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PhD Thesis

**NKG2D-dependent cross talk between
NK cells and CD4 T cells in chronic hepatitis B**

Author: Wei-Chen Huang

Supervisor: Prof. Mala K. Maini

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Contents

Declaration of Authorship	5
Acknowledgements	6
List of Abbreviations	7
List of Figures	10
List of Tables	12
Abstract	13
Chapter 1 Background of the Study	15
1.1 Overview of Hepatitis B Virus Infection	15
1.2 Immune Response Defects in HBV Infection	22
1.3 Overview of Human NK Cells	29
1.4 Regulation and Interactions of the NKG2D Pathway	37
Chapter 2 Materials and Methods	46
2.1 Patients and Healthy Controls	46
2.2 PBMC and Intrahepatic Lymphocyte Isolation	47
2.3 Flow Cytometric Analysis and Gating Strategies	48
2.4 HLA-A2-restricted Multimer Staining	50
2.5 Intranuclear FoxP3 Staining	52

2.6	Cell Lines	52
2.7	NK cell and CD4 T cell Isolation	53
2.8	NKG2D Pathway Blockade.....	53
2.9	NK / Target cell Co-culture System	54
2.10	Short-Term Stimulation.....	55
2.11	Overnight Stimulation	56
2.12	Hydrogen Peroxide Stimulation	56
2.13	Recombinant Human Interferon- α Stimulation.....	56
2.14	Statistical Analysis	57
Chapter 3	Chronic hepatitis B renders T cells vulnerable to NKG2D-mediated regulation	58
3.1	Introduction: Dysfunctional T cells in HBV infection.....	58
3.2	Aim and Hypotheses	64
3.3	Results	65
3.3.1	NKG2D-L upregulation on T cells in chronic HBV infection.....	65
3.3.2	Enrichment of NKG2D-L expression on T cells activated by HBV infection.....	70
3.3.3	Oxidative stress in CHB as an underlying mechanism of NKG2D-L induction on T cells....	75
3.3.4	Restoration of activated/HBV-specific T cells upon NKG2D blockade or NK cell depletion	81

3.4	Conclusions and Discussion.....	86
Chapter 4	NKG2D-mediated NK cell Regulation in Chronic HBV Infection	95
4.1	Introduction: NK cell-mediated T cell regulation in virus disease	95
4.2	Aim and Hypotheses	100
4.3	Results	101
4.3.1	NK cell effector function driven by NKG2D-L expressing cells.....	101
4.3.2	Potent NKG2D-mediated cytotoxic degranulation of NK cells in chronic HBV infection ..	106
4.3.3	NK cell activation responds to stressed T cells through NKG2D pathway in CHB.....	110
4.3.4	Promotion of NKG2D dependent regulation during interferon therapy of CHB patients	114
4.4	Conclusions and Discussion.....	119
Chapter 5	Future Outlook and Potential Directions.....	125
References	133
List of Achievements and Experiments Conducted	169

Declaration of Authorship

‘I, Wei-Chen Huang, affirm that the work presented in this thesis is my own and where information has been derived from other sources, I have so indicated.’

Signature: Wei-Chen Huang

Date: 08 / 04 / 2016

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List of Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AHB	Acute Hepatitis B
ALT	Alanine transaminase
APC	Antigen presenting cells
APOBEC3	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related
Bim	Bcl2-interacting mediator
cccDNA	Covalently closed circular DNA
CD	Cluster of differentiation
CHB	Chronic hepatitis B
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
CXCR	Chemokine receptor
DC	Dendritic cell
Dex	Dextramer
DDR	DNA-damage response
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
Fas-L	Fas-ligand
FcR	FcR blocking reagent
FCS	Fetal calf serum
FOXP3	Forkhead box P3
Grb2	Growth factor receptor-bound protein 2
H₂O₂	Hydrogen peroxide
HAI	Histological activity index
HAV	Hepatitis A virus

HBV	Hepatitis B virus
HBsAb	Hepatitis B virus e antigen-specific antibody
HBsAg	Hepatitis B e antigen
HBsAb	Hepatitis B virus surface antigen-specific antibody
HBsAg	Hepatitis B surface antigen
HBx	Hepatitis B virus X protein
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCMV	Human cytomegalovirus
HDV	Hepatitis delta virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICS	Intracellular staining
IFN	Interferon
IL	Interleukin
IHL	Intrahepatic lymphocytes
KIR	Killer cell immunoglobulin-like receptors
KO	Knockout
LCMV	Lymphocytic choriomeningitis virus
LSS	Lymphoid stress-surveillance
mAb	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MCMV	Murine cytomegalovirus
MDSC	Myeloid-derived suppressor cells
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MICA/B	MHC Class I-Related Chain A and B
miRNA	MicroRNA
MMP	Metalloproteinase
mRNA	Messenger RNA
NCAM	Neural cell adhesion molecule
NCR	Natural cytotoxicity receptor
NFκB	Nuclear factor κB
NK cell	Natural killer cell
NKG2A	Natural killer group 2, member A

NKG2C	Natural killer group 2, member C
NKG2D	Natural killer group 2, member D
NKG2D-L	NKG2D ligands
NKT cell	Natural killer T cell
Nrf2	Nuclear factor erythroid-2-related factor 2
NTCP	Sodium taurocholate cotransporting polypeptide
OLP	HBV overlapping peptides
OS	Oxidative stress
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed death 1
pDC	Plasmacytoid dendritic cells
PD-L1	Programmed death ligand 1
PI3K	Phosphoinositide 3-kinase
PEG-IFN-α	Pegylated Interferon- α
PMA	Phorbol 12-myristate 13-acetate
rcDNA	Relaxed circular DNA
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper cells
Tim-3	T cell immunoglobulin- and mucin-domain-containing molecule 3
TNF-α	Tumor necrosis factor- α
TRAIL	TNF-related apoptosis inducing ligand
TRAIL-R2	TRAIL receptor 2
Treg	Regulatory T cells
T_{FH}	Follicular helper T cells
Type-I IFN	Type I interferon
ULBP	UL16 binding protein
UT	Untreated

List of Figures

Figure 1.1	Schematic depiction of the HBV replication cycle.....	16
Figure 1.2	Natural course of chronic HBV infection.....	19
Figure 1.3	Schematic depiction of immune responses during virus infection.....	23
Figure 1.4	Schematic representation of three major families of NK cell receptors.....	32
Figure 1.5	Schematic diagram of the hepatic sinusoid structure and its cellular components.....	34
Figure 1.6	Human NKG2D and its ligands.....	38
Figure 1.7	Mechanisms of NKG2D/DAP10 regulation.....	40
Figure 1.8	Mechanisms of NKG2D-L regulation.....	42
Figure 2.1	Representative gating strategies.....	50
Figure 2.2	Representative gating strategy for <i>ex vivo</i> dextramer staining.....	51
Figure 2.3	Representative gating strategy for C1R/C1R-MICA cell staining.....	52
Figure 2.4	Representative plots of NK cell and CD4 T cell isolation.....	53
Figure 2.5	Representative plots of efficacy and specificity of NKG2D blockade.....	54
Figure 3.1	Evaluation of NKG2D-L expression on T cells.....	66
Figure 3.2	Evaluation of NKG2D-L expression on intrahepatic T cells.....	67
Figure 3.3	Evaluation of proliferation status of MICA/B ⁺ T cells.....	68
Figure 3.4	Phenotype analysis of MICA/B ⁺ T cells.....	69
Figure 3.5	Evaluation of NKG2D-L expression on activated T cells.....	70
Figure 3.6	Evaluation of NKG2D-L expression on intrahepatic activated T cells.....	71
Figure 3.7	Evaluation of NKG2D-L expression on HBV-specific T cells.....	72
Figure 3.8	Evaluation of NKG2D-L expression on HBV/CMV-specific CD8 T cells.....	73
Figure 3.9	Evaluation of NKG2D-L expression on intrahepatic HBV-specific T cells.....	74

Figure 3.10	NKG2D-L expression on intrahepatic T cells within activated/HBV-specific subsets. .	75
Figure 3.11	NKG2D-L induction on purified CD4 T cells upon TCR signalling or OS stimulation..	77
Figure 3.12	NKG2D-L induction upon oxidative stress stimulation of purified CD4 T cells.	78
Figure 3.13	Evaluation of NKG2D-L induction on T cells upon oxidative stress stimulation.	79
Figure 3.14	Upregulation of Fas/PD-1 on NKG2D-L expressing CD4 T cells upon OS stimulation.	80
Figure 3.15	The impact of NKG2D pathway and NK cells on activated T cells in CHB.	82
Figure 3.16	The impact of NKG2D pathway and NK cells on HBV-specific T cells in CHB.	83
Figure 3.17	The impact of NKG2D pathway on intrahepatic HBV-specific T cells in CHB.	84
Figure 4.1	NK cell activation driven by NKG2D-L expressing cells.....	102
Figure 4.2	NK cell CD107a/IFN- γ /TRAIL expression induced by NKG2D-L expressing cells....	103
Figure 4.3	NK cell HLA-DR/CD107a induced by target cells with various NKG2D-L levels	104
Figure 4.4	Target cell viability changes in the co-culture of isolated NK cells	105
Figure 4.5	NKG2D engagement induced IFN- γ /HLA-DR changes in NK cells.....	107
Figure 4.6	NKG2D engagement induced CD107a changes in NK cells.....	108
Figure 4.7	NKG2D engagement induced viability changes in NK/target cell co-culture.	109
Figure 4.8	NKG2D expression of NK cells in chronic HBV infection.	111
Figure 4.9	HLA-DR of NK cells in chronic HBV infection.....	112
Figure 4.10	The correlation between NK cell HLA-DR and T cell MICA/B expression in CHB....	113
Figure 4.11	Effects of PEG-IFN α therapy on T cell NKG2D-L induction.	115
Figure 4.12	Longitudinal evaluation of PEG-IFN α therapy on T cell NKG2D-L induction.	116
Figure 4.13	IFN- α effects on NK cell degranulation activity.....	117
Figure 4.14	Effects of IFN- α on NK cell killing towards NKG2D-L expressing cells.....	118
Figure 4.15	Relationship between HBV infection and host immunity in the NKG2D axis.....	124

Figure 5.1 NKG2D-L expression on CD4 Treg cells in CHB patients. 126

Figure 5.2 NK cell NKG2D down-regulation by cell-bound NKG2D-L..... 131

List of Tables

Table 1. Clinical data of study controls and patients47

Table 2. List of anti-human mAb used for flow cytometric analysis49

Table 3. List of MHC class I-restricted multimers used for flow cytometric analysis51

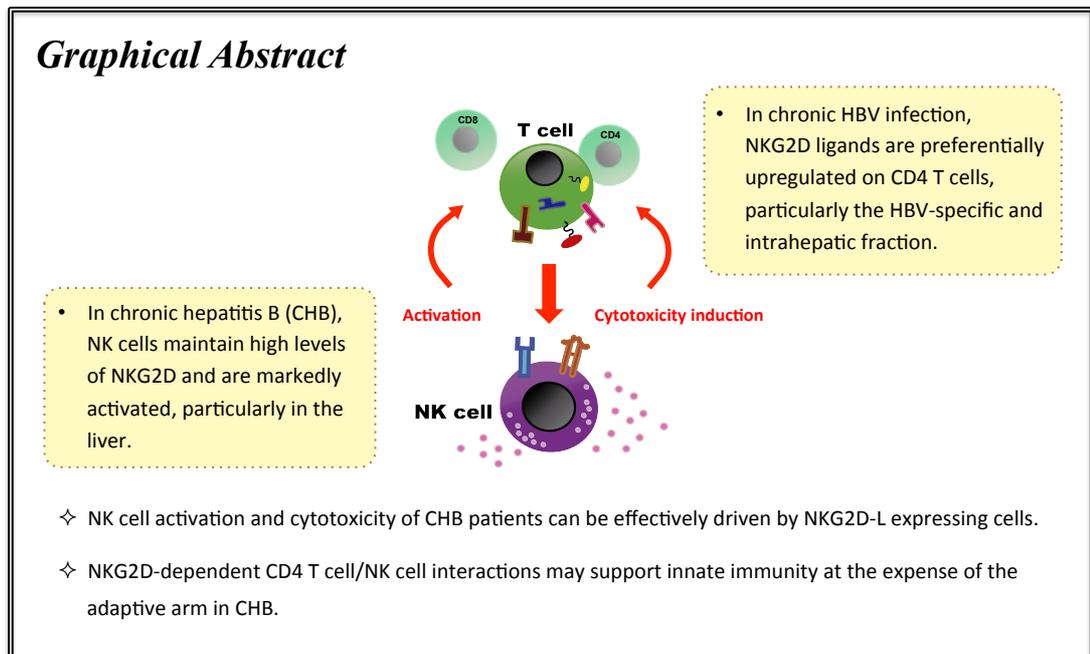
Abstract

NK cells are emerging as potent regulators of adaptive immunity in virus infection. Our group recently documented the partially TRAIL-dependent deletion of HBV-specific T cells by NK cells. For this study, we investigated the underlying interactions between NK cells and T cells through the NKG2D pathway in chronic HBV infection.

In this study, we observed that activated and HBV-specific T cells, especially the CD4 fraction, expressed NKG2D ligands (NKG2D-L) not normally seen on T cells. NKG2D-L upregulation was further enriched on CD4 T cells in HBV-infected livers compared to the circulation and control livers. Oxidative stress, one noteworthy pathogenic feature of HBV infection, was demonstrated to recapitulate the T cell NKG2D-L upregulation pattern seen in patients with chronic hepatitis B (CHB). NK cells from patients with CHB maintained NKG2D expression and their increased activation and cytotoxicity could be driven by NKG2D-L expressing cells. In line with the distinctive features of T cells and NK cells in CHB, we discovered a positive correlation between activation of NKG2D⁺NK cells and the NKG2D-L (MICA/B) levels on CD4 T cells. Additionally, the pro-inflammatory cytokine IFN- α , used in HBV treatment, was shown to favour for NKG2D-mediated regulation.

To conclude, we provide the first *ex vivo* evidence that human T cells, particularly those sequestered within tissues, can become visible to the stress surveillance system by the induction of NKG2D-L. We show that in active CHB, T cells upregulate NKG2D-L which can drive NK cell activation and cytotoxicity via the NKG2D

pathway. These interactions may be triggered by aberrant oxidative stress and result in a homeostatic response of "damage removal", thereby limiting T cell antiviral immunity. Therefore, efforts to manipulate the HBV-infected liver milieu in order to decrease T cell oxidative stress and diminish constraints from NK cells and the NKG2D pathway should be considered to reduce HBV pathogenesis and promote immunity.



Chapter 1 Background of the Study

1.1 Overview of Hepatitis B Virus Infection

Hepatitis B virus and its global health impact

HBV is a serious global health issue with roughly 30% of the worldwide population presenting serological evidence of ongoing or previous HBV infection. According to the WHO 2015 report, it is estimated that around 240 million people are chronic HBV carriers and more than 700,000 annual deaths are attributed to the resultant liver diseases, cirrhosis and hepatocellular carcinoma (2015; Trepo et al., 2014). HBV is usually transmitted via blood or semen. Therefore, vertical transmission from infected mothers to neonates, or horizontal transmission from unprotected sexual behavior, contaminated needles, instruments, or blood product exposure are all common infection sources (2015; Trepo et al., 2014).

HBV is a hepatotropic, partially double-stranded DNA virus belonging to the Hepadnaviridae family. Acting as a "stealth virus" with non-cytopathic properties, HBV can efficiently infect a large proportion of hepatocytes and establish persistent liver infection (Protzer et al., 2012; Seeger and Mason, 2000; Yoo et al., 1987).

The HBV virion enters hepatocytes through the sodium taurocholate cotransporting polypeptide (NTCP) and releases its nucleocapsid into the cell cytoplasm (Yan et al., 2012; Zoulim and Locarnini, 2009) (Figure 1.1). The virus relaxed circular DNA (rcDNA) is then transported to the nucleus, where the HBV genome is transformed into a transcriptional template called covalently closed circular DNA (cccDNA)

(Seeger and Mason, 2000; Urban et al., 2010). HBV cccDNA residing within the hepatocyte nucleus is fundamental for the virus maintenance and replication.

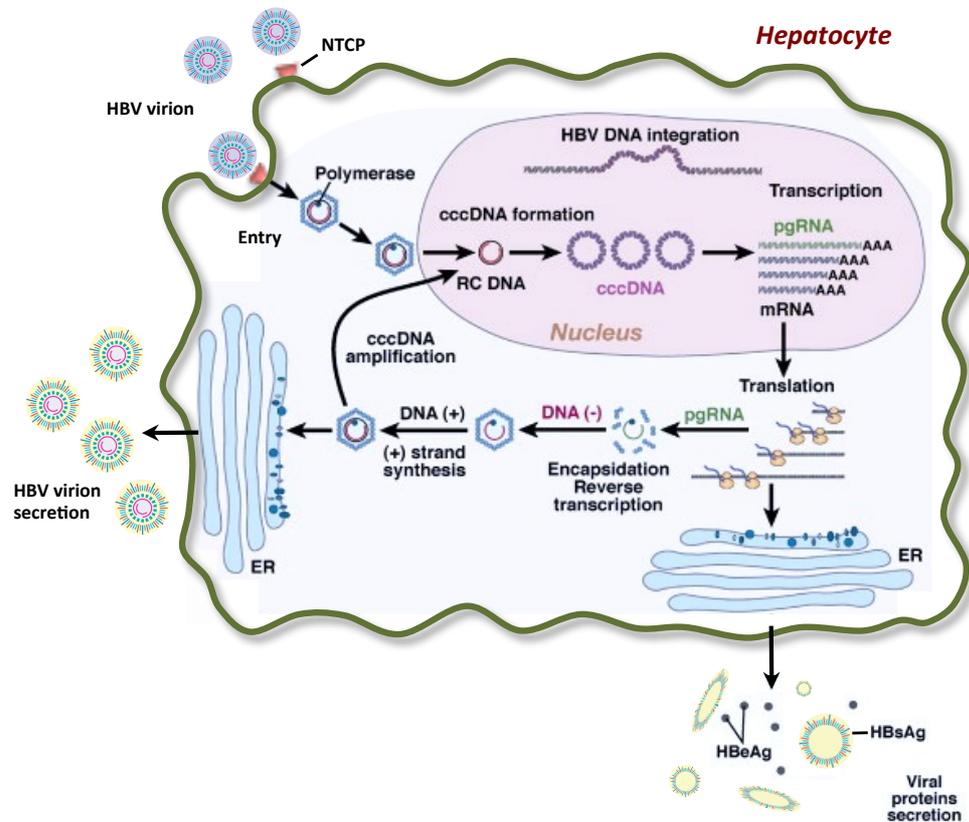


Figure 1.1 Schematic depiction of the HBV replication cycle (*figure modified from Zoulim and Locarnini, 2009).

During replication, HBV reverse transcriptase converts pre-genomic RNA intermediates into virus DNA, an error-prone step with high mutation tendency. The viral messenger RNA (mRNA) codes for the various structural and non-structural proteins. The pre-core protein is processed in the endoplasmic reticulum (ER) and secreted as HBeAg. A small non-particulate protein, HBxAg, capable of transactivating the transcription of both cellular and viral genes, is important for viral replication and carcinogenesis. The envelope protein presenting as the outer surface of the virion or in smaller tubular and spherical forms is referred to as HBsAg. The

core protein is essential for assembly of nucleocapsid particles which contain HBV DNA and DNA polymerase (Beck and Nassal, 2007; Seeger and Mason, 2000, 2015; Yokosuka and Arai, 2006). A typical feature of HBV life cycle is the persistently high-level viral protein secretion (Ganem and Prince, 2004), with different clinical implications. By definition, patients who remain HBsAg-positive longer than 6 months are chronic HBV carriers. On the other hand, serum HBsAg usually becomes undetectable with protective HBsAb appearance within 6 months in acute resolved patients. The secreted nucleocapsid protein, HBeAg was widely used as an indicator for viral replication and infectivity; however there are now many patients with eAg-negative mutants (see below) who can also have high levels of viral replication (Ganem and Prince, 2004; Trepo et al., 2014).

Clinical course of HBV infection

The outcome of HBV infection is mainly dependent on age; infection during adulthood is usually resolved spontaneously with lifelong immunity development and only less than 5% of cases result in chronicity. On the other hand, around 95% of neonates and 30% of children become long-term carriers, bearing the risk of liver complications and cancer progression (Trepo et al., 2014).

Clinical symptoms of acute hepatitis B (AHB) can be slight or subclinical, especially in children or young adults (Trepo et al., 2014). Non-specific symptoms such as malaise, anorexia, abdominal discomfort, or jaundice are common complaints. Due to the prolonged and clinically indistinct incubation period of HBV infection, human

immunological studies in acute or pre-symptomatic patients are challenging and difficult to access (Dunn et al., 2009; Webster et al., 2000). Patients with chronic HBV infection can be broadly divided into four clinical phases (Figure 1.2) (Liaw and Chu, 2009; Lok, 2007). Particularly, when HBV is acquired before early childhood, patients will firstly experience **the asymptomatic immunotolerant phase** characterized by extremely high viral loads (HBV DNA usually $>10^7$ IU/mL) but normal serum alanine aminotransferase (ALT), and no/minimal liver pathology. This period can last for years until unknown triggers precipitate the development of **the immune clearance phase**, showing a decrease of HBV DNA with active hepatitis and liver function abnormalities. Some patients going through this phase successfully suppress HBV replication to a lower viral load ($<10^3$ IU/mL) and move into **the inactive carrier phase**, presenting long-term normalisation of ALT and seroconversion of HBeAg to HBeAb (Liaw and Chu, 2009; Lok, 2007). Generally, the prognosis of patients in the inactive carrier phase is favorable, particularly if this state is achieved early. However, in spite of HBeAg seroconversion, roughly 30% of patients will experience HBV reactivation with fluctuating viral loads and intermittent hepatic flares. In **the HBV reactivation phase**, these HBeAg-negative patients with precore or basal core promoter mutations have an increased risk of liver cirrhosis and cancer development (Liaw and Chu, 2009; Lok, 2007).

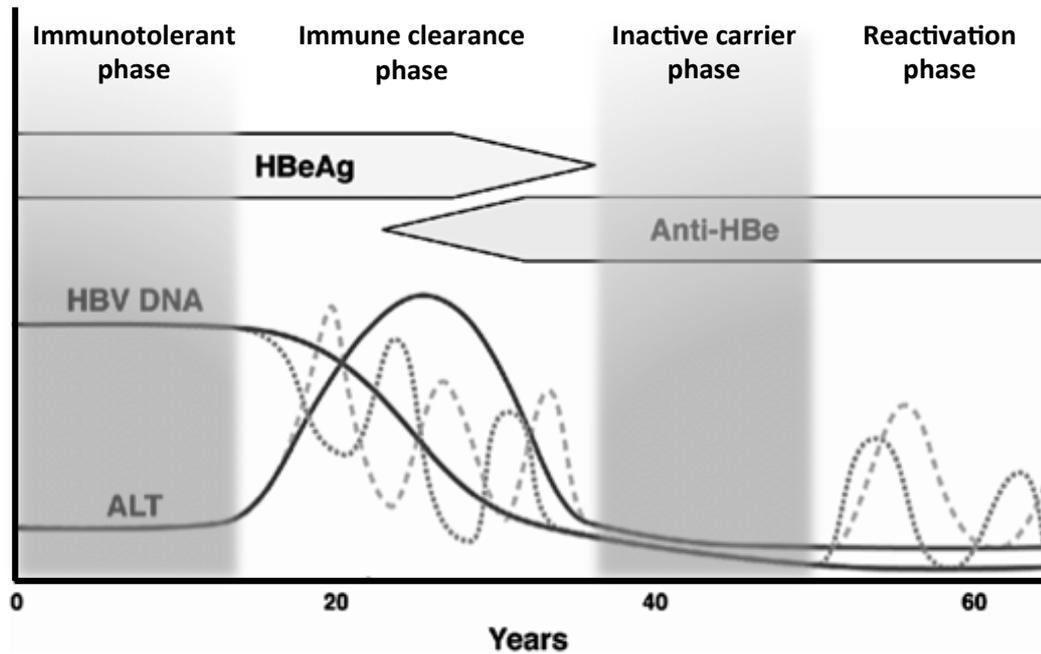


Figure 1.2 Natural course of chronic HBV infection (*figure modified from Lok AS, 2007).

Current treatment and limitations

Although several potent, first-line anti-HBV drugs are available nowadays, including interferon-based regimens (conventional or pegylated-interferon α , PEG-IFN α) and nucleos(t)ide analogues (lamivudine, adefovir, entecavir, telbivudine, and tenofovir), complete HBV eradication and sustained off-treatment responses are still rarely achieved ([Papatheodoridis et al., 2008](#)).

Interferon has both anti-viral and immunomodulatory effects. Recent research has illustrated that interferon- α is able to promote non-cytotoxic cccDNA eradication in HBV-infected cells through activating the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A (APOBEC3A) cytidine deaminase ([Lucifora et al., 2014](#)). However, many adverse effects, such as fatigue, influenza-like symptoms,

bone marrow suppression and even severe hepatitis flares have limited interferon clinical usage ([Papatheodoridis et al., 2008](#); [Trepo et al., 2014](#)).

On the other hand, nucleos(t)ide analogues are generally safe and well-tolerated in patients. These anti-viral regimens can efficiently suppress HBV replication by inhibiting HBV polymerase/reverse transcriptase activity. Hence, without the advantage of directly targeting cccDNA, virus relapse after nucleos(t)ide analogue discontinuation is common. Furthermore, in view of the HBV high mutation and error-prone tendency, drug resistance development is also another key concern of the nucleos(t)ide analogue treatment ([Papatheodoridis et al., 2008](#); [Trepo et al., 2014](#)), although tenofovir and entecavir, the mainstay of HBV treatment in the UK, have high genetic barriers to resistance. The long-term or even life-long maintenance therapy usually required with nucleos(t)ide analogues carries a huge cost burden as well as risks of non-compliance and longterm toxicity ([Lampertico et al., 2015](#)).

Interferon-based and nucleos(t)ide analogue regimens have divergent effects on immune responses ([Lampertico et al., 2015](#); [Thimme and Dandri, 2013](#)). Work from our group has shown that PEG-IFN α therapy significantly enhanced the pro-proliferative cytokine IL-15 induction and CD56^{bright} NK cell expansion. Within expanded NK cells, boosting effects of PEG-IFN α were also reflected in increased IFN- γ production, activating receptor NKp46 and TRAIL upregulation. Moreover, the functional CD56^{bright} NK cell expansion correlated with patient peak virological responses, further suggesting that PEG-IFN α anti-viral effects involve augmentation of innate immunity ([Micco et al., 2013](#)).

Conversely, a marked reduction of CD8 T cells was noticed during PEG-IFN α therapy. These findings, along with other group results, revealed that PEG-IFN α did not improve HBV-specific T cell response and had no effect on the inhibitory molecule (PD-1 or CTLA-4) expression of T cells (Micco et al., 2013; Penna et al., 2012). Therefore, interferon therapy appears to have relatively limited benefits on T cell immunity.

Unlike PEG-IFN α , long-term treatment with nucleos(t)ide analogues can contribute to improvement of HBV-specific CD8 T cell functions. As in results reported by Boni et al in 2012, greater expansion potential of HBV-specific CD8 T cells was observed, at least in the few patients with complete antigen clearance (HBsAg seroconversion), upon nucleos(t)ide analogue treatment compared to those in untreated patients or patients who had not seroconverted (Boni et al., 2012). Differently, NK cell functional recovery was not noted in patients undertaking nucleos(t)ide analogues (Peppas et al., 2010).

In summary, PEG-IFN α and nucleos(t)ide analogues seem to have different but complementary capabilities to restore impaired innate and adaptive immunity in chronic HBV infection. Thus, considering the complex nature of the virus and unsatisfactory treatment outcomes, a breakthrough in HBV management is in urgent demand and requires further study.

1.2 Immune Response Defects in HBV Infection

Tolerogenic liver environment exploited by HBV

The liver is a vital organ, carrying out numerous important functions, such as metabolism, detoxification, and immunomodulation to maintain homeostasis and health (Crispe, 2009; Protzer et al., 2012; Sheth and Bankey, 2001). Due to the constant exposure to gastrointestinal antigens derived from food, the liver has immunotolerant features, presumably to avoid reacting to harmless antigenic material. In fact, various cells within the liver together with the cytokine milieu predispose the tolerogenic microenvironment to dampen humoral and cellular immunity (Crispe, 2003; Kmiec, 2001; Knolle et al., 1999; Tu et al., 2008). Nevertheless, this physiological trait can be exploited by hepatotropic pathogens to establish persistent infection.

Hepatitis B virus (HBV) is one of the most well known intrahepatic pathogens, which can efficiently infect hepatocytes and lead to chronic liver pathology and lethal complications, including liver cirrhosis and cancer (Protzer et al., 2012). By virtue of previous research, host-virus interactions following infection could be briefly depicted in a model involving the rapid anti-viral cytokine responses, then the early innate cellular defence, and the final viral clearance by mature adaptive immunity development (Figure 1.3A) (Alexandre et al., 2014; Murphy et al., 2012; Openshaw and Tregoning, 2005).

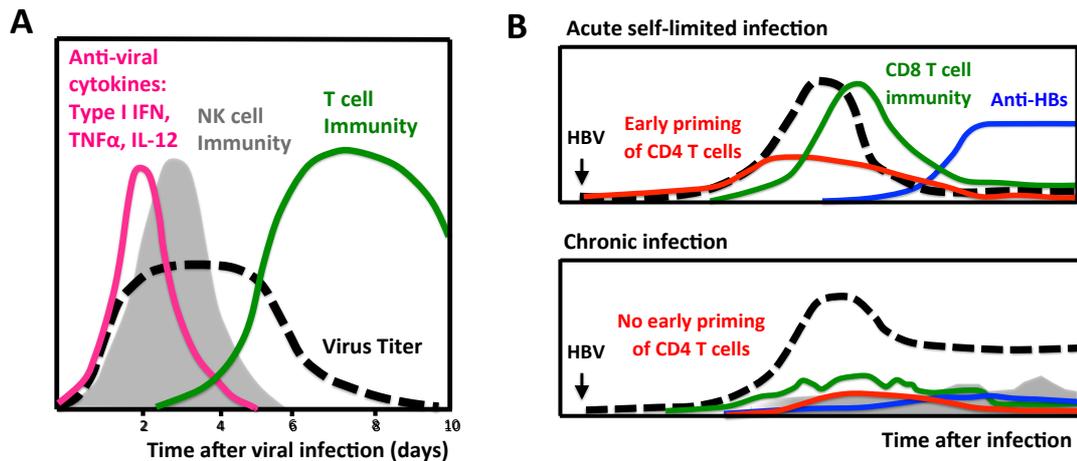


Figure 1.3 Schematic depiction of immune responses during virus infection.

A Time course of virus titer and immune responses during MCMV infection (virus titer-dashed black line; cytokine response-pink line; NK cell response-gray area; T cell response-green line; *figure modified from Murphy et al. Janeway's Immunobiology, 8th edition).

B Time course of virus titer and immune responses during acute and chronic HBV infection (virus titer-dashed black line; NK cell response-gray area; CD4 T cell response-red line; CD8 T cell response-green line; antibody response-blue line; *figures modified from Isogawa M and Tanaka Y, 2015).

However, HBV is a cunning virus, one that has evolved to elude and debilitate the host immunity in diverse ways and can establish long-term infection in the liver. Thus, in view of the widespread devastating influence of HBV and current unsatisfactory treatment options, it is crucial to understand these defects in liver-specific immunity for better virus control and disease resolution.

HBV as a “stealth virus” slipping through the front-line cytokine defence

The hallmark of early defence against viral infection is the production of type I interferons (IFNs) which can directly inhibit virus and also support NK cell function, promote dendritic cell maturation, and assist adaptive T cell responses (Dalod et al., 2003; Lanier, 2008; Tough et al., 1996). However, unlike the common immune

response to acute viral infection, previous *in vivo* chimpanzee studies revealed IFN-I responsive genes in the liver were barely detectable during the early HBV entry and expansion phase (Wieland et al., 2004). Similarly, the serum IFN- α concentrations remained low with no significant upregulation observed at the time of peak viraemia in acute HBV patients compared to HBV resolved patients and healthy controls (Dunn et al., 2009). In contrast, self-limiting hepatitis A virus (HAV) infection presenting higher serum IFN- α levels; thus HBV acts like a “stealth virus” showing mild/minimal clinical symptoms and defective IFN responses in the early stage of infection (Protzer et al., 2012; Wieland and Chisari, 2005). Since the extreme sensitivity of HBV to IFN- α has been proven in transgenic mouse models (McClary et al., 2000; Wieland et al., 2000), this inherent strategy for evading innate recognition probably reflects HBV replication within the nucleus, shielded from many pattern recognition receptors. In addition, recent research suggests there may be a component of active suppression of innate immunity during HBV infection (Bertoletti et al., 2010).

