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INNATE IMMUNITY IN THE IMMUNOPATHOGENESIS OF CHRONIC VIRAL INFECTION

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Karolinska Institutet

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Innate Immunity in the Immunopathogenesis of Chronic Viral Infection

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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So much universe, and so little time

Terry Pratchett

ABSTRACT

Natural killer (NK) cells have a key role in control and clearance of viral infections. To carry out this function NK cells are capable of recognising infected cells and responding with induction of apoptosis in these cells, and production of pro-inflammatory cytokines. Target cells are recognised through various stress or infection related activating signals alongside 'missing self' recognition of down-regulation of human leukocyte antigen molecules. Additional stimulation for NK cells comes in the form of the type I interferon (IFN), IFN-α, which is released by infected cells and the immune system's sentinels, the dendritic cells (DC). As well as stimulating NK cells, IFN-α induces an anti-viral state in cells through up-regulation of expression of IFN-stimulated genes.

Infections with hepatitis B, C, and δ viruses (HBV, HCV, HDV) cause viral hepatitis and are major risk factors for developing liver fibrosis. Despite these infections causing similar clinical manifestations, and until recently treatment for all three utilising IFN-α, these three viruses differ greatly. Human immunodeficiency virus (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS). This disease is characterised by loss of CD4 expressing cells resulting in a diminished immune system that leaves the host susceptible to opportunistic infections. HIV-1 is the cause of the AIDS pandemic, while HIV-2 associates with a slower disease progression.

Each of these viruses is able to cause chronic infection. Part of the pathology of chronic infection, particularly HIV infection, is persistent immune activation and microbial translocation. In attempting to clear the infection the immune system can become perpetually activated, a condition associated with immune dysfunction. During this phase the immune response can contribute to disease progression through off-target effects, or appear as exhausted and dysfunctional.

In this thesis I will show that the phenotype and function of NK cells are altered during infection, primarily dependent upon the stage of hepatitis infection irrespective of infecting virus. For HIV infections NK cell activation is dependent upon the level of viral replication. During IFN-α therapy for hepatitis infections there is an increase of the chronic inflammation and microbial translocation marker sCD14, while NK cell function is altered due to fluctuations in intracellular STAT signalling.

LIST OF SCIENTIFIC PAPERS

My thesis consists of 3 publications and 1 manuscript. The individual papers are referred to by roman numerals.

I. Lunemann S, **Malone DF**, Hengst J, Port K, Grabowski J, Deterding K, Markova A, Bremer B, Schlaphoff V, Cornberg M, Manns MP, Sandberg JK, Ljunggren HG, Björkström NK, Wedemeyer H. Compromised Function of Natural Killer Cells in Acute and Chronic Viral Hepatitis. Journal of Infectious Diseases, 2014 May 1, 209(9), 1362-73

II. Sebastian Lunemann*, **David FG Malone***, Jan Grabowski, Kerstin Port, Vivien Béziat, Birgit Bremer, Karl-Johan Malmberg, Michael P Manns, Johan K Sandberg, Markus Cornberg, Hans-Gustaf Ljunggren, Heiner Wedemeyer, Niklas K Björkström. Effects of HDV infection and pegylated interferon α treatment on the natural killer cell compartment in chronically infected individuals. Gut, 2015 Mar, 64(3), 469-82. *Contributed equally

III. **Malone DFG**, Falconer K, Weiland O, Sandberg JK. The Dynamic Relationship between Innate Immune Biomarkers and Interferon-Based Treatment Effects and Outcome in Hepatitis C Virus Infection is Altered by Telaprevir. PLoS One, 2014 Aug 26, 9(8), e105665

IV. Susanna M Bächle*, **David FG Malone***, Marcus Buggert, Annika Karlsson, Per-Erik Isberg, Antonio Biague, Hans Norrgren, Patrik Medstrand, the SWEGUB CORE group, Markus Moll, Johan K Sandberg, Marianne Jansson. Elevated levels of iNKT cell and NK cell activation correlate with disease progression in HIV-1 and HIV-2 infections. Manuscript submitted. *contributed equally

CONTENTS

LIST OF MAJOR ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ART	Anti-retroviral therapy
cccDNA	Covalently closed circular deoxyribonucleic acid
CHB	Chronic hepatitis B
CHC	Chronic hepatitis C
CHD	Chronic hepatitis δ
DAA	Direct acting antivirals
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
GzB	Granzyme B
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDV	Hepatitis δ virus
HIV	Human immunodeficiency virus
HSC	Haematopoietic stem cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
iNKT	Invariant natural killer T cell
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
KIR	Killer cell immunoglobulin-like receptor
LPS	Lipopolysaccharide
MAIT	Mucosa-associated invariant T cell
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex

NASH	Non-alcoholic steatohepatitis
NCR	Natural cytotoxicity receptor
NK	Natural killer cell
PCA	Principle component analysis
pDC	Plasmacytoid dendritic cell
PRR	Pattern recognition receptor
pSTAT	Phosphorylated signal transducer and activator of transcription
RNA	Ribonucleic acid
TCR	T cell receptor
TF	Transcription factor
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand

1 INTRODUCTION

Infection with some microorganisms leads to disease [1], and our bodies' way of fighting these pathogens is with the immune system [2,3] Unlike most human systems such as the nervous system consisting of the brain, spinal cord, and peripheral nerves, the immune system is not made from specific tissue or composed of a few large organs. Rather the immune system is formed from the lymphatic system, white blood cells (leukocytes), and complement proteins found throughout the body.

The term immunity derives from the Latin immūnis (meaning exempt from tribute or taxation [4]), however the functions of the immune system go beyond protecting us from disease. Thought leukocytes are known to play parts in reproduction and development [5,6], and homeostasis [7-9], the immune system is primarily seen as the system for fighting pathogens. In performing this function it utilises many different leucocytes. All leucocytes derive from the haematopoietic stem cell (HSC) (Fig. 1), and will go on to permeate every system and organ in our body to varying degrees.

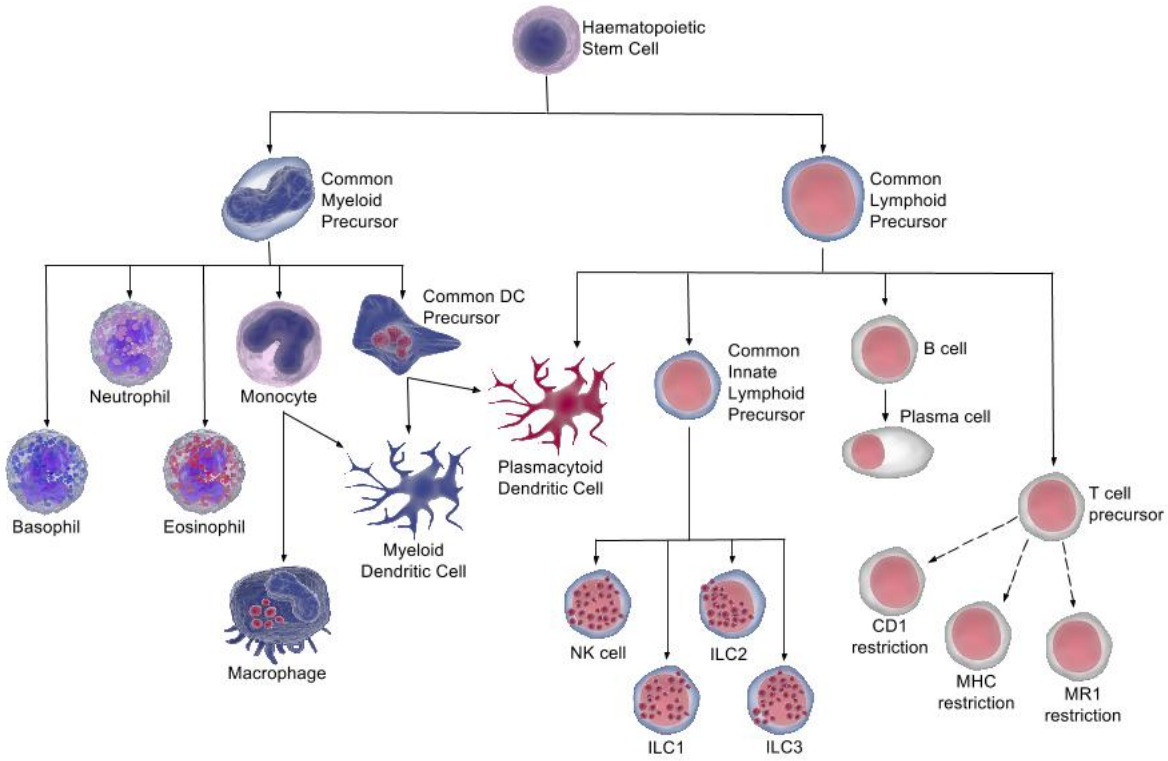

Figure 1: Lineage of leukocytes in the Immune System

The immune system was traditionally separated into two factions: innate and adaptive. These terms have blurred over the years with a number of leukocytes displaying both innate and adaptive features [10-12]. When responding to pathogens the immune system has a spectrum of defence mechanisms at its disposal, and deployment of these is dependant upon the microbe encountered [13-15]. Leukocytes also exhibit different functions depending upon the organ in which they reside [16-18].

The immune response is therefore restricted in some instances. In the intestines, for example, immediate aggressive responses to gut microbiota would be counterproductive and so the immune system has a degree of tolerance [19,20]. Furthermore, along with pathogens causing disease themselves, some diseases can be caused or exacerbated due to excessive or inappropriate immune responses [21,22].

1.1 IMMUNOLOGY

During initial development from the HSC, leukocytes split into either myeloid or lymphoid cells [23-25]. The myeloid basophils and eosinophils are involved in defence against parasites, while neutrophils are involved in bacterial defence. There are two different types of dendritic cell (DC) [26]; the myeloid DC (mDC) is primarily involved in detecting bacterial or viral infections and initiating adaptive immune responses [27], while plasmacytoid DCs (pDC) also detect infections and are a major source of type I interferon (IFN) [27]. Monocytes are able to mature into macrophages, a phagocytic cell type, and the mDC.

Lymphoid cells include B cells, which following stimulation can develop into antibody producing plasma cells. T cells that have diverse functions as helpers, regulators and effectors of the immune system, and some pDCs are derived from the common lymphoid progenitor [28]. Also of lymphoid origin are the innate lymphoid cells (ILCs) [29], comprising lymphoid tissue inducers that are involved in the formation of lymph nodes, ILCs 1-3 [7] that help direct the immune response depending upon stimulus, and natural killer (NK) cells that are capable of directly killing transformed cells and producing inflammatory cytokines [30,31].

Virtually every cell in our body has some role to play in immunity to pathogens. Most cells will display what type of proteins they contain through the major histocompatibility complex (MHC) class I antigen presentation pathway [32]. MHC class I molecules are able to present peptides to the T cell receptor (TCR) of T cells, if a self-peptide is presented the T cell will generally not react, as they have gone through a tolerogenic selection process [33] and are not receiving any additional inflammatory signals. However if the peptide is non-self, and there is an additional inflammatory stimulus, then the T cell will become active and kill the target [34].

MHC class II proteins are expressed by major antigen-presenting cells such as DCs and macrophages, which are specialised in presenting antigens from the extracellular environment. The captured pathogens are broken down inside the cell and the peptides loaded onto the MHC class II molecules for display on the cell surface [35]. Upon engulfing the pathogen, the DC will mature and head to a local lymph node to initiate an adaptive immune response [36].

Whilst MHC molecules present peptides, additional MHC-like molecules can present different cellular products. MR1 molecules present vitamin B2 metabolites to the recently discovered mucosa-associated invariant T (MAIT) cells [37,38], while CD1 molecules display

glycolipids to the TCR of natural killer T (NKT) cells [39]. MAIT cells have a semi-invariant TCR, as do type II NKT cells, whereas type I NKT cells have an invariant TCR and are often referred to as iNKT cells [40]. Together with their rapid response to infection, the invariant TCRs of both MAIT and NKT cells instils them with innate characteristics [41], and they can help induce and regulate the adaptive immune responses.

Adaptive immunity generally refers to a response involving rearrangement of genetic material in a leukocyte, commonly B and T cells. These adaptive cells can further develop into memory cells which are capable of a specific and relatively fast immune response upon repeat infections [42]. Innate cells tend to be short lived and do not undergo such gene rearrangements. However, it appears that innate cells can possess memory characteristics caused by alterations of the cells' epigenetics, sometimes referred to as training [43,44].

1.1.1 Viral Infection Response

If a virus penetrates the frontline defences of skin or mucosa and infects a cell, the immune system has multiple methods to initially recognize an infection, and then attempt to clear the infection. Our cells utilize a litany of receptors that recognize pathogen associated molecular patterns (PAMPs), and damage associated molecular patterns (DAMPs) called pattern recognition receptors (PRRs) [45-47], which can either be membrane bound or intracellular. There are different families of these receptors – Toll-like receptors (TLRs) [48], C-type lectin receptors (CLRs) [49], nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) [50], and retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs) [51,52]. Each of these is used in the identification of some pathogenic motif such as double stranded RNA or un-methylated CpG motifs in DNA that are associated with viruses. Signalling of the PRRs leads to a cascade of molecular interactions resulting in the induction of anti-viral responses [53], dependent upon which PRR is activated.

