on total NK cells, and both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets is significantly higher in CHB patients than HC, suggesting a potential role for this receptor in this chronic viral infection. The receptor is also expressed at much higher frequency than other receptors. Perhaps there is higher LAIR-1 expression on NK cells as they differentiate and mature in the bone marrow where they can encounter stromal cells, and potentially; as these bone marrow stromal cells produce collagen, circulating NK cells have a higher LAIR-1 expression in line with their requirement for a high threshold for activation. This hypothesis however requires further investigation.

There was no correlation with clinical parameters such as age, disease phase or fibrosis stage. However, the inhibitory receptor LAIR-1 was increased on $CD56^{bright}$ NK cells from patients with CHB. One of a number of ways NK cells can contribute to viral persistence in CHB is via an impaired/exhausted $CD56^{bright}$ NK cell cytokine response failing to promote responses of lymphocytes of the adaptive immune system (Peppa et al., 2010). Functional inhibition of NK cells by LAIR-1 (as shown previously by Lebbink et al) may occur in CHB as supported by our finding of an inverse correlation between NK cell LAIR-1 expression and effector function.

A number of studies demonstrate higher expression of activatory receptors (NKp30, NKp46 and NKG2C) and lower inhibitory receptor expression on peripheral NK cells in CHB compared with HCV-infected patients and healthy controls (Rehermann, 2013). In general (not specific to viral infection) intrahepatic NK cells are more activated (Huang et al., 2017), showing higher cytotoxic capacity than peripheral NK cells (Fasbender et al., 2016). Interestingly, intrahepatic NK cells had a different phenotype compared to circulating NK cells, with downregulation of LAIR-1, and LAIR-1 was strikingly further downregulated on liver resident versus non-resident NK cells. LAIR-1 is also preferentially expressed on $CD56^{bright}$ NK cells within the HBV-infected liver, where we could speculate local collagen could promote inhibition of NK cells through this inhibitory receptor.

These data point to a role for the liver milieu downregulating LAIR-1, either by promoting internalisation or shedding. In Chapter four we probed whether this downregulation was due to exposure to collagen, the cytokine milieu or crosstalk with HSCs. NK cells are believed to maintain liver homeostasis/tolerant environment by their interaction with other cell types and the presence of soluble factors like cytokines can influence the phenotype of NK cells (Shi et al., 2011). Findings from murine models suggest that cytokines may impair NK cell function by modifying their surface receptor expression. Lassen et al demonstrate this by showing that IL-10 contributed to the regulation of intrahepatic NK cells by maintaining a higher percentage of the less responsive $NKG2A^{+}Ly49^{-}$ subset of NK cells (Lassen et al., 2010). Antiviral therapy in CHB has been linked to the downregulation of NKG2A and partial recovery of NK cell IFNγ production (Tjwa et al., 2011). The impaired function of NK cells in CHB has also been attributed to the capacity of TGFβ to downregulate the inhibitory NKG2D/DAP10 and 2B4/SAP pathways (Sun et al., 2012). Shi et al demonstrated that in HBV, plasmacytoid dendritic cells (pDC) could impair NK cell IFNγ production without affecting NK cell cytotoxicity (Shi et al., 2012). This cross talk of immune cells could be a potential escape mechanism for the virus, perpetuating chronicity.

7.1.2. Aim 2: Investigate effect on LAIR-1 expression on NK cell function in CHB.

In this study no consistent data was identified supporting collagen or liver-relevant cytokines in downregulating the surface expression of NK cell LAIR-1. When we further explored whether NK cell function changed in the presence of collagen, preliminary experiments on PBMC from CHB patients demonstrated a downregulation of CD107a on NK cells upon collagen pre-coated plates. This is consistent with previous studies by Lebbink et al demonstrating collagens can inhibit immune cell function by binding to LAIR-1 (Lebbink et al., 2006).