Pathogenic role rather than protective role of the NK cell

NK cells, the largest (30-40%) component of intrahepatic lymphocytes, can regulate various immune interactions and immunopathology (Doherty et al., 1999; Shi et al., 2011). Unlike in the peripheral counterpart, liver NK cells are highly activated and comprise a larger CD56^{bright} population. Recent murine research implicates those intrahepatic NK cells with high CXCR6 expression and a relative immature phenotype as a particular liver-resident lineage, which could mediate “memory-like”

responses to viruses or haptens (O'Leary et al., 2006; Paust et al., 2010). Since HBV infects and replicates within the liver, NK cell immunity is likely to play a crucial role in this hepatotropic virus invasion. However, though NK cells have been shown to efficiently inhibit HBV replication in several animal models (Guidotti et al., 1999; Kakimi et al., 2000; Yang et al., 2002), delayed and suppressed NK cell effector functions were witnessed in patients during the early replication phase of HBV infection which, coincidentally, were in line with the immunosuppressive cytokine, IL-10 levels (Dunn et al., 2009; Fisicaro et al., 2009).

Indeed, despite NK cell frequency appearing unaffected in some studies, their responses in HBV infection are dysfunctional and skewed (Bonorino et al., 2009; Peppia et al., 2010). First of all, defective cytokine production by NK cells has been observed in several studies of CHB patients, with impaired IFN- γ production directly reducing NK cell anti-viral activity as well as diminishing their support for T cell immunity (Oliviero et al., 2009; Peppia et al., 2010; Tjwa et al., 2011). A number of pathologic factors in chronic HBV infection have been suggested to be responsible for NK cell functional defects, such as disturbed pDC/NK cell interplay by HBV, Kupffer cell suppression via galectin-9, IL-10, and TGF- β , or aberrant activating/inhibitory signals for NK cell receptors (Ju et al., 2010; Nebbia et al., 2012; Peppia et al., 2010; Shi et al., 2012; Sun et al., 2012; Tu et al., 2008).

However, in addition to dysfunctional cytokine production, the preserved cytotoxicity of NK cells has been shown to participate in various immunopathology (Peppia et al., 2010; Zhang et al., 2011b). In CHB, NK cells can cause liver injury in

a TNF-related apoptosis inducing ligand (TRAIL) dependent manner (Dunn et al., 2007). Besides the TRAIL pathway, Fas and NKG2D pathways were also implicated in NK cell-mediated hepatocyte damage (Okazaki et al., 2012; Zou et al., 2010). Furthermore, recent work from our group also illustrated that through TRAIL-dependent cytotoxicity, NK cells can kill HBV-specific CD8 T cells to constrain anti-viral immunity in patients with chronic HBV infection (Peppia et al., 2013). Thus, considering the current evidence, the NK cell seems to play a more pathogenic rather than protective role in HBV infection.

Crucial but defective anti-viral T cells in chronic HBV infection

It is widely accepted CD4 T cells are crucial for supporting competent adaptive immunity in viral infection. Consistently, current evidence also suggests a close link between CD4 T cell immunity and the outcome of HBV infection (Chisari and Ferrari, 1995; Guidotti et al., 2015b). In contrast to the vigorous and multi-specific CD4 T cell responses to HBV in resolved patients, CD4 T cells in chronic HBV carriers have a weak and narrow repertoire of anti-viral responses and poor proliferative capacity (Figure 1.3B) (Ferrari et al., 1991; Ferrari et al., 1990).

The pivotal role of CD4 T cells is also clearly supported by the *in vivo* clinical observation, where HIV-infected, low CD4 T cell patients are more prone to develop HBV chronicity in horizontal transmission cohorts (Hadler et al., 1991; Puoti et al., 2006). Additionally, the previous *in vivo* chimpanzee models even more specifically demonstrated the necessity of CD4 T cells in HBV infection. Accordingly, although

CD4 T cell depletion at the peak of HBV viraemia did not hinder the eventual disease resolution, depletion of CD4 T cells prior to HBV infection did both quantitatively and qualitatively impair HBV-specific CD8 T cells and lead to consequent HBV persistence (Asabe et al., 2009; Thimme et al., 2003). Therefore, these results point to CD4 T cells indirectly contributing to virus control, probably through priming and supporting anti-viral immunity, such as by IL-2 secretion or by promoting APC co-stimulation for HBV-specific CD8 T cells (Bertolino et al., 2001; Castellino and Germain, 2006; Limmer et al., 2000).

Nevertheless, some studies also suggest CD4 T cells can modulate the inflammatory response. The recently defined T-helper 17 (Th17) CD4 T cell subset increases in CHB patients and can produce IL-17 and IL-22 to exacerbate disease activity (Zhang et al., 2010; Zhang et al., 2011a). By contrast, regulatory CD4 T cells (Treg) with the ability to suppress T cell expansion and survival are implicated as having anti-inflammatory properties (Manigold and Racanelli, 2007; Xu et al., 2006).

Apart from CD4 T cells, CD8 T cells also have decisive significance in HBV infection. The most solid evidence provided in HBV chimpanzee models reveals that depletion of CD8 T cells at the peak of infection impeded HBV clearance until T cell re-induction (Thimme et al., 2003). Additionally, human and chimpanzee studies demonstrated that CD8 T cells could potentially eliminate HBV via non-cytolytic anti-viral cytokines such as IFN- γ and TNF- α (Guidotti et al., 1999; Maini et al., 2000; Phillips et al., 2010).

Similar to the weak and narrowly focused CD4 T cell responses to HBV, CD8 T cells in CHB patients lack the vigorous and multi-specific anti-viral immunity required for HBV clearance (Figure 1.3B) (Bertoletti and Ferrari, 2012; Boni et al., 2007; Fisicaro et al., 2010; Rehermann et al., 1995). Moreover, the cytopathic activity of CD8 T cells has been described to cause liver damage (Ando et al., 1993; Isogawa et al., 2005; Maini et al., 2000). Surrounded by persistent, high antigen load and bombarded with excessive inhibitory signals, liver-infiltrating lymphocytes are driven to anti-viral exhaustion and in turn become pathogenic in the HBV-infected liver. Noticeably, evidence from transgenic mouse and human studies showed that when HBV-specific CD8 fail to control virus they initiate liver damage, which is then mainly caused by the non-HBV-specific subset (Guidotti and Chisari, 2006; Isogawa et al., 2005; Maini et al., 2000; Nakamoto et al., 1997). Thus, defective and inappropriate CD8 T cell responses can be a key factor in the failure of HBV control and varying degrees of liver pathology. Hence, to improve HBV control, the future therapeutic approaches should aim to reverse negative suppression and re-direct the anti-viral immunity of T cells.

1.3 Overview of Human NK Cells

NK cell development and features

NK cells, as a part of innate immunity, are large granular lymphocytes having the ability to kill targets without the need for specific sensitisation or immunization. Indeed, experiments in mice have shown NK cells can rapidly exert effector functions to contain virus infections, providing more time for adaptive immunity to fully develop and mature (Caligiuri, 2008; Vivier et al., 2008). In contrast, a lack of NK cells in the early phase of infection can lead to greater virus replication and serious consequences (Lee et al., 2007; Orange, 2002; Scalzo et al., 2007).

Intriguingly, recent research has extended the border of NK cells to the gray zone between innate and adaptive immunity, since their "memory-like" behavior exhibits antigen-specific features (O'Sullivan et al., 2015; Sun et al., 2011). Moreover, recent studies indicate that NK cells do not always react in an invariant manner, but can potentially adapt to the surrounding environment and modify their functions correspondingly (Vivier et al., 2011). Thus, the sophisticated capabilities of NK cells highlight their immune significance in pathogen defence and tumour surveillance.

Generally, the surface phenotype of human NK cells lacks CD3 but possesses CD56, a neural cell adhesion molecule (NCAM). While the phenotype of NK cells varies extensively across species, one highly conserved natural cytotoxicity receptor (NCR) NKp46 serves as a good marker for mammalian NK cells (Caligiuri, 2008; Walzer et al., 2007).

It is widely recognised that human NK cells primarily develop within the bone marrow. However, current evidence points out some NK precursor cells with differentiation potential can be found in certain secondary lymphoid organs including the liver (Eissens et al., 2012; Galy et al., 1995; Miller et al., 1994; Moroso et al., 2011; Shibuya et al., 1993). Although the development of human NK cells has not been fully understood, recent research has depicted their differentiation pathway based on the sequential acquisition of functional capabilities and specific phenotypic markers. These include the differentiation progression from bone marrow-derived hematopoietic stem cells to the pro-NK (CD34⁺CD117⁻CD94⁻) stage, pre-NK (CD34⁺CD117⁺CD94⁻) stage, immature iNK (CD34⁻CD117⁺CD94⁻) stage, and the stage of NK cells presenting CD34⁻CD117^{+/-}CD94⁺ corresponding to the NK cell CD56^{bright} subset (Freud et al., 2005; Freud et al., 2006; Sun and Lanier, 2011).

CD56^{bright} NK cells have greater levels of adhesion molecules L-selectin (CD62L) and lymphoid homing receptor CCR7 and are preferentially enriched in secondary lymphoid tissue (SLT), such as lymph nodes, tonsils, and the liver. In fact, CD56^{bright} NK cells are conventionally regarded as the principal source of NK cell-derived cytokines, such as IFN- γ , TGF- β , IL-10, but lack the low-affinity Fc γ receptor CD16 and cytotoxic granules perforin and granzymes (Caligiuri, 2008; Cooper et al., 2001; Vivier et al., 2008).

In contrast, the CD56^{dim} subset, comprising the majority of NK cells in the bone marrow, blood and the spleen displays dominant cytolytic capacity in line with its abundance of perforin and granzymes, as well as high levels of CD16 relevant to

antibody-dependent cellular cytotoxicity (ADCC) (Caligiuri, 2008; Cooper et al., 2001; Vivier et al., 2008).

The findings of recent definitive studies support that CD56^{dim} NK cells represent a phenotypically more mature subset and are directly derived from CD56^{bright} NK cells (Chan et al., 2007; Lanier et al., 1986; Ouyang et al., 2007; Romagnani et al., 2007). However, considering their disparate phenotype features and different functional behaviors, whether CD56^{bright} and CD56^{dim} NK cells can be satisfactorily defined into two successive differentiation stages remains unanswered.

NK cell function and regulation

Human NK cells in the circulation are relatively short-lived with an estimated turnover time of about 2 weeks (Zhang et al., 2007). Although in viral infection, NK cells can offer rapid non-specific defence before adaptive immunity is fully activated, their effector functions are mainly dependent on the background cytokine milieu and the integrated signals received from the surface activating and inhibitory receptors (Vivier et al., 2008).

IL-2 is well-known to be able to promote proliferation, cytotoxicity and cytokine production of NK cells, whilst other cytokines such as type I IFN, IL-12, IL-15, and IL-18 are potent activators for NK cell effector function as well (Vivier et al., 2004; Vivier et al., 2008; Walzer et al., 2005).

Distinct surface receptors also tightly regulate NK cell activity. NK cells have a great variety of germline-encoded activating and inhibitory receptors (Bryceson et al.,

2006a; Lanier, 2005). Broadly, there are three major families of natural killer receptors (NKR). One superfamily is killer cell immunoglobulin-like receptors (KIR) recognising classical major histocompatibility complex (MHC) class I molecules, like HLA-A, B, and C. Another is the C-type lectin-like receptors, consisting of seven NKG2 family members (NKG2A, B, C, D, E, F and H), which recognise MHC class-I like molecules or non-classical MHC class-I molecules. Thirdly, the natural cytotoxicity receptor (NCR) superfamily has members such as NKp30, NKp44, NKp46, and NKp80 (Figure 1.4).

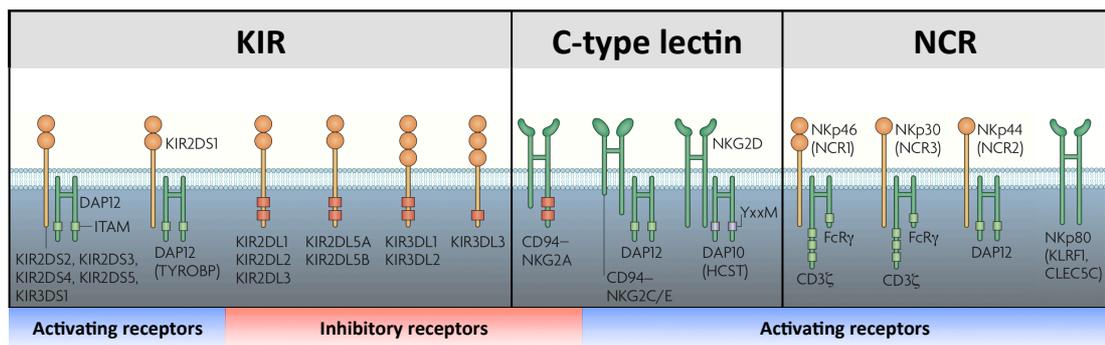


Figure 1.4 Schematic representation of three major families of NK cell receptors and their structure (*figure modified from the poster of Eric Vivier and Sophie Ugolini in Nature Reviews).

In general, NK cell cytotoxicity is adequately controlled by the balance of signals from NK cell receptors to ensure elimination of targets, but not healthy cells. Ordinarily, killing of healthy "self" cells by NK cells is prevented by dominant inhibitory receptors recognising "self" MHC class I molecules on the cells. This MHC class I based self-tolerance attribute is essential for the NK cell education process before NK cells achieve functional competence (Caligiuri, 2008; Hoglund and Brodin, 2010; Kim et al., 2005; Vivier et al., 2008).

NK cells perform a complementary role to cytotoxic T cells in host immunity, effectively targeting "altered-self" cells with downregulated self-MHC class I molecules and recognising cells with stimulatory signals surpassing the inhibitory threshold (Caligiuri, 2008; Long et al., 2013; Sun and Lanier, 2011; Vivier et al., 2004). While facing dangerous assaults and pathologic stress, NK cells can exert cytotoxicity by exocytosis of perforin and granzymes or by expressing death ligands, such as the TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (Fas-L) to eradicate affected cells (Smyth et al., 2005).

Immune perspective of liver NK cells

Current evidence indicates that to maintain regional health and local immune homeostasis, organ-specific NK cells carry divergent phenotypic and functional features different from bone marrow-derived conventional NK cells (Shi et al., 2011; Yokoyama et al., 2013).

Unlike peripheral NK cells that only account for a minor fraction of total circulating lymphocytes, hepatic NK cells represent the largest (30-40%) lymphocyte population in the human liver (compared to 5-10% in mouse liver) (Doherty et al., 1999; Shi et al., 2011). With preferential residence within the hepatic sinusoidal space, liver NK cells intimately contact other lymphocytes and non-parenchymal cells to modulate liver immunology (Figure 1.5) (Krueger et al., 2011).

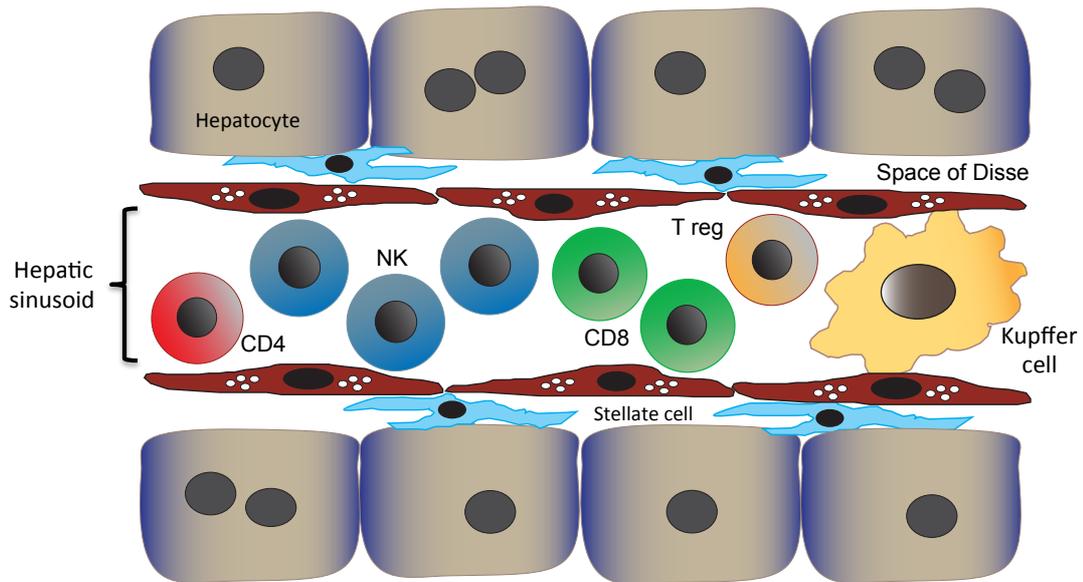


Figure 1.5 Schematic diagram of the hepatic sinusoid structure and its cellular components (*figure kindly provided by Dr. Anna Schurich, Maini group).

Since the liver is constantly exposed to gut-derived antigens, hepatic NK cells are usually maintained in a functionally hyporesponsive state (Tu et al., 2008). In fact, many studies have proposed that the inhibitory receptor NKG2A on hepatic NK cells may contribute to the tolerogenic liver environment (Krueger et al., 2011). Research in patients with chronic hepatitis C suggests the increased NKG2A on NK cells may unfavorably facilitate HCV persistence by restraining anti-viral T cell immunity (Jinushi et al., 2004; Nattermann et al., 2006). Similarly, previous murine HBV models also demonstrated that elevated NKG2A on hepatic NK cells positively correlates with viral loads and can impede HBV clearance (Li et al., 2013).

Additionally, the intrahepatic cytokine milieu greatly impacts NK cell function. For example, IL-10 secreted from Kupffer cells has been shown to suppress NK cell activation and effector function (Jinushi et al., 2007; Krueger et al., 2011). However,

this IL-10-mediated NK cell suppression has been implicated in avoiding excessive hepatic damage during liver inflammation.

On the other hand, various cytokines in the inflamed liver are able to activate NK cells and drive their cytotoxicity (Burt et al., 2009; Kim et al., 2005; Shi et al., 2011). Evidence has revealed either endogenous, or therapeutic IFN- α can induce TRAIL upregulation on NK cells which could contribute to HBV or HCV elimination, but also collaterally inflict liver damage (Dunn et al., 2007; Stegmann et al., 2010). Moreover, in a TRAIL-dependent manner, NK cells have been known to kill HBV-specific T cells and hepatic stellate cells (Glassner et al., 2012; Peppia et al., 2013).

Notably, recent studies observed that a particular CXCR6-positive subset of hepatic NK cells manifests antigen-specific recall behaviors (Paust et al., 2010). Although difficulty accessing healthy liver tissue limits human liver research, many animal models have shed light on NK cell "memory-like" responses in this field. Specifically, upon haptens, virus-like particles, or vaccinia virus sensitisation, hepatic, but not splenic, NK cells exhibit distinct reactions akin to immunological memory (O'Leary et al., 2006; Paust et al., 2010; Paust and von Andrian, 2011). This phenomenon could be detected in mice lacking T cells and B cells, in which these "primed" NK cells remained long-lived and were able to mount a secondary response when re-exposed to the same stimuli. Furthermore, preferential clonal-expansion of NKG2C-positive NK cells in HCMV infection also suggests the existence of NK cell memory in humans (Foley et al., 2012; Lopez-Verges et al., 2011). However, the

immunobiological implications of this virus-specific proliferation are not yet fully determined, since NK cells in NKG2C-null patients are still competent to control HCMV infection ([Beziat et al., 2013](#); [Schlums et al., 2015](#)). Therefore, more detailed mechanisms, such as identifying HCMV-mediated recognition molecules or clarifying the human NK cell specificity to other viruses apart from HCMV, require further investigation ([O'Sullivan et al., 2015](#)).

In conclusion, it would be beneficial to consider all the particular features together, such as NK cell response to background circumstances or NK cell cross talk with other cells, to better understand pathology mechanisms and disease management.

1.4 Regulation and Interactions of the NKG2D Pathway

NKG2D on human NK cells

NKG2D (natural-killer group 2, member D) is one of the major activating receptors on NK cells, which can trigger cytokine production and cytotoxicity induction and participate in immunoregulation of infection, tumour surveillance, autoimmunity and transplantation (Bauer et al., 1999; Burgess et al., 2008). The significance of this receptor has come to light since diverse mechanisms for evading NKG2D-mediated recognition have been discovered in various viral infections and tumours (Lopez-Larrea et al., 2008; Obeidy and Sharland, 2009).

Generally, effector functions of NK cells are defined by the cytokine milieu and integrated signals from inhibitory and activating receptors of NK cells. However, due to the activating signal upon NKG2D engagement surpassing other inhibitory inputs, NKG2D is viewed as a "master switch" and can predominantly determine the NK cell activation state (Obeidy and Sharland, 2009; Watzl, 2003).

NKG2D is a C-type lectin-like receptor, first identified on NK cells and also expressed by NKT cells, $\gamma\delta$ T cells, and most CD8 T cells, though not normally seen on CD4 T cells (Bauer et al., 1999; Eagle and Trowsdale, 2007; Wu et al., 1999). In humans, the structure of NKG2D consists of a disulphide-linked homodimer, in which each monomer associates with two DAP10 adaptor proteins (Figure 1.6A). Unlike mouse NKG2D containing DAP10 or DAP12, the activity of human NKG2D is exclusively mediated through DAP10 intracellular signalling domains (Eagle and Trowsdale, 2007; Gilfillan et al., 2002).

Following engagement with NKG2D ligands, DAP10 transduces extracellular signals by phosphorylation of YXXM tyrosine-based motifs, resulting in recruitment and activation of the P85 subunit of phosphoinositide 3-kinase (PI3K) and the growth factor receptor-bound protein 2 (Grb2). The sequential signalling of PI3K and Grb2 can trigger the downstream MEK/ERK pathway to induce cytotoxicity, cytokine production, and cell proliferation (Burgess et al., 2008; Eagle et al., 2009; Vivier et al., 2004).

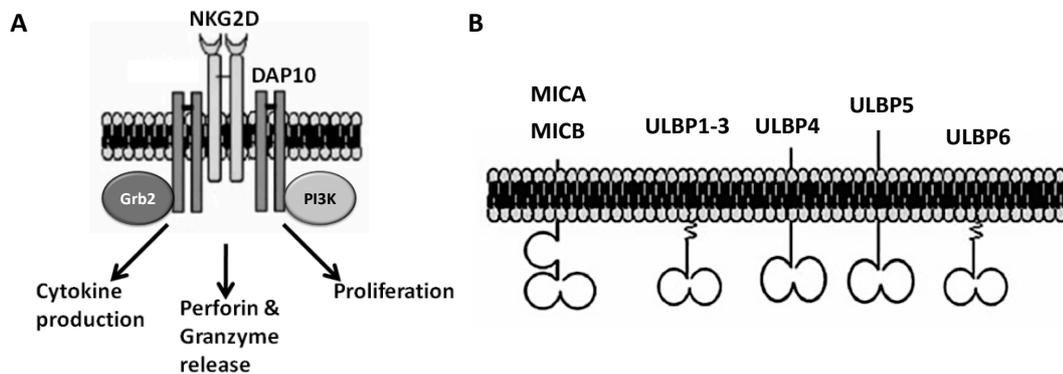


Figure 1.6 Human NKG2D and its ligands (*figures modified from Eagle RA et al. 2009).

A Structure of human NKG2D and its functions.

B Structure of human NKG2D ligands (NKG2D-L).

Nevertheless, while stimulation signals transduce through NKG2D, functional outcomes differ depending on various cell types and synergistic inputs (Diefenbach et al., 2002; Raulet et al., 2013). More specifically, NKG2D can serve as a primary activating receptor for activated NK cells in which NKG2D engagement alone is sufficient to induce NK cell-mediated cytotoxicity. Whereas, while NK cells are in a resting state, mere NKG2D stimulation is not enough to trigger full effector function. Thus, NKG2D signalling together with either other synergistic stimulation or

cytokine priming are necessary to fully activate natural cytotoxicity and cytokine secretion of naïve, resting NK cells (Bryceson et al., 2006b).

In NK cells, once NKG2D-mediated exocytosis is triggered, cytotoxic granules such as perforin and granzymes can efficiently cause apoptotic and necrotic death of target cells through direct cytolysis, cellular caspase activation, or caspase-independent pathways (Hayakawa and Smyth, 2006).

Although the expression of NKG2D on NK cells is observed in healthy individuals as well as in patients with CHB, previous findings have demonstrated various mechanisms influencing cellular NKG2D expression (Figure 1.7) (Burgess et al., 2008; Oliviero et al., 2009; Zhang et al., 2011b; Zhao et al., 2012).

Crucial for NK cell effector function and proliferation, IL-15 has been demonstrated to upregulate NKG2D on human NK cells (Dann et al., 2005). Moreover, *in vitro* stimulation with the pro-inflammatory cytokine, IFN- α was also shown to induce NKG2D expression on NK cells (Konjevic et al., 2010). On the other hand, IFN- γ and TGF- β were illustrated to have inhibitory effects on surface NKG2D (Castriconi et al., 2003; Lee et al., 2004; Zhang et al., 2004). Additionally, IL-21, secreted by activated CD4 T cells, has been shown to dampen DAP10 translation, thereby consequently decreasing NKG2D on NK cells (Burgess et al., 2006; Takaki et al., 2005).

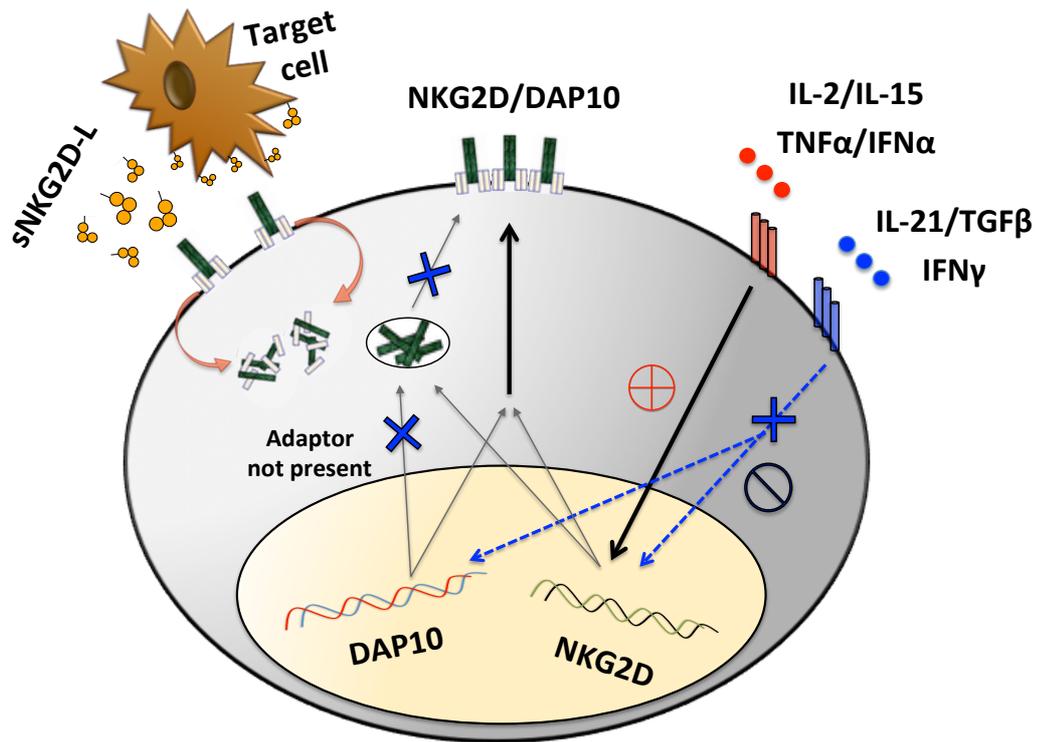


Figure 1.7 Schematic depiction of NKG2D/DAP10 regulation, in which IL-2, IL-15, TNF- α , and IFN- α are favourable for NKG2D expression, whereas IL-21, TGF- β , IFN- γ , and chronic stimulation of soluble/membrane-bound NKG2D-L can limit NKG2D/DAP10 expression (*figure modified from Burgess SJ et al. 2008).

In humans, another noteworthy mechanism responsible for NKG2D down-regulation is overexposure to NKG2D ligands. Since NKG2D is a vital component of tumour surveillance, some tumours may evade immune recognition by shedding or secreting soluble NKG2D ligands to induce endocytosis or degradation of NKG2D in NK cells (Holdenrieder et al., 2006a; Raffaghello et al., 2004; Salih et al., 2002). Previous research suggests placenta-derived soluble MICA/B in pregnant women utilizes a similar mechanism to protect and promote fetal survival by reducing unwanted NKG2D-mediated cytotoxicity (Mincheva-Nilsson et al., 2006). Consistently, chronic exposure to membrane-bound NKG2D ligands from tumour cells is also known to mediate down-regulation of NKG2D expression and reduction of cytotoxic

activity of NK cells (Coudert et al., 2005; Molfetta et al., 2014; Oppenheim et al., 2005; von Lilienfeld-Toal et al., 2010). Therefore, a more comprehensive understanding of NKG2D regulation in infection or cancer settings is useful for therapeutic strategy development for restoration of normal NK cell function and immunity.

Regulation and interactions of NKG2D-L

In humans, eight NKG2D ligands, including MHC class I-chain related proteins A and B (MICA and MICB) and UL16 binding protein 1 through 6 (ULBP1-6) have been identified so far (Figure 1.6B) (Cosman et al., 2001; Groh et al., 1996). These ligands, with structure similarity to MHC class I proteins, are encoded by highly polymorphic genes and have different affinities and activating capabilities for NK cells (Eagle and Trowsdale, 2007; Raulet et al., 2013).

Rarely expressed on healthy cells, NKG2D ligands are viewed as "stress molecules" due to the variety of pathologic stimuli responsible for their upregulation, such as infection, inflammation, tumour transformation as well as numerous metabolic and cellular stresses (Gonzalez et al., 2008; Shafi et al., 2011). However, to ensure stressed cell elimination without destruction of healthy cells, NKG2D ligand expression is tightly regulated at several stages, including transcriptional, post-transcriptional and post-translational levels (Figure 1.8) (Mistry and O'Callaghan, 2007; Raulet et al., 2013).

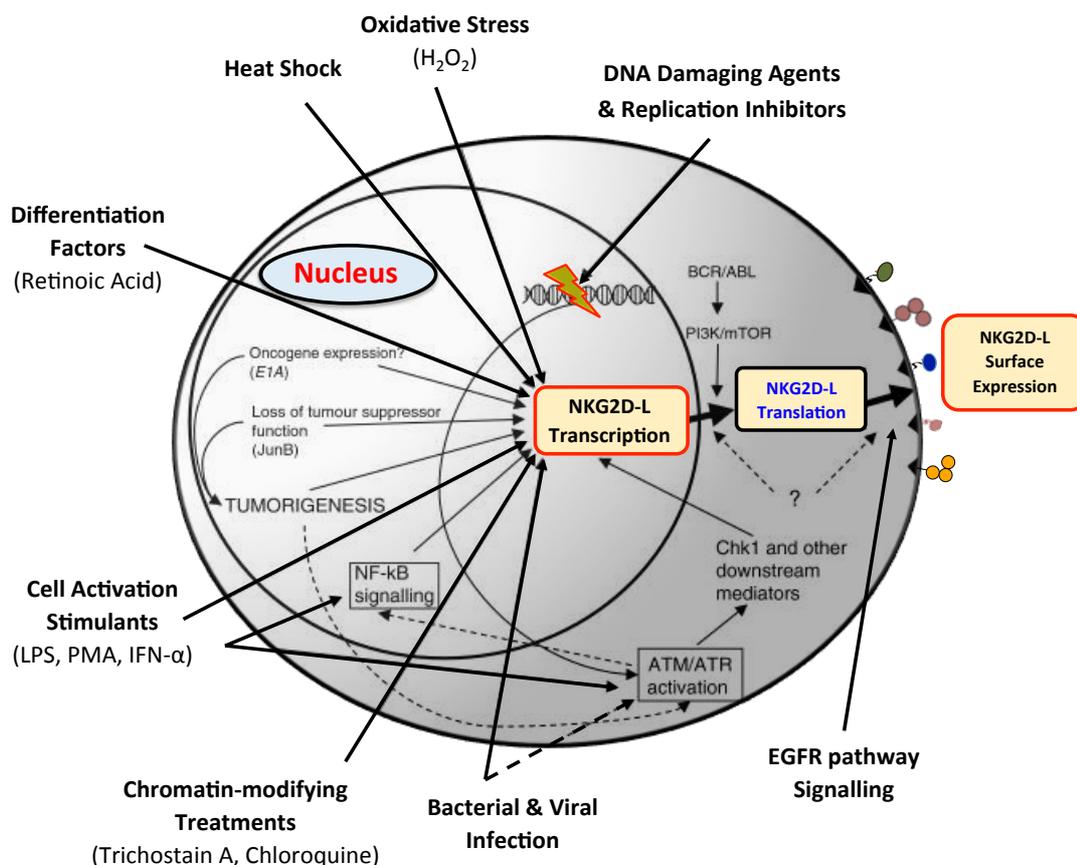


Figure 1.8 Mechanisms of NKG2D-L regulation (*figure modified from Mistry and O'Callaghan, 2007).