DCs endocytose bacteria and viral particles from their surrounding environment to present their peptides to $CD4^+$ T cells via MHC class II [35], or to $CD8^+$ T cells with MHC class I molecules [54]. Upon the proteolytic degradation of microorganisms in the endosomal compartment, various parts of the genetic material are exposed to TLRs. TLR3 will detect double stranded RNA and signal via TRIF to activate interferon regulatory factor 3 (IRF3) [55-57]. TLR7 recognizes single stranded RNA [58], and TLR9 binds CpG islands on DNA [59]. Both signal through MyD88 to activate IRF5 and IRF7 [60,61]. All these IRFs will translocate to the nucleus of the cell to induce the production of type I IFNs [57,62] (Fig. 2).

Viral infection and replication in cells will lead to the presentation of viral peptides via MHC class I proteins, which can be engaged by $CD8^+$ cytotoxic T cells specific for that peptide [63]. Additionally PRRs on the surface of cells can also induce activation of IRFs, while cytosolic PRRs can induce a form of controlled cell death (apoptosis) of the infected cell via caspase 3 [64]. In these ways viruses are able to be readily recognized, resulting in protective mechanisms of involving IFN production, signalling to cytotoxic cells, and apoptosis of

infected cells. However, many viruses have evolved to evade the immune system by disrupting many of these mechanisms such as causing down-regulation of MHC class I [65,66] or CD1 [67], and interfering with IFN signalling [68,69].

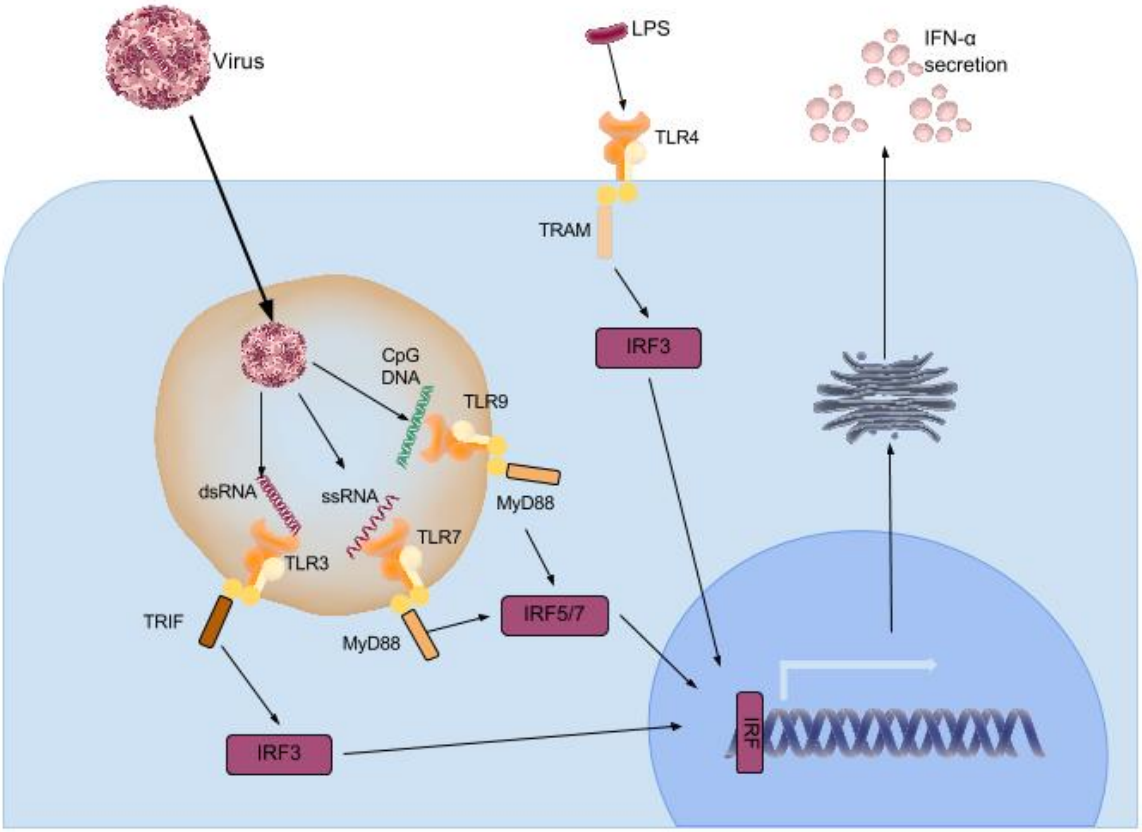

Figure 2: Virus induction of IFN-α production via various TLRs

1.1.2 Liver Immunology

All organs in the body have their own unique microenvironment in which the immune system operates. The liver not only takes blood from the circulation and removes toxins, but also receives blood from the gut via the portal vein. Incoming blood from the gut contains bacterial products, which under normal circumstances need to be tolerated lest you get a strong inflammatory response in the liver that would cause damage and possibly lead to fibrosis.

Despite being a rather homogenous organ, the liver performs a large range of functions, including but not limited to bile production, glycogen storage, and blood detoxification. 75% of the livers blood supply originates from the gut via the portal vein, with the remaining quarter from the hepatic artery. Blood enters the sinusoidal spaces, from portal triads, and leaves via the hepatic vein (Fig. 3), allowing the blood plasma and associated soluble factors to permeate into the liver tissue.

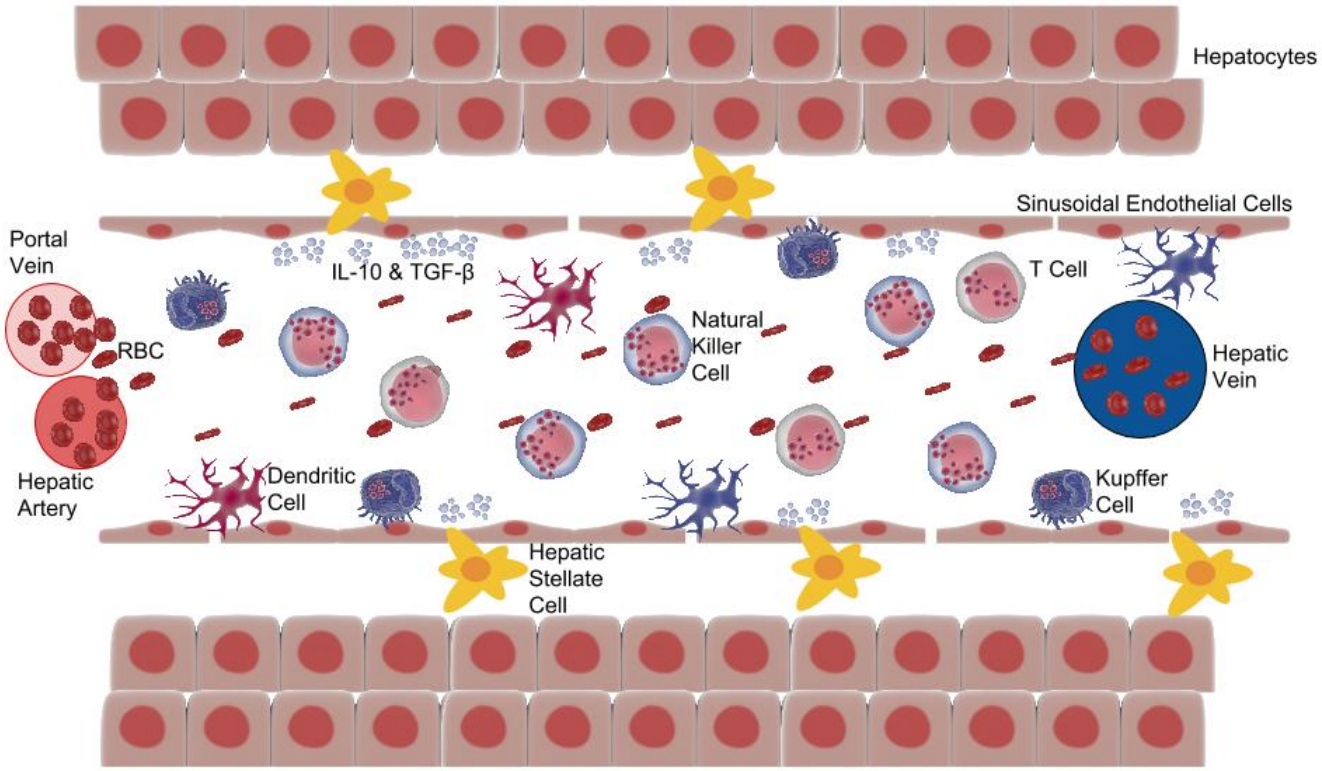

Figure 3: Sinusoidal space in the liver

Many different immune cell types reside in the liver, including Kupffer cells (the macrophages of the liver), DCs, MAIT cells, ILCs, and NK cells [70] (Fig 3). The liver sinusoidal endothelial cells (LSEC) help create a tolerogenic environment through their constitutive production of transforming growth factor (TGF)-β and interleukin (IL)-10 [71], thus any antigenic material that the DCs digest and present will culminate in the induction of regulatory T cells. TGF-β and IL-10 also induce tolerance in Kupffer cells, and NK cells [71-74], however their effect on the MAIT cells is unknown.

Although maintenance of a tolerogenic hepatic state is necessary, it is still important that the balance can be tipped in favour of an inflammatory response when there is an infection. To ensure this can happen the sinusoidal cells and hepatocytes express many PRRs [70,75] so that an infection can be readily detected, and production of pro-inflammatory cytokines can occur. Thus, with mDCs, pDCs, and Kupffer cells constantly sampling the environment, tolerogenic DCs can be induced to mature and, particularly pDCs, produce IFN-α [28,76].

During clearance of a viral infection the liver may be damaged due to destruction of hepatocytes, by either a cytopathic virus or non-specific killing caused by cytotoxic $CD8^+$ T cells and NK cells [77]. If the infection can be cleared in a timely fashion then the damage will be limited and due to the regenerative capacity of the liver [78,79], will be repaired in time. On the other hand, if the infection and killing persists, then in place of the hepatocytes the hepatic stellate cells will lay down extracellular matrix (fibrotic tissue) [80,81]. With enough build up of the fibrotic tissue, a patient will develop fibrosis that may develop into to cirrhosis (Fig. 4).

Infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) are significant risk factors for the development of liver cirrhosis; in turn cirrhosis is a major risk factor for developing hepatocellular carcinoma (HCC). Effective therapy of hepatitis viral infections can lead to a reversal of fibrosis and cirrhosis [82,83]. However, removal of the underlying infection does not completely abate the risk of HCC development [84-86].

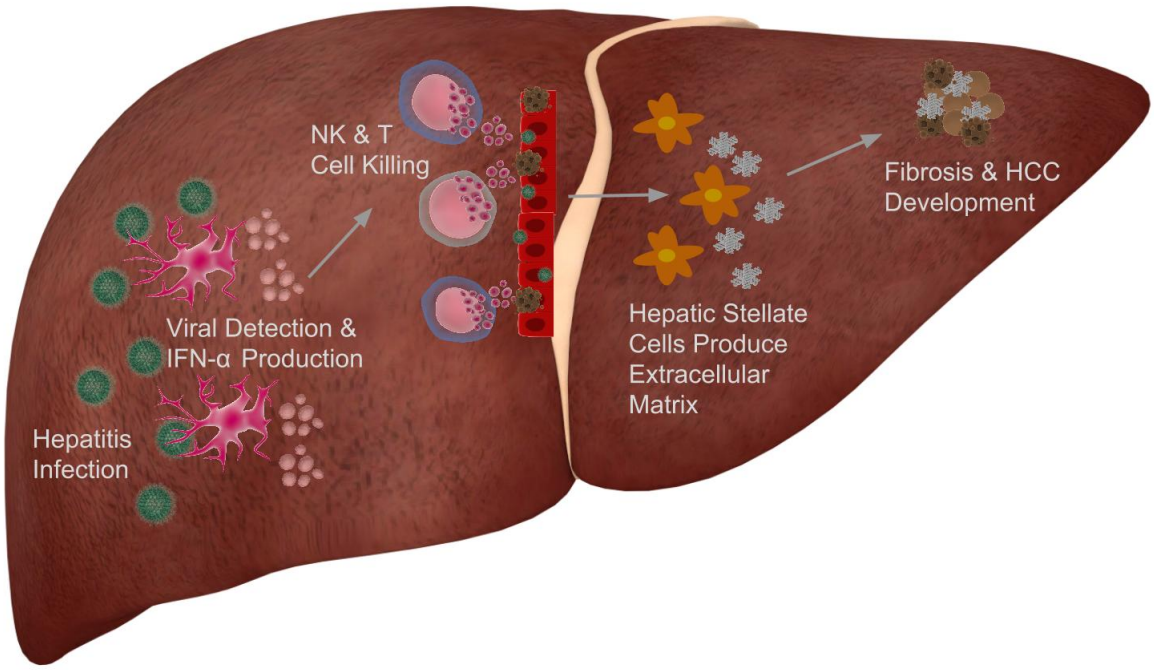

Figure 4: Progression from viral infection to fibrosis

1.2 INNATE IMMUNE RESPONSES

Immunity to viruses first involves barriers to infection such as skin or mucus and immunoglobulin (Ig) A. If a virus can survive or bypass these barriers through for example a cut (which it-self can produce a response by signalling DAMPs) then recognition of a virus is paramount to initiating a response. There are numerous PRRs, such as TLRs, that can recognize various non-self signatures. Most cells in the body also present what proteins they produce via the MHC molecules. After recognition of a viral threat, type I IFNs including IFN-α will be produced to induce anti-viral responses in the local environment, and activate immune cells that can kill virally infected cells such as NK cells.