Further work would be to look specifically at LAIR-1 positive NK cells and other aspects of cytotoxicity to observe whether pre-incubation with blocking anti-hLAIR-1 $F(ab')_2$ (8A8) fragments reverses inhibition. It may be difficult however to block this interaction because there is no commercially available blocking antibody and only a very limited supply from one lab. To overcome this hurdle we may need to consider use of small interfering RNA (siRNA) for knock-down instead. Son et al who transfected human monocytes with LAIR-1 siRNA with a moderate 40% efficiency used this technique (Son and Diamond, 2015). Kang et al silenced the expression of LAIR-1 *in vitro* by introducing Lentivirus-encoded shRNA into human leukaemia lines (Kang et al., 2015). Both techniques have been used previously with regards to LAIR-1 and have their respective limitations, but nevertheless are important in furthering the understanding of the biology of this receptor.

7.1.3. Aim 3: Investigate whether there is cross talk between collagen producing cells in the liver (HSCs) and LAIR-1 on NK cells.

NK cells are enriched in the liver and there is growing evidence that they impact the function of HSCs in liver fibrosis, playing a protective role by removing activated HSCs to limit fibrosis (Fasbender et al., 2016). In experimental mouse models, depletion of NK cells leads to fibrosis. NK cells act via crosstalk with other immune cells and cytolytic attack against HSCs can prevent collagen deposition and fibrosis (Friedman, 2008). Furthermore we know NK cells are impaired in function in chronic liver injury, and a number of studies have shown their antifibrotic function is impaired through downregulation of receptors such as NKG2D and 2B4. It has been suggested that collagen fibre scaffolds laid down in progressive fibrosis prevent NK cells from accessing their target cells (Fasbender et al., 2016).

In Chapter four we described experiments involving co-culture of PBMC with pHSC and pHSC supernatant. There was no change in LAIR-1 surface expression, but it is possible that the function of

NK cells could be altered if there was ligand engagement. This would be of interest for investigation in due course.

In Chapter five we explored the expression of LAIR-1 on circulating NK cells from patients with HCC and NAFLD. LAIR-1 expression was significantly higher in the HCC cohort and preferentially expressed on $CD56^{dim}$ NK cells. In HCC the inflammatory microenvironment can stimulate and exacerbate injury. Most HCC occur in the context of chronic inflammation and liver injury, which has led to cirrhosis. There is strong evidence that NK cells in HCC and in liver cirrhosis are functionally impaired. Early observations by Chuang et al in the 1990s showed that in a small subset of twenty-nine cirrhotic patients, impaired NK cell activity in severe liver disease demonstrated with cytotoxicity assays, have been further corroborated in later studies (Chuang et al., 1991). In the diseased liver and stromal tumour microenvironment HSCs play an important role in depositing collagen fibres. Impairment of NK cell function in fibrosis and HCC, and the observation of high expression of LAIR-1 on NK cells in HCC led us speculate whether these changes could be attributed to collagen deposition by HSCs and a potential role of NK cell LAIR-1-collagen interaction. However, the experiments in chapter four did not find a cause for downregulation of LAIR-1 on intrahepatic NK cells. We were unable to show in co-culture experiments that collagen derived from HSCs influences the expression of LAIR-1 on NK cells and this remains to be determined.

Finally in chapter six we were the first to look at LAIR-2 as a biomarker in the urine and serum of CHB patients. Similar to previous studies LAIR-2 is detected in healthy individuals. However, there was no difference in serum or urine LAIR-2 concentrations between CHB and healthy controls. It would be interesting to re-examine LAIR-2 levels during marked hepatic flares of CHB, and whether more significant inflammation resulted in differences.