Previous research has demonstrated that oxidative stress can enhance MICA/B transcription in colon carcinoma cells (Yamamoto et al., 2001). In addition, other studies also revealed oxidative stress participated in NKG2D-L upregulation on T cell blasts, human small bowel mucosa and airway epithelial cells (Allegretti et al., 2013; Borchers et al., 2006; Rabinovich et al., 2000).

Moreover, various genotoxic stimuli such as ionizing radiation, cisplatin or hydroxyurea treatment were also shown to induce NKG2D-L upregulation in human fibroblast cells (Gasser et al., 2005). Provoking a DNA-damage response (DDR) is widely recognised as an important contributor to NKG2D-L upregulation. Thus,

inhibition of DDR pathway mediators, ataxia-telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3-related (ATR) kinases, can suppress NKG2D-L induction in experimental settings ([Mistry and O'Callaghan, 2007](#); [Raulet et al., 2013](#)).

Epidermal growth factor receptor (EGFR), another important post-transcriptional checkpoint for human NKG2D-L, has received great attention recently. Consistent with the positive correlation between the hyper-activated EGFR pathway and high expression of NKG2D-L on human carcinoma cells, EGFR activation can stabilize NKG2D-L mRNAs by relocalization of AUF1 proteins to subsequently secure post-transcriptional ligand expression ([Vantourout et al., 2014](#)).

In addition, during the infection process, multiple NKG2D-L regulatory mechanisms can be initiated and altered diversely at different biogenesis and expression stages.

As demonstrated in one previous transgenic HBV murine model, infected hepatocytes expressed higher NKG2D-L rendering them susceptible to NKG2D-mediated lysis ([Chen et al., 2007](#)). Contrary to this, recent research in a human hepatoma cell line revealed that HBsAg can induce several suppressive microRNAs to target MICA/B 3'-untranslated regions, thereby repressing their surface expression and avoiding NKG2D-mediated elimination ([Wu et al., 2014](#)).

Another example is HCMV infection, in which viral immediate-early gene (IE1 or IE2) products are capable of inducing MIC gene transcription and surface MICA and MICB expression ([Venkataraman et al., 2007](#)). However, despite the induction effects of IE proteins, HCMV has evolutionarily acquired the ability to evade and suppress surface NKG2D-L levels via specific viral products, such as UL16, UL112,

UL142, US18, and US20 ([Cosman et al., 2001](#); [Dunn et al., 2003](#); [Fielding et al., 2014](#)).

Similarly, human immunodeficiency virus (HIV) has also developed evasion strategies to counteract NKG2D-L surface manifestation. The HIV protein Vpr has been shown to induce ATR kinase-dependent DDR and thereby upregulate NKG2D-L expression ([Ward et al., 2009](#)). However, HIV Vif can partly decrease DDR-mediated NKG2D-L induction to evade NK cell immune recognition ([Norman et al., 2011](#)). Moreover, HIV-1 Nef protein can also down-modulate surface expression of MICA, ULBP1 and ULBP2 on infected cells to escape NK cell-mediated killing ([Cerboni et al., 2007a](#)). These mechanisms to evade NKG2D-L expression further underscore the influential relevance of this pathway in host-virus interplay.

Notably, cellular NKG2D-L expression can also be down-regulated by surface metalloproteinase (MMP) cleavage. This proteolytic shedding results in releasing various soluble NKG2D ligands, which have been detected in the sera of different cancer patient types ([Raulet et al., 2013](#); [Salih et al., 2006](#); [Salih et al., 2002](#); [Waldhauer and Steinle, 2006](#)). Indeed, numerous cancer cells can adopt this strategy to evade NKG2D-mediated tumour surveillance, since this mechanism can not only directly decrease the NKG2D-L on the affected cells, but also lead to NKG2D internalization or lysosomal degradation by engaging with cleaved soluble ligands ([Holdenrieder et al., 2006a](#); [Mistry and O'Callaghan, 2007](#); [Raffaghello et al., 2004](#)). Moreover, circulating soluble ligands have been shown to correlate with poor

prognosis in certain cancers; the presence of soluble NKG2D-L in the sera may therefore serve as a prognostic indicator ([Holdenrieder et al., 2006b](#); [Paschen et al., 2009](#)).

In summary, these regulation mechanisms altogether highlight the importance of the appropriate immune-alert system in maintaining host health.

Chapter 2 Materials and Methods

2.1 Patients and Healthy Controls

Blood samples were collected from 113 patients with CHB recruited from the Mortimer Market Clinic (NHS Trust), the Royal Free Hospital, University College London Hospital, and the Royal London Hospital (Barts Health NHS Trust). A total of 46 healthy control blood samples were obtained from staff or students at University College London. Surplus liver tissue was obtained from 36 patients with CHB who underwent diagnostic liver biopsies. Control liver samples were obtained from 11 non-HBV infected patients undergoing liver resections for colorectal metastases. Nine sets of transplant perfusates from cadaveric donor livers were collected during liver transplant surgery under the standard graft preparation protocols. The study was approved by the relevant ethical review boards and informed consent was obtained in writing from each individual or legally authorised representative. All patients with CHB were HBsAg and HBV DNA positive longer than 6 months and were negative for HCV, HDV, and HIV infection. With the exception of 8 patients selected for longitudinal PEG-IFN α study, all patients were treatment naïve (Table 1). Liver inflammation was determined by serum alanine transaminase (ALT).

Table 1: Clinical data of study controls and patients.

	HC (n=46) (PBMC)	CHB (n=69) (PBMC, ALT≤60)	CHB (n=44) (PBMC, ALT>60)	CHB (n=8) (Pre-Tx Baseline)	CHB (n=8) (*On PEG-IFN α Tx)	CHB liver (n=36) (Biopsy)	Control liver (n=9) (Perfusate)
Age, years [mean±SEM]	32.4±1.4	37.8±1.3	37.1±1.8	30.6±3.3	30.6±3.3	37.8±1.7	42.2±6.2
Gender, F/M (%)	56.5/43.5	36.1/63.9	52.3/47.7	62.5/37.5	62.5/37.5	33.3/66.7	44.4/55.6
ALT IU/L [median (IQ range)]	n.a.	30.1 (21-37)	142.4 (80-168)	190 (96.3-299)	97.5 (46.3-230)	32.5 (24.3-76.8)	n.a.
HBeAg, Positive/Negative (%)	n.a.	17.4/82.6	56.8/43.2	37.5/62.5	37.5/62.5	25/75	n.a.
HBV DNA cps/ml [median (range)]	n.a.	1.4×10 ³ (20-3.2×10 ⁸)	4.1×10 ⁶ (860-1.1×10 ⁹)	6.1×10 ⁷ (7.8×10 ⁴ -4.5×10 ⁸)	1.2×10 ³ (540-5.5×10 ⁶)	4.5×10 ³ (20-6.5×10 ⁵)	n.a.

Abbreviations: SEM: standard error of mean; IQ: interquartile; n.a.: not applicable; HC: healthy controls; CHB: chronic hepatitis B; Tx: treatment
* On 3-4 months of PEG-IFN α treatment

2.2 PBMC and Intrahepatic Lymphocyte Isolation

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by standard gradient centrifugation on Ficoll-paque Plus (GE Healthcare). Intrahepatic lymphocytes (IHL) were obtained following gentle mechanical disruption of liver tissue prior to passing through a 70 μ m cell strainer (BD Bioscience) and multiple washes with RPMI 1640 (Invitrogen) as previously described (Dunn et al., 2007; Peppas et al., 2010). IHL from transplant perfusates were retrieved by density-gradient centrifugation as PBMC isolation. All cells in this study were maintained in complete RPMI medium composed of 500ml RPMI 1640 (Invitrogen) + 10% fetal calf serum (FCS, Invitrogen) + 100U/ml Penicillin/Streptomycin (Invitrogen) + MEM essential amino acid (Invitrogen) + MEM non-essential amino acids (Invitrogen) + hydroxyethyl piperazineethanesulfonic acid (HEPES: Invitrogen) + β -mercaptoethanol (Sigma-Aldrich) + sodium pyruvate (Invitrogen).

2.3 Flow Cytometric Analysis and Gating Strategies

For flow cytometric analysis, PBMC and IHL were washed with PBS and stained with a fixable Live/Dead dye (Invitrogen) at 4°C for 10 min, prior to Fc-Receptor blocking with FcR blocking reagent (Miltenyi Biotec, Germany). Surface staining was performed at 4°C for 30 minutes in the presence of saturating concentrations of monoclonal antibodies (mAbs), or isotype matched controls (Figure 2.1A and B). After fixation and permeabilisation with Cytofix/Cytoperm (BD Biosciences), intracellular staining (ICS) was conducted by using monoclonal antibodies in the presence of 0.1% saponin for 30 minutes at 4°C in the dark. Table 2 details anti-human monoclonal antibodies (mAb) used for the sequential gating strategy in this study. In general, NK cells, CD4 T cells, and CD8 T cells were identified as CD3⁻/CD56⁺, CD3⁺/CD4⁺, and CD3⁺/CD8⁺ populations, respectively (Figure 2.1A). Specific cell groups, such as MICA/B⁺, HLA-DR⁺, and IFN- γ ⁺ populations were identified by using MICA/B mAb, HLA-DR mAb and IFN- γ mAb and the majority, negative stained cells, were viewed as global populations (Figure 2.1C). All samples were acquired on a LSR Fortessa or BD LSRII using BD FACSDiva6.0 (BD Bioscience). Data were analysed using FlowJo v.8 (TreeStar, Ashland, OR, USA).

Table 2: List of anti-human mAb used for flow cytometric analysis.

Antibody	Fluorochrome	Company	Dilution	Type of staining
Fixable live/dead	Blue dye	Invitrogen	1.5:1000	Surface
CD3	PE-Cy7	eBioscience	1:100	Surface
CD4	APC-eFluor780	eBioscience	1:100	Surface
CD8	AlexaFluor700	eBioscience	1:100	Surface
CD14	V500	BD Biosciences	1:100	Surface
CD19	APC-eFluor780	eBioscience	1:100	Surface
CD19	V500	BD Biosciences	1:100	Surface
CD25	PerCP-Cy5.5	eBioscience	2:50	Surface
CD25	PE-Cy7	eBioscience	2.5:50	Surface
CD27	APC	BioLegend	1:100	Surface
CD39	BV421	BioLegend	1:50	Surface
CD45RA	eFluor450	eBioscience	1.5:100	Surface
CD56	ECD	Beckman Coulter	1.5:50	Surface
CD69	APC	BD Biosciences	2.5:50	Surface
CD107a	APC	BD Biosciences	2.5:50	Surface
CD127	PerCP-Cy5.5	BioLegend	1:100	Surface
HLA-DR	V500	BD Biosciences	0.5:50	Surface
NKG2D	Alexa Fluor488	R&D systems	1:50	Surface
MICA/B	PE	eBioscience	3:50	Surface
MICA/B	PE	BD Biosciences	2:50	Surface
ULBP1	PE	R&D systems	1.5:50	Surface
ULBP2/5/6	APC	R&D systems	2.5:50	Surface
ULBP3	PE	R&D systems	1.5:50	Surface
TRAIL	V450	BD Biosciences	1:50	Surface
TRAIL-R2	PE	R&D systems	2:50	Surface
Fas/CD95	eFluor450	eBioscience	1:50	Surface
PD-1	PerCP-Cy5.5	BioLegend	1:50	Surface
Ki67	FITC	BD Biosciences	5:50	Intracellular
IFN- γ	V450	BD Biosciences	1:50	Intracellular
Caspase 3	PE	BD Biosciences	10:50	Intracellular
FoxP3	eFluor660	eBioscience	5:50	Intranuclear

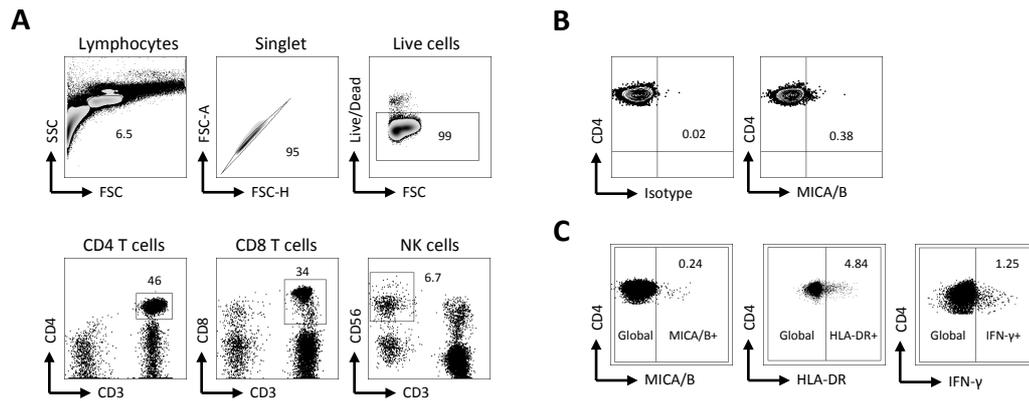


Figure 2.1 Representative gating strategies.

- A Sequential gating strategy used to identify CD4 T cells (left lower panel), CD8 T cells (middle lower panel), and NK cells (right lower panel).
- B Representative plots for NKG2D-L staining by using MICA/B mAb (right panel) and the matched IgG2a isotype (left panel).
- C Representative plots for gating MICA/B⁺ T cells (left panel), HLA-DR⁺ T cells (middle panel), IFN- γ ⁺ T cells (right panel) and their counter parts, global T cells.

2.4 HLA-A2-restricted Multimer Staining

The frequencies of virus-specific CD8 T cells from HLA-A2 positive individuals were evaluated directly *ex vivo* by multimer staining as previously described (Pallett et al., 2015). Total PBMC were stained with APC-labelled CMV pp65 495-504 or HBV core 18-27, envelope 183-191, envelope 335-343, envelope 348-357, polymerase 455-463, and polymerase 502-510 dextrans (Immudex, Denmark) at 37°C for 20 minutes in RPMI plus 10% fetal calf serum (Table 3). For gating virus-specific CD8 T cells, CD14/CD19 cells were first excluded and a control dextramer was used to identify true populations (Figure 2.2). Afterward, the cells were washed and pelleted for the sequential surface or intracellular staining as mentioned above.

Table 3: List of MHC class I-restricted multimers used for flow cytometric analysis.

HLA-A2 peptide sequence	Multimer type	Dilution	Fluorochrome	Viral protein	Amino acid region	Type of staining
FLPSDFFPSV	Dextramer	1:40	APC	HBV core	18-27	Surface
FLLTRILTI	Dextramer	1:40	APC	HBV envelope	183-191	Surface
WLSLLVPFV	Dextramer	1:40	APC	HBV envelope	335-343	Surface
GLSPTVWLSV	Dextramer	1:40	APC	HBV envelope	348-357	Surface
GLSRYVARL	Dextramer	1:40	APC	HBV polymerase	455-463	Surface
KLHLYSHPI	Dextramer	1:40	APC	HBV polymerase	502-510	Surface
NLVPMVATV	Dextramer	1:40	APC	CMV pp65	495-504	Surface
Control	Dextramer	1:40	APC	irrelevant protein	not applicable	Surface

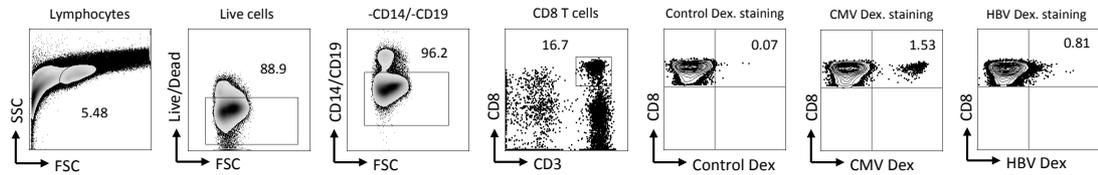


Figure 2.2 Representative gating strategy for *ex vivo* dextramer staining.

Representative plots of sequential gating strategy used to identify virus-specific CD8 T cells by staining of control dextramer (panel 5), CMV-specific dextramer (panel 6), and HBV-specific dextramers (panel 7).

2.5 Intranuclear FoxP3 Staining

After surface staining, cells were first fixed with FoxP3 Buffer A (BD Bioscience) for 10 minutes at room temperature (RT). Following fixation, cells were then incubated in FoxP3 Buffer C (BD Bioscience) for 30 minutes at RT prior to the intranuclear FoxP3 mAb staining. After complete staining, cells were washed twice with PBS before flow cytometric acquisition.

2.6 Cell Lines

A B-lymphoblastoid cell line, C1R transfected with MICA*008 (C1R-MICA), was used as a source of cell-bound MICA (Tieng et al., 2002). A control cell line lacking MICA, C1R, served as a comparison (kindly provided by Professor Antoine Toubert, France). The MICA expression was verified by MICA/B mAb staining as described previously (Figure 2.3).

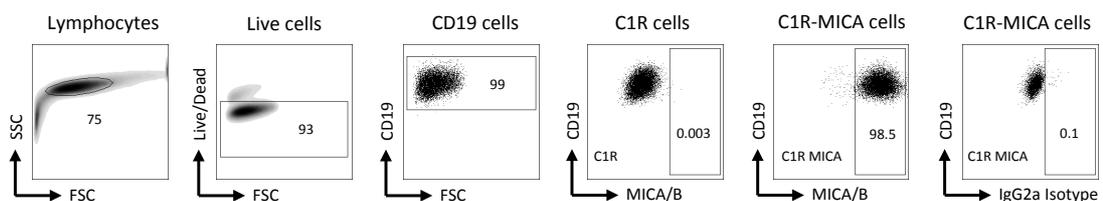


Figure 2.3 Representative gating strategy for C1R/C1R-MICA cell staining.

Representative plots of gating strategy for C1R/C1R-MICA cells and their MICA expression by using MICA/B mAb (panels 4 and 5) and the matched IgG2a isotype (panel 6).

2.7 NK cell and CD4 T cell Isolation

Purification of NK and CD4 T cells or depletion of NK cells from PBMC was achieved by using NK cell or CD4 T cell Isolation Kits (Miltenyi Biotec) reaching 95% mean depletion rate and purity, as per manufacturer's instructions (Figure 2.4A and B).

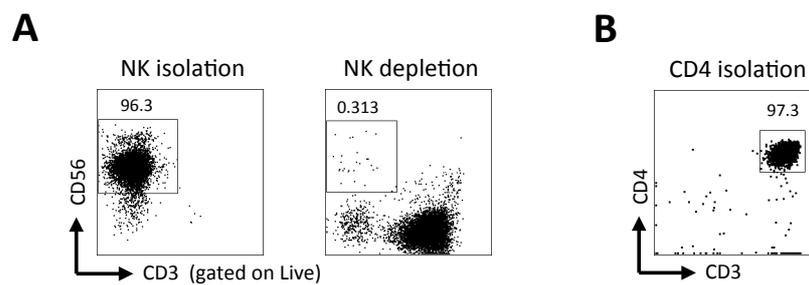


Figure 2.4 Representative plots of NK cell and CD4 T cell isolation.

- A Examples of NK cell purification (left panel) and NK cell depletion from PBMC (middle panel).
- B Example of CD4 T cell isolation (right panel).

2.8 NKG2D Pathway Blockade

NKG2D blockade was conducted by using anti-NKG2D mAb (0.5 μ g/ml, eBiosciences) without interference in NK cell viability. The efficacy and specificity were checked in the described C1R-MICA/NK cell co-culture system and after short-term stimulation of PBMC (Figure 2.5).

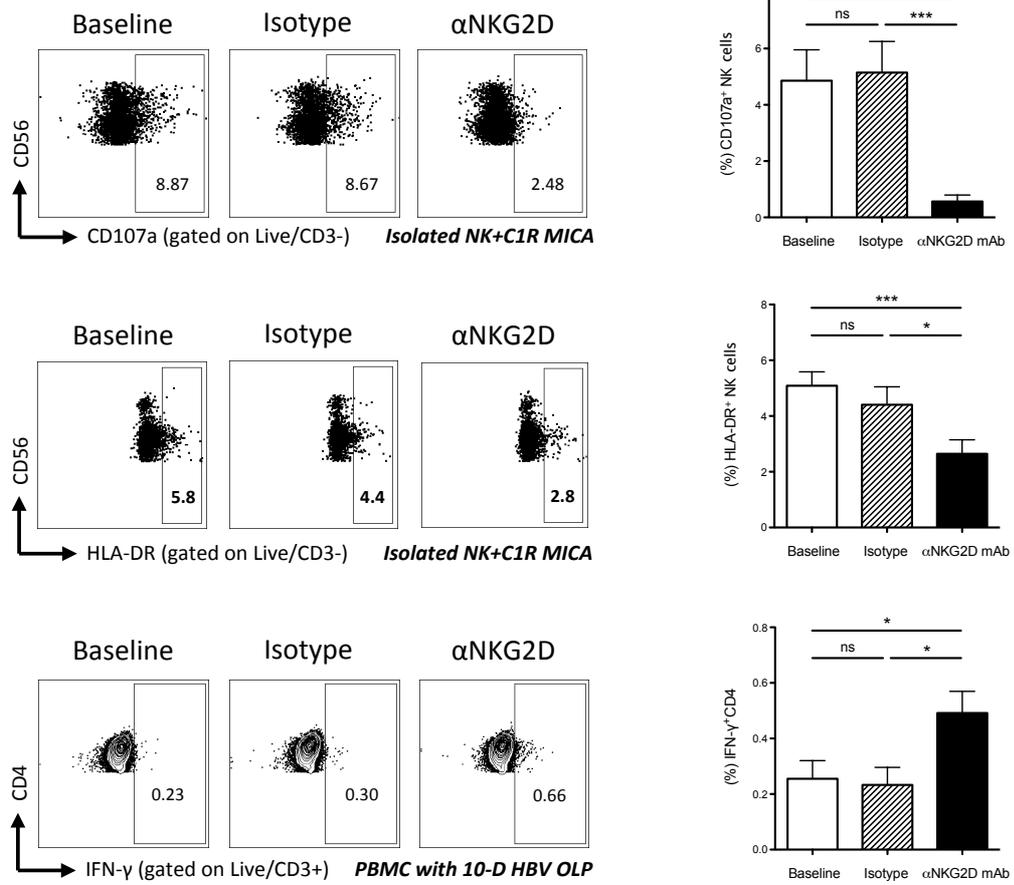


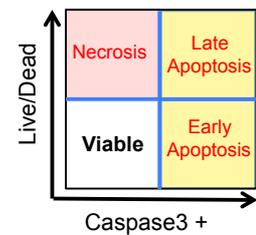
Figure 2.5 Representative plots of efficacy and specificity of NKG2D blockade.

Examples of NKG2D blockade with anti-NKG2D mAb and summary results: CD107a response of purified NK cells upon NKG2D blockade in NK/C1R-MICA cell co-culture system (upper horizontal panel; n=10), HLA-DR response of purified NK cells upon NKG2D blockade in NK/C1R-MICA cell co-culture system (central horizontal panel; n=10), IFN- γ response of CD4 T cells upon NKG2D blockade in PBMC short-term stimulation system (lower horizontal panel; n=9); data analysed by Friedman test.

2.9 NK / Target cell Co-culture System

To measure NK cell degranulation capacity, purified NK cells were co-cultured at 1:1 ratio with target cells (C1R/C1R-MICA) at 37°C for 6 hours, in the presence of anti-CD107a-APC (BD Biosciences). Brefeldin A (1 μ g/ml, Sigma-Aldrich) and Monensin (1 μ g/ml, Sigma-Aldrich) were added after 1 hour of incubation. Where

indicated an anti-NKG2D blocking antibody or a control isotype antibody (0.5µg/ml, eBiosciences) was added at the onset of culture. NK cell cytotoxicity of target cells was evaluated in the same co-culture system using ICS for Caspase 3 in the presence of fixable Live/Dead staining (Invitrogen). Target cells were identified by CD19 staining and their cytotoxicity was calculated as the sum of dead and Caspase 3+ populations, as shown in the illustration on the right.



2.10 Short-Term Stimulation

Where indicated, PBMC or NK-depleted PBMC were stimulated with HBV overlapping peptides (OLP) (15mer peptides overlapping by 10 residues spanning genotype D HBV core protein, 1µg/ml, JPT) in the presence of 40IU IL-2 (Miltenyi Biotec) in RPMI complete medium for 10 days at 37°C. IL-2 and medium were refreshed on day 4 of culture. On day 9, PBMC were re-stimulated with 1µg/ml HBV OLP overnight (16hr), in the presence of Brefeldin A (1µg/ml). In selected experiments a physiological ratio of freshly isolated NK cells was re-added in the culture at the onset of stimulation. Following short-term culture with HBV OLP, virus-specific T cells were identified for IFN-γ production via ICS. To examine the effect of NKG2D blockade on virus-specific responses, an NKG2D blocking mAb (0.5µg/ml, eBiosciences) was added with HBV OLP at the onset of culture and cells treated as described above.

2.11 Overnight Stimulation

For overnight (16hr) culture of PBMC or IHL, 10 µg/ml HBV OLP were added and the cells were incubated at 37°C in the presence of Brefeldin A (1µg/ml). Where indicated these experiments were repeated in the presence of an NKG2D blocking antibody (0.5µg/ml, eBiosciences) added at the time of HBV OLP stimulation. HBV-specific T cells were identified as IFN-γ positive T cells by ICS.

2.12 Hydrogen Peroxide Stimulation

To investigate *in vitro* NKG2D-L stimulation, hydrogen peroxide (H₂O₂) solution (30%, BDH) was used to induce oxidative stress in cell culture. Purified CD4 T cells or PBMC from healthy controls were rested for 48 hours prior to the addition of H₂O₂ solution at varying concentrations (0.25mM-1mM) for the indicated time periods ranging from 0.5hr to 2hr. The NKG2D-L expression was examined by surface staining for NKG2D-L as mentioned above.

2.13 Recombinant Human Interferon-α Stimulation

NK cells isolated from PBMC of CHB patients or healthy controls were treated with 1000 U/ml IFN-α (Alpha 2A, PBL InterferonSource) for four hours ([Dunn et al., 2007](#); [Lucifora et al., 2014](#)). Degranulation capability of NK cells was assessed directly after the IFN-α stimulation by CD107a staining. To examine the IFN-α induced NKG2D cytotoxicity of NK cells, untreated or IFN-α treated NK cells were

co-cultured with C1R-MICA cells at 1:1 ratio for 6 hours. Control C1R cells were also used to exclude other unspecific or allogeneic reactions.

2.14 Statistical Analysis

The non-parametric Mann-Whitney U test (for 2 groups), the Wilcoxon signed rank test (for 2 paired groups), Kruskal-Wallis (for >2 non-paired groups) or Friedman (for >2 paired groups) one-way tests were used as appropriate. Correlations between variables were analysed using Spearman's rank correlation coefficient (r). P value < 0.05 was considered to be significant for all tests. All figures are labelled as follows:

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$.

Chapter 3 Chronic HBV Infection renders T cells vulnerable to NKG2D-mediated regulation

3.1 Introduction: Dysfunctional T cells in HBV infection

Hepatitis B virus (HBV) is the most prevalent virus responsible for persistent liver infection and liver disease worldwide (Trepo et al., 2014). Although numerous anti-viral regimens are available for chronic HBV infection nowadays, sustained off-treatment responses with thorough virus control are rarely achieved (Lampertico et al., 2015). Nevertheless, unlike neonatal/perinatal HBV infection causing chronicity, the successful self-resolution of most adult-acquired HBV indicates that the mature immune system is capable of dealing with this virus, but is somehow restrained in chronic carriers (Trepo et al., 2014).

Compared to the vigorous and multi-specific antiviral T cell immunity in HBV resolved patients, chronic HBV carriers have profoundly exhausted and depleted HBV-specific CD4 and CD8 T cell responses (Ferrari et al., 1990; Maini et al., 2000; Rouse and Sehrawat, 2010; Wang and Zhang, 2009). The importance of T cells in control of HBV suggested by these clinical observations has been supported by *in vivo* chimpanzee studies, which demonstrated that CD4 T cell depletion prior to HBV inoculation precluded adequate T cell priming leading to quantitatively and qualitatively compromised HBV-specific CD8 T cells and consequent HBV persistence (Asabe et al., 2009). Moreover, the fundamental role of HBV-specific CD8 T cells has also been established by the loss of virus control in acute HBV

infection following the CD8 T cell depletion in the chimpanzee model ([Thimme et al., 2003](#)).

Since the defective nature of antiviral T cells is crucial to the outcome of HBV infection, multiple virus and host factors contributing to T cell failure have been intensively studied for future therapeutic advancement ([Maini and Schurich, 2010](#); [Ye et al., 2015](#)).

A hallmark of HBV infection is the large amount of viral particles and antigenic proteins, such as HBsAg and HBeAg, produced during the virus replication cycle. Persistent exposure to high antigen loads in the HBV-infected liver is postulated to be a key factor detrimental to T cell function ([Maini and Schurich, 2010](#); [Ye et al., 2015](#)). The rarely detectable HBV-specific CD8 T cells in patients with high viral load ($>10^7$ copies/ml) increased transiently following HBV suppression by anti-viral therapy ([Boni et al., 2003](#); [Boni et al., 2001](#); [Webster et al., 2004](#)) and in a more consistent manner within those achieving clearance of viral antigens associated with HBsAg seroconversion ([Boni et al., 2012](#); [Boni et al., 2015](#)). In accord with a progressive and hierarchical loss of T cell effector function driven by the viral antigen load and stimulation duration, T cells in chronic HBV carriers display a pattern of poor proliferation, weak cytokine production and impaired cytotoxicity induction ([Boni et al., 2007](#); [Fiscaro et al., 2010](#); [Maini et al., 2000](#); [Wherry et al., 2003](#); [Younes et al., 2003](#)).

Pronounced metabolic stress, including arginine deprivation and oxidative stress accumulation, is also a recognized feature of the chronically HBV-infected and inflamed liver milieu (Li et al., 2015; Pallett et al., 2015; Protzer et al., 2012; Shimoda et al., 1994). Recently, arginine depletion in HBV infection resulting from the injured hepatocytes and arginase-producing myeloid-derived suppressor cells (MDSC) has been shown to suppress T cell proliferation and IL-2 production as well as downregulate the CD3- ζ -chain of the TCR signalling complex (Das et al., 2008; Pallett et al., 2015). Furthermore, as a signature of HBV pathology, aberrant oxidative stress originating from HBV replication, viral products and chronic inflammation, may also have a negative impact on T cell function and survival (Ardolino et al., 2011; Hildeman et al., 1999; Kesarwani et al., 2013; Rabinovich et al., 2000). Collectively, these pathways of compromised T cell immunity highlight the importance of metabolic immunopathology in the setting of CHB.

Apart from the abundant virus and pathogenic liver milieu background, the T cells themselves also upregulate various co-inhibitory molecules in chronic HBV infection, such as programmed cell death-1 (PD-1), T-cell immunoglobulin domain and mucin domain 3 (TIM-3), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4/CD152), and CD244/CD48 (Maini and Schurich, 2010; Ye et al., 2015). Encountering overwhelming HBV antigens without adequate intrahepatic APC co-stimulation, outweighed by multiple co-inhibitory signals, could drive T cell exhaustion and hyporesponsiveness in the tolerogenic liver microenvironment (Isogawa et al., 2005; Maini and Schurich, 2010). Therefore, efforts to boost T cell responses by blocking

those co-inhibitory pathways have been widely researched, particularly the distinct progression on the PD-1/PD-L1 pathway.

CD4 T cells have the essential role of supporting CD8 T cell responses and supporting the development of neutralizing antibodies. The exhausted characteristics of HBV-specific CD4 T cells, with notably high PD-1 expression, have been recently defined using DRB1*01-restricted MHC class II tetramers; their weak cytokine production and proliferation ability could be enhanced by PD-L1/2 neutralization ([Raziorrouh et al., 2014](#)). Along with CD4 T cells, HBV-specific CD8 T cells have also been proven to possess higher PD-1, particularly the intrahepatic fraction, showing greater restoration of anti-viral immunity upon PD-1/PD-L1 blocking via anti-PD-L1 antibodies ([Boni et al., 2007](#); [Fisicaro et al., 2010](#); [Fisicaro et al., 2012](#)).

Besides functional exhaustion, the pro-apoptotic phenotype of HBV-specific CD8 T cells has also been revealed by gene microarray analysis. In CHB patients, the diminished and barely detectable HBV-specific CD8 T cells upregulate the apoptosis-inducing protein Bim (Bcl2-interacting mediator), increasing their susceptibility to death ([Lopes et al., 2008](#)). Through downstream blockade of the Bim pathway, recovery of HBV-specific CD8 T cells was achieved *in vitro* and directly *ex vivo* ([Lopes et al., 2008](#)). Thus, Bim-mediated apoptotic deletion is another important contribution to insufficient anti-HBV immunity and viral persistence.