1.2.1 Interferon α

IFNs were first identified as secreted proteins which were capable of interfering with influenza virus infections [87]. A major player in anti-viral responses, immune activation and hepatitis therapies is the type I IFN, IFN-α. When PRRs detect an infection they can lead to the production of IFN-α, additionally pDCs produce large quantities of IFN-α during infections [88,89]. IFN-α will help produce an anti-viral state by stimulating surrounding cells to induce interferon-stimulated genes (ISGs) [62,89], and activate NK cells [90]. Until very recently IFN-α was the mainstay of treatment for HCV [91], and despite hopes of new therapies is still used in treatment of HBV and hepatitis δ virus (HDV) infections [92-94].

IFN-α signals through a heterodimer receptor complex, consisting of the subunits IFN-α receptor 1 (IFNAR1) and IFNAR2. Binding of the receptor triggers an intracellular signalling cascade involving the JAK/STAT pathway culminating in the formation of IFN-stimulated gene factor 3 (ISGF3) that moves to the nucleus to induce ISGs [95] (Fig. 5). General effects of the ISGs are to increase expression of PRRs, and antiviral effectors that will inhibit viral entry and replication [62,89]. Exact functions enhanced by induction of the ISGs are dependent upon which and what concentrations of the 13 IFN-α subtypes bind the receptor, and the affinity of the binding [96]. Hence in this way an immune response can be fine-tuned.

1.2.2 Natural Killer Cells

A part of the wider ILC family [7], natural killer (NK) cells are an early responding lymphocyte important for cytotoxicity and pro-inflammatory cytokine production. NK cells were originally described over 40 years ago for their ability to kill tumour cells without any prior sensitization [97,98]. It would be another decade before the use of Leu-19 (NCAM-1, and henceforth CD56) to positively identify the majority of NK cells [99-101] superseding the use of HNK-1 (CD57) [102,103], which was also present on a large population of T cells.

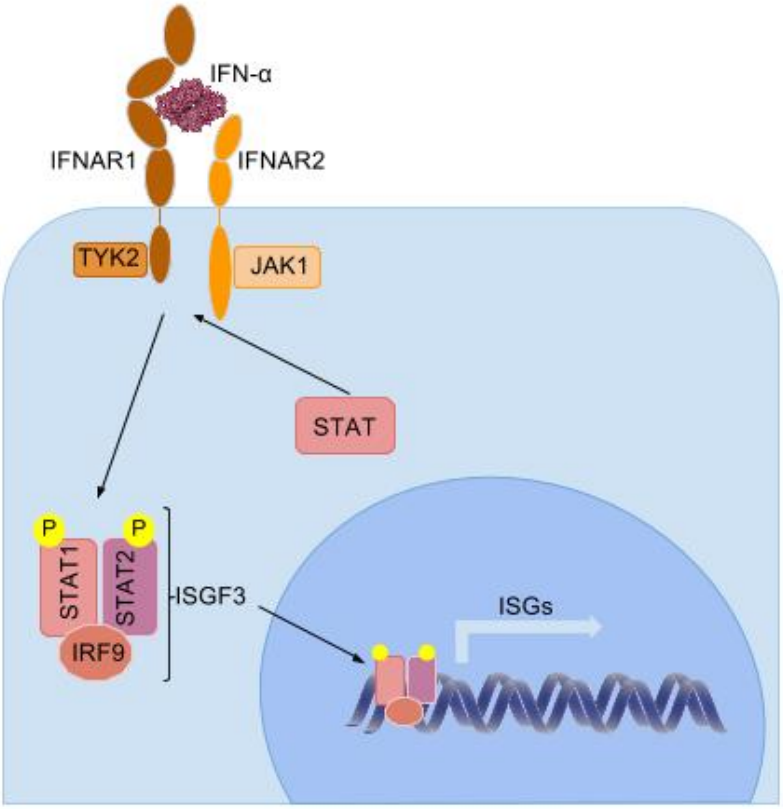

Figure 5: IFN-α induction of IFN-stimulated genes

Based upon their expression level of CD56, NK cells are traditionally split into $CD56^{bright}$ and $CD56^{dim}$ subsets [104]. It is currently a matter of some debate if these are two separate lineages of NK cells [105,106] or if the $CD56^{bright}$ NK cells mature into the $CD56^{dim}$ NK cells [107-110]. Although these subsets are both capable of releasing cytokines and cytotoxicity, $CD56^{bright}$ NK cells do respond more readily to cytokine stimulation and the $CD56^{dim}$ NK cells to cellular activation [107,111-113]. A third NK cell subset, lacking CD56 expression, has been observed in viremic human immunodeficiency virus (HIV) and HCV infections [114,115]. This $CD56^{neg}$ NK cell subpopulation is also present in healthy individuals but in a much lower frequency. The function, and origin of these NK cells are still under investigation [116].

NK cells develop from the common lymphoid progenitor partially through the action of the transcription factors (TF) EOMES and T-bet [117-119]. Recent literature suggests that EOMES is an important TF with regards to NK cell cytotoxicity and can be used to help segregate NK cells from ILC-1, while T-bet is involved in cytokine production [119,120].

Given that NK cells do not require previous encounters with a target to be able to perform their cytotoxic function, the requirements for NK cell activation were unclear until the proposal of the 'missing self' hypothesis [121]. Here it was proposed that the hosts MHC molecules actively inhibit NK cells. When such self-MHC molecules are missing, due to viral infections or tumour transformation, the NK cells are no longer inhibited and will kill their target. Subsequent studies revealed that NK cell activation comes about through the

integration of a plethora of inhibitory and activating signals, be they cell-cell interactions, cytokines, or antibody recognition [122-124].

Major inhibitory pathways for NK cells include the cell surface killer immunoglobulin-like receptors (KIR) and NKG2A, whose ligands are indeed MHC class I molecules. The cytokines IL-10 and TGF-β have also been implicated in NK cell inhibition. Common activating cellular receptors include natural cytotoxicity receptors (NCR), NKG2C, NKG2D, DNAM-1, and some KIRs, with MHC class I molecules again acting as ligands alongside various stress-induced molecules like MIC-A. Pro-inflammatory cytokines IFN-α, IL-12, and IL-18 are also potent activators of NK cells. In addition $CD56^{dim}$ and $CD56^{neg}$ NK cells have the IgG Fc receptor CD16. Activation leading to a functional response is dependent upon which combinations of receptors are stimulated [125,126], and prior epigenetic reprogramming [127,128].

There is the potential for a multitude of receptor combinations on NK cells, and with advancing technology it has been shown that if you were so inclined, NK cells could be subdivided into thousands of subsets [129]. Presumably, each potential subset will react slightly differently depending upon their receptor combination, the stimulus they receive, and the environment in which they find themselves. This wide range of receptor combinations does appear to diminish as a person grows older, hypothesized to be due to the number of infections encountered over the course of your lifetime [130].

The potential strength of NK cells responses can be altered by the inhibitory receptors on their surface in a series of processes termed education/licensing/arming/tuning [131-134]. During NK development, interaction of an inhibitory receptor NKG2A, KIR etc. with its cognate ligand will functionally mature the NK cell. This allows for an increased level of activation, if the cell can overcome the increased inhibition. Those NK cells that lack either the inhibitory receptor or cognate ligands still play an important role in controlling viral infection [135]. Expression of the KIRs is a stochastic event [136,137] that will ultimately tune the NK response [134,138].

1.2.3 NK Cell Functions

Due to the uniqueness of their activation pathway, NK cells are important in early cytopathic response to transformed cells such as cancerous or virally infected cells. Their most evident responses are release of IFN-γ and their cytotoxic response. Following recognition of a target cell [126] and lacking inhibition [139], NK cells will form an immunological synapse [140,141], into which they will release perforin [142] and granzyme B (GzB) [141] (Fig. 6). Perforin perforates the cell, while GzB [143] enters the target and initiates apoptosis [144,145]. NK cells are also able to induce apoptosis in target cells through use the tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), and Fas-ligand [146].

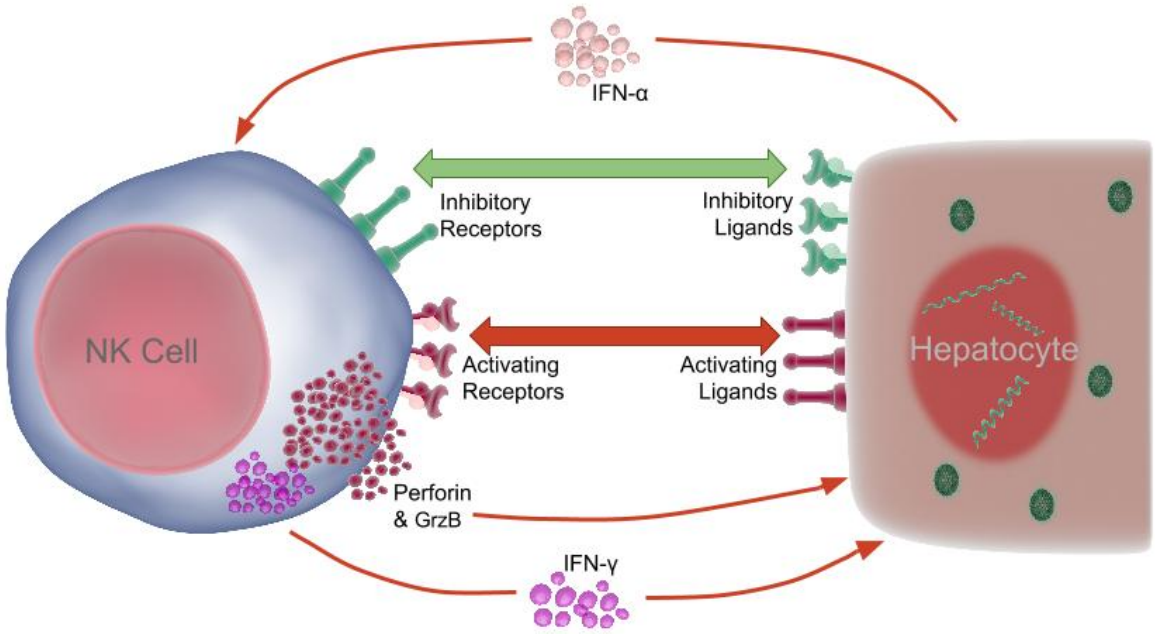

Figure 6: NK cell interaction with infected target cell

Recognition of IgG, produced by plasma cells, by CD16 is another important pathway in NK cell activation, as it does not require additional stimulation. Once activated via CD16, NK cells will perform antibody-dependent cell-mediated cytotoxicity (ADCC) [147], irrespective of their education [148]. Thus this function forms a part of the B cell adaptive response.

NK cells have a number of key roles during viral infections [149], including hepatitis and HIV infections that shall be discussed in detail later. Although the major function of NK cells is to kill targets, it is the nature of the target that defines the role they play in immune responses. By targeting $CD4^+$ helper T cells [150], and $CD8^+$ cytotoxic T cells [151], NK cells are able to halt the adaptive immune response, an effect that can be avoided with type I IFN [152,153]. NK cells can further regulate the immune response by targeting and lysing DCs [154,155].

1.3 INFECTIOUS DISEASES

A number of major discoveries have helped us in our battle with infectious disease, ranging from improved hygiene [156], a better understanding of how microorganisms can spread [157], and vaccinations [158]. However, despite these advances, infectious diseases affect billions of people. Since 1990 over 400 million disability-adjusted life years (sum of years of healthy life lost) attributably to communicable diseases have been saved [159] (Fig. 7). With future improvements in our understanding about infectious agents, and how our immune system reacts to them we should be able to develop more effective vaccines and treatment that could further lower the global impact of infectious diseases.

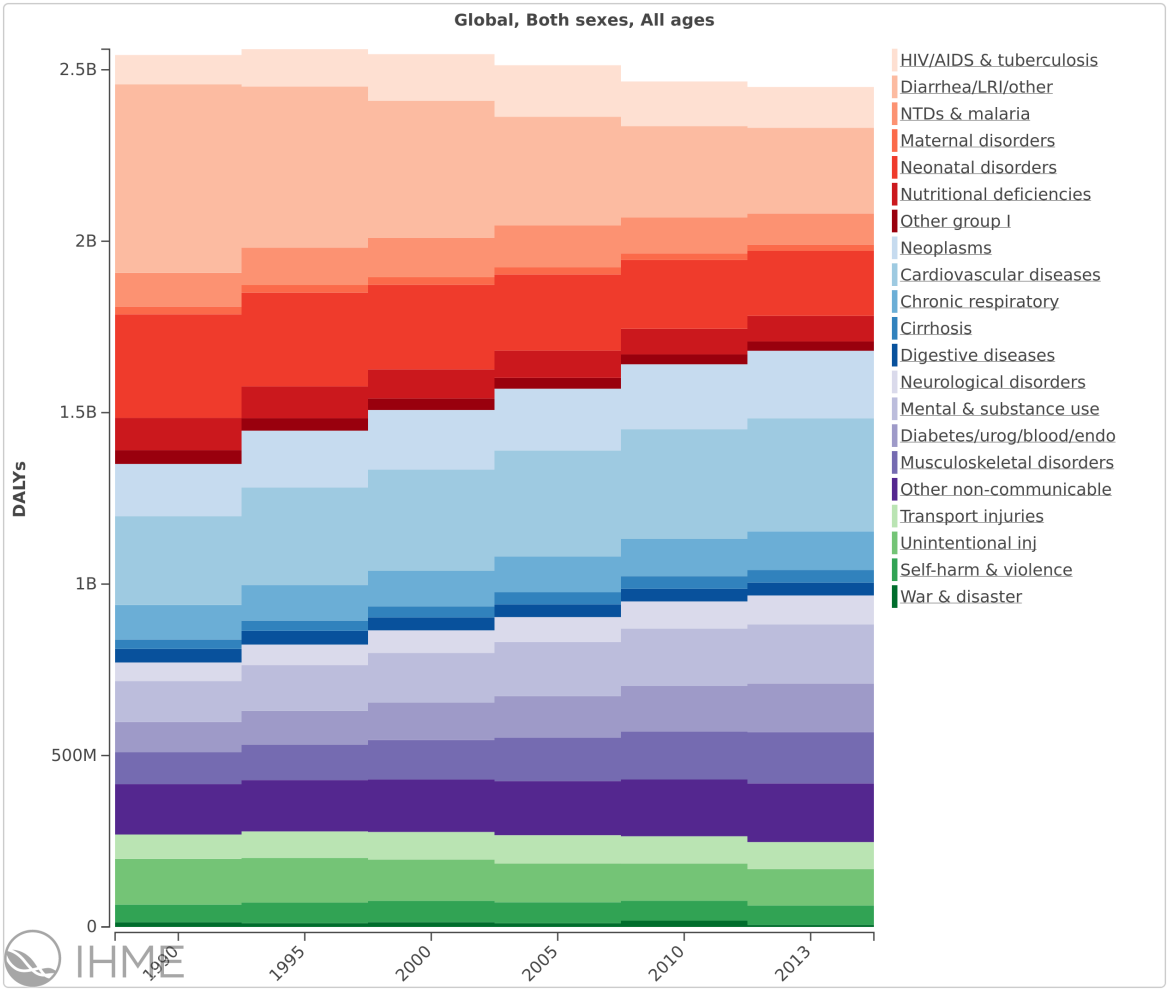

Figure 7: Causes of disability-adjusted life years; from www.healthdata.org [159]