7.2 Questions and future direction

7.2.1. Investigating role of immune complex bound complement component C1q as another LAIR-1 ligand in CHB

The interplay between NK cells and dendritic cells (DC) has been shown to be crucial to innate and adaptive responses against infection (Andoniou et al., 2005). Plasmacytoid DC (pDC) are a subpopulation of cells that can directly inhibit viral replication via their production of type 1 interferons. They can also prime and recruit NK cells to become more cytotoxic in vivo. Early work by Poggi et al showed engagement of LAIR-1 inhibited differentiation of peripheral blood monocytes toward dendritic cells in vitro (Poggi et al., 1998). At this time the ligand of LAIR-1 had not been identified and therefore mAb was used for cross-linking, these experiments have not been repeated since the discovery of collagen as the ligand of LAIR-1.

A number of years later preliminary experiments by Son et al showed human pDCs produced less IFNα with the addition of C1q (Son et al., 2012). In the knowledge that monocytes have high expression of LAIR-1, Son et al set out to investigate how C1q mediates suppression of function of human monocytes via LAIR-1. Their experiments involved using synthetic oligonucleotides that contain CpG motifs, which would normally mimic the activity of CpG motifs in bacterial DNA to trigger monocytes. In their follow-up paper they were able to show addition of C1q and anti-LAIR-1 mAb suppressed production of type 1 interferons by monocytes. They demonstrated the importance of LAIR-1 since C1q-mediated suppression did not occur in cell lines transfected with LAIR-1 siRNA. Interestingly, they also observed the engagement between C1q and LAIR-1 inhibits toll-like receptor (TLR) signalling (Son and Diamond, 2015, Son et al., 2017).

Focusing specifically on what we know about the role of complement in influencing cells of the adaptive immune system, complement has been demonstrated to regulate CD4+ and CD8+ T cell functions but there is no literature regarding regulation of NK cells (Stoermer and Morrison, 2011). Could C1q binding to LAIR-1 have a role in the regulation of NK cells? C1q bound to immune complexes merits investigation as a potential ligand for LAIR on NK cells in CHB.

Of interest investigating the role of immune complex bound complement component C1q in CHB could also be highly relevant to CHB due to the presence of circulating immune complexes in the periphery, and whether this regulates LAIR-expressing NK cells in the periphery rather than the liver.

Shi et al demonstrated that in HBV pDC could impair NK cell IFNγ production but without affecting NK cell cytotoxicity (Shi et al., 2012). It would be interesting to investigate if this was due to the higher expression of LAIR-1 on $CD56^{bright}$ NK cells. Cross talk between immune cells could be a potential escape mechanism for virus persistence.

7.2.2. LAIR-1 in other diseases

LAIR-1 captured our interest as the ligand is abundant in the diseased liver in CHB. Collagen is implicated to play a role in several rheumatological diseases and autoimmune conditions. Researchers have speculated that the LAIR-1-collagen interaction may have a role in these diseases, however, little has been published regarding this.

Organ failure due to fibrosis is the main cause of morbidity and mortality in patients with systemic sclerosis (LeRoy et al., 1988). Samples have shown increased accumulation of types one and three collagen in gastric wall of systemic sclerosis patients. Gastrointestinal (GI) involvement affects 9 out of 10 patients with SSc. Interestingly in these patients the liver is not affected. It has not previously been investigated whether LAIR-1 could be a biomarker of GI involvement in systemic sclerosis or correlate with severity of involvement. The presence of myofibroblasts in the skin of patients with SSc has been extensively investigated (Abraham and Varga, 2005); it would be interesting to see whether there is a functional role for LAIR-1 or whether this marker is indicative of severity or progression.

7.2.3. Functional consequences of LAIR-1

It would be useful to look at histopathology sections for LAIR-1 staining (by immunohistochemistry) to determine the spatial relationship between LAIR-1 expressing NK cells and HSCs. It is well described that cytotoxicity is a less efficient process compared to cytokine-mediated antiviral effects, as the former requires one-to-one interaction between cells. It would be interesting to focus on the spatio-temporal relationships between HSCs and NK cells when architectural changes occur in liver damage in fibrosis and HCC.