Various suppressive cytokines and cellular interactions also take part in T cell regulation. Although the comprehensive role of liver-resident macrophages (Kupffer cells) in hepatitis B is still under investigation, they are known to predispose to liver immune tolerance and T cell suppression by PD-L1 expression and production of the immunosuppressive cytokine, IL-10 (Bilzer et al., 2006; Breous et al., 2009; Wu et al., 2009). Oxidative stress generated by Kupffer cells can also interfere with metabolic immune regulation by disrupting the local redox balance (Gabrilovich and Nagaraj, 2009; Hagen et al., 1994; Kesarwani et al., 2013)

The contribution of classical FoxP3⁺ CD4 Treg (regulatory T cells) to T cell exhaustion is still controversial. Many studies have revealed that Treg, which can suppress HBV-specific T cells, are significantly higher in chronic HBV carriers than in resolved patients and controls (Manigold and Racanelli, 2007; Stoop et al., 2005; Yang et al., 2007). However, an inverse correlation between HBV disease activity and Treg frequency implies Treg may have beneficial anti-inflammatory effects (Manigold and Racanelli, 2007; Xu et al., 2006).

NK cells are the largest component of intrahepatic lymphocytes and participate in early control of invading viral pathogens (Shi et al., 2011; Vivier et al., 2008). However, a newly growing focus is the dominant immunomodulatory capacity of NK cells in viral infection (Crome et al., 2013; Lang et al., 2012; Waggoner et al., 2012; Waggoner et al., 2010). In the HBV-infected liver, NK cells have been demonstrated to cause liver damage through the TNF-related apoptosis inducing

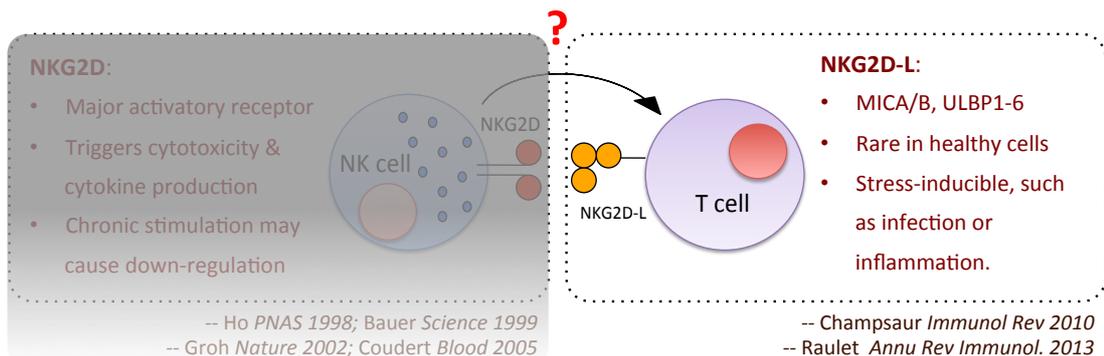
ligand (TRAIL) dependent manner ([Dunn et al., 2007](#)). Moreover, our group recently found that *in vitro* NK cell depletion contributes to the enhancement of CD8 T cell immunity against HBV but not other control viruses (EBV, CMV, influenza virus). This suppressive regulation is a rapid and contact-dependent killing of HBV-specific T cells mediated by activated NK cells. Although the HBV-specific T cell deletion was shown to be TRAIL-dependent, the partial restoration upon TRAIL blockade of NK cells indicates other pathways were also involved in T cell killing and required further investigation ([Peppas et al., 2013](#)).

Considering the current unsatisfactory anti-HBV treatment regimens and the pivotal role of anti-viral T cells in HBV control, novel immunotherapeutic strategies for revitalizing exhausted and pro-apoptotic T cells are in urgent demand. Since T cells are subjected to metabolic stress and closely regulated by cellular interactions in the liver sinusoidal microvasculature, potent immunomodulatory mechanisms targeting both aspects, such as the NKG2D pathway ([Lang et al., 2012](#); [Shafi et al., 2011](#); [Strid et al., 2011](#)), could be a promising approach to improve HBV control.

3.2 Aim and Hypotheses

Aim of the study:

Through study of the NKG2D pathway, we aimed to investigate the role of stress surveillance of T cells in the HBV-infected liver.



Hypothesis:

1. We hypothesized that persistent HBV infection may lead to stress molecule (NKG2D-L) upregulation on T cells.
2. We hypothesized that the increased expression of NKG2D-L on T cells may render them susceptible to NKG2D-mediated stress surveillance.

3.3 Results

3.3.1 NKG2D-L upregulation on T cells in chronic HBV infection

The expression of NKG2D ligand (NKG2D-L) is low in healthy tissues, but it can be induced by stress, such as infection, inflammation or tumour transformation (Champsaur and Lanier, 2010; Raulet et al., 2013). We hypothesised that T cells in CHB may upregulate NKG2D-L expression, thereby rendering them susceptible to killing by NKG2D⁺NK cells. To investigate NKG2D-L expression, CD4 and CD8 T cells from healthy controls and CHB patients were evaluated by flow cytometry using a panel of mAb specific for MICA/B, ULBP1, ULBP2/5/6 (stained with a single mAb), and ULBP3. Out of this panel of ligands, MICA/B was increased on T cells in CHB patients and further significantly upregulated in those patients with ongoing liver inflammation ALT>60 IU/L (Figure 3.1A and B); ULBP1 was also selectively elevated on T cells from patients with HBV-related liver inflammation (Figure 3.1C). However, significant upregulation of ULBP2/5/6 and ULBP3 on T cells in CHB patients was not observed (data not shown; this work credited to Dr. Xin-Zi Tang, Maini group 2011).

To further determine the impact of chronic HBV infection, we next evaluated the T cell expression of NKG2D-L within the liver, the site of disease pathology. Intrahepatic lymphocytes (IHL) were isolated from surplus liver biopsy tissue and compared with matched PBMC samples from CHB patients. IHL of non-HBV infected controls isolated either from healthy tissue of liver metastasis resections or from transplant perfusates of deceased donor livers were also used for comparison.

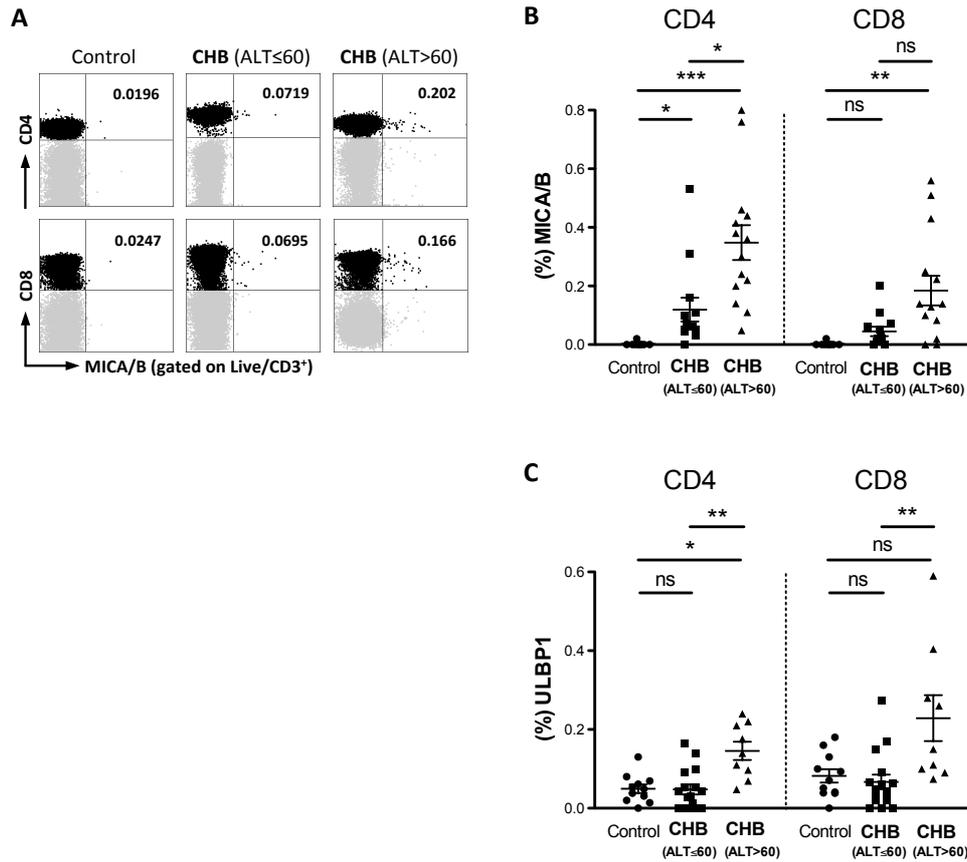


Figure 3.1 Evaluation of NKG2D ligand expression on T cells from healthy controls and CHB patients. (*This work credited to Dr. Xin-Zi Tang, 2011)

- A Representative plots of *ex vivo* MICA/B staining of T cells from healthy controls, CHB patients with ALT \leq 60 IU/L, and CHB patients with ALT $>$ 60 IU/L.
- B Summary results of panel A (HC n=9; CHB (ALT \leq 60) n=13; CHB (ALT $>$ 60) n=14; gates set by control isotype staining; analysed by Kruskal-Wallis test).
- C Summary results of *ex vivo* ULBP1 expression of T cells from healthy controls, and CHB patients (HC n=11; CHB (ALT \leq 60) n=16; CHB (ALT $>$ 60) n=9; analysed by Kruskal-Wallis test).

In *ex vivo* staining, the proportion of T cells expressing MICA/B was markedly increased in the liver compared to the periphery of CHB patients (Figure 3.2A and B). Moreover, the MICA/B expression of intrahepatic T cells was considerably higher in HBV-infected livers compared to the control livers (Figure 3.2A and B). In

this cohort of experiments, preferential enrichment of MICA/B on CD4 T cells in both the periphery and liver was also noted (Figure 3.2C and D).

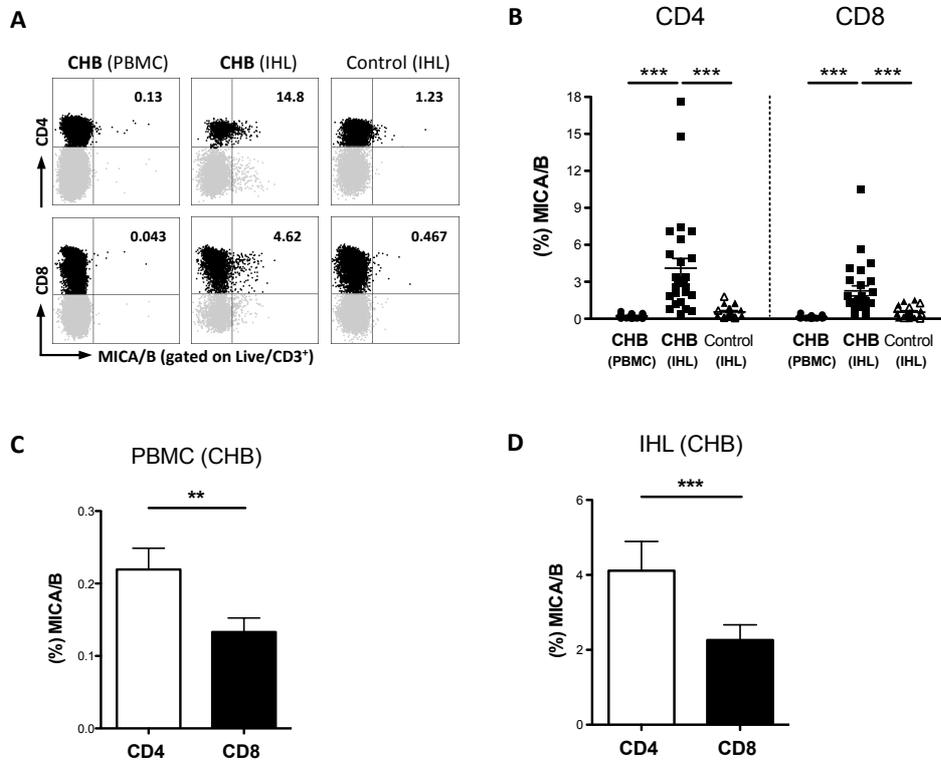


Figure 3.2 Evaluation of NKG2D ligand expression on intrahepatic T cells from healthy controls and CHB patients.

- A Representative plots of *ex vivo* MICA/B staining of paired peripheral and intrahepatic T cells from CHB patients, and intrahepatic T cells from non-HBV infected control livers.
- B Summary results of panel A (CHB PBMC n=27; CHB IHL n=27; Control IHL n=20; black triangles (n=11) indicate intrahepatic lymphocytes isolated from healthy part of liver resection tissue; clear triangles (n=9) indicate intrahepatic lymphocytes isolated from transplant perfusates of deceased donor livers; analysed by Kruskal-Wallis test).
- C Comparison summary of MICA/B expression on peripheral CD4 and CD8 T cells from CHB patients (n=27; analysed by Wilcoxon signed rank test).
- D Comparison summary of MICA/B expression on intrahepatic CD4 and CD8 T cells from CHB patients (n=27; analysed by Wilcoxon signed rank test).

Since antigen exposure and cellular proliferation can contribute to NKG2D-L induction (Zingoni et al., 2012), we further examined the potential influence of HBV

viral load, HBeAg status, and T cell proliferation (Ki67⁺ staining) in CHB patients. However, NKG2D-L expression on T cells in our patient cohort did not stratify significantly with HBV viral load or HBeAg status (data not shown). In addition, our *ex vivo* findings did not indicate increased proliferation of MICA/B expressing T cells than of global T cells (Figure 3.3A and B).

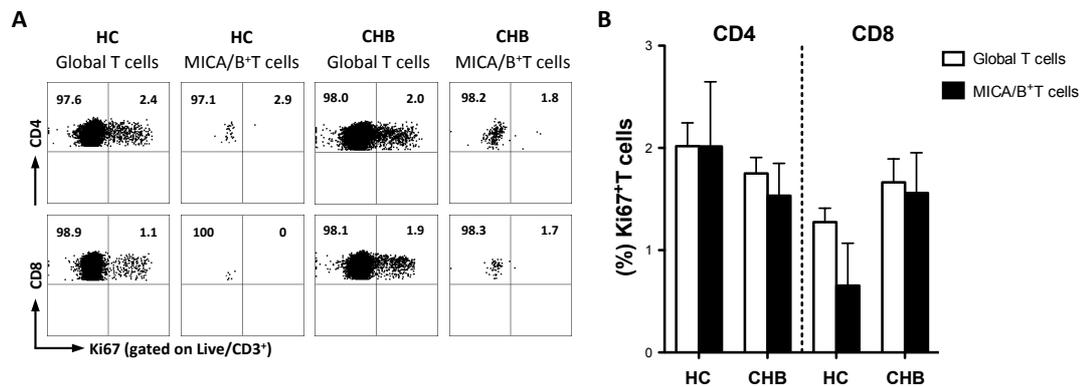


Figure 3.3 Evaluation of proliferation status of MICA/B⁺ T cells from healthy controls and CHB patients.

- A Representative plots of *ex vivo* Ki67 staining of global (MICA/B⁻)T cells and MICA/B⁺ T cells from healthy controls and CHB patients.
 B Summary results of panel A (HC n=4; CHB n=10).

For a closer inspection of MICA/B expressing T cells, we applied CD27 and CD45RA *ex vivo* staining to evaluate phenotypic features. In both PBMC and IHL of CHB patients, we observed that MICA/B-expressing T cells were composed of relatively fewer effector memory (EM) and effector memory revertant (EMRA) populations than global T cells (Figure 3.4A-D). Similar to the differentiation pattern of TRAIL-R2 positive T cells, MICA/B expressing CD4 and CD8 T cells were slightly enriched for naïve or central memory populations respectively (Peppas et al., 2013).

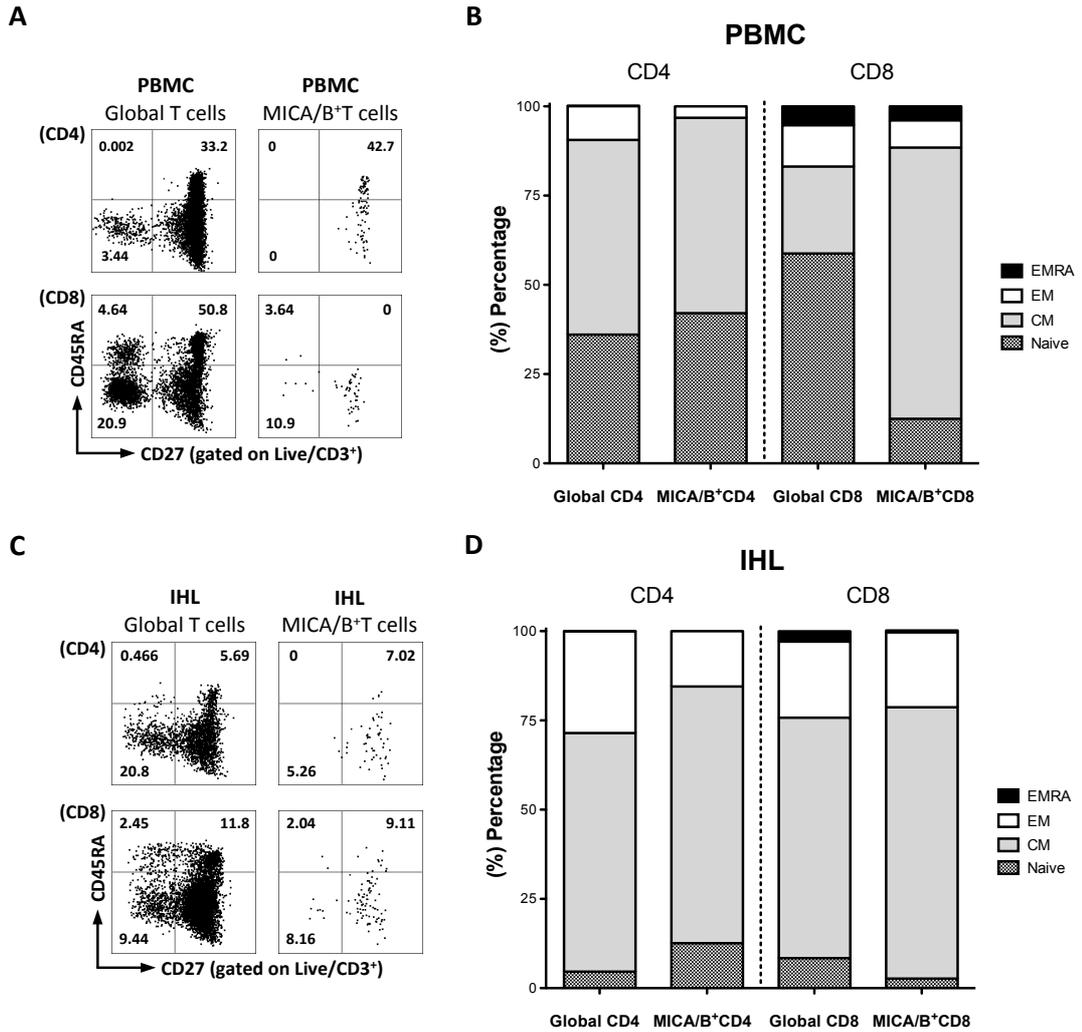


Figure 3.4 Phenotype analysis of MICA/B⁺ T cells from CHB patients.

- A Representative plots of differentiation status of global (MICA/B⁻) T cells and MICA/B⁺ T cells from PBMC of CHB patients by *ex vivo* CD27 and CD45RA staining. (Upper right quadrant, lower right quadrant, lower left quadrant, and upper left quadrant indicate naïve, central memory (CM), effector memory (EM), and effector memory re-expressing CD45RA (EMRA) populations, respectively).
- B Summary analysis of panel A (n=10).
- C Representative examples of phenotype analysis of global (MICA/B⁻) and MICA/B⁺ T cells from IHL of CHB patients by *ex vivo* CD27 and CD45RA staining.
- D Summary analysis of panel C (n=8).

To conclude, our results illustrate that NKG2D ligands are upregulated on T cells in CHB, especially in patients with pronounced liver inflammation and on intrahepatic T cells.

3.3.2 Enrichment of NKG2D-L expression on T cells activated by HBV infection

Although NKG2D ligands were consistently upregulated on T cells from patients with CHB, the percentage of expressing cells was low, suggesting that a particular subset of T cells was affected. We hypothesised that NKG2D-L might be preferentially induced on those T cells activated by HBV infection. By using the activation marker HLA-DR, we compared MICA/B expression on activated (HLA-DR⁺) and global (HLA-DR⁻) T cells. *Ex vivo* staining of PBMC from patients revealed greater enrichment of MICA/B expression on activated T cells (Figure 3.5A and B).

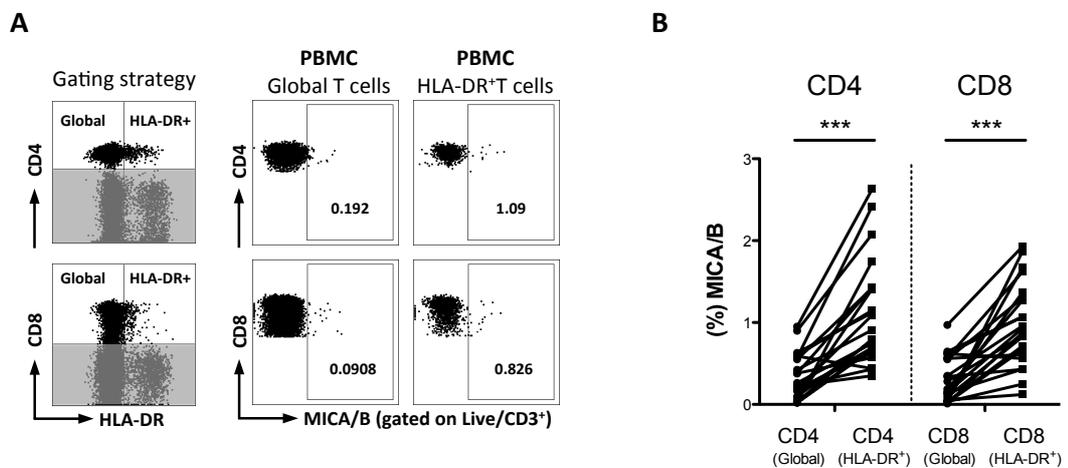


Figure 3.5 Evaluation of NKG2D ligand expression on activated T cells from CHB patients.

- A Representative plots of *ex vivo* MICA/B staining of global (HLA-DR⁻) T cells and activated (HLA-DR⁺) T cells from PBMC of CHB patients.
- B Summary results of panel A (n=22; analysed by Wilcoxon signed rank test).

Similarly, the MICA/B upregulation on intrahepatic activated (HLA-DR⁺) T cells was more apparent than on the global (HLA-DR⁻) fraction (Figure 3.6A and B).

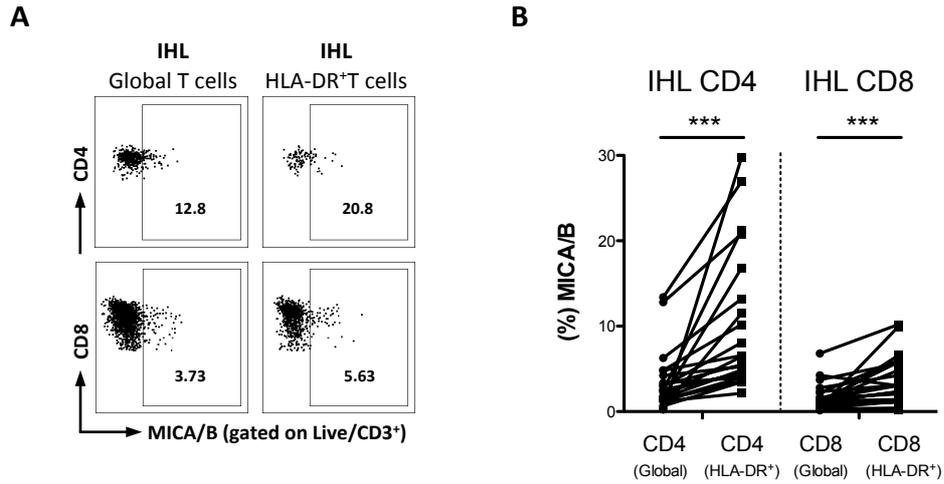


Figure 3.6 Evaluation of NKG2D ligand expression on intrahepatic activated T cells from CHB patients.

- A Representative plots of *ex vivo* MICA/B staining of global (HLA-DR⁻) and activated (HLA-DR⁺) T cells from IHL of CHB patients.
- B Summary results of panel C (n=21; analysed by Wilcoxon signed rank test).

Activated T cells in CHB likely consist of a mixture of HBV-specific T cells and other bystander T cells. Therefore, we next investigated the NKG2D-L expression on HBV-specific T cells identified by anti-viral cytokine, IFN- γ production. Following 10-day expansion and stimulation with overlapping peptides spanning the HBV core protein, we found MICA/B was expressed at much higher levels on HBV-specific (IFN- γ ⁺), compared to the global (IFN- γ ⁻), CD4 and CD8 T cells (Figure 3.7A and B).

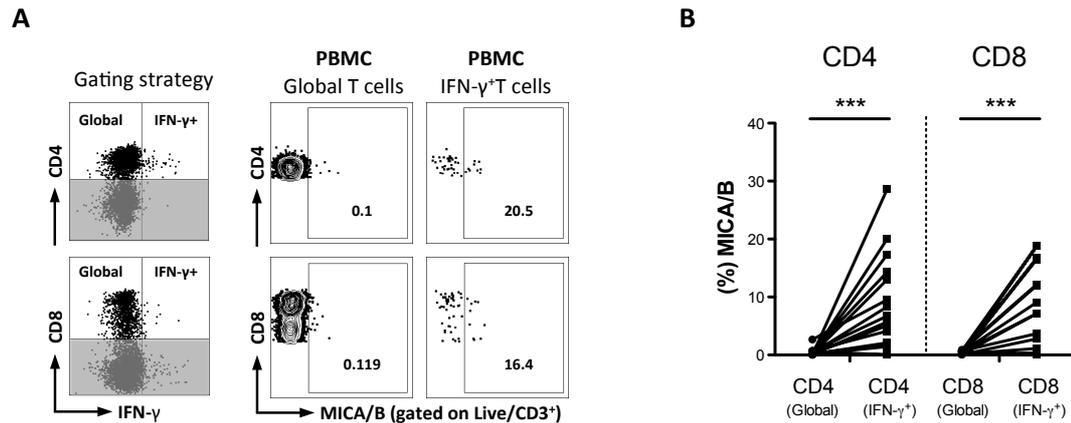


Figure 3.7 Evaluation of NKG2D ligand expression on HBV-specific T cells from CHB patients

- A. HBV-specific T cells were identified by IFN- γ production following short-term expansion and stimulation with overlapping peptides (OLP) spanning HBV core protein. Representative plots of MICA/B staining of global (IFN- γ ⁻) T cells and HBV-specific (IFN- γ ⁺) T cells.
- B. Summary results of panel A (n=17; analysed by Wilcoxon signed rank test).

Probing whether MICA/B was upregulated on HBV-specific T cells, and not just secondary to *in vitro* HBV overlapping peptide (HBV OLP) stimulation, we assessed *ex vivo* HLA-A2/HBV peptide dextramer (HBV Dex) staining. Consistently, the results also showed HBV-specific (HBV Dex⁺) CD8 T cells presented significantly higher levels of MICA/B than global (HBV Dex⁻) T cells (Figure 3.8A and B). Furthermore, in a parallel comparison of the same cohort, *ex vivo* MICA/B expression was preferentially enriched on HBV-specific (HBV Dex⁺) T cells but not on CMV-specific (CMV Dex⁺) T cells (Figure 3.8C).

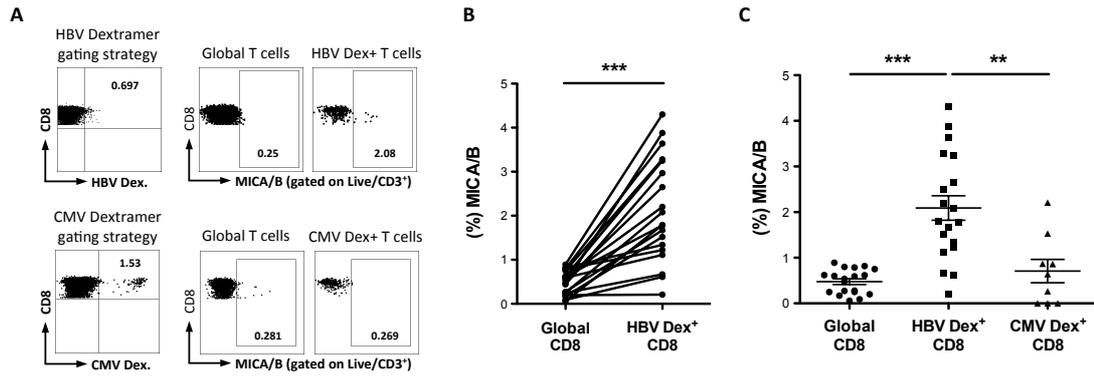


Figure 3.8 Evaluation of NKG2D ligand expression on HBV and CMV-specific CD8 T cells from CHB patients.

- A HBV or CMV-specific CD8 T cells were identified by HLA-A2/HBV peptide dextramer (HBV Dex) or HLA-A2/NLV-dextramer (CMV Dex) staining. Representative plots of *ex vivo* MICA/B staining on global (Dex⁻) T cells, HBV-specific (HBV Dex⁺) T cells, and CMV-specific (CMV Dex⁺) T cells (Gates set using control dextramer staining).
- B Summary results of *ex vivo* MICA/B expression on global (Dex⁻) T cells, HBV-specific (HBV Dex⁺) T cells (n=19; analysed by Wilcoxon signed rank test).
- C Extended comparison and summary of *ex vivo* MICA/B expression on global (Dex⁻) T cells, HBV-specific (HBV Dex⁺) T cells, and CMV-specific (CMV Dex⁺) T cells from a subset of the same cohort (Global CD8 n=19; HBV Dex⁺ CD8 n=19; CMV Dex⁺ CD8 n=9; analysed by Kruskal-Wallis test).

To examine whether intrahepatic HBV-specific T cells can also upregulate NKG2D-L expression, lymphocytes obtained from the livers of CHB patients were stimulated with HBV OLP overnight. Similar to the periphery, MICA/B levels were considerably higher on intrahepatic HBV-specific (IFN- γ^+) CD4 and CD8 T cells than on global (IFN- γ^-) T cell fractions (Figure 3.9A and B). In parallel, MICA/B levels were noted to be further enriched on intrahepatic HBV-specific T cells than those from matched peripheral blood samples (Figure 3.9C).

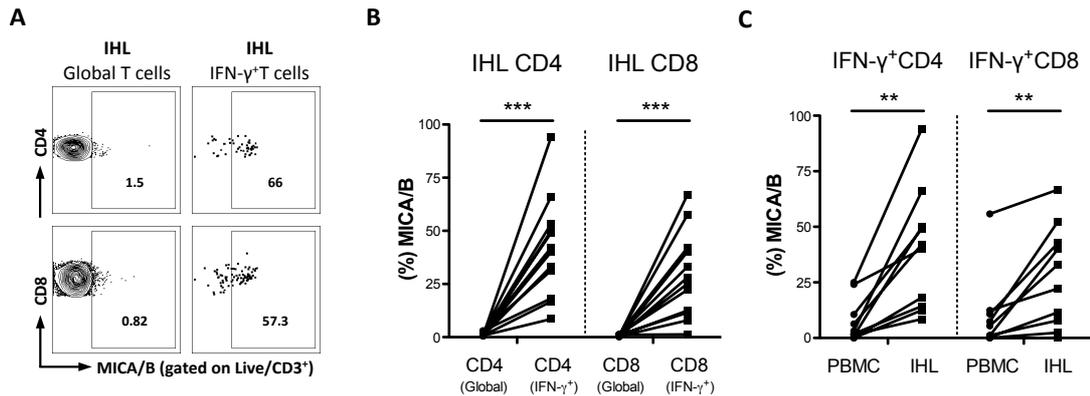


Figure 3.9 Evaluation of NKG2D ligand expression on intrahepatic HBV-specific T cells from CHB patients.

- Intrahepatic HBV-specific T cells were identified by IFN- γ production following overnight stimulation with OLP spanning HBV core protein. Representative plots of MICA/B staining of intrahepatic global (IFN- γ^-) T cells and intrahepatic HBV-specific (IFN- γ^+) T cells.
- Summary results of panel A (n=12; analysed by Wilcoxon signed rank test).
- Summary results of MICA/B expression on HBV-specific (IFN- γ^+) T cells from paired PBMC and IHL following overnight stimulation with OLP spanning HBV core protein (n=10; analysed by Wilcoxon rank test).

Notably, in the HBV-infected liver, both activated (HLA-DR⁺) and HBV-specific (IFN- γ^+) CD4 T cells showed markedly higher MICA/B than their CD8 counterparts (Figure 3.10A and B).

In conclusion, our study demonstrates for the first time that NKG2D-L can be expressed by non-virally infected human T cells and are preferentially enriched on activated and HBV-specific T cells.