Hepatitis in general is inflammation of the liver that can have a number of causes including alcohol, fatty liver, drug toxicity, autoimmunity, or the predominant cause of viral infection. Infections with the hepatitis viruses are major risk factors for fibrosis and cirrhosis, and HCC [22,160,161]. Hepatitis B, C, and δ viruses are transmitted via infected bodily fluids parenterally, usually through shared needles of intravenous drug users (IDUs), perinatally, or during sexual intercourse [162]. Routes of infection vary in different settings and in different parts of the world. Each virus is very different but linked in that they infected hepatocytes. HIV-1 and HIV-2 are the causative agents of acquired immunodeficiency syndrome (AIDS),

with the HIV-1 pandemic resulting in 1.5 million deaths per year [162]. As with HBV, HCV, and HDV, HIV-1 and HIV-2 infect new hosts via infected bodily fluids [162].

1.3.1 Hepatitis B Virus

1.3.1.1 HBV & Disease

HBV is highly infectious, with over 2 billion people infected at some point [162]. The ability to clear the virus without medical intervention depends upon when you are infected. 90% of infected adults are able to clear the virus without intervention [162], and adult infection is often acquired by sexual transmission or use of contaminated needles. However, 90% of perinatal infections will result in chronic hepatitis B (CHB) [162].

HBV has a double stranded DNA genome that contains four overlapping reading frames, which code for seven proteins [163,164]. Amongst these are three envelope proteins that help bind the virus to hepatocytes for infection [165]. Once endocytosed within the hepatocyte HBV still has to gain access to the cell nucleus to replicate, which it achieves through use of a nuclear localization signal found in its core protein [166]. Once in the nucleus the virus can begin transcription of its genome and replication by converting from a relaxed circular DNA structure to covalently closed circular (ccc) DNA [163,167].

The cccDNA structure is very stable facilitating viral persistence [168], meaning that unless the infection is cured it has to be suppressed indefinitely. Current attempts to cure HBV infection still rely upon pegylated IFN-α [169], but drugs targeting the HBV reverse transcriptase are in development [170]. If a curative treatment is unsuccessful, patients often require lifelong drug treatment with Tenofovir to suppress the virus [171].

It has been suggested that the size of the inoculum contributes to the outcome of the infection [172]. A large inoculation may be cleared eventually, a moderate inoculum may result in rapid clearance, while a small inoculum may lead to a large spread and persistent infection. This model suggests that a small HBV inoculum that does not break the livers immune tolerance will persist, while larger inoculums that break this tolerance will eventually be cleared. While HBV infection is mainly considered to be non-cytopathic [163,173], there are some arguments against this notion [174]. It is likely that non-antigen specific T cells contribute to liver damage via the Fas/Fas-ligand pathway [175], and NK cells via TRAIL [176,177].

1.3.1.2 HBV Immunology

Despite the intrinsic ability of the HBV core protein to activate the immune system [178,179], the early stages of infection are characterized by an induction of IL-10 production, rather than type I IFN, resulting in attenuated responses of NK and T cells [180,181]. This lack of a pro-inflammatory response may be due to HBV altering TLR3 signalling and subsequent IFN-α production [48,182]. Along with the already tolerogenic nature of the liver, the

immune system thus requires a large insult to activate multifunctional virus-specific CD8+ T cells that are crucial for viral clearance [177,183].

IFN-α stimulation of NK cells is useful during initial stages of infection due to their production of IFN-γ and TNF. It has recently been shown that T cell derived IFN-γ and TNF are able to induce the decay of the HBV cccDNA [184]. Hence it may be possible that early induction of these pro-inflammatory cytokines by NK cells could halt persistent infection. Certainly they help provoke an anti-viral environment, and lyse infected cells if NK cells can overcome the inhibitory MHC signals [185]. However, if overactive then NK cells may have off-target effects and kill uninfected hepatocytes [173,176]. If IFN-α is inadequately produced, in combination with IL-10 up-regulation, then NK cells may kill pDCs, and CD8+ T cells allowing for further viral spread [163].

TRAIL expression of NK cells is seen to be up-regulated in CHB patients [176], and is thought to play a role in killing hepatocytes [186] and anti-viral CD8 T cells [187] during hepatitis infections. Despite their increased expression of TRAIL, these NK cells have a reduced capacity to produce IFN-γ potentially caused by increased levels of IL-10 [188]. Although the NK cells have increased TRAIL expression the HBV core protein is able to reduce expression of the TRAIL ligand DR5 (TRAIL-R2) on hepatocytes [189], thus reducing the potential for virally infected cells to be targeted.

1.3.2 Hepatitis δ Virus

1.3.2.1 HDV & Disease

First identified by Rizzetto et al in 1977 [190,191], HDV was named thus due to the extra (delta) antigen involved in HBV infection. An estimated 20 million people are infected with HDV [162], which is considered the most severe form of viral hepatitis, as progression to cirrhosis is faster, and the risk of HCC greater [192].

HDV has since been discovered to be a sub-viral virion, the only one known to infect humans [193]. Other known viroids infect plants, which has led to some debate as to the origin of HDV [194]. Viable reproduction only occurs in a cell already infected with a hepadnavirus, most commonly HBV, and can occur as a simultaneous co-infection or subsequent super-infection [195]. The HDV consists of a HBV envelope and contains a single strand of circular RNA [196] that codes for one protein, the delta protein.

HDV is likely to use the same viral entry mechanisms as HBV due to the HBV envelope proteins, however replication is separate [197], and well reviewed by Taylor 2015 [198]. The HDV RNA translocates to the cell nucleus were the genetic material is copied in a double rolling-circle replication system reliant on host cell replication factors [197,198]. This results in production of HDV mRNA, leading to the production of delta protein, and genomic HDV

RNA. Delta protein and HDV RNA are combined in HDV ribonucleoprotein, which is packaged in HBV envelope proteins in the Golgi, prior to secretion.

Due to the use of HBV envelope, the same pathways of infected bodily fluids spread HDV. Similarly infection can be effectively vaccinated against using the HBV vaccine. Treatment is still restricted to pegylated IFN-α therapy [94,199] which has a 25% response rate [200]. However, trials such as the HIDIT studies [201] are aimed at further developing the therapy.

1.3.2.2 HDV Immunology

A relatively rare viral hepatitis infection, the immunology of HDV infection is little studied. It must also be kept in mind that HDV infection occurs within the context of active HBV infection. HDV is also considered to be non-cytopathic, hence it is thought that the immune system causes the majority of the disease pathogenesis [202].

We know that HDV is capable of inhibiting IFN-α signalling [203], adding to the tolerogenic environment created by HBV. In chronic hepatitis δ (CHD) the virus-specific T cell responses are weak [202], and prior to our work there were very few investigations of NK cells during HDV infection [204,205]. These studies identified NK activity in response to IFN-α therapy as important in HDV clearance.

1.3.3 Hepatitis C Virus

1.3.3.1 HCV & Disease

HCV, like the other two hepatitis viruses described, is blood borne and infects roughly 130-150 million people globally [205]. Of those infected about 30% are able to clear the infection spontaneously within six months and will not progress to chronic hepatitis C (CHC), and the majority are asymptomatic. Effects of HCV infection were first observed in the 1970s due to transfusion patients contracting hepatitis. After the development of assays to detect hepatitis A virus (HAV), and HBV, it was determined that a new agent, originally termed non-A non-B hepatitis, was the cause [206].

HCV has since been discovered to be a positive-sense single-stranded RNA virus in the family of Flaviviridae. Viral endocytosis into hepatocytes is mediated by clathrin along with a host of co-receptors [207,208], including CD81 binding the HCV E2 protein [209]. Once in the cell the endosomal pH triggers release of the viral RNA into the cytoplasm, the host machinery can then translate the genome; viral proteases then proceed to cut the polyprotein into 10 viral proteins [210].

The history of HCV and IFN therapy have recently been well reviewed by Pawlotsky et al 2015 [211], and Heim 2013 [91], detailing the history of the use of IFN-α as a therapy and the dawn of the new direct acting antiviral (DAA) drugs [212]. IFN-α therapy is believed to work by activating the immune system mechanisms acting to eradicate viral infection. However,

the use of IFN-α as a therapy alone has poor treatment response rates that have been increased with the addition of ribavirin to the treatment regimen, and the pegylation of IFN-α [91]. The new DAAs are in effect a game changer in HCV therapy. A slew of studies can be summed up by saying effectiveness now ranges from 90-99% [213-215], depending on HCV genotype [216].

The appearance of $CD56^{neg}$ NK cells has been observed during HCV infection and appears to be indicative of IFN-α treatment response [115], as does the patients' IL-28b genotype [217]. A very good predictor of response to IFN-α therapy was shown to be the activity of the ISGs in the liver of patients, with low ISG activity predicting good response [218,219]. This heavily implies that IFN-α therapy works by initiating an immune response that is not occurring, possibly due to a lack of danger signals or blockage by viral proteins [220].

1.3.3.2 HCV Immunology

IFN-α is known to be critical during HCV infection [220], however the HCV has a number of immune evasion mechanisms. Viral protein E2 can halt pDC IFN-α production [221,222], while NS3 inhibits TLR3 signalling [223]. HCV is also non-cytopathic, hence liver damage probably originates from the immune responses' attempts to control and eradicate the infection. Again this damage comes from the action of the cytotoxic lymphocyte subsets $CD8^+$ T cells and NK cells [224]. While these responses are needed to clear the infection, they are required to be specific responses and therefore the off target effects need to be limited [225].

NK cells play a crucial role in HCV infection [226]. Particularly the inhibitory KIR2DL3 on NK cells and its cognate ligand human leukocyte antigen (HLA)-C1 are reportedly involved in clearance of HCV [227], as is IFN-γ production [228]. These studies highlight NK cells as a significant effector cell in this context. However, HCV infection can cause NK cells to down regulate the activating receptor NKp30 [229], while NKp46 expressing NK cells appear ineffective against infection [230].

1.3.4 Human Immunodeficiency Viruses

1.3.4.1 HIV & AIDS

AIDS is a disease caused by infection with either HIV-1 or HIV-2, and characterized by the progressive destruction of immune cells. This destruction, particularly of the CD4 T cells, leaves patients susceptible to many opportunistic infections that are generally well controlled in healthy individuals such as *T. gondii*, cryptococcus, *P. jirovecii*, or cytomegalovirus. There is also an increased incidence of some cancers like Karposi's sarcoma. Thus in the end it is not HIV that directly kills the host, rather the host succumbs to other ailments.

Clinically, Gottlieb first described AIDS in 1981 [231], due to its appearance and apparent restriction to the homosexual population in San Francisco and Los Angeles. However, AIDS had long been observed in Africa [232,233] and was referred to as 'slim disease' due to the

wasting effects of the disease. The causative agent, HIV, was initially isolated in 1983 [234], by this stage it was known that the disease could be spread sexually, perinatally and through contaminated blood products.

HIV can infect many different immune cells including T cells, dendritic cells, and macrophages due to their expression of the CD4 receptor [235] and co-receptor CCR5 or CXCR4 [236]. Once in the cell cytoplasm, the viral RNA genome can begin to be transcribed by reverse transcriptase into DNA. This viral DNA is then transported to the nucleus and integrated into the host genome, where it may lie dormant or begin to replicate [237,238].

HIV-1 and HIV-2 viral genomes have approximately 60% sequence homology [239] and differ in a number of ways. HIV-2 appears to be less transmissible [240], and fewer of those infected progress to AIDS [241]. For these reasons infection with HIV-2 is seen by some to act as an attenuated form of HIV, while HIV-1 is responsible for the AIDS pandemic.

Treatment of HIV infection has progressed rapidly over the last few decades, as AIDS became a major global problem and received dedicated research funding. Antiretroviral therapy (ART) has helped transform treatment and vastly increased the life span of people infected with the virus, saving countless lives and reducing the burden of disease [242]. Currently treatment with ART is suggested to start upon diagnosis of the disease irrespective of a patients CD4 T cell count [243]. Although we have effective treatment we still lack a practicable cure [244], with stem cell transplantation not a viable option for large-scale implementation [245]. An effective vaccine is also still a distant goal. There are other effective methods to help stop the spread of the disease, with condoms and pre-exposure prophylaxis (PrEP) [246] able to significantly reduce infection from sexual encounters.