In CHB virus-specific T cells are deleted or become dysfunctional and their role in suppressing virus infection is compromised. One of the mechanisms by which this occurs is via co-inhibitory receptors like PD-1. Kennedy et al analysed the expression of LAIR-1 on circulating global CD4+ and CD8+ T cells (Kennedy et al., 2012), but it would also be pertinent to look at LAIR-1 on virus-specific T and B cells in CHB to see if it is constraining these.

We did not have the opportunity to perform collagen functional studies with intrahepatic lymphocytes. There is no known role for sLAIR-2 in directly inhibiting immune cells and it is possible that sLAIR-2 can affect the interaction of LAIR-1 with collagen and indirectly affect NK cell function. Blockade studies with $F(ab')_2$ fragments would help to dissect this. In a similar way, an experimental design using matrix metalloproteases or collagenase to break down collagen on the pre-coated plate may also help.

A more sophisticated but complex experimental plan (currently in progress) involves using three-dimensional liver scaffolds in collaboration with Pinzani & Mazza. These scaffolds can be designed to have collagen in the correct spatial configuration to bind LAIR-1 and repopulated with HSCs and HCC cell lines to observe their effect on NK cell function through LAIR-1. The process of

decellularisation maintains the scaffold microarchitecture but destroys immune cells within the matrix, enabling a 'clean slate' for observational and functional studies. Mazza et al have previously shown decellularised human liver scaffolds could be repopulated with liver resident cell lines and recreated a bioartificial environment *in vitro*. Collagen type I, III and IV were preserved following decellularisation (Mazza et al., 2015). Exciting preliminary data show a clear down-regulation of NK cell LAIR-1 upon engagement with collagen in this more physiological configuration.

Based on the current knowledge, this study has explored LAIR-1 in CHB for the first time. The novel findings of high expression on NK cells in CHB and a correlation between expression and effector function suggest this inhibitory receptor could have a potential role in exhaustion of NK cells in CHB. We found NK cell function downregulated upon collagen interaction and ongoing work with liver scaffolds has shown LAIR-1 is downregulated on NK cells in this environment supporting the ex vivo observations on intrahepatic NK cells. This further work confirms our hypothesis that the hepatic collagen matrix can shape NK cells through LAIR-1. Additional experiments to determine the specific functional relevance of LAIR-1 downregulation are underway.

Figure 7.1 is an overview of the functional significance of potential LAIR-1-collagen interactions.

Future research should investigate the potential role of high expression of LAIR-1 in HCC and whether the tumour microenvironment exploits the LAIR-1-collagen interaction dampening anti-tumour responses. Further work may facilitate our understanding of the complexities and interplay between immune cells and potential use for therapeutic approaches.

7.2.4. Targeting LAIR-1 as a therapeutic strategy

The ultimate end-point would be to develop a novel approach targeting LAIR-1, similar to PD-1 and CTLA-4 checkpoint inhibitors. For example it would be of interest to explore LAIR blockade as a potential therapeutic strategy in HCC as an adjunct to existing checkpoint inhibitors, which are only successful in a fraction of cases. To elaborate further it would be helpful to explore whether LAIR-1 is also increased on T cells in HCC and whether expression is also increased on tumour-infiltrating NK and T cells in HCC.

Some collagen inhibitors have been developed for use in liver fibrosis such as Hsp47 but efficacy has not yet been demonstrated. It is important to understand the mechanisms of ligand receptor interactions between HSCs and NK cells as several other ligands have been noted to be present on HSCs as well as hepatocytes (MIC-A, TRAIL receptor, FAS) but we need to be mindful that therapeutic strategies that activate NK cells could result in hepatocytes also being targeted which may lead to further liver damage.

The advantage of targeting LAIR-1 is that therapy would not be affecting collagen per se, rather simply the interaction of LAIR-1 on immune cells. Risks would be related to loss of tolerance. However, reassuringly debilitating autoimmunity has not precluded the use of PD-1 blockade as a therapeutic strategy.