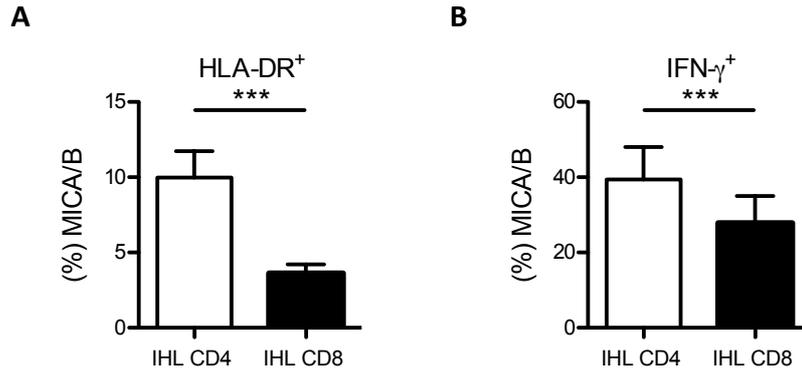


Figure 3.10 Comparison of NKG2D ligand expression on intrahepatic CD4 and CD8 T cells within activated and HBV-specific subsets.

- A Comparison summary of *ex vivo* MICA/B expression on activated (HLA-DR⁺) CD4 and CD8 T cells from IHL of CHB patients (n=21; analysed by Wilcoxon rank test).
- B Comparison summary of MICA/B expression on intrahepatic HBV-specific (IFN- γ ⁺) CD4 and CD8 T cells following overnight stimulation with OLP spanning HBV core protein (n=12; analysed by Wilcoxon rank test).

3.3.3 Oxidative stress in CHB as an underlying mechanism of NKG2D-L induction on T cells

NKG2D-L expression is tightly regulated and normally rare on healthy cells. However, in response to various stimuli or cellular stress, such as virus infection, genotoxic or oxidative stress, these "stress ligands" can be upregulated to initiate corresponding immune interactions (Champsaur and Lanier, 2010; Rabinovich et al., 2000; Raulet et al., 2013; Zingoni et al., 2012). In CHB, persistent HBV infection and chronic liver inflammation can cause continuing antigenic stimulation and pathogenic oxidative stress (Hagen et al., 1994; Shimoda et al., 1994). Therefore, we investigated whether *in vitro* Ag-induced TCR signalling or oxidative stress could contribute to T cell NKG2D-L upregulation. We used plate-bound α CD3 antibody (0.5 μ g/ml) to mimic transmembrane signalling upon TCR activation (Gimmi et al.,

1991; Ledbetter et al., 1985; Martin et al., 1986). Additionally, hydrogen peroxide (H_2O_2), a cellular metabolic product of physiological reactive oxygen species (ROS) (Nathan and Cunningham-Bussel, 2013; Valko et al., 2007) was also applied for oxidative stress stimulation. Purified CD4 T cells isolated from healthy donors or CHB patients were either rested or treated with plate-bound α CD3 antibody for 48 hours and/or 1mM H_2O_2 for 1 hour. In this exploratory study, we found striking MICA/B upregulation, analogous to that seen on intrahepatic T cells, following H_2O_2 treatment; by contrast, α CD3 stimulation did not significantly induce MICA/B either alone or in combination with H_2O_2 (Figure 3.11A and B). Similarly, no significant MICA/B induction was observed in experiments using either α CD3/ α CD28 for full T cell activation or α CD28/PMA treatment for signalling activation bypassing the TCR/CD3 complex (Molinero et al., 2002) (data not shown). Throughout preliminary screenings, other relevant stimuli were also tested for T cell MICA/B induction in control PBMC, including IFN- α , TNF- α , IL-2, IL-10, IL-15, IL-17, EGF, as well as ER stress inducer thapsigargin, but without positive results (data not shown). Although slight upregulation of MICA/B on T cells was observed upon IFN- α stimulation (500IU/ml for 4 hours), it failed to reproduce intrahepatic expression on peripheral T cells (data not shown).

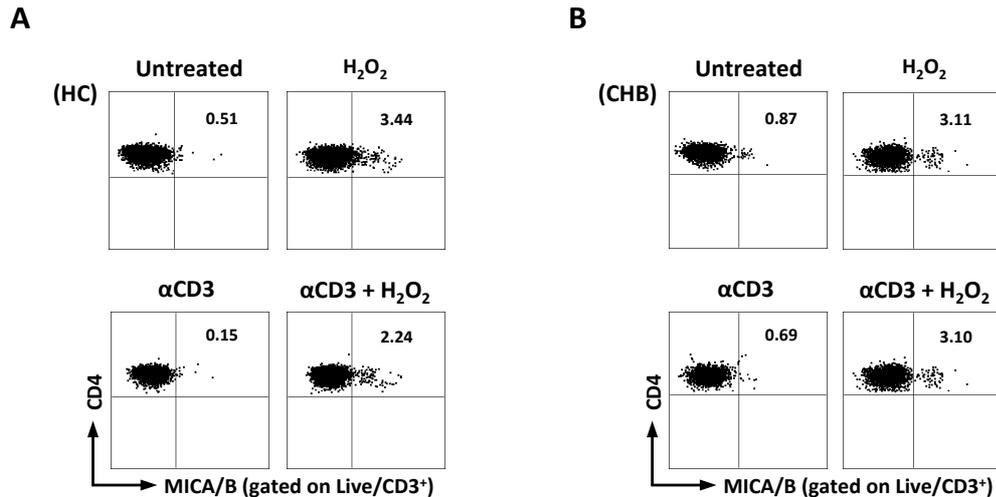


Figure 3.11 Evaluation of NKG2D ligand induction on purified CD4 T cells upon TCR signalling or oxidative stress stimulation.

A Purified CD4 T cells from a healthy donor were treated with plate-bound anti-CD3 (0.5µg/ml) or rested for 48hr and/or stimulated 1-hour with 1mM H₂O₂. Representative plots show the changes of MICA/B expression on purified CD4 T cells following different *in vitro* stimuli.

B Purified CD4 T cells from a CHB patient were treated with plate-bound anti-CD3 (0.5µg/ml) or rested for 48hr and/or stimulated 1-hour with 1mM H₂O₂. Representative plots show the changes of MICA/B expression on the purified CD4 T cells following different *in vitro* stimuli.

We then examined the time-course and dose-response of H₂O₂ induction of MICA/B. As before, purified CD4 T cells isolated from healthy individuals were in resting incubation for 48 hours to minimise other unwanted interactions. In the final 2.0, 1.0, or 0.5 hours, oxidative stress was induced by the addition of varying concentrations of H₂O₂. From this, we concluded that 1-hour stimulation with 0.5mM H₂O₂ optimally induced MICA/B expression on CD4 T cells from healthy individuals (Figure 3.12A and B). We then tested purified CD4 T cells from 14 healthy donors with the same 1hr stimulation at 0.5mM H₂O₂, finding a striking and consistent induction of MICA/B in all donors, with a mean increase of 5-fold (Figure 3.12 C).

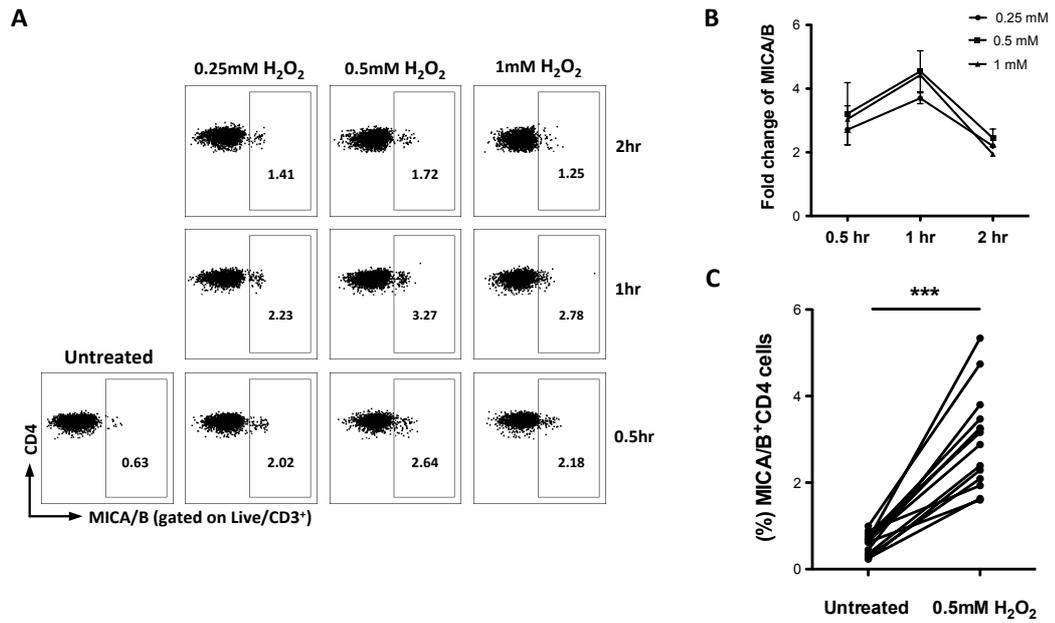


Figure 3.12 Evaluation of NKG2D ligand induction upon oxidative stress stimulation of purified CD4 T cells.

- A Purified CD4 T cells isolated from healthy individuals were treated with H₂O₂ for different durations and at different concentrations. Representative plots show the MICA/B changes from untreated CD4 T cells (baseline) to those with short-term H₂O₂ treatment at 2.0, 1.0, and 0.5 hours.
- B Summary results of panel A (n=2).
- C Summary of MICA/B expression on untreated CD4 T cells compared to those with 1-hour 0.5mM H₂O₂ treatment (n=14; analysed by Wilcoxon signed rank test).

Next, we checked the MICA/B, ULBP1, ULBP2/5/6, and ULBP3 induction on T cells from paired PBMC and purified CD4 T cells simultaneously in the same experimental setting. After the same 1-hour 0.5mM H₂O₂ stimulation, we found ULBP1, ULBP2/5/6, and ULBP3 were also significantly increased on purified CD4 T cells, even though, the upregulation levels of ULBP2/5/6 was notably less than the other NKG2D-L (Figure 3.13A-D). Moreover, in the parallel PBMC experiments, we found the increments of all tested NKG2D-L, especially ULBP2 and ULBP3, were lower on both CD4 and CD8 T cells compared to the upregulation of NKG2D-L on purified CD4 T cells. This implies oxidative stress stimulation may initiate other

counter-regulatory pathways in whole PBMC to diminish NKG2D-L expressing T cells; these findings are compatible with the possibility that NKG2D-L-expressing T cells are preferentially deleted in the presence of NK cells within PBMC cultures (Figure 3.13A-D).

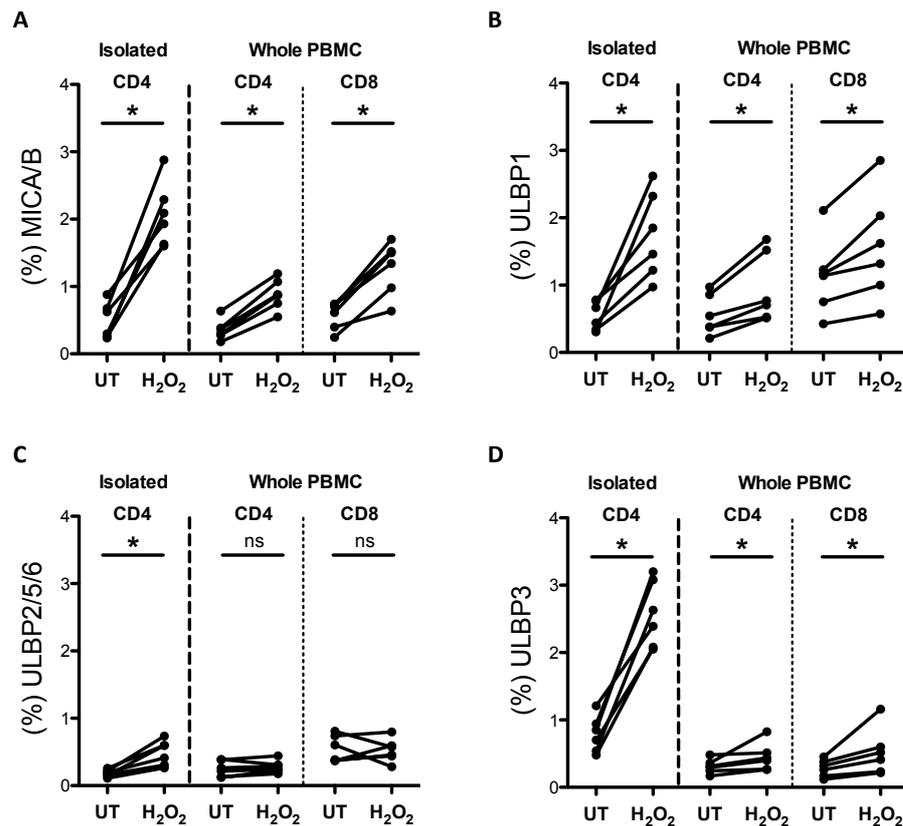


Figure 3.13 Evaluation of NKG2D-L induction on T cells upon oxidative stress stimulation.

- A Parallel comparison of H₂O₂ effects on T cell MICA/B induction in isolated CD4 T cells and in whole PBMC (UT: untreated; n=6; analysed by Wilcoxon signed rank test).
- B Parallel comparison of H₂O₂ effects on T cell ULBP1 induction in isolated CD4 T cells and in whole PBMC (UT: untreated; n=6; analysed by Wilcoxon signed rank test).
- C Parallel comparison of H₂O₂ effects on T cell ULBP2/5/6 induction in isolated CD4 T cells and in whole PBMC (UT: untreated; n=6; analysed by Wilcoxon signed rank test).
- D Parallel comparison of H₂O₂ effects on T cell ULBP3 induction in isolated CD4 T cells and in whole PBMC (UT: untreated; n=6; analysed by Wilcoxon signed rank test).

To explore additional possible co-regulatory pathways for limiting NKG2D-L expressing T cells upon oxidative stress, we assessed the co-expression of death receptors (Fas/CD95 and TRAIL-R2) and the exhaustion marker PD-1 on isolated MICA/B expressing CD4 T cells in parallel experiments. Extended H₂O₂ stimulation experiments revealed that both Fas and PD-1 were preferentially increased on MICA/B expressing CD4 T cells, though no significant changes on TRAIL-R2 levels were noted (Figure 3.14A-C and data not shown). These findings suggest oxidative stress has the ability to manipulate T cell Fas and PD-1 in addition to NKG2D-L.

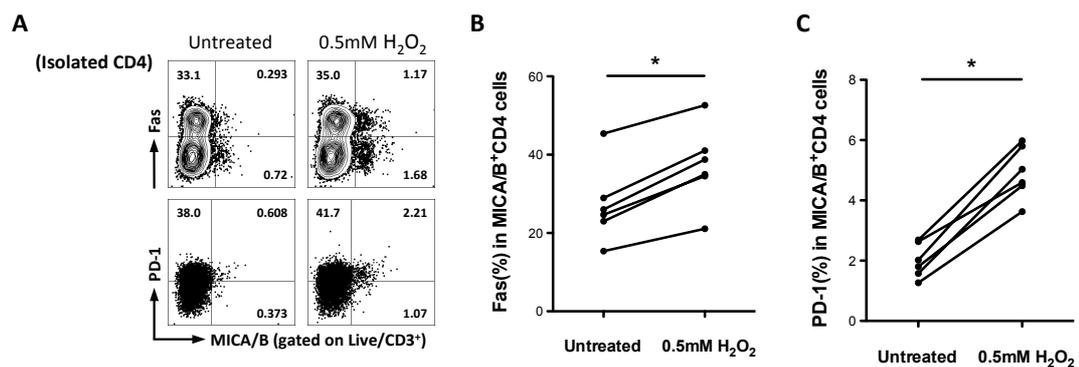


Figure 3.14 Evaluation of upregulation of death receptor (Fas) and exhaustion marker (PD-1) on NKG2D-L expressing CD4 T cells upon oxidative stress stimulation.

- A Purified CD4 T cells isolated from healthy individuals were rested for 48hr and treated in the final hour with 0.5mM H₂O₂. Representative plots of co-expression of Fas or PD-1 on MICA/B expressing CD4 T cells.
- B Summary results of Fas expression on MICA/B⁺CD4 T cells upon oxidative stimulation (n=6; analysed by Wilcoxon signed rank test).
- C Summary results of PD-1 expression on MICA/B⁺CD4 T cells upon oxidative stimulation (n=6; analysed by Wilcoxon signed rank test).

In conclusion, our results indicate that oxidative stress could be a factor driving NKG2D-L upregulation on T cells, rendering stressed T cells susceptible to immune regulation in CHB.

3.3.4 Restoration of activated or HBV-specific T cells upon NKG2D blockade or NK cell depletion

We postulated that the expression of NKG2D-L by T cells in CHB would render them susceptible to engagement with NKG2D-expressing NK cells, triggering their deletion. Although this process was likely to have already been engaged *in vivo* in the HBV-infected liver and therefore not easily interrupted *in vitro*, we tested whether any T cell responses could still be rescued by *in vitro* blockade. To probe the impact of the NKG2D pathway and NK cells on activated and HBV-specific T cells, we therefore performed NKG2D blockade and NK cell depletion by using NKG2D blocking mAb and NK cell isolation kits, respectively. PBMC of CHB patients were cultured short-term (10 days) with overlapping peptides (OLP) spanning HBV core protein and activated T cells were identified by HLA-DR staining. Following NK cell depletion, significant restoration of activated CD4 and CD8 T cells was noticed. However, only partial rescue of activated T cells was observed upon NKG2D blockade (Figure 3.15A and B), in line with our previous work indicating that other pathways such as TRAIL are involved in NK cell killing of activated T cells ([Peppas et al., 2013](#)).

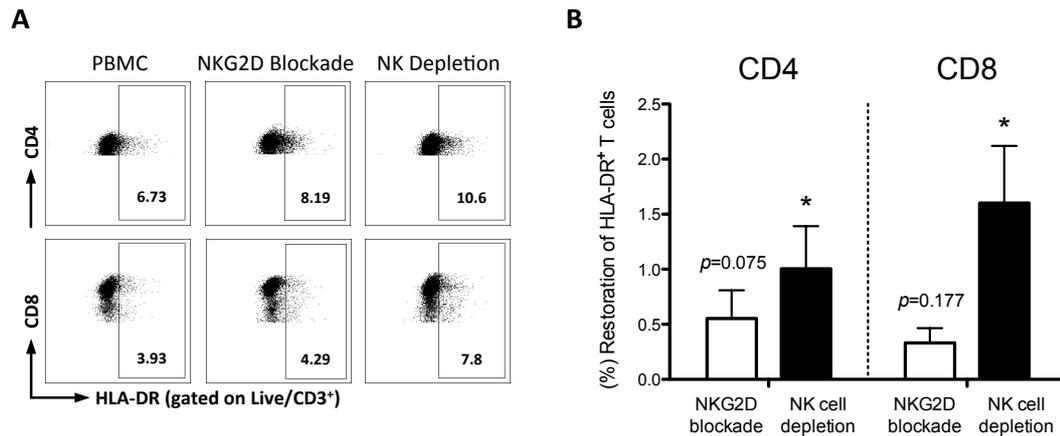


Figure 3.15 Evaluation of the impact of NKG2D pathway and NK cells on activated T cells in CHB.

- A Following short-term culture with overlapping peptides spanning HBV core protein, the percentage of activated (HLA-DR⁺) T cells was evaluated from PBMC (baseline), PBMC with NKG2D blockade by anti-NKG2D mAb, and PBMC with NK cells depleted by NK cell isolation kits. Representative plots of activated (HLA-DR⁺) T cell restoration upon NKG2D blockade and NK cell depletion.
- B Summary results of panel A, showing percentage restoration after subtraction of baseline (n=8; analysed by Wilcoxon rank test).

In the same culture setting, we proceeded to evaluate the effects of NKG2D blockade and NK cell depletion on HBV-specific T cells. PBMC isolated from CHB patients were cultured short-term (10 days) with HBV OLP, and HBV-specific T cells were identified by IFN- γ production. Following NK cell depletion and NKG2D blockade, restoration of HBV-specific CD4 T cells by both methods was observed in patients with ongoing liver inflammation (ALT>60 IU/L), but not in patients with ALT \leq 60 IU/L (Figure 3.16A-C). In addition, the rescue effect upon NK cell depletion was suppressed by re-adding NK cells at a physiological ratio in the beginning of the short-term culture (Figure 3.16A and B).

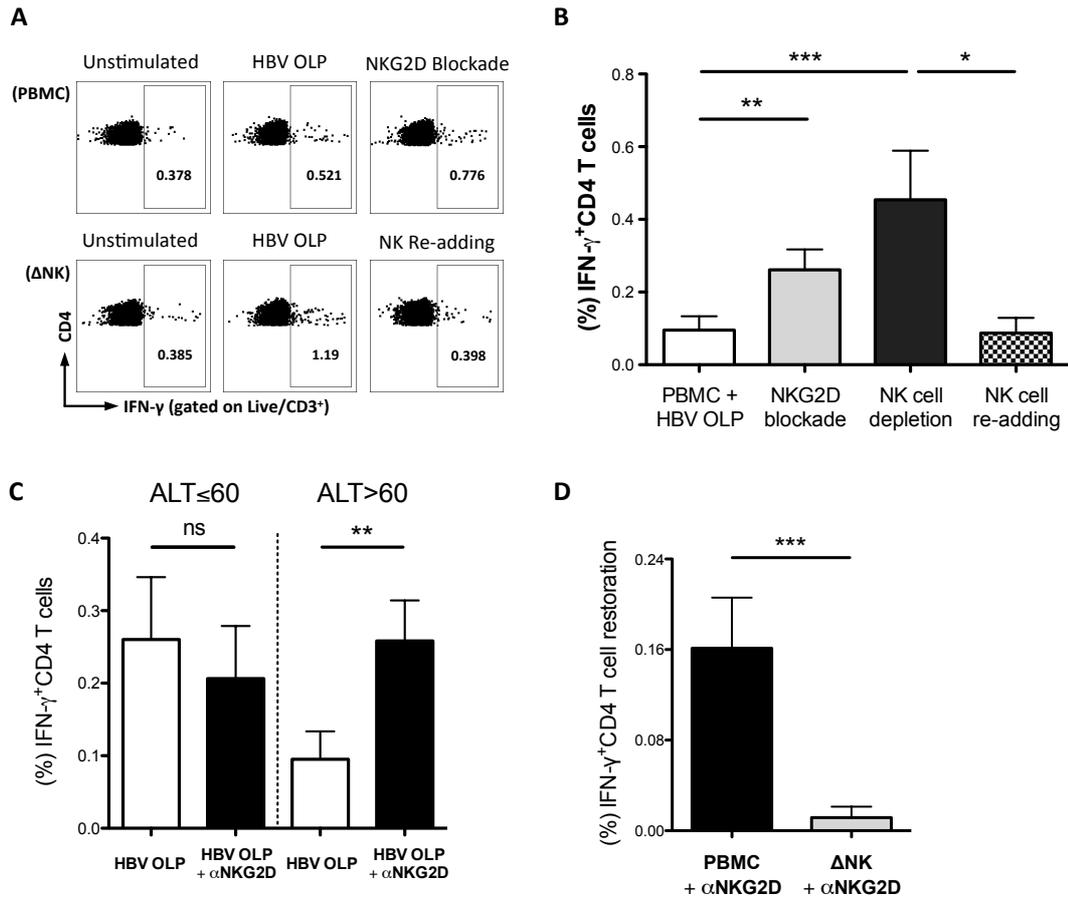


Figure 3.16 Evaluation of the impact of NKG2D pathway and NK cells on HBV-specific T cells in CHB.

- A Following short-term culture with overlapping peptides (OLP) spanning HBV core protein, the percentage of HBV-specific (IFN- γ ⁺) CD4 T cells was evaluated from PBMC, PBMC with NKG2D blockade, PBMC with NK cell depletion (Δ NK), and NK cell-depleted PBMC with NK cells re-added. Representative plots of HBV-specific (IFN- γ ⁺) CD4 T cell changes upon NKG2D blockade, NK cell depletion, and NK cell re-adding.
- B Summary results of panel A from patients with ALT > 60 IU/L (n=15; percentage is after subtraction of baseline IFN- γ production without OLP stimulation; analysed by Friedman test).
- C Extended comparison of HBV-specific (IFN- γ ⁺) CD4 T cell restoration upon NKG2D blockade in patients with ALT ≤ 60 IU/L and patients with ALT > 60 IU/L (ALT ≤ 60 n=11; ALT > 60 n=15; analysed by Wilcoxon signed rank test).
- D Extended comparison of HBV-specific (IFN- γ ⁺) CD4 T cell restoration upon NKG2D blockade between PBMC and NK cell-depleted PBMC (Δ NK) from patients with ALT > 60 IU/L (PBMC n=15; NK cell-depleted PBMC n=13; percentage is after subtraction of baseline IFN- γ production without OLP stimulation; analysed by Mann Whitney test).

Since the NKG2D receptor is also found on other cells, such as NKT cells, $\gamma\delta$ T cells, and activated CD8 T cells (Eagle and Trowsdale, 2007), we examined NKG2D blockade effects on NK cell-depleted PBMC in our parallel panel simultaneously. Notably, rescue of HBV-specific T cells was not seen upon NKG2D blockade in the absence of NK cells, implying an NK cell-dependent NKG2D effect (Figure 3.16D).

Consistent with the enhancement of peripheral anti-viral responses upon NKG2D blockade, intrahepatic HBV-specific CD4 T cells, but not CD8, were also significantly restored by blocking the NKG2D pathway following overnight HBV OLP stimulation (Figure 3.17A and B).

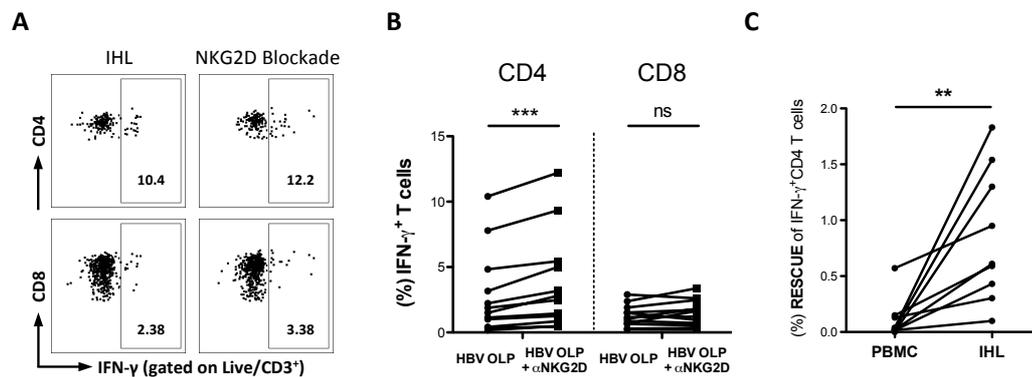


Figure 3.17 Evaluation of the impact of NKG2D pathway on intrahepatic HBV-specific T cells in CHB.

- A Following overnight stimulation with overlapping peptides (OLP) spanning HBV core protein, the percentage of intrahepatic HBV-specific (IFN- γ)⁺ T cells was evaluated from intrahepatic lymphocytes (IHL), and IHL with NKG2D blockade. Representative plots of intrahepatic HBV-specific (IFN- γ)⁺ T cell restoration upon NKG2D blockade.
- B Summary results of panel A (n=12; analysed by Wilcoxon signed rank test).
- C Comparison summary of HBV-specific (IFN- γ)⁺ CD4 T cell restoration upon NKG2D blockade from paired PBMC and IHL following overnight HBV OLP stimulation (n=9; analysed by Wilcoxon signed rank test).

When comparisons were made between intrahepatic lymphocytes and paired peripheral lymphocytes in parallel, our experiments demonstrated NKG2D blockade preferentially rescued HBV-specific CD4 T cells in the liver rather than the periphery (Figure 3.17C).

Taken together, these results indicate that NK cells have suppressive effects on activated and HBV-specific T cells. Furthermore, HBV-specific CD4 T cells, particularly the intrahepatic fraction, are susceptible to NK cell dependent NKG2D killing in the setting of active CHB, resulting in constraints on antiviral immunity.

3.4 Conclusions and Discussion

Antiviral T cells are crucial in immune control of HBV (Maini et al., 2000; Thimme et al., 2003); however, many intrinsic and extrinsic factors have been discovered to cause T cell functional exhaustion in patients with CHB (Maini and Schurich, 2010; Protzer et al., 2012). Upregulation of pro-apoptotic protein Bcl2-interacting mediator (Bim) also drives susceptibility of HBV-specific T cells to profound apoptotic deletion (Lopes et al., 2008). Therefore, an important consideration in improving current HBV therapy is to thoroughly define the potential negative impacts on anti-viral T cells.

In humans, eight NKG2D ligands including MICA/B and ULBP1-6 have been identified so far (Champsaur and Lanier, 2010; Raulet et al., 2013). Due to the normally rare but inducible expression upon various types of cellular stress, these ligands constitute part of the lymphoid stress-surveillance (LSS) system (Shafi et al., 2011; Strid et al., 2011). Moreover, the fine-tuned and diverse expression of the NKG2D-L with their highly polymorphic character limits tumour or virus evasion underscoring their non-redundant immune significance (Cosman et al., 2001; Eagle and Trowsdale, 2007; Gonzalez et al., 2008; Mistry and O'Callaghan, 2007). Firstly, our *ex vivo* study clearly demonstrated that human T cells are capable of expressing selective NKG2D-L. This is the first time, to our knowledge, that non-virally infected human T cells have been found to express NKG2D-L directly *ex vivo*. Previous virus research has shown that NKG2D-L can be delicately adjusted at multiple transcriptional and post-transcriptional levels to intricately regulate their

expression on certain cell populations, such as stromal cells (Fielding et al., 2014; Lam et al., 2013; Norman et al., 2011; Raulet et al., 2013; Richard et al., 2010). Likewise, the disproportionate upregulation of NKG2D-L on activated and HBV-specific T cells pointed to differential regulation. In CHB, T cells susceptible to TRAIL-mediated NK cell killing in common with MICA/B positive T cells, showed a less senescent or terminal differentiated phenotype, comprised largely of naïve and central memory cells (Peppas et al., 2013). Unlike CMV-specific T cells, HBV-specific T cells, with profoundly exhausted and apoptotic qualities, also show striking induction of NKG2D-L and TRAIL-R2, and are sensitive to NKG2D and TRAIL mediated depletion (Peppas et al., 2013). Taken together, these findings indicate that chronic HBV infection can mold T cells to present multiple features making them vulnerable to immune regulation.

To investigate the underlying mechanisms of NKG2D-L upregulation on T cells, we first stratified our patient cohort by different virological and clinical parameters. In contrast to the preferential enrichment of NKG2D-L expression on T cells from patients with pronounced liver inflammation (ALT>60 IU/L), the link between T cell NKG2D-L upregulation with either HBeAg or viral load did not appear statistically significant. In view of the considerably higher levels of NKG2D-L expression on intrahepatic T cells, we next considered various factors relevant to HBV-related liver inflammation. Although our *ex vivo* and *in vitro* studies were unable to support antigen-driven activation and proliferation as a trigger for T cell NKG2D-L

induction, they did reveal that oxidative stress can strikingly upregulate NKG2D-L expression of peripheral T cells, achieving the levels seen in the HBV-infected liver.

Reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical, and hydrogen peroxide, are products of cellular metabolism as well as important signalling molecules with broad biological effects (Fridovich, 1999; Nathan and Cunningham-Bussel, 2013; Nordberg and Arner, 2001). Recent advances in such redox mechanisms have demonstrated that ROS can regulate T cell function, differentiation, and survival by modulating several transcription factors and mitogen-activated protein kinases, including p53, nuclear factor κ B (NF κ B), nuclear factor erythroid-2-related factor 2 (Nrf2), extracellular signal-regulated kinase (ERK) pathway, and p38-MAPK pathway (Devadas et al., 2002; Kesarwani et al., 2013; Kim et al., 2008a; Kim et al., 2008b; Polyak et al., 1997; Suzuki et al., 1997; Trachootham et al., 2008). Moreover, imbalance of cellular redox state and oxidative stress caused by inappropriate accumulation of ROS have been implicated in aging, genetic damage, infection, inflammation and carcinogenesis (Nathan and Cunningham-Bussel, 2013; Reuter et al., 2010; Valko et al., 2007). Substantial evidence has shown that oxidative stress, resulting from persistent viral infection (Higgs et al., 2014; Schwarz, 1996; Severi et al., 2006) and chronic inflammation (Jaeschke, 2011; Mittal et al., 2014; Reuter et al., 2010), is increased in CHB patients and even more prominent within the liver, the origin of HBV pathology (Bolukbas et al., 2005; Fujita et al., 2008; Hagen et al., 1994; Li et al., 2015; Shimoda et al., 1994). Hydrogen peroxide, a relatively weak endogenous ROS being widely used for

oxidative stress induction, has physiological significance including the capability of diffusing through biological membranes and as an intermediate in generating other more reactive oxidizing products (Fridovich, 1997; Massey, 1994; Robbins and Zhao, 2004; Winterbourn et al., 2000). Currently, cellular production of hydrogen peroxide has been regarded as a damage signal of tissue injury and inflammation (van der Vliet and Janssen-Heininger, 2014; Wittmann et al., 2012), since it contributes to Ca^{2+} influx and ATP release along with various signalling cascades, such as ERK and NF κ B pathway activation as well as IL-2/IL-2R α expression (Devadas et al., 2002; Kesarwani et al., 2013; Los et al., 1995). Thus, by using hydrogen peroxide *in vitro*, we demonstrated that oxidative stress could be an underlying mechanism of NKG2D-L induction on T cells in CHB (Allegretti et al., 2013; Borchers et al., 2006; Rabinovich et al., 2000). Moreover, we observed that the heterogeneous NKG2D-L responses to oxidative stress were similar to the *ex vivo* pattern of selectively upregulated MICA/B and ULBP1 on T cells in CHB patients. In agreement with previous documented research showing a positive association between oxidative stress and the severity of HBV-related liver disease (Bolukbas et al., 2005; Duygu et al., 2012; Fujita et al., 2008; Kitada et al., 2001), our study demonstrated higher upregulation of NKG2D-L on T cells from patients with greater liver injury (ALT > 60 IU/L). On a background of aberrant oxidative stress, all newly generated cells encountering their antigen in the liver might be expected to be affected, which could explain the detection of NKG2D-L expressing T cells in our patient cohort. However, the discrepancy of NKG2D-L upregulation between whole PBMC and isolated CD4 T cells *in vitro* suggests NKG2D-L expressing T cells are tightly constrained and that

counter-regulatory pathways may also be initiated by oxidative stress. Besides a broad spectrum of immune responses, oxidative stress and cellular redox changes can trigger many cellular interactions, such as perforin-mediated and DNAM-1-dependent NK cell killing, to regulate T cell function and survival (Ardolino et al., 2011; Kesarwani et al., 2013; Rabinovich et al., 2000). Therefore, the selective NKG2D-L expression on T cells in CHB could be viewed as a highly orchestrated phenomenon, reflecting complex immune networks.