1.3.4.2 HIV Infection & Immunity

HIV entry through a mucosal surface will be encountered by DCs, which will take up the virus to present it to CD4 T cells. At the same time a local immune response to the infection can begin. However, DCs can pass the virus onto CD4 T cells via the receptor DC-SIGN and other surface lectins [247,248]. Once an infected DC arrives in a lymph node, the infection can spread systemically [249,250]. If HIV enters intravenously then the virus can travel to any tissue and immediately begin to infect T cells, and DCs.

In infected cells, viral RNA and DNA intermediates will stimulate the PRRs leading to a cytokine storm of pro-inflammatory cytokines during acute infection [251,252] (Fig. 8), including a substantial and rapid increase in IFN-α. After infection HIV has a number of mechanisms to evade immune recognition controlled by accessory proteins, which differ between HIV-1 and HIV-2. These proteins are not directly necessary for replication but do target host immune proteins thereby enabling effective and efficient transmission and replication [253]. The accessory HIV-1 protein Vpu, is known to interrupt the cycling of

MHC-like molecules [66] and IRF3 [254], while the HIV-2 Vpx protein can inhibit IRF5 [255].

Declining CD4 T cells, which typifies AIDS progression, occurs either due to activation-induced cell death, killing by anti-viral cytotoxic cells, or a form of virally induced programmed cell death: pyroptosis. CD8 T cells are able to recognize and kill infected cells [256,257] but also lyse uninfected CD4 T cells [258], while NK cells are also capable of killing infected cells [259]. Recently it has been suggested that pyroptoptic cell death drives CD4 T cell depletion [260], which occurs when HIV genetic material is detected in the cytoplasm of naïve CD4 T cells [261].

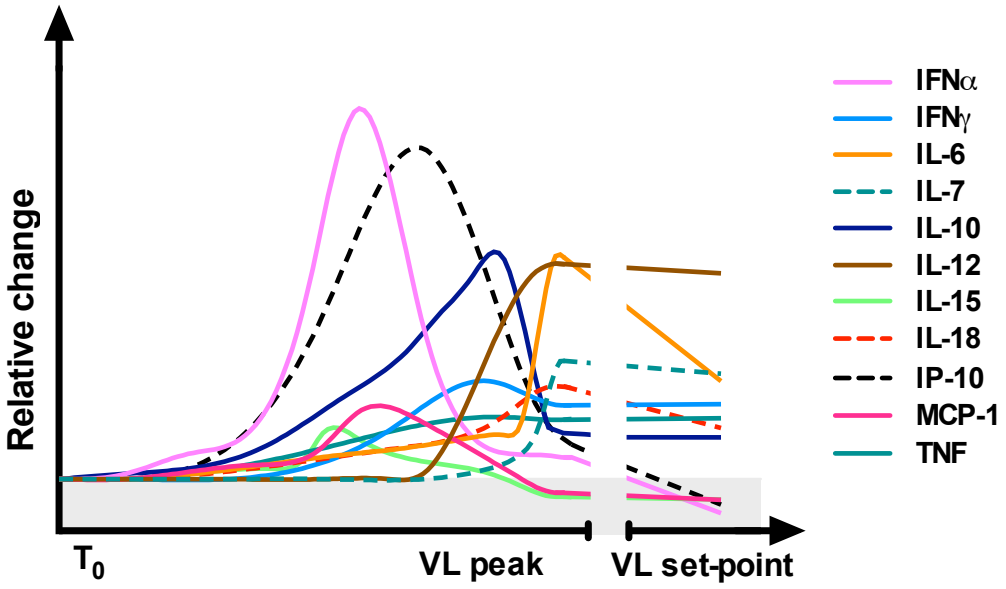

Figure 8: Cytokine storm in acute HIV infection [252]

Innate immunity, involving iNKT and NK cells, also plays an important role in pathogenesis [254]. During HIV-1 infection there is a significant reduction of $CD4^+$ iNKT cells [262], with the remaining iNKT cells exhibiting functional impairment with reduced IFN-γ production, and increased PD-1 expression suggesting exhaustion [263]. Loss of iNKT cell regulation of the immune response during HIV-1 infection may hinder effective NK cell and DC function [264].

NK cells are activated during viremic HIV-1 infection [265], and such activation has been associated with disease progression [266]. However, lysis of infected targets may require pDC derived IFN-α [267], and the activating receptors NKp46 and NKG2D [268]. During HIV-1 infection there is a vast increase in the $CD56^{neg}$ NK cells, particularly in viremic patients [116]. The origin of these cells in HIV-1 infection has been suggested to be the $CD56^{dim}$ NK cells [269]. Regrettably the role of NK cells during HIV-2 infection has not been well studied. The one study previously performed identified the viral load during HIV-2 infection to be associated with NK cell function [270].

1.4 CHRONIC VIRAL INFECTION

There appears to be a window in which the cellular immune response can eradicate a viral infection. At this juncture NK cells switch their intracellular signalling pathways in response to IFN-α stimulation [271], and T cells will start to be killed by NK cells [272]. Thus if a virus can persist during this initial period, either due to an underactive immune response or unmanageable viral burden, the infection may become chronic. Despite failing to clear various infections some persistent infections are kept in check by the immune system and remain asymptomatic. In some chronic cytomegalovirus infections there is an expansion of $NKG2C^+$, self-KIR^+ NK cells [273], which may be involved in keeping the virus in check.

Following failure to clear a viral infection the immune system may persist in its responses, with increased pro-inflammatory, and anti-inflammatory signals. With both these competing signals increased, the immune system can become dysfunctional and exhausted. Hence in predominantly non-cytopathic infections such as hepatitis, off target activation can cause severe damage to the liver [274], while in HIV you find over-activated and functionally impaired NK cells. Of course in both hepatitis and HIV infections the virus can cause degrees of cell death, the amount of which may well help determine how long it will take to progress to cirrhosis or AIDS.

1.4.1 Chronic Immune Activation

A big part of the pathology of chronic viral infection is the associated immune activation. During HIV-1 infection this chronic activation can have a number of causes, including pyroptotic cell death and microbial translocation [275]. This activation causes aging of the immune system [276] and also causes co-morbidities such as liver steatosis and cardiovascular disease [275,277]. Levels of pro-inflammatory cytokines are increased [252], and T cells have been observed to have a persistently activated and exhausted phenotype [275]. Additionally, NK cells are continually activated and are likely to contribute to the pro-inflammatory cytokine milieu with IFN-γ [278]. Even during ART NK cells display a partly activated phenotype [279].

Chronic immune activation in the liver can eventually lead to severe cirrhosis. Numerous studies have linked the rising levels of pro-inflammatory cytokines to worsening stages of liver disease [280,281]. The recent Icona Foundation study showed that high levels of TNF correlated with an increased risk in advancing to cirrhosis [282], and increased sCD163, signifying macrophage activation, is associated with the fibrosis stage [283]. During chronic hepatitis activated NK cells are able to alleviate fibrosis by killing non-virus-specific CD8 T cells and hepatic stellate cells via TRAIL. Despite this protective action however, NK cells will also continue to produce IFN-γ and kill hepatocytes [22]. It has not been fully elucidated if the new DAA therapy for HCV is able to alleviate the immune activation.

1.4.2 Microbial Translocation

A major contributor to chronic immune activation is microbial translocation from the gut. Immune activation in the gut, with involvement of TNF and IFN-γ, can result in aberrant enterocyte turnover and destruction of tight junctions [284]. With the loss of structural integrity the gut epithelium becomes more permeable permitting the movement of relatively large quantities of bacterial material into the tissues, which can then drain to the liver via the portal vein.

Microbial translocation can be measured as presence of lipopolysaccharide (LPS) levels in the general circulation, while the effects can be observed in the increased levels of soluble CD14 (sCD14) in the blood [281]. CD14 is a co-receptor for LPS, alongside TLR4, found on the surface of macrophages that can stimulate activation. Upon activation, membrane bound CD14 will be shed as sCD14, and sCD14 can also be secreted. Hepatocytes are reported to express and secrete CD14 in response to pro-inflammatory signals [285], suggesting that microbial translocation can induce sCD14 from both macrophages (possibly Kupffer cells as well), and hepatocytes. Conceivably there are also other routes leading to sCD14 release as patients with non-alcoholic steatohepatitis (NASH) [286], and common variable immune deficiency (CVID) [287] have increased sCD14 plasma levels. A high level of sCD14 in the blood has been reported to be a negative predictor of IFN-α treatment of HCV in HIV/HCV co-infection [288].

In both HIV-1 and HIV-2 infections the level of microbial translocation, as measured by LPS [289], is indicative of CD4 count and viral load [290]. Likewise in hepatitis infections, the level of LPS, and by extension microbial translocation, correlates with disease severity in HBV and HCV [281]. In both HIV and hepatitis infections, immune activation and microbial translocation cause and perpetuate each other, increasing disease severity (Fig 9). Understanding and breaking this vicious cycle could lead to effective new treatments [291].

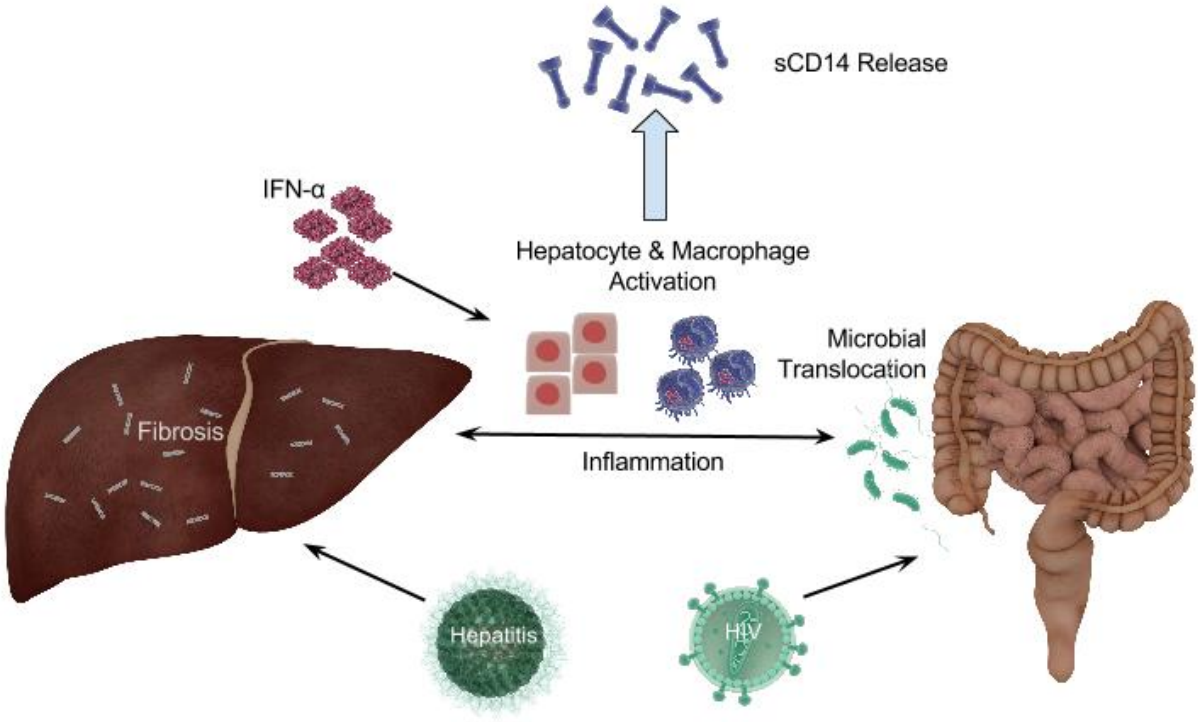

Figure 9: interplay of immune activation and microbial translocation

2 AIMS

As is to be expected from basic science research the precise aims of my work have fluctuated over the years depending upon results and collaboration opportunities. However, the over-arching theme has remained true – improve our understanding of the role of NK cells in viral infections. To this end the work included in this thesis explores how the phenotype and function of NK cells differ between stages of hepatitis infection and between the virus infections themselves. Furthermore, I have investigated if the activation level of NK and iNKT cells differs between HIV infections. In addition we asked the question of what effect IFN-α therapy of hepatitis has upon innate immune systems, studying the role of sCD14 as a biomarker in HCV therapy and the alterations of NK cells in HDV therapy.

3 RESULTS AND DISCUSSION

Paper I: "Compromised Function of Natural Killer Cells in Acute and Chronic Viral Hepatitis".

Paper II: "Effects of HDV infection and pegylated interferon α treatment on the natural killer cell compartment in chronically infected individuals".

Paper III: "The Dynamic Relationship between Innate Immune Biomarkers and Interferon-Based Treatment Effects and Outcome in Hepatitis C Virus Infection Is Altered by Telaprevir".

Paper IV: "Elevated levels of iNKT cell and NK cell activation correlate with disease progression in HIV-1 and HIV-2 infections".

3.1 ALTERATIONS OF NK CELLS DURING VIRAL INFECTIONS

Papers I and **IV** principally concern the alterations and activation of NK cells that occur in viral infections. During viral hepatitis infections major changes in NK cell phenotype and function occur progressively throughout acute and chronic infections. These alterations are shared between infections regardless of the infecting virus. Along the same lines as this, we observed no principle difference in NK cell activation levels between HIV-1 and HIV-2 infections, with changes in activation more associated with disease progression and closely linked to viral loads.