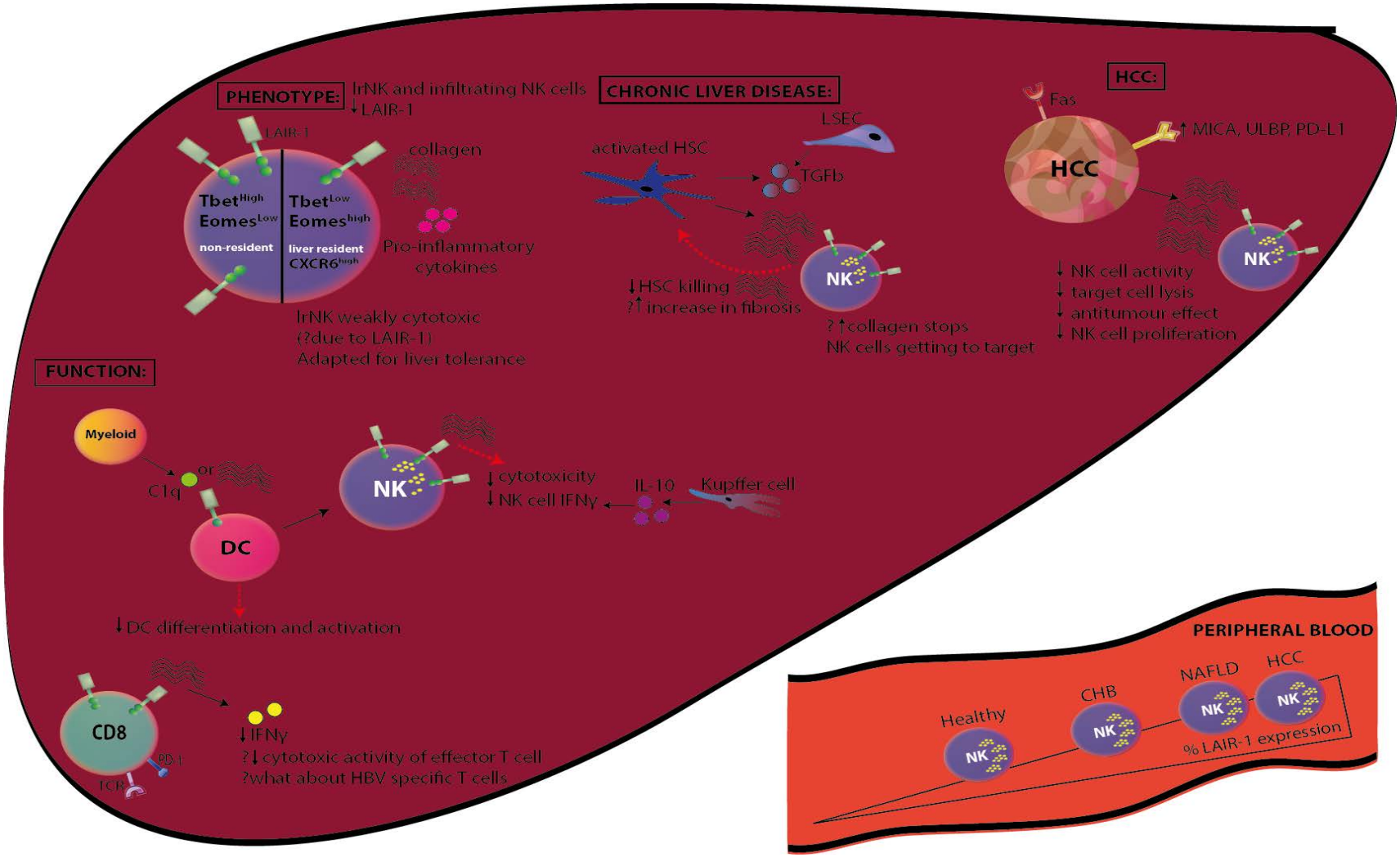


Figure 7.1: Overview of the potential LAIR-1-collagen-mediated interactions

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