Within the chronically HBV-infected and inflamed liver, T cells are directly exposed not only to overwhelming HBV antigenic stimulation, but also to other cellular and metabolic stressors (Guidotti et al., 2015b; Protzer et al., 2012). Consistent with our results illustrating more marked upregulation of NKG2D-L in the liver than the periphery, pathogenic NK cell responses, arginase-producing myeloid cells causing L-arginine deprivation, and oxidative stress are all more prominent within the HBV-infected liver (Hagen et al., 1994; Pallett et al., 2015; Peppas et al., 2013). In line with greater HBV-specific T cell restoration upon NKG2D blockade in the liver compared to the periphery, we suggest that the NKG2D pathway is more influential in the HBV-infected liver, especially in the presence of ongoing inflammation.

Similar to the preferential NKG2D-L upregulation on CD4 T cells in other chronic viral infections (Schuster et al., 2014), our study also demonstrated CD4 T cells expressed higher MICA/B than their CD8 counterparts, particularly the activated and HBV-specific fractions within the HBV-infected liver. As a part of the complicated

NKG2D-L control system, the epidermal growth factor receptor (EGFR) signalling pathway can stabilize the cellular NKG2D-L expression and further shape the immunological visibility of cells (Vantourout et al., 2014). Our findings of NKG2D-L upregulation in chronic HBV infection are consistent with previous studies suggesting prominent activation of the EGFR pathway in CHB patients (Barreiros et al., 2009). However, unlike CD4 T cells, CD8 T cells have very low/undetectable EGFR, the receptor for EGF (Dai et al., 2014; Zaiss et al., 2013). This could possibly account for why we find that CD8 T cells have less capability to maintain NKG2D-L expression and explain the preferential enrichment of NKG2D-L on CD4 T cells. In addition, unlike CD4 T cells, CD8 T cells usually possess NKG2D (Eagle and Trowsdale, 2007). Very little is known about possible pathophysiological interactions between NKG2D-L and NKG2D expressed on the same cells. The possibility of either direct self-activation or ligand-internalization on CD8 T cells might also account for the reduced NKG2D-L displayed on CD8 T cells in our study.

The preferential susceptibility of CD4 T cells to NK cell interactions may have specific implications in viral immunity (Rehermann, 2013; Rydyznski et al., 2015; Schuster et al., 2014; Waggoner et al., 2012; Waggoner and Kumar, 2012). Sufficient anti-viral CD8 T cell responses together with neutralizing antibody development crucial for the outcome of HBV infection both depend on adequate CD4 T cell help (Chisari and Ferrari, 1995; Guidotti et al., 2015b). As previous research in murine viral models demonstrated, since CD8 T cells express higher CD48 that can engage

the inhibitory receptor CD244 on NK cells, CD4 T cells are predominant targets for NK cell killing (Crome et al., 2013; Rehermann, 2013; Waggoner et al., 2012; Waggoner and Kumar, 2012). The seminal mouse LCMV study from Waggoner et al. demonstrated that NK cells serve as rheostats of disease activity in different viral load settings by primarily eliminating activated CD4 T cells and subsequently hindering CD8 T cell immunity (Waggoner et al., 2012). Our findings of NKG2D-L expression and rescue of antiviral immunity by NKG2D blockade in active CHB patients, suggest that NKG2D-mediated interactions may serve as a homeostatic response for surveillance of stressed cells, thereby collaterally constraining adaptive immunity in chronic HBV infection.

Despite the clear-cut upregulation of NKG2D-L on T cells in chronic HBV infection, dramatic restoration of antiviral immunity upon NKG2D blockade was not observed. This may be due to the following reasons. First, in the pathogenic and inflammatory milieu, many inhibitory or apoptotic molecules and regulatory interactions may be triggered (Kesarwani et al., 2013; Maini and Schurich, 2010; Protzer et al., 2012; Rabinovich et al., 2000). Corresponding to our findings, as a pathological signature of chronic hepatitis B (Ha et al., 2010; Hagen et al., 1994; Poli, 2000), oxidative stress can induce both PD-1 and Fas expression on NKG2D-L expressing T cells. Therefore, the majority of highly activated and stressed T cells may be depleted or prone to apoptosis, leaving few of them amenable to rescue following *in vitro* NKG2D blockade. Second, other pathways may also participate in NK cell-mediated deletion. Indeed, the chronic HBV-infected and inflamed liver is favorable for

DNAM-1 and TRAIL-dependent NK cell regulation ([Ardolino et al., 2011](#); [Schuster et al., 2014](#)). According to previous HBV research on the TRAIL pathway, despite significant rescue of HBV-specific T cells following TRAIL-R blockade, the recovery was incomplete ([Peppas et al., 2013](#)). For the optimized restoration of antiviral immunity, dissecting all possible regulatory mechanisms could be a worthwhile pursuit. Since many pathways have synergistic effects and are required to fully activate NK cells, it is possible that the NKG2D pathway not only can drive activation and cytotoxicity of NK cells, but also synergise killing ability through other pathways ([Bryceson et al., 2006b](#)). Unlike the TRAIL pathway whose effector function can be terminated by TRAIL pathway blockade, the degranulation cytotoxicity triggered by NKG2D-L is unable to be fully abolished once the NKG2D receptor is engaged. As a result, when the killing cascade is initiated, NK cell mediated deletion cannot be fully reversed by NKG2D blockade alone. Furthermore, the highly polymorphic NKG2D-L have unique functional heterogeneity and may engage with an undefined receptor on T cells other than NKG2D to suppress T cell proliferation ([Kriegeskorte et al., 2005](#); [Raulet et al., 2013](#)). Thus, further investigating all aspects of the NKG2D pathway may provide a better understanding of T cell survival in CHB. Overall, we would anticipate that *in vivo* NKG2D blockade would rescue substantially greater HBV-specific T cells than what was achievable *in vitro*. However, it is also likely that this pathway is merely a small portion of the pathological regulatory mechanisms in CHB pathology and therefore not sufficient to fully decode the complexity of this immune network.

To conclude, these key points sum up our research. First of all, our study clearly demonstrated in chronic HBV infection, T cells, especially the intrahepatic and HBV-specific fractions, upregulate selective NKG2D-L, allowing them to engage in NKG2D-dependent NK cell regulation. Additionally, the NKG2D-L expression pattern of T cells can be recapitulated by oxidative stress, a feature of HBV pathology. This implies that in the HBV-infected and inflamed liver, NKG2D-dependent T cell/NK cell interactions may serve as a homeostatic response for stress surveillance, resulting in constraints on antiviral immunity. Therefore, to further optimize antiviral responses and improve HBV therapy, efforts to minimize HBV-related oxidative stress should be considered.

Chapter 4 NKG2D-mediated NK cell Regulation in Chronic HBV Infection

4.1 Introduction: NK cell-mediated T cell regulation and the immunopathology implications in virus disease

NK cells are an important cellular component of innate immunity. The conventional view was that NK cells directly participate in the first-line defence against viral infection and provide support for adaptive immunity to achieve viral control. However, there is growing recognition that NK cells can also act as regulatory cells to restrain T cell anti-viral immunity. In fact, NK cells have the potential to diversely modulate T cell responses in autoimmune disease or transplantation settings, as well as regulating pathological sequelae in viral infection ([Crome et al., 2013](#)).

In CHB, inadequate priming support from intrahepatic antigen-presenting cells (APC) may be a key driver of T cell exhaustion and hypo-responsiveness ([Isogawa et al., 2005](#); [Maini and Schurich, 2010](#)). Recent research demonstrates the ability of NK cells to modulate APC function and interfere with adaptive immunity against various pathogens ([Crome et al., 2013](#); [Sun and Lanier, 2011](#)). In one MCMV study, NK cells have been reported to hinder anti-viral T cell responses by limiting the effectiveness of APC priming for T cells in a perforin-mediated manner ([Andrews et al., 2010](#)). Moreover, the augmentation of antigen presentation efficacy following NK cell depletion in LCMV-infected mice also suggests NK cell-mediated APC modulation could be an influential mechanism to alter anti-viral T cell immunity ([Su et al., 2001](#)).

One mechanism by which NK cells can regulate T cells is cytokine production. For example, previous murine research has shown, in response to MCMV infection, NK cells can suppress CD8 T cells by secreting the immunosuppressive cytokine, IL-10. The subsequent enhancement of T cell anti-viral immunity upon either diminishing IL-10 from NK cells or directly neutralizing IL-10 further validated the unappreciated immune restraints from the NK cell-mediated cytokine milieu (Lee et al., 2009).

Apart from cytokine production and APC priming modulation, NK cells can directly kill CD4 and CD8 T cells to suppress adaptive immunity. In CHB, NK cells can exert TRAIL-mediated killing not only of HBV-infected hepatocytes, but also of HBV-specific CD8 T cells (Dunn et al., 2007; Peppas et al., 2013). The significant restoration of HBV-specific CD8 T cells achieved by NK cell depletion, TRAIL or TRAIL-R2 blockade, demonstrated that TRAIL-dependent NK cell killing adversely impaired adaptive immunity in CHB (Peppas et al., 2013). Besides human HBV studies, in MCMV models, activated CD4 T cell killing by TRAIL positive NK cells was also reported to constrain viral-induced autoimmunity (Schuster et al., 2014). Taken together, these findings implicate the TRAIL pathway as a potent effector arm of NK cells which can significantly affect T cell immunity and tissue pathology.

In patients with chronic HBV infection, the receptor 2B4/CD244 has been reported to be one driver of exhaustion, which inhibits the proliferation and cytotoxicity of

HBV-specific T cells in the periphery and liver ([Raziorrouh et al., 2010](#)). Likewise, in mouse LCMV models, 2B4 is considered as an inhibitory molecule not only participating in CD8 T cell exhaustion ([Blackburn et al., 2009](#); [Wherry et al., 2007](#)), but also providing self-tolerant signalling for NK cells ([Waggoner et al., 2010](#)). When mice lacking 2B4 were infected with LCMV, 2B4-deficient NK cells caused long-lasting pathology and the consequent virus persistence. Moreover, deletion of activated CD8 T cells by NK cells also developed in either 2B4-deficient NK cell or CD48-KO T cell settings ([Waggoner et al., 2010](#)). Overall, these findings indicate that an imbalanced interaction between 2B4 and CD48 can disturb innate and adaptive immunity in viral infection disease.

As a part of the lymphoid stress surveillance system, NKG2D-positive NK cells can promptly respond to stress-induced ligands in various pathologic situations, such as infection, inflammation and tumour transformation ([Eagle and Trowsdale, 2007](#); [Shafi et al., 2011](#)). In LCMV infected mice, NK cells with upregulated NKG2D were shown to directly kill CD8 T cells by NKG2D-mediated perforin lysis. After NK cell depletion or NKG2D blockade, the subsequent improvement of T cell immunity and rapid virus control both confirmed the negative impact of NKG2D-dependent regulation ([Lang et al., 2012](#)). Moreover, recent *in vitro* studies also discovered that the NKG2D pathway is responsible for NK cell killing of activated human CD4 T cells ([Cerboni et al., 2007b](#); [Nielsen et al., 2012](#)).

Taking these discoveries into account, we can conclude that NK cells not only contribute to first-line defence against virus infection, but also have regulatory functions to modulate adaptive anti-viral immunity. Through impairing APC priming, releasing immunosuppressive cytokines or direct killing of T cells, NK cells can compromise T cell immunity and potentially lead towards viral chronicity. On the other hand, NK cell-mediated T cell regulation may have protective homeostatic value. Viral infection, in some cases, can arouse T cell-mediated autoimmune responses to exacerbate disease progression (Getts et al., 2013; McKinney et al., 2015). Such self-destructive reactions have been revealed to cause tissue pathology, vital organ damage, and even lethal consequences (Getts et al., 2013). Thus, NK cells may serve as an important homeostatic control to inhibit T cell-mediated autoimmunity at the expense of antiviral immunity and viral persistence (Schuster et al., 2014).

In another aspect, NK cells can act as rheostats of disease activity by modulating T cell immunity. Specifically, Waggoner et al. showed that in the face of overwhelming virus infection, NK cell killing of CD4 T cells caused the subsequent anti-viral CD8 T cell exhaustion and LCMV chronicity. Nevertheless, in this case, the NK cell cytotoxicity was regarded as protective to host survival, since the vigorous but harmful T cell responses in the absence of NK cell suppression eventually led to extensive tissue damage and host death (Waggoner et al., 2012). Together, these results suggest that in certain instances of viral infection, while

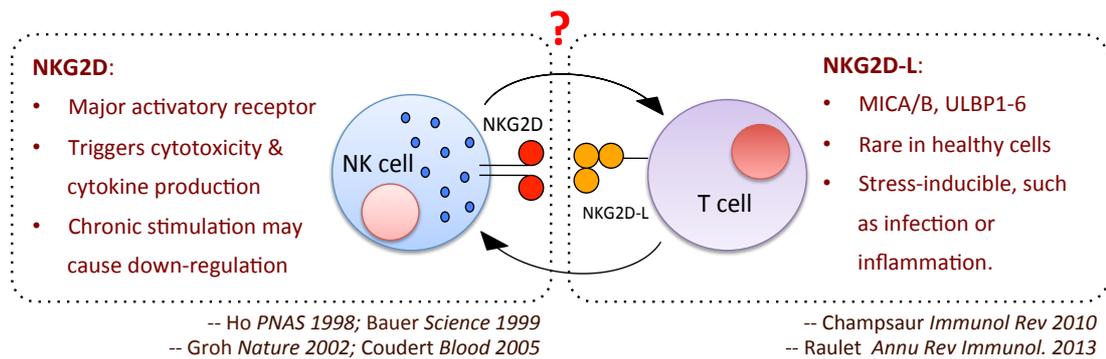
determining host homeostasis or devastating complications, T cells could become expendable sacrifices of NK cell regulation.

To conclude, recent research has demonstrated NK cell-mediated T cell regulation has multiple facets in viral infection. Whether the effects are direct or indirect, harmful or protective, they all display the complexity of the immune networking system. Here, we chose to focus our study on the NKG2D pathway, postulating that within the HBV-infected liver, it constitutes an important part of NK cell immune communication as well as stress surveillance. Therefore, by investigating NK cell responses and exploring cross talk with T cells through the NKG2D axis, we aimed to shed light on a new aspect of HBV immunology of potential relevance for therapies to combat this infection.

4.2 Aim and Hypotheses

Aim of the study:

Through study of the NKG2D pathway, we aimed to investigate the potential driving forces for pathogenic immunoregulation of NK cells in CHB.



Hypothesis:

1. We hypothesized that NKG2D-L expressing cells could activate cytotoxicity of NK cells in CHB.
2. We hypothesized that the potent immunomodulatory cytokine, IFN- α may augment NK cell regulation via the NKG2D pathway.

4.3 Results

4.3.1 NK cell activation, degranulation, and cytokine production driven by NKG2D-L expressing cells

In the previous chapter, our results showed that T cells upregulate NKG2D-L in CHB. We postulated that this would allow them to engage the NKG2D receptor on NK cells and drive NK cytotoxicity. To assess the impact of NKG2D engagement of NK cells from patients with CHB, we employed a B-lymphoblastoid cell line transfected with MICA*008 (C1R-MICA) as our target cells (kindly provided by Antoine Toubert, INSERM). A control cell line lacking MICA (C1R) served as a comparison ([Chauveau et al., 2014](#); [Tieng et al., 2002](#)). Isolated NK cells from CHB patients were co-cultured with target cells at 1:1 ratio for 6 hours. In this study, we compared the activation, degranulation, and cytokine production of NK cells while co-culturing with C1R or C1R-MICA cells to assess the increment in NK cell responses upon NKG2D engagement. Considering TRAIL induction on NK cells is a potent regulation pathway involved in HBV-specific T cell deletion ([Peppia et al., 2013](#)), we also evaluated TRAIL expression in the same co-culture setting.

Our results revealed that encounters with NKG2D-L expressing cells could drive activation of NK cells from CHB patients. Notably, compared to other activation markers (CD25 or CD69), HLA-DR acted as a sensitive indicator to reflect NKG2D engagement responses in both total NK cells and, more strikingly, in the NKG2D positive fraction of NK cells (Figure 4.1A-C). The increase in activation of total NK

cells was consistent with the fact that a large proportion of NK cells express NKG2D.

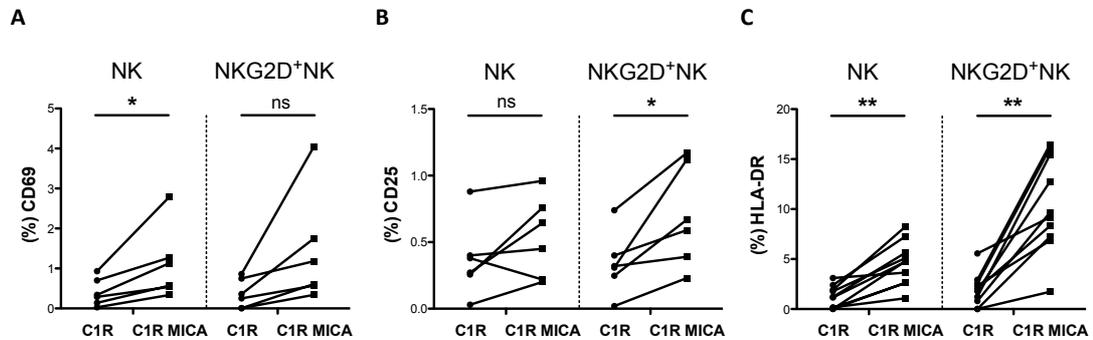


Figure 4.1 Evaluation of NK cell activation driven by NKG2D-L expressing cells.

- A Comparison summary of activation marker (CD69) changes of NK/NKG2D⁺NK cells in the setting of isolated NK cells co-cultured with C1R or C1R-MICA cells (CHB NK n=6; baseline levels of isolated NK cells were subtracted; analysed by Wilcoxon signed rank test).
- B Comparison summary of activation marker (CD25) changes of NK/NKG2D⁺NK cells in the setting of isolated NK cells co-cultured with C1R or C1R-MICA cells (CHB NK n=6; baseline levels of isolated NK cells were subtracted; analysed by Wilcoxon signed rank test).
- C Comparison summary of activation marker (HLA-DR) changes of NK/NKG2D⁺NK cells in the setting of isolated NK cells co-cultured with C1R or C1R-MICA cells (CHB NK n=10; baseline levels of isolated NK cells were subtracted; analysed by Wilcoxon signed rank test).

Moreover, by comparing responses of isolated NK cells in co-culture with control C1R or C1R-MICA cells, our results also showed that NKG2D engagement could effectively activate degranulation (CD107a) and IFN- γ production in total NK cells and NKG2D⁺NK cells (Figure 4.2A and B). TRAIL ligand, by contrast, was not significantly induced by NKG2D engagement. (Figure 4.2C).

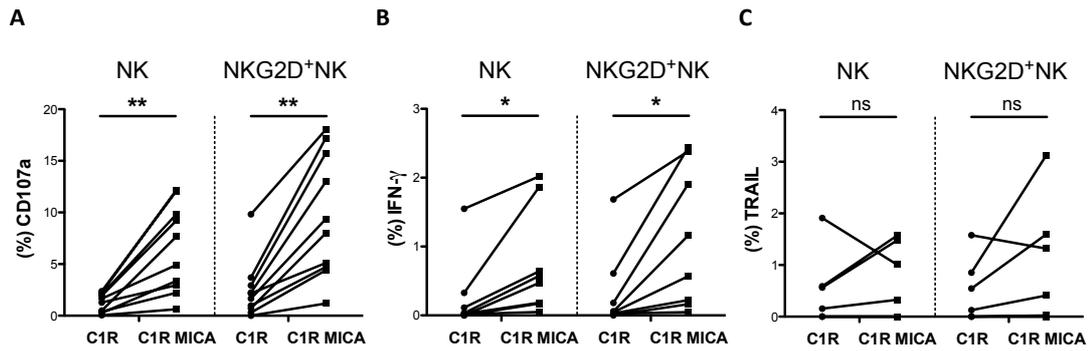


Figure 4.2 Evaluation of NK cell degranulation (CD107a), cytokine production (IFN- γ), and death ligand (TRAIL) expression induced by NKG2D-L expressing cells.

- A Comparison summary of degranulation (CD107a) changes of NK/NKG2D⁺NK cells in the setting of isolated NK cells co-cultured with C1R or C1R-MICA cells (CHB NK n=10; baseline levels of isolated NK cells were subtracted; analysed by Wilcoxon signed rank test).
- B Comparison summary of cytokine production (IFN- γ) changes of NK/NKG2D⁺NK cells in the setting of isolated NK cells co-cultured with C1R or C1R-MICA cells (CHB NK n=8; baseline levels of isolated NK cells were subtracted; analysed by Wilcoxon signed rank test).
- C Comparison summary of death ligand (TRAIL) expression changes of NK/NKG2D⁺NK cells in the setting of isolated NK cells co-cultured with C1R or C1R-MICA cells (CHB NK n=6; baseline levels of isolated NK cells were subtracted in the representative data; analysed by Wilcoxon signed rank test).

Knowing that the NKG2D pathway as a stress surveillance system can trigger cytotoxicity towards NKG2D-L expressing targets (Eagle and Trowsdale, 2007; Shafi et al., 2011), we next examined whether NK cells from CHB patients would properly react to target cells with different NKG2D-L levels. Isolated NK cells from CHB patients were co-cultured with target cells at 1:1 ratio for 6 hours. Different MICA levels of target cells were manipulated by mixing C1R cells and C1R-MICA cells at different ratios. HLA-DR and CD107a staining were used for evaluating NK cell activation and degranulation activity, respectively. Live/Dead and Caspase 3 were stained for determining target cell viability.

In this experiment, we found that the cell activation and cytotoxic degranulation of NKG2D⁺NK cells positively correlated with the MICA levels of target cells (Figure 4.3A-C).

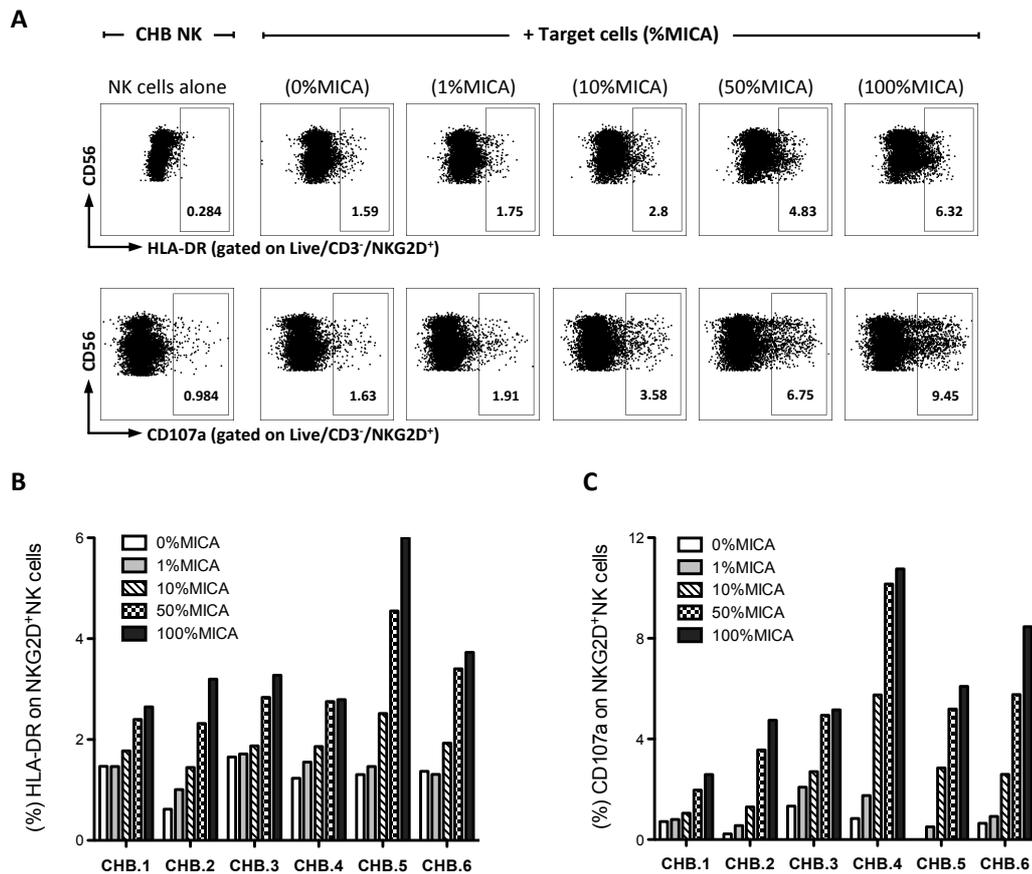


Figure 4.3 Evaluation of NK cell activation (HLA-DR) and degranulation (CD107a) induced by target cells with different NKG2D-L levels.

- A Representative plots of NKG2D⁺NK cell activation (HLA-DR) and cytotoxicity (CD107a) in isolated NK cells (baseline) and in those co-cultured with target cells expressing different levels of MICA (MICA expression levels of target cells were manipulated by mixing C1R cells and C1R-MICA cells at different mix ratios).
- B Summary results of NKG2D⁺NK cell activation (HLA-DR) changes upon co-culture of isolated NK cells and target cells with different MICA expression levels (Baseline levels were subtracted).
- C Summary results of NKG2D⁺NK cell degranulation (CD107a) changes upon co-culture of isolated NK cells and target cells with different MICA expression levels (Baseline levels were subtracted).

Simultaneously, we also evaluated the corresponding target cell viability. In line with the increased activation and degranulation of NK cells, the induced target cell cytotoxicity (all apoptotic and dead cells) also positively correlated with their MICA levels (Figure 4.4A and B).

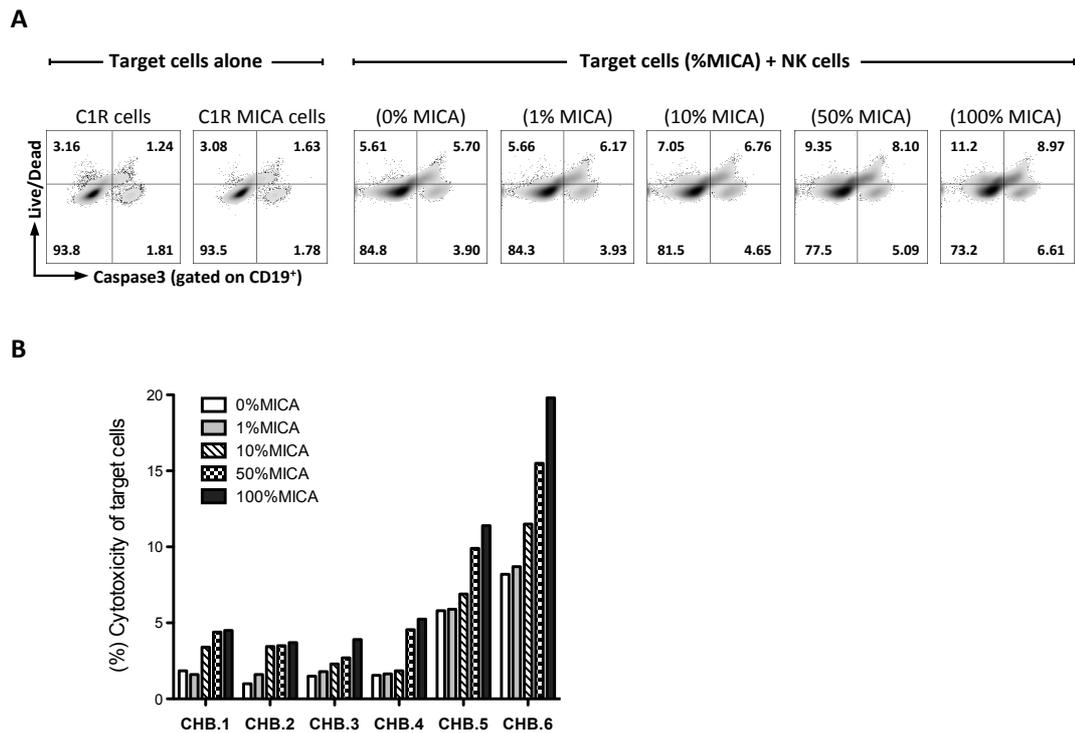


Figure 4.4 Evaluation of target cell viability changes in the co-culture of isolated NK cells and target cells with different NKG2D-L levels.

- A Cell viability was evaluated by Live/Dead and Caspase 3 staining; lower left, lower right, upper right and upper left quadrant indicate viable, early apoptotic, late apoptotic, and necrotic populations, respectively. Representative plots of baseline viability in original target cells (C1R/C1R-MICA) and viability changes in different MICA expressing target cells co-cultured with NK cells.
- B Target cell cytotoxicity summary of panel A (Target cell cytotoxicity was the sum of all apoptotic and dead cells after subtraction of levels seen in baseline C1R/C1R-MICA cells).

To sum up, this co-culture study illustrates that activation and degranulation activity of NK cells from CHB patients can be triggered according to the NKG2D-L levels of

target cells, which consequently leads to proportional apoptosis and death of target cells induced by the NKG2D pathway.

4.3.2 Potent NKG2D-mediated cytotoxic degranulation of NK cells in chronic HBV infection

In view of the noticeable increases of activation, degranulation, and cytokine production by NK cells following NKG2D engagement, we then compared responses in healthy controls and CHB patients. Isolated NK cells from healthy individuals or patients were co-cultured with either C1R cells or C1R-MICA cells at 1 to 1 ratio for 6 hours. Enhanced responses from NK cells co-cultured with C1R-MICA cells compared to those co-cultured with C1R cells were regarded as NKG2D engagement effects. Additionally, background levels in isolated NK cells were evaluated to exclude unspecific or allogeneic reactions.

MICA expressing target cells could significantly trigger the IFN- γ production and activation (HLA-DR) of NKG2D⁺NK cells in both healthy controls and CHB patients (Figure 4.5A-D). In parallel comparison of the increments, we found that the NKG2D-induced IFN- γ production and HLA-DR in patients were comparable to those in healthy controls (Figure 4.5A-D and data not shown).