3.1.1 NK Cells During Acute & Chronic Hepatitis Infections

In **Paper I,** utilising material collected by our collaborators at Hannover Medical School, we were able to perform a comprehensive examination of NK cells during both acute and chronic phases of HBV and HCV infection, and chronic phase of HDV infection. The first thing to notice is a substantial increase in the percentage of $CD56^{bright}$ NK cells during the acute infections relative to both healthy individuals and the chronic phases. Levels of $CD56^{dim}$ NK cells were only increased in chronic HCV and HDV infections, not HBV infection.

Decreases in expression of several NK cell surface markers during infection did not appear to be specific to either NK cell subset. We did however see differences between the acute and chronic phases, with reductions of CD25 and DNAM-1 on $CD56^{dim}$ NK cells in chronic hepatitis, while reduction of CD48 on $CD56^{bright}$ NK cells was again associated with chronic infection. Despite these changes in subset distribution and receptor expression between the acute and chronic phases, there were minimal alterations of maturation-associated makers (i.e. CD57, KIRs, and NKG2A) on $CD56^{dim}$ NK cells.

Induction of *in vitro* functional responses, following stimulation with K562 target cells with addition of IFN-α, were generally reduced in NK cells from hepatitis patients compared to controls. NK cells did however retain a multifunctional profile (Fig. 10). One noticeable

difference between acute and chronic HBV infection is that NK cells in the former condition produced less macrophage inflammatory protein (MIP)-1β, an effect not seen for HCV infection.

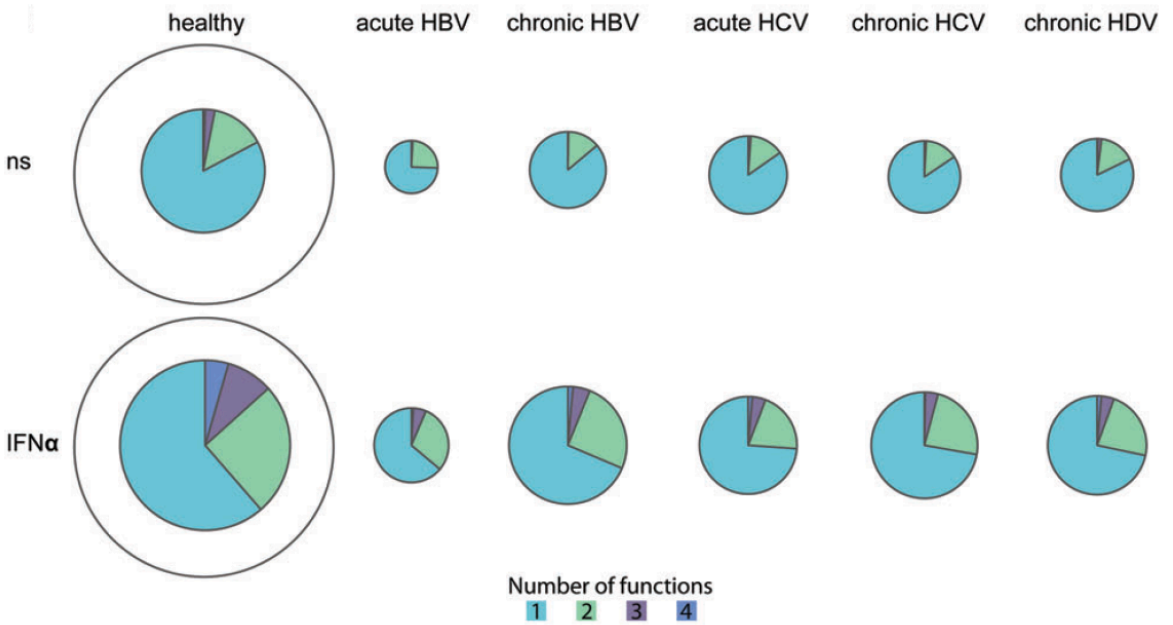

Figure 10: NK cell multi-functional responses to K562 +/- IFN-α during different phases of hepatitis infections (Paper I)

We performed a principle component analysis (PCA) of the NK cell profile to assess which combination of NK cell markers were significantly different between the viral infections and disease stages. Our PCA indicated relatively little difference in phenotype and function of NK cells between the viral infections; rather it was between the acute and chronic infections that we found the major alterations of the NK cells. Highly significant changes in the NK cells were a shift in subset distribution towards $CD56^{bright}$ NK cells, $\%NKG2A^{+}$ NK cells, and TRAIL expression on all NK cells. Expression levels of NKG2A are likely to be related to the increased size of the $CD56^{bright}$ subset (Fig. 11). Although our study did not consider $CD56^{neg}$ NK cells and how their appearance may affect NK cell subset distribution, as our results were based upon NK levels as a percent of lymphocytes this guards against the loss of $CD56^{dim}$ NK cells appearing as an increase in $CD56^{brights}$ NK cells.

Regrettably in **Paper I** we did not explore alterations of the activating NCRs NKp30, NKp44, and NKp46, or NKG2C on NK cells. In 2011, Alter et al [292] showed that HCV infections were associated with a decrease in NKp30, and NKp46 expression on NK cells, although distribution of these markers between $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets or expression levels were not explored. The role of NKp46 in CHC in particular is perplexing with reports indicating that it may be involved in suppressing HCV replication [293] but that $NKp46^{+}$ NK cells are pathologically activated and irresponsive to therapy [230]. Additionally in HBV infection Zaho et al [294] reported increases of NCR expression on NK cells, particularly during the acute phase.

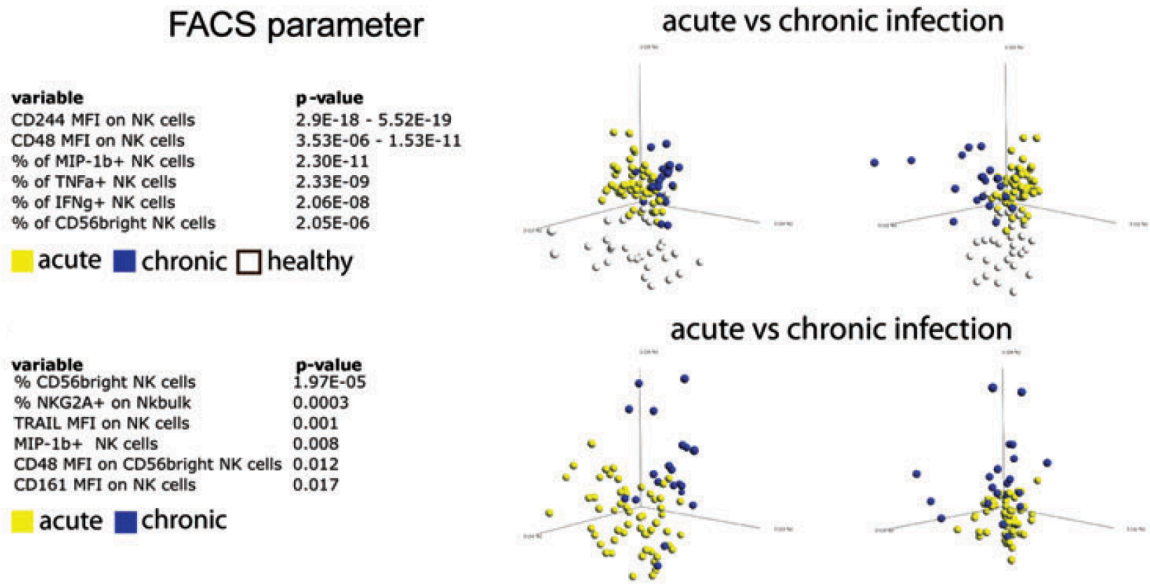

Figure 11: PCA of NK cell parameters between acute and chronic hepatitis infections (Paper I)

In 2009, Oliviero et al [295] examined expression levels of the NCRs and NKG2C of NK cells during CHB and CHC infections. They described that NKG2C was significantly increased on NK cells from CHB but not CHC infection, while levels of NCR expression were unchanged on NK cells that expressed NCRs. Interestingly their functional assays indicated that NK cells from patients with CHC had greater degranulation responses to P815 target cells with cytokine stimulation, as compared to patients with CHB infection or healthy controls. However when switching to K562 cell targets NK cells from CHC subjects had the same CD107a response as healthy controls, while NK cells from CHB had a lower degranulation response than both. When using anti-NCR stimulation and again P815 targets they saw an increase in the CD107a response from NK cells from CHC infected patients. These digressions from our results intonates that the target cell line and cytokine mix used for functional assays may lead to differing results, although differences between patient cohorts are hard to exclude. Furthermore, the differing receptor expression on NK cells from the various viral infections may also have a bearing on the functional responses, and be a key factor affecting disease outcome.

In our study, pan-KIR expression was not altered due to infection, nor did it differ between the infections. Oliviero et al [295] observed a slight decrease in inhibitory KIR expression in CHC compared to CHB and controls, while similar to us Alter et al [292] saw no difference in NK cell KIR expression between acute or chronic HCV compared to controls. However, in a more detailed examination of the KIR profile Oliviero et al observed that the reduction was due to reduced KIR3DL1, and a trend for reduced KIR2DL1 [295], similar to our unpublished results currently being compiled for publication.

3.1.2 NK Cell Activation During HIV Infections

For **Paper IV**, as part of a wider collaboration of HIV-2 studies in a prospective cohort in Guinea-Bissau, we were able to demonstrate that activation levels exhibited by NK cells is

associated more with disease activity and progression, rather than with the specific type of HIV infection. Additionally by comparing viremic versus non-viremic HIV-2 infections we were able to delineate if altered activation levels of the NK cells was due to infection or disease progression.

Similar to prior studies of HIV-1 infection [296], we observed a trend for fewer $CD56^{dim}$ NK cells in the blood and a significant reduction in the number of $CD56^{bright}$ NK cells. Looking within the HIV-2 infected group we observed that the reduction of $CD56^{dim}$ NK cells occurred in viremia, while the reduced numbers of $CD56^{bright}$ NK cells appeared to be related to infection as it also occured in non-viremic persons. In 2004 Alter et al suggested that NK cell numbers in HIV-1 infections were related to viremia [265], implying that in both HIV-1 and HIV-2 infections depletion of NK cells is associated with viremia.

Our study utilized the expression of CD38 as a marker for cellular activation [297]. HIV-1 infection was associated with greater $CD56^{dim}$ NK cell activation when compared to community-matched healthy controls and HIV-2 infected persons. Along with studies on HIV-1 [265,266,298], our HIV-2 viremia results indicate this increase in activation is driven by viral replication. $CD56^{bright}$ NK cells have a higher activation level in all settings of HIV infection compared to non-infected controls, with HIV-1 infection exhibiting greater activation than HIV-2 infection. Viremia in HIV-2 infection associates with increased activation of the $CD56^{bright}$ NK cells, but even the non-viremic HIV-2 infected persons have higher activation than the controls indicating that both infection status and viral replication are involved in the generation of activated of $CD56^{bright}$ NK cells (Fig. 12).

Similar to previous studies [262], $CD4^{+}$ iNKT cell numbers were reduced in HIV-1 infections relative to controls, and in HIV-2 infections the reduction of cell numbers was related to viremia. Also paralleling prior studies [263], the activation status of iNKT cells in all HIV infections was increased and correlated with increasing viral loads and declining CD4 T cells. The activation levels of iNKT and NK cells in our study were very closely correlated, particularly $CD4^{+}$ iNKT cells and $CD56^{bright}$ NK cells. Additionally, iNKT and NK cell activation was concurrent with CD4 T cell activation (Fig. 13), implying that the same mechanisms may be causing activation of these cell types.

Due to the limited sample availability, our analysis of NK cell populations was disappointingly limited, and we could not perform a more extensive phenotypic or functional assessment in the different infections. In particular it would be fascinating to examine NCR expression of NK cells in HIV-2 relative to HIV-1 [299], and relate this information to their functional capabilities. Despite these gaps in the literature, the findings in **Paper IV** along with other HIV studies [266,270,300] indicate that activation of NK cells during HIV infection is concomitant with other immune activation events (Fig. 13). It is likely that this apparent increase in NK cell activation and limited functional capacity [270,298] reflects

chronic immune activation, which may also be the underlying cause of the increase in CD56^{neg} NK cells.