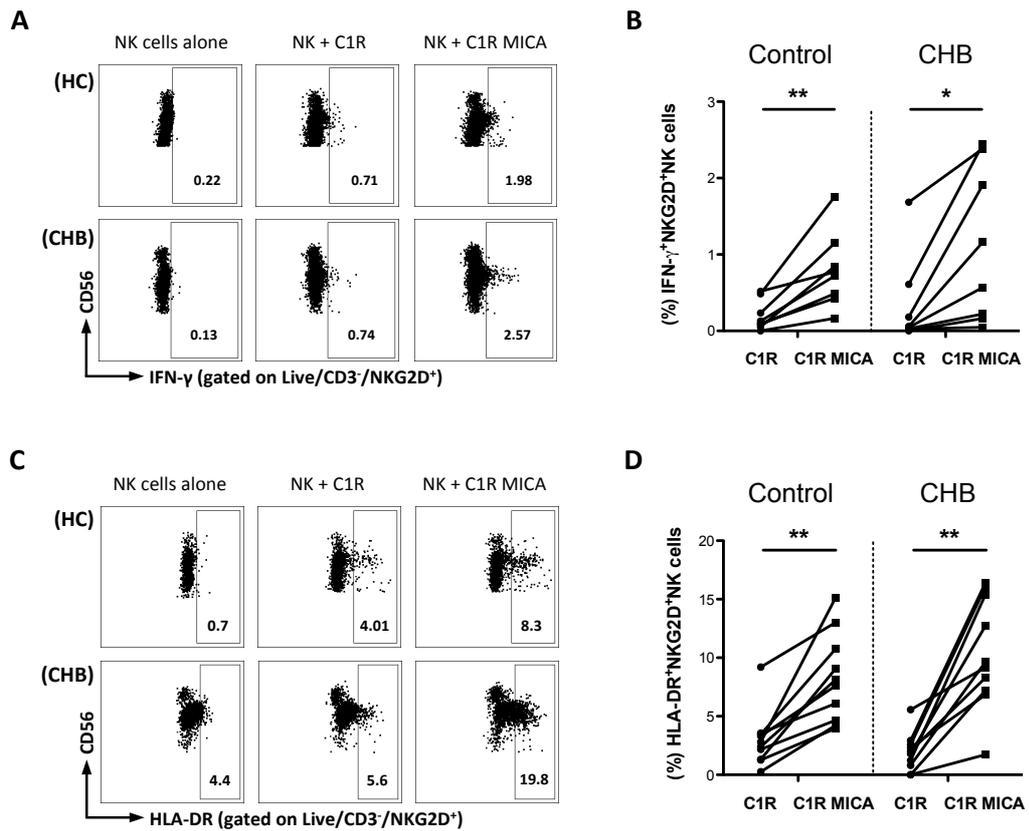


Figure 4.5 Evaluation of NKG2D engagement induced cytokine production (IFN- γ) and activation (HLA-DR) changes in NK cells from healthy controls and CHB patients

- A Representative plots of NKG2D⁺NK cell IFN- γ production in isolated NK cells (baseline) and in those co-cultured with C1R or C1R-MICA cells.
- B Summary results of panel A (HC n=8; CHB n=8; baseline levels were subtracted; analysed by Wilcoxon signed rank test).
- C Representative plots of NKG2D⁺NK cell activation (HLA-DR) in isolated NK cells (baseline) and in those co-cultured with C1R or C1R-MICA cells.
- D Summary results of panel C (HC n=10; CHB n=10; baseline levels were subtracted; analysed by Wilcoxon signed rank test).

In the same study arrangement, we also examined the degranulation activity (CD107a) of NK cells. Even though the MICA-expressing cells could effectively enhance CD107a in both healthy controls and CHB patients, the NKG2D engagement-induced degranulation of NK and NKG2D⁺NK cells was more pronounced in CHB patients (a mean 2.4-fold higher, Figure 4.6A-C).

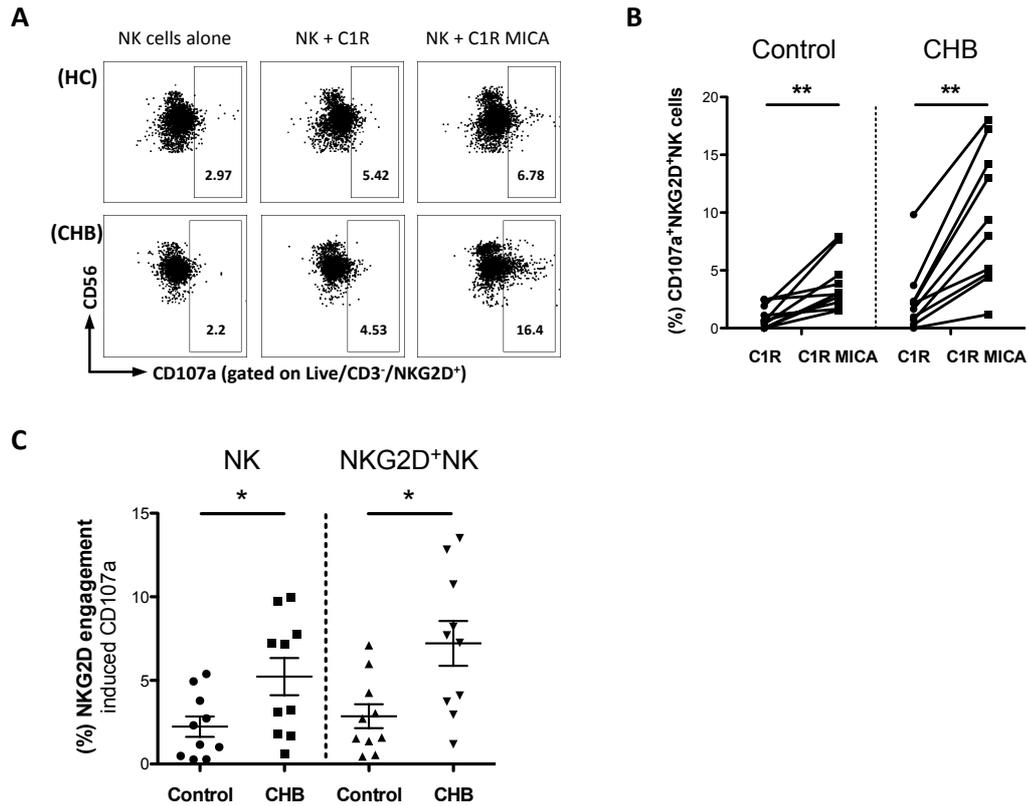


Figure 4.6 Evaluation of NKG2D engagement induced degranulation (CD107a) changes in NK cells from healthy controls and CHB patients

- A Representative plots of NKG2D⁺NK cell degranulation (CD107a) in isolated NK cells (baseline) and in those co-cultured with C1R or C1R-MICA cells.
- B Summary results of panel A (HC n=10; CHB n=10; baseline levels were subtracted; analysed by Wilcoxon signed rank test).
- C Difference in enhanced levels of total NK/NKG2D⁺NK cell degranulation (CD107a) from NK cells co-cultured with C1R cells to those co-cultured with C1R-MICA cells (HC n=10; CHB n=10; analysed by Mann Whitney test).

Next, we evaluated target cell viability in the same co-culture setting to compare the NKG2D-induced cytotoxicity by NK cells of healthy controls and CHB patients. In agreement with their potent degranulation activity upon NKG2D engagement, NK cells from CHB patients caused greater apoptosis and death of the MICA-expressing target cells (Figure 4.7A-C). Moreover, the induced apoptosis and death of C1R-MICA cells could be effectively reversed by NKG2D blockade via

anti-NKG2D mAb, indicating a direct effect from NKG2D/NKG2D-L interactions (Figure 4.7A and B).

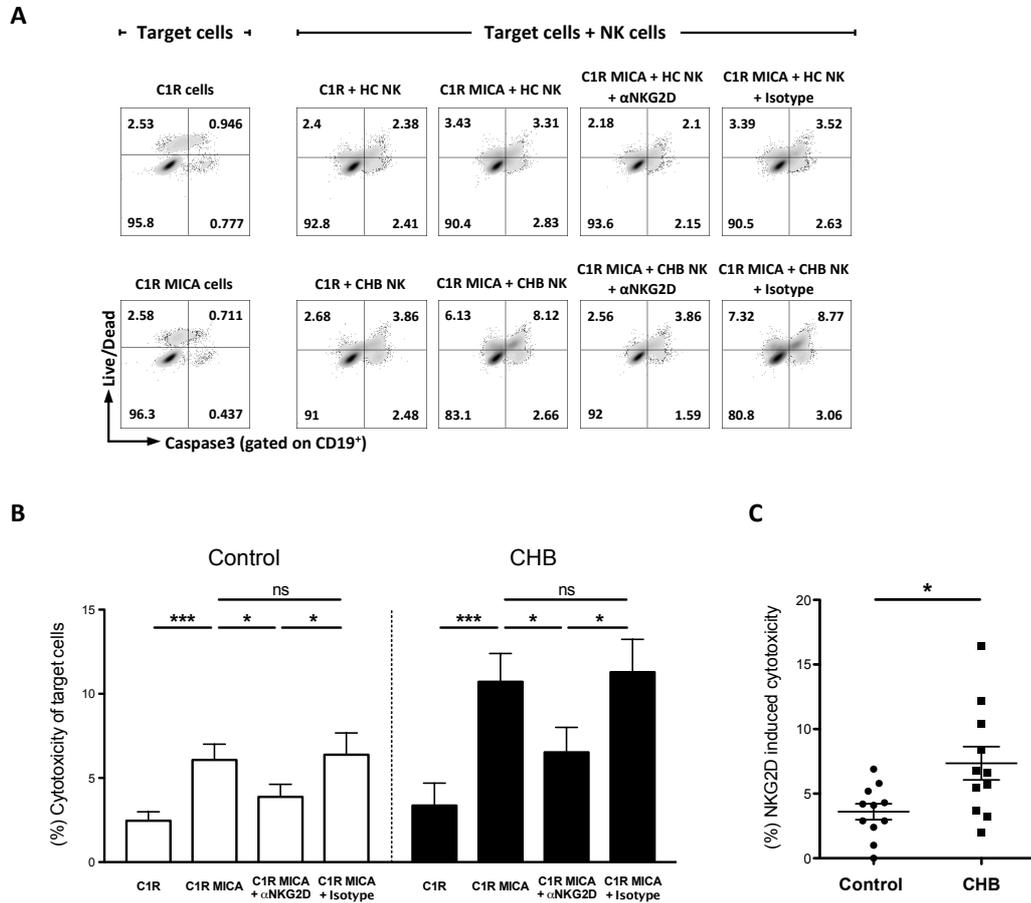


Figure 4.7 Evaluation of NKG2D engagement induced viability changes in target cells co-cultured with isolated NK cells from healthy controls or CHB patients.

- A Representative plots of target cell viability in original cell lines (C1R/C1R-MICA) and in those co-cultured with NK cells from healthy controls or CHB patients. Rescue of target cell apoptosis and death was conducted by adding NKG2D blocking mAb, using the IgG1 control isotype for comparison.
- B Target cell cytotoxicity summary of panel A (HC n=11; CHB n=11). Target cell cytotoxicity was the sum of all apoptotic and dead cells; baseline levels of C1R cells or C1R-MICA cells were subtracted; analysed by Friedman test).
- C Comparison summary of induced target cell cytotoxicity from C1R cells to C1R-MICA cells while co-cultured with NK cells (HC n=11; CHB n=11; analysed by Mann Whitney test).

In conclusion, our study demonstrates that cytotoxic immune responses by NK cells can be more effectively stimulated by NKG2D engagement in patients with CHB than healthy controls. Moreover, NK cells from CHB patients can cause potent cytotoxicity of target cells through the NKG2D pathway.

4.3.3 NK cell activation responds to stressed T cells through NKG2D pathway in chronic HBV infection

In CHB, the infected and inflamed liver milieu may alter NK cell phenotype and function. Down-regulation of the NKG2D receptor has been reported to occur in some situations of pathologic over-stimulation ([Coudert et al., 2005](#); [Groh et al., 2002](#); [Oppenheim et al., 2005](#)). Considering that NKG2D expression may be affected by chronic HBV infection, we therefore evaluated the NKG2D levels on NK cells in PBMC and IHL from CHB patients. For comparison, we also used PBMC from healthy individuals and IHL of non-HBV infected controls, either from healthy tissue from liver metastasis resections or from transplant perfusates of deceased donor livers. Results showed both peripheral and intrahepatic NK cells from CHB patients maintained high levels of NKG2D similar to those from non-HBV infected controls (Figure 4.8A and B). Moreover, when compared to their peripheral counterparts, the levels of NKG2D were, surprisingly, even higher on intrahepatic NK cells of CHB patients (Figure 4.8A and B).

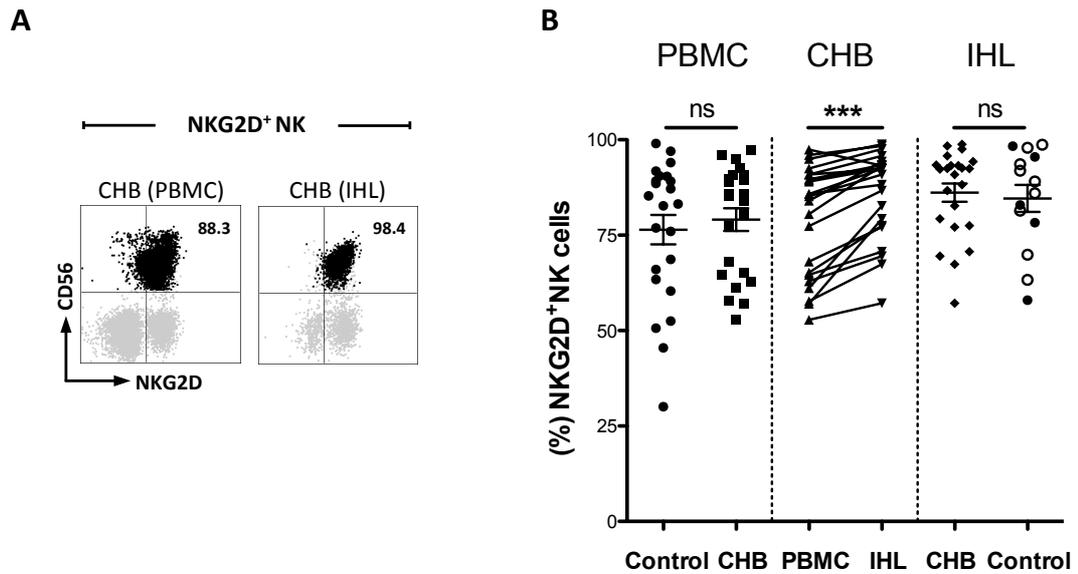


Figure 4.8 Evaluation of NKG2D expression of NK cells in chronic HBV infection.

- A Representative plots of *ex vivo* NKG2D expression on NK cells from paired blood and liver samples of a CHB patient.
- B Summary results of *ex vivo* NKG2D expression on NK cells of PBMC from healthy controls (n=23), paired PBMC and IHL from CHB patients (n=23), and IHL from non-HBV infected livers (n=14; filled circles (n=5) indicate intrahepatic lymphocytes isolated from healthy part of liver resection tissue; open circles (n=9) indicate intrahepatic lymphocytes isolated from transplant perfusates of deceased donor livers; analysed by Mann Whitney test and Wilcoxon signed rank test)

Since our results showed that HLA-DR levels could sensitively reflect the activation responses of NK cells upon NKG2D engagement, we next evaluated the HLA-DR expression of NK cells directly *ex vivo* in PBMC and IHL from CHB patients and non-HBV infected controls. HLA-DR levels were significantly elevated on NK cells from CHB patients, especially the intrahepatic fraction, compared to those from PBMC or IHL of controls (Figure 4.9A and B). Upon further investigation of NKG2D⁺NK cell activation status, we also noticed that the HLA-DR levels were consistently upregulated on the NKG2D⁺ fraction of circulating and particularly intrahepatic NK cells from CHB patients, compared to those from non-HBV infected controls (Figure 4.9A and C).

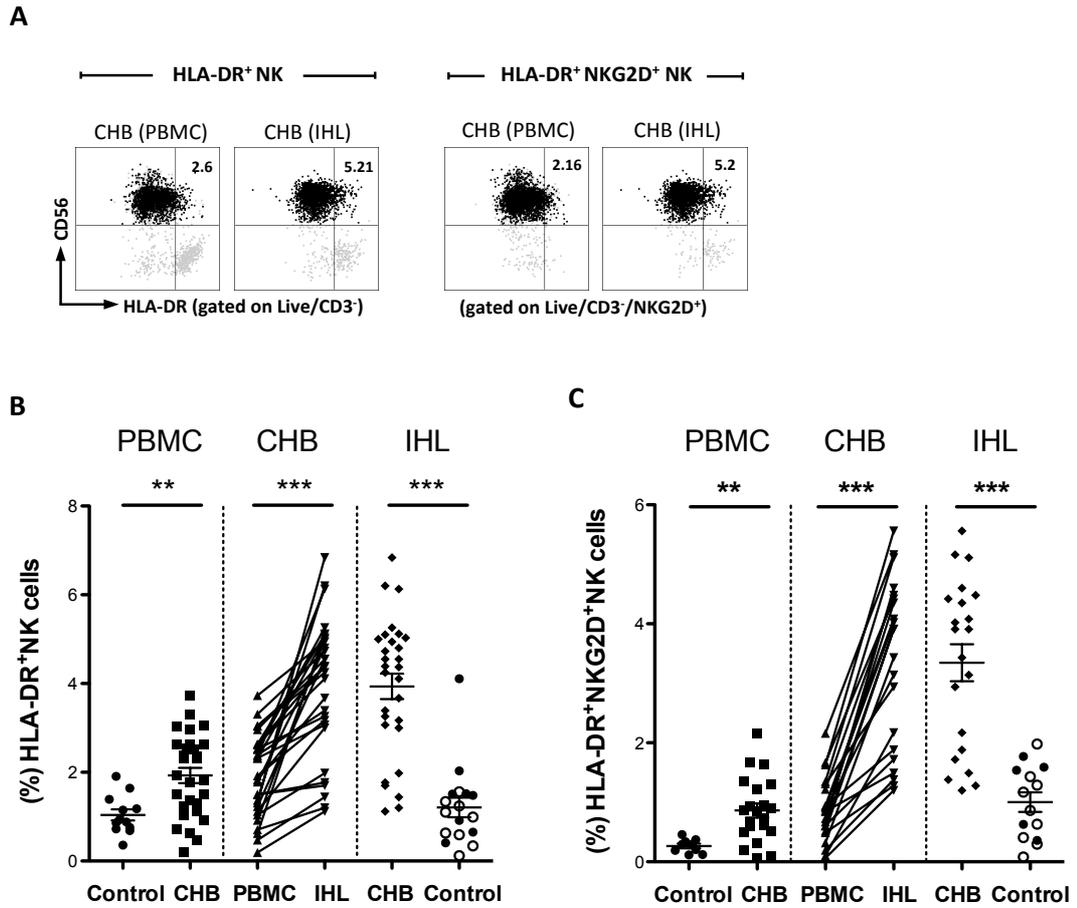


Figure 4.9 Evaluation of activation (HLA-DR) of NK cells in chronic HBV infection.

- A Representative plots of *ex vivo* HLA-DR staining of total NK/NKG2D⁺NK cells from paired blood and liver samples of a CHB patient.
- B Summary results of *ex vivo* HLA-DR levels on NK cells of PBMC from healthy controls (n=12), paired PBMC and IHL from CHB patients (n=29), and IHL from non-HBV infected livers (n=17; filled circles (n=8) indicate intrahepatic lymphocytes isolated from healthy part of liver resection tissue; open circles (n=9) indicate intrahepatic lymphocytes isolated from transplant perfusates of deceased donor livers; analysed by Mann Whitney test and Wilcoxon signed rank test)
- C Summary results of *ex vivo* HLA-DR levels on NKG2D⁺NK cells of PBMC from healthy controls (n=9), paired PBMC and IHL from CHB patients (n=21), and IHL from non-HBV infected livers (n=14; filled circles (n=5) indicate intrahepatic lymphocytes isolated from healthy part of liver resection tissue; open circles (n=9) indicate intrahepatic lymphocytes isolated from transplant perfusates of deceased donor livers; analysed by Mann Whitney test and Wilcoxon signed rank test)

We next sought possible correlations between T cell expression of NKG2D-L and associated activation of NK cell responses within patients with CHB. In the extended analysis of our *ex vivo* study, we found that the proportion of activated (HLA-DR) NKG2D⁺NK cells positively correlates with periphery and liver MICA/B levels on CD4 T cells in CHB patients (Figure 4.10A and B). Since the majority of NK cells were NKG2D⁺, we also observed a similar positive, but weaker correlation between activation of total NK cells and MICA/B levels on CD4 T cells in the CHB liver. However, no such correlation was found with MICA/B on CD8 T cells (data not shown).

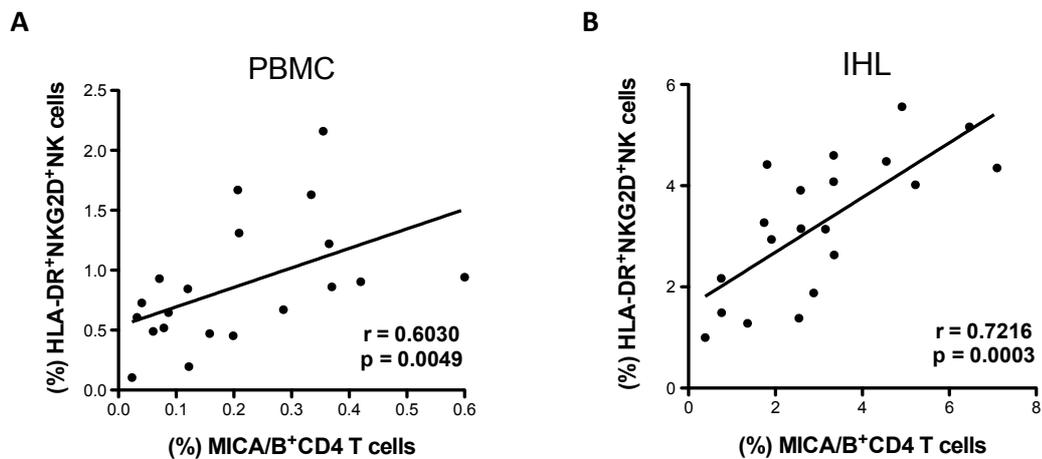


Figure 4.10 Evaluation of NK cell activation and the associated correlation with T cell MICA/B expression in chronic HBV infection.

- A Correlation summary of *ex vivo* MICA/B expression on peripheral CD4 T cells and HLA-DR levels of NKG2D⁺NK cells in CHB patients. (n=20; analysed by Spearman rank correlation test).
- B Correlation summary of *ex vivo* MICA/B expression on intrahepatic CD4 T cells and HLA-DR levels of NKG2D⁺NK cells in CHB patients (n=20; analysed by Spearman rank correlation test).

Results of these experiments led us to conclude that NK cells in CHB maintain high NKG2D levels and increased activation, particularly intrahepatic NK cells. The positive correlation between *ex vivo* NK cell activation and CD4 T cell NKG2D-L also implicates NK cell cross talk with T cells via the NKG2D pathway in CHB.

4.3.4 Promotion of NKG2D dependent regulation during interferon therapy of CHB patients

To date, interferon-alpha (IFN- α) with both immunomodulatory and anti-viral properties has been widely used as a first-line therapy for CHB ([Lampertico et al., 2015](#); [Lucifora et al., 2014](#); [Trepo et al., 2014](#)). Nevertheless, undesirable side effects and frequent ALT flares ([Nair and Perrillo, 2001](#); [Perrillo, 2009](#); [Saracco et al., 1994](#); [ter Borg et al., 2008](#)) limit the clinical usage of IFN- α therapy. Additionally, recent research has shown that IFN signalling drives oxidative stress-mediated liver injury in viral hepatitis ([Bhattacharya et al., 2015](#)), which is consistent with the transient increases in serum and intrahepatic IFN- α observed in CHB patients with hepatitis flares ([Dunn et al., 2007](#); [Fang et al., 1994](#); [Wu et al., 2013](#)). We therefore postulated that IFN- α therapy may impact NKG2D-dependent regulation in CHB.

To probe this possibility, we first compared the MICA/B expression on T cells of patients with CHB, both before and during pegylated interferon- α (PEG-IFN- α) therapy. By *ex vivo* staining of paired samples, we observed that T cell MICA/B expression increased when patients underwent PEG-IFN- α treatment (Figure 4.11A and B).

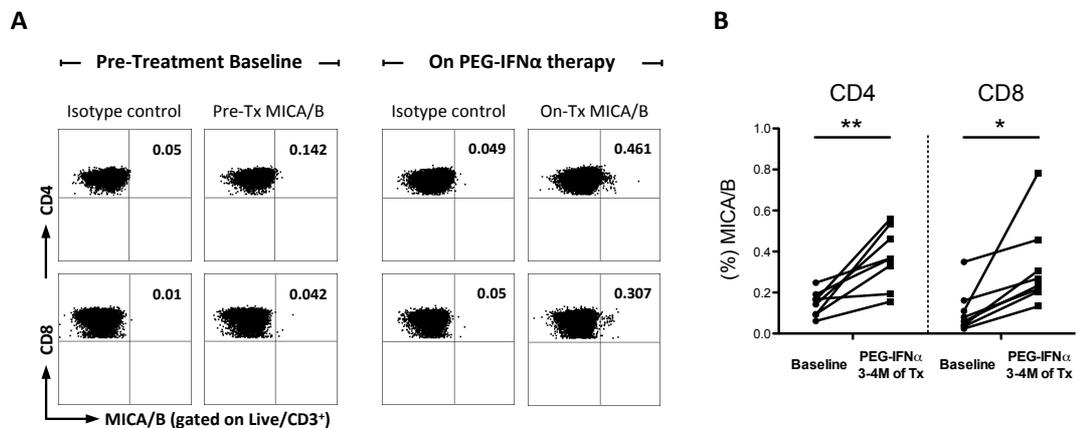


Figure 4.11 Evaluation of effects of pegylated interferon- α (PEG-IFN α) therapy on T cell NKG2D-L induction.

- A Representative plots of *ex vivo* MICA/B expression on CD4 and CD8 T cells from a CHB patient before and during PEG-IFN- α treatment.
- B Comparison summary of panel A (n=8; gates set by control isotype staining; analysed by Wilcoxon signed rank test).

Moreover, in a thorough longitudinal study of one patient, we found other NKG2D ligands, such as ULBP1, ULBP2/5/6 and ULBP3 also increased gradually during PEG-IFN- α therapy as well as MICA/B. (Figure 4.12A and B).

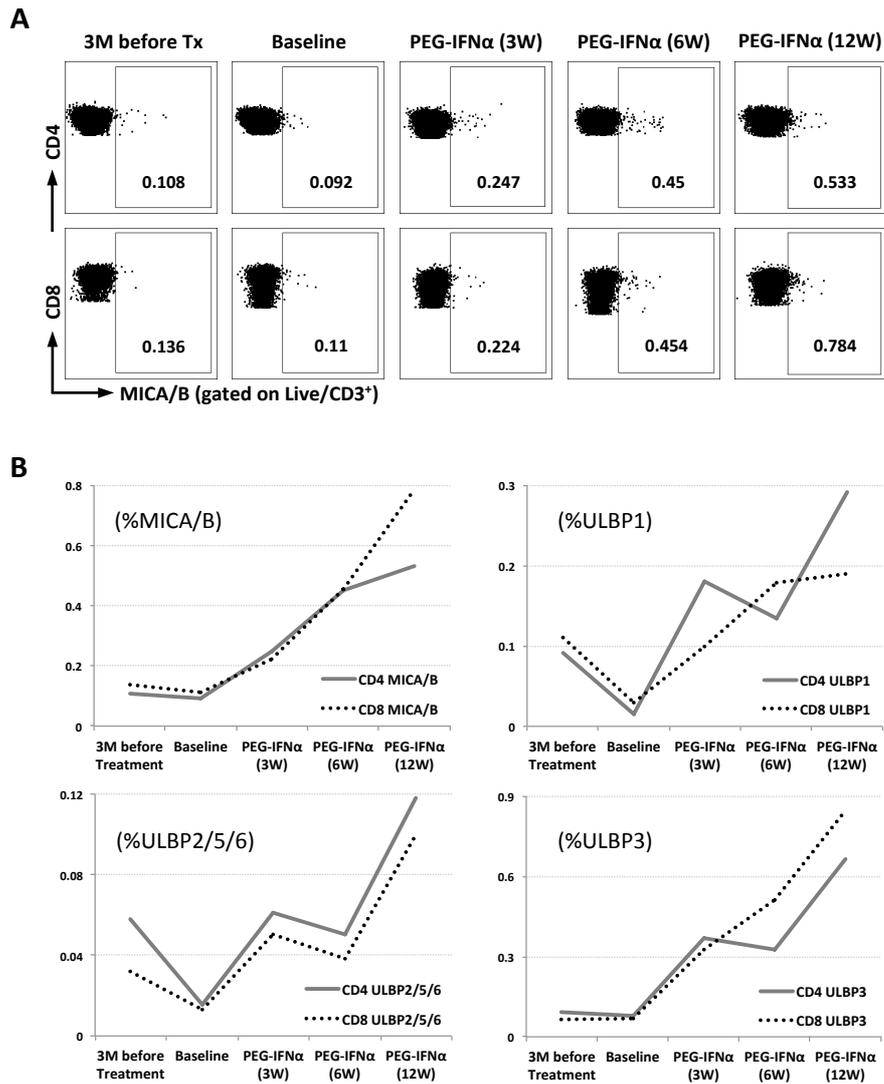


Figure 4.12 Longitudinal evaluation of pegylated interferon- α (PEG-IFN α) therapy on T cell NKG2D-L induction.

- A Longitudinal study of a CHB patient undergoing PEG-IFN- α therapy. Representative plots of *ex vivo* T cell MICA/B expression at 5 different intervals: 3 month pre-treatment, baseline pre-treatment, week three, week six, and week twelve of treatment.
- B Summary results of MICA/B, ULBP1, ULBP2/5/6, and ULBP3 expression on T cells from the same CHB patient undergoing PEG-IFN- α therapy.

Next, we examined the direct effect of IFN- α on NK cell degranulation activity (CD107a). Purified NK cells isolated from a healthy donor were treated with interferon- α at different concentrations for 4 hours. Consistent with the general consensus (Ahlenstiel et al., 2010; Vivier et al., 2008), we observed that IFN- α

treatment could effectively enhance the cytotoxic degranulation of NK cells, especially the NKG2D positive fraction in a dose-dependent manner (Figure 4.13).

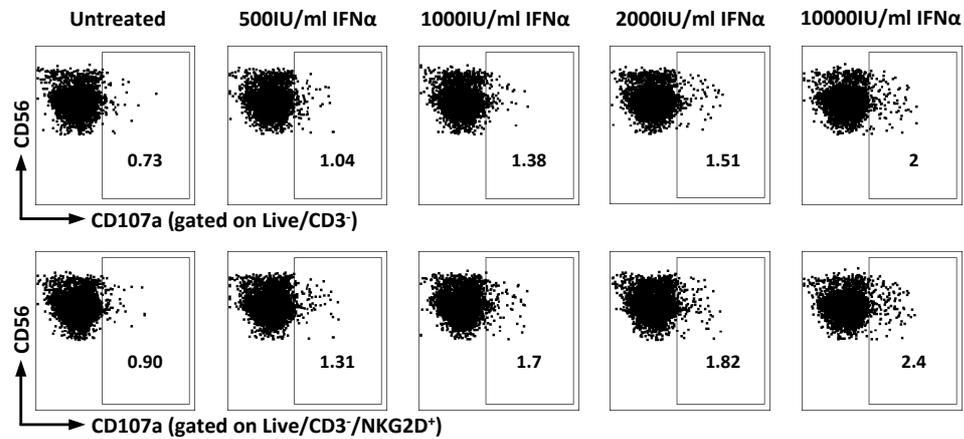


Figure 4.13 Evaluation of interferon- α effects on NK cell degranulation activity.

Purified NK cells isolated from a healthy donor were treated with interferon- α at different concentrations for 4 hours. Representative plots of NK cell (upper panels) and NKG2D⁺NK cell (lower panels) degranulation (CD107a) changes in untreated and interferon- α treated NK cells.

Subsequently, we further investigated whether IFN- α treatment can augment NK cell killing of NKG2D-L expressing target cells. NK cells isolated from PBMC of CHB patients or healthy controls were treated with 1000 U/ml IFN- α for 4 hours. After two washes, untreated or IFN- α treated NK cells were co-cultured with C1R-MICA cells at 1:1 ratio for 6 hours. Target cells (C1R/C1R-MICA) without NK cell co-culture were also tested to exclude other unspecific or allogeneic reactions. By adding anti-NKG2D blocking mAb, NKG2D-mediated cell apoptosis and death were reversed in both co-culture conditions (Figure 4.14A). However, compared to the IFN- α untreated (UT) condition, we noticed an enhancement of NKG2D-mediated killing of targets in the IFN- α pre-treatment setting (Figure 4.14A and B). These

findings suggest that IFN- α pre-treatment could intensify NK cell killing towards target cells via the NKG2D pathway in both healthy controls and CHB patients.

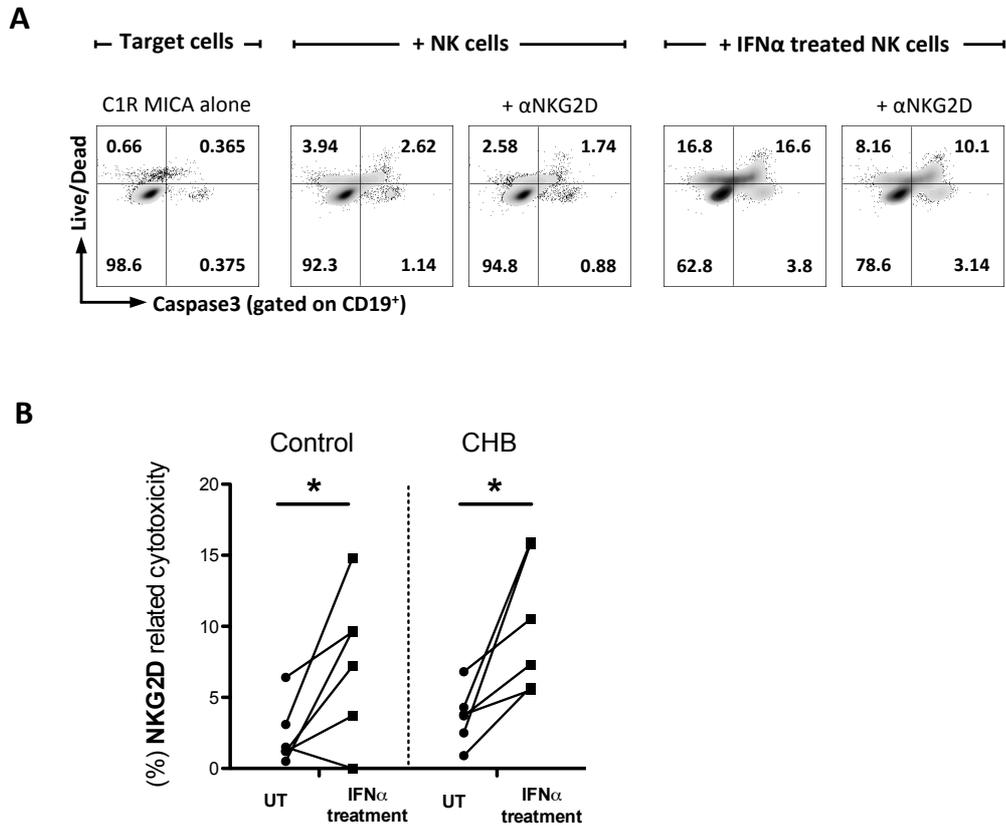


Figure 4.14 Evaluation of effects of interferon- α on NK cell killing towards NKG2D-L expressing cells.

- A Representative plots of target cell viability in C1R-MICA cells alone and in those co-cultured with untreated or IFN- α pretreated NK cells with/without NKG2D blockade.
- B Summary of NKG2D pathway related target cell cytotoxicity in untreated (UT) and IFN- α pretreated conditions (HC n=6; CHB n=6. NKG2D pathway related cytotoxicity was determined by viable cell (lower left quadrant) restoration upon NKG2D blockade in the co-culture of C1R-MICA cells with untreated or IFN- α pretreated NK cells; analysed by Wilcoxon signed rank test).

To sum up, our results imply that through inducing NKG2D-L on T cells or boosting NK cell cytotoxicity, IFN- α signalling pathway is able to enhance NKG2D-mediated regulation.

4.4 Conclusions and Discussion

It is known that NK cells participate in early cellular defence to virus infection (Vivier et al., 2008). However, accumulating evidence has also established their immune regulatory role (Crome et al., 2013; Lang et al., 2012; Waggoner et al., 2010). In chronic viral infection, NK cells have been reported to mediate disease pathology and compromise anti-viral immunity (Rehermann, 2013; Rydyznski et al., 2015; Schuster et al., 2014; Waggoner et al., 2012; Waggoner and Kumar, 2012). Indeed, NK cells have various dysfunctional traits in chronic HBV infection including defective non-cytolytic anti-viral function, and the capacity to impair antiviral T cell responses or mediate liver damage (Dunn et al., 2007; Ju et al., 2010; Peppia et al., 2010; Shi et al., 2012; Zhang et al., 2011b). Recently, our group has shown that NK cells can eliminate HBV-specific CD8 T cells via the TRAIL pathway as well as causing liver damage by killing HBV-infected hepatocytes (Peppia et al., 2013). Since NKG2D is a major activating receptor of NK cells with an important role in immune regulation and lymphoid stress surveillance (LSS) (Shafi et al., 2011; Strid et al., 2011), we chose to focus our study on the NKG2D axis.

We first examined the reactions of NK cells following NKG2D engagement with a cell line transfected with MICA to allow a careful dose-response analysis. In our *in vitro* co-culture study, we observed that NKG2D-L expressing cells could effectively drive various effector responses of patient NK cells. In line with the general consensus that NKG2D is a highly sensitive stress sensor for NK cells (Shafi et al., 2011), our study also demonstrated dose-dependent activation and cytotoxicity

towards NKG2D-L expressing cells. In the context of MICA- or ULBP2-transfected CHO cells, it has been shown that NKG2D-expressing NK cells can respond to relatively small changes in target cell NKG2D-L expression (Shafi et al., 2011). This was confirmed by our experiments titrating a MICA-expressing cell line and observing that as few as 10% MICA-expressing cells could activate NKG2D-dependent NK cell cytotoxicity. This frequency is likely to be fairly representative of the situation found within the liver sinusoids, where hepatic NK cells would first encounter HBV-specific T cells (Guidotti et al., 2015a; Peppas et al., 2013), a large fraction of which expressed NKG2D-L. Additionally, NK cells from CHB patients presented greater degranulation activity upon NKG2D engagement compared to those from control subjects. The subsequent viability assays revealing greater NKG2D-induced cytotoxicity of target cells by patient NK cells supported this finding. This first demonstration that NK cells from CHB patients show enhanced responsiveness to NKG2D-L merits further investigation. As illustrated in recent human NK cell research, clusters of the inhibitory receptor KIR2DL1 on the surface of resting NK cells can influence signal thresholds and integration, thereby altering NKG2D responses of NK cells (Pageon et al., 2013). Moreover, previous studies also confirm that full effector function of naïve NK cells from healthy individuals can not be achieved merely by NKG2D stimulation alone, but required other cytokines or “priming” through other receptors (Bryceson et al., 2006b). Therefore, our results could not be accounted for simply by the levels of NKG2D expression, but instead suggest that NK cells from CHB patients may have different

thresholds of responsiveness to NKG2D engagement compared to naïve NK cells from healthy individuals.

Secondly, we examined whether the HBV-infected and pathogenic liver milieu could interfere with NK cell phenotype and function (Maini and Peppas, 2013). Recent literature suggests that in some pathologic stimulations, over-exposure to soluble or membrane-bound NKG2D ligands may cause NKG2D down-regulation (Coudert et al., 2005; Groh et al., 2002; Oppenheim et al., 2005). However, in line with previous HBV research (Boni et al., 2015; Oliviero et al., 2009; Zhang et al., 2011b; Zhao et al., 2012), our study revealed NK cells from CHB patients maintained high levels of NKG2D and were highly activated compared to those from healthy controls. By extensive analysis of paired blood and liver samples, we showed for the first time that, paradoxically, NKG2D is even more highly expressed on intrahepatic than peripheral NK cells. Similarly, high levels of NKG2D were noted in uninfected livers, indicating that the hepatic niche supports NKG2D expression, although there was considerable unexplained inter-donor variability. Further studies in our group will investigate the mechanisms favouring NKG2D expression in the liver. Possible candidates are cytokines such as IL-15 and IFN- α which can accumulate within the HBV-infected liver (Fang et al., 1994; Wu et al., 2013; Zhang et al., 2011b) and are known to enhance effector function and NKG2D expression of NK cells (Burgess et al., 2008; Dann et al., 2005; Konjevic et al., 2010; Nielsen et al., 2012; Tang et al., 2013). Collectively, our results reveal that NK cells maintain high NKG2D expression and NKG2D-mediated regulation is fostered in CHB.

NK cells are known to be more activated and cytotoxic in the HBV-infected liver but factors driving this have not been previously identified (Dunn et al., 2007; Zhang et al., 2011b). Here we have identified a selective increase in activation of the NKG2D⁺ fraction of intrahepatic NK cells, pointing to a key role for this major activating receptor. We go on to demonstrate that NKG2D-expressing NK cells have an exaggerated activation of cytotoxicity in response to NKG2D-L and to identify a source of NKG2D-L expressing T cells that would be expected to come into close contact with NK cells within the hepatic vasculature. We provide *ex vivo* evidence suggesting that induction of NKG2D-L on liver-infiltrating CD4 T cells is a factor driving the activation of intrahepatic NK cells in the HBV-infected liver.

Interferon-alpha (IFN- α) is a first-line regimen and the only currently available treatment that can target HBV cccDNA (Lampertico et al., 2015; Lucifora et al., 2014). However, the undesirable side effects and frequent ALT flares (Nair and Perrillo, 2001; Perrillo, 2009; Saracco et al., 1994; ter Borg et al., 2008) are legitimate concerns in clinical usage. Indeed, recent research has pointed out that type I IFN signalling activation can drive oxidative liver damage in murine viral hepatitis (Bhattacharya et al., 2015). In accordance with our previous study suggesting oxidative stress in CHB could be a factor for NKG2D-L induction, patients undergoing PEG-IFN- α therapy exhibited elevated levels of NKG2D-L on T cells. Moreover, the type I IFN signalling pathway has been shown to interfere with T cell immunity and consequently contribute to persistent viral infection (Osokine et al., 2014; Teijaro et al., 2013; Wilson et al., 2013). Our group has previously shown

that HBV IFN- α therapy has divergent effects, which leads to significant loss of T cells, but conversely boosts NK cell effector activity (Micco et al., 2013; Thimme and Dandri, 2013). Accordingly, besides the augmentation of NK cell activation, proliferation, and cytokine production, many studies documented both endogenous and therapeutic IFN- α can induce TRAIL upregulation on NK cells which could promote their killing of hepatocytes and T cells (Dunn et al., 2007; Edlich et al., 2012; Micco et al., 2013; Stegmann et al., 2010; Thimme and Dandri, 2013). Corresponding to our findings showing IFN- α could drive cytotoxic degranulation of NK cells, we also observed IFN- α could amplify NKG2D-mediated NK cell killing of target cells. Previous work has similarly shown that upregulation of MICA/B on IFN- α stimulated cells (Jinushi et al., 2003a; Jinushi et al., 2003b; Zhang et al., 2008) and IFN- α -driven enhancement of both NKG2D expression and NKG2D-mediated degranulation of NK cells (Burgess et al., 2008; Konjevic et al., 2010; Nielsen et al., 2012; Zhang et al., 2005). Collectively, these results, together with the Lang et al. murine LCMV study (Lang et al., 2012), all imply that IFN- α could play a key role in NKG2D-related NK cell regulation. Thus, to enhance anti-HBV responses and minimize unwanted inhibitory immunomodulation, manipulation of the NKG2D pathway may need to be considered in combination with IFN- α therapy.

To sum up, our research implies a paradoxical triangular relationship between HBV infection, innate immunity, and adaptive immunity. The inflammatory HBV-infected liver milieu with aberrant stress could lead to stress ligand upregulation on T cells, such as the NKG2D-L observed in our study. Upregulated NKG2D-L could drive

NK cell activation and cytotoxicity induction and subsequently contribute to NK cell-mediated T cell deletion via the NKG2D pathway. This pathogenic cellular communication could compromise anti-viral immunity and further facilitate persistent HBV infection as well as long-term liver inflammation. Therefore, to optimize hepatitis B treatment, attempts to diminish HBV replication and minimize hepatic oxidative stress should be prioritized, thereby breaking the vicious destructive cycle (Figure 4.15).

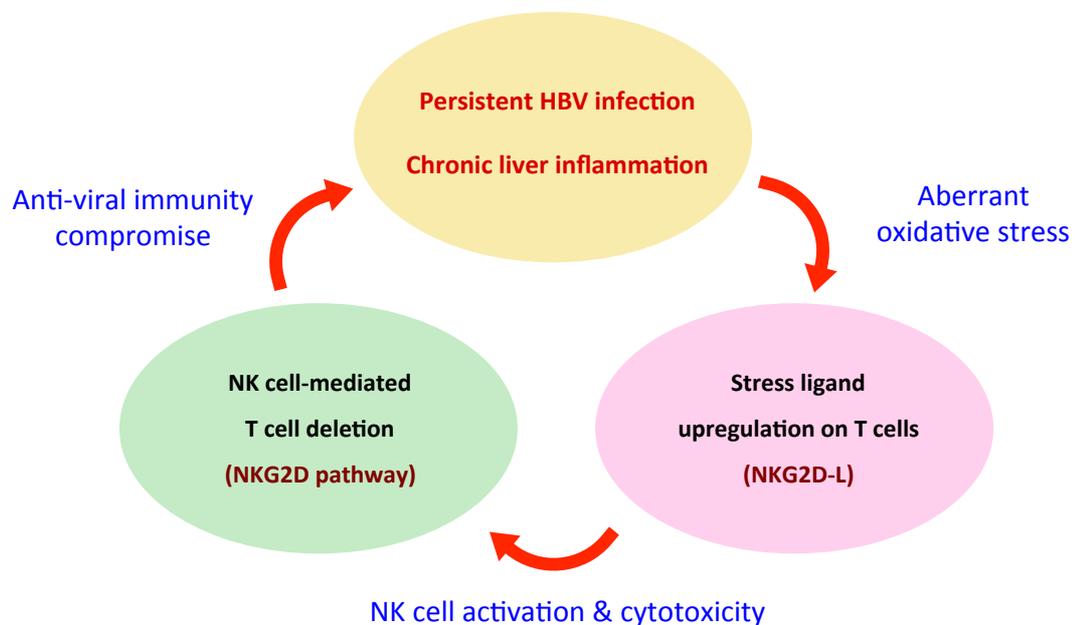


Figure 4.15 Depiction of the paradoxical relationship between HBV infection, innate immunity, and adaptive immunity in the NKG2D axis.

Chapter 5 Future Outlook and Potential Directions

Evaluating other CD4 T cell subsets in CHB

In our study, we have shown NKG2D-L preferentially enriched on CD4 T cells in CHB patients. In particular, activated and HBV-specific CD4 T cells expressed higher levels of NKG2D-L compared to other T cell populations. Comprising many different subsets, CD4 T cells have diverse immunological functions in viral infection. For example, regulatory CD4 T cells (Treg) possessing immunosuppressive properties have been implicated in suppression of anti-viral immunity and liver inflammation in HBV infection ([Manigold and Racanelli, 2007](#); [Xu et al., 2006](#)). In the extended analysis of our *ex vivo* study, we observed that CD25^{hi}CD127^{lo}Foxp3⁺CD4 Treg cells expressed slightly higher MICA/B than the general CD4 population (Figure 5.1A and B). However, the immunological implications of this observation still remain to be determined.

Moreover, in murine virus research, follicular helper CD4 T cells (T_{FH}) have been documented to be suppressed by NK cells, which leads to a weakened germinal centre response and compromised humoral immunity ([Rydyznski et al., 2015](#)).

Therefore, further investigation of CD4 T cell sub-populations in relation to the NKG2D axis or NK cell regulation may improve our understanding of immune defects in HBV chronicity.

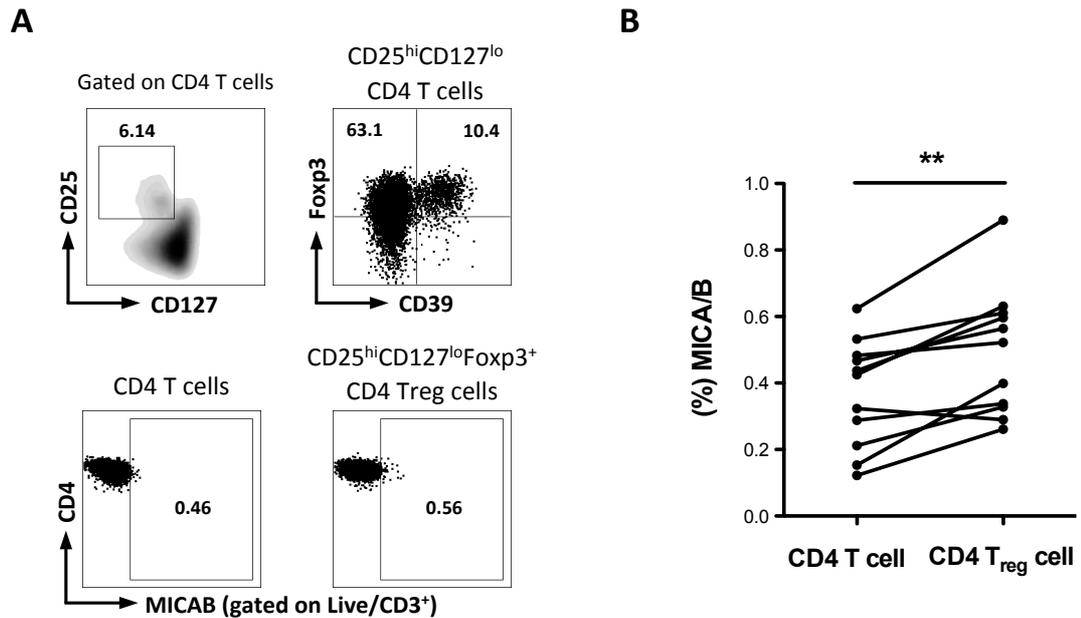


Figure 5.1 Evaluation of NKG2D-L expression on CD4 Treg cells in CHB patients.

- A CD4 Treg cells were identified by CD25^{hi}CD127^{lo} and Foxp3⁺ staining. Representative plots of *ex vivo* MICA/B expression on CD4 T cells and CD4 Treg cells from CHB patients.
- B Summary results of panel A (n=11; analyzed by Wilcoxon signed rank test).

Evaluating other pathways of NK cell regulation in CHB

Our results illustrated that NK cells have immune regulatory effects via the NKG2D pathway in chronic HBV infection. However, other pathways, such as DNAM-1 can also contribute to NK cell killing of T cells (Ardolino et al., 2011; Zingoni et al., 2012). Indeed, DNAM-1 and NKG2D are two mechanisms sharing many similarities in NK cell-mediated T cell regulation, particularly since ligands for both of these activating receptors can be induced on T cells by oxidative stress (OS) (Ardolino et al., 2011; Rabinovich et al., 2000; Zingoni et al., 2012). Furthermore, recent research also demonstrated that OS-induced DNAM-1 ligand expression is related to the

DNA damage responses (DDR) ([Ardolino et al., 2011](#)). Thus, evaluation of DNAM-1 pathway involvement and evidence of a T cell DDR (such as γ -H2AX or p38 phosphorylation assessment) in CHB patients would be interesting. From a therapeutic standpoint, using reactive oxygen species scavengers (such as N-acetylcysteine) or DDR inhibitors (such as ATM/ATR kinase inhibitors) to diminish NK cell regulation could be a promising approach.

Evaluating the T/NK cell interplay by longitudinal follow-up and murine models

HBV is a non-cytopathic, hepatotropic virus that persistently infects the majority of hepatocytes in chronic carriers ([Seeger and Mason, 2000](#); [Yoo et al., 1987](#)). Within an HBV-infected patient, the host immunity usually stays in a precarious balance between virus control and tissue damage ([Guidotti and Chisari, 2006](#); [Guidotti et al., 2015b](#)). Recent data suggest that through the course of HBV infection, NK cells and T cells can respond conversely, presenting an inverse correlation ([Boni et al., 2015](#); [Grimm et al., 2013](#); [Isogawa and Tanaka, 2015](#); [Peppia et al., 2013](#)). As such, in naïve CHB patients, NK cells with “inflammatory” phenotypes having regulatory activity show increased activation, proliferation and TRAIL expression ([Boni et al., 2015](#); [Peppia et al., 2013](#); [Zhang et al., 2011b](#)). In contrast, virus-suppressed and ALT-normalized patients with NK cells showing “resting” profiles have reciprocal improvement of the HBV-specific T cell immunity following nucleos(t)ide analogue treatment ([Boni et al., 2012](#); [Boni et al., 2015](#); [Thimme and Dandri, 2013](#)). In agreement with evidence indicating the pathogenic regulation of NK cells positively

corresponds with the hepatitis severity in CHB patients (Dunn et al., 2007; Zhang et al., 2011b), research also demonstrates the preserved T cell function in CHB patients with no/minimal liver inflammation in the immune-tolerant phase (Kennedy et al., 2012). Therefore, continuing our NKG2D research and further investigating T/NK cell interplay by longitudinal evaluation of patients with hepatitis flares or those undergoing anti-HBV treatment may provide substantial information for re-directing pathogenic immunomodulation towards favorable virus elimination.

Alternatively, using *in vivo* murine models (such as transgenic knockout or *in vivo* blockade) may offer better opportunities to promptly reflect actual cellular interactions, since *in vitro* functional assessments are difficult to reproduce real biological conditions for NK cells and T cells. However, the distinctly different NKG2D systems across species, and the fact that HBV is unable to replicate in mouse hepatocytes, make establishing an ideal animal/infection model for *in vivo* studies in this field an ongoing challenge (Allweiss and Dandri, 2016).

Evaluating other correlations between liver pathology and host immunity

In view of the fact that NK cells account for the largest fraction (30~40%) of intrahepatic lymphocytes, they have a key role in immunity and pathology modulation (Doherty et al., 1999; Dunn et al., 2007; Maini and Peppas, 2013). Our study has illustrated that the prominent NKG2D-L upregulation on T cells in the HBV-infected liver can contribute to the activation and cytotoxicity induction of NK cells to subsequently constrain adaptive immunity. However, while probing other

possible connections between the liver histological activity index (HAI) with NKG2D-L of T cells or activation of NK cells, we did not observe significant correlations in our patient cohort. This may be partly due to the lack of diversity in our subjects with a narrow homogeneous pattern of clinical parameters (ALT max 150; ISHAK fibrosis score 1~3; HAI 3~7).

Actually, this is a common issue in human liver research, since patients with greater severity of cirrhosis or liver function impairment are likely excluded from the invasive procedure of liver biopsy (Duygu et al., 2012). Moreover, the accessibility of human tissue also limits studies for other intrahepatic cell types (Kupffer cells, hepatocytes, or stellate cells) in the context of various liver diseases, such as HCV hepatitis, nonalcoholic steatohepatitis, or autoimmune hepatitis. Therefore, further research and expanding our data are essential and may reveal noteworthy links between liver pathology and host immunity in the future.

Evaluating NKG2D regulation of NK cells in CHB

As illustrated in our co-culture experiments, NKG2D expression on NK cells could be down-regulated by NKG2D-L expressing target cells (membrane-bound NKG2D-L) (Figure 5.2A and B). However, we did not witness this phenomenon in our *ex vivo* study. On the contrary, NK cells from CHB patients were highly activated and maintained high levels of NKG2D. One possible explanation could be that the cytokine milieu of the HBV-infected liver, such as the local expression of IL-15, can enhance NKG2D expression and also activate NK cells (Dann et al., 2005;

Tang et al., 2013; Zhang et al., 2011b). Alternatively, if soluble NKG2D-L (sNKG2D-L) are shed in CHB, they may participate in the maintenance of NKG2D expression. Although engagement by sNKG2D-L has been suggested to cause down-regulation of NKG2D on NK cells in most human research (Holdenrieder et al., 2006a; Raffaghello et al., 2004; Raulet et al., 2013; Salih et al., 2002), one recent murine study showed sNKG2D-L from tumor-associated cells could maintain NKG2D expression and boost NK cell activation to promote tumor rejection (Deng et al., 2015). Certainly, the NKG2D system in different species involving different receptors, ligand affinities and activating capabilities may cause opposing results in mice and humans. However, further investigation of the impact on NKG2D of various cytokines and sNKG2D-L from serum or hepatocytes is necessary and currently being carried out by our group.

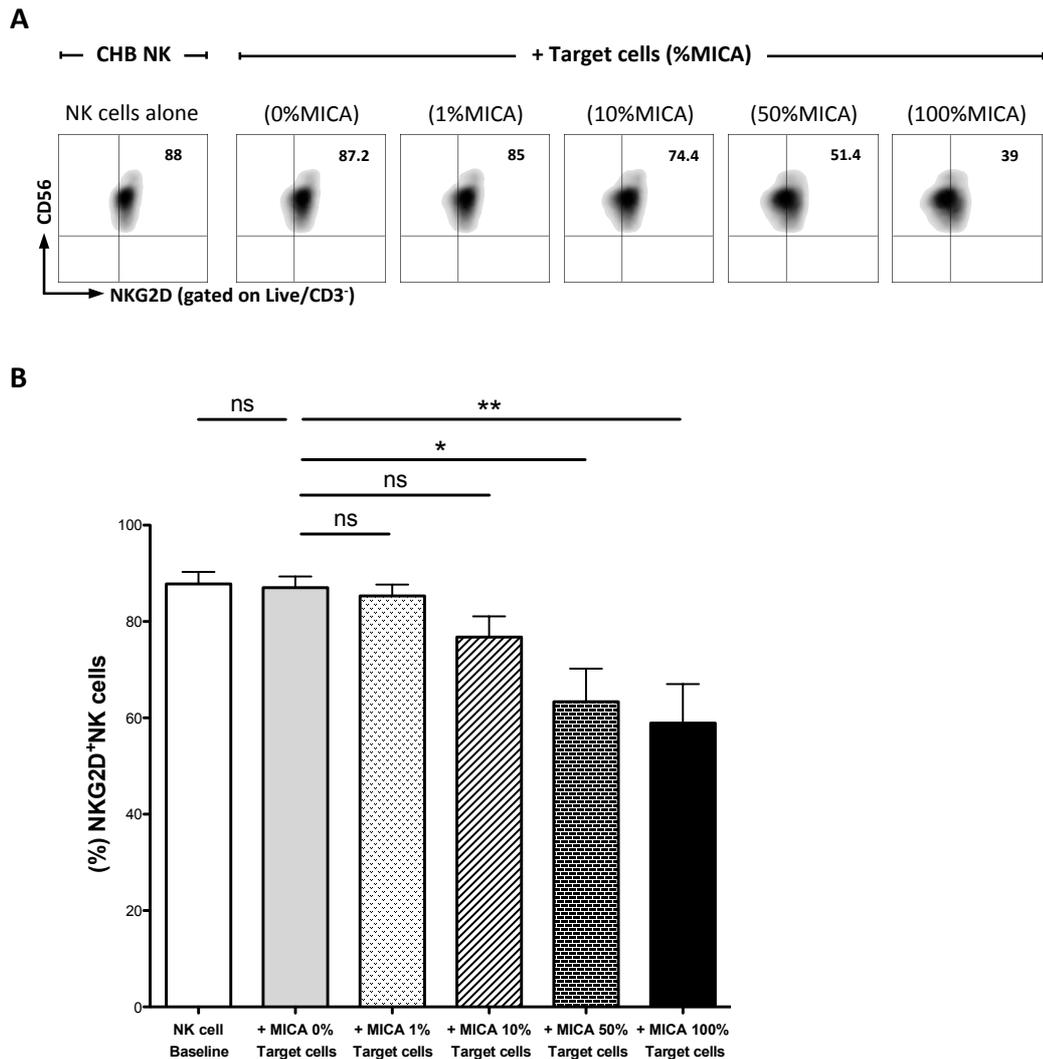


Figure 5.2 Evaluation of NK cell NKG2D down-regulation by cell-bound NKG2D-L.

- A Isolated NK cells from CHB patients were co-cultured with target cells at 1:1 ratio for 6 hours. Different MICA levels of target cells were manipulated by mixing C1R cells and C1R-MICA cells at different mix ratios. Representative plots of NKG2D on isolated NK cells (baseline) and on NK cells co-cultured with target cells.
- B Summary of panel A (n=6; analyzed by Friedman test).

Evaluating the NKG2D axis in HBV-related liver cancer or cirrhosis settings

Our research points out that the NKG2D pathway is a potent arm of NK cell immunity with sensitive responses to targets in chronic HBV infection. The robust

NKG2D-driven cytotoxicity of NK cells may have important homeostatic significance. As we know, multiple factors triggering NKG2D-L upregulation, such as repeated liver destruction and regeneration, genotoxin and DNA damage, persistent antigenic stimulation, and aberrant oxidative stress, can all exist in the HBV-infected liver (Cougot et al., 2005; Guerrieri et al., 2013; Raulet et al., 2013). These detrimental forces can ubiquitously and continuously affect different kinds of cells and promote carcinogenesis and pathogenesis (Cougot et al., 2005; Guerrieri et al., 2013). Thus, to restore homeostasis in such high oncogenetic and pathogenic situations, NK cells need to be equipped with competent NKG2D effector function (Oliviero et al., 2009). Moreover, since tumour transformation and cell stress responses share the same features of NKG2D-L induction, the NKG2D pathway must be crucial for both “cancerous” and “stressed” cell surveillance. Therefore, further assessment of the NKG2D axis in the settings of HBV-related liver cancer or cirrhosis is necessary.

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List of Achievements and Experiments Conducted

1. Evaluation of NKG2D ligand expression on intrahepatic T cells from healthy controls and CHB patients.
2. Evaluation of proliferation status (Ki67) of MICA/B⁺T cells from healthy controls and CHB patients.
3. Phenotype analysis of peripheral and intrahepatic MICA/B⁺T cells from CHB patients.
4. Evaluation of NKG2D ligand expression on CD4 Treg cells from CHB patients.
5. Evaluation of NKG2D ligand expression on peripheral and intrahepatic HLA-DR⁺T cells from CHB patients.
6. Evaluation of NKG2D ligand expression on peripheral and intrahepatic HBV-specific (IFN- γ ⁺) T cells from CHB patients
7. Assessment of NKG2D ligand expression on peripheral HBV and CMV-specific (dextramer⁺) CD8 T cells from CHB patients.
8. Preliminary assessment of NKG2D ligand expression on intrahepatic HBV and CMV-specific (dextramer⁺) CD8 T cells from CHB patients (inconclusive results and insufficient data, n=2; poor quality of intrahepatic dextramer staining).
9. Preliminary assessment of T cell MICA/B induction by using IFN- α , TNF- α , IL-2, IL-10, IL-15, IL-17, EGF, α CD3, α CD28, PMA, ionomycin, L-arginine depleted medium, hypoxic incubation, HBcAg, HBsAg, and

CHB patient serum as well as ER stress inducer thapsigargin (all without positive results).

10. Evaluation of NKG2D ligand induction on purified CD4 T cells upon TCR signalling or oxidative stress (H₂O₂) stimulation.
11. Assessment of time-course and dose-response of NKG2D ligand induction upon oxidative stress (H₂O₂) stimulation of purified CD4 T cells.
12. Comparison of NKG2D ligand induction upon oxidative stress (H₂O₂) stimulation in purified CD4 T cells and in whole PBMC.
13. Evaluation of upregulation of death receptor (Fas) and exhaustion marker (PD-1) on NKG2D-L expressing CD4 T cells upon oxidative stress (H₂O₂) stimulation.
14. Preliminary phosflow assessment of T cell γ -H2AX phosphorylation from CHB patients (technical problems: the recommended methanol permeabilization buffer influenced the surface staining).
15. Evaluation of NKG2D-L responses upon H₂O₂ stimulation with/without ATM kinase inhibitor (preliminary results revealed ATM kinase inhibitor was unable to prevent OS-mediated NKG2D-L induction; n=6).
16. Evaluation of the impact of NKG2D pathway and NK cells on activated T cells in patients with CHB.
17. Evaluation of the impact of NKG2D pathway and NK cells on HBV-specific (IFN- γ ⁺) T cells in patients with CHB.

18. Comparison of the impact of NKG2D pathway and NK cells on HBV and CMV-specific (IFN- γ^+) T cells in patients with CHB (inconclusive results and insufficient data, n=2).
19. Evaluation of the impact of NKG2D pathway on intrahepatic HBV-specific T cells in patients with CHB.
20. Preliminary assessment of HBV-specific (IFN- γ^+) T cell restoration upon NKG2D blockade together with TRAIL blockade (inconclusive results and insufficient data, n=1; poor viability was noted in cells with double pathway blockade).
21. Preliminary assessment of HBV-specific (IFN- γ^+) T cell restoration upon MICA blockade (inconclusive results and insufficient data, n=1; poor viability was noted in cells treated with MICA blocking mAb).
22. Evaluation of NK cell activation (HLA-DR, CD38, CD69, CD25) driven by NKG2D-L expressing cells.
23. Evaluation of NK cell degranulation (CD107a), cytokine production (IFN- γ), and death ligand (TRAIL) expression induced by NKG2D-L expressing cells.
24. Evaluation of NK cell activation (HLA-DR) and degranulation (CD107a) induced by target cells with different NKG2D-L levels.
25. Evaluation of target cell viability changes in the co-culture of isolated NK cells and target cells with different NKG2D-L levels.

26. Evaluation of NKG2D engagement induced cytokine production (IFN- γ) and activation (HLA-DR) changes in NK cells from healthy controls and CHB patients
27. Evaluation of NKG2D engagement induced degranulation (CD107a) changes in NK cells from healthy controls and CHB patients
28. Evaluation of NKG2D engagement induced viability changes in target cells co-cultured with isolated NK cells from healthy controls or CHB patients.
29. Assessment of the activation status (HLA-DR) and NKG2D expression of peripheral and intrahepatic NK cells from CHB patients and non-HBV infected controls.
30. Evaluation of NK cell activation and the associated correlation with T cell MICA/B expression in chronic HBV infection.
31. Evaluation of effects of pegylated interferon- α (PEG-IFN α) therapy on T cell NKG2D-L induction.
32. Preliminary floccytometry assessment of 2'-5'-oligoadenylate synthetase 1 of T cells from patients with CHB (technical problems: the recommended methanol permeabilization buffer influenced the surface staining).
33. Evaluation of IFN- α effects on NK cell degranulation activity.
34. Evaluation of effects of IFN- α on NK cell killing towards NKG2D-L expressing cells.
35. Evaluation of NK cell NKG2D down-regulation by cell-bound NKG2D-L (C1R-MICA cells).

36. Preliminary evaluation of NK cell NKG2D changes upon soluble NKG2D-L (sULBP1) and IL-15 stimulation (inconclusive results and insufficient data, n=1).