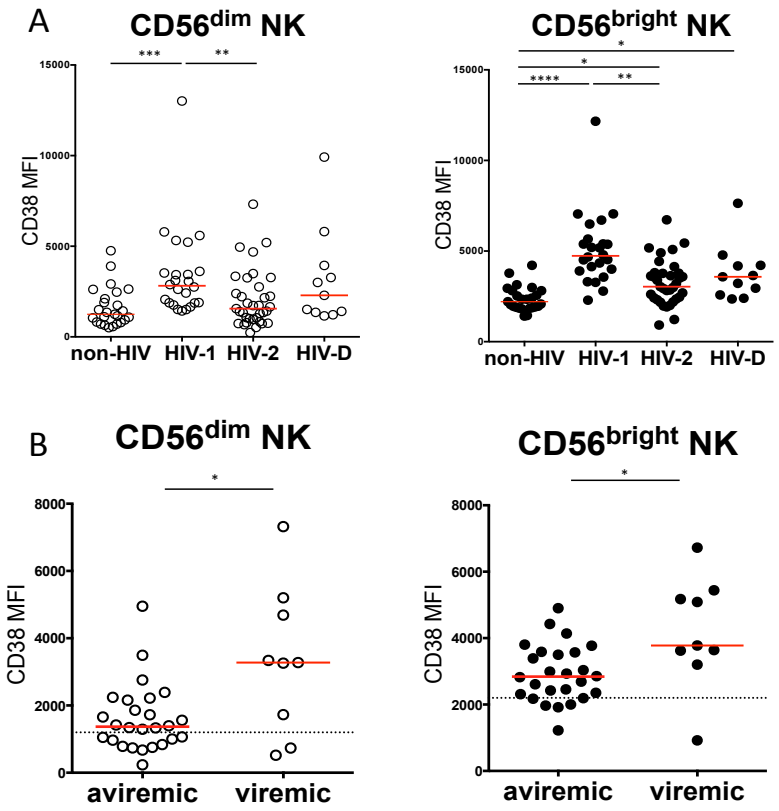

Figure 12: Activation levels of NK cell subsets in A) HIV infections, and B) HIV-2 non-viremic (aviremic) vs viremic infections; *Significance (Paper IV)

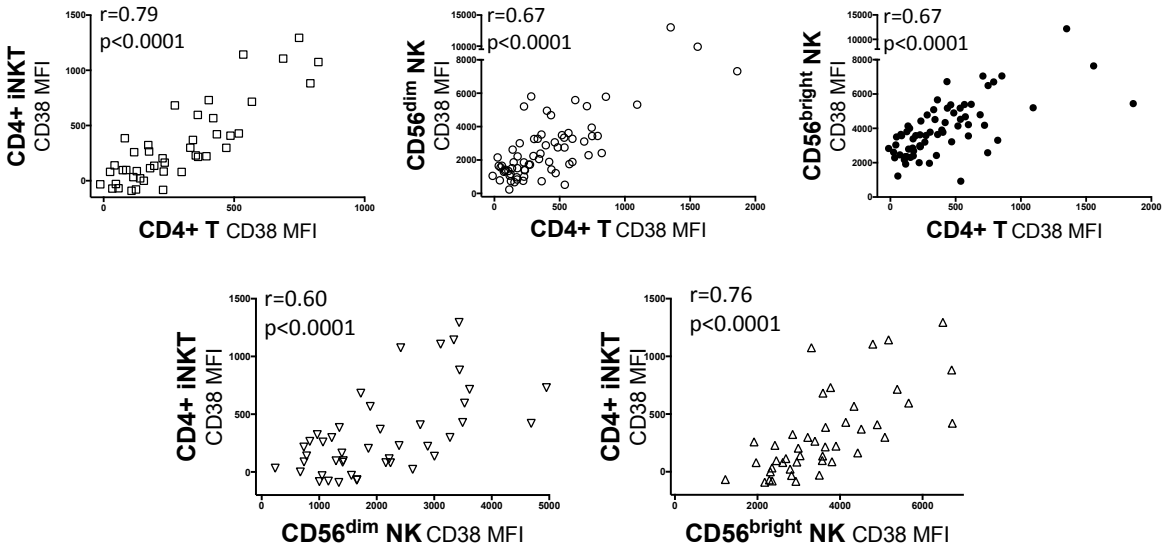

Figure 13: Concomitant cellular activation during HIV infection (Paper IV)

3.1.3 NK Cells in Chronic Viral Infections

Throughout these studies it has been difficult to ascertain which alterations of the NK cells represents a response to chronic infection, and which are resultant from general broad-based chronic inflammation. A further caveat of our studies is that we have access only to blood samples, and thus can only surmise on how organ-resident NK cells are altered. This will pose a problem if they respond or are affected differently in tissues such as the liver [301].

Proportions of NK cells within peripheral blood appear to differ between hepatitis and HIV infections with levels of NK cells increasing in hepatitis and decreasing in HIV. Comparative studies of HIV and HCV have shown that NK cells decline in both infections at the expense of $CD56^{dim}$ cells [296], most likely due to a transition of $CD56^{dim}$ to $CD56^{neg}$ NK cells [269], a subset we did not measure. As we observe that the levels of $CD56^{bright}$ NK cells are affected in HIV infected patients, and levels of $CD56^{dim}$ NK cells are associated with markers of disease progression, we could surmise that differences in NK cell levels between these infections are due to differing levels of chronic inflammation. Regrettably we were unable to stratify our chronic hepatitis patients by fibrosis stage to examine if this affected NK cell subset distribution. We do know that chronic inflammation is linked to more severe fibrosis in viral hepatitis and disease progression in HIV infections, thus it would be interesting to assess distribution of NK cell subsets along with inflammatory status between these infections and non-viral inflammatory conditions such as NASH. In this way we could attempt to reconcile our results with others showing declining NK cell numbers in both HIV and HCV [296].

Having only measured CD38 in our HIV study, we are unable to comprehensively compare the NK cell phenotype between the HIV infections. As NK cells from HIV-1 long-term non-progressors do not appear to differ from viremic HIV-1 persons [298], we could hypothesise that the less likely to progress HIV-2 infection also does not cause major phenotypic changes in NK cells. Certainly NCR expression would be fascinating to assess due to the role of these receptors in mediating and regulating NK cell function in HIV infection [299] as well as viral hepatitis [295].

During both HIV and hepatitis infection there is an increased activation of the NK cells accompanied with a reduced functional capacity. Thus it appears that in chronic infection NK cells may be less responsive to the chronic inflammatory conditions in which they reside. It would be intriguing to further explore the functional differences of NK cells between chronic infections, while controlling for levels of inflammation.

3.2 EFFECTS OF IFN-α IN HEPATITIS THERAPY

Pegylated IFN-α and ribavirin has been the basis for hepatitis treatment for many years, and its use afforded us the opportunity to examine the effects of IFN-α upon the immune system. Both **Papers II** and **III** explore the modulatory effects of IFN-α therapy in chronic hepatitis upon innate immunity. **Paper II**, and its associated work, shows how the NK cell response to IFN-α therapy is altered, while **Paper III** examines sCD14 levels over the course of IFN-α therapy, with or without the new DAA drugs.

3.2.1 Effects of IFN-α Therapy on NK Cells

In **Paper II** we studied the effects that IFN-α therapy has upon NK cells during chronic HDV infection. In our cohort of HDV patients we observed that, compared to healthy donors, there were higher proportions of both $CD56^{dim}$ and $CD56^{bright}$ NK cells in the peripheral blood. Following 12 weeks of IFN-α therapy there was a significant decrease in the number of $CD56^{dim}$ NK cells; those that were left had a more immature phenotype than at baseline with increased NKG2A and less CD57 expression (Fig. 14). Similar to observations in HBV [302] and HCV therapy [303,304] the numbers of $CD56^{bright}$ NK cells significantly increase after 12 weeks of therapy, implicating IFN-α in arresting NK cell maturation.

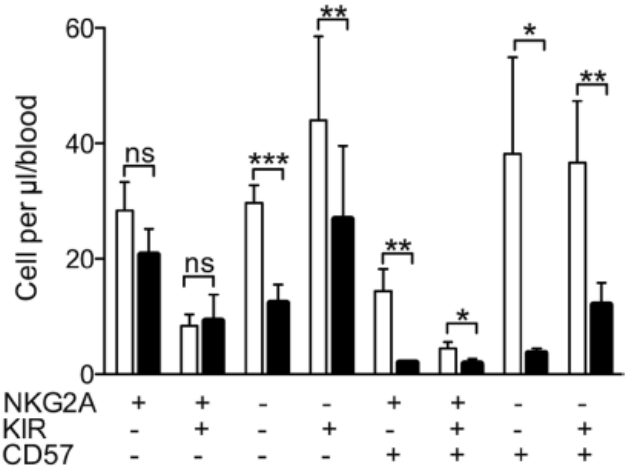

Figure 14: Number of $CD56^{dim}$ NK cells exhibiting maturation markers in CHD patient before (white bars) and after (black bars) 12 week IFN-α therapy; *Significance (Paper II)

NK cell phenotypes were significantly altered after 12 weeks of IFN-α therapy in our HDV cohort. Following therapy, $CD56^{dim}$ NK cells expressed less CD16, NKG2D, and less of the inhibitory receptor Siglec-7. While $CD56^{bright}$ NK cells also expressed less CD16, and NKp30, their expression of Siglec-7 was increased. The decrease in NKp30 levels was not seen in a previous study of IFN-α therapy of HCV [303]; the same study also reports significant decreases in Siglec-7 expression on NK cells but failed to clearly distinguish between the $CD56^{dim}$ and $CD56^{bright}$ NK cells. However, in another study of HBV and HCV infection, Siglec-7 negative NK cells were associated with reduced expression of CD16 and NCRs, and suggested to be dysfunctional [305].

IFN-α therapy had profound effects upon the functionality of NK cells. Not only was responsiveness to K562 target cells reduced significantly, induction of functions by *in vitro* IFN-α stimulation was also diminished. NK cells, from week 12 samples, displayed limited increase in CD107a expression in response to IFN-α, and no increase in production of pro-inflammatory cytokines (Fig. 15). Induction of TRAIL expression was also stunted in response to IFN-α, however as post therapy levels of TRAIL were higher than those observed at baseline, the level of TRAIL following IFN-α stimulation was very similar between baseline and week 12 samples.

A possible explanation for the stunted maturation and refractory response to IFN-α is the changes in NK cell intracellular signalling caused by therapy. Following IFN-α therapy we found significantly increased levels of phosphorylated signal transducer and activator of transcription (pSTAT) 1 and pSTAT4 in the NK cells. However, the *in vitro* potential for IFN-α to stimulate the induction of pSTAT4 was severely reduced following therapy (Fig. 16), suggesting that IFN-α therapy predisposes NK cells to function primarily through the pSTAT1 pathway, which is involved in NK cell maturation and degranulation. This may help explain why IFN-α could no longer induce production of pro-inflammatory cytokines, a predominantly pSTAT4-dependent pathway, and the stunted maturation. The timing of this switch may have some bearing on treatment outcome [306].

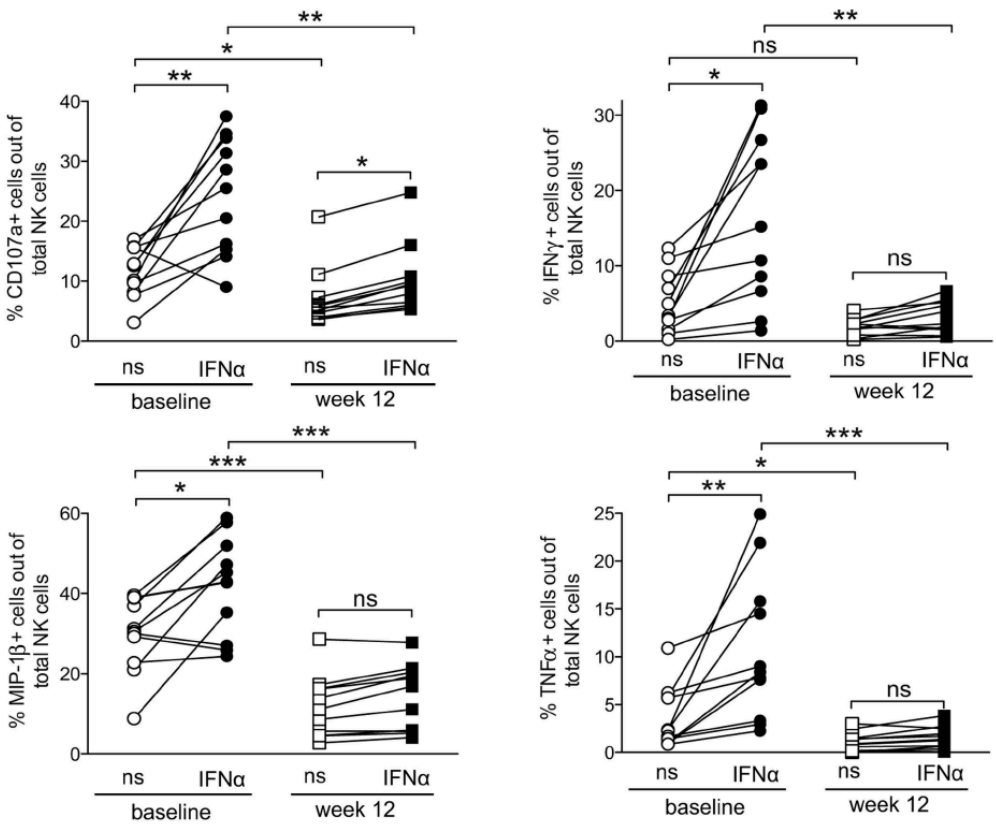

Figure 15: Functional response of NK cells to K562 +/- IFN-α before and after IFN-α therapy for CHD; *Significance (Paper II)

Clearly, prolonged therapy with IFN-α has varied effects upon NK cells with $CD56^{dim}$ NK cells generally decreasing activating and inhibitory surface receptors, with the exception of NKG2A; while the $CD56^{bright}$ population displays both increases and decreases of receptor expressions. We also observed, although did not publish, the changes occurring in the $CD56^{neg}$ NK cell population during IFN-α therapy. Lower levels of this abnormal NK cell subset, prior to IFN-α therapy of HCV, is associated with a better treatment response [307]. During IFN-α therapy for HDV these $CD56^{neg}$ NK cells appear to recover their phenotype towards that of $CD56^{dim}$ NK cells with restored expression of NKp46, NKG2D, and increased TFs EOMES and T-bet (Fig. 17). We were unable to assess if this potential recovery was linked to treatment success but this topic is certainly worthy of future study. However, a high baseline proportion of $CD56^{dim}$ NK cells was associated with a good treatment response; these higher levels of $CD56^{dim}$ NK cells possibly correlate with fewer $CD56^{neg}$ NK cells, a pattern which was previously linked to better treatment responses in HIV/HCV co-infections [288].

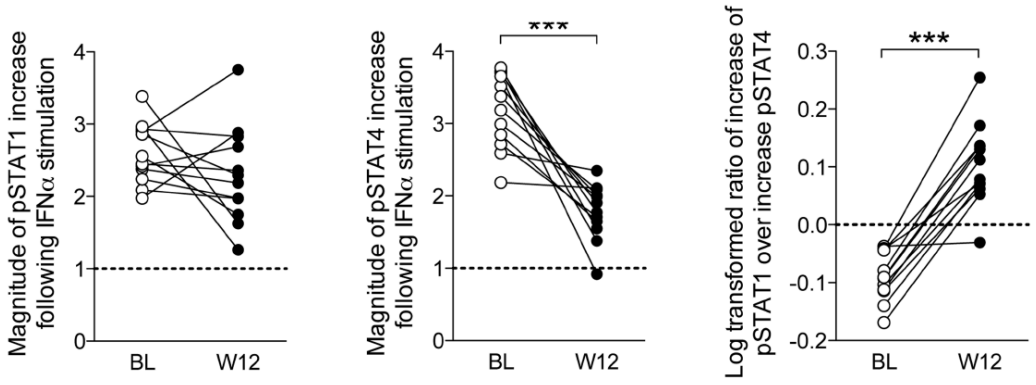

Figure 16: Reduction of pSTAT4 inducibility in NK with IFN-α following CHD therapy with IFN-α; *Significance (Paper II)

3.2.2 Biomarker Alterations During IFN-α Therapy

For **Paper III** we were fortunate enough to be able to obtain prospectively collected HCV plasma samples from the Karolinska University Hospital bio-bank before and during IFN-α therapy. Using these samples we measured the levels of sCD14 and IL-18 for patients receiving either, at the time standard, dual therapy of IFN-α and ribavirin, or the new triple therapy that had the addition of the DAA protease inhibitor, Telaprevir.

Patients undergoing therapy with IFN-α experienced significantly increased plasma levels of sCD14 relative to pre-treatment levels, but the measurable dynamic range of sCD14 was limited. Previously it has been shown that high levels of sCD14 associate with advanced liver disease in HBV, and HCV infections [281]. Consonant with this we observed in our dual therapy cohort that the lowest baseline levels of sCD14 associated with treatment response; this association has also been made in treatment of HIV-1/HCV co-infection [288]. However, in our study, this relationship was ablated by the addition of Telaprevir to the treatment regimen (Fig. 18). For the triple therapy cohort a different pattern emerged, as a larger

increase of sCD14 after 4 weeks of therapy associated with treatment response. This observation adds weight to the thought that Telaprevir improves the effectiveness of IFN-α therapy by restoring IFN-α sensitivity [91].

An on therapy increase of pro-inflammatory cytokine IL-18 was observed in our dual therapy cohort; this was not seen in the triple therapy cohort where the patients exhibited elevated baseline IL-18 levels. Potentially, these higher IL-18 levels in the triple therapy cohort were due to over half the group having stage 4 liver fibrosis. In contrast, we observed an increase of IL-18 after 4 weeks of dual therapy that was not seen for triple therapy. Furthermore, the lower baseline levels of IL-18 were associated with dual therapy response, but again the effect was not observed in triple therapy. Given the high baseline levels of IL-18 in the triple therapy cohort, it was difficult to interpret if Telaprevir affects the patient's IL-18 response to IFN-α therapy.

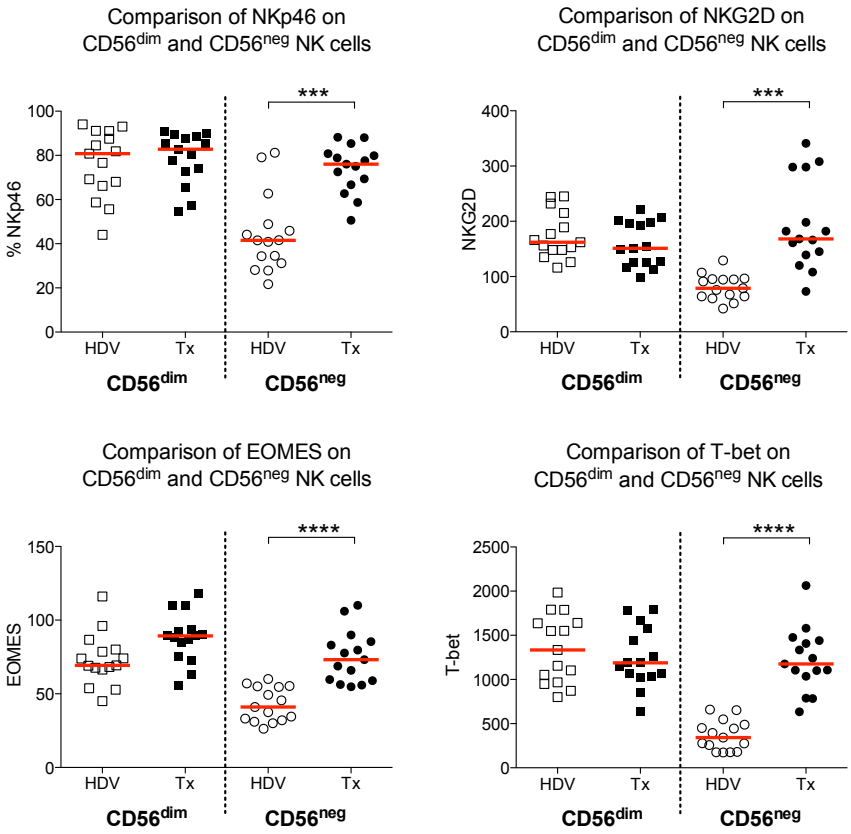

Figure 17: Normalisation of CD56^{neg} NK cell phenotype to that of CD56^{dim} NK cells following IFN-α therapy for CHD; *Significance (unpublished data)

3.2.3 Predicting IFN-α Therapy Response

A number of soluble and cellular markers have previously been suggested to be linked to disease progression or help predict response to IFN-α therapy. The presence of CD56^{neg} NK cells has been mentioned previously; sCD163 is linked to fibrosis in HBV and HCV infections [283,308], as well as being associated with IL-28B (IFN-λ3) genotype [309] which is predictive of IFN-α therapy outcome [310]. Education of NK cells through KIR2DL3 is associated with

viral clearance [227]. More recently, levels of soluble Siglec-7 and the presence of Siglec-7 negative NK cells have been correlated with disease severity in hepatitis [305]. Additionally, the levels of TRAIL on NK cells induced by therapy was linked to a favourable outcome [311], while NKp46^{+} NK cells were reported to not respond to therapy [230]. The strongest reported predictor of IFN-α treatment response for HCV is the hepatic expression of four ISGs [218], including ISG15 that has also been linked to treatment response in HBV infection [312].

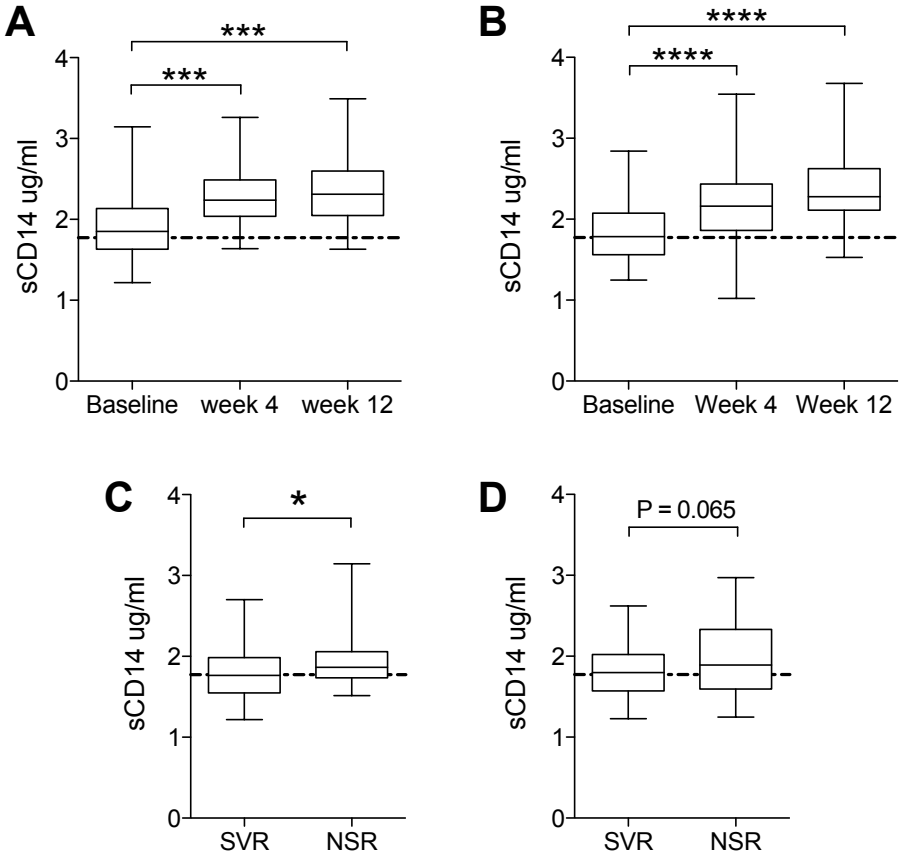

Figure 18: Increased sCD14 during A) dual therapy and B) triple therapy. Baseline levels of sCD14 associated with treatment response in C) dual therapy but D) not triple therapy; *Significance (Paper III)

The two biomarkers that we have reported on here, i.e. high baseline levels of CD56^{dim} NK cells and low levels of plasma sCD14, give credence to the idea that IFN-α therapy requires the innate immune system to not be in a state of over-activation or exhaustion to be effective. With the myriad of innate biomarkers linked to HCV infection resolution and treatment response, we can begin to build a picture of the NK cell compartment ideally suited to help IFN-α therapy work effectively. However, what appears to be a crucial factor in treatment response is the ability of IFN-α to further induce ISG expression in the liver, which may only be possible if the system has not been exhausted by systemic chronic immune activation.

3.3 NK CELLS IN THE AGE OF DAA

Fibrosis stage and chronic inflammation are good indicators of how a patient will respond to IFN-α therapy. Therefore it is of great interest that in **Paper III** the effectiveness of triple therapy was not confined to patients with the lowest levels of sCD14, and by implication not as affected by chronic inflammation. Thus we can speculate whether there is an active role for NK cells in the new DAA therapy or if they are superfluous.

At the time of writing there are two prominent published articles examining NK cells during IFN-free DAA therapies for HCV infection. Serti et al [313] reported that the increased levels of NKp30, NKp46, and NKG2A returned to healthy control levels after 8 weeks of treatment. Functional responses of NK cells also normalised to levels exhibited by healthy controls with decreases in K562 degranulation response and TRAIL expression, and increases in IFN-γ secretion. A possible underlying cause of these changes may be declining levels of pSTAT1 in NK cells during DAA therapy, unlike IFN-α therapy.

Recent results by Spaan et al [314] confirm the decreased expression of NKp30, NKp46, NKG2A, and TRAIL on NK cells over the course of therapy. This study also suggested that DAA therapy was associated with a decrease of ISG mRNA expressed by leukocytes. Regrettably, neither study examined the $CD56^{neg}$ NK cells, but nonetheless these papers suggest that DAA therapy restores NK cell phenotype and function to that observed in healthy controls. The mechanisms behind this action still require investigation, but a reasonable hypothesis would be that DAA in halting viral replication alleviates chronic inflammation and unburdens the NK cells, allowing them to function normally and potentially aid viral clearance.

4 CONCLUDING REMARKS

This thesis aimed to explore how NK cells and innate immune activation change during viral infections, and what effect IFN-α therapy had upon them. The data and conclusions from the papers presented show that NK cell distribution and activity are important during chronic viral infection, and that IFN-α as a therapy fundamentally alters these aspects of NK cell biology. Furthermore, broad-based persistent activation of the innate immune system occurs during chronic viral infection, and during IFN-α therapy. The main conclusion from each paper is as follows:

- Alterations of NK cell phenotype and functional capabilities in viral hepatitis infections are dependent upon disease stage (**Paper I**)
- IFN-α therapy of CHD results in refractory NK cell responses to IFN-α, along with a propensity to signal through pSTAT1 rather than pSTAT4 (**Paper II**)
- Baseline levels of sCD14 are predictive of IFN-α treatment of CHC, an effect ablated by DAAs (**Paper III**)
- Activation levels of NK, iNKT, and CD4 T cells increase concomiantly with increased HIV replication (**Paper IV**)

Together these findings help illustrate the roles that chronic infection and IFN-α therapy have in altering NK cells and the wider innate immune system. These conclusions only allude to potential mechanisms that require further study.

4.1 FUTURE DIRECTIONS

Despite my conjecture $CD56^{neg}$ NK cells still hold many mysteries. The precise origin of these cells in chronic infections, and if they do have a function, remains to be ascertained. However a suitable phenotypic characterization of these cells in healthy individuals must be initially completed to allow for comparative studies to be performed.

Precise effects of chronic inflammation upon NK cells still remain to be separated from those caused by chronic infection. Therefore studies comparing NK cells from different hepatitis fibrosis stages and NASH could be performed to provide answers in this area. To complement such studies it could be useful to further examine the effects of DAA therapy upon NK cells. As we see a normalization of NK cell phenotype and function in IFN-free therapy it would be of interest to examine which intracellular mechanisms are affected during these new DAA therapies. Particularly parameters such as pSTAT levels and inducibility, along with potential epigenetic changes would shed light on how this effective therapy affects the immune system.

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"*I may not have gone where I intended to go, but I think I have ended up where I needed to be*". Thanks to all my family and friends for their continued support in helping me get there.